The Development of Assays and their Application

in Elaborating the Pharmacokinetic Disposition

of Cocaine and its Metabolites

in the Mature Dog

by

© Riccardo L. Boni

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

Department of Chemistry and Faculty of Pharmacy

University of Manitoba

Winnipeg, Manitoba, Canada

July, 1986

THE DEVELOPMENT OF ASSAYS AND THEIR APPLICATION
IN ELABORATING THE PHARMACOKINETIC DISPOSITION
OF COCAINE AND ITS METABOLITES
IN THE MATURE DOG

BY

RICCARDO L. BONI

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

© 1986

ABSTRACT

Analytical methods were developed for the determination
of cocaine and its hydrolysis products: ecgonine methyl es-
ter, benzoylecgonine, and ecgonine in plasma and urine.
Ecgonine methyl ester, benzoylecgonine, and ecgonine were
quantitated by gas chromatography with a flame ionization
detector with a minimal detectable level of 1-2 μg/mL plasma
or urine. Cocaine was quantitated by gas chromatography
with a nitrogen-phosphorus detector with a minimal detecta-
ble level of 10-20 ng/mL plasma or urine. The analytical
methods were applied to elaborating the pharmacokinetics of
the disposition of cocaine and its individual hydrolysis
products in the dog.

In separate pharmacokinetic experiments, cocaine, ecgo-
nine methyl ester, benzoylecgonine, and ecgonine at dosage
levels of 5, 25, 10, and 10 mg/kg, respectively, were admin-
istered to the same mature female Beagle dog. The disposi-
tions of cocaine, benzoylecgonine, and ecgonine were best
described by a two-compartment open model whereas the dis-
postion of ecgonine methyl ester was best described by a
one-compartment open model. The elimination half-lives of
cocaine, ecgonine methyl ester, benzoylecgonine, and ecgo-
nine were 1.6, 2.0, 4.0, and 1.2 hours, respectively.

Injected ecgonine and benzoylecgonine were not metabolized but were eliminated primarily via excretion in the urine. Injected ecgonine methyl ester underwent _in vivo_ hydrolysis to form ecgonine. Only 34% of the administered ecgonine methyl ester dose was recovered in the urine as ecgonine methyl ester and ecgonine, 19% and 15%, respectively.

Following the intravenous administration of cocaine to the dog benzoylecgonine, ecgonine methyl ester, ecgonine, and unchanged cocaine were recovered in the urine. Benzoylecgonine represented the largest percentage (11%) and unchanged cocaine represented the smallest percentage (0.6%) of the cocaine dose recovered. Only a total of 24% of the dose was recovered in the urine as unchanged cocaine and its hydrolysis products.

In separate _in vitro_ experiments, the spontaneous hydrolysis of cocaine and of ecgonine methyl ester in urine at 37° C was demonstrated. Ecgonine methyl ester was hydrolyzed to ecgonine. Cocaine was hydrolyzed to benzoylecgonine, ecgonine, and ecgonine methyl ester with benzoylecgonine as the chief product.

- ii -

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER III. EXPERIMENTAL

A. ANALYTICAL PROCEDURES

    1. Analysis of Ecgonine Alkaloids

    2. Determination of Ecgonine in Water, Plasma, and Urine

    3. Determination of Benzoylecgonine and Ecgonine in Water, Plasma, and Urine

    4. Determination of Ecgonine Methyl Ester and Ecgonine in Water, Plasma, and Urine

    5. Determination of Cocaine in Water, Plasma, and Urine

B. PHARMACOKINETICS

    1. Intravenous Ecgonine

    2. Intravenous Benzoylecgonine

LIST OF FIGURES

LIST OF TABLES

Chapter I

INTRODUCTION

I-A. HISTORY OF COCAINE USE

Cocaine is an alkaloid occurring naturally in the leaves of <u>Erythroxylum</u> <u>coca</u> and closely related species of plants native to the Andes Mountains and the Amazon River basin regions of South America. Cocaine is present in significant quantities only in the leaves of the cultivated species: <u>Erythroxylum</u> <u>coca</u>, <u>Erythroxylum</u> <u>novogranatense</u> and <u>Erythroxylum</u> <u>novogranatense</u> var. <u>truxillense</u>. The cocaine content of air-dried leaves of these varieties of coca is approximately 0.5-1.0% by weight, composing 50-80% of the leaves' total alkaloid content (1,2,3).

Human experience with coca leaves and cocaine can be traced back almost 5000 years. Archeological evidence unearthed in Ecuador and Peru suggests that coca was important in a number of ancient shamanistic rites and that cocaine derived from coca leaves may have been used as a local anesthetic in complicated "trephining" surgical procedures (3,4). The cultivation and use of coca spread through the emerging civilizations of western South America and played a central role in the sophisticated theocratic society of the

extensive Inca Empire (1200-1553 AD). With the coming of the Spanish Conquistadores and the destruction of the Incan civilization in the middle of the sixteenth century, the cultivation of coca and the habit of coca-chewing spread rapidly among the native populations. At present it is estimated that over 90% of Andean Indians chew coca leaves (5,6,7,8).

The German chemist Friedrich Gaedecke was the first to isolate an alkaloid from coca leaves in 1855. Four years later, Albert Niemann independently isolated and chemically characterized the alkaloid which he named cocaine. Thomas Moreno Y Maiz and Vasili von Anrep in 1868 and 1880, respectively, conducted the first systematic experimental studies on the pharmacological actions of cocaine confirming the reported stimulant and local anesthetic effects (4,5,9). In 1884, Carl Koller proposed the use of cocaine as a topical anesthetic in ophthalmic surgery. The use of cocaine as a topical anesthetic in otolaryngology remains the only recognized medical use of cocaine today, with cocaine being the local anesthetic of choice in nasal surgery (10,11).

Koller became interested in cocaine as a result of Sigmund Freud's highly influential series of papers, "On Coca", an extensive review of the knowledge of cocaine up to that time. Based on the experience of other physicians as well as his own repeated self-experimentation, Freud suggested that cocaine's stimulant properties could be of great ben-

efit in a variety of conditions especially in the cure of morphine addiction (4,5,6). It soon became apparent, however, that cocaine had serious abuse potential as increasing numbers of cases of strong psychological dependencies to cocaine were reported. Legislation, notably the Harrison Tax Act of 1914, was introduced in the United States classifying cocaine and coca as narcotics and banning their use except on prescription. Medicinal use of cocaine dwindled and the many "cocainized nostrums" popular at the time disappeared from common use (6).

The illicit use of cocaine continued, spreading through the criminal and musical subcultures and dramatically re-emerging in the "glitter crowd" of the 1970's (6). Cocaine has become fashionable as a "recreational drug" among users in the 1980's with an estimated 4.2 million Americans taking cocaine regularly and 28% of all people ages 18-25 having tried the drug according to the National Institute of Drug Abuse (12).

As the abuse of cocaine becomes increasingly widespread there is renewed interest in the study of cocaine and its actions. There has been considerable research into the biochemical mechanisms through which cocaine exerts its pharmacological actions, especially the local anesthetic and sympathomimetic effects. Cocaine's stimulation of the central nervous system, however, is poorly understood. There are also many gaps in our knowledge of the biological fate of

cocaine. Much of present-day cocaine research involves the elucidation of the qualitative and quantitative aspects of the disposition of cocaine in man and laboratory animals (4,10).

II-B. CHEMISTRY AND ANALYSIS OF COCAINE AND RELATED
ECGONINE ALKALOIDS

Cocaine is identified as 8-azobicyclo[3.2.1]octane-2-carboxylic acid 3-benzoyloxy-8-methyl methyl ester [1R-(exo-exo)] in the Chemical Abstracts. Cocaine is a member of the tropane family of alkaloids. The gross structure of cocaine was established through total synthesis by Willstätter in 1923. The correct stereochemical structure of cocaine was resolved by Hardeggar and Ott in 1955 (4,13). Cocaine contains three functional groups: a benzoyl ester, a methyl ester, and a tertiary methyl amino group. Selective hydrolytic cleavage of one or both of the ester functions results in the production of one of the related ecgonine alkaloids. Hydrolysis of the C3 benzoyl ester function of cocaine produces ecgonine methyl ester whereas hydrolysis of the C2 methyl ester function of cocaine will result in benzoylecgonine. Hydrolysis of both the ester functions of cocaine results in the production of ecgonine. The structures of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine are presented in Figure 1.
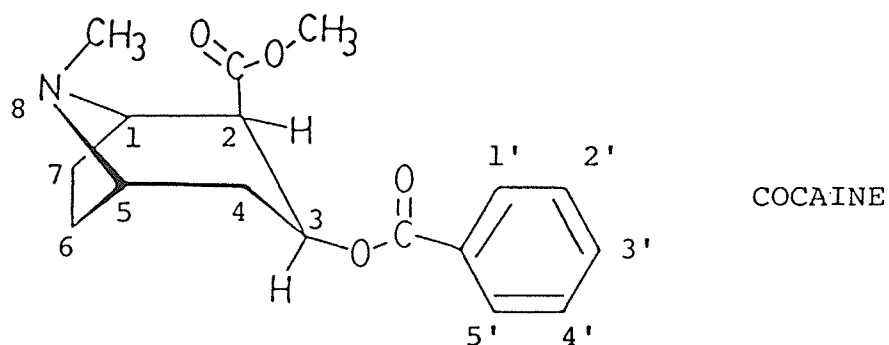
5

8-azobicyclo[3.2.1]octane-2-carboxylic acid
3-benzoyloxy-8-methyl methyl ester [1R-(exo-exo)]

COCAINE

ECGONINE METHYL ESTER

BENZOYLECGONINE
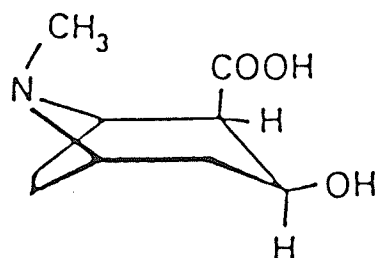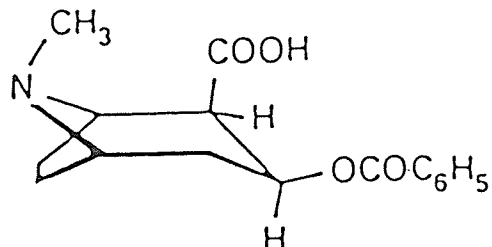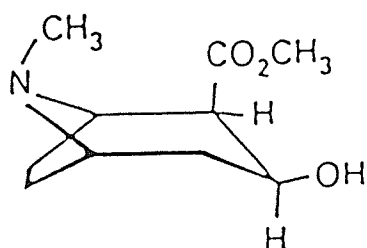
ECGONINE

Figure 1:   Stereochemical structures of cocaine and its hy-
drolysis products ecgonine methyl ester, benzoylecgonine,
and ecgonine.

Cocaine is a tertiary amine. Removal of cocaine's N-methyl group produces norcocaine. Similar to cocaine, norcocaine can be selectively de-esterified to produce a series of norecgonine alkaloids: norecgonine methyl ester, benzoylnorecgonine, and norecgonine (13).

Cocaine is prepared from the crude alkaloid extracted from the leaves of <u>Erythroxylum</u> <u>coca</u> or by methylation and benzoylation of ecgonine (3). Cocaine (free base mp 98° C) is an organic base, pKa 8.65, which is only very slightly soluble in water but very soluble in alcohol, chloroform, and ether. Commercially, cocaine is most commonly available as the hydrochloride salt (mp 197° C) which is very soluble in water, alcohol, and chloroform, but insoluble in ether (14-17).

Ecgonine methyl ester is prepared from ecgonine by methylation (12,15,18). The free base is an oil at room temperature and has a pKa of 9.16 (17). Usually obtained as the hydrochloride salt (mp 212° C), ecgonine methyl ester has solubility properties very similar to those of cocaine (15).

Benzoylecgonine is prepared from cocaine by hydrolysis (14,18). Benzoylecgonine is an amphoteric compound containing both the basic tertiary amine function as well as a free carboxylic acid group. Benzoylecgonine free base (anhydrous mp 195° C) has a pKa of 11.80 ("mixed constant") and is soluble in hot water, ethanol, and dilute acid and alkali so-

lutions. Benzoylecgonine is insoluble in ether, chloroform, and other organic solvents (14,15).

Ecgonine hydrochloride (mp 246° C) is prepared by total hydrolysis of cocaine hydrochloride in aqueous hydrochloric acid at reflux temperature (19). Similar to benzoylecgonine, ecgonine is an amphoteric compound with a pKa ("mixed constant") of 11.15 (17). Both ecgonine free base and the hydrochloride salt are soluble in water, ethanol, and methanol, but insoluble in ether, chloroform, and other organic solvents (3,15,16).

The chemical and physical properties of cocaine and related ecgonine alkaloids have been extensively studied. Important physico-chemical, spectrophotometric (ultraviolet, visible, infrared), nuclear magnetic resonance, and mass spectrometric data have been collected and evaluated (13,14). A number of analytical techniques have been developed to detect, identify, and quantitate cocaine and its metabolites in biological fluids. The principal methods which have been used are colorimetry, immunologic assays, thin-layer chromatography, high-performance liquid chromatography, gas chromatography, and gas chromatography-mass spectrometry (20,21).

Colorimetry was one of the first methods developed to measure cocaine in biological fluids. Bromocresol purple will bind with cocaine (and other alkaloids) to form a col-

ored complex which can be quantified spectrophotometrically. Woods et al. (22) employed this procedure to measure plasma, tissue, and urinary levels of cocaine after administration of cocaine by a variety of routes to dogs and rabbits (23). Colorimetric methods lack the sensitivity and specificity required to quantitate cocaine in the plasma after the relatively low doses typically administered to humans and cannot be used to detect the other ecgonine alkaloids (20,21).

Immunoassay methods, notably radioimmunoassay (RIA), enzyme multiplied immunoassay technique (EMIT), and hemagluttination inhibition (HI), have been developed for the detection of benzoylecgonine in the urine and are commercially available (24-30). These assays are very sensitive allowing the direct detection of low levels of benzoylecgonine in urine but lack absolute specificity. Immunoassays, especially EMIT, are of primary use in the large-scale screening of urines for evidence of cocaine use (20).

Numerous thin-layer chromatographic (TLC) methods have been developed to separate and purify cocaine and its chemical and metabolic congeners following solvent extraction from biological fluids (14,28,31,32,33). The TLC systems developed by Misra et al. (31) coupled with liquid scintillation spectroscopy have been used extensively in the study of the metabolism of tritium ring-labelled cocaine in experimental animals (34,35,36). The TLC systems of Noirfalise and Mees (32) and Wallace et al. (33) have been used to

isolate cocaine and benzoylecgonine from extracted human urine samples. The alkaloids were detected by treatment with Dragendorf-20% $H_2SO_4-I_2$ (33) or Ludy Tenger's (31) reagents. These TLC systems employ organic solvents of varying polarities and are typically alkaline, containing significant proportions of ammonia. In contrast, Munier and Drapier (37,38) have developed TLC solvent systems which employ highly polar neutral solvents avoiding any hydrolysis problems associated with alkaline systems. In itself TLC represents an efficient method for the isolation and purification of cocaine and related alkaloids after extraction from biological fluids. It must be coupled with a sensitive and specific analytical technique if quantitation is desired.

High-performance liquid chromatography utilizing reverse phase columns and UV detectors has been used to determine cocaine and benzoylecgonine in extracts of biological fluids (39,40,41). Reported methods have high sensitivity, specificity, and the added advantage that derivatization is not required for the UV detection of cocaine and benzoylecgonine. The corresponding demethylated compounds, norcocaine, and benzoylnorecgonine, are also easily separated and detected. The debenzoylated ecgonine alkaloids ecgonine methyl ester and ecgonine, however, do not show sufficient UV absorption to permit their sensitive detection (39).

Gas chromatography (GC) employing flame ionization (FID), nitrogen-phosphorus (NPD), or electron capture (ECD) detectors has been the method of choice for the quantitation of cocaine and its metabolites in biological fluids. Several GC-FID methods have been developed and used to simultaneously measure levels of cocaine and benzoylecgonine in urine (42-46). Unlike nonpolar cocaine, benzoylecgonine and ecgonine are highly polar and require derivatization before they can be analyzed by GC. Alkylation, acylation, and silylation have been commonly used to form derivatives of benzoylecgonine and ecgonine which can be analyzed (14). Moore (47) described a qualitative method for the determination of both benzoylecgonine and ecgonine after derivatization with N,O-bis-(trimethylsilyl)-acetamide (BSA). This method was modified by Roscoe (48) for the quantitation of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine extracted from aqueous media.

Flame ionization detectors are not sufficiently sensitive to measure the concentration of cocaine in the plasma after the relativlely low doses administered to man. Jatlow and Bailey (49) used a nitrogen-phosphorus detector to measure plasma cocaine concentrations as low as 5-10 ng/mL. The greater sensitivity and specificity of GC-NPD techniques have been used to advantage in the measurement of cocaine plasma levels subsequent to cocaine administered by various routes (44,49-52).

Gas chromatography with an electron capture detector (GC-ECD) is another highly sensitive and specific method which has been used for the determination of cocaine in biological fluids. Cocaine can be reduced and acylated to form a fluorinated derivative (53). This technique has been adapted to the quantitation of cocaine in blood, plasma, and urine (54) as well as the determination of benzoylecgonine in plasma and urine (46).

The combination of gas chromatography and mass spectrometry (GC-MS) provides the most sensitive and specific assay for cocaine and its metabolites (20). Gas chromatography-mass spectrometry has been used to quantitate cocaine in biological fluids and has been used to detect and identify a number of metabolites of cocaine including norcocaine, benzoylecgonine, ecgonine methyl ester, and ecgonine (55-59). Even though GC-MS methods are the most reliable for the quantitation of very low levels of cocaine in biological fluids they are not simple or cheap. Elaborate extraction and derivatization schemes are still required in the analysis of cocaine's polar metabolites.

I-C. ABSORPTION AND DISPOSITION OF COCAINE AND RELATED
     ECGONINE ALKALOIDS

The proper understanding of the pharmacological, toxicological, and behavioral effects of cocaine depend on an

accurate assessment of the qualitative and quantitative aspects of its disposition in biological systems. The absorption, distribution, metabolism, and excretion of cocaine and its putative ecgonine-related metabolites have been the subjects of considerable research but much remains unclear.

Cocaine rapidly reaches high concentrations in plasma after intravenous injection but its rate of absorption after administration via other routes is varied and dependent on a number of factors. Woods et al. (23) measured plasma cocaine concentrations after subcutaneous administration of cocaine hydrochloride solution to dogs and rabbits. Plasma cocaine concentration peaked at 45 minutes in dogs and between 60 and 90 minutes in rabbits and remained elevated for 5-6 hours. Nayak et al. (34) found that the disappearance of cocaine from the subcutaneous injection sites of acutely and chronically cocaine-treated rats was rapid but the peak cocaine plasma levels were attained 2-4 hours after injection. The prolonged absorption of cocaine may be due in part to the local vasoconstriction caused by cocaine (60).

The absorption of cocaine from mucosal membranes differs with the area of application. Adriani and Campbell (61) reported peak levels of cocaine in the blood 4 to 6 minutes after application of a cocaine solution to the pharynx of dogs. Cocaine was more rapidly absorbed after tracheal instillation and when inhaled in a finely nebulized form. No cocaine was detected in the blood when an aqueous

cocaine solution was applied topically to unbroken skin. Cocaine was absorbed, however, when it was applied in either a water-soluble ointment base or in aqueous solution to abraded skin. Peak levels of cocaine in the blood occurred 6-10 minutes after administration. Van Dyke et al. (62) measured plasma cocaine concentrations after application of a cocaine hydrochloride solution to the nasal mucosa of cardiovascular and dental surgery patients. Peak cocaine levels occurred approximately 60 minutes after nasal instillation but cocaine remained on the nasal mucosa for as long as 3 hours after administration possibly due to local vasoconstriction limiting absorption. In a subsequent study, the same workers obtained similar plasma cocaine profiles in healthy subjects after intranasal administration of cocaine hydrochloride solution and crystalline cocaine hydrochloride with mean times to peak of 47 and 35 minutes, respectively (63).

It has been wrongly assumed that cocaine is poorly absorbed orally as a consequence of it being susceptible to hydrolysis in the gastrointestinal tract (60). Van Dyke et al. (64) detected cocaine in the plasma 30 minutes after oral administration to healthy subjects. Peak plasma cocaine levels occurred 1 hour after oral administration. In a later study, the same workers found the oral absorption of cocaine was best descibed by a single first order rate constant with a lag time of 30 minutes. They attributed this

lag to the time it takes orally administered cocaine to reach the relatively mild alkaline environment of the small intestine where it is predominantly non-ionized and as such can be absorbed (64).

Holmstedt et al. (65) were able to measure cocaine in the blood of volunteer subjects after administering coca according to the traditional methods practiced by the Indians of the Peruvian Andes and Amazon basin. Unlike cocaine administered in capsule form, coca chewing produced measurable cocaine levels in plasma 5 minutes after chewing coca leaves began suggesting that a certain amount of cocaine is locally absorbed. Peak plasma cocaine concentrations occurred between 0.4 and 2.0 hours after administration and the estimated absorption half-life ranged from 0.2 to 0.6 hours.

Little is known about the systemic distribution of cocaine in humans. Following intravenous administration of cocaine in rats, peak levels of cocaine in plasma and tissues occurred within 15 minutes. The highest concentrations of cocaine were observed in the spleen, kidneys, lungs, brain, and testes. Lower concentrations of cocaine were measured in the liver and in fat tissue with the lowest cocaine concentrations found in muscle, heart, and plasma. Cocaine's rapid distribution to the brain is a result of its high lipid solubility. The brain/plasma ratio in rats ranged between 8 and 12 after intravenous injection. The slow distribution of cocaine to fat tissue is probably due

to the relatively poor supply of blood to fat depots. Higher concentrations of cocaine, however, were present in the fat tissue of rats chronically treated with cocaine. Cocaine was found to be poorly bound (approximately 36%) to plasma proteins in both acutely and chronically cocaine-treated rats. Cocaine levels in all tissues declined sharply with time and no cocaine was detected in brain, testes, muscle, heart, and plasma 6 hours after intravenous administration. Low levels of cocaine were detected in liver, lungs, kidney, spleen and intestine (34).

Similarly, 30 minutes after intravenous administration of cocaine to dogs, the highest cocaine concentrations were found in liver, kidneys, lungs, and brain. The lowest cocaine concentrations were present in heart, muscle, and plasma. Brain/plasma ratios ranged between 5 and 8. The mobilization of cocaine from fat tissue was found to be much faster in the dog than in the rat (35).

In monkeys, the highest concentrations of cocaine were found in pancreas, lungs, spleen, brain, and kidney after intravenous administration. As was the case for rats and dogs, muscle, heart and plasma of the cocaine-treated monkeys contained the lowest cocaine concentrations (36).

The distribution of the individual cocaine metabolites: ecgonine, benzoylecgonine, and benzoylnorecgonine has been investigated after intravenous administration of each to

rats (66,67,68). The highest concentrations of ecgonine were found in the kidney, plasma, and liver and the lowest ecgonine concentrations were found in the brain. Very little benzoylecgonine or benzoylnorecgonine entered the brain of rats after intravenous administration. The highly polar nature of these cocaine metabolites prevents them from crossing the blood-brain barrier to any significant extent (66,67,68).

Cocaine is rapidly and extensively metabolized in man and laboratory animals. The metabolites of cocaine which have been identified in man and animals are listed in Figure 2 (13,21,69,70,71). De-esterification and demethylation represent the two major pathways for the biotransformation of cocaine. Among the major metabolites are included ecgonine methyl ester, benzoylecgonine, and ecgonine as well as norcocaine, benzoylnorecgonine, and norecgonine (13,21). Minor metabolites: cocaethylene, ecgonine ethyl ester, ecgonidine methyl ester, and the arylhydroxycocaines have been identified in multiple intoxication and overdose cases (21,69,70,71).

De-esterification of cocaine repesents the major route of cocaine metabolism. Valenzuela-Ortiz (72) identified benzoylecgonine and ecgonine in the urine of cocaine-treated rats. Nayak et al. (34) identified benzoylecgonine, benzoylnorecgonine, and ecgonine in the urine of acutely and chronically cocaine-treated rats. The same workers identi-
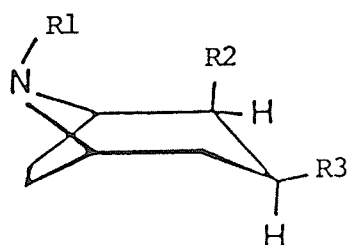
	R1	R2	R3
cocaine	$-CH_3$	$-C(O)OCH_3$	$-OC(O)C_6H_5$

Major Metabolites

	R1	R2	R3
ecgonine methyl ester	$-CH_3$	$-C(O)OCH_3$	$-OH$
benzoylecgonine	$-CH_3$	$-C(O)OH$	$-OC(O)C_6H_5$
ecgonine	$-CH_3$	$-C(O)OH$	$-OH$
norcocaine	$-H$	$-C(O)OCH_3$	$-OC(O)C_6H_5$
benzoylnorecgonine	$-H$	$-C(O)OH$	$-OC(O)C_6H_5$
norecgonine	$-H$	$-C(O)OH$	$-OH$

Minor Metabolites

	R1	R2	R3
cocaethylene	$-CH_3$	$-C(O)OC_2H_5$	$-OC(O)C_6H_5$
ecgonine ethyl ester	$-CH_3$	$-C(O)OC_2H_5$	$-OH$
ecgonidine methyl ester	$-CH_3$	$-C(O)OCH_3$	$-OH$ (2,3-dehydro)
arylhydroxy-cocaines	$-CH_3$	$-C(O)OCH_3$	$-OC(O)C_6H_4(OH)$
	$-CH_3$	$-C(O)OCH_3$	$-OC(O)C_6H_3(OCH_3)(OH)$
	$-CH_3$	$-C(O)OC_2H_5$	$-OC(O)C_6H_3(OH)_2$
	$-CH_3$	$-C(O)OC_2H_5$	$-OC(O)C_6H_3(OCH_3)(OH)$

Figure 2: Major and minor metabolites of cocaine identified in biological fluids of man and laboratory animals.

fied ecgonine, benzoylnorecgonine, benzoylecgonine, norco-
caine, norecgonine, and ecgonine methyl ester as the urinary
metabolites of cocaine in the dog. The bulk of the metabo-
lized cocaine was present as ecgonine, benzoylnorecgonine,
and benzoylecgonine representing 35, 8, and 3 % of adminis-
tered cocaine, respectively (35). Benzoylnorecgonine,
benzoylecgonine, norcocaine, ecgonine methyl ester, and
ecgonine were identified in the urine of monkeys chronically
treated with cocaine. The highest percentage of adminis-
tered cocaine was present in the urine as benzoylnorecgonine
and benzoylecgonine, 35-40% and 7-12%, respectively (36).
The half-life of cocaine in the plasma following intravenous
administration in rats, dogs, and monkeys was 0.3, 1.2, and
1 hour, respectively (34,35,36).

De-esterification accounts for most of the metabolism
of cocaine in man. Fish and Wilson (73) identified benzoyl-
ecgonine as the chief urinary metabolite in man after an in-
tramuscular dose of cocaine; 35-54% of the daily adminis-
tered dose was recovered as benzoylecgonine. Valanju et al.
(28) identified benzoylecgonine and ecgonine in the urine of
cocaine abusers. Ecgonine was present only in those urine
samples which also contained benzoylecgonine. Benzoyl-
ecgonine, however, was often present in the absence of ecgo-
nine. Benzoylecgonine has also been identified as the major
urinary metabolite of cocaine by a number of other workers
(39,43,45,46,56,74).

In contrast, recent research has indicated that de-es-
terification of cocaine to ecgonine methyl ester, not to
benzoylecgonine, may be the major pathway for the biotrans-
formation of cocaine in man. Ecgonine methyl ester account-
ed for 32-49% of the urinary metabolites after oral inges-
tion of cocaine (75). Ambre et al. (76) found that ecgonine
methyl ester is the most prominent urinary metabolite of co-
caine in man after illegal use of cocaine with an average of
20 times as much ecgonine methyl ester in the urine as co-
caine. Taylor et al. (77) investigated the hydrolysis of
cocaine in human serum _in vitro_. They found cocaine was me-
tabolized to ecgonine methyl ester, benzoylecgonine, and
ecgonine. At 1 hour, de-esterification of cocaine to ecgo-
nine methyl ester accounted for 66% of the hydrolyzed co-
caine. After 4 hours, however, a larger proportion of
benzoylecgonine and ecgonine was found as compared to ecgo-
nine methyl ester.

Stewart et al. (78) demonstrated that the _in vitro_ hy-
drolysis of cocaine to ecgonine methyl ester was mediated by
cholinesterase. The hydrolyzing activity was inhibited by
eserine and DFP and partially inhibited by fluoride. A
highly purified preparation of human plasma cholinesterase
showed the same cocaine hydrolyzing activity. They did not
observe the hydrolysis of cocaine to benzoylecgonine and
concluded that previously reported _in vitro_ and _in vivo_ hy-
drolysis of cocaine may have been due to the spontaneous

non-enzymatic hydrolysis in aqueous alkaline solutions which they were also able to demonstrate. In a later study (79), the same workers observed that human liver contains an esterase which can hydrolyze cocaine to ecgonine methyl ester. The liver esterase appeared to be a different enzyme from that found in serum. No evidence was found for the enzymatic formation of benzoylecgonine by either the serum or the liver esterases. They also found that cholinesterase had a low but detectable activity in the hydrolysis of benzoylecgonine to ecgonine. They suggested that the presence of ecgonine as a metabolite of cocaine in the urine may result from enzymatic hydrolysis of benzoylecgonine or from the non-enzymatic breakdown of ecgonine methyl ester.

Even though quantitatively N-demethylation may represent a minor pathway for cocaine biotransformation it is important because the product formed, norcocaine, has potent CNS stimulant and local anesthetic properties (67,80). Norcocaine has been identified as a urinary metabolite of cocaine in dogs (35) and monkeys (36). Norcocaine has also been identified as a metabolite of cocaine in the brain of rats (81) and monkeys (82). The N-demethylation of cocaine involves the mixed-function oxidase systems which are present predominantly in the liver. Both _in vitro_ and _in vivo_ studies in rats have shown that cocaine is metabolized in the liver to form norcocaine. This N-demethylation can be inhibited by SKF-525A, a microsomal enzyme inhibitor (83).

Inaba et al. (75) demonstrated that only a small frac-
tion (2.4-6.2%) of cocaine is converted to norcocaine in
man. The N-demethylation of cocaine is limited by the rapid
de-esterification of cocaine by plasma and liver esterases.
It is likely that the more polar metabolites of cocaine re-
sulting from cocaine hydrolysis do not have access to the
nonpolar sites of N-demethylation in the endoplasmic reticu-
lum (75). In addition, the small percentage of norcocaine
formed may undergo subsequent hydrolysis by liver and serum
esterases. This limits the amount of pharmacologically-ac-
tive norcocaine which can reach the brain (79).

Very little unchanged cocaine is excreted in the urine
and feces of man and laboratory animals after cocaine admin-
istration by a variety of routes. In rats only 0.04% of an
intravenous dose of cocaine was excreted in the bile as un-
changed cocaine. The total amount of cocaine and metabolite
excreted in the bile was 36%. Approximately 1% of a subcu-
taneous dose of cocaine was excreted unchanged in the urine
and feces of acutely-treated rats with maximum excretion oc-
curring within 24 hours after injection. Total cocaine and
metabolites excreted in the urine and feces were 49% and
22%, respectively (34). Approximately 3% of an intravenous
dose of cocaine was excreted unchanged in the urine of
acutely-treated dogs and approximately 1% was excreted un-
changed in the feces within 24 hours. Excretion of metabo-
lites continued for several days with a total of 49% and 17%

of the dose excreted in the urine and feces, respectively
(35). In acutely-treated monkeys, 0.2% of an intravenous
dose of cocaine was recovered unchanged in urine and 0.2%
unchanged in feces. Total excretion of cocaine and metabo-
lites represented 42% and 10% of the injected dose in urine
and feces, respectively. The majority of excretion occurred
within 24 hours (36).

Fish and Wilson (73) studied the excretion of cocaine
and benzoylecgonine after intravenous administration of co-
caine to human subjects. Only 1-9% of the dose was excreted
unchanged in the urine and 35-54% was excreted as benzoyl-
ecgonine. The cumulative excretion of cocaine appeared to
depend on urinary pH. Wallace et al. (43) measured the uri-
nary excretion of cocaine and benzoylecgonine in patients
receiving cocaine topical anesthesia prior to nasal surgery.
They found that very little unchanged cocaine was excreted
in the urine and that the excretion of cocaine and benzoyl-
ecgonine occurred mostly within the first 24 hours after co-
caine administration. Hamilton et al. (74) found that maxi-
mal urinary excretion of unchanged cocaine occurred within 2
hours of nasal inhalation and decreased rapidly thereafter.
Maximal urinary excretion of benzoylecgonine occurred 4-8
hours following cocaine inhalation and diminished slowly
over the next few days. Ambre et al. (84) measured cocaine,
benzoylecgonine, and ecgonine methyl ester excretion in the
urine after intravenous and intranasal administration of co-

caine. Benzoylecgonine and ecgonine methyl ester excretion rates were found to decline logarithmically by 4-8 hours after the dose of cocaine. The half-lives of ecgonine methyl ester and benzoylecgonine excretions averaged 4.2 and 5.1 hours, respectively. Total urinary recovery averaged 93% with ecgonine methyl ester accounting for 26-60% of the cocaine dose.

The vast majority of studies have dealt with the qualitative aspects of the disposition of cocaine and related ecgonine alkaloids. Only with recent technical advances have reliable data on the concentrations of cocaine and metabolites in biological fluids become available. This has led to some studies into the pharmacokinetics of cocaine following administration in man.

Van Dyke et al. (62) measured the plasma cocaine concentration in human subjects undergoing cardiovascular and dental surgery. Subjects were administered cocaine intranasally at a dose of 1.5 mg/kg. Cocaine concentrations in plasma peaked at approximately 60 minutes and then decreased in a log-linear fashion with an apparent half-life in the plasma of 2.5 hours. The same workers determined plasma cocaine concentrations in four subjects with histories of previous cocaine use following intranasal nd oral administration of 2.0 mg/kg cocaine hydrochloride. Peak plasma cocaine concentrations occurred at 50-90 minutes and 60-120 minutes after intranasal and oral administration, respec-

tively. After the peak concentration was reached the levels of cocaine decreased in a log-linear fashion with the apparent half-life in plasma of 1.3 hours after intranasal and 0.9 hour after oral administration (64). In follow-up study, Wilkinson et al. (63) determined plasma cocaine levels after intranasal doses of 0.19-2.0 mg/kg and oral cocaine doses of 2.0 and 3.0 mg/kg. Intranasal cocaine kinetics were described by a 1-compartment open model with 2 consecutive first-order input steps and first-order elimination. The mean elimination half-life for cocaine in 7 subjects was 75 minutes. Oral cocaine disposition was described by a 1-compartment open model with a lag time of approximately 30 minutes followed by a single first-order input phase and first-order elimination. The mean half-life after oral administration was 48 minutes.

Javaid et al. (85) were the first to study the plasma half-life of cocaine after intravenous administration in human subjects. The half-life of cocaine disappearance from plasma varied from 16 to 87 minutes. Kogan et al. (46) presented data on plasma and urine concentrations of cocaine and benzoylecgonine in three human subjects who had received 1.0-1.9 mg/kg cocaine hydrochloride intravenously. Cocaine concentrations in plasma peaked at 5 minutes and then declined in a multi-exponential fashion over the next 5-6 hours. The distributional half-life of cocaine in plasma was 20-40 minutes and the mean biological half-life of co-

caine in the plasma was 2.8 hours. Barnett et al. (86) in-
vestigated the pharmacokinetics of cocaine following in-
travenous administration of 100 mg cocaine hydrochloride to
three subjects and 200 mg cocaine hydrochloride to two sub-
jects. The plasma cocaine concentration-time data obtained
following intravenous administration of 100 mg was best de-
scribed by a mono-exponential equation in all three sub-
jects. For the 200 mg doses the data obtained was best de-
scribed by a mono-exponential equation in one subject and by
a bi-exponential equation in the other. The calculated
biological half-life of cocaine ranged from 40-91 minutes.
The distributional half-life of cocaine in the subject ex-
hibiting bi-exponential decline of plasma cocaine levels was
13 minutes. Terminal plasma half-life and total plasma
clearance were found to have a strong dose dependence over
the range 1-3 mg/kg administered. The authors concluded
that these findings must be viewed as tentative in that the
intravenous studies were carried out on small number of
subjects (86).

There is very little information available on the ki-
netics of the individual metabolic and excretory steps ac-
counting for the elimination of cocaine in man and animals.
Johns et al. (25) determined the concentration of benzoyl-
ecgonine in the serum after topical application of cocaine
during nasal surgery. They found that cocaine was rapidly
hydrolyzed in the serum to form benzoylecgonine which was

detected by RIA 15 minutes after cocaine administration and that peak benzoylecgonine levels occurred 4.5 hours after administration. The half-life of benzoylecgonine in the serum was 5.5 hours. Ambre et al. (84) determined the extent and rate of ecgonine methyl ester and benzoylecgonine excretion following intravenous and intranasal administration of cocaine. Recovery data indicated that total recovery of cocaine, ecgonine methyl ester, and benzoylecgonine in the urine averaged 93% of the dose of cocaine administered with 1-15%, 26-60%, and 33-55% recovered as cocaine, ecgonine methyl ester, and benzoylecgonine, respectively. There was no apparent effect of increasing dose on the pattern of metabolite excretion. The elimination half-lives of ecgonine methyl ester and benzoylecgonine averaged 4.2 and 5.1 hours, respectively.

For ethical reasons, pharmacokinetic studies of cocaine in humans have been carried out in subjects receiving cocaine during surgery or in subjects with histories of previous cocaine use (50,86). Previous and concomitant use of cocaine and other drugs may have influenced the results of these studies. Detailed information on the pharmacokinetics of cocaine in experimental animals is lacking. Much more research on the pharmacokinetics of cocaine in a suitable animal model is required to aid in the understanding of the disposition of cocaine in man.

Chapter II

RESEARCH OBJECTIVES

II. GENERAL

Cocaine abuse has increased dramatically within the last decade. This has led to renewed interest in the research of the pharmacology of cocaine, in particular its pharmacodynamic and its psychopharmacological actions. A proper understanding of the pharmacology of cocaine requires fundamental knowledge of the biological fate of cocaine and its metabolites. Early studies of the disposition of cocaine and related alkaloids have been hampered by the specificity and sensitivity limitations of available analytical technology. In man cocaine is rapidly and extensively metabolized. After administration of non-toxic doses, cocaine is present in the blood in very low concentrations (ng/mL levels) for a short period of time. In addition, unless precautions are taken in the collection, storage, and analysis of biological samples, cocaine can undergo enzymatic and non-enzymatic hydrolysis in vitro to produce the same compounds which are formed in vivo (49,77,78,79,87,88, 89).

Improvements in analytical methodology have facilitated the study of the quantitative aspects of the disposition of

cocaine in man.   A number of researchers have studied the
pharmacokinetics of cocaine in man following administration
by a variety of routes (46,62,63,64,85,85,86).   Some esti-
mates of the pharmacokinetic parameters which can be used to
describe the absorption and disposition of cocaine in man
have been obtained.   For ethical reasons these studies have
been carried out on subjects with previous histories of co-
caine use or who received cocaine during surgery.   It is
possible that prior use of cocaine or its concomitant use
with other drugs may have some influence on the results ob-
tained.

Experiments carried out with a suitable animal model
would be of great benefit in clarifying our understanding of
the biological fate of cocaine. The physiological distribu-
tion and metabolism of cocaine has been examined in rats,
dogs, and monkeys (34,35,36).   The animals were acutely and
chronically treated with radiolabelled cocaine and after ap-
propriate periods of time the animals were sacrificed and
collected plasma and tissue samples analyzed. The half-lives
of cocaine in plasma and tissues were calculated from the
combined animal data.   Metabolites of cocaine in plasma,
tissue homogenates, and urine were identified by TLC.   The
findings of these studies may have been compromised by the
methods employed for sample collection, storage, and analy-
sis in light of recently reported results on the in vitro
stability of cocaine (20,88,89).

The main objective of the present research was to develop reliable, sensitive analytical methods which can be applied to the investigation of the disposition of cocaine and its hydrolysis products: ecgonine methyl ester, benzoylecgonine, and ecgonine in the mature dog. For practical and theoretical reasons the dog is ideally suited as an animal model for experiments with cocaine (35,90). Procedures were developed which allowed the quantitation of cocaine and its de-esterified metabolites in both dog plasma and urine.

As very low levels of cocaine were expected in the plasma and urine following the administration of a single non-toxic dose of cocaine, a sensitive gas chromatographic method employing a nitrogen-phosphorus detector (GC-NPD) was used to quantitate cocaine extracted from plasma and urine samples. A less sensitive gas chromatographic method employing a flame ionization detector (GC-FID) was used to measure the amounts of ecgonine methyl ester, benzoylecgonine, and ecgonine excreted in the urine following intravenous cocaine.

A compartmental model was constructed to describe the disappearance of cocaine from plasma following intravenous administration of a single dose of cocaine to the mature dog. Pharmacokinetic equations derived from this model can be used to describe the distribution, metabolism, and excretion of cocaine in the dog. Estimates of the rate constants

and other pharmacokinetic parameters used to quantitate these processes were obtained by fitting theoretical curves through the plasma cocaine concentration-time data.

The relative amounts of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine excreted in the urine provided information on the relative importance of each de-esterification pathway in the metabolism of cocaine in the dog. Urine data alone, however, were not sufficient to obtain accurate estimates of the rate constants associated with each metabolic conversion. Also, the trace amounts of each metabolite in the plasma could not be accurately measured. In order to obtain more reliable estimates of the individual pharmacokinetic parameters associated with the disposition of these de-esterified metabolites of cocaine, a series of separate experiments in which each of the metabolites: ecgonine methyl ester, benzoylecgonine, and ecgonine was administered alone intravenously in the same animal were carried out. As these ecgonine congeners are devoid of pharmacological activity (67) each could be administered in doses large enough to allow their quantitation by GC-FID. Compartmental models were constructed and equations derived to describe the disposition of each of these compounds. The fitting of theoretical curves through the plasma alkaloid concentration-time data provided estimates of relevant pharmacokinetic parameters.

An attempt was made to determine to what extent the pharmacokinetic parameters determined for cocaine and individually for its putative metabolites may be combined to synthesize an integrated description of the overall fate of injected cocaine and its metabolites.

II-A. ANALYTICAL PROCEDURES

1. ANALYSIS OF ECGONINE ALKALOIDS

Gas Chromatography. Cocaine can be quantitated rather easily by GC without derivatization. Gas chromatographic methods employing flame ionization and nitrogen-phosphorus detectors have been used to measure microgram and nanogram quantities of cocaine extracted from plasma and urine (42-46,48,50,51,52). Recently a GC method has been reported for the analysis of ecgonine methyl ester without derivatization but a mass spectrographic detector was required to measure ecgonine methyl ester concentrations as low as 0.1 μg/mL (76).

The polar metabolites of cocaine, benzoylecgonine and ecgonine, must be derivatized before they can be analyzed by GC. Alkylation, acylation, and silylation have been used to derivatize benzoylecgonine and ecgonine prior to analysis by GC (14). Moore (47) described a qualitative method for the simultaneous analysis of cocaine, benzoylecgonine, and ecgonine in commercial cocaine hydrochloride samples. Trimethylsilyl derivatives of benzoylecgonine and ecgonine

32

were formed by reaction with N,O-Bis-(trimethylsilyl)-acet-
amide (BSA). Derivatized benzoylecgonine and ecgonine as
well as underivatized cocaine were separated on a 10% OV-101
column and detected by FID. This method was modified by
Roscoe (48) who used it to separate and simultaneously quan-
titate cocaine, ecgonine methyl ester, benzoylecgonine, and
ecgonine extracted from aqueous media. This method was
adopted for the present work as it was desirable for theo-
retical and practical reasons to have a single method capa-
ble of quantitating all four compounds at the same time.
Modifications were made to improve the sensitivity of this
method and its suitability for the analysis of biological
samples. GC standard calibration curves were constructed
and used to assess the sensitivity and linearity of the
GC-FID method. This modified GC-FID method was used to
quantitate cocaine, ecgonine methyl ester, benzoylecgonine,
and ecgonine in preliminary experiments designed to evaluate
the behavior of these alkaloids in various extraction and
thin-layer chromatographic systems. The developed GC-FID
method was also used to quantitate ecgonine methyl ester,
benzoylecgonine, and ecgonine in dog plasma and urine fol-
lowing intravenous administration of these compounds.

Extraction Studies. Cocaine is readily extracted from
aqueous media into organic solvents cyclohexane, chloroform,
and ether under moderately alkaline conditions (14,28,33).

There are little data available on the extraction behavior of ecgonine methyl ester although it has been extracted along with cocaine into chloroform:isopropanol at pH 8.5-9 (76). The recovery of ecgonine methyl ester was lower than that of cocaine probably due to the presence of the free hydroxyl group. The extraction of benzoylecgonine and ecgonine from aqueous media is extremely difficult due to the highly polar, amphoteric nature of these alkaloids. Mixed organic solvents of relatively high polarity, high pH, and saturation of the aqueous phase to achieve "salting-out" are required to obtain good recovery of benzoylecgonine and to a lesser extent ecgonine (14,20,28,30).

A preliminary study of the extraction behavior of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine was carried out. All four ecgonine alkaloids were added to a series of aqueous buffer solutions of increasing pH and immediately extracted with one of a number of organic solvents which were tested. The extraction recovery of each alkaloid in each solvent/pH system was determined by GC-FID. The recovery data aided in the development of extraction schemes which were used in the analysis of cocaine and metabolites in plasma and urine.

Based on the results of the extraction study a solvent was chosen which can extract both cocaine and ecgonine methyl ester at a moderately alkaline pH. It was hoped that such an extraction could be used in the analysis of cocaine

and ecgonine methyl ester in plasma and urine without the need of a clean-up step prior to GC-FID analysis. Additionally, finding a solvent which efficiently extracts cocaine and ecgonine methyl ester at moderately alkaline pH would minimize possible chemical hydrolysis of these compounds during analysis (78,80,81,82). Benzoylecgonine and ecgonine remaining in the aqueous phase could then be recovered by a second extraction at a higher pH with a mixed solvent system of higher polarity and saturation of the aqueous phase with electrolye to salt-out the alkaloids.

Thin-layer Chromatography. As previously mentioned the extraction of benzoylecgonine and ecgonine requires the use of relatively polar mixed organic solvents and saturation of the aqueous phase with a suitable electrolyte. When applied to biological fluids and in particular urine, these salting-out techniques produce extracts containing large amounts of co-extractable impurities. Subsequent isolation and purification of the extracted benzoylecgonine and ecgonine is necessary before they can be derivatized and quantitated by GC (14). Unfortunately, benzoylecgonine and ecgonine are not readily absorbed on styrene divinyl-benzene copolymers (i.e. XAD-2) or ion-exchange resins (79,83,84).

Thin-layer chromatography (TLC), however, has been used to isolate and purify metabolites of cocaine extracted from urine prior to derivatization and quantitation by GC

(48,79,85) and for the separation and identification of ra-
diolabelled cocaine and metabolites in biological fluids
(34,35,36). Numerous solvent systems for TLC development
are available for the separation of cocaine from other basic
drugs but benzoylecgonine and ecgonine do not migrate appre-
ciably in these systems (20). These systems have been modi-
fied to allow optimum isolation and purification of benzoyl-
ecgonine, ecgonine, as well as other metabolites of cocaine
(31,32,33). These TLC systems employ mixed solvents of var-
ying polarity and often contain a significant proportion of
ammonia and may be sufficiently alkaline to result in the
chemical hydrolysis of cocaine and ecgonine methyl ester.
In contrast, Munier and Drapier (37,38) have described TLC
of cocaine and related alkaloids with highly polar, neutral
solvents avoiding problems due to alkaline hydrolysis.

In the present work, TLC procedures to separate and
isolate ecgonine and benzoylecgonine from other co-extracta-
ble ecgonine alkaloids and impurities present in plasma and
urine were developed. Based on the results reported by Mu-
nier and Drapier (37), methanol:water (70:30) was tested as
developing solvent. Initial experiments were carried out to
characterize the TLC behavior of ecgonine and benzoyl-
ecgonine in this solvent system. The conditions which pro-
vided optimum separation of ecgonine and benzoylecgonine
were determined. In addition, the recoveries of ecgonine
and benzoylecgonine eluted from silica scraped from TLC

plates after development were determined by GC-FID. The recoveries of ecgonine and benzoylecgonine were sufficient to ensure that each alkaloid could be quantitated by GC-FID following TLC clean-up of plasma and urine extracts. More importantly, the linearity of results obtained from GC-FID analysis of ecgonine and benzoylecgonine eluted from TLC plates was assessed to ensure that appropriate calibration curves could be constructed and used to quantitate ecgonine and benzoylecgonine in plasma and urine following extraction/TLC/GC-FID analysis.

2. DETERMINATION OF ECGONINE IN WATER, PLASMA, AND URINE

Analytical procedures were developed for the determination of ecgonine in the plasma and urine of a dog following intravenous administration of a single dose of ecgonine. Ecgonine was extracted from biological fluids according to a modification of the method of Misra et al. (67). Prior to extraction, proteins were precipitated and the supernatant washed to remove acid and neutral compounds. Ecgonine was extracted into organic solvent after saturation of the aqueous phase with potassium carbonate, and purified by the developed TLC procedure. The isolated ecgonine was eluted from the silica and quantitated by GC-FID.

Initial experiments in which water samples were spiked with increasing concentrations of ecgonine and analyzed by extraction/TLC/GC-FID were carried out to evaluate the pro-

cedure. The recovery of ecgonine from each spiked water sample was determined and modifications made in the procedure to maximize the recovery. Similarly, dog plasma and urine samples were spiked with ecgonine, analyzed by extraction/TLC/GC-FID, and the recovery of ecgonine determined. In addition, the linearity of the procedures developed for the analysis of ecgonine in plasma and urine was assessed. Plasma and urine ecgonine concentration calibration curves were constructed from the GC-FID data.

The sensitivity of this method was limited by a number of factors including: inefficient extraction of ecgonine into the organic solvent, incomplete elution of ecgonine from the silica after TLC development, procedural losses due to sample manipulation and dilution factors, as well as the sensitivity of the GC-FID to detect recovered ecgonine. Lack of sensitivity was not a serious problem as relatively high concentrations of ecgonine were expected in the plasma following the intravenous administration of ecgonine in the dog. The concentration of ecgonine in the urine was much higher than that in the blood. Experimental _in vivo_ urine samples were diluted immediately prior to analysis to bring the ecgonine concentration within the range of the ecgonine concentration calibration curves with the added advantage that the concentration of compounds which might interfere with the GC-FID analysis of ecgonine were greatly reduced.

Much more critical than sensitivity was the linearity of the extraction/TLC/GC-FID method for ecgonine. It is doubtful that a compound with the necessary comparable properties which could be used as an internal standard added at the beginning of the procedure could have been easily found. In the present work an internal standard was added to the ecgonine extract eluted from the silica immediately prior to derivatization and was used as a reference in the subsequent GC-FID analysis of ecgonine. Care was taken to ensure that all volumes taken at each step of extraction and TLC purification of ecgonine were accurately measured and that all conditions were kept as consistent as possible.

The developed extraction/TLC/GC-FID procedure for ecgonine was used to determine the concentration of ecgonine in dog plasma and urine samples obtained after the intravenous administration of ecgonine. Blank plasma and urine spiked with ecgonine served as known standards. Plasma and urine ecgonine concentration calibration curves were constructed from data obtained from the extraction/TLC/GC-FID analysis of ecgonine-spiked samples and used to calculate the concentration of ecgonine in experimental _in vivo_ samples.

3. DETERMINATION OF BENZOYLECGONINE AND ECGONINE IN WATER,
    PLASMA, AND URINE

The procedure used for the extraction/TLC/GC-FID analy-
sis of ecgonine was used with minor modification to deter-
mine the concentrations of both benzoylecgonine and ecgonine
in the plasma and urine of a dog following the intravenous
administration of benzoylecgonine. As was the case for
ecgonine, initial experiments were carried out to evaluate
the recovery of benzoylecgonine from water samples spiked
with benzoylecgonine and ecgonine. It was possible that the
highly alkaline conditions and high electrolyte concentra-
tion used in the extraction might have led to hydrolysis of
benzoylecgonine. Such hydrolysis of benzoylecgonine with
the formation of ecgonine during analysis would have had a
great effect on the accuracy of the analysis of experimental
in vivo samples. It would have resulted in an underestima-
tion of benzoylecgonine concentrations and an overestimation
of ecgonine concentrations. In a separate experiment, the
possible conversion of benzoylecgonine to ecgonine during
analysis was investigated. Water samples spiked with high
concentrations of benzoylecgonine alone were carried through
the entire extraction/TLC procedure and any ecgonine gener-
ated quantitated by GC-FID.

The recoveries of both benzoylecgonine and ecgonine
from spiked plasma and diluted urine samples were evaluated.
Blank plasma and diluted urine were spiked with increasing

concentrations of benzoylecgonine and ecgonine, analyzed by extraction/TLC/GC-FID, and the recoveries of benzoylecgonine and ecgonine calculated. The GC-FID data were used to construct plasma and urine calibration curves for benzoylecgonine and ecgonine.

The sensitivity of the extraction/TLC/GC-FID method for the determination of benzoylecgonine was limited by the same factors which limited the sensitivity of the determination of ecgonine. Relatively high concentrations of benzoylecgonine were expected in the plasma and urine following intravenous administration of benzoylecgonine. The plasma ecgonine concentrations, however, were expected to be very low following intravenous benzoylecgonine unless the in vivo conversion of benzoylecgonine to ecgonine was rapid and extensive. The concentration of ecgonine in the urine would be much higher allowing accurate measurement and estimation of the in vivo conversion of benzoylecgonine to ecgonine.

As discussed previously, the linearity of the plasma and urine calibration curves was essential for the determination of the concentrations of benzoylecgonine and ecgonine in experimental in vivo samples. Spiked plasma and urine samples were used as known standards and analyzed alongside experimental samples. The concentrations of benzoylecgonine and ecgonine in experimental samples were calculated from the concentration calibration curves constructed from data obtained from the spiked plasma and urine standards.

4. DETERMINATION OF ECGONINE METHYL ESTER AND ECGONINE IN WATER, PLASMA AND URINE

A method was developed for the determination of ecgonine methyl ester and ecgonine in the plasma and urine of a dog following the administration of intravenous ecgonine methyl ester. Ecgonine methyl ester was extracted separately and quantitated by GC-FID analysis. The selection of solvent and pH used for the extractions was based on the results of the study of the extraction behavior of the ecgonine alkaloids. After extraction of ecgonine methyl ester, ecgonine remaining in the aqueous phase was extracted and purified according to previous extraction/TLC techniques modified accordingly. The amounts of ecgonine methyl ester and ecgonine recovered were determined by GC-FID analysis.

In preliminary experiments, water samples were spiked with ecgonine methyl ester and ecgonine and analyzed. The recoveries of ecgonine methyl ester and ecgonine were calculated. In a separate experiment the possible conversion of ecgonine methyl ester to ecgonine during analysis was investigated. Water samples spiked with ecgonine methyl ester alone were carried through the entire analytical procedure and any ecgonine generated quantitated by GC-FID.

Previously discussed sensitivity and linearity considerations are equally applicable to the determination of ecgonine methyl ester. It was very difficult to predict

ahead of time whether low plasma concentrations would have been encountered in the dog experiment as data on plasma ecgonine methyl ester concentrations following its administration have not been reported. This problem was circumvented by increasing the dose of ecgonine methyl ester administered as it has been shown to be without observable pharmacological effect even after large doses (200 mg/kg iv in the rat) (67). Very little ecgonine was expected in the plasma following intravenous administration of ecgonine methyl ester but concentrations in the urine were high enough to allow accurate determination. The plasma and urine concentration curves based on co-analyzed spiked samples as known standards were linear over the range of concentrations examined allowing their use in the calculation of the concentrations of ecgonine methyl ester and ecgonine in experimental samples.

Blank plasma and diluted urine samples were spiked with ecgonine methyl ester and ecgonine, analyzed, and their recoveries determined. Spiked plasma and urine samples served as known standards in the construction of plasma and urine ecgonine methyl ester and ecgonine concentration calibration curves. The concentration calibration curves were used to calculate the concentrations of ecgonine methyl ester and ecgonine in experimental in vivo samples.

It has been suggested that ecgonine methyl ester may undergo non-enzymatic hydrolysis to form ecgonine and that

this may account for the small amounts of ecgonine found in human urine (79). Even though precautions were taken to prevent urine collected following the intravenous administration of ecgonine methyl ester from becoming alkaline it was necessary to conduct an in vitro experiment to assess the stability of ecgonine methyl ester in urine.

5. DETERMINATION OF COCAINE IN WATER, PLASMA, AND URINE

The disposition of cocaine in dogs following administration of a 5 mg/kg dose has been reported (35). Plasma cocaine levels declined rapidly from a peak value of 0.64 μg/mL to 0.03 μg/mL after 6 hours. It is not possible to accurately measure concentrations of cocaine this low with conventional GC-FID methods. In addition, to increase the dose of cocaine was not possible because convulsions and death usually result from the intravenous administration of doses greater than 10 mg/kg in the dog (23,89). For these reasons a gas chromatograph equipped with a highly sensitive nitrogen-phosphorus detector was used to determine cocaine in plasma and urine of a dog dosed at the low level of cocaine.

A Hewlett-Packard Model 5880 GC equipped with NPD was used for the quantitation of cocaine. Calibration mixtures containing increasing amounts of cocaine and a fixed amount of internal standard were used to adjust chromatograph and detector operating parameters so as to achieve optimum re-

tention and response charcteristics. The HP 5880 was operated in the multi-level internal calibration mode wherein measured response factors were used by the instrument along with corresponding concentration information on separate calibration mixtures to create an internal calibration curve. The absolute amount of cocaine in subsequently injected unknown samples containing the internal standard was automatically printed out. The results of analyzed GC calibration mixtures were monitored to evaluate the sensitivity and linearity of the method.

A modification of the extraction/GC-NPD procedure of Jatlow and Bailey (49) for plasma was used for the determination of cocaine in both plasma and urine. Initial experiments were carried out with cocaine-spiked water samples to determine the recovery of cocaine by the modified method.

Cocaine is rapidly hydrolyzed in vitro in serum, plasma, and blood (49,77,79,86,87,88). Loss of cocaine can be prevented by the addition of sodium fluoride to inhibit plasma cholinesterase (78,86,88). Experiments were conducted in order to evaluate the recovery of cocaine from spiked plasma samples and to assess the stability of cocaine during blood collection, plasma separation, and storage.

The concentrations of cocaine in dog plasma and urine samples obtained after intravenous administration of cocaine were determined according to the developed extraction/GC-NPD

method.   Cocaine-spiked plasma and urine samples served as
the known standards used to calibrate the gas chromatograph.
The multi-level internal calibration curve produced allowed
the automatic computation of the cocaine concentration of
experimental in vivo samples analyzed.   Data obtained from
the analysis of cocaine plasma and urine standards were mon-
itored to ensure that adequate sensitivity and linearity was
maintained.

A slow but significant hydrolysis of cocaine in alka-
line urine in vitro has been reported (88). No hydrolysis
was found when cocaine was added to acidic urine and incu-
bated.   Even though precautions were taken to ensure that
urine collected after the administration of cocaine did not
become alkaline it became necessary to carry out an experi-
ment in vitro to assess the hydrolysis of cocaine in urine.

II-B.   PHARMACOKINETICS

1. DISPOSITION OF COCAINE AND ITS DE-ESTERIFIED METABOLITES

Cocaine is rapidly and extensively metabolized in man
and various laboratory animals.   De-esterification and N-
demethylation are the two main pathways for the biotransfor-
mation of cocaine. De-esterification via enzymatic and non-
enzymatic hydrolysis represents the major route of cocaine
metabolism.   Taylor et al.   (77) described the metabolism of
cocaine in serum in vitro to ecgonine methyl ester, benzoyl-
ecgonine, and ecgonine.   The hydrolysis pathways of cocaine
are depicted in Figure 3.
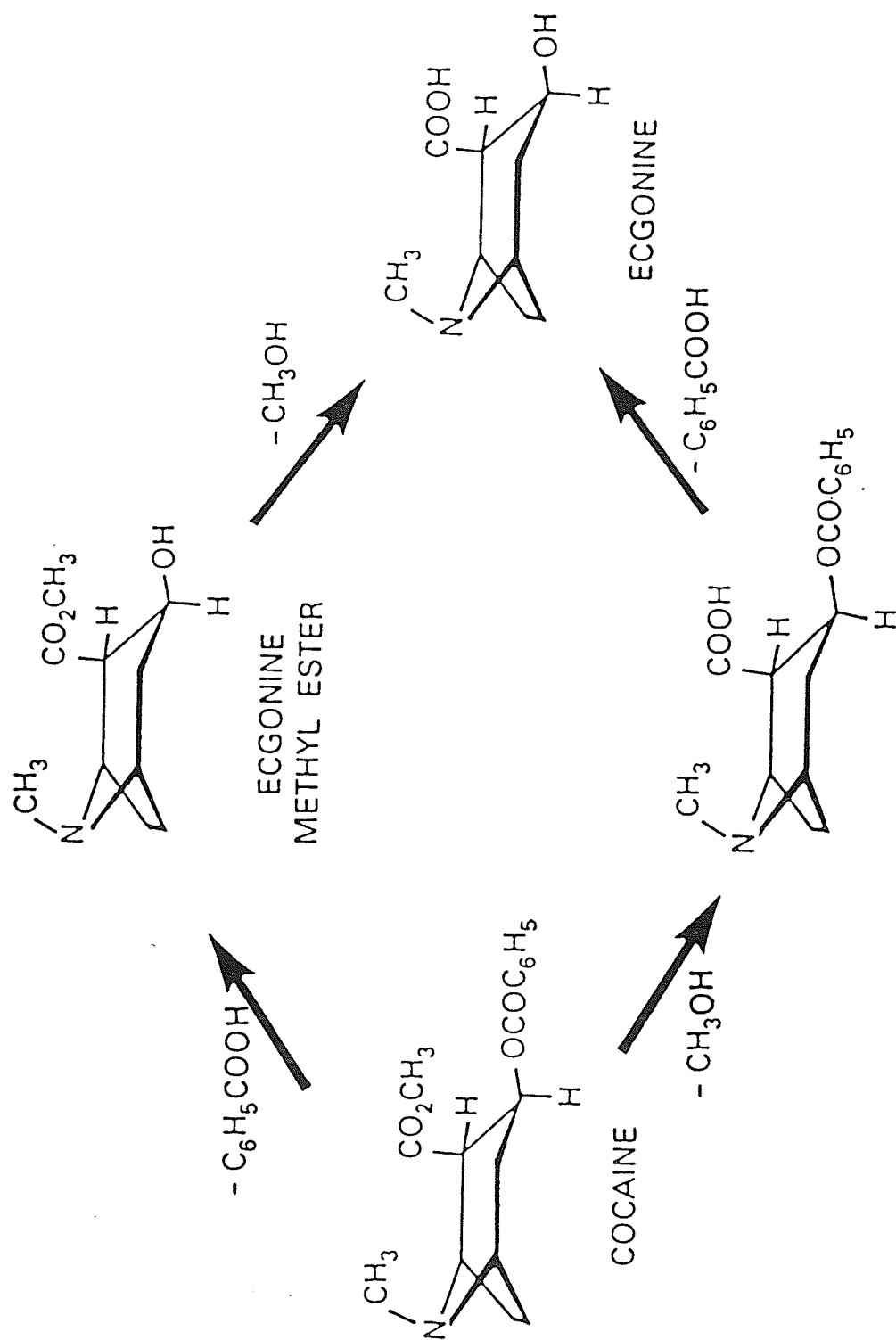
Figure 3: Proposed pathways for the hydrolysis of cocaine to ecgonine methyl ester, benzoylecgonine, and ecgonine.

Stewart et al. (78) demonstrated the enzymatic hydrolysis of cocaine to ecgonine methyl ester in human plasma in vitro and proved that the enzyme responsible was cholinesterase. They found no evidence of enzymatic hydrolysis of cocaine to benzoylecgonine in plasma at pH 7.4. A very limited conversion occurred but was not significantly different from that observed in pH 7.4 buffer in the absence of plasma. The spontaneous conversion of cocaine to benzoylecgonine was accelerated at pH 8.3. These authors suggested that cholinesterase may be able to convert benzoylecgonine to ecgonine and that the conversion of ecgonine methyl ester to ecgonine through spontaneous hydrolysis would occur slowly at physiological pH. In a later study Stewart et al. (79) were able to demonstrate a low but detectable hydrolysis of benzoylecgonine to ecgonine by cholinesterase. They also showed the enzymatic hydrolysis of cocaine to ecgonine methyl ester in vitro by liver esterase. The liver esterase appeared to be a different enzyme from that in serum. The affinity of the liver esterase was lower (higher Km value) and the Vmax was higher for the liver enzyme compared to the serum esterase. At a cocaine concentration of 0.3 μM (a concentration comparable to levels found in vivo) cocaine is hydrolyzed at comparable rates in serum and liver. No evidence of enzymatic conversion of cocaine to benzoylecgonine by either serum or liver esterases was found. Cocaine incubated at 37° C did, however, undergo spontaneous hydrolysis to benzoylecgonine (42.3% after 24 hours).

In the present work the developed analytical methods were used to investigate the disposition of cocaine and products of its _in vivo_ de-esterification i.e. ecgonine methyl ester, benzoylecgonine, and ecgonine following the intravenous administration of cocaine in the mature dog. Estimates were obtained for the relevant pharmacokinetic parameters associated with the distribution and elimination of cocaine.

For reasons previously discussed it was not possible to obtain accurate estimates of the pharmacokinetic parameters which described the disposition of cocaine's de-esterified metabolites directly.

In separate experiments single doses of ecgonine, benzoylecgonine, and ecgonine methyl ester were administered individually to the same animal in order to study the disposition of each. The plasma alkaloid concentrations following intravenous administration were determined with the developed analytical procedures. Fitting theoretical curves through the plasma alkaloid concentration-time data provided estimates of the pharmacokinetic parameters which described the distribution and elimination of each ecgonine alkaloid in the dog. In addition, the determination of absolute and relative amounts of parent drug and metabolite excreted in the urine provided information on the metabolism and excretion of each of the ecgonine alkaloids tested.

Cocaine was administered intravenously to the same animal and the time-course of the plasma cocaine concentrations determined with the developed analytical procedures. The fitting of theoretical curves through the plasma cocaine concentration-time data allowed the estimation of the pharmacokinetic parameters which described the distribution and elimination of cocaine in the dog. The absolute and relative amounts of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine excreted in the urine were determined. It was hoped that the results obtained from the study of the disposition of cocaine together with the results of the studies with the individual cocaine metabolites would allow a comprehensive description of the fate of these compounds in the dog.

2. INDIVIDUAL PHARMACOKINETIC EXPERIMENTS

Intravenous Ecgonine. Very little is known of the disposition of ecgonine in laboratory animals. Misra et al. (66) studied the distribution and metabolism of radiolabelled ecgonine following intravenous administration of 10 mg/kg to the rat. Plasma ecgonine concentrations declined rapidly from a peak of 23.02 μg/mL at 0.25 h to 0.53 μg/mL at 6 h. Apparently, ecgonine was not metabolized in the rat; 86.2% of the dose was excreted in the urine within 24 hours.

The pharmacokinetics of ecgonine following intravenous administration were studied in the mature dog. A dose equivalent to 10 mg ecgonine base per kg dog body weight was administered and blood samples drawn at various times after ecgonine administration.

The plasma ecgonine concentrations of collected blood samples were determined with the developed extraction/TLC/ GC-FID method. The plasma ecgonine concentration-time data were analyzed according to established pharmacokinetic techniques to determine whether the data was best described by equations derived from a one- or two-compartment model. Nonlinear regression analysis was used to obtain estimates of the pharmacokinetic parameters associated with the more appropriate model.

Urine samples collected following intravenous ecgonine administration were analyzed and the total amount of ecgonine excreted in the urine determined. The percent ecgonine recovered in the urine relative to the dose administered was calculated.

Intravenous Benzoylecgonine. Misra et al. (67) have investigated the disposition of radiolabelled benzoylecgonine following the intravenous administration 10 mg/kg to rats. Plasma benzoylecgonine concentrations declined from 4150 ng/mL at 0.25 h to 28 ng/mL at 6 h in an apparent

log-linear fashion. A plasma half-life of 0.8 h was report-
ed. Benzoylecgonine, ecgonine, and minor amounts of two un-
identified metabolites were detected in the urine of rats
following the administration of benzoylecgonine, 200 mg/kg,
intraperitoneally. The concentrations or amounts of each
alkaloid excreted were not determined.

After a suitable period of time to allow full recovery,
a dose of 10 mg/kg benzoylecgonine was administered intrave-
nously to the same dog which had previously received ecgo-
nine. Blood samples were drawn periodically following
benzoylecgonine administration and urine was collected for
72 hours in a metabolic cage.

The plasma benzoylecgonine concentration of drawn blood
samples were determined with the developed extraction/TLC/
GC-FID method. Plasma samples were also analyzed for ecgo-
nine in order to evaluate the extent of in vivo conversion
of benzoylecgonine and ecgonine. The plasma benzoylecgonine
concentration-time data were analyzed according to estab-
lished pharmacokinetic techniques to determine whether the
disposition of benzoylecgonine in the dog was best descirbed
by a one- or two-compartment model. The fitting of theoret-
ical curves through the plasma benzoylecgonine concentra-
tion-time data was accomplished with nonlinear regression
analysis. Estimates of the parameters associated with the
appropriate pharmacokinetic model were obtained.

The amounts of benzoylecgonine and ecgonine excreted in the urine following intravenous administration of benzoylecgonine were determined. The percent of benzoylecgonine dose recovered in the urine as benzoylecgonine and ecgonine provided valuable information on the relative importance of metabolism and excretion on the disposition of benzoylecgonine in the dog.

Intravenous Ecgonine Methyl Ester. The administration of ecgonine methyl ester to laboratory animals has not been reported. A dose equivalent to 25 mg ecgonine methyl ester base per kg body weight was administered to the same mature dog which had received the benzoylecgonine and ecgonine. Blood samples were drawn at various times following the ecgonine methyl ester intravenous injection, plasma separated and stored.

The ecgonine methyl ester concentrations of plasma samples were determined with the developed extraction/GC-FID procedure. Pharmacokinetic analysis of the resulting plasma ecgonine methyl ester concentration-time data was carried out in order to determine the best compartmental model for the disposition of ecgonine methyl ester following intravenous administration in the dog. Estimates of the pharmacokinetic parameters associated with this model were determined by least-squares nonlinear regression analysis of the plasma data. Selected plasma samples were analyzed for ecgonine and

the *in vivo* conversion of ecgonine methyl ester to ecgonine assessed.

The amounts of ecgonine methyl ester and ecgonine excreted in the urine following intravenous administration of ecgonine methyl ester were determined. The percent of dose recovered in the urine as ecgonine methyl ester and ecgonine were calculated and used to determine the extent of *in vivo* hydrolysis of ecgonine methyl ester.

Intravenous Cocaine. Misra et al. (35) reported plasma cocaine concentrations following the intravenous administration of radiolabelled cocaine, 5 mg/kg, to dogs. The half-life of cocaine in the plasma of acutely-treated dogs was 1.2 hours. In the present study a dose of cocaine hydrochloride equivalent to 5 mg base per kg body weight was administered after a suitable period of time to the same animal which had previously received ecgonine, benzoylecgonine, and ecgonine methyl ester. Plasma samples were collected at intervals following administration and the cocaine concentrations determined by extraction/GC-NPD. A pharmacokinetic analysis of the plasma cocaine concentration-time data allowed the selection of the appropriate compartmental model to describe the distribution and elimination of cocaine in the dog.

The amount of unchanged cocaine excreted in the urine was determined by extraction/GC-NPD analysis. The amounts of ecgonine methyl ester, benzoylecgonine, and ecgonine excreted in the urine were determined by extraction/TLC/GC-FID. The percent of the dose of cocaine administered recovered as cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine were calculated.

It was hoped that the results of the intravenous cocaine experiment in combination with results of the individual dispositional studies of ecgonine methyl ester, benzoylecgonine, and ecgonine would lead to a better understanding of the various processes involved in the metabolism and excretion of cocaine and its _in vivo_ hydrolysis products in the dog. Of particular interest was the relative importance of two major competing hydrolysis pathways to the overall metabolic elimination of cocaine. In addition, these results would help in elucidating the origin of ecgonine excreted in the urine after the administration of cocaine.

Chapter III

EXPERIMENTAL

III-A. ANALYTICAL PROCEDURES

1. ANALYSIS OF ECGONINE ALKALOIDS

    a. Materials and Equipment

Cocaine hydrochloride (Lot #84030,   Code K90780) pur-
chased from BDH Chemical, Toronto, Ont., Can. was recrystal-
lized from 2-propanol, dried and stored over phosphorus pen-
toxide.   Anhydrous theophylline NF also purchased from BDH
Chemicals was recrystallized from hot aqueous solution,
dried and stored over phosphorus pentoxide.

Ecgonine hydrochloride was synthesized from cocaine hy-
drochloride by an established method (19).   It was recrys-
tallized from a hot aqueous solution containing ethanol; the
crystals were dried and stored over phosphorus pentoxide.

Benzoylecgonine prepared from cocaine hydrochloride
(18), was recrystallized from water, dried and stored over
phosphorus pentoxide.

Ecgonine methyl ester hydrochloride was prepared from
cocaine hydrochloride by an established method (18). It was

precipitated from a hot methanolic solution with the addition of acetone. This "crude" ecgonine methyl ester hydrochloride was purified for assay use in the following manner:

To 1.75 g of the "crude" ecgonine methyl ester hydrochloride in a 100 mL centrifuge tube was added a minimum of triple-distilled water (approximately 2.5 mL) to dissolve it. To this aqueous solution 75 mL of a cyclohexane:methylene chloride mixture (1:1) was added. A saturated solution of sodium phosphate was added drop-wise to adjust the pH of the aqueous phase to pH 9-10. Approximately 10 mL were needed. The tube contents were mixed and centrifuged at 2000 rpm for 5 minutes to separate the phases. The upper organic layer was transferred to a fresh tube and dried by passing through a coarse sintered glass filter funnel containing about a 1 cm layer of anhydrous sodium sulfate. The "dried" organic phase was collected in a fresh 100 mL centrifuge tube. To the tube, placed under a stream of helium, was added 0.6 N HCl in 2-propanol, 1-2 mL at a time with swirling to mix until the exiting stream of helium was acidic to moist pH paper. A total of approximately 12 mL of the acid-alcohol was required. The stream of helium was continued until all the cyclohexane:methylene chloride was removed and a white crystalline mass remained. The final residue of ecgonine methyl ester hydrochloride material was recrystallized from a hot methanolic solution containing acetone. The pure crystalline material was dried and stored over phosphorus pentoxide.

The n-propyl ester of benzoylecgonine (n-PEBE) was synthesized from benzoylecgonine and 1-propanol with acetyl chloride as catalyst (91). It was recrystallized from hot n-propanol with the addition of double-distilled water. The final crystalline product was dried and stored over phosphorus pentoxide.

The melting point of each compound was determined and compared to its literature value. In addition, the purity of each alkaloid was checked by gas chromatography and thin-layer chromatography.

The methanol, 2-propanol, methylene chloride, and cyclohexane used in the preparation and purification of the ecgonine alkaloids were purchased as certified 99+mol% solvents from Fisher Scientific Ltd., Winnipeg, Man., Canada. Sodium phosphate, tribasic (ACS grade, Fisher) and triple-distilled water were used to prepare a saturated solution of sodium phosphate. Anhydrous sodium sulfate (ACS, Fisher) was used to dry organic extracts prepared during synthesis and purification of the ecgonine alkaloids.

Methanol (pesticide grade, Fisher) was used to prepare the alkaloid standard solutions. Purified constant-boiling hydrochloric acid was made by distillation of the diluted (6 N) concentrated acid (ACS, Fisher). The constant-boiling hydrochloric acid was diluted 10 times with 2-propanol (pesticide grade, Fisher) to produce the 0.6 N HCl in 2-propanol used in the preparation of gas chromatographic samples.

Gas chromatographic (GC) analyses were performed on a Hewlett-Packard Model HP 5710 gas chromatograph equipped with a flame ionization detector (FID). Empty coiled glass columns, 6 ft long x 1/8 in OD, were purchased from Chromatographic Specialities Ltd., Brockville, Ont., Canada, and before packing were washed and pre-treated with 5% dichloro-dimethylsilane (Pierce Chemical Co., Rockford, Ill., USA) in toluene (ACS grade, Fisher). The columns were packed initially with 10% OV-101 on 80/100 mesh Gas Chrom Q and later with 3% OV-101 on 80/100 mesh Chromosob W HP, both of which were purchased from Chromatographic Specialties. Packed columns were conditioned and maintained with periodic 25 μL injections of Silyl-8® (Pierce). In addition, commercially-packed coiled glass columns, 6 ft long x 1/8 in OD, 1.8 mm ID, configuration 1A P/W, containing 3% SP-2100 on 80/100 mesh Supelcoport purchased from Supelco Inc., Bellafonte, PA, USA, were employed.

The derivatizing agents, N,O-Bis-(trimethylsilyl)-acetamide (BSA) was purchased from the Pierce Chemical Co., and N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Regis Chemical Co., Morton Grove, Ill., USA. The Silyl-8®, BSA, and BSTFA were stored at 4° C.

The chloroform, methylene chloride, cyclohexane, and 2-propanol used in the extraction studies were certified 99+mol%, purchased from Fisher. All certified ACS grade chemicals (Fisher Scientific Ltd): potassium phosphate mo-

nobasic, sodium phosphate dibasic, sodium bicarbonate, and anhydrous sodium carbonate were used to prepare aqueous buffers.

Thin-layer chromatography (TLC) was carried out on Fisher Redi-Plates®, 5 x 20 cm pre-coated with a 250 μm thickness of Silica Gel G containing calcium sulfate as binder. The TLC streaking solvent (methanol:water, 70:30) was prepared with pesticide grade methanol (Fisher) and triple-distilled water, and the TLC developing solvent (methanol:water, 70:30) was prepared with 99+mol% methanol (Fisher) and double-distilled water. The TLC elution solvent (methanol: water, 80:20) was prepared with pesticide grade methanol (Fisher) and triple-distilled water.

TLC plates were pre-run and activated before use as follows: the plates were placed in a TLC tank and 200 mL of TLC developing solvent added. The tank was covered and the solvent allowed to run toward the top of each plate (approximately 90 min). The plates were removed, air-dried for about 10 min and activated in an oven at 110° C for 1 hour. The plates were removed and kept in a desiccator over anhydrous calcium sulfate until used.

Drummond® 50 μL WIRETROL Micropipets purchased from Fisher were used to streak the TLC plates. The TLC plates were run in T.L.C. CHROMATANK®s manufactured by Shandon of England. The alkaloids on TLC reference plates were visual-

ized using Ludy Tenger's Reagent (31) prepared immediately prior to use. Samples were prepared for streaking in custom-made conical bottom culture tubes, 16 mm x 90 mm. Small, short-stemmed glass funnels (60 mm top diameter, 30 mm stem length) were used to transfer silica bands scraped off TLC plates into screw-capped test tubes. Both the conical tubes and funnels were silanized by treatment with a 5% solution of dichlorodimethylsilane in toluene for 15 min.

Except for the custom-made conical bottom culture tubes all other sample manipulations were carried out in Teflon-lined screw-capped test tubes, 16 mm x 125 mm (Kimax® brand, Fisher). Before use, the tubes were cleaned by soaking overnight in 50% nitric acid, thoroughly rinsed with water and air dried.

Parafilm® manufactured by the American Can Co., Greenwich, CT, USA, was used to seal test tubes, culture tubes, and volumetric flasks.

Samples were vortexed on a Vortex Jr. Mixer, manufactured by Scientific Industries Inc., Springfield, Mass., USA. A Modular Dri-Bath Model DB-16525, distributed by the Thermolyne Corp., Dubuque, Iowa, USA, was used to heat samples for the evaporation of solvents. Centrifugation was carried out in either a Dynac Centrifuge (Clay Adams, Parsippany, NJ, USA) or an International Centrifuge Model UV (International Equipment Co., Needham, Mass., USA). A

flatbed shaker, manufactured by the Eberbach Corp., Ann Arbor, Mich., USA, was used in extraction procedures.

b. Methods

GC-FID Standard Calibration Curves. Quantitation of the individual alkaloids: cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine was accomplished according to a modification of the gas chromatographic-FID procedure first used by Moore (47). Gas chromatographic standards containing one or more of the ecgonine alkaloids were routinely prepared and analyzed by GC-FID. The data obtained were used to construct GC standard calibration curves which served as primary references for the quantitation of each ecgonine alkaloid in all samples analyzed by GC-FID.

In initial GC-FID analyses, columns were packed with 10% OV-101 on 80/100 mesh Gas Chrom Q. Also, theophylline was used as the internal standard in combination with the GC standards containing all four ecgonine alkaloids for the GC standard calibration curves. N,O-Bis-(trimethylsilyl)-acetamide (BSA) was used as derivatizing agent and injecting solvent.

Individual Primary Standards of each of the ecgonine alkaloids: cocaine hydrochloride, ecgonine methyl ester hydrochloride, benzoylecgonine, and ecgonine hydrochloride were prepared in methanol at a concentration of 200 μg base-equivalents per mL. A Mixed Working Standard containing all

four ecgonine alkaloids was prepared by adding 5.0 mL of each Primary Standard to a 50.0 mL volumetric flask and diluting to volume with methanol to produce a final concentration of 20 μg/mL of each of the four alkaloids calculated as free bases. The theophylline Internal Standard, 60 μg/mL in methanol, was prepared by dissolving 30.0 mg theophylline in sufficient methanol to make a 500.0 mL solution.

Individual GC standard calibration curves for cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine were obtained as follows. Measured volumes of the Mixed Working Standard: 50, 100, 200, or 400 μL were added to a series of test tubes, followed by 100 μL of theophylline Internal Standard (60 μg/mL) and 25 μL of 0.6 N HCl in 2-propanol. The tubes were vortexed and the contents taken to dryness under a stream of nitrogen. Fifty microliters (50 μL) of derivatizing agent BSA were added to each tube which was immediately capped and vortexed. These GC standards were heated at 70° C for 10 min, and after cooling to room temperature centrifuged at 2500 rpm for 5 min. A 1 μL aliquot of each standard was injected into the gas chromatograph.

The gas chromatograph was a HP 5710 gas chromatograph equipped with FID and a glass column packed with 10% OV-101 on 80/100 mesh Gas Chrom Q. The instrument was operated under the following conditions: gas flow rates of 40 mL/min nitrogen as carrier gas, 30 mL/min hydrogen and 240 mL/min air. The oven temperature was programmed from 170° to 270°

C at a rate of 8°/min. The initial temperature was held for 2 minutes. The electrometer was set at range=10 and attenuation=8, equivalent to 4.0×10^{-10} A for full scale deflection. Chart speed was set at 0.25 in/min.

The peak heights obtained for the ecgonine methyl ester, ecgonine, theophylline, cocaine, and benzoylecgonine standards were measured manually. The peak height ratio (PHR) corresponding to alkaloid peak height/theophylline peak height was calculated for each of the ecgonine alkaloids in the GC standards, and a GC calibration curve constructed by plotting the PHR's obtained versus the corresponding amounts (ng) of alkaloid injected. A least-squares linear regression was calculated to obtain the best-fitting calibration curve.

The GC-FID procedure with theophylline as internal standard was used to investigate the extraction efficiency of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine from various aqueous buffers into a number of different organic solvent systems.

The GC-FID procedure outlined above was modified for all subsequent experiments requiring a GC standard calibration curve. The n-propyl ester of benzoylecgonine (n-PEBE) replaced theophylline as the internal standard and BSTFA replaced the BSA as the derivatizing reagent. The gas chromatograph was equipped with a column packed with 3% SP-2100 on

80/100 mesh Supelcoport in place of the 10% OV-101 column.
Gas chromatographic conditions modified accordingly.

In the modified GC-FID procedure GC standards contain-
ing all four ecgonine alkaloids were prepared and analyzed
as follows: measured volumes of the Mixed Working Standard:
50, 100, 200, or 400 μL were added to separate test tubes.
Two hundred fifty microliters (250 μL) of n-PEBE Working
Standard were added to each tube followed by 25 μL of 0.6 N
HCl in 2-propanol. (The n-PEBE Working Standard (20 μg/mL)
was prepared from the n-PEBE Primary Standard (200 μg/mL) by
dilution with methanol.) The tubes were vortexed and the
contents taken to dryness at 50° C under a stream of helium.
Fifty microliters (50 μL) of BSTFA was added to each tube to
effect derivatization as previously described. A 1 μL ali-
quot of each GC standard was injected into the chromato-
graph.

Gas chromatographic analyses of GC standards containing
n-PEBE as internal standard were also carried out on the HP
5710 gas chromatograph but which was equipped with a 6 ft
column packed with 3% SP-2100 on 80/100 mesh Supelcoport.
(The latter is equivalent to 3% OV-101 on 80/100 mesh Chro-
mosorb W HP.) Gas flow rates remained the same: 40 mL/min
nitrogen carrier gas, 30 mL/min hydrogen and 240 mL/min air.
The oven temperature was programmed from 135° C to 270° C at
a rate of 16°/min with a 2 min hold on the initial and final
temperatures. Electrometer setting and chart speed were the
same as previously described.

The peak heights of ecgonine methyl ester, ecgonine, cocaine, benzoylecgonine, and the internal standard n-PEBE were measured and the peak height ratios (PHR), alkaloid/n-PEBE, calculated for each of the alkaloids in the GC standards. A GC calibration curve was constructed for each ecgonine alkaloid by plotting PHR obtained vs. the corresponding amounts (ng) of alkaloid injected. The best-fitting calibration curve was obtained by least-squares linear regression analysis.

With the exception of the extraction studies all quantitation by GC-FID analysis was carried out using n-PEBE as internal standard, BSTFA as derivatizing agent, and a column packed with 3% SP-2100 on 80/100 Supelcoport (or its equivalent 3% OV-101 on 80/100 mesh Chromosorb W HP).

Extraction Studies. The partitioning of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine between various aqueous buffers and organic solvents was investigated in order to find the best combination of solvent(s) and pH for optimum extraction of each alkaloid. The GC procedure with theophylline as internal standard was used to assess the efficiency of extraction of the four alkaloids. A series of buffers with pH values of 6, 7, 8, 9, 10, and 11 was prepared. Buffers pH 6, 7, and 8 were prepared with mixtures of 0.5 M monobasic potassium phosphate and 0.5 M dibasic sodium phosphate while buffers pH 9, 10, and 11 were prepared with 1.0 M sodium bicarbonate and 1.0 M sodium

carbonate (92). The volumes of each solution given in Table I were added to a 1.0 L volumetric flask and made up to volume to produce the buffer of appropriate pH.

An alkaloid solution containing the equivalent of 200 μg each of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine per mL (as free base) was prepared by dissolving the appropriate weight of cocaine hydrochloride, ecgonine methyl ester hydrochloride, benzoylecgonine, and ecgonine hyrochloride in 50.0 mL methanol.

For each pH/solvent(s) combination tested, 1.0 mL of a specific buffer was added to a test tube followed by 8.0 mL of organic solvent. Twenty microliters (20 μL) of the alkaloid solution equivalent to 4 μg of each ecgonine alkaloid were then added. Each tube was immediately capped and shaken 15 times by hand followed by shaking on the flatbed shaker for 5 min on the LOW setting (approximately 180 cycles/min). All samples were then centrifuged for 5 minutes at 2500 rpm. A 7.0 mL aliquot of the organic phase of each tube was transferred to a fresh tube containing 100 μL of 0.6 N HCl in 2-propanol. The extracts were vortexed and taken to dryness at 50° C under a stream of nitrogen. Methanol (100 μL) was used to rinse down the sides of each tube. One hundred microliters (100 μL) of theophylline Internal Standard were added to each tube. After mixing, the contents were once again taken to dryness. The residue from each sample was derivatized with 50 μL BSA and assayed by

Table I: Composition of Buffers Used for pH/Solvent Extrac-
tion Experiments (92).

pH at 25° C	Volumes of Solutions (mL) Diluted to 1.0 Liter			
	KH_2PO_4[a]	Na_2HPO_4[a]	$NaHCO_3$[b]	Na_2CO_3[b]
6	142	19.5	–	–
7	39	53.6	–	–
8	2.8	32.4	–	–
9	–	–	76.8	7.74
10	–	–	24.6	25.1
11	–	–	1.8	32.2

[a]0.5 M

[b]1.0 M

gas chromatography as previously described. The peak heights of each ecgonine alkaloid and the theophylline internal standard were measured and the peak height ratios of alkaloid to theophylline calculated. The amount of each alkaloid (ng) present in the 1 μL injected was calculated from the daily GC standard calibration curve. The total amount and the percent (%) recovery of each alkaloid was calculated for each pH/solvent combination investigated.

Thin-Layer Chromatography. Thin-layer chromatographic procedures were developed to separate and isolate ecgonine and benzoylecgonine from other co-extractable ecgonine alkaloids and impurities present in plasma and urine extracts which would otherwise interfere with their GC-FID analysis.

Initial experiments were conducted to characterize the TLC behavior of ecgonine and benzoylecgonine, and to assess the efficiency of the TLC procedure for the elution of the two alkaloids from the silica bands in developed TLC plates. Test samples were prepared from a combined benzoylecgonine/ ecgonine Working Standard (each 20 μg/mL) corresponding to 4, 8, and 16 μg of both benzoylecgonine and ecgonine. Along with the test samples, TLC reference samples were also included, which would be used to help locate the relative positions of the alkaloid bands in the TLC test plates after development. The TLC reference samples consisted of 50 μL of both ecgonine and benzoylecgonine TLC reference solutions corresponding to 50 μg of each alkaloid.

The TLC procedure was carried out as follows: the test and reference samples containing both ecgonine and benzoyl-ecgonine were added to short, conical culture tubes. The tubes were taken to dryness at 50° C under a stream of helium. After the addition of 100 μL of TLC streaking solvent to each tube, the tubes were sealed with Parafilm®, vortexed, and centrifuged at 3000 rpm for 5 min. The entire volume of solvent in each tube was streaked onto separate pre-run, activated plates 23 mm from the bottom and allowed to evaporate. Care was taken to keep the streaks narrow and not disturb the silica surface.

The dry streaked TLC plates were placed in a clean, dry TLC tank and 200 mL of TLC developing solvent carefully added to the bottom of the tank. The tank was covered and the plates allowed to develop for 75 min. At the end of this period, the solvent fronts were marked, and the plates removed and allowed to air dry for approximately 10 min.

The benzoylecgonine and ecgonine bands on the reference TLC plates were visualized with freshly prepared Ludy Tenger's Reagent. The distances from the origin to the middle of the ecgonine band and to the middle of the benzoyl-ecgonine band were measured. With the distances as a guide two separate areas of silica were chosen on the test plates, one corresponding to the ecgonine band, the other the benzoylecgonine band. Each area was scraped off by means of a single-edge razor blade and collected in a separate test

tube by means of a funnel. Both areas of silica were adja-
cent to each other and each measured 2 cm high x 5 cm wide
(the latter corresponding to the entire width of the plate).

To the separate test tubes containing the silica pow-
der, 8.0 mL of TLC eluting solvent were added to elute the
ecgonine or benzoylecgonine. The tubes were capped, vor-
texed, and centrifuged at 3000 rpm for 30 min. A 7.0 mL al-
iquot of eluate was taken from each sample and transferred
to a fresh test tube. (Care was taken to avoid aspirating
any silica into the pipet.) The tube contents were taken to
dryness at 70° C under a stream of helium. A rinse of 1 mL
distilled ethanol followed by 5 min centrifugation at 3000
rpm was used to collect any residue at the bottom of the
tube. Two hundred fifty microliters (250 μL) of n-PEBE
Working Standard (20 μg/mL) and 25 μL of 0.6 N HCl in
2-propanol were added to each tube.

All samples were again taken to dryness, this time at
50° C, under a stream of helium. The samples were deriva-
tized with BSTFA and assayed using the modified GC-FID meth-
od previously described. Peak heights for ecgonine,
benzoylecgonine, and n-PEBE were measured and the peak
height ratios of alkaloid to n-PEBE calculated. The amounts
and recoveries of eluted benzoylecgonine and ecgonine were
calculated by comparison of their peak height ratios with
those used to plot GC standard calibration curves for
benzoylecgonine and ecgonine. In addition, the linearities

of peak height ratios of the eluted benzoylecgonine and ecgonine vs. amounts streaked were evaluated.

2. DETERMINATION OF ECGONINE IN WATER, PLASMA, AND URINE

a. Materials and Equipment

Ecgonine hydrochloride and the n-propyl ester of benzoylecgonine (n-PEBE) were prepared and purified as previously described. Primary Standards (200 μg/mL) of ecgonine and of n-PEBE, and Working Standards (20 μg/mL) of ecgonine and of n-PEBE were prepared as described and stored at $-15°$ C.

The 6.5 N perchloric acid used to deproteinize plasma and urine samples was prepared from 60% Perchloric Acid (ACS grade, Fisher) and triple-distilled water.

Anhydrous diethyl ether, reagent grade, was purchased from Fisher Scientific Ltd. The solvent used to extract spiked water, plasma, and urine samples was a 1:1:1 mixture of chloroform:methylene chloride:2-propanol (all pesticide grade, Fisher). Anhydrous potassium carbonate (ACS, Fisher) was used to adjust the pH in the extractions.

Blood and urine were obtained from mature female Beagle dogs, approximately 4 years of age and weighing approximately 15 kg. The dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Mich., USA.

The plasma used for assay development was separated from dog blood obtained by venipuncture using 10 mL glass syringes and 1 1/2" 21G Yale hypodermic needles manufactured

by Becton-Dickinson, Mississauga, Ont., Canada. The blood was collected and centrifuged in heparin-treated sodium fluoride collection tubes. The collection tubes were prepared by adding 0.5 mL 1.3% sodium fluoride (ACS grade, Fisher) in triple-distilled water to Kimble® disposable 13 x 100 mm glass culture tubes and then dried in an oven at approximately 100° C. The heparin was added to prevent clotting just prior to blood collection as 100 μL of Heparin Sodium Injection USP, 1000 IU/mL, Allen & Hanburys, Toronto, Ont., Canada. The plasma obtained by centrifugation was transferred to Kimble® 13 x 100 mm culture tubes covered with Parafilm® and stored at -30° C until used.

Urine used for assay development was collected overnight from fasted Beagle dogs placed in a dog metabolic cage (70 cm L x 84 cm W x 84 cm H) equipped to collect separately urine and feces. The urine was collected in a 950 mL amber wide-mouthed bottle containing 2.0 mL of dilute sulfuric acid solution (2 mL concentrated sulfuric acid, ACS Fisher, diluted to 10 mL with double-distilled water) as preservative. The pH, and specific gravity of the collected urine were measured, and the urine filtered before storage at -30° C in 500 mL Nalgene® bottles (Fisher Scientific Ltd.).

All other materials and equipment used have been previously described.

b. Methods

Extraction/TLC/GC Procedure.   An extraction/TLC/GC-FID
procedure was developed for the determination of ecgonine in
dog plasma and urine.   Preliminary experiments with water,
plasma and urine samples spiked with ecgonine hydrochloride
were carried out to evaluate the procedure.

Spiked Water Samples.   A measured volume: 0, 200, 400,
or 800 μL of ecgonine Working Standard corresponding to 0,
4,   8,   or 16 μg of ecgonine,respectively, as free base was
added to individual test tubes.   The samples were taken to
dryness at 50° C under a stream of helium.

To each tube,   3.5 mL of triple-distilled water were
added followed by gentle hand swirling to mix.   The tube
contents were acidified by adding 300 μL of 6.5 N perchloric
acid.   Samples were hand swirled and then placed in an ice
bath for 1 hour.   After cooling all samples were centrifuged
15 min at 3000 rpm, and the supernatants transferred to
fresh test tubes. Approximately 8 mL of ether were added to
each sample, the tubes capped and shaken 40 times by hand.
Following 5 min centrifugation of the tubes at 3000 rpm, the
upper ether layer was aspirated and discarded.

A 3.0 mL aliquot of the remaining aqueous phase of each
sample was transferred to a fresh test tube for extraction.
To each tube were added 8.0 mL chloroform:methylene chlo-
ride:2-propanol followed by 4.0 g of anhydrous potassium

carbonate. The tubes were immediately capped and each shaken 40 times by hand. After each sample had been processed individually in this manner, all the tubes were placed on the flatbed shaker and extracted for an additional 4 min at the HIGH setting (approximately 240 cycles/min). All the samples were centrifuged for 5 min at 3000 rpm and a 7.0 mL aliquot of each organic phase transferred to a short, conical bottom culture tube. The extracts were taken to dryness at 70° C under a stream of helium. The resulting residues were each washed down to the bottom of the tubes with 1 mL distilled ethanol. All tubes were centrifuged 5 min at 3000 rpm, and again taken to dryness at 70° C under helium. A measured volume (100 μL) of TLC streaking solvent was added to each tube which was then covered with Parafilm® and vortexed to dissolve the residue.

Along with the test samples TLC reference samples were also prepared. These were made by adding 50 μL of ecgonine TLC reference solution (1000 μg/mL) to the bottom of fresh culture tubes and taking them to dryness at 50° C under a stream of helium. The dry samples were dissolved in 100 μL of TLC streaking solvent. All reconstituted test and reference samples were streaked onto individual pre-run, activated TLC plates and developed for 75 min as previously described. At least one TLC reference sample was included in each tank of TLC test plates developed.

The developed reference plates were sprayed with fresh-
ly prepared Ludy Tenger's Reagent to visualize the ecgonine
bands. With the position (± 1 cm) of ecgonine on the refer-
ence plate as a guide, two centimeter high bands of silica
on test plates corresponding to ecgonine were scraped into
test tubes and the ecgonine eluted from the silica as be-
fore. These eluates were taken to dryness, rinsed down with
methanol, and after the the addition of n-PEBE again taken
to dryness. The final residues derivatized with BSTFA were
assayed for ecgonine by gas chromatography as previously de-
scribed.

The peak heights of ecgonine and n-PEBE were measured
and peak height ratios of ecgonine to n-PEBE calculated.
From the peak height ratios, the amount and percent (%) re-
covery of ecgonine from the spiked water samples were esti-
mated by comparison with an ecgonine GC standard calibration
curve of peak height ratios vs. the nanograms of ecgonine
injected.

Ecgonine GC standards for the experiments on the recov-
ery of ecgonine from the spiked water samples were prepared
and analyzed as follows: measured volumes of ecgonine Work-
ing Standard: 50, 100, 200, and 400 μL containing 1, 2, 4,
and 8 μg of ecgonine, respectively, were added to separate
test tubes. A 250 μL aliquot of n-PEBE Working Standard (20
μg/mL) and 25 μL of 0.6 N HCl in 2-propanol were also added
to each tube. The tubes were vortexed and taken to dryness

at 50° C under a stream of helium.   The residues were deri-
vatized with 50 μL of BSTFA and analyzed by gas chromatogra-
phy as previously described.   The plot of the least-squares
linear regression of peak height ratios (ecgonine/n-PEBE)
against nanograms ecgonine injected was used as the GC stan-
dard calibration curve to calculate the recovery of ecgonine
from the spiked water samples.

    <u>Spiked Plasma Samples</u>.   Recovery of   ecgonine from
spiked plasma samples was estimated in a similar manner.
Individual plasma samples were spiked with ecgonine hydro-
chloride and assayed as follows: measured volumes 0,   200,
400,   or 800 μL, of ecgonine Working Standard were trans-
ferred to test tubes and taken to dryness, 50°/helium.   Tri-
ple-distilled water (2.5 mL) was added to each tube with
gentle swirling to dissolve the ecgonine hydrochloride resi-
due.   To each tube, 1.0 mL of plasma was added and the tube
swirled again to mix.   Perchloric acid, 300 μL of 6.5 N, was
then added to precipitate the plasma proteins.   A thin glass
stirring rod was used to stir the resulting suspension with
special care to break up any large protein aggregates.   The
plasma samples were placed in an ice bath for 1 hour fol-
lowed by centrifugation at 3000 rpm for 15 min.   The super-
natants were then transferred by means of Pasteur pipets to
fresh tubes with care to exclude any solid material.   Each
aqueous supernatant was washed by hand-shaking with 8.0 mL
of fresh ether followed by centrifugation to separate the

two phases. After removal of the ether by aspiration, 3.0 mL of the aqueous phase were transferred to a fresh test tube. The ether-washed samples were further processed by extraction of the ecgonine into organic solvent, TLC separation and isolation in the manner previously described for spiked water samples. The ecgonine was quantitated by GC using n-PEBE as internal standard. Peak heights of ecgonine and n-PEBE were measured and peak height ratios calculated. The amount of ecgonine found and the percent (%) recovered were calculated from the GC standard calibration curve of peak height ratios vs. ng/mL injected.

Spiked Urine Samples. The recovery of ecgonine from spiked urine samples was determined in a similar manner. Spiked urine samples were prepared using blank urine diluted 1:50 with triple-distilled water. The spiked urine samples containing 0, 4, 8, or 16 μg ecgonine per mL of diluted urine were prepared in the same way as the spiked plasma samples. The spiked urine samples, however, formed negligible precipitate on addition of 300 μL of 6.5 N perchloric acid. The extraction/TLC/GC-FID procedures were carried out as before with the plasma samples. Ecgonine and n-PEBE peak heights were measured, peak height ratios calculated. The recoveries of ecgonine from these spiked urine samples were calculated from the GC standard calibration curve.

Experimental (IN VIVO) Dog Plasma and Urine Samples. The concentration of ecgonine in dog plasma and urine sam-

ples obtained after intravenous administration of ecgonine hydrochloride was determined using the analytical procedures described above. The ecgonine concentrations of experimental $\underline{in}$ $\underline{vivo}$ samples were calculated from the concentration calibration curves based on spiked plasma or urine samples as standards. These concentration calibration curves were obtained by least-squares linear regression analysis of the peak height ratio (ecgonine/n-PEBE) vs. μg ecgonine per mL of spiked plasma or diluted urine sample.

3. DETERMINATION OF BENZOYLECGONINE AND ECGONINE IN WATER, PLASMA, AND URINE

   a. Materials and Equipment

Benzoylecgonine and ecgonine hydrochloride were prepared and purified as previously described. Primary Standards (200 μg/mL) of benzoylecgonine, ecgonine, and n-PEBE as well as a mixed benzoylecgonine/ecgonine Working Standard (each 20 μg/mL) and a n-PEBE Working Standard (20 μg/mL) were prepared and stored at $-15°$ C when not in use.

The plasma used for assay development was obtained from fresh blood collected as previously described. Urine used for assay development was obtained from fasted Beagle dogs kept in a metabolic cage. The urine was collected in 950 mL amber wide-mouthed bottles containing 100 mL dilute sulfuric acid solution (0.4 mL of concentrated sulfuric acid, ACS Fisher, diluted to 100 mL with double-distilled water). The sulfuric acid solution served as preservative in all subsequent urine collections unless stated otherwise.

All other materials and equipment used were as previously described.

b. Methods

Extraction/TLC/GC-FID Procedure. The analytical proce-
dures for the determination of benzoylecgonine and ecgonine
both in water, plasma, and urine were very similar to the
method described for ecgonine alone.

Spiked Water Samples.   The recovery of benzoylecgonine
in spiked water samples was evaluated.   Measured volumes of
the benzoylecgonine/ecgonine Working Standard: 0, 200, 400,
or 800 μL added to individual tubes were taken to dryness at
50° C under a stream of helium. Triple-distilled water (3.5
mL) was added to each tube and the tubes swirled to dissolve
the benzoylecgonine/ecgonine residue. The spiked water sam-
ples received the same perchloric acid treatment and ether
wash described for ecgonine.   A 3.0 mL aliquot of aqueous
phase from each ether-washed sample was transferred to a
fresh   tube and 8.0   mL of chloroform:methylene chlo-
ride:2-propanol, 1:1:1, added. The assay for benzoyl-
ecgonine differed from that of ecgonine at this point in
that all samples were capped and immersed in an ice bath for
5 min before extraction into organic solvent.   Four grams
P4.0 g) of anhydrous potassium carbonate were added to each
cooled sample which already contained the extraction solvent
and was immediately shaken 40 times by hand. Extraction was
completed with an additional 4 min shaking on the flatbed
shaker set at HIGH (approximately 240 cycles/min) and then
all samples centrifuged at 3000 rpm for 10 min.

A 7.0 mL aliquot of the organic phase from each sample was transferred to a short, conical bottom culture tube and the solvent allowed to evaporate at 70° C under a stream of helium. The residue on the sides of each tube was washed down with 1 mL distilled ethanol, taken to dryness, and the resulting residue taken up in 100 µL of TLC streaking solvent. Thin-layer chromatography was carried out as previously described. Bands of silica 2.0 cm wide corresponding to the benzoylecgonine streak as visualized on a TLC reference plate were scraped into fresh test tubes. The benzoylecgonine was eluted from the silica and estimated by gas chromatography with n-PEBE as internal standard. The recovery of benzoylecgonine was estimated from a GC standard calibration curve based on peak height ratios benzoylecgonine to n-PEBE.

In a separate experiment, the possible conversion of benzoylecgonine to ecgonine by hydrolysis during the benzoylecgonine assay procedure was examined. Regular spiked water samples containing both benzoylecgonine and ecgonine were prepared with 0, 200, 400, and 800 mL of benzoylecgonine/ecgonine Working Standard (each 20 µg/mL). In addition, companion samples containing benzoylecgonine alone were prepared with 200 µL of benzoylecgonine Primary Standard (200 µg/mL). All samples were carried through the complete extraction/TLC procedure. In the case of each test sample the bands of silica corresponding to the individual

areas of benzoylecgonine and ecgonine were checked for alkaloid content.   The amount of ecgonine generated during the assay of water samples spiked with benzoylecgonine alone was quantitated by GC-FID.

Spiked Plasma and Urine Samples.   Recovery studies were also carried out for benzoylecgonine and ecgonine both in spiked plasma and diluted urine (1:25) samples.   Deproteinization with perchloric acid and the ether washing were carried out as previously described.

The deproteinized, ether-washed samples were extracted and subjected to TLC in the same manner as previously described for the spiked water samples. At the same time separate companion samples prepared from benzoylecgonine TLC reference solution and ecgonine TLC reference solution were processed directly by TLC (one TLC reference plate was used per TLC tank).   The TLC reference plates were sprayed with Ludy Tenger's Reagent to locate the position of the bands of benzoylecgonine and ecgonine.   The two corresponding bands of silica were taken from the plates for the spiked plasma or urine samples, eluted and assayed by GC. The peak height ratio, alkaloid to n-PEBE, was used to determine the amount of benzoylecgonine or ecgonine injected from the appropriate GC standard calibration curve.   The recoveries of benzoylecgonine and ecgonine from the spiked plasma and urine samples were calculated.

<u>Experimental (IN VIVO) Dog Plasma and Urine Samples</u>.
The benzoylecgonine and ecgonine content of plasma and urine
samples obtained after intravenous administration of
benzoylecgonine was determined according to the analytical
procedures described above. The benzoylecgonine and ecgo-
nine concentrations of test samples were calculated from the
concentration calibration curves based on spiked plasma or
urine samples as known standards. Least-squares linear re-
gression calculations of the peak height ratio vs. alkaloid
concentration spiked were used to prepare these concentra-
tion calibration curves.

4. DETERMINATION OF ECGONINE METHYL ESTER AND ECGONINE IN
   WATER, PLASMA, AND URINE

   a. Materials and Equipment

Ecgonine methyl ester hydrochloride, ecgonine hydro-
chloride, and n-propyl ester of benzoylecgonine were syn-
thesized and purified as described in a previous section.
Primary Standards (200 μg/mL) of ecgonine methyl ester,
ecgonine, and n-PEBE as well as a n-PEBE Working Standard
(20 μg/mL) were prepared as previously described. An ecgo-
nine methyl ester/ecgonine Working Standard (each 20 μg/mL)
was prepared by diluting a mixture of the ecgonine methyl
ester and ecgonine Primary Standards with methanol. All
standards were stored at -15° C when not in use.

A saturated solution of potassium carbonate (ss K_2CO_3)
was prepared with anhydrous potassium carbonate (certified
ACS, Fisher) and triple-distilled water. The solution was
filtered through a coarse sintered-glass filter funnel and
stored at room temperature.

A 1:1 mixture of cyclohexane and methylene chloride
(pesticide grade, Fisher) was used to extract ecgonine
methyl ester, while a 1:1:1 mixture of chloroform, methylene
chloride, and 2-propanol (pesticide grade, Fisher) was used
to extract ecgonine.

All other materials and equipment used have been previously described.

b. Methods

Extraction/TLC/GC-FID Procedures.   Procedures were developed for the extraction/GC-FID analysis of ecgonine methyl ester and the extraction/TLC/GC-FID analysis of ecgonine in the plasma and urine of a dog after the administration of ecgonine methyl ester hydrochloride intravenously. The two alkaloids were extracted separately in successive extractions and quantitated by GC-FID. The ecgonine extract was purified by TLC prior to GC-FID analysis.   Water, plasma, and urine samples spiked with ecgonine methyl ester hydrochloride and ecgonine hydrochloride were analyzed to evaluate the method.

Spiked Water Samples.   Initial assay development was carried out with spiked water samples.   These samples were spiked to contain 0, 4, 8, or 16 μg of ecgonine methyl ester and ecgonine per tube.   To each test tube 3.5 mL of triple-distilled water was added and a measured volume of ecgonine methyl ester/ecgonine Working Standard: 0, 200, 400, or 800 μL. Sufficient methanol was added to each sample so that the final volume of all the spiked water samples was the same.

In the case of the determination of ecgonine methyl es-
ter, to each spiked water sample, 300 μL of 6.5 N perchloric
acid were added, the contents were swirled to mix, and the
tube was capped and incubated in an ice bath for 1 hour. At
the end of this time all spiked water samples were centri-
fuged for 15 min at 3000 rpm and the supernatants trans-
ferred to fresh test tubes. (This centrifugation and trans-
fer was unnecessary for spiked water samples but was
included as it would be required in the analysis of plasma
and urine samples.)

Each perchloric acid-treated sample was then washed by
mixing with 8 mL of ether which after centrifugation was re-
moved by aspiration. A 3.0 mL aliquot of each aqueous phase
was transferred to a fresh test tube, and 8.0 mL cyclohex-
ane:methylene chloride, 1:1, added. The pH of each spiked
water sample was adjusted to approximately pH 10 with the
addition of 300 μL of saturated solution of potassium carbo-
nate. Each sample was immediately capped and shaken 40
times by hand. All samples were then shaken on the flatbed
shaker for an additional 4 min on the HIGH setting (approxi-
mately 240 cycles/min) to complete extraction. At the end
of this period all samples were centrifuged at 3000 rpm for
10 min. A 7.0 mL aliquot of the upper organic layer con-
taining the ecgonine methyl ester was transferred to a fresh
test tube. The lower aqueous phase was retained for subseq-
uent extraction of the ecgonine.

To each 7.0 mL aliquot of the ecgonine methyl ester containing-cyclohexane:methylene chloride, 100 μL of 0.6 N HCl in 2-propanol were added. The tubes were swirled to mix and taken to dryness at 50° C under a stream of helium. Approximately 1 mL of methanol was used to rinse any ecgonine methyl ester residue to the bottom of each tube. The internal standard, 250 μL of n-PEBE Working Standard (20 μg/mL) and 25 μL of 0.6 N HCl in 2-propanol were added to each tube which was again taken to dryness, 50° C/helium.

The final residues were taken up and derivatized with BSTFA. Just prior to the addition of BSTFA, however, it was most important to leave the test tubes containing the final ecgonine methyl ester residues uncapped for 10 min permitting exposure of the residues to room atmosphere. Derivatization was carried out in the usual manner.

Fifty microliters (50 μL) of BSTFA were added to each tube, the tubes were capped, vortexed, and heated at 70° C for 10 min. Samples were centrifuged for 5 min at 3000 rpm and 1 μL analyzed by gas chromatography as previously described. The peak heights of ecgonine methyl ester and n-PEBE were measured and the peak height ratios of ecgonine methyl ester to n-PEBE calculated. From the peak height ratios, the amount and percent (%) ecgonine methyl ester recovered from each spiked water sample were estimated by comparison with an ecgonine methyl ester GC standard calibration curve of peak height ratios vs. ng ecgonine methyl ester per μL injected.

In the case of the determination of ecgonine, the aqueous phases remaining after the extraction of ecgonine methyl ester from the spiked water samples were re-extracted prior to analysis for their ecgonine content. First, however, the aqueous fractions remaining after their first extraction were frozen by immersing the uncapped test tubes in a dry ice/acetone bath, and the residual cyclohexane:methylene chloride from the ecgonine methyl ester extraction was removed by aspiration. Then, 8.0 mL chloroform:methylene chloride:2-propanol were added to each tube. The tube contents were allowed to thaw but were kept cold in an ice-bath prior to pH adjustment by the addition of 4.0 g solid potassium carbonate. After shaking to effect extraction followed by centrifugation, the ecgonine extracts were processed through the TLC/GC-FID procedure previously described. The (%) recovery of ecgonine from each spiked water sample was calculated from the peak height ratio (ecgonine to n-PEBE) obtained by comparison with peak height ratios vs. ng ecgonine per μL injected off the ecgonine GC standard calibration curve.

Separate experiments were conducted to investigate the possible conversion of ecgonine methyl ester to ecgonine by hydrolysis during the extraction/TLC assay procedure. Regular spiked water samples containing 0, 4, 8, and 16 μg of ecgonine methyl ester and ecgonine were prepared. Additionally, four companion samples containing 40 μg of ecgonine

methyl ester alone were prepared by adding 200 μL of ecgonine methyl ester Primary Standard (200 μg/mL) and 600 μL methanol to 3.5 mL triple-distilled water. These four test samples along with the spiked water samples were carried through the entire extraction/TLC procedure. The amount of ecgonine generated in the test samples was quantitated by GC-FID as previously described.

Spiked Plasma and Urine Samples. The method developed for spiked water samples was used with little modification to determine both ecgonine methyl ester and ecgonine in plasma and urine. Plasma and urine samples were first diluted by adding 2.5 mL of triple-distilled water to 1.0 mL of either plasma or dilute urine (1:10). The diluted samples were spiked with ecgonine methyl ester and ecgonine in the manner previously described. In the case of spiked plasma samples, the proteinaceous precipitate formed on addition of 300 μL of 6.5 N perchloric acid was broken up by stirring with a glass rod. Agitation was unnecessary in the assay of the spiked urine samples. The rest of the procedure was as described for the spiked water samples. The percent recoveries of ecgonine methyl ester and ecgonine from plasma and urine were calculated using the ecgonine methyl ester and ecgonine GC standard calibration curves.

The possible hydrolytic conversion of ecgonine methyl ester to ecgonine in unacidified urine at 37° C was investigated in vitro. Freely-voided urine was obtained from a

fasted dog (ID# XA59) kept in a metabolic cage. The urine was collected in a 950 mL bottle containing 3-4 crystals of thymol as preservative. The pH and specific gravity of the urine were measured, which was then filtered and stored at -30° C until assayed.

The ecgonine methyl ester stability test samples were prepared by combining in each tube 5.0 mL of blank urine (urine preserved with thymol) and 200 μL of a solution of ecgonine methyl ester in methanol (1000 μg base per mL). Controls containing 5.0 mL of unacidified urine and 200 μL methanol were also prepared. All tubes were heated in the Dribath at 37° C for 24 hours. The tube contents were then allowed to cool to room temperature. The concentration of ecgonine methyl ester was determined in 1.0 mL aliquots of each of the incubated samples. For each sample, the aqueous phase remaining after the initial extraction of ecgonine methyl ester with cyclohexane:methylene chloride was analyzed for ecgonine to determine the extent of hydrolysis of the original ecgonine methyl ester. A set of blank urine samples spiked in the usual way with the combined ecgonine methyl ester/ecgonine Working Standard (each 20 μg/mL) were used as standards for the urine ecgonine methyl ester and ecgonine concentration calibration curves. The concentrations of ecgonine methyl ester and ecgonine were determined in the hydrolysis test samples. The extent of ecgonine methyl ester hydrolysis was compared with the amount of ecgonine produced.

Experimental (IN VIVO) Dog Plasma and Urine Samples. The ecgonine methyl ester and the ecgonine concentrations of plasma and urine samples obtained from a dog after intravenous administration of ecgonine methyl ester were determined according to the analytical procedures described above. The concentrations of test samples were calculated from the concentration calibration curves based on spiked plasma or urine samples as known reference standards, taken as 100%. Least-squares linear regressions of the peak height ratios vs. spiked alkaloid concentration were used as the basis of these concentration calibration curves.

5. DETERMINATION OF COCAINE IN WATER, PLASMA, AND URINE

a. Materials and Equipment

For use in the gas chromatographic (GC) procedure, Primary Standards of cocaine base (212 μg/mL) and n-propyl ester of benzoylecgonine (n-PEBE, 200 μg/mL) were prepared in methanol (pesticide grade, Fisher). In addition, a cocaine base Secondary Standard (21.2 μg/mL) was prepared by diluting the Primary Standard with methanol.

The cocaine base was prepared from cocaine hydrochloride as follows: to a 10% solution of cocaine hydrochloride in distilled water 5 times the volume of cyclohexane was added. A saturated solution of potassium carbonate was added dropwise until the addition of a drop of potassium carbonate failed to produce a milky precipitate in the aqueous phase. The aqueous phase was separated from the organic phase and discarded. The organic phase was washed three times with small volumes of distilled water, and dried over anhydrous sodium sulfate. The solvent was removed by evaporation in a roto-evaporator (water bath temperature 45-50° C), and the crystalline cocaine base was dried over phosphorus pentoxide under vacuum at room temperature. Crystalline n-PEBE was synthesized and purified as previously described.

Reagent grade heptane and iso-amyl alcohol used for extraction of cocaine were distilled prior to use. For pH adjustment a solid carbonate buffer mixture was formulated by

triturating 20 parts anhydrous sodium carbonate and 17.5
parts sodium bicarbonate (both ACS grade, Fisher). The
buffer was used both as a powder and as a solution prepared
immediately prior to use by adding 700 mg of solid carbonate
buffer mixture to 10 mL of triple-distilled water. For the
back-extraction of cocaine into water 50 mmol/L sulfuric
acid solution (1.4 mL concentrated sulfuric acid (ACS, Fish-
er) made up to 500 mL with double-distilled water) was used.

Final heptane:iso-amyl alcohol extracts of cocaine were
dried by passage through drying tubes containing anhydrous
sodium sulfate (ACS grade, Fisher). The drying tubes were
easily made by removing the tips from 5 3/4" long Disposable
Pasteur Pipettes (Fisher), plugging the bottom with glass
wool, and filling with approximately 1 g anhydrous sodium
sulfate.

In order to measure the low levels of cocaine expected
in extracts of dog plasma and urine, gas chromatographic
analysis was carried out on a Hewlett-Packard Model 5880
equipped with the highly sensitive nitrogen-phosphorus de-
tector (GC-NPD). Empty coiled glass columns, 6 ft x 1/4"
OD, 2 mm ID, purchased from Hewlett-Packard Ltd., Mississau-
ga, Ont., Canada, were washed and treated with 5% dichloro-
dimethylsilane in toluene. Columns were packed with 3%
OV-101 on 80/100 mesh Chromosorb W HP purchased from Chroma-
tographic Specialities. Helium was used as the carrier gas
with a flow rate of 40 mL/min. Flow rates of hydrogen and

medical air were 5 and 100 mL/min, respectively. Both the injector and detector temperatures were maintained at 300° C The oven temperature was programmed from 200 to 260° C at a rate of 15°/min with a final hold of 2 min at 260° C The auxiliary temperature to the NPD ceramic element was raised 190-210° C to obtain an offset between 20 and 22 x 10^{-12} A above baseline. Chart speed was set a 2 cm/min.

Withdrawal of venous blood from dogs for stability studies of cocaine on collection and storage was facilitated by placement of an in-dwelling IV catheter (Longdwell Catheter Needle, 20G, 1 1/2" purchased from Becton-Dickinson) fitted with an access plug (IV Catheter Plug, manufactured by McGaw Laboratories, purchased from American Hospital Supply Canada, Inc., Winnipeg, Man., Canada). The catheter was kept patent with a heparin lock, consisting of a solution of 1000 IU/mL Heparin USP diluted 1 to 6 with normal saline (0.9% Sodium Chloride Injection, Squibb, Montreal, Que., Canada). Blood was drawn into glass syringes and then transferred to Kimble® disposable 13 x 100 mm glass culture tubes (Fisher) to which were added 200 μL of 4% sodium fluoride solution and 100 μL of heparin solution (1000 IU/ mL) per tube.

To help minimize the hydrolysis of cocaine in collected blood/plasma samples, a portable refrigerated centrifuge was employed for the separation of plasma from the NaF/heparin-treated blood samples. Construction of the centrifuge unit

was improvised as follows: the Dynac Centrifuge referred to in a previous section was placed in a cardboard box (54 cm L x 41 cm W x 26 cm H) which in turn was placed inside another larger box (69 cm L x 53 cm W x 39 cm H). The boxes were of sufficient size to provide an air space of 6-8 cm between walls and 10-12 cm between the two lids. Several 5-7 cm holes were cut into the walls of the inner box to permit free flow of air around the centrifuge. For insulation, 1" thick Styrofoam® sheeting was glued to the outer surface of the box fitted with a snug lid of the same material. To cool the centrifuge commercially available freezer packs (ICEPAK®, Stanbel Ltd., Montreal, Que., Can.) were frozen at −30° C overnight and placed in the space between the walls of the two containers. Test water samples placed in the centrifuge were cooled to near 4° C and remained at that temperature for several hours.

Plasma separated from blood was transferred to Vacutainer® Evacuated Glass Tubes, 16 x 127 mm, with a silicone interior coating (Becton-Dickinson), frozen in dry-ice and stored at −30° C in a deep-freeze.

All other materials and equipment used have been previously described.

b. Methods

HP 5880 GC Multi-Level Internal Calibration Method.
For the quantitation of cocaine in unknown plasma and urine

samples, the HP 5880 GC equipped with NPD was operated in the multi-level internal calibration mode, whereby the instrument prints out not only basic GC parameters for both cocaine and the internal standard (n-PEBE) but also the absolute amount of cocaine found in the injected sample. Briefly, the calibration procedure is as follows: a set of calibration mixtures was prepared each with increasing amounts of cocaine and a fixed amount of n-PEBE included as internal standard. The concentration range for cocaine was chosen to cover the expected range in the final unknown samples prepared for GC quantitation: 2.7, 5.3, 10.6, 21.2, 42.4, and 63.6 ng cocaine base, each with 30 ng n-PEBE per 2 μL volume of methanol injected. Samples of the calibration mixtures were run under the same conditions used for the unknown samples. At the end of each run the instrument provides a direct print-out of the retention times, peak heights and peak areas of the cocaine and of the n-PEBE along with the response factors quantifying as a ratio the amount of each compound (cocaine and n-PEBE) relative to its peak height or peak area. The response factors are stored in memory and together with corresponding concentration information for the separate calibration mixtures create an internal calibration curve. On injecting an unknown sample with added n-PEBE as internal standard the gas chromatograph prints out directly the absolute amount of cocaine present in the injected sample (93).

98

<u>Extraction/Gas Chromatographic (GC)-NPD Procedure</u>. A modification of the GC-NPD procedure of Jatlow and Bailey (49) was developed for the determination of cocaine in plasma and urine. Briefly, the procedure consists of an extraction of cocaine, after pH adjustment of samples to 9, into heptane:iso-amyl alcohol, a clean-up back-extraction into dilute sulfuric acid and lastly re-extraction at pH 9 into the organic solvent. The residue left on evaporation of the solvent is dissolved in methanol and its cocaine content quantitated directly (without derivatization) by injection into the calibrated gas chromatograph.

The n-PEBE was used as internal standard in the procedure in one of two ways: (a) as a "modified" internal standard by adding the n-PEBE to the final reconstituted methanolic solutionof cocaine before the sample is is injected into the gas chromatograph, or (b) as a "true" internal standard whereby the n-PEBE was added dissolved in the extracting solvent to the original sample and extracted along with the cocaine.

<u>Spiked Water Samples</u>. The "modified" internal standard procedure was used to determine the recovery of cocaine from spiked water samples. Five Cocaine Extraction Standards were prepared according to Table II. One hundred microliters (100 µL) of each of the five Extraction Standards were transferred to test tubes along with 2.0 mL of triple-distilled water. (An additional test tube containing 2.0 mL of

Table II: Cocaine Extraction Standards.

| Number | Composition | | Concentration |
	Sec Std[a] (mL)	Methanol (mL)	(ng/100 μL)
#1	0.3	10.0	62
#2	0.6	10.0	120
#3	1.3	10.0	244
#4	3.0	10.0	490
#5	5.0	10.0	1060

[a]Cocaine base Secondary Standard, 21.2 μg/mL.

triple-distilled water and 100 μL of methanol served as a blank.) Ten milliliters (10.0 mL) of heptane:iso-amyl alcohol, 98:2, and 0.5 mL of carbonate buffer solution were added to each sample. Each tube was immediately capped, shaken 40 times by hand and centrifuged for 5 min at 2000 rpm. The upper organic phase was transferred to a fresh test tube and 1.0 mL of 50 mmol/L sulfuric acid solution added. All sample tubes were capped, shaken 40 times by hand, and centrifuged for 5 min at 2000 rpm. The upper organic phase in each tube was aspirated off and discarded. The remaining aqueous phase was washed with 3.0 mL heptane:iso-amyl alcohol which after centrifugation was removed by aspiration. To each tube containing the aqueous phase, 2.0 mL of fresh heptane:iso-amyl alcohol were added followed by 400 mg of solid carbonate buffer mixture. Each tube was immediately capped and shaken 40 times by hand. All samples were then centrifuged for 5 min at 2000 rpm. The upper layer of each of these final extracts was dried by percolation through approximately 1 g anhydrous sodium sulfate retained in a Pasteur Pipette on a glass-wool plug. The sodium sulfate was rinsed with approximately 1 mL of heptane:iso-amyl alcohol.

The combined dried organic extract and rinse were taken to dryness at room temperature under a stream of helium. Fifty microliter (50 μL) of n-PEBE solution (30 ng/2 μL) were added to each test tube. After mixing and centifugation, all samples were analyzed by GC-NPD. Two microliters

(2 μL) of the final extract were injected into the HP 5880 gas chromatograph calibrated with standard cocaine/n-PEBE mixtures. The GC gave a direct readout of the ng cocaine per 2 μL injected for each extracted spiked water sample. The total amount of cocaine and percent (%) recovered from each spiked water sample were calculated.

The "true" internal standard method was also used to estimate the recovery of cocaine from spiked water samples. In this procedure one hundred microliters (100 μL) of each of the Extraction Standards (Table II) were added to test tubes containing 2.0 mL of triple-distilled water. Blank water samples were prepared by adding 100 μL of methanol to 2.0 mL of triple-distilled water. To each of the water samples were added 10.0 mL of heptane:iso-amyl alcohol containing 750 ng n-PEBE, and in turn 0.5 mL of carbonate buffer solution for extraction. These initial extracts were back-extracted into 1.0 mL of 50 mmol/L sulfuric acid solution. All the tubes were centrifuged, the organic phases aspirated off, and the aqueous phases washed with 3.0 mL plain heptane:iso-amyl alcohol (without n-PEBE). After treatment with 400 mg solid carbonate buffer mixture, the aqueous phases were re-extracted with 2.0 mL plain heptane:iso-amyl alcohol. The final extracts were dried by passage through anhydrous sodium sulfate and taken to dryness as previously described. The final residues were dissolved in 50 μL of methanol. Quantitation by GC-NPD analysis of these samples

was carried out as previously described. The percent (%) recovery of cocaine from each spiked water sample was calculated.

Spiked Blood and Plasma Samples. The "modified" internal standard method was used to determine the recovery of cocaine from spiked plasma samples as well as assess the stability of cocaine during blood collection, plasma separation and storage. Venous blood was collected from a single mature female Beagle dog which was fasted overnight. Three sets of samples were analyzed: blank plasma samples, cocaine-spiked plasma samples, and cocaine-spiked blood samples.

To prepare each of the blank plasma samples approximately 5 mL of freshly drawn blood were added to a culture tube containing 200 μL of 4% sodium fluoride solution and 100 μL of 1000 IU/mL heparin. The culture tubes were covered with Parafilm®, inverted several times to mix, cooled in an ice bath, and centrifuged in the portable refrigerated centrifuge at 2700 rpm for 10 min. The separated plasma was transferred to a separate Vacutainer tube and immediately frozen on dry ice.

For each of the spiked plasma samples, approximately 5 mL of freshlyl drawn blood were added to a culture tube containing 200 μL 4% sodium fluoride solution and 100 μL of 1000 IU/mL heparin. The tube contents were cooled in an ice

bath and then centrifuged for 10 min at 2700 rpm. Exactly 2.5 mL of plasma were transferred from each tube to a separate Vacutainer tube to which were added 50 μL of cocaine base Secondary Standard (21.2 μg/mL). The tubes were immediately sealed and inverted several times to mix. The tube contents were frozen on dry ice.

In preparing each of the spiked blood samples, exactly 5.0 mL of blood were transferred to a culture tube kept in an ice bath. One hundred microliters (100 μL) of cocaine base Secondary Standard (21.2 μg/mL) were added. The spiked blood sample was inverted to mix and centrifuged for 10 min at 2700 rpm. Exactly 2.5 mL of the separated plasma were transferred to a Vacutainer tube and immediately frozen on dry ice.

As part of the stability study of cocaine the three sets of plasma samples were stored at -30° C for four weeks and then assayed for cocaine.

Immediately prior to assay the three sets of plasma sampls were thawed out at room temperature. Two milliters (2.0 mL) of each blank plasma, spiked plasma, and plasma obtained from spiked blood sample were transferred to separate test tubes. Forty microliters (40 μL) of cocaine base Secondary Standard (21.2 μg/mL) were added to each of the blank plasma samples and the tubes swirled to mix their contents. The cocaine content of all samples was determined by GC-NPD

based on the "modified" internal standard method. The HP 5880 gave a direct readout of the amount of cocaine injected for each spiked plasma sample according to the internal calibration curve established with GC standards. The recovery of cocaine from each sample was calculated and the mean recoveries of each set compared.

Spiked Urine Samples. The method for the determination of cocaine in spiked water samples was employed with little modification to determine cocaine in experimental urine samples. In the case of the latter the modification simply involved dilution of the urine samples to concentration levels within the sensitivity range of the GC-NPD method. Blank urine samples spiked with increasing levels of cocaine base were carried through the entire extraction/GC-NPD procedure with n-PEBE as internal standarsd. These GC data were used as standards to calibrate the HP 5880.

As in the case of ecgonine methyl ester the stability of cocaine in unacidified urine at 37° C was investigated in a separate experiment. Two hundred microliters (200 μL) of a cocaine base solution, 1000 μg/mL in methanol, were added to 5.0 mL of thymol-preserved blank urine and incubated at 37° C for 24 hours. Control urine samples were prepared by adding 200 μL of methanol to methanol to 5.0 mL thymol-preserved urine and incubated 37° C for 24 hours.

At the end of the incubation period the concentration of cocaine in the test samples was determined by the extraction/GC-NPD method. A 1.0 mL aliquot of each of the incubated hydrolysis test samples was diluted to 10.0 mL with triple-distilled water. The concentration of cocaine in 250 µL aliquots of these samples was determined. A set of incubated control blank urine samples was diluted and spiked with the cocaine base Working Standards and carried through the entire procedure to calibrate the HP 5880 gas chromatograph. The concentrations of cocaine in the incubated urine test samples were compared with the spiked incubated control blank urine samples. The fraction of cocaine hydrolyzed was calculated for each test sample.

The concentrations of ecgonine methyl ester, benzoylecgonine, and ecgonine in these in vitro urine stability test samples were determined according to the extraction/TLC/GC-FID method previously described. Ecgonine methyl ester was extracted from each of the undiluted urine stability test sample into cyclohexane:methylene chloride, derivatized and quantitated by GC-FID using the HP 5710A. Benzoylecgonine and ecgonine were extracted from the remaining aqueous phases. After the TLC clean-up of the residues from these extracts, the amounts of benzoylecgonine and ecgonine were determined by GC-FID. The concentrations of benzoylecgonine, ecgonine, and ecgonine methyl ester in the urine samples were calculated from concentration calibration

curves for urine obtained from analyses of control urine samples spiked in the usual manner with the Mixed Working Standard (20 μg/mL cocaine, ecgonine methyl ester, benzoyl-ecgonine, and ecgonine bases). The fraction of the cocaine in the urine converted by incubation to each of its hydrolysis products was calculated.

Experimental (IN VIVO) Dog Plasma and Urine Samples. The concentration of cocaine was determined in plasma and urine samples obtained from an adult dog after intravenous administration of cocaine hydrochloride according to the extraction/GC-NPD analytical procedure outlined above. Accurately measured volumes of plasma or diluted urine, 2.0 mL or less, were added to test tubes and, if necessary, made up to 2.0 mL volume with triple-distilled water. A set of standard plasma or urine samples was prepared with 2.0 mL blank plasma or diluted urine containing 100 μL of one of the cocaine base Working Standards descibed in Table III. All test samples and standards were analyzed for cocaine by GC-NPD with n-PEBE as the "true" internal standard. The standard plasma or urine samples were used to generate an internal calibration curve in the HP 5880 which allowed automatic computation of the concentration of cocaine injected. These data were used to calculate the final amounts and concentrations of cocaine in the test plasma and urine samples.

Table III: Cocaine Base Working Standards

Number	Composition		Working Standards	
	Sec Std[a]	Methanol	Conc	Amt Injected
	(mL)	(mL)	(ng/100 μL)	(ng/2 μL)
#1	0.0	10.0	0	0.0
#2	0.2	9.8	40	1.6
#3	0.4	9.6	80	3.2
#4	0.8	9.2	160	6.4
#5	1.6	8.4	320	12.8
#6	3.2	6.8	640	25.6
#7	6.4	3.6	1280	51.2

[a]Cocaine base Secondary Standard, 20 μg/mL.

III-B. PHARMACOKINETICS

1. INTRAVENOUS ECGONINE

a. Materials and Equipment

A female Beagle dog, ID# XA59, approximately 4 years of age, weighing 15.7 kg was used for the intravenous ecgonine experiment. The animal was purchased from Laboratory Research Enterprises Inc., Kalamazoo, Mich., USA.

A stock solution of ecgonine for injection,   40 mg/mL ecgonine base, was prepared by dissolving in 6.0 mL of normal saline 287.3 mg of ecgonine hydrochloride.

The materials and equipment used for sampling of blood, preparation and separation of plasma, and collection of urine and their subsequent assay for ecgonine content have been described in detail in previous sections.

Nonlinear least-squares fitting of theoretical curves to plasma ecgonine concentration-time data was carried out by means of the NONLIN computer program (94) obtained from the Upjohn Drug Co., Kalamazoo, Mich., USA, and programmed into the University of Manitoba high-speed digital computer (Almdahl 580 system).

b. Methods

Ecgonine Administration. On the morning of the experiment the dog was removed from its holding cage, placed on a

table and allowed to adjust to its surroundings. A
Longdwell catheter was inserted into the cephalic vein of
the right foreleg and flushed with fresh heparin-lock solu-
tion to keep it patent. A control blood sample was taken as
follows: approximately 3 mL of catheter fluid were first
removed and discarded, and then a 10 mL sample of whole
blood was drawn. The blood was divided between two sodium
fluoride collection tubes each containing 100 μL of 1000 IU/
mL heparin. The tubes were sealed with Parafilm® and placed
in an ice bath. The catheter was flushed with about 2 mL of
fresh heparin-lock solution to keep it patent.

At zero-time (t=0 h) a dose equivalent to 10 mg ecgo-
nine free base per kilogram body weight was administered to
the dog intravenously. The required dose of 157 mg ecgonine
base contained in 3.93 mL of ecgonine stock solution was in-
jected into the cephalic vein of the left foreleg (contrala-
teral to the catheter-bearing foreleg) over a period of 10
seconds.

Blood and Urine Collection. Blood samples (10 mL each)
were drawn at 2.5, 7.5, 17.5, 30, 45, 60, 90, 120, and 180
min after ecgonine administration. After the 60 min sample,
the dog was placed in the metabolic cage equipped to collect
urine. The dog was allowed food and water ad lib. The dog
was carefully watched to ensure it did not disturb the in-
dwelling catheter. After the last two blood samples were
taken, the catheter was removed and the dog returned to the

metabolic cage.   At the end of the 48 h urine collection period the dog was returned to its holding cage.   Any residual urine remaining on the collection tray was rinsed into a separate 950 mL bottle with double-distilled water.

On the same day of the ecgonine experiment blood was drawn from a separate, fasted dog and transferred to sodium fluoride collection tubes containing 100 μL 1000 IU/mL heparin.   These tubes were sealed with Parafilm® and kept on ice with the other blood samples until the time the plasma was separated.   Blank urine was also collected from the dog and used to prepare spiked urine samples as standards for the ecgonine concentration calibration curve.

All blood samples were centrifuged for 10 min at 3000 rpm and the plasma transferred to fresh 13 x 100 mm disposable culture tubes, sealed with Parafilm®, and stored at -30° C until assayed.

The volumes, pH, and specific gravities of the urine collections over the 48 h period and accompanying rinses were measured.   The urine and rinses were filtered and stored in 500 mL Nalgene® bottles at -30° C until assayed.

Plasma Analysis. The concentration of ecgonine in each plasma sample was determined by the extraction/TLC/GC method previously described for ecgonine in spiked plasma samples. Experimental plasma samples were thawed immediately prior to assay. Plasma standards containing 4, 8, and 16 μg of ecgo-

nine per mL were prepared by spiking blank plasma samples with 200, 400, and 800 μL of ecgonine Working Standard (20 μg/mL). One milliliter (1.0 mL) aliquots of the plasma standards were analyzed and the peak height ratios (ecgonine to n-PEBE) obtained were used to construct a ecgonine concentration calibration curve. The plasma standards were assayed along with 1.0 mL aliquots of the experimental plasma samples. Plasma samples containing greater than 16 μg/mL ecgonine were diluted with triple-distilled water prior to assay. The concentration of ecgonine in the experimental plasma samples was determined from the ecgonine-spiked plasma calibration curve.

Pharmacokinetic Analysis. A semi-logarithmic plot of the natural log of the plasma concentrations of ecgonine (μg/mL) versus time (h) at the midpoint of the blood collection period was constructed. The curve obtained was resolved into two exponential components by the method of residuals (96,97). Accordingly, the data were fitted by the general biexponential equation:

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \qquad (1)$$

Initial estimates obtained for the coefficients C_1 and C_2, and the rate constants λ_1, and λ_2 were used to calculate initial estimates of model parameters V_1, k_{12}, k_{21}, and K for a two-compartment open model with rapid IV input described by Equation 2.

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \qquad (2)$$

The model used to derive equations (1) and (2) and defini-
tion of terms are presented in the Appendix. Final least-
squares estimates of the several pharmacokinetic parameters
were obtained by NONLIN (94) analysis of the ecgonine plasma
concentration-time data.

Data analysis according to equation (1) and (2) was
completed with different weightings for the plasma concen-
tration, y: 1.0y, 1/y, and $1/y^2$. The goodness of fit of
the theoretical curves to the observed data points was
judged by the magnitude and distribution of the %deviation
between observed and computer calculated values of plasma
concentrations. Also considered were the test statistics,
"r^2", taken as equal to 1 $- \Sigma dev^2/\Sigma obs^2$ and "cor" taken as
the correlation coefficient of the linear regression, pre-
dicted y vs. observed y.

Urine Analysis. The concentration of ecgonine in the
urine collected over the 48 hours after drug administration,
and the accompanying rinses was determined according to the
extraction/TLC/GC method developed for ecgonine in spiked
urine samples. The urine was thawed immediately prior to
assay and diluted 1:50 with triple-distilled water. The
concentration of ecgonine was determined from the ecgonine
urine concentration calibration curve based on spiked urine

standards.   The   total amount of ecgonine excreted in the
urine and percent of dose excreted unchanged were calculat-
ed.

2. INTRAVENOUS BENZOYLECGONINE

a. Materials and Equipment

Benzoylecgonine was administered to Beagle dog XA59, the same animal which had received the ecgonine hydrochloride intravenously several months previously. At the time of the present experiment the dog had a fasted weight of 15.7 kg.

Benzoylecgonine was prepared and purified as previously described. The benzoylecgonine stock solution for injection (40 mg/mL) was prepared by dissolving 240.0 mg of benzoylecgonine base in 6.0 mL of normal saline.

All materials and equipment used in the administration of the benzoylecgonine, the drawing of whole blood and collection of urine were the same as those used for the intravenous ecgonine experiment. Also, all materials and equipment used for the assay of plasma and urine for benzoylecgonine levels have also been previously described.

b. Methods

Benzoylecgonine Administration. The dog fasted overnight was dosed at a rate of 10 mg/kg benzoylecgonine as free base. Prior to drug administration a 10 mL control blood sample was drawn. At zero-time, t=0 h, 3.9 mL of drug stock solution containing the required total dose of 157 mg were injected into the cephalic vein of the left foreleg over a period of 10 seconds.

Blood and Urine Collection. A blood sample was drawn at 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 360 min after drug administration. For blood samples up to and including the 90 min sample, 10 mL samples were taken. A volume of 15 mL of blood was collected for each of the five remaining sampling times. Each blood sample was divided into 5 mL portions in sodium fluoride tubes containing heparin. The plasma was separated and stored at −30° C until assayed.

After the 60 min blood sample, the dog was placed into a metabolic cage except during the remaining sampling times. After the last blood sample the dog was allowed food and water ad lib. The animal was kept in the metabolic cage for urine collection. A 0-48 h urine collection was obtained. Any urine remaining on the tray was rinsed into a separate wide-mouthed bottle. The volumes, pH, and specific gravities of the urine collections and rinses were measured prior to filtration and storage at −30° C.

Plasma and Urine Analyses. The benzoylecgonine and ecgonine concentrations in the plasma and urine of the dog receiving benzoylecgonine intravenously were determined by the analytical procedures previously described for the two alkaloids. Blank plasma and blank urine obtained from a separate untreated, fasted dog were used to prepare spiked plasma and spiked urine samples to be used as standards for the concentration calibration curves. These plasma and

urine samples were spiked at concentration levels of 4, 8, and 16 μg each of benzoylecgonine and ecgonine per milliliter. In the case of experimental samples found to contain greater than 16 μg/mL benzoylecgonine, volumes less than 1.0 mL were re-analyzed.

The 0-48 h urine was diluted 1:25 and 1.0 mL samples analyzed for benzoylecgonine and ecgonine. Undiluted 0-48 h urine rinse samples were also analyzed.

Pharmacokinetic Analysis. The plasma concentration-time data obtained in this experiment were analyzed in the same way as that for intravenous ecgonine. Similarly, a semi-log plot of the plasma benzoylecgonine concentration-time data was resolved into its two exponential components to obtain initial estimates of the coefficients and pharmacokinetic parameters for the general biexponential equation (Equation 1). These initial estimates and the size of the dose were used to estimate the remaining pharmacokinetic parameters of Equation 2 which applies to a two-compartment open model with IV drug administration.

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \tag{1}$$

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \tag{2}$$

Final estimates of the parmeters were obtained by nonlinear least-squares fitting (NONLIN) of a theoretical curve to the plasma benzoylecgonine concentration-time data.

The total amount of benzoylecgonine and ecgonine excreted in the urine as well as the fraction of the dose excreted as each compound were calculated.

3. INTRAVENOUS ECGONINE METHYL ESTER

    a.   Materials and Equipment

The ecgonine methyl ester hydrochloride was synthesized and purified as previously described. An ecgonine methyl ester stock solution for injection (67.8 mg/mL) was prepared by dissolving 480 mg of ecgonine methyl ester hydrochloride in 6.0 mL of normal saline.

In the present experiment the same dog, XA59, which received benzoylecgonine intravenously in the last experiment, was after some intervening months, given ecgonine methyl ester intravenously at a dosage rate of 25 mg ecgonine methyl ester base per kilogram body weight. The dog weighed 14.1 kg.

All other materials and equipment used have been previously described.

    b.   Methods

Ecgonine Methyl Ester Administration. Prior to drug administration the dog was fasted overnight. Also, a Longdwell catheter was inserted into the cephalic vein of the left foreleg and a control blood sample (10 mL) was collected. At zero-time, 5.2 mL of the drug stock solution equivalent to 352.5 mg of ecgonine methyl ester base were injected into the lateral saphenous vein of the left hindleg over a period of 10 seconds. The dog received 25 mg/kg ecgonine methyl ester base.

Blood and Urine Collection.   A blood sample was drawn at 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 360 min after drug administration. The plasma was separated and stored at -30° C until assayed as previously described.

The dog was placed in a metabolic cage and urine was collected as for the benzoylecgonine experiment. Twenty-four hour urine collections were made over a 72 hour period. The volumes, pH's, and specific gravities of the urine collections and rinses were measured, which were then filtered and stored in the deep-freeze until assayed.

Plasma Analysis. The concentrations of ecgonine methyl ester and ecgonine in the plasma samples were determined according to the procedures as previously outlined.   At least a 1.0 mL sample was necessary to assay for ecgonine methyl ester. Because of the relatively low concentration of ecgonine methyl ester expected in the 360 min plasma sample, duplicate 1.0 mL plasma samples were extracted,   and the two 7.0 mL aliquots of the cyclohexane:methylene chloride extracts containing the ecgonine methyl ester were pooled and taken to dryness in a single tube.   Ecgonine methyl ester was quantitated as previously described.

The ecgonine remaining in the aqueous phase of each plasma sample after the initial extraction of ecgonine methyl ester was re-extracted into chloroform:methylene chloride:2-propanol after alkalinization with solid K_2CO_3.

This second organic extract was assayed for ecgonine as described previously. Blank plasma samples spiked with the combined ecgonine methyl ester/ecgonine Working Standard (each 20 μg/mL) were used to establish the plasma ecgonine methyl ester and ecgonine concentration calibration curves.

Pharmacokinetic Analysis. A semi-log plot of the natural log of the ecgonine methyl ester concentrations (μg/mL) vs. time at the midpoint of blood collection period was constructed. Initial estimates of the pharmacokinetic parameters for the monoexponential equation (Equation 3) which characterizes a one-compartment open model after intravenous administration were obtained directly from the plot (see Appendix).

$$C = [D/V]\exp(-Kt) \tag{3}$$

Final estimates were obtained by nonlinear fitting of the ecgonine methyl ester plasma concentration-time data by application of the NONLIN computer program.

Urine Analysis. The concentrations of ecgonine methyl ester and ecgonine in the urine collections and rinses were determined according to the methods developed for spiked urine samples. Blank urine spiked with ecgonine methyl ester/ecgonine Working Standard (each 20 μg/mL) served as standards for the urine ecgonine methyl ester and ecgonine concentration calibration curves. The total amounts of each alkaloid and the fractions of the administered dose of ecgonine methyl ester excreted were calculated.

4. INTRAVENOUS COCAINE

a. Materials and Equipment

The same female Beagle dog, XA59, used in the previous experiments was used in the present experiment on intravenous cocaine. The fasting weight of the animal was 15.2 kg.

The cocaine hydrochloride administered was synthesized and purified as previously described. A cocaine stock solution for injection (20 mg/mL) was prepared by dissolving 112.8 mg of cocaine hydrochloride in 5.0 mL normal saline.

All other materials and equipment used have been previously described.

b. Methods

Cocaine Administration. Before dosing the dog was fasted overnight. In preparation for drug administration and blood sampling Longdwell catheters were inserted into the cephalic veins of the right and left forelegs and kept open by instilling heparin lock solution into the catheters. A control blood sample was drawn from the catheter in the left foreleg. At zero-time, t=0 h, the dog was dosed at a rate of 5 mg/kg cocaine base by injecting through the catheter in the right foreleg 3.8 mL of the drug stock solution for injection over a period of 10 seconds. The catheter in the left foreleg was reserved for sampling blood after drug administration.

122

Blood and Urine Collections.   Blood samples (approximately 10 mL each) were drawn at 5, 10, 45, 60, 90, 120, 180, 240, and 360 min following the intravenous administration of cocaine hydrochloride.   All but the last two blood samples were taken through the in-dwelling catheter. To avoid in situ clotting likely to occur in long-implanted venous catheter, the 240 and 360 min blood samples were drawn directly by venipuncture of the lateral saphenous vein of the left hindleg.

Each blood sample was divided between two collection tubes, each containing 200 μL of 4% sodium fluoride solution and 100 μL of 1000 IU/mL heparin solution. The samples were sealed with Parafilm®, inverted several times to mix, and immediately placed on ice.   Immediately after cooling the samples were centifuged for 10 min at 2700 rpm in the refrigerated centrifuge.   The two plasma fractions from the same divided blood sample were pooled in a single Vacutainer tube, frozen on dry ice,   and subsequently stored in the deep-freeze at -30° C.

After the 60 min blood sample the animal was kept in a metabolic cage between sampling times.   Urine was collected in 950 mL wide-mouth bottles containing 100 mL 0.4% sulfuric acid solution.   Two urine collections were made, 0-48 h and 48-96 h, and accompanying rinses were processed for assay as previously described.

Plasma Analysis.   The concentrations of cocaine in the plasma separated from the blood samples were determined by the extraction/GC-NPD procedure with n-PEBE as internal standard already described.   Measured volumes of freshly thawed plasma, 1.0 mL or less, were analyzed.   Triple-distilled water was added to bring the total initial volume of each sample to 2.0 mL.   Blank plasma (2.0 mL) spiked with 100 μL of the cocaine base Working Standards and carried through the entire assay procedure were used to calibrate the gas chromatograph.   The internal calibration allowed a direct measurement of the nanogram level of cocaine per 2 μL of the extracted experimental plasma samples.

Pharmacokinetic Analysis.   The plasma cocaine concentration-time data from the intravenous cocaine experiment was subjected to the same pharmacokinetic analysis as was used for the intravenous ecgonine and benzoylecgonine experiments.   A semi-log plot of the natural log plasma cocaine concentration-time data was resolved into its two exponential components to obtain initial estimates of the coefficients and constants of the general biexponential equation:

$$C = C_1\exp(-\lambda_1 t) + C_2\exp(-\lambda_2 t) \qquad (1)$$

These estimates were used to calculate initial estimates of the pharmacokinetic parameters V_1, k_{12}, k_{21}, and K of Equation 2 for a two-compartment open model with rapid IV input:

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \qquad (2)$$

Final estimates of the several constants were obtained by least-squares fitting of the original plasma cocaine concentration-time data according to Equation 1 and Equation 2 by means of the NONLIN computer program.

Urine Analysis. The concentration of cocaine in the two 48 h urine collections and accompanying rinses was also determined by the extraction/GC-NPD method. Measured volumes (1.0 mL) of the 0-48 h urine collections and the 0-48 h rinse (2.0 mL) were analyzed. Blank urine samples spiked with increasing levels of cocaine base Working Standards were used to to calibrate the. HP 5880.

The concentrations of ecgonine methyl ester, benzoylecgonine, and ecgonine in the 0-48 h urine and accompanying rinse were also determined. A combination of the extraction/TLC/GC-FID procedures previously described for the determination of ecgonine methyl ester, benzoylecgonine, and ecgonine in urine was employed. Measured volumes of urine were diluted, and after perchloric acid treatment and ether washing, ecgonine methyl ester was extracted into cyclohexane:methylene chloride and its concentration determined as previously described. Benzoylecgonine and ecgonine were extracted from the remaining aqueous phases into chloroform:methylene chloride:2-propanol. After solvent evapora-

tion and TLC clean-up of the residues, benzoylecgonine and ecgonine were quantitated by GC-FID as previously described.

The total amount and percent of the dose of cocaine found in the urine as ecgonine methyl ester, benzoyl-ecgonine, and ecgonine in the urine were calculated.

Chapter IV

RESULTS

IV-A.   ANALYTICAL PROCEDURES

1. ANALYSIS OF ECGONINE ALKALOIDS

The melting points of the alkaloids used in the present experiments compared favorably to their literature values: cocaine hydrochloride (197° C), ecgonine hydrochloride (246° C), benzoylecgonine (195° C),   and ecgonine methyl ester (215° C). Gas chromatographic (GC) and thin-layer chromatographic (TLC) analysis of Primary Standard solutions of each of the alkaloids in methanol (200 μg/mL) revealed only single peaks and bands, respectively.

The melting points of the compounds used as internal GC standards, theophylline and the n-propyl ester of benzoylecgonine (n-PEBE), also compared favorably to their literature values: 272° C and 79° C, respectively. The purity of the Primary Standard solutions of each internal standard in methanol was verified by GC-FID analysis.

Gas Chromatography-FID.   A   GC-FID procedure with N,O-Bis-(trimethylsilyl)-acetamide (BSA)   as derivatizing agent was used for the separation and quantitation of co-

caine, ecgonine methyl ester, benzoylecgonine, and ecgonine in the same sample. A typical gas chromatogram obtained from the analysis of a GC standard containing 80 ng of each of the ecgonine alkaloids as well as 120 ng of the internal standard theophylline per microliter injected is given in Figure 4. Ecgonine methyl ester, theophylline, and benzoyl-ecgonine are present as their trimethylsilyl (TMS) deriva-tives. Ecgonine is present as the (TMS)$_2$ derivative (car-boxyl and hydroxyl groups are easily silylated with BSA according to the manufacturer). Cocaine remains underiva-tized under the reaction conditions employed. The retention times for the peaks corresponding to ecgonine methyl ester, ecgonine, theophylline, cocaine, and benzoylecgonine were 3.6, 4.6, 7.1, 10.7, and 11.6 min, respectively.

Theophylline was used as internal standard and BSA as derivatizing agent in the GC-FID method for quantifying the ecgonine alkaloids in the studies on the efficiency of sev-eral organic solvent systems in extracting the alkaloids from various aqueous buffers.

For all subsequent experiments n-PEBE replaced theo-phylline as the internal standard and N,O-Bis-(trimethyl-silyl)-trifluoroacetamide (BSTFA) replaced BSA as derivatiz-ing agent. A typical gas chromatogram obtained from the analysis of a GC standard containing 80 ng of each of the four ecgonine alkaloids and 100 ng of the internal standard n-PEBE per microliter injected is given in Figure 5. Ecgo-
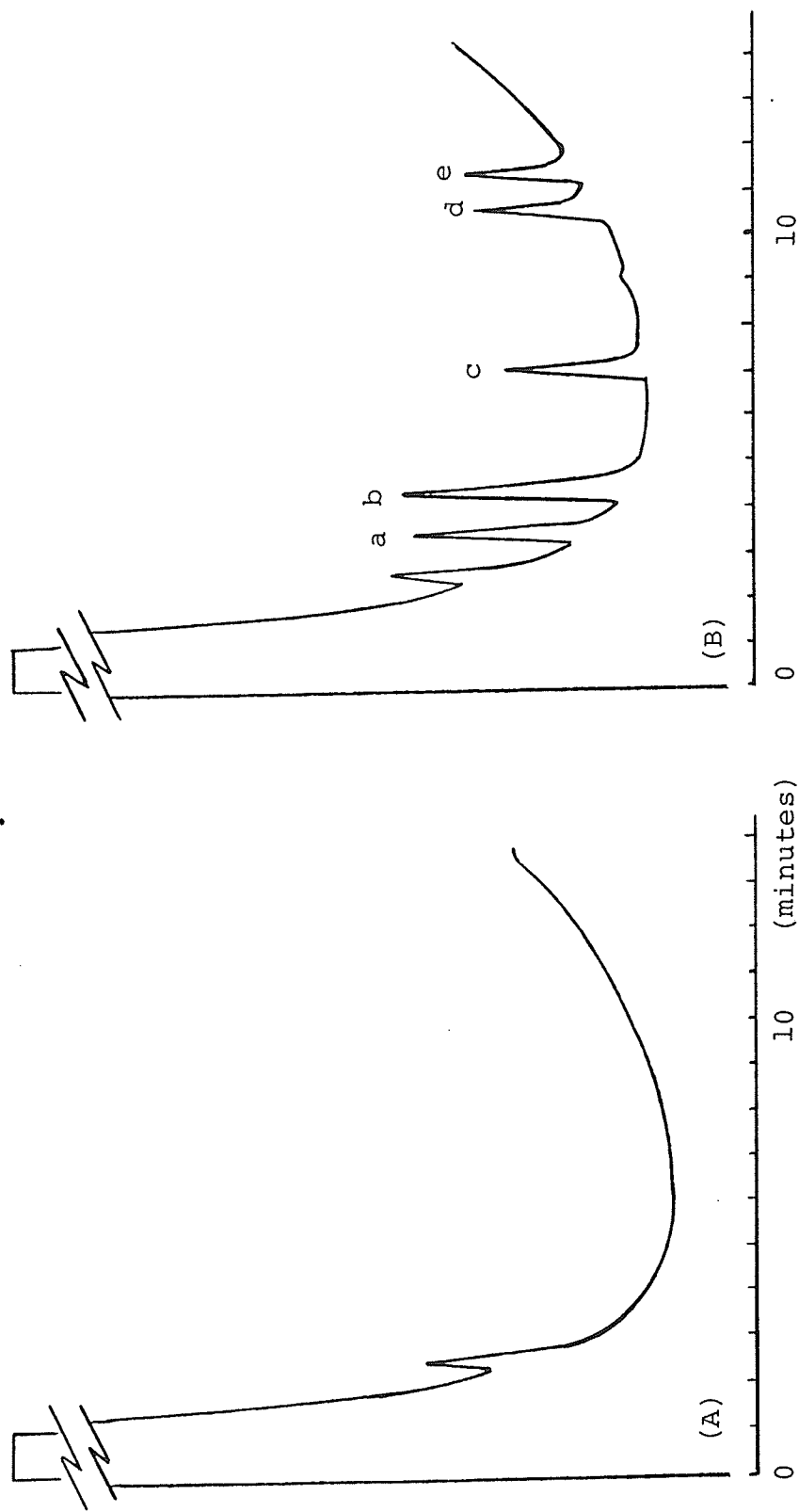
Figure 4: Gas chromatographic analysis of BSA-derivatized ecgonine alkaloids on 10% OV-101 on Gas Chrom Q.

Key: (A) N,O-Bis-(trimethylsilyl)-acetamide (BSA) blank; (B) GC standard containing 80 ng of each ecgonine alkaloid and 120 ng theophylline as internal standard per μL injected.
a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = theophylline-TMS, d = cocaine, e = benzoylecgonine-TMS
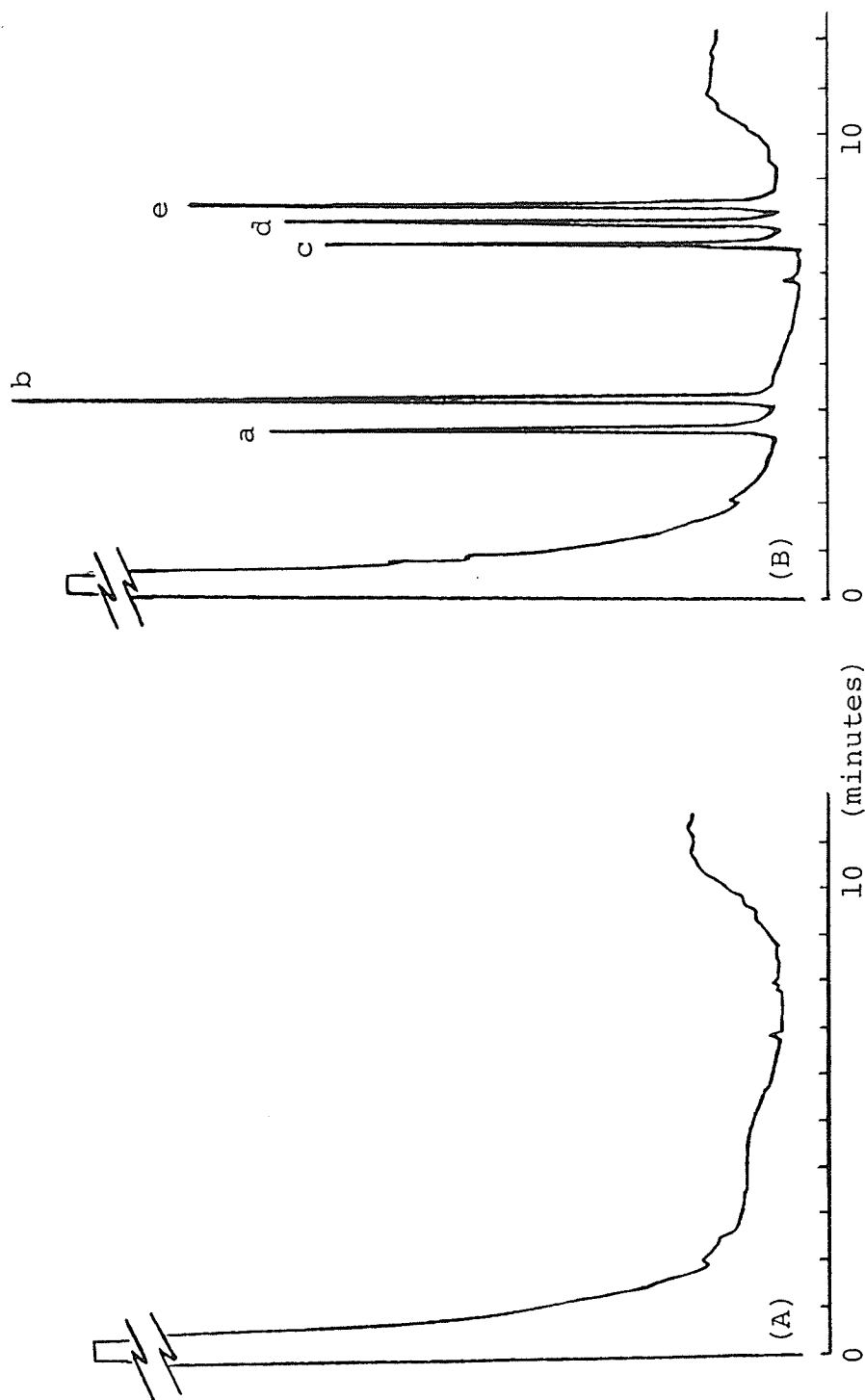
Figure 5: Gas chromatographic analysis of BSTFA-derivatized ecgonine alkaloids on 3% SP-2100 on Supelcoport.

Key: (A) N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) blank; (B) GC standard containing 80 ng of each alkaloid and 100 ng n-PEBE as internal standard per μL injected.
a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = cocaine, d = benzoylecgonine-TMS, e = n-propyl ester of benzoylecgonine

nine methyl ester and benzoylecgonine are present as their
TMS derivatives. Ecgonine is present as the $(TMS)_2$ deriva-
tive. Cocaine and n-PEBE are not derivatized by BSTFA under
the conditions described. The retention times for ecgonine
methyl ester, ecgonine, cocaine, benzoylecgonine, and n-PEBE
were 3.6, 4.3, 7.9, 8.2, and 8.6 min, respectively. Gas
chromatograms of samples prepared with BSTFA as derivitazing
agent and injection solvent were consistently cleaner than
those prepared with BSA.

GC-FID Standard Calibration Curves. GC standard cali-
bration curves constructed from data obtained by GC-FID
analysis of Working Standard solutions of the four ecgonine
alkaloids were used as primary references for quantitating
the amount of each ecgonine alkaloid in all samples analyzed
by GC-FID.

GC standards containing all four ecgonine alkaloids
were prepared and analyzed by GC-FID in order to evaluate
the sensitivity and linearity of the method. Separate GC
standard calibration curves were constructed for each alka-
loid by plotting the peak height ratio (PHR) of alkaloid
peak height to internal standard peak height vs. the corre-
sponding amounts of alkaloid injected. Examples of GC stan-
dard calibration curves for cocaine, ecgonine methyl ester,
benzoylecgonine, and ecgonine with theophylline as internal
standard are shown in Figure 6. Each data point on each GC
standard calibration curve represents the mean ± SD of four
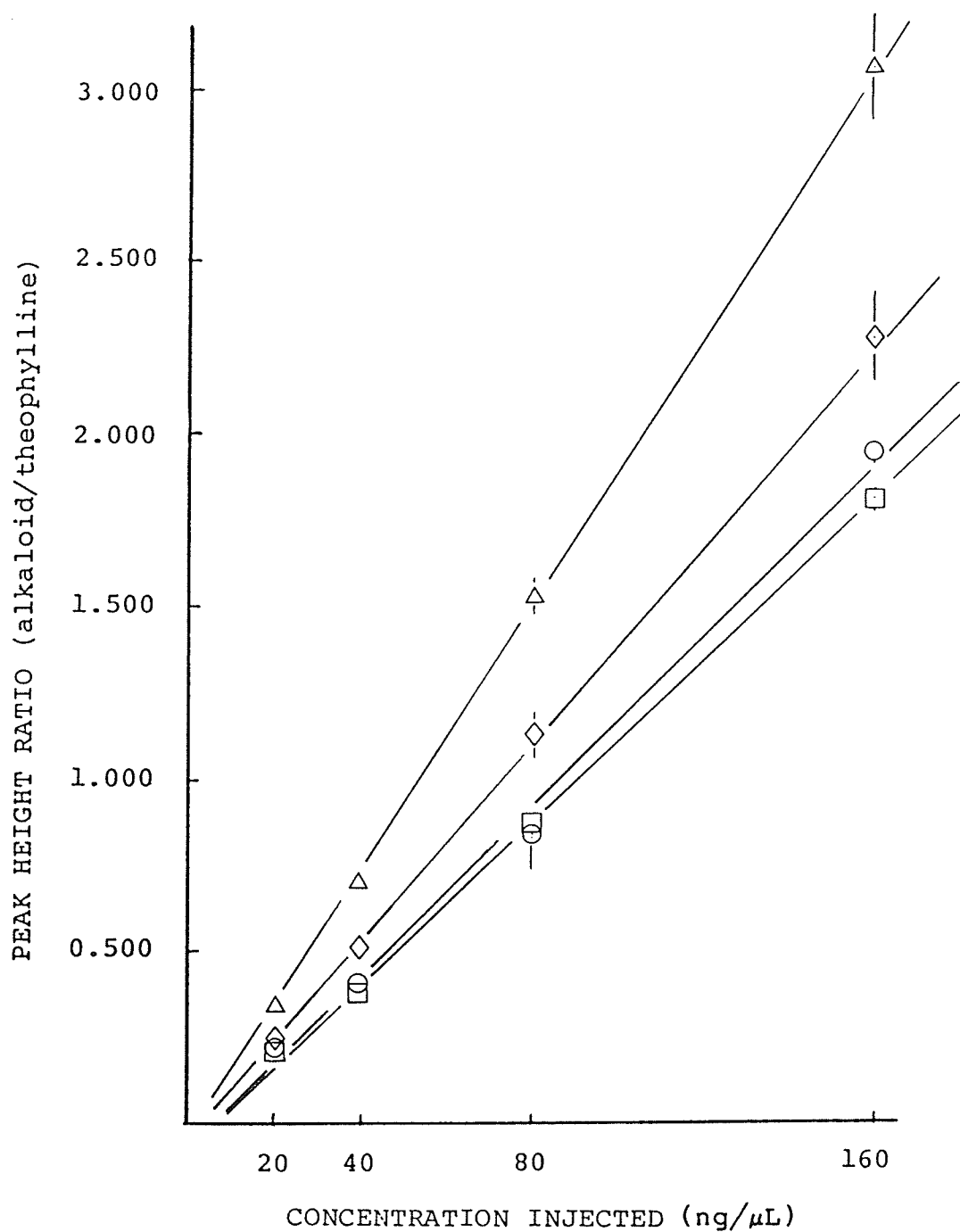
Figure 6:   GC concentration calibration curves with theo-
   phylline as internal standard.   (Derivatizing agent:
   BSA, GC column: 10% OV-101 on Gas Chrom Q.)

   Key: (◇) ecgonine methyl ester; (△) ecgonine; (□) co-
   caine; and (○) benzoylecgonine.

replicate GC standards. Linearity was excellent over the range of concentrations examined. The correlation coefficients, r, of the least-squares linear regression calculated for calibration curves for ecgonine methyl ester, ecgonine, cocaine, and benzoylecgonine were 0.996, 0.997, 0.999, and 0.995, respectively.

As internal standard, n-PEBE more consistently gave reproducible peak heights than theophylline. A GC standard calibration curve for each of the ecgonine alkaloids with n-PEBE as internal standard based on peak height ratio, alkaloid/n-PEBE, vs. nanograms alkaloid per microliter injected is presented in Figure 7. Each data point on each GC standard calibration curve represents the mean ± SD of four replicate samples of GC standard analyzed. The linearity of each curve over the range of concentrations was excellent with correlation coefficients, r, of 0.999, 0.999, 0.999, and 0.998 for ecgonine methyl ester, ecgonine, cocaine, and benzoylecgonine, respectively.

Extraction Studies. The percent(%) recoveries for the extraction of the ecgonine alkaloids into the separate solvent systems: chloroform, methylene chloride, cyclohexane, chloroform:cyclohexane, methylene chloride:cyclohexane, and chloroform:methylene chloride:2-propanol are depicted graphically in Figures 8, 9, 10, 11, 12, and 13, respectively. Each data point represents a single pH/solvent combination tested.
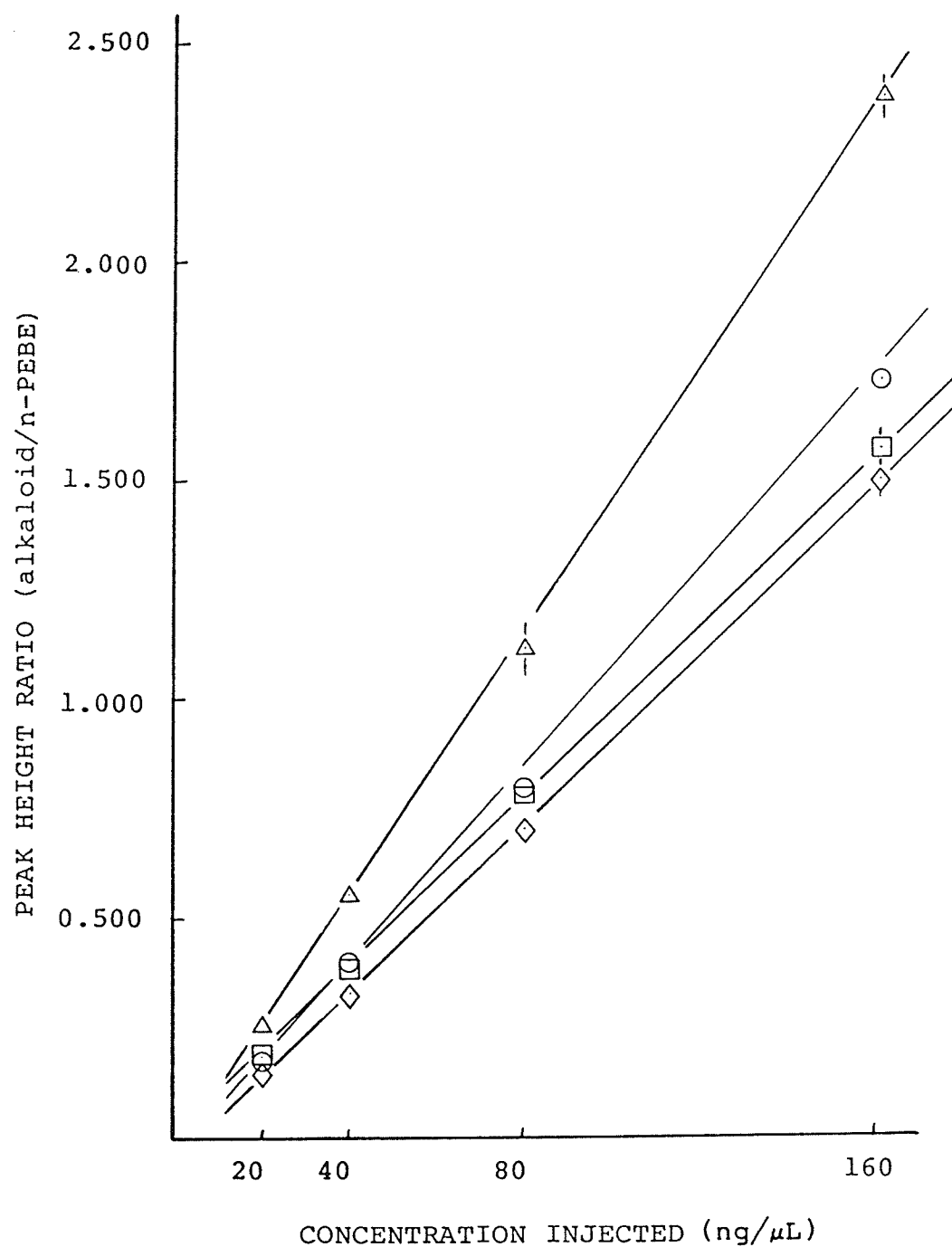
Figure 7: GC concentration calibration curves with n-propyl ester of benzoylecgonine (n-PEBE) as internal standard. (Derivatizing agent: BSTFA, GC column: 3% SP-2100 on Supelcoport.)

Key: (◇) ecgonine methyl ester; (△) ecgonine; (□) cocaine; and (○) benzoylecgonine.

Chloroform was able to extract 90-100% of cocaine at pH 7 through 11 and 90-100% of ecgonine methyl ester at pH 9 and 10 (Figure 8). Extraction of benzoylecgonine was considerably lower. Between pH 7 and 10, 50-60% was extracted; at pH 11 the extraction fell to ca. 30%. Ecgonine was not extracted into chloroform under the conditions tested.

The extraction of cocaine into methylene chloride was consistently 95-100% over the range of pH 6 to 11 (Figure 9). Ecgonine methyl ester was not extracted from the pH 6 buffer. The extraction of ecgonine methyl ester increased from 30% at pH 7 to a maximum of 75-80% between pH 9 and 11. The recovery of benzoylecgonine extracted with methylene chloride was 50-60% over the range of pH examined. Ecgonine was not extracted with methylene chloride.

Cocaine and ecgonine methyl ester exhibited variable extraction into cyclohexane (Figure 10). The recovery of cocaine varied between 75 and 90% at pH 7 to 10 and increased to ca. 100% at pH 11. Ecgonine methyl ester was not extracted at pH 7 or 8. The recovery of ecgonine methyl ester increased from 19% at pH 9 to 29% at pH 11. Both benzoylecgonine and ecgonine were not extracted into cyclohexane.

Chloroform:cyclohexane (1:1) was able to extract 90-100% of cocaine at pH 6 to 10 (Figure 11). Ecgonine methyl ester was not extracted at pH 6 or 7. Recovery of
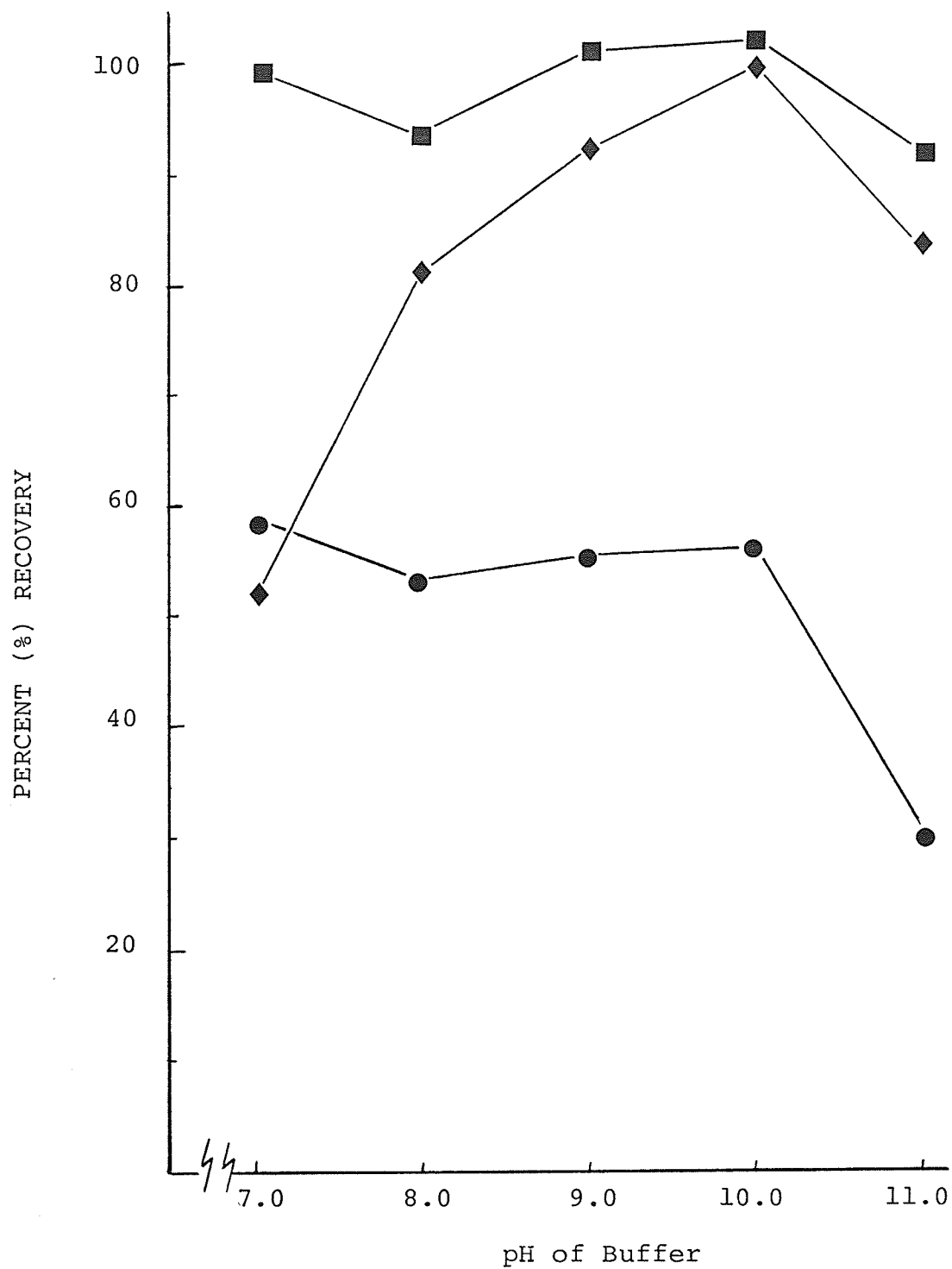
Figure 8:   Recovery of ecgonine alkaloids extracted from
   aqueous buffers, pH 7.0 - 11.0, into chloroform.

   Key: (♦) ecgonine methyl ester; (■) cocaine; and (●)
   benzoylecgonine. (Ecgonine not extracted.)

136
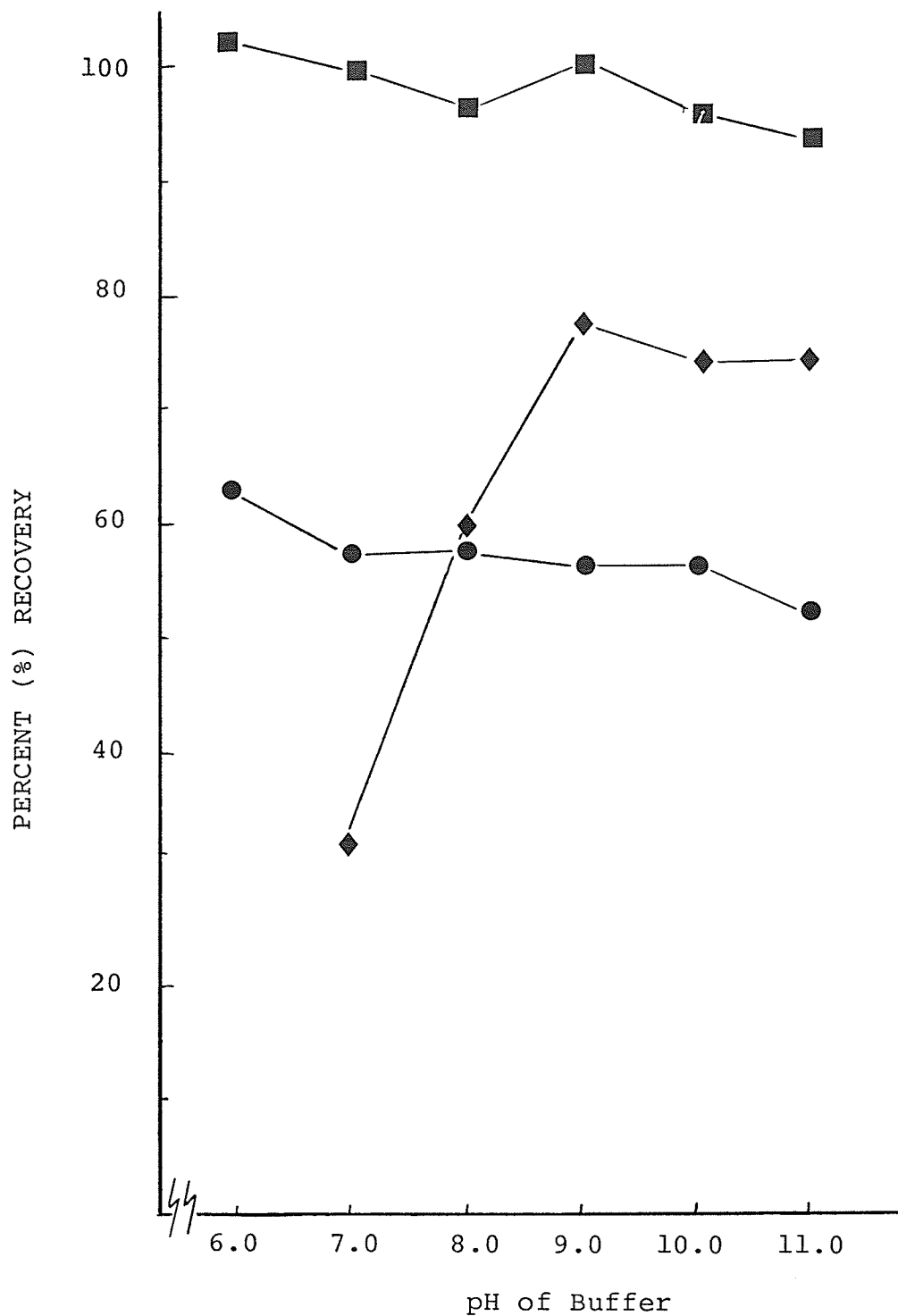
Figure 9:   Recovery of ecgonine alkaloids extracted from
  aqueous buffers, pH 6.0 - 11.0, into methylene chloride.

  Key: (◆) ecgonine methyl ester; (■) cocaine; and (●)
  benzoylecgonine. (Ecgonine not extracted.)
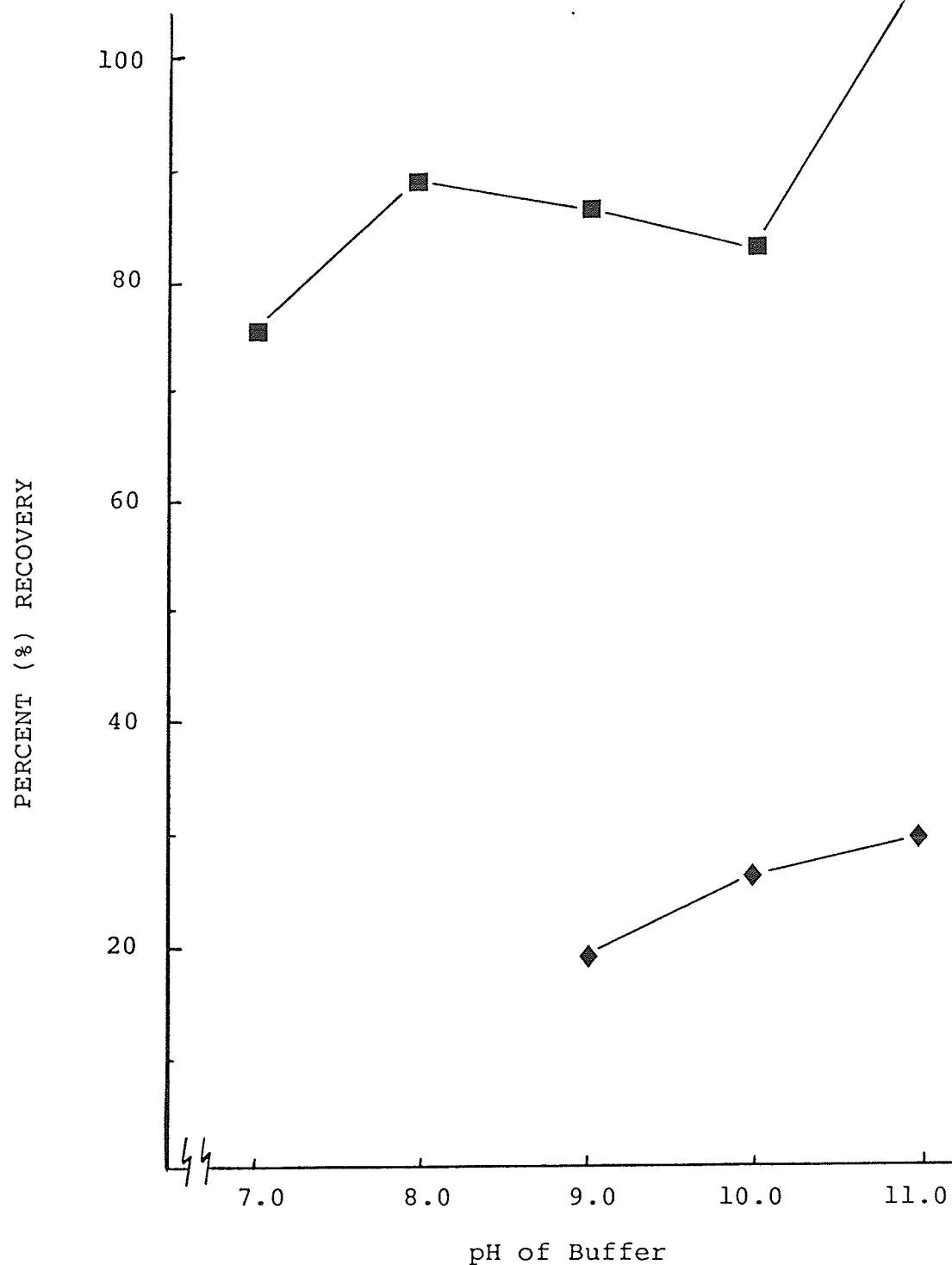
Figure 10:   Recovery of ecgonine alkaloids extracted from
    aqueous buffers, pH 7.0 – 11.0, into cyclohexane.

    Key: (◆)   ecgonine methyl ester; (■)   cocaine.
    (Benzoylecgonine and ecgonine not extracted.)
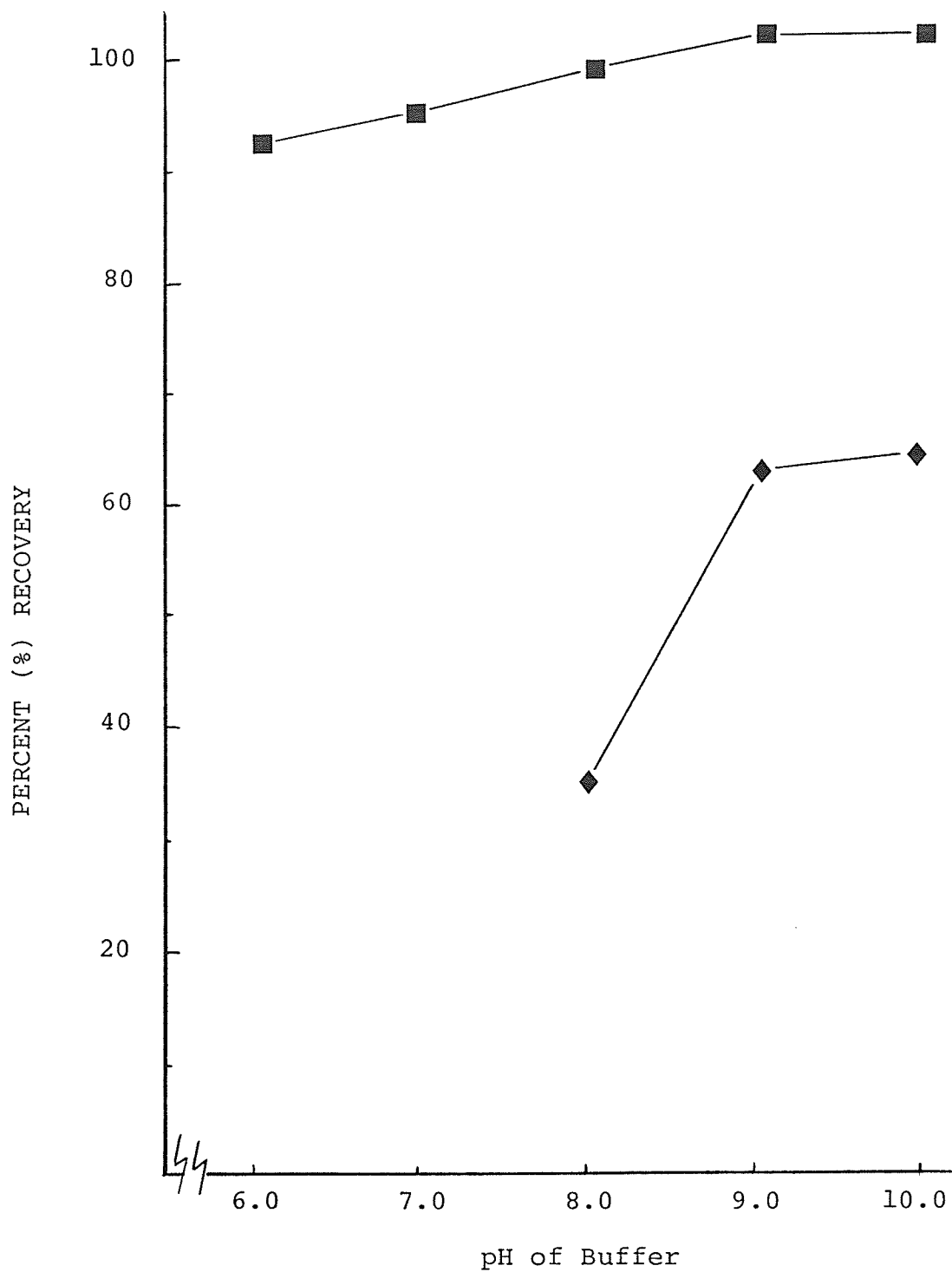
Figure 11:   Recovery of ecgonine alkaloids extracted from
   aqueous buffers, pH 6.0 - 10.0, into chloroform:cyclohex-
   ane, 1:1.

   Key: (◆)   ecgonine methyl ester; (▦)   cocaine.
   (Benzoylecgonine and ecgonine not extracted.)

ecgonine methyl ester increased from 36% at pH 8 to a maximum of 64% at pH 9 and 10. Benzoylecgonine and ecgonine were not extracted.

The recovery of cocaine extracted from aqueous buffers pH 6-10 into methylene chloride:cyclohexane (1:1) was 90-100% (Figure 12). Ecgonine methyl ester was not extracted at pH 6 or 7, but its recovery increased from 35% at pH 8 to a maximum of 74% at pH 10. Benzoylecgonine and ecgonine were not extracted into methylene chloride:cyclohexane from aqueous buffers pH 6 to 10.

The mixture of chloroform:methylene chloride:2-propanol (Figure 13) was able to extract 95-100% of cocaine at pH 6 to 11 (Figure 13). The recovery of benzoylecgonine was slightly lower, approximately 90% throughout the pH range of 6 to 11. The recovery of ecgonine methyl ester was 20% at pH 6 increasing to 70-75% at pH 8 to 11. No ecgonine was extracted at any pH tested.

Thin-Layer Chromatography. The present TLC procedure was quite adequate in separating and isolating ecgonine and benzoylecgonine from other co-extractable ecgonine alkaloids and impurities in plasma and urine extracts. A diagram of a Ludy Tenger's reagent-sprayed TLC reference plate is given in Figure 14. Ecgonine and benzoylecgonine are shown as each would appear, as colored-visualized bands. Also indicated are the separate broader areas of silica associated
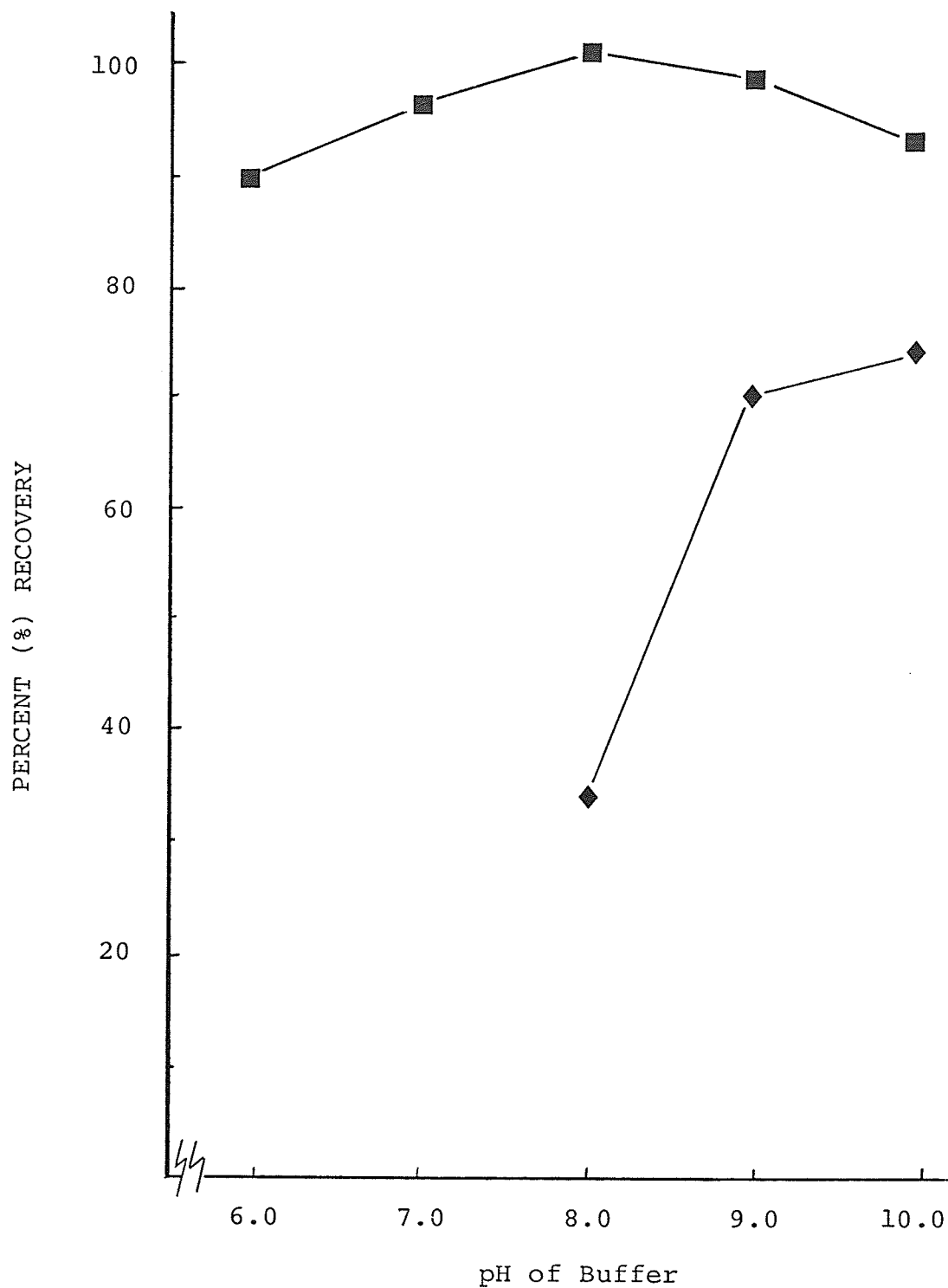
Figure 12: Recovery of ecgonine alkaloids extracted from aqueous buffers, pH 6.0 - 10.0, into methylene chloride:cyclohexane, 1:1.

Key: (◆) ecgonine methyl ester; (■) cocaine. (Benzoylecgonine and ecgonine not extracted.)

141
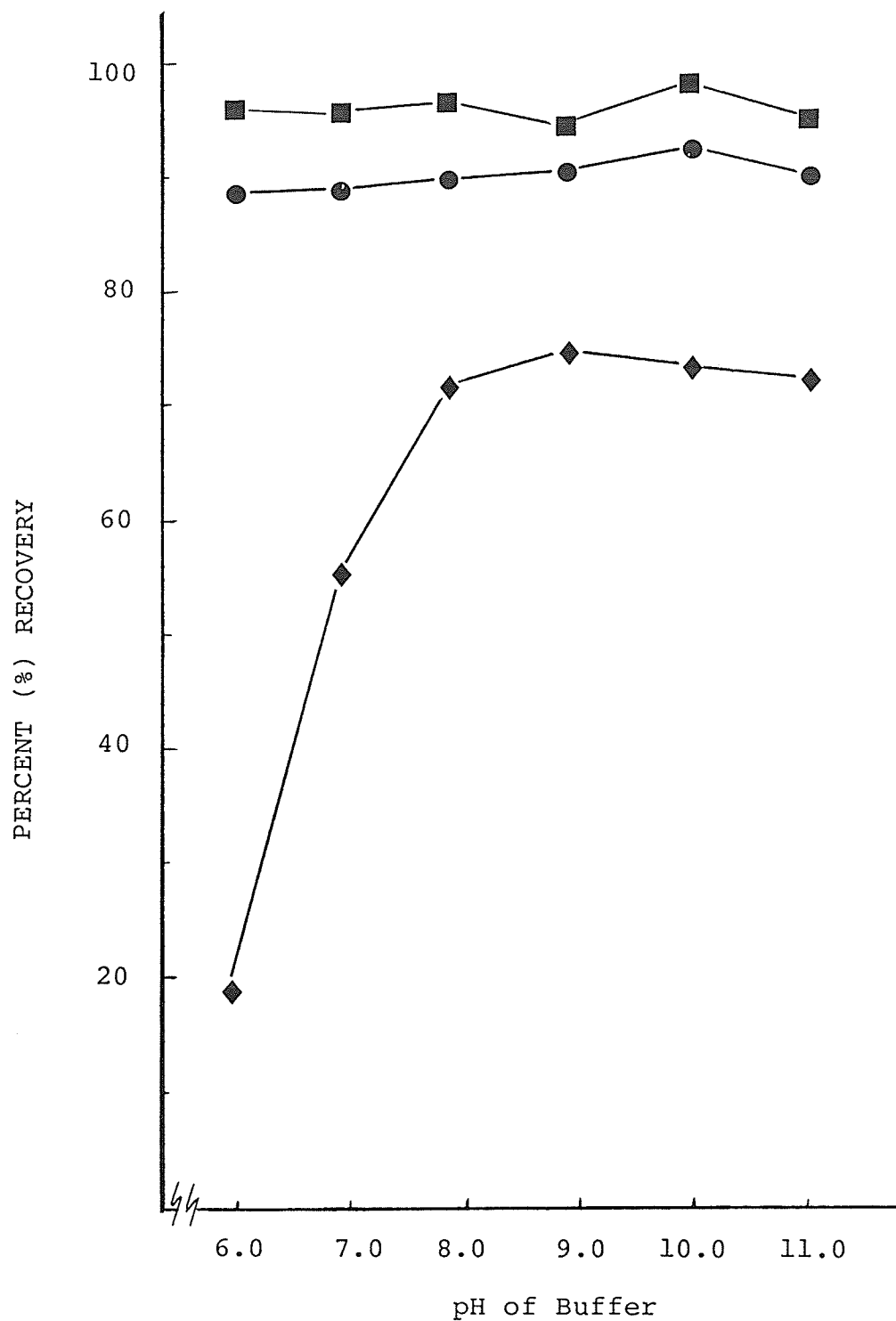
Figure 13:   Recovery of ecgonine alkaloids extracted from
    aqueous buffers, pH 6.0 - 11.0, into chloroform:methylene
    chloride:2-propanol, 1:1:1.

    Key: (◆) ecgonine methyl ester; (■) cocaine; and (●)
    benzoylecgonine. (Ecgonine not extracted.)
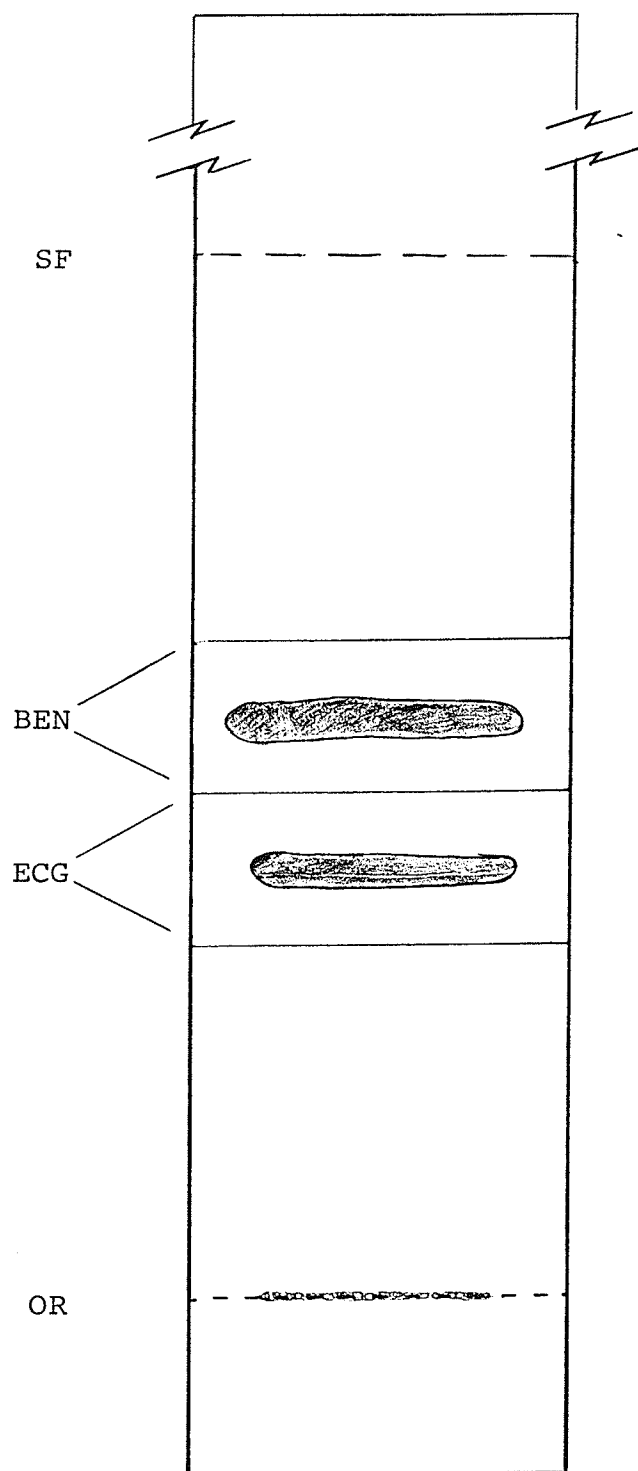
Figure 14:   Separation of ecgonine and benzoylecgonine on
   TLC reference plate, each streaked at a load of 50 μg.

   Key: (SF) solvent front; (OR) origin;   (ECG) silica area
   with ecgonine band;   and (BEN) silica area with benzoyl-
   ecgonine band.

with each alkaloid band which are removed from test plates for the isolation and quantitation of individual alkaloids in unknown samples. The orange-colored benzoylecgonine band appeared almost immediately on spraying while the orange-brown color of the ecgonine band required a few minutes to fully develop. The slightly broader benzoylecgonine band migrated 76 mm from the origin in 75 minutes while the narrower ecgonine band migrated only 56 mm from the origin in the same time. The two bands were well separated with approximately 2.0 cm between them.

The percent (%) recoveries of ecgonine and benzoylecgonine eluted from developed TLC test plates previously streaked with varying known amounts of each alkaloid were calculated. The mean recovery data are given in Table IV. Individual recoveries ranged from 62-97% for ecgonine and 79-97% for benzoylecgonine.

Table IV: Percent Recovery[a] of Ecgonine and Benzoylecgonine
    Eluted from Silica of Developed TLC Test Plates.

Amount (μg)	Ecgonine (%)	Benzoylecgonine (%)
4.0	62.86 ± 1.75	87.77 ± 12.74
8.0	77.91 ± 3.03	83.47 ± 6.93
16.0	93.95 ± 4.86	88.29 ± 0.47

[a]$\bar{X}$ ± SD.

2. DETERMINATION OF ECGONINE IN WATER, PLASMA, AND URINE

Extraction/TLC/GC-FID Procedure.   An analytical procedure was successfully developed for the determination of ecgonine in plasma and urine.   Initial experiments carried out with water samples spiked with ecgonine hydrochloride helped establish baseline recovery data.

Spiked Water Samples.   Individual water samples spiked with ecgonine were carried through the entire extraction/ TLC/GC-FID procedure.   Typical gas chromatograms of GC-FID analysis of water samples are given in Figure 15.   There was minimal interference from extraneous peaks with the ecgonine and the internal standard (n-PEBE) peaks.

The amount of ecgonine recovered was calculated by comparing the peak height ratios, ecgonine/n-PEBE, of analyzed spiked water samples with the peak height ratios established for the ecgonine GC standard calibration curve.   The recovery of ecgonine from individual spiked water samples ranged from 65-77% as given in Table V.

Spiked Plasma Samples.   Similarly, individual plasma samples were spiked with ecgonine hydrochloride and carried through the extraction/TLC/GC-FID procedure.   Typical gas chromatograms obtained from the analysis of plasma samples spiked with ecgonine are presented in Figure 16.   There was minimal interference from extraneous peaks with the ecgonine and the n-PEBE peaks.
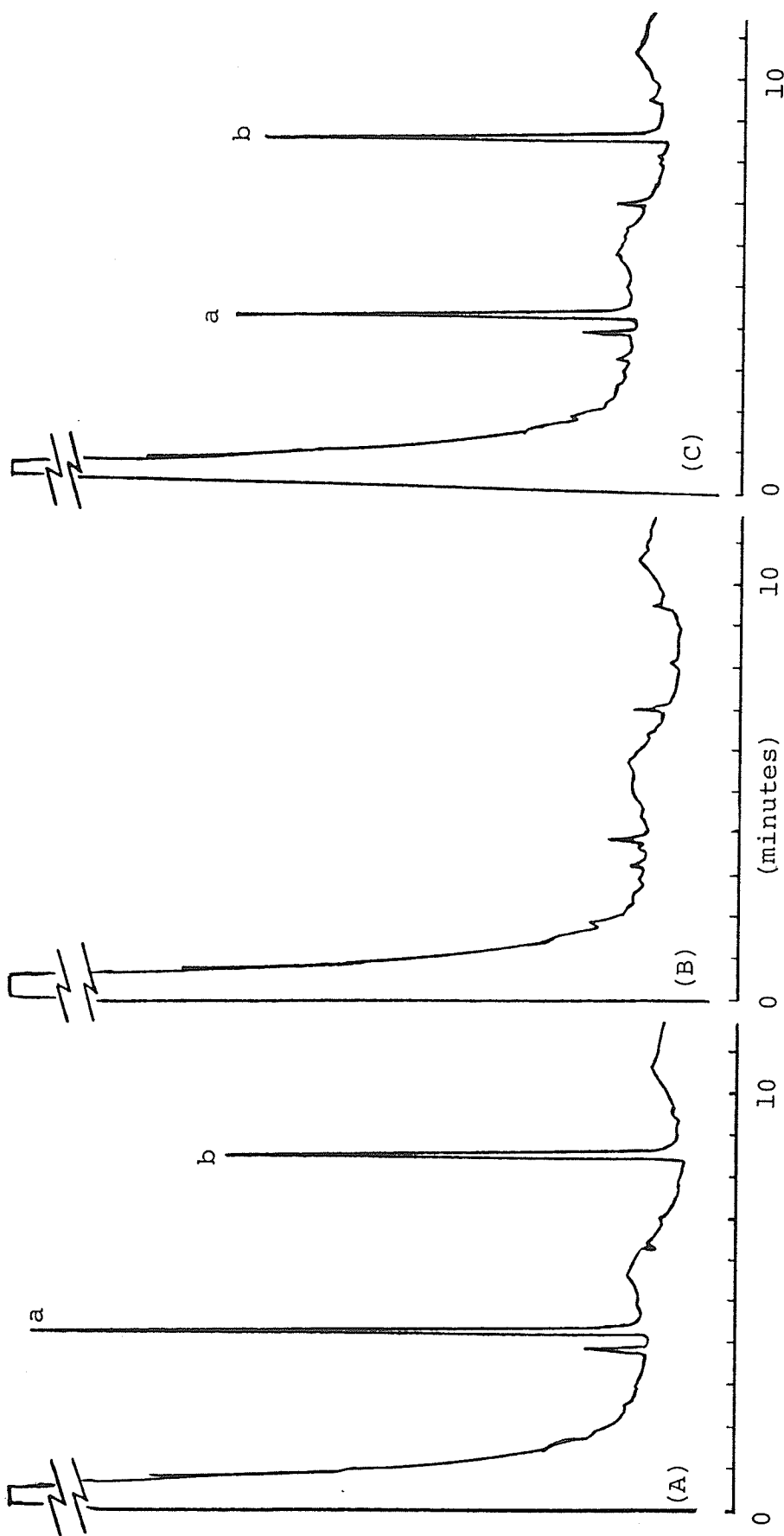
Figure 15: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine-spiked water samples.

Key: (A) GC standard containing 80 ng ecgonine and 100 ng n-PEBE as internal standard per µL injected; (B) ecgonine-free water sample with no n-PEBE; and (C) water sample spiked with ecgonine (8.0 µg/mL), and with n-PEBE as internal standard. a = ecgonine-(TMS)$_2$, b = n-PEBE

Table V: Percent Recovery[a] of Ecgonine from Spiked Water, Plasma, and Urine Samples.

Initial Ecgonine Conc. (μg/mL)	Recovery from:		
	Water (%)	Plasma (%)	Urine[b] (%)
4.0	66.87 ± 3.03	65.55 ± 6.14	73.38 ± 7.79
8.0	65.18 ± 0.71	68.69 ± 6.80	65.91 ± 1.97
16.0	75.12 ± 2.79	73.60 ± 8.94	71.89 ± 2.43

[a]$\bar{X}$ ± SD.

[b]Diluted urine, 1:50
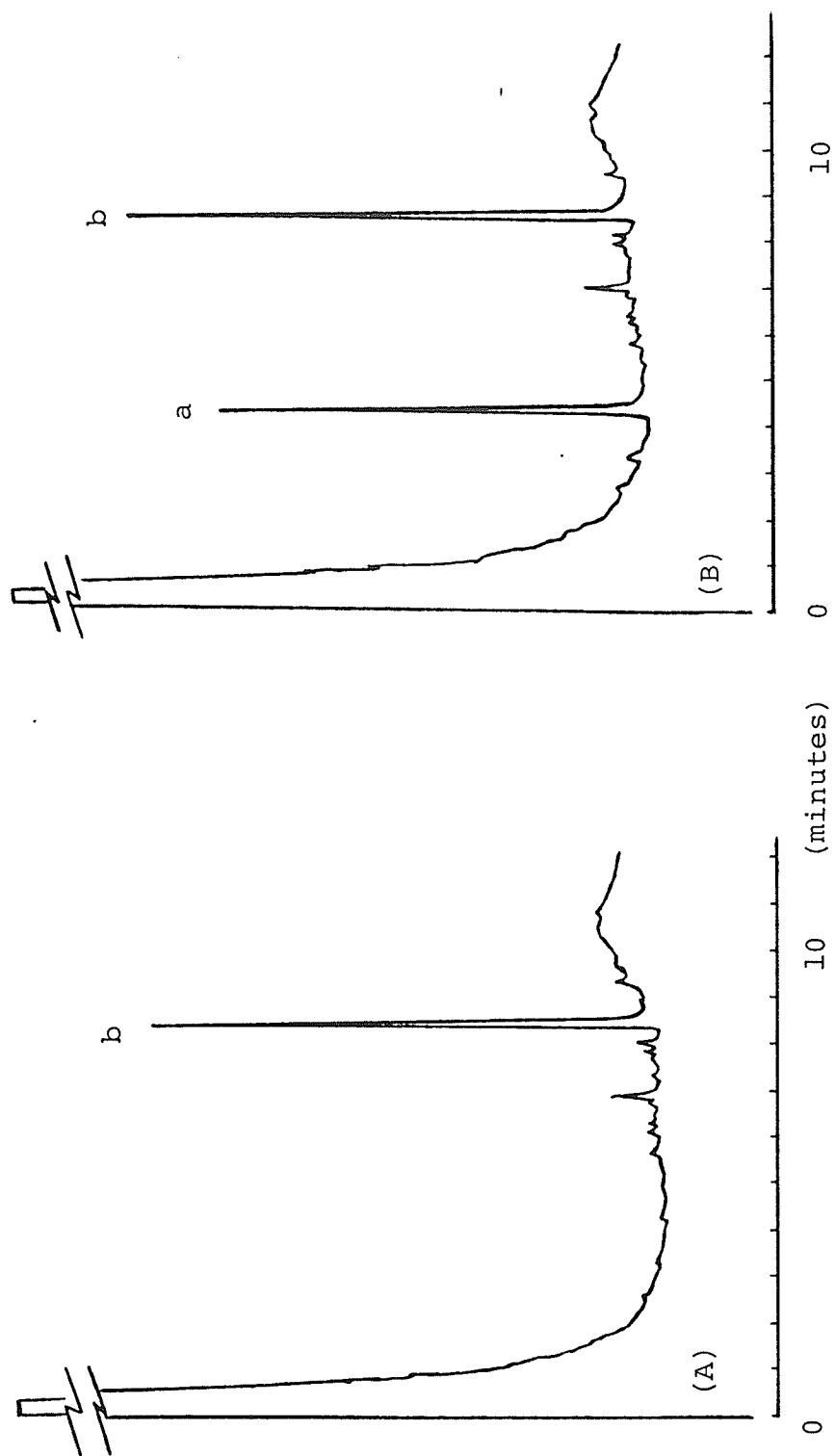
Figure 16: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine-spiked plasma samples.

Key: (A) ecgonine-free plasma sample with only n-PEBE; (B) plasma sample spiked with ecgonine (8.0 µg/mL), and with n-PEBE as internal standard.
a = ecgonine-(TMS)$_2$, b = n-PEBE

The recovery of ecgonine from individual spiked plasma samples ranged from 58-86%. The mean recovery data are given in Table V.

Spiked Urine Samples. Individual samples of urine first diluted with triple-distilled water were spiked with ecgonine hydrochloride and carried through the entire extraction/TLC/GC-FID procedure in order to estimate the recovery of ecgonine from urine. Gas chromatograms obtained from the analysis of urine samples are shown in Figure 17. The ecgonine and n-PEBE peaks were virtually free of any significant interference by background noise from impurities.

The recovery of ecgonine from individual spiked urine samples ranged from 65-79%. The mean recovery data are presented in Table V.

Experimental (In Vivo) Plasma and Urine Samples. The concentrations of ecgonine in dog plasma and urine samples obtained after intravenous administration of 10 mg/kg ecgonine were determined according to the extraction/TLC/GC-FID method. Examples of gas chromatograms from the analysis of experimental plasma and urine samples are presented in Figure 18. The tracings showing well-defined ecgonine and n-PEBE peaks were similar to those obtained from the analysis of spiked plasma and urine samples.
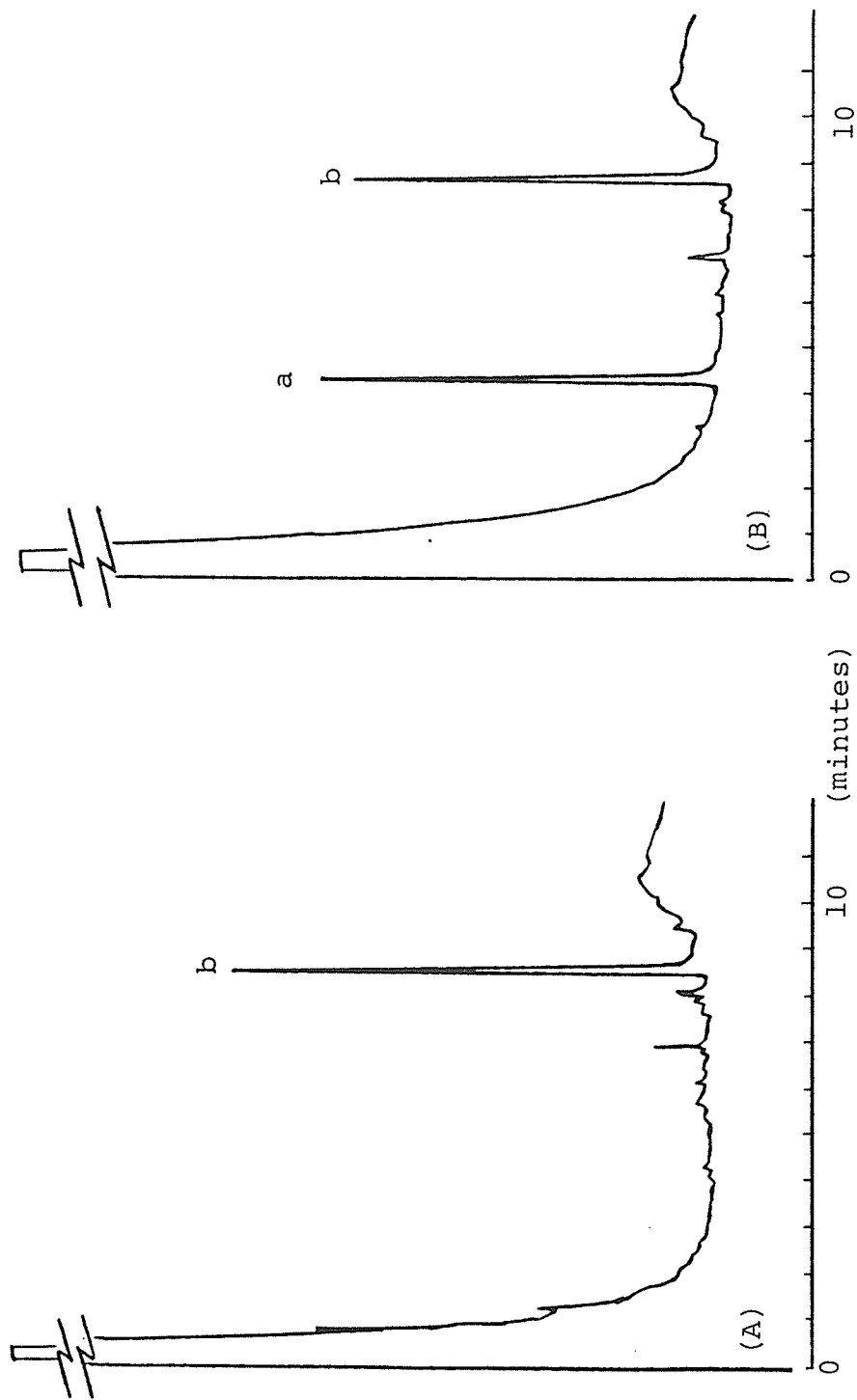
Figure 17: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine-spiked urine samples.

Key: (A) ecgonine-free diluted urine (1:25) sample with only n-PEBE; (B) diluted urine (1:50) sample spiked with ecgonine (8.0 μg/mL), and with n-PEBE as internal standard. a = ecgonine-(TMS)₂, b = n-PEBE
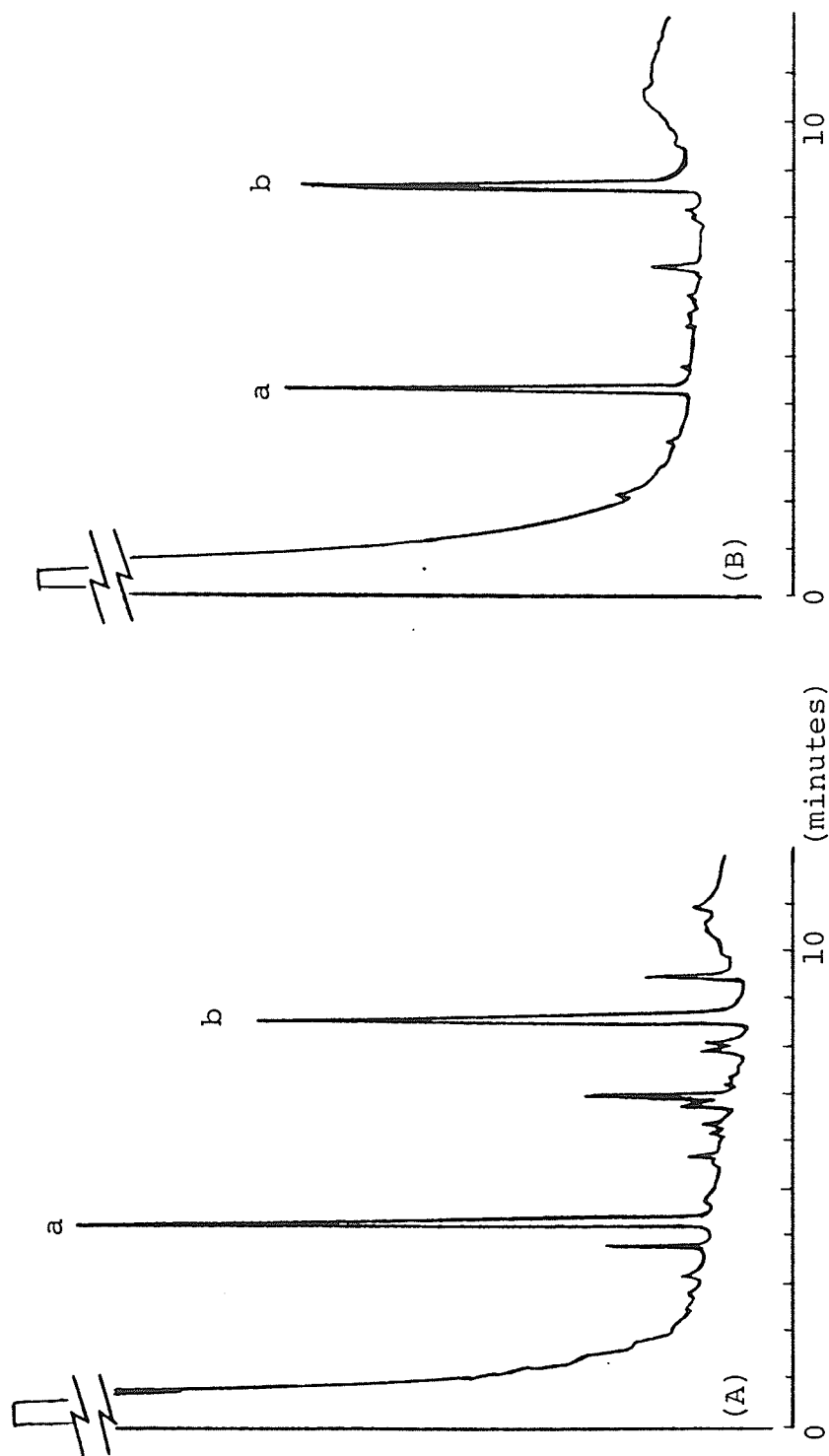
Figure 18: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of dog plasma and urine samples obtained after intravenous administration of a single dose of ecgonine, 10 mg/kg, to a female Beagle dog.

Key: (A) plasma sample, t=2.02 h; (B) diluted urine (1:50) sample, 0-48 h. a = ecgonine-$(TMS)_2$, b = n-PEBE

152

Ecgonine-spiked plasma samples served as standards for the ecgonine concentration calibration curves used to determine the levels of ecgonine in experimental (in vivo) plasma samples. The curve obtained by least-squares linear regression analysis of the peak height ratio, ecgonine/n-PEBE, vs. μg ecgonine per mL of spiked plasma is given in Figure 19. The ecgonine concentration calibration curves was linear over the range of concentrations examined. The correlation coefficients, r, of the least-squares regression line for spiked plasma samples was 0.990.

Similarly, as for the plasma samples, the ecgonine concentration calibration curve for determining the levels of ecgonne in experimental (in vivo) urine samples was based on ecgonine-spiked urine samples serving as standards. The ecgonine concentration calibration curve shown in Figure 20 was linear over the concentration range examined. The correlation coefficient, r, of the least squares regression line for spiked urine samples was 0.996.
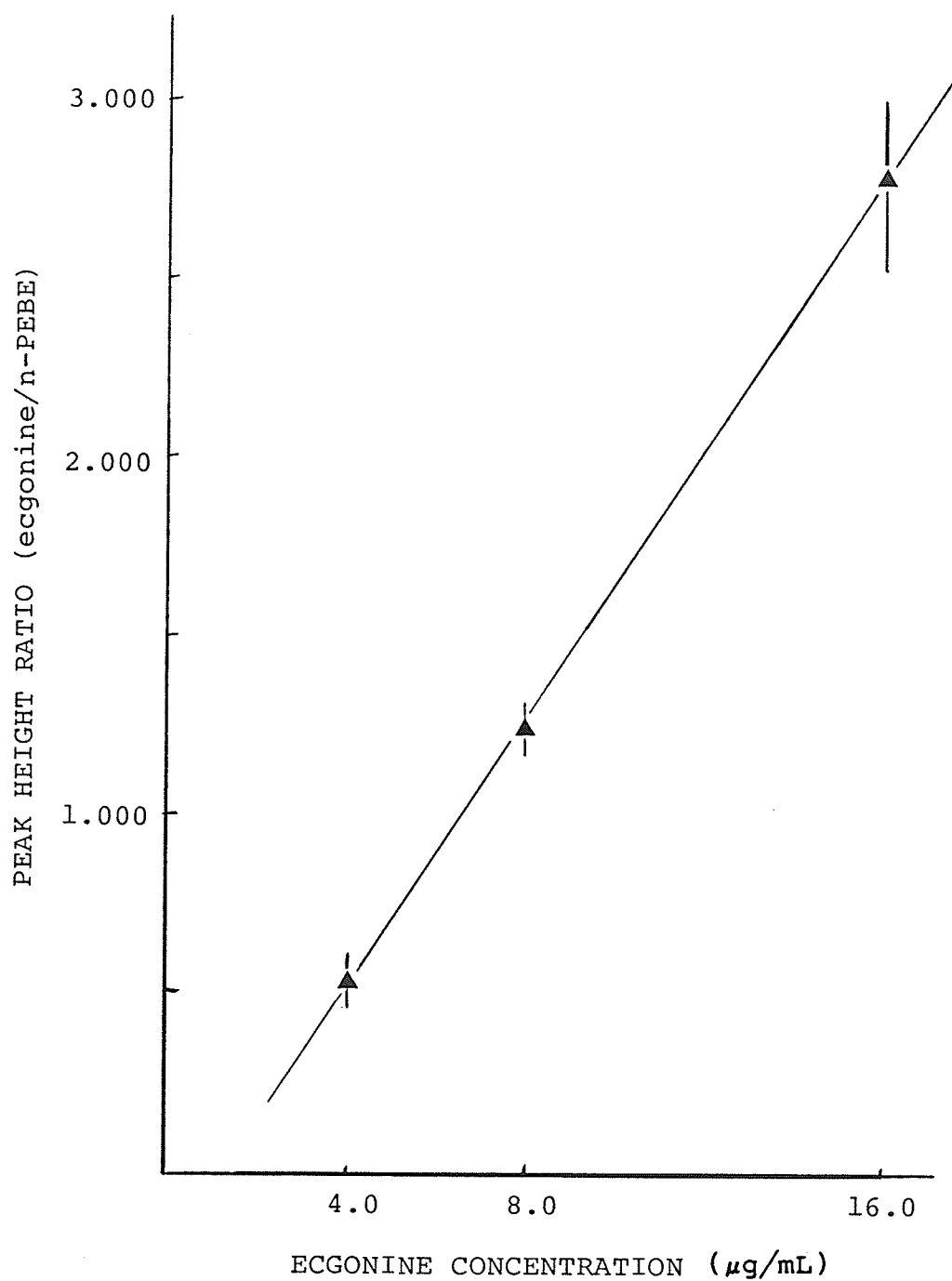
Figure 19: GC concentration calibration curve for the determination of ecgonine in plasma.
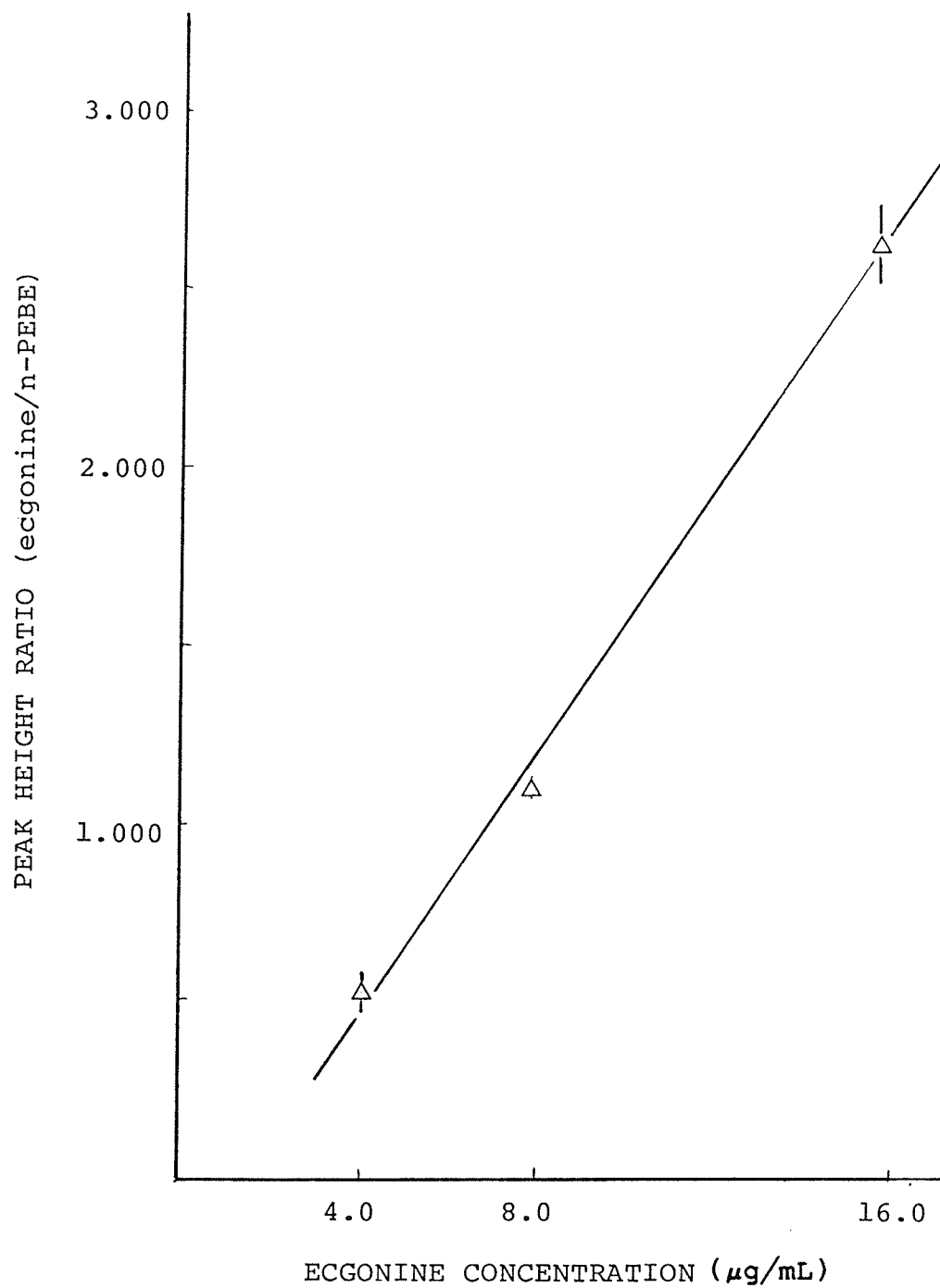
Figure 20:   GC concentration calibration curve for the de-
   termination of ecgonine in diluted urine (1:50).

3.   DETERMINATION OF BENZOYLECGONINE AND ECGONINE IN WATER,

PLASMA, AND URINE

Extraction/TLC/GC-FID Procedure. A suitable analytical procedure was developed for the determination of benzoyl-ecgonine and ecgonine in plasma and urine.

Spiked Water Samples. Initial experiments were carried out with water samples spiked with both benzoylecgonine and ecgonine, but the extraction/TLC/GC-FID assay procedure was applied to measure benzoylecgonine content alone. Gas chromatograms obtained from the analysis of spiked water samples for benzoylecgonine are given in Figure 21. Interference with peaks corresponding to benzoylecgonine and to the internal standard n-PEBE was minimal. There was no evidence of a spurious ecgonine peak.

The total amount of benzoylecgonine recovered from each spiked water sample was calculated by comparing the peak height ratio, benzoylecgonine/n-PEBE, with the peak height ratios of GC standards used to prepare a benzoylecgonine GC calibration curve. The percent (%) benzoylecgonine recovered from indiviudal spiked water samples ranged from 67-82%. The mean percent recoveries of benzoylecgonine from spiked water samples are given in Table VI.

In a separate experiment, the possible conversion of benzoylecgonine to ecgonine by chemical hydrolysis during
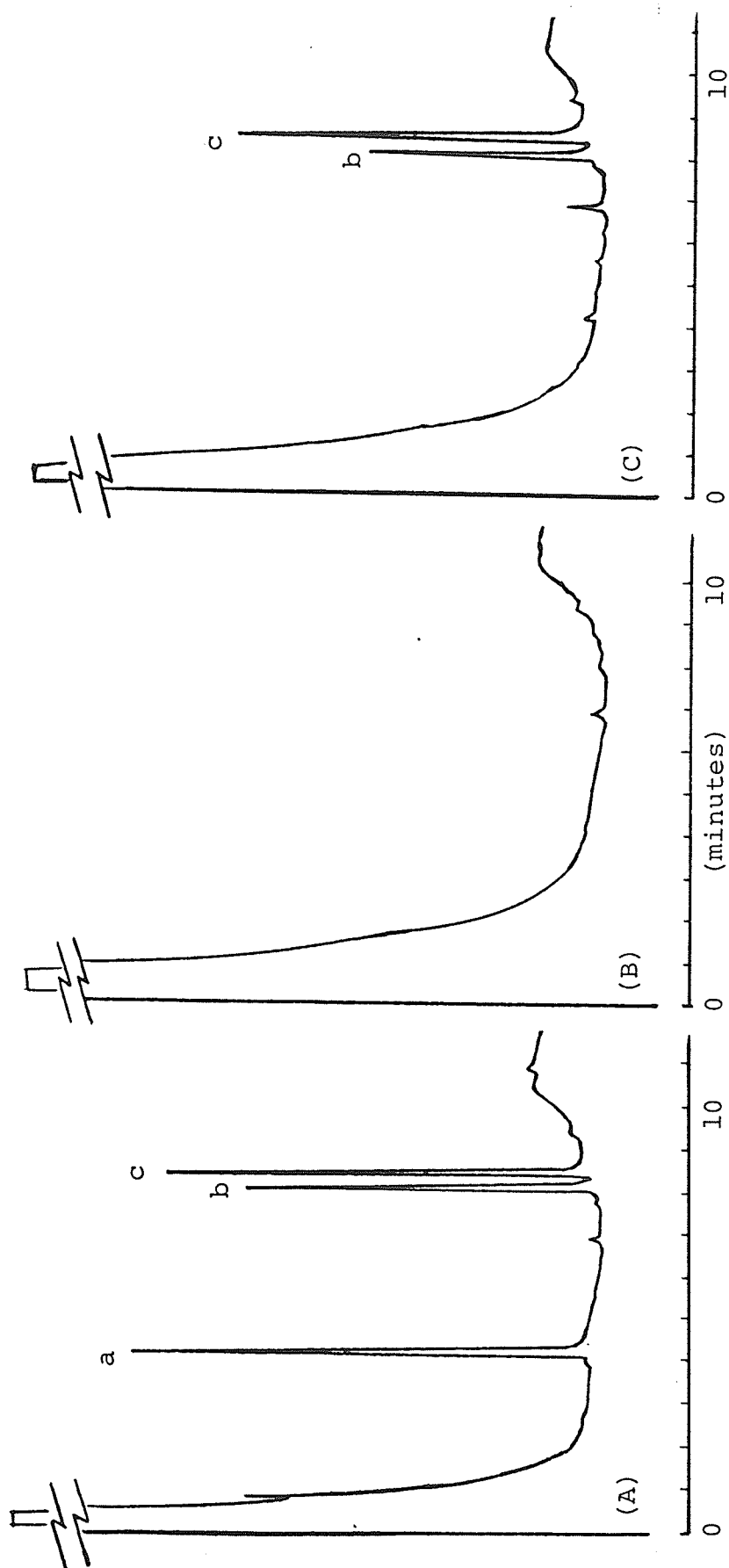
Figure 21: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of benzoylecgonine- and ecgonine-spiked water samples.

Key: (A) GC standard containing 80 ng ecgonine, 80 ng benzoylecgonine, and 100 ng n-PEBE as internal standard per µL injected; (B) benzoylecgonine-free water sample with no n-PEBE; and (C) water sample spiked with benzoylecgonine (8.0 µg/mL), and with n-PEBE as internal standard.

a = ecgonine-(TMS)$_2$, b = benzoylecgonine-TMS, c = n-PEBE

Table VI: Percent Recovery[a] of Benzoylecgonine from Water,
   Plasma, and Urine Samples Spiked with Benzoylecgonine and
   Ecgonine.

Initial Benzoyl-ecgonine Conc. (μg/mL)	Recovery from:		
	Water (%)	Plasma (%)	Urine[b] (%)
4.0	68.93 ± 2.72	69.23 ± 9.27	69.32 ± 1.44
8.0	71.58 ± 0.69	66.73 ± 5.33	73.82 ± 2.97
16.0	80.45 ± 1.86	70.20 ± 3.78	81.08 ± 1.31

[a] $\bar{X}$ ± SD.

[b] Diluted urine, 1:25.

the extraction/TLC/GC-FID assay procedure for benzoyl-ecgonine was examined. The amount of ecgonine generated during the analysis of test water samples spiked with 40 μg of benzoylecgonine alone was insignificant. The mean ± standard deviation amount of ecgonine generated in four replicate test samples was 0.01 μg ± 0.02 μg.

Spiked Plasma and Urine Samples. Individual samples of plasma or diluted urine were spiked with both benzoyl-ecgonine and ecgonine and analyzed by the extraction/TLC/GC-FID procedure. Gas chromatograms obtained from the analysis of spiked plasma and urine samples for both benzoylecgonine and ecgonine content are given in Figure 22. There was no interference with peaks corresponding to benzoylecgonine and the internal standard n-PEBE.

The mean percent recoveries of benzoylecgonine from plasma and urine samples spiked with benzoylecgonine and ecgonine are given in Table VI. Mean percent recoveries of ecgonine from similarly spiked samples are given in Table VII.

Experimental (In Vivo) Plasma and Urine Samples. The concentrations of benzoylecgonine and ecgonine in dog plasma and urine samples obtained after intravenous administration of benzoylecgonine were determined by the extaction/TLC/GC-FID method. Examples of gas chromatograms obtained in the determination of benzoylecgonine in experimental plasma and

Table VII: Percent   Recovery[a] of Ecgonine from Plasma and
     Urine Samples Spiked with Benzoylecgonine and Ecgonine.

| Initial Ecgonine Conc. (μg/mL) | Recovery from: | |
	Plasma (%)	Urine[b] (%)
4.0	60.20 ± 11.69	42.62 ± 8.01
8.0	56.21 ± 9.05	40.31 ± 2.58
16.0	61.59 ± 4.48	45.41 ± 3.50

[a]$\bar{X}$ ± SD.

[b]Diluted urine, 1:25.
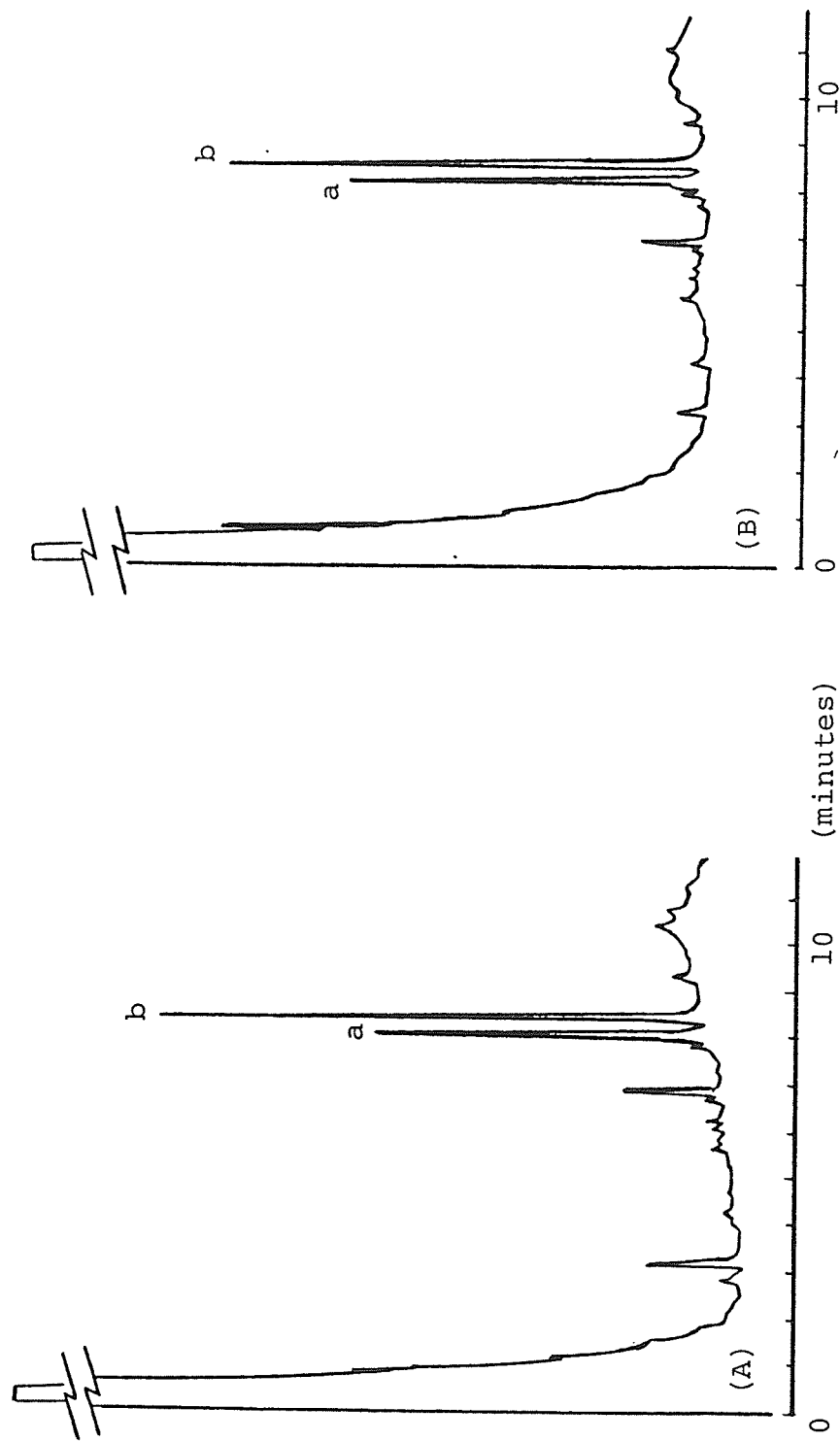
Figure 22: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of benzoylecgonine- and ecgonine-spiked plasma and urine samples.

Key: (A) plasma sample spiked with benzoylecgonine (8.0 μg/mL), and with n-PEBE as internal standard; (B) diluted urine (1:25) sample spiked with benzoylecgonine (8.0 μg/mL), and with n-PEBE as internal standard.
a = benzoylecgonine-TMS, b = n-PEBE

160

urine test samples are given in Figure 23. Gas chromato-
grams of analyzed experimental plasma and urine samples were
not appreciably different from those of analyzed spiked
plasma and urine samples. No ecgonine was detected in any
of the plasma or urine samples analyzed.

Benzoylecgonine concentration calibration curves (peak
height ratio, benzoylecgonine/n-PEBE, vs. μg benzoylecgonine
per mL of spiked plasma or diluted urine) were used to cal-
culate the benzoylecgonine concentration of experimental (in
vivo) samples. Benzoylecgonine concentration calibration
curves based on spiked plasma and urine samples are given in
Figure 24. The benzoylecgonine concentration calibration
curves were linear over the range of concentrations exam-
ined. The correlation coefficients, r, of the least-squares
regression lines for the plasma and urine concentration cal-
ibration curves were 0.992 and 0.999, respectively.
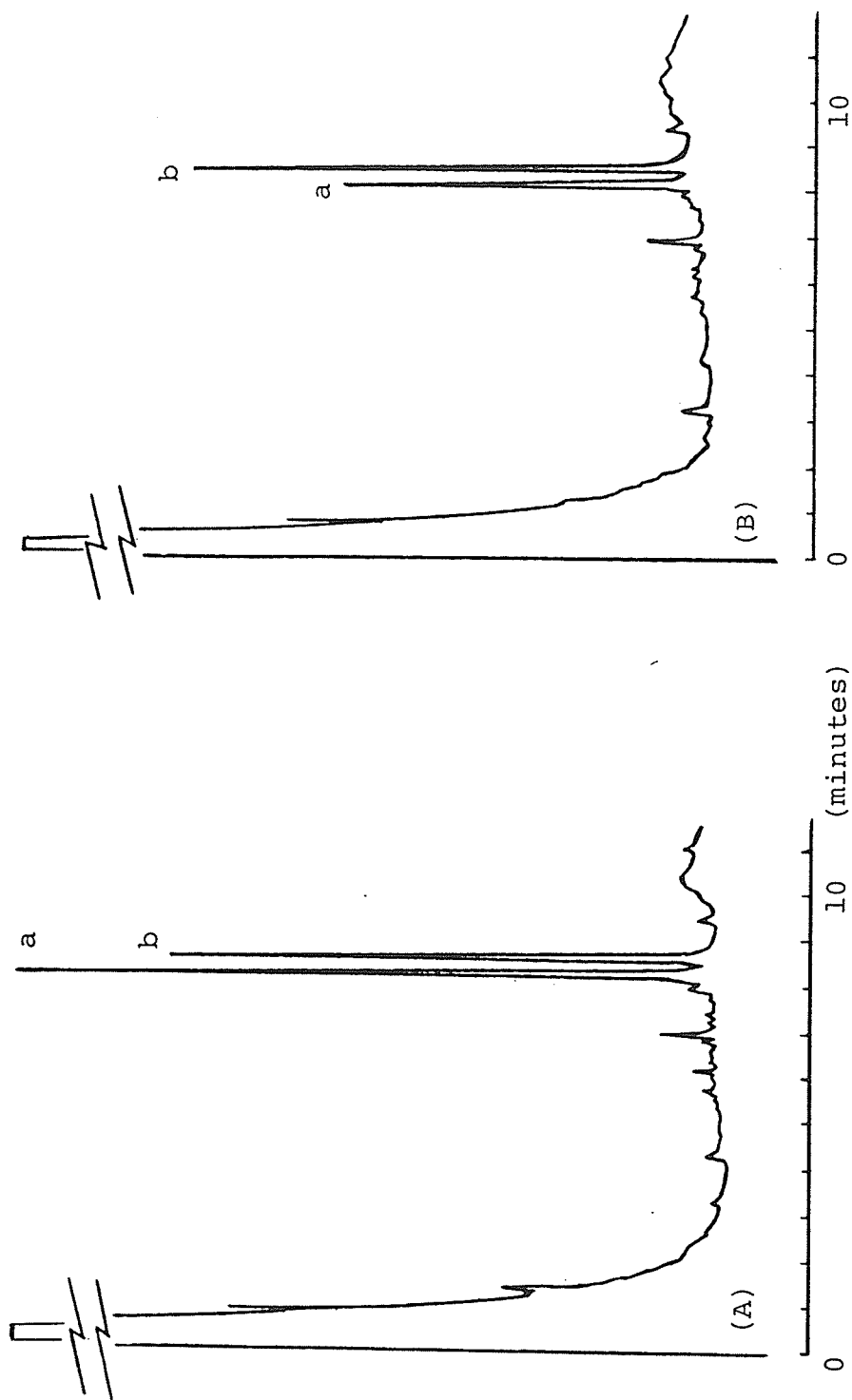
Figure 23: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of dog plasma and urine samples obtained after intravenous administration of a single dose of benzoylecgonine, 10 mg/kg, to a female Beagle dog.

Key: (A) plasma sample, t=4.02 h; (B) diluted urine (1:25) sample, 0-48 h.
a = benzoylecgonine-TMS, b = n-PEBE
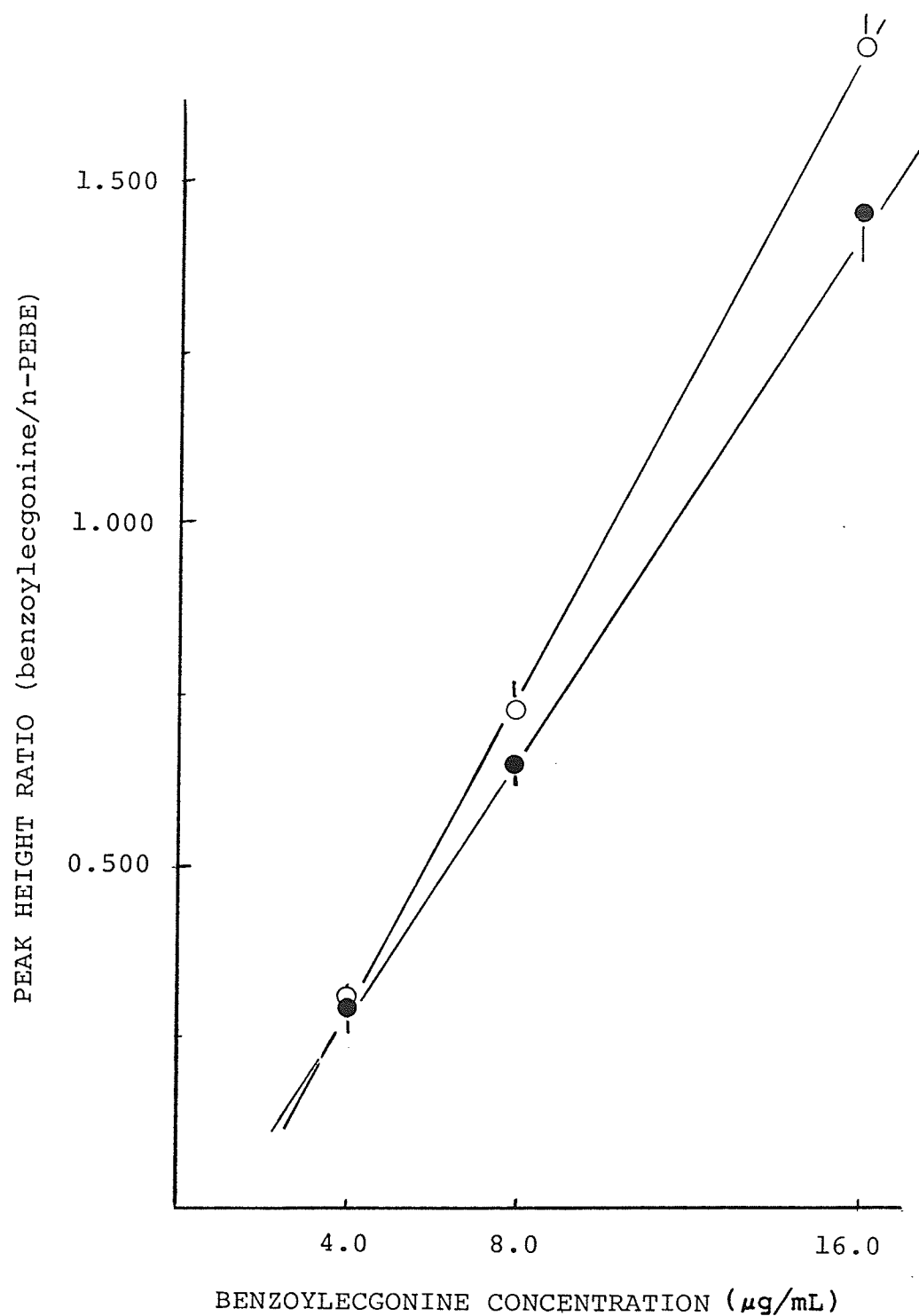
Figure 24:   GC concentratin calibration   curves for the de-
   termination   of   benzoylecgonine   in   spiked   plasma   and
   urine.

   Key: (●) plasma; (○) diluted urine, 1:25.

4. DETERMINATION OF ECGONINE METHYL ESTER AND ECGONINE IN WATER, PLASMA, AND URINE

Extraction/TLC/GC-FID Procedure. An analytical procedure was developed for the determination of both ecgonine methyl ester and ecgonine in the same plasma and urine samples. Initial assay development was carried out with spiked water samples.

Spiked Water Samples. Individual water samples were spiked with increasing amounts of both ecgonine methyl ester and ecgonine. The ecgonine methyl ester was extracted and quantitated by GC-FID. Typical gas chromatograms are shown in Figure 25. There was no interference with the ecgonine methyl ester and n-PEBE peaks.

The amounts of ecgonine methyl ester recovered from spiked water samples were calculated by comparing the peak height ratios, ecgonine methyl ester/n-PEBE, of the analyzed spiked water samples with the peak height ratios from the ecgonine methyl ester GC standard calibration curve. The mean data for the recovery of ecgonine methyl ester from spiked water samples are given in Table VIII.

The aqueous phases remaining after the extraction of ecgonine methyl ester were re-extracted in order to quantitate ecgonine. Typical gas chromatograms obtained from the GC-FID analysis of the ecgonine extracts from spiked water samples are presented in Figure 26.

Figure 25: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine methyl ester- and ecgonine-spiked water samples for the determination of ecgonine methyl ester.

Key: (A) GC standard containing 80 ng ecgonine methyl ester, 80 ng ecgonine, and 100 ng n-PEBE as internal standard per μL injected; (B) ecgonine methyl ester-free water sample with no n-PEBE; and (C) water sample spiked with ecgonine methyl ester (8.0 μg/mL), and with n-PEBE as internal standard.
a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = n-PEBE

Table VIII: Percent Recovery[a] of Ecgonine Methyl Ester from Water, Plasma, and Urine Samples Spiked with Both Ecgonine Methyl Ester and Ecgonine.

Initial Ecgonine Methyl ester Conc. (μg/mL)	Recovery from:		
	Water (%)	Plasma (%)	Urine[b] (%)
4.0	61.53 ± 2.88	64.41 ± 10.83	59.62 ± 6.12
8.0	66.69 ± 3.67	68.50 ± 4.59	65.03 ± 5.09
16.0	71.93 ± 5.51	78.70 ± 2.74	71.45 ± 3.91

[a]$\bar{X}$ ± SD.

[b]Diluted urine, 1:10.

167
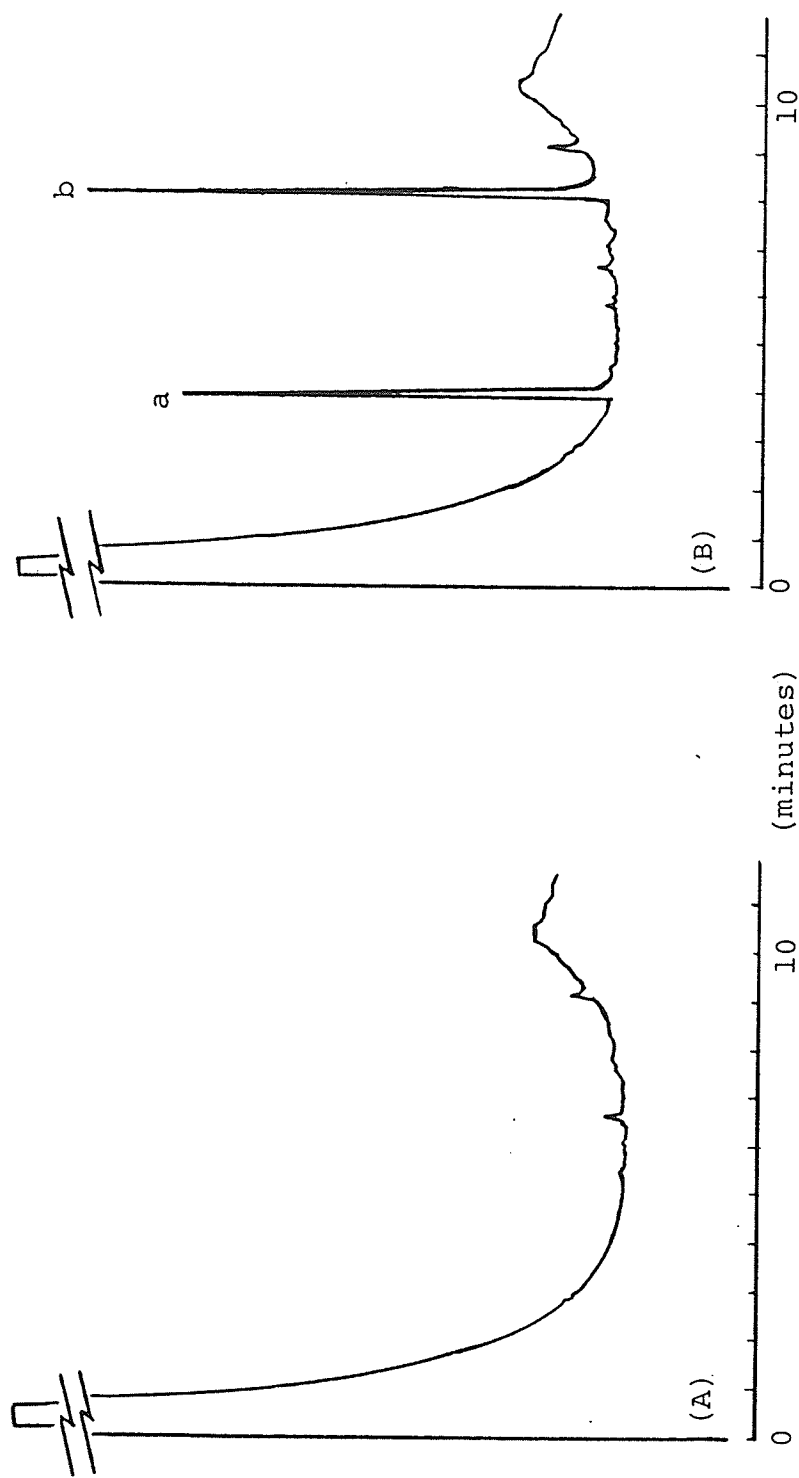
Figure 26: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine methyl ester- and ecgonine-spiked water samples for the determination of ecgonine.

Key: (A) ecgonine-free water sample with no n-PEBE and (B) water sample spiked with ecgonine (8.0 μg/mL), and with n-PEBE as internal standard. a = ecgonine-(TMS)$_2$, b = n-PEBE

The percent (%) recovery of ecgonine from spiked water samples was calculated by comparing the peak height ratio, ecgonine/n-PEBE, of analyzed spiked water samples with the peak height ratios from the ecgonine GC standard calibration curve. The mean percent (%) recoveries of ecgonine from water samples spiked with both ecgonine methyl ester and ecgonine are given in Table IX.

In a separate experiment the possible conversion of ecgonine methyl ester to ecgonine by chemical hydrolysis during the extraction/TLC/GC-FID procedure was examined. The amount of ecgonine generated during the analysis of test water samples spiked with 40 μg of ecgonine methyl ester alone was insignificant. The mean ± standard deviation of four replicate test samples was 0.06 ± 0.21 μg.

Spiked Plasma and Urine Samples. The recoveries of ecgonine methyl ester and ecgonine from spiked plasma and urine samples were determined. Typical gas chromatograms obtained from the analysis of spiked plasma and spiked urine samples are given in Figures 27 and 28, respectively. Gas chromatograms of ecgonine methyl ester fractions showed no interference in the ecgonine methyl ester and n-PEBE regions. There was minimal interference with peaks corresponding to ecgonine and n-PEBE in chromatograms of the analyzed ecgonine fractions of spiked plasma and urine samples.

Table IX: Percent Recovery[a] of Ecgonine from Water, Plasma, and Urine Samples Spiked with Both Ecgonine Methyl Ester and Ecgonine.

Initial Ecgonine Conc. (μg/mL)	Recovery from:		
	Water (%)	Plasma (%)	Urine[b] (%)
4.0	53.22 ± 5.45	51.73 ± 6.53	59.37 ± 4.98
8.0	55.46 ± 7.32	54.22 ± 3.82	54.29 ± 4.50
16.0	61.01 ± 3.73	58.36 ± 3.05	63.47 ± 2.86

[a] $\bar{X}$ ± SD.

[b] Diluted urine, 1:10.
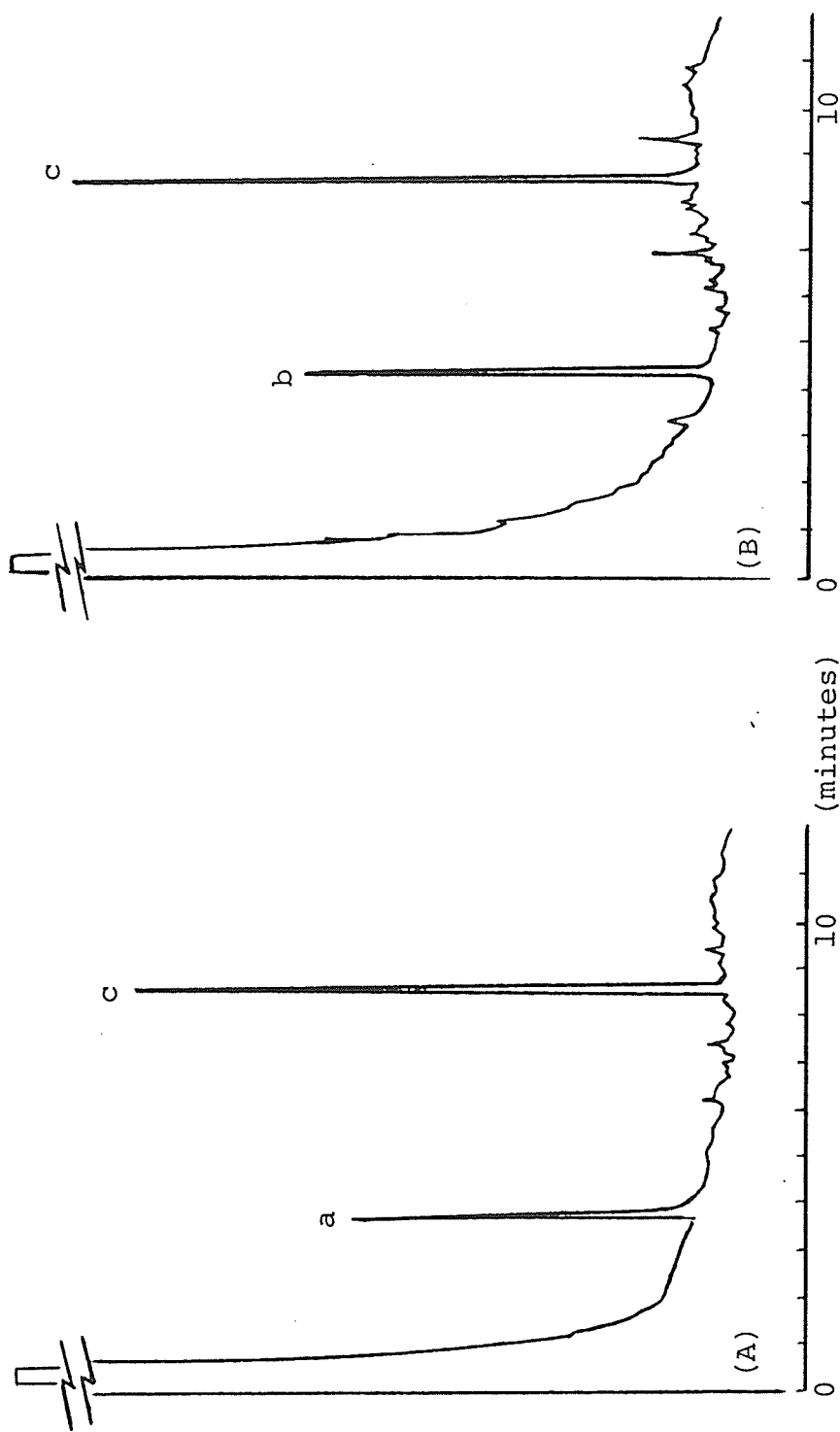
Figure 27: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine methyl ester- and ecgonine-spiked plasma samples.

Key: (A) ecgonine methyl ester fraction and (B) ecgonine fraction, both fractions from plasma sample spiked with ecgonine methyl ester (8.0 μg/mL) and ecgonine (8.0 μg/mL). a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = n-PEBE (internal standard).

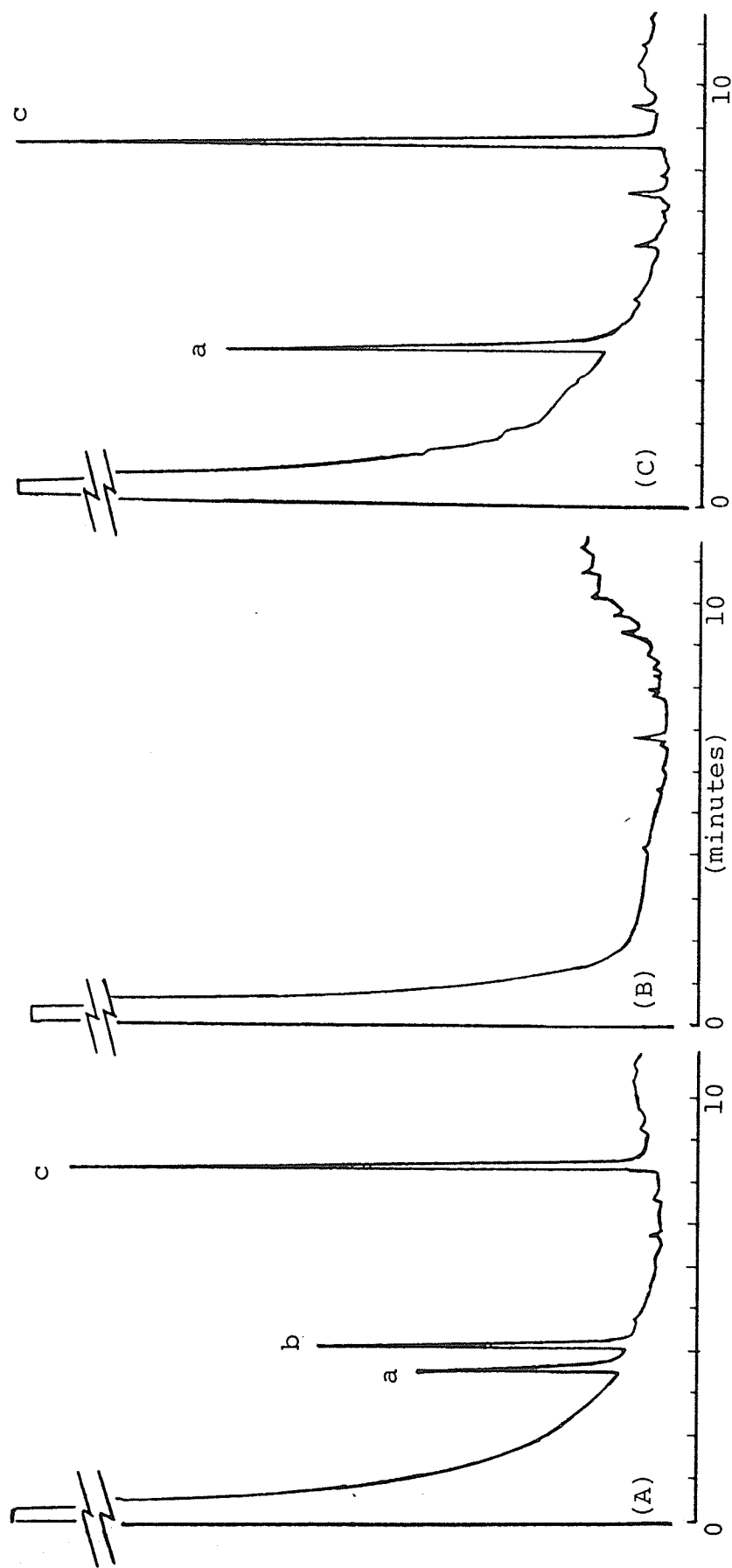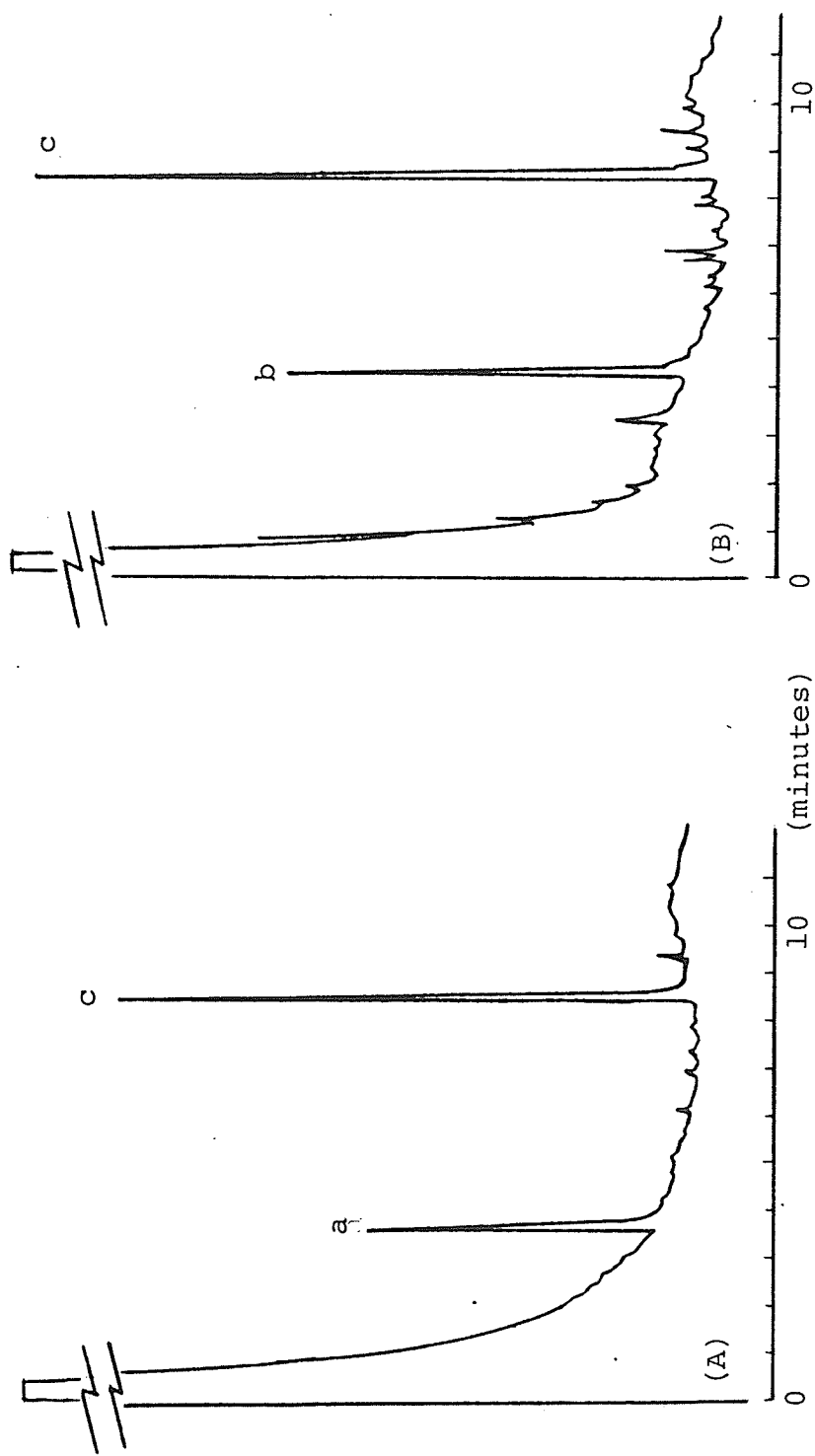Figure 28: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of ecgonine methyl ester- and ecgonine-spiked urine samples.

Key: (A) ecgonine methyl ester fraction and (B) ecgonine fraction, both fractions from diluted urine (1:10) sample spiked with ecgonine methyl ester (8.0 μg/mL) and ecgonine (8.0 μg/mL).

a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = n-PEBE (internal standard)

The percent (%) recoveries of ecgonine methyl ester from plasma and urine samples spiked with both ecgonine methyl ester and ecgonine are given in Table VIII. The percent (%) recoveries of ecgonine from similarly spiked plasma and urine samples are given in Table IX. Overall, the recoveries of ecgonine averaged about 10% lower than those for ecgonine methyl ester.

The possible conversion of ecgonine methyl ester to ecgonine in unacidified urine at 37° C was investigated in vitro. Ecgonine methyl ester test samples were prepared with undiluted dog urine preserved with thymol, which had an initial pH of 7.57 and a specific gravity of 1.040. The urine test samples were spiked with ecgonine methyl ester at a concentration of 38.46 μg/mL, calculated as the free base, equivalent to 193.0 μmol/L. The mean final concentration of ecgonine methyl ester in the test samples after incubation at 37° C for 24 hours was 7.25 μg/mL, equivalent to 36.40 μmol/L, corresponding to 18.86% of the original ecgonine methyl ester concentration. The mean final concentration of ecgonine found in the test samples after incubation at 37° C for 24 h was 23.82 μg/mL, equivalent to 128.6 μmol/L or 66.63% conversion of the original ecgonine methyl ester to ecgonine.

Experimental (In Vivo) Dog Plasma and Urine Samples. The concentrations of both ecgonine methyl ester and ecgonine were determined in dog plasma and urine samples ob-

tained after intravenous administration of ecgonine methyl ester hydrochloride. Examples of gas chromatograms obtained from the analysis of ecgonine methyl ester and ecgonine in experimental dog plasma and urine samples are given in Figures 29 and 30, respectively.

The concentrations of ecgonine methyl ester and ecgonine in experimental plasma and urine samples were calculated from corresponding ecgonine methyl ester and ecgonine concentration calibration curves based on spiked plasma and urine samples as standards. The ecgonine methyl ester plasma and urine concentration calibration curves used are shown in Figure 31. The concentration calibration curves, peak height ratio ecgonine methyl ester/n-PEBE vs. ecgonine methyl ester concentration in μg/mL, were linear over the range of concentrations examined. The two curves for plasma and urine were quite similar. The correlation coefficients, r, calculated for ecgonine methyl ester plasma and urine concentration calibration curves were 0.996 and 0.994, respectively.

The ecgonine plasma and urine concentration calibration curves were also linear over the range examined. The correlation coefficients, r, for the plasma and urine concentration calibration curves were 0.994 and 0.992, respectively. The two calibration curves are reproduced in Figure 32.
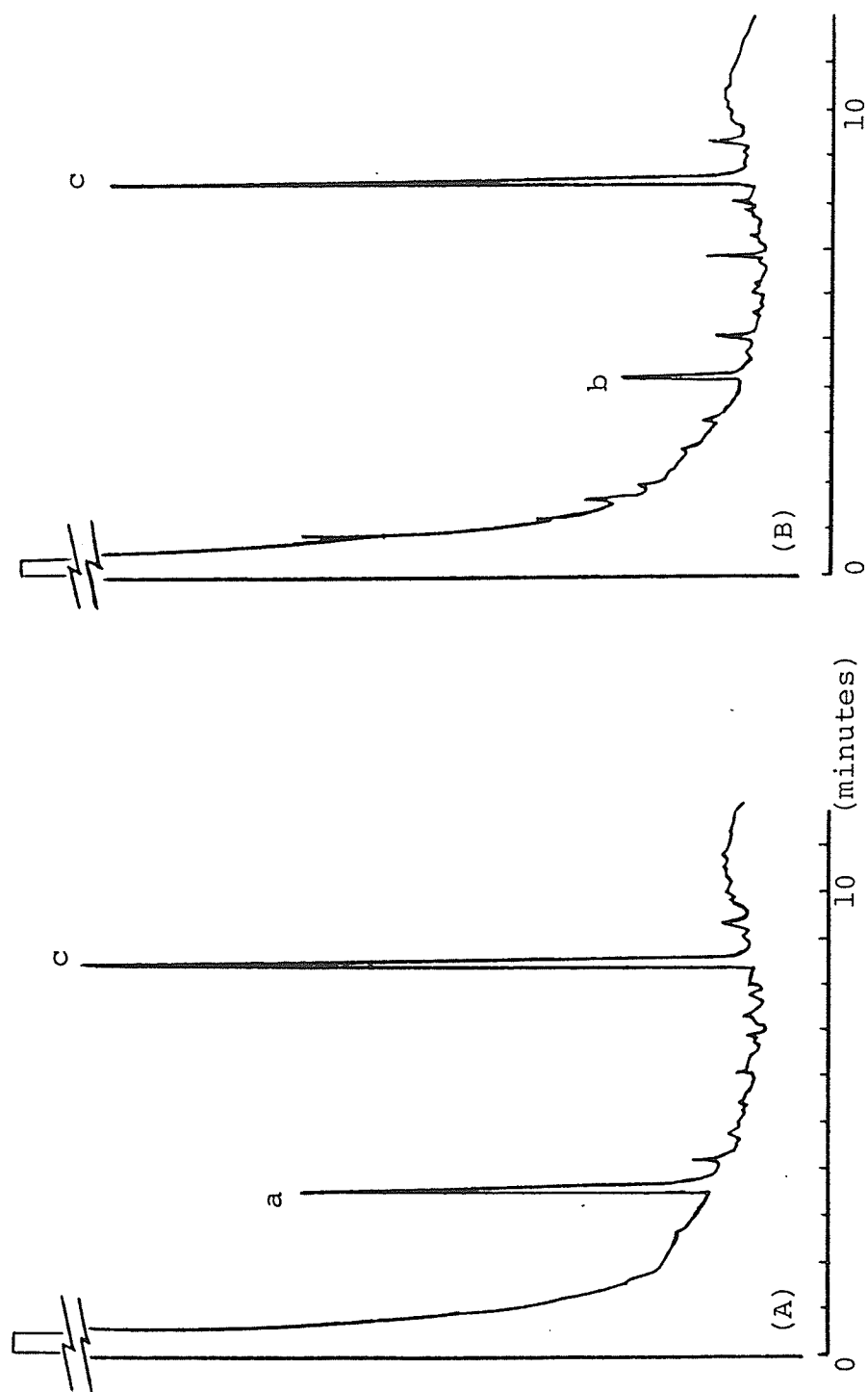
Figure 29: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of dog plasma samples after intravenous administration of a single dose of ecgonine methyl ester, 25 mg/kg, to a female Beagle dog.

Key: (A) ecgonine methyl ester and (B) ecgonine from plasma sample, t=2.00 h.
a = ecgonine methyl ester-TMS, b = ecgonine-$(TMS)_2$, c = n-PEBE (internal standard)
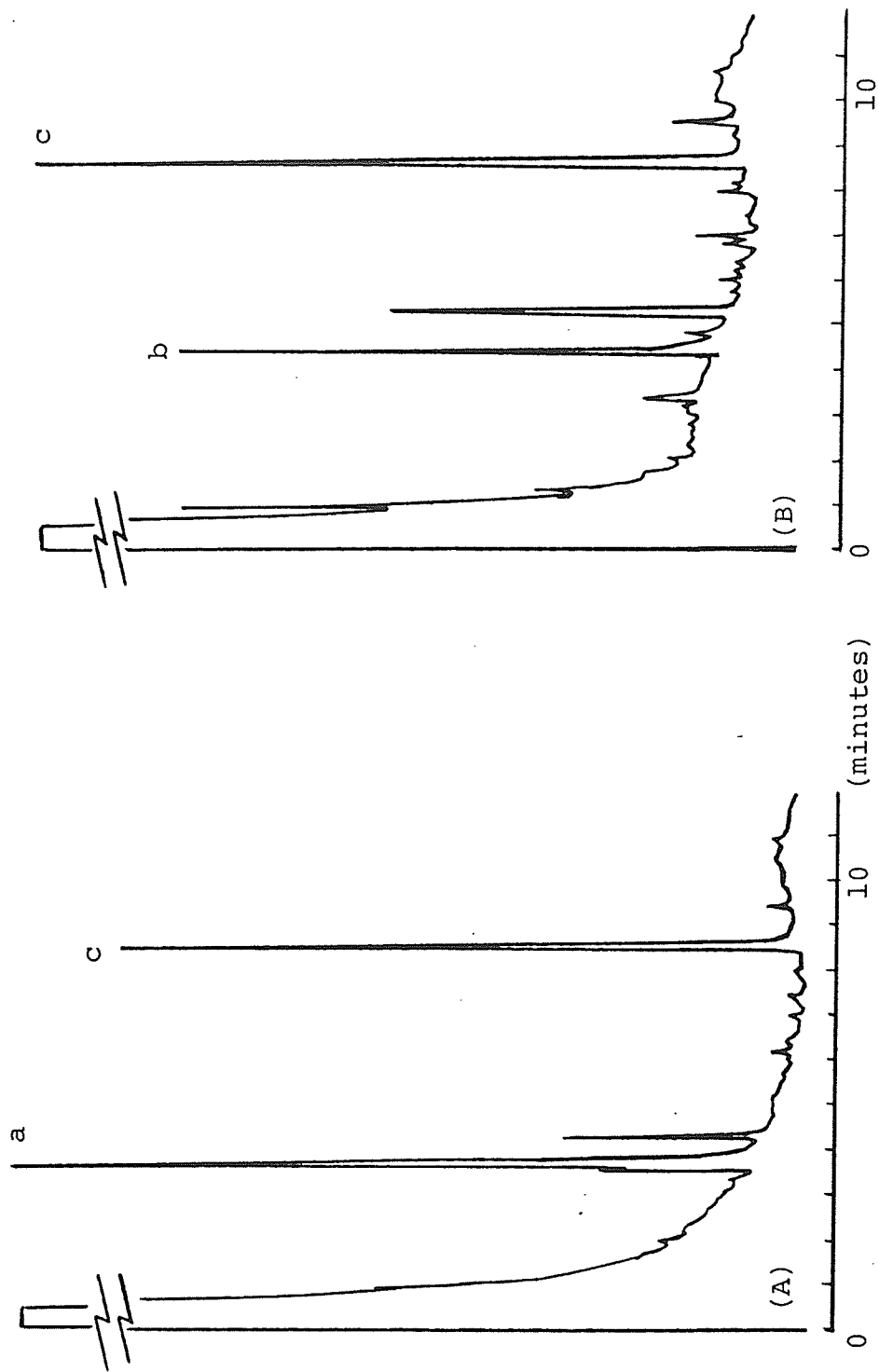
Figure 30: Sample gas chromatograms from the extraction/TLC/GC-FID analysis of dog urine samples after intravenous administration of a single dose of ecgonine methyl ester, 25 mg/kg, to a female Beagle dog.

Key: (A) ecgonine methyl ester and (B) ecgonine from diluted urine (1:10) sample, 0-48 h.
a = ecgonine methyl ester-TMS, b = ecgonine-(TMS)$_2$, c = n-PEBE (internal standard)
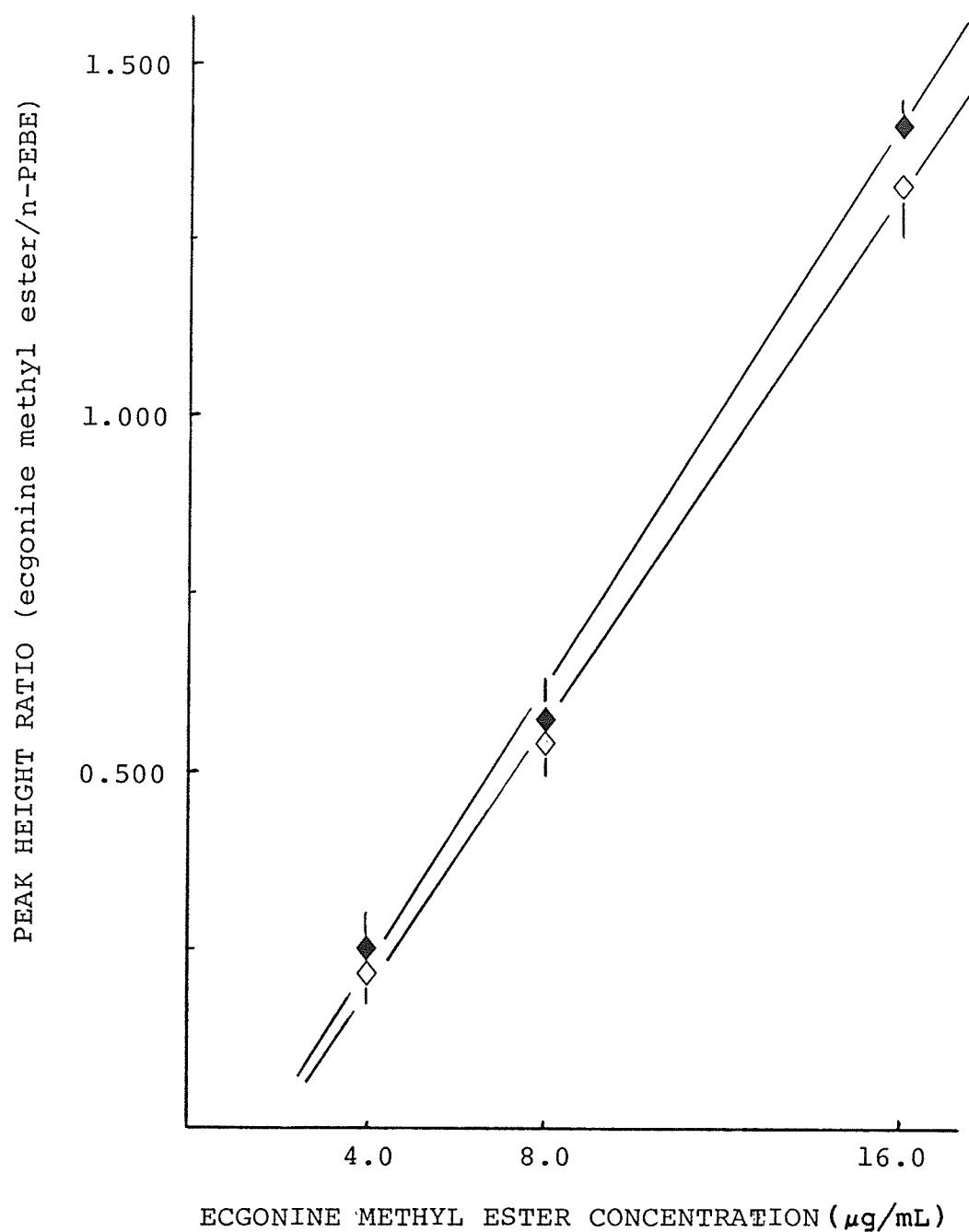
Figure 31:   GC concentration calibration curves for the determination of ecgonine methyl ester in spiked plasma and urine.

Key: (◆) plasma; (◇) diluted urine, 1:10.
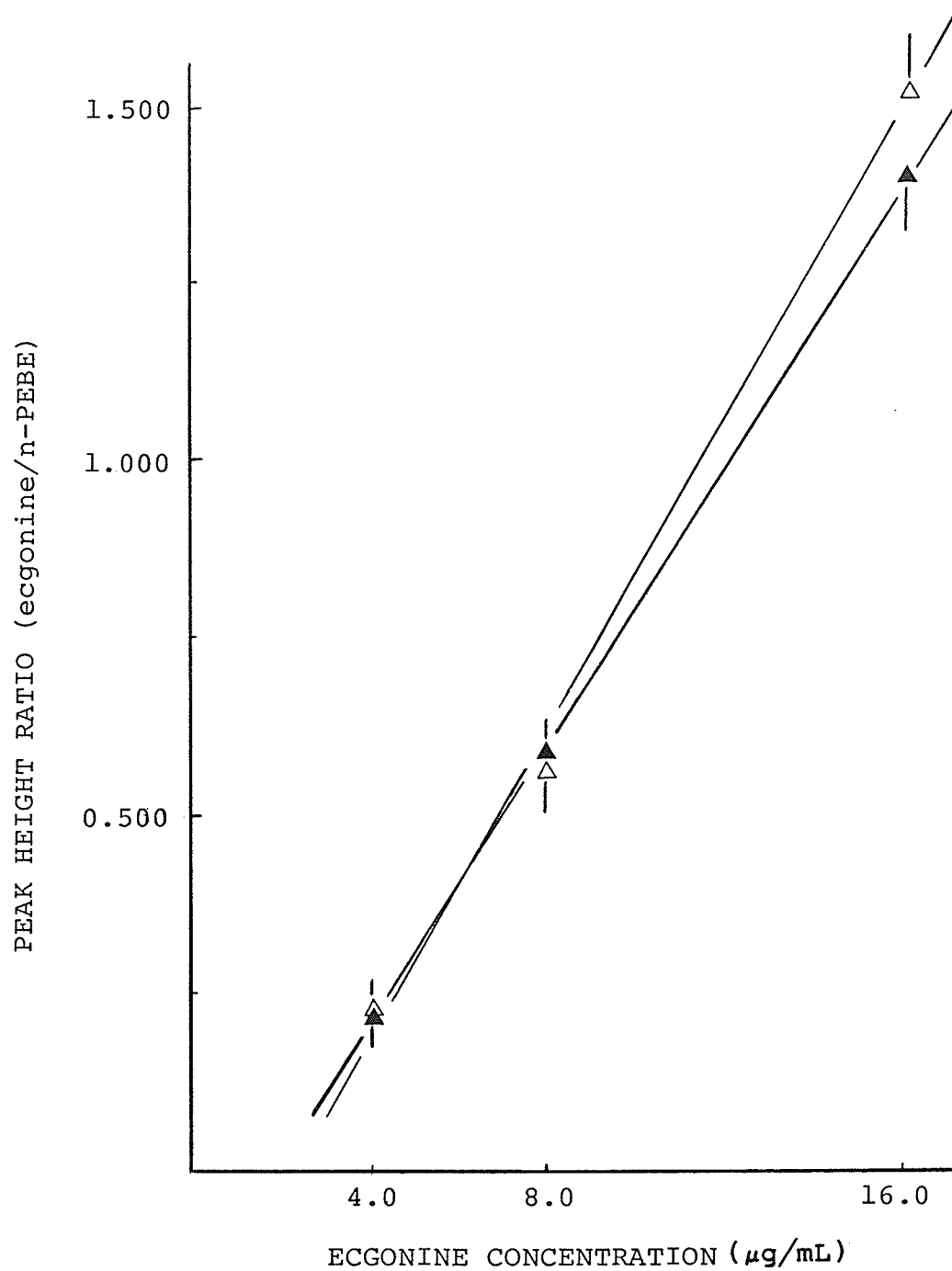
Figure 32: GC concentration calibration curves for the determination of ecgonine in spiked plasma and urine.

Key: (▲) plasma; (△) diluted urine, 1:10.

5. DETERMINATION OF COCAINE IN WATER, PLASMA, AND URINE

Cocaine base was prepared from cocaine hydrochloride. The melting point of the base compared favorably with the literature value (98° C). A solution of cocaine base in methanol showed only a single peak when analyzed by gas chromatography ·equipped with a nitrogen–phosphorus detector (GC–NPD).

HP 5880 GC Multi-Level Calibration Method. A GC–NPD procedure was developed for the quantitation of cocaine base. Cocaine and the internal standard n–PEBE were easily separated by the method developed. The retention times of cocaine and n–PEBE were 3.2 and 4.0 min, respectively. Typical gas chromatograms of GC standards containing 0.0 and 10.6 ng cocaine base and each 30 ng n–PEBE per 2 μL injected are shown in Figure 33.

A set of calibration mixtures each with increasing amounts of cocaine and a fixed amount of n–PEBE included as internal standard were analyzed by GC–NPD in order to calibrate the HP 5880. On the basis of this internal multi-level calibration curve, the concentrations of injected unknown cocaine samples were determined automatically. The peak height ratios, cocaine/n–PEBE, of GC standards which were used to calibrate the instrument were monitored in order to evaluate the sensitivity and linearity of the method. A co-
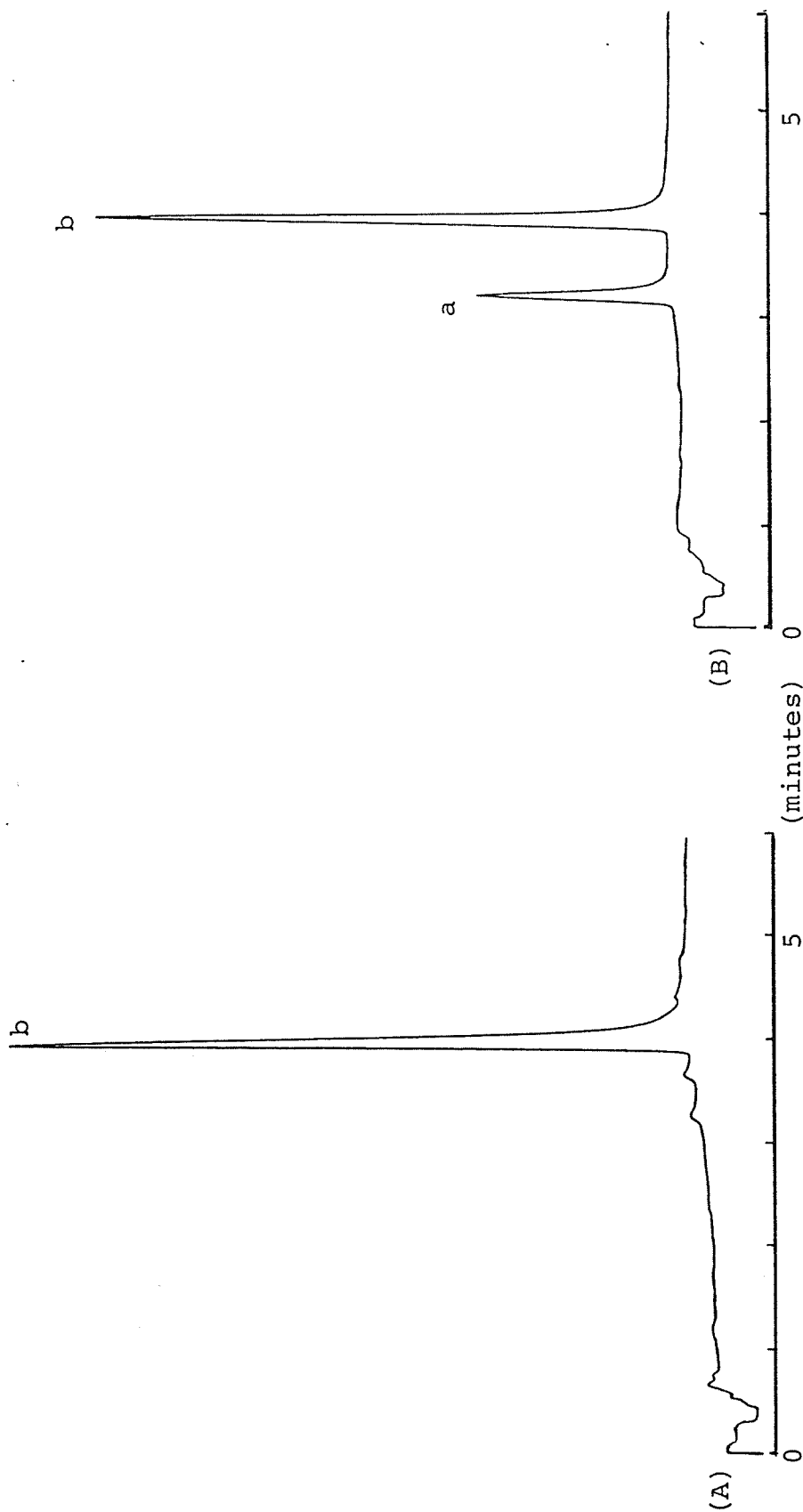
Figure 33: Sample gas chromatograms of cocaine base standards analyzed by GC-NPD.

Key: (A) cocaine-free standard containing 30 ng n-PEBE per 2 µL injected; (B) cocaine standard containing 10.6 ng cocaine and 30 ng n-PEBE as internal standard per 2 µL injected.
a = cocaine, b = n-PEBE

caine GC standard calibration curve is given in Figure 34. Each data point represents the mean of two or three replicate determinations. The linearity of the cocaine GC standard calibration curve was excellent, the correlation coefficient, r, of the least-squares line was 0.999.

Extraction/GC-NPD Procedure. A modification of the extraction/GC-NPD procedure of Jatlow and Bailey (49) was developed for the determination of cocaine in plasma and urine. Initial experiments were carried out with spiked water samples.

Spiked Water Samples. The recovery of cocaine from spiked water samples was determined according to the modified internal standard method in which the internal standard, n-PEBE, is added immediately prior to injection for GC-NPD analysis. Water samples spiked with varying amounts of cocaine base were extracted and the amount of cocaine quantitated by GC-NPD. The HP 5880 gave a direct readout of the ng cocaine base per 2 μL injected based on the instrument's internal calibration curve. The percent (%) recovered from each spiked water sample ranged between 83 and 103 %. Mean recovery data are given in Table X.

The recovery of cocaine from spiked water samples was also determined according to the true internal standard method whereby n-PEBE is added to the organic solvent used for the initial extraction of cocaine. A typical gas chro-
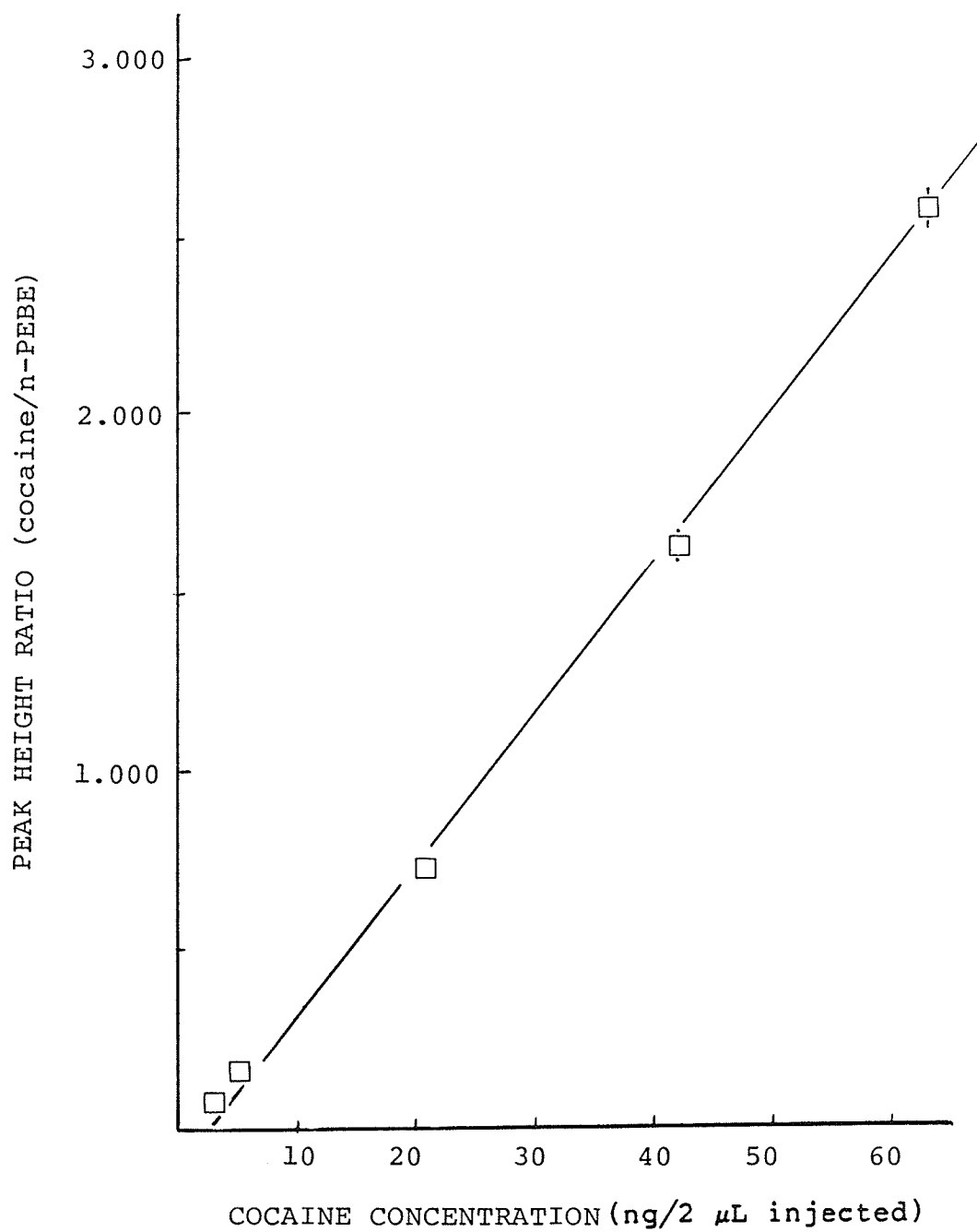
Figure 34: Concentration calibration curve for the GC-NPD analysis of cocaine.

Table X: Percent Recovery[a] of Cocaine from Spiked Water
   Samples Analyzed by GC-NPD according to the "Modified"
   and "True" n-PEBE Internal Standard Methods.

Initial Cocaine Conc. (ng/2 mL)	"Modified" (%)	"True" (%)
62	99.4 ± 4.2	106.6 ± 1.4
120	90.3 ± 1.0	104.4 ± 1.5
244	85.6 ± 2.2	99.8 ± 6.7
490	88.9 ± 0.3	96.9 ± 3.0
1060	85.1 ± 2.4	97.0 ± 2.0

[a] $\bar{X}$ ± SD.
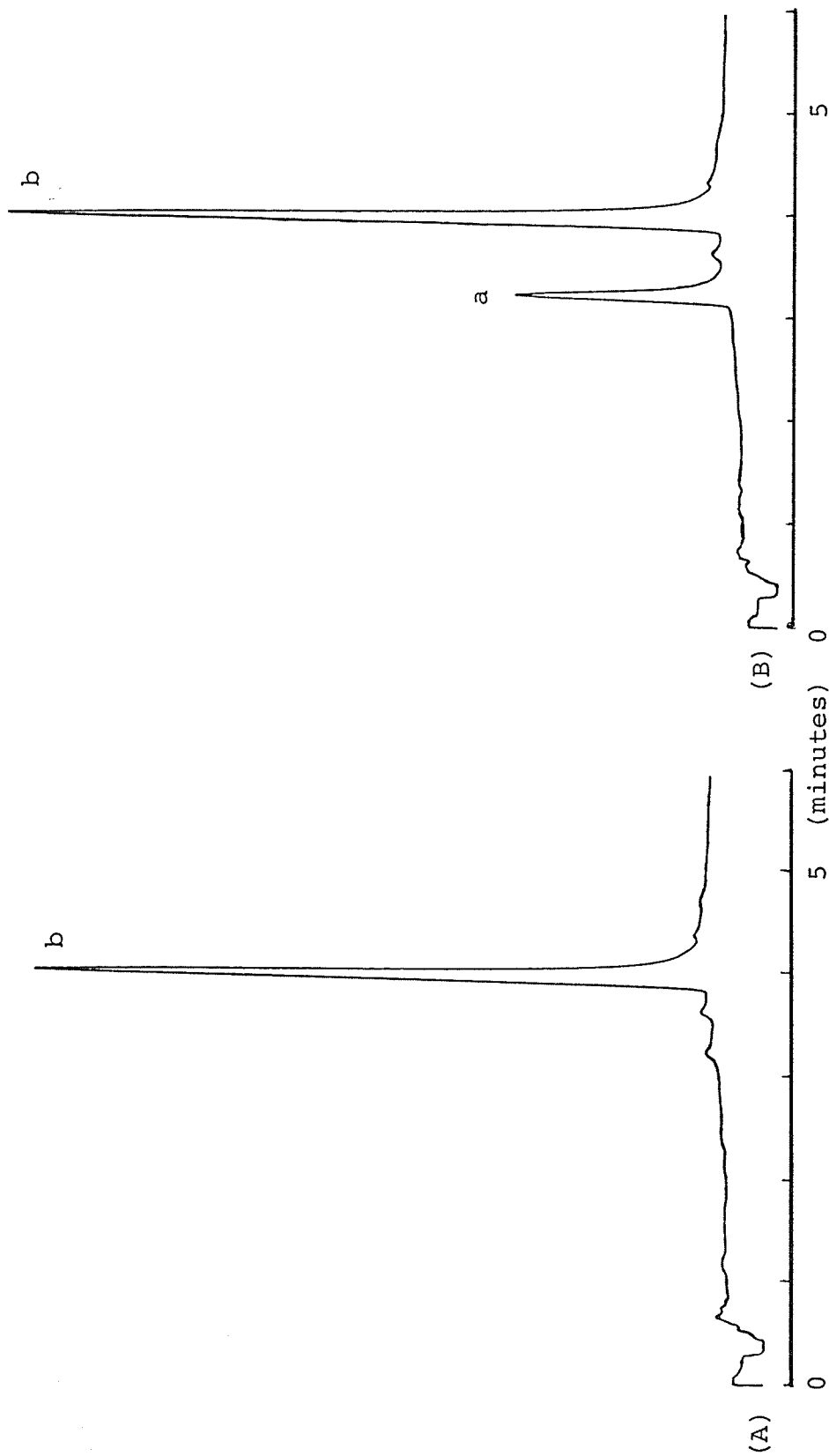
Figure 35: Sample gas chromatograms from the extraction/GC-NPD analysis of cocaine-spiked water samples.

Key: (A) cocaine-free water sample with only n-PEBE; (B) water sample spiked with cocaine (118 ng/mL) and with n-PEBE as internal standard. a = cocaine, b = n-PEBE

matogram is given in Figure 35. The cocaine and the n-PEBE
peaks appear to be free of any foreign peaks.

The percent (%) recovery of cocaine from individual
spiked water samples was determined by extraction/GC-NPD
with n-PEBE as the true internal standard ranged between
95-108%. Mean percent (%) recovery data are given in Table
X.

Spiked Blood and Plasma Samples. The recovery of co-
caine from spiked plasma samples as well as the stability of
cocaine during blood collection, plasma separation and stor-
age were evaluated according to the extraction/GC-NPD method
with n-PEBE as modified internal standard. Three sets of
cocaine-plasma samples each prepared differently were ana-
lyzed: (a) blank plasma samples spiked immediately prior to
analysis, (b) freezer-stored cocaine-spiked plasma samples,
and (c) freezer-stored plasma from cocaine-spiked blood sam-
ples.

Blank plasma samples prepared as previously described
had been stored at -30° C for four weeks. The samples were
thawed and spiked with cocaine base immediately prior to
analysis. The mean percent ± standard deviation of the co-
caine recovered from three replicate spiked blank plasma
samples was 78.0% ± 1.4%.

The freezer-stored cocaine-spiked plasma samples were
prepared by adding cocaine base to plasma after separation

from freshly drawn blood, immediately freezing the spiked plasma samples, and storing them at -30° C for four weeks. The concentration of cocaine was determined at the end of the storage period. The mean percent recovery ± standard deviation of cocaine from three cocaine-spiked plasma samples after storage at -30° C for four weeks was 74.8% ± 3.5%.

In the case of the third set of cocaine-plasma samples, freshly-drawn blood samples were spiked with cocaine base, the plasma separated, immediately frozen, and stored at -30° C for four weeks. The concentration of cocaine in the plasma from each cocaine-spiked blood sample was determined by extraction/GC-NPD. The mean percent recovery ± standard deviation of cocaine from the spiked blood samples was found to be 67.8% ± 1.6%.

The percent recoveries of cocaine from the three sets of cocaine-plasma samples were: (a) 78.0% for the plasma samples spiked with cocaine immediately prior to analysis, (b) 74.8% for cocaine-spiked plasma sample, and (c) 67.8% for the plasma from cocaine-spiked blood samples.

Blank plasma samples were spiked with increasing levels of cocaine base, carried through the entire extraction/GC-NPD procedure with n-PEBE as true internal standard, and used to calibrate the HP 5880 for plasma analysis. Examples of gas chromatogram profiles obtained for spiked plasma sam-
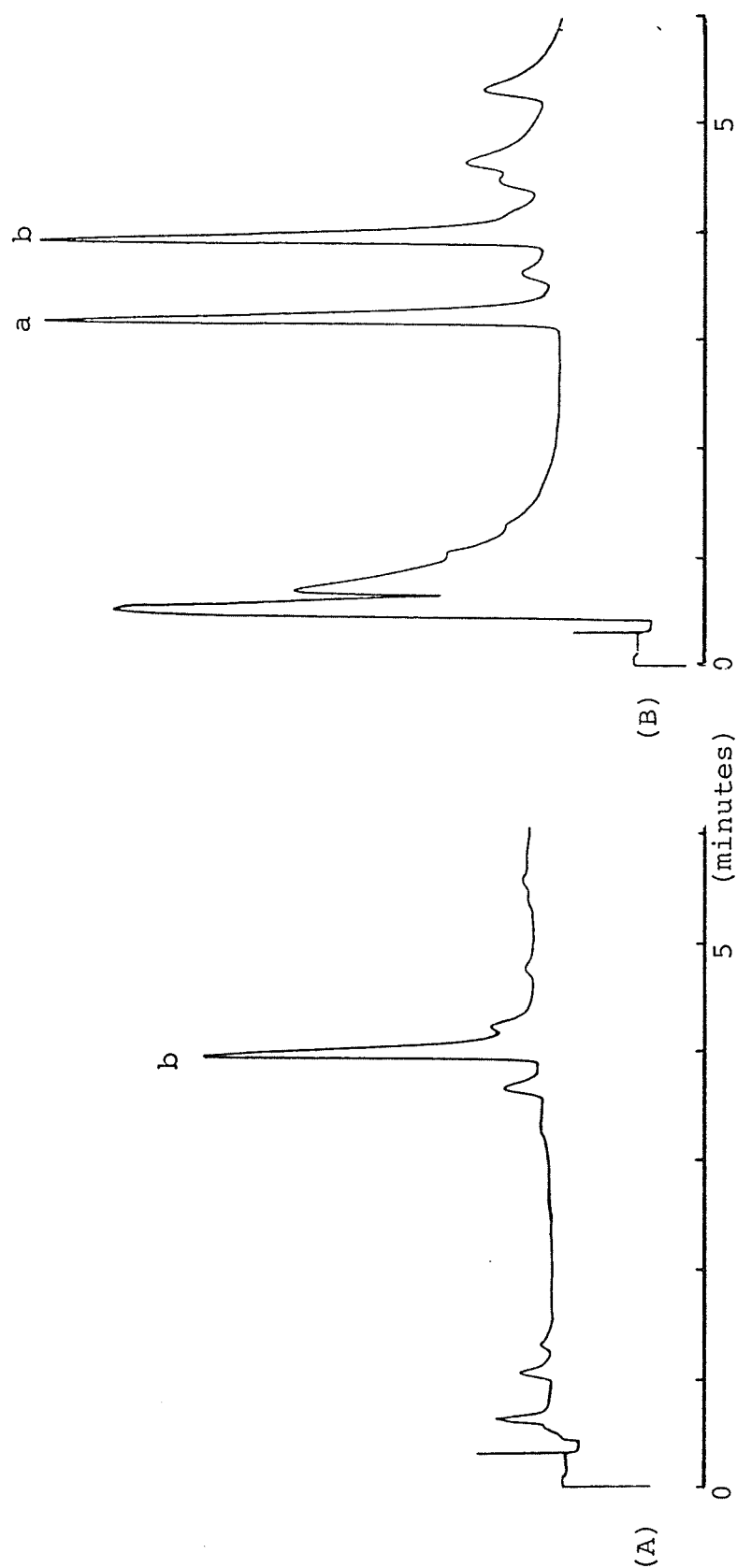
Figure 36: Sample gas chromatograms from the extraction/GC-NPD analysis of cocaine-spiked plasma samples.

Key: (A) cocaine-free plasma sample with only n-PEBE; (B) plasma sample spiked with cocaine (320 ng/mL), and with n-PEBE as internal standard. a = cocaine, b = n-PEBE

ples are given in Figure 36. There was consistently minimal interference with the cocaine and n-PEBE peaks.

Spiked Urine Samples. Blank urine samples spiked with increasing levels of cocaine base and carried through the entire extraction/GC-NPD procedure with n-PEBE as internal standards were used to calibrate the HP 5880 for urine analysis. Examples of gas chromatograms of spiked urine samples analyzed for cocaine are given in Figure 37. Tracings were cleaner than those of analyzed plasma samples in the cocaine and n-PEBE regions.

The possible conversion in vitro of cocaine to ecgonine methyl ester, benzoylecgonine, and ecgonine in unacidified urine at 37° C was investigated. Cocaine test samples were prepared by adding cocaine to undiluted dog urine (pH 7.57, specific gravity = 1.040, preserved with a few crystals of thymol) to a concentration of 38.46 μg/mL, equivalent to 126.8 μmol/L. After incubation at 37° C for 24 hours, the cocaine concentration was found to be 8.224 μg/mL, equivalent to 27.11 μmol/L; only 21.38% of the original cocaine concentration remained in the urine test samples.

The final concentrations of ecgonine methyl ester, benzoylecgonine, and ecgonine were also determined in the incubated test samples. The mean final concentrations of benzoylecgonine, ecgonine, and ecgonine methyl ester found in the urine test samples were 83.86, 20.76, and 4.41 μmol/
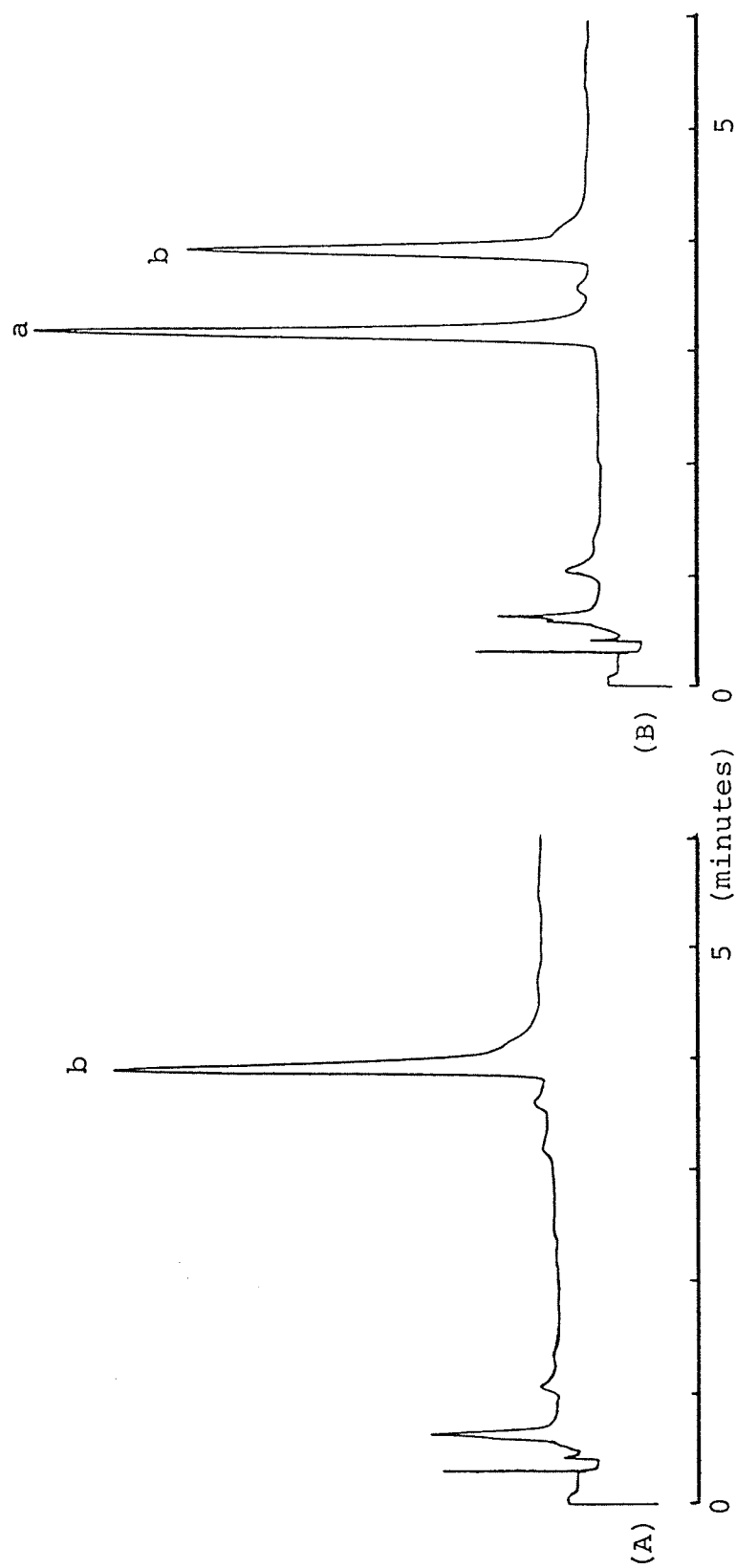
Figure 37: Sample gas chromatograms from the extraction/GC-NPD analysis of cocaine-spiked urine samples.

Key: (A) cocaine-free diluted urine (1:10) sample with only n-PEBE; (B) diluted urine (1:10) sample spiked with cocaine (320 ng/mL), and with n-PEBE as internal standard. a = cocaine, b = n-PEBE

L, respectively, representing 66.14, 16.37, and 3.48 % conversion of the spiked cocaine to benzoylecgonine, ecgonine and ecgonine methyl ester, respectively, after 24 h at 37° C.

Experimental (In Vivo) Dog Plasma and Urine Samples. The concentration of cocaine in dog plasma and urine samples obtained after intravenous administration of cocaine hydrochloride was determined according to the extraction/GC-NPD method with n-PEBE used as true internal standard. Examples of gas chromatograms obtained from the GC-NPD analysis of cocaine in experimental dog plasma and urine samples are given in Figure 38. The peak height ratios, cocaine/n-PEBE, of analyzed spiked plasma and urine samples used as standards to calibrate the gas chromatograph were monitored to ensure that the required sensitivity and linearity were maintained. Examples of cocaine plasma and urine concentration calibration curves are given in Figure 39. The cocaine concentration calibration curves for plasma and urine were linear over the range of concentrations examined with correlation coefficents, r, of each equal to 0.999.
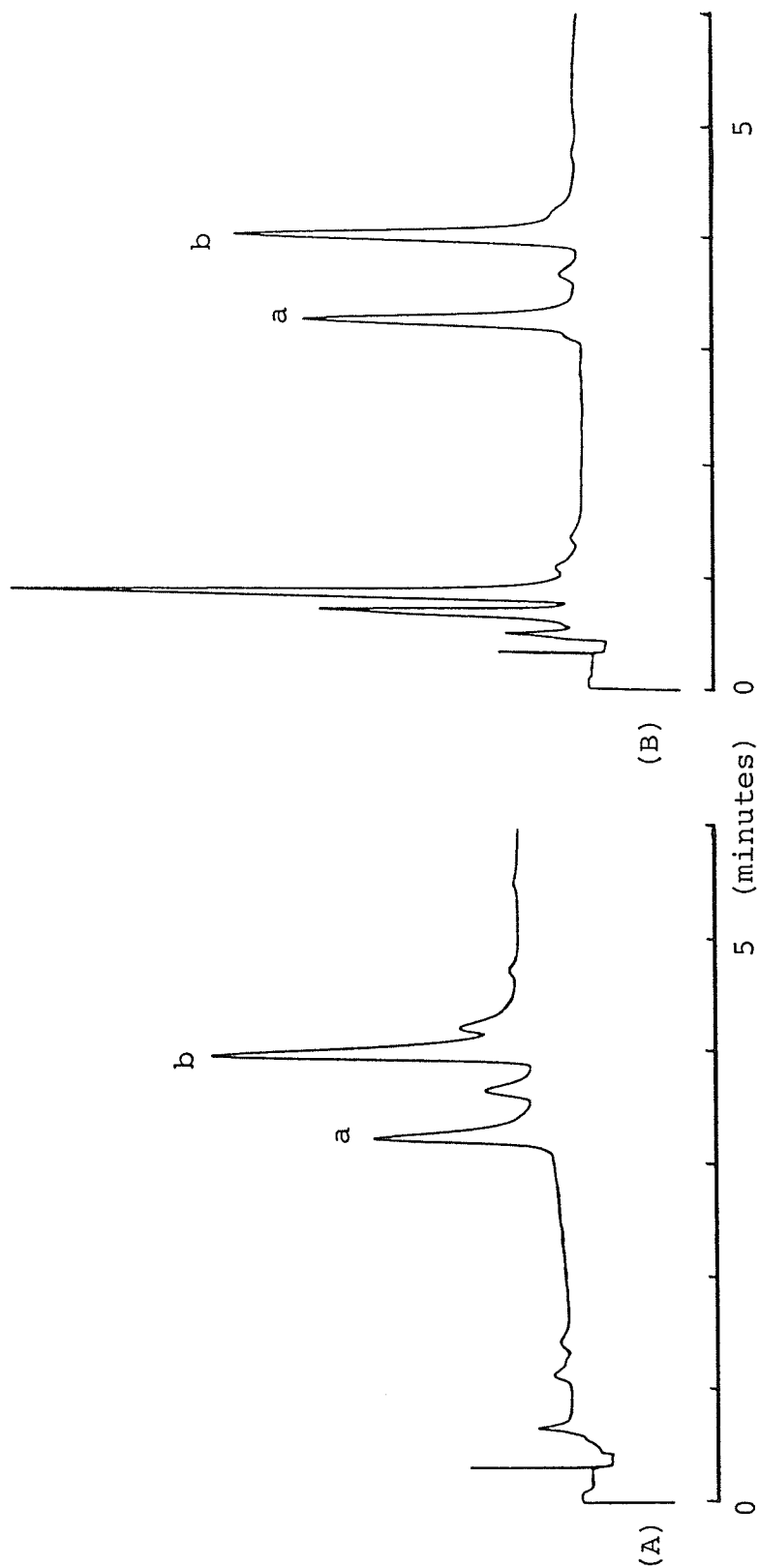
Figure 38: Sample gas chromatograms from the extraction/GC-NPD analysis of dog plasma and urine samples obtained after the intravenous administration of a single dose of cocaine, 5 mg/kg, to a female Beagle dog.

Key: (A) plasma sample, t=1.51 h; (B) diluted urine (1:4), 0-48 h.
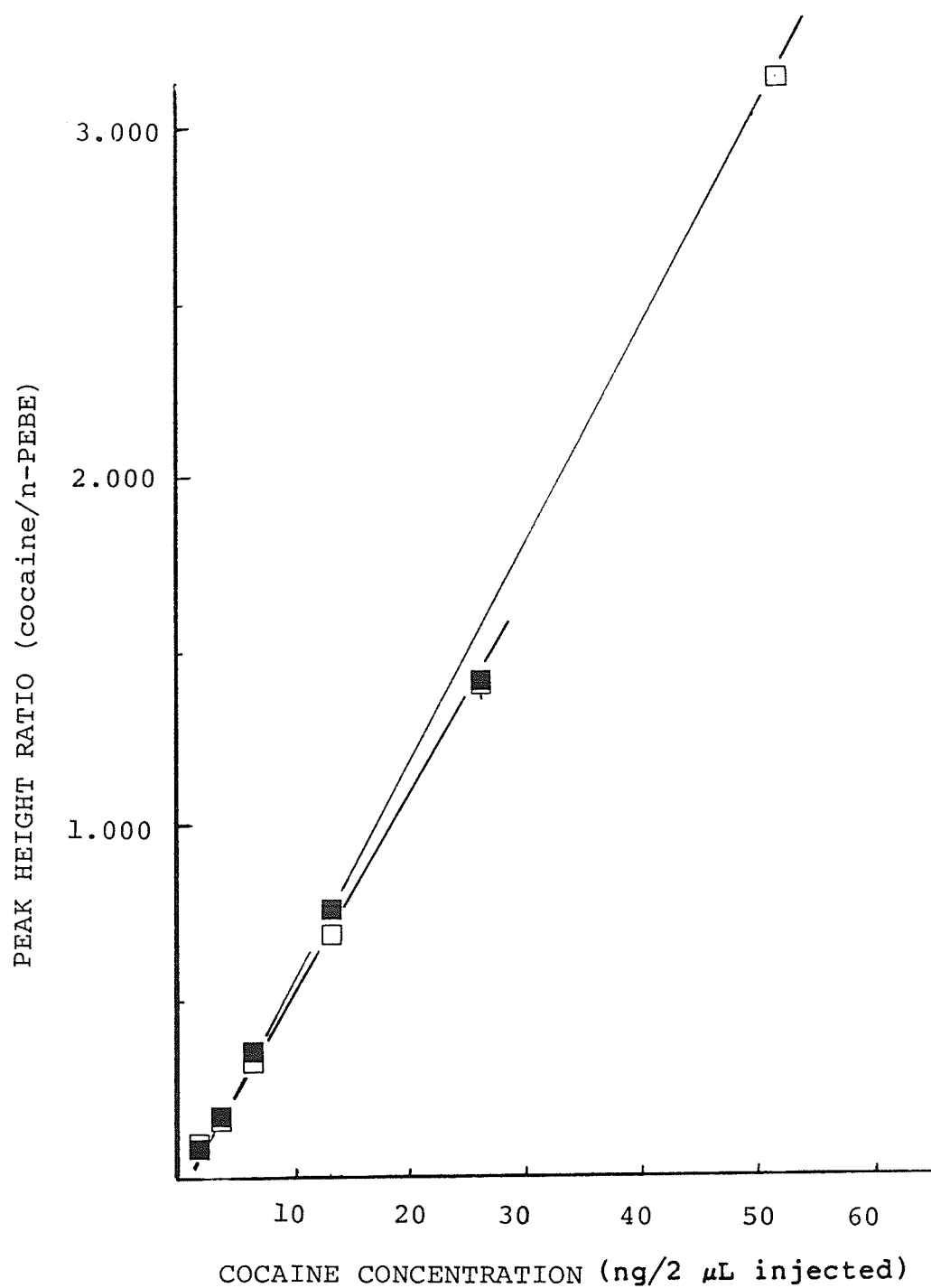
Figure 39: GC concentration calibration curves for the determination of cocaine in plasma and urine.

Key: (■) plasma; (□) diluted urine, 1:4.

IV-B. PHARMACOKINETICS

1. INTRAVENOUS ECGONINE

A dose of ecgonine hydrochloride equivalent to 10 mg ecgonine base per kg body weight was administered intravenously to a female Beagle dog, XA59. A series of blood samples were drawn up to 3 h following administration of the dose. Except for brief discomfort associated with insertion of an in-dwelling venous catheter, the dog experienced no observable ill effects.

A 0-48 h urine sample, volume=335 mL, pH=6.32, and specific gravity=1.038, was collected from the dog kept in a metabolic cage. An accompanying tray rinse, volume=431 mL, was also collected. A small volume of urine (approximately 10 mL) was lost when the dog was removed from the metabolic cage for the last blood sampling.

Plasma Analysis. The concentration of ecgonine in each plasma sample was determined according to the extraction/ TLC/GC-FID method. Gas chromatograms of control plasma samples did not show any peaks which would interfere with the measurement of ecgonine. The plasma ecgonine concentration-time data are given in Table XI.

Pharmacokinetic Analysis. A semi-logarithmic plot of the natural log of the plasma ecgonine concentration (μg/mL) vs. time (h) was constructed. The resulting curve was re-

193

Table XI: Plasma Ecgonine Concentrations following Intrave-
nous Administration of a Single Dose of Ecgonine, 10 mg/
kg, to a Female Beagle Dog.

Time[a] (h)	Concentration[b] (μg/mL)
0.05	53.1 ± 12.2
0.14	39.0 ± 2.7
0.30	36.3 ± 4.5
0.56	24.5 ± 1.8
0.76	23.3 ± 1.0
1.01	17.0 ± 0.4
1.51	12.1 ± 0.8
2.02	8.4 ± 0.2
3.03	4.9 ± 0.4

[a] Mid-point of blood collection period.

[b] $\bar{X}$ ±SD.

solved by the method of residuals into two exponential components corresponding to the two terms of the general biexponential equation:

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \qquad (1)$$

Initial estimates of the coefficients, C_1 and C_2, and the rate constants, λ_1 and λ_2, were obtained directly from the "stripped" semi-log plot. These initial estimates for C_1, C_2, λ_1, and λ_2 were used to calculate initial estimates of the pharmacokinetic parameters V_1, k_{12}, k_{21}, and K of Equation 2:

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \qquad (2)$$

which describes a two-compartment open model with rapid IV input. The initial estimates of the several coefficients and pharmacokinetic parameters are given in Table XII.

The initial estimates were used as starting values to obtain final estimates of the pharmacokinetic parameters by least-squares nonlinear regression analysis of the plasma ecgonine concentration-time data. Data analyses according to Equations 1 and 2 were completed with different weightings for the plasma ecgonine concentration, y: $1.0y$, $1/y$, and $1/y^2$. Weighting the plasma ecgonine concentration-time data according to $1/y^2$ gave the best fit as judged by the test statistics generated by NONLIN. All subsequent nonli-

Table XII: Initial and Final Estimates of Pharmacokinetic
    Parameters for Ecgonine after Intravenous Administration
    of a Single Dose, 10 mg/kg, to a Female Beagle Dog.

Parameter	Initial Estimate[a]	Final Estimate[b]	± SD
C_1 (μg/mL)	16.71	26.23	7.78
λ_1 (h^{-1})	3.180	2.410	1.116
C_2 (μg/mL)	35.13	26.15	8.79
λ_2 (h^{-1})	0.6707	0.5578	0.1228
V_1 (mL)	3029	3001	232
k_{12} (h^{-1})	0.5802	0.5718	0.3070
k_{21} (h^{-1})	2.371	1.443	0.855
K (h^{-1})	0.8995	0.9029	0.0874

[a]Estimate by graphical analysis.

[b]NONLIN estimate.

196

near regressions were carried out with weighting the plasma concentration data according to $1/y^2$.

Final estimates of the pharmacokinetic parameters are given in Table XII. There was good agreement between the predicted plasma ecgonine concentrations and the observed ecgonine plasma concentrations as can be seen by inspection in Figure 40. The test statistics "r^2" = 0.998 and "cor" = 0.990 also indicate very good agreement between the observed and predicted concentration values.

Urine Analysis. The amount of ecgonine found in the urine collected over the 48 hours after drug administration and the accompanying rinse are presented in Table XIII. A total of 125.1 mg of ecgonine was recovered in the urine representing 79.68% of the dose of ecgonine administered intravenously.
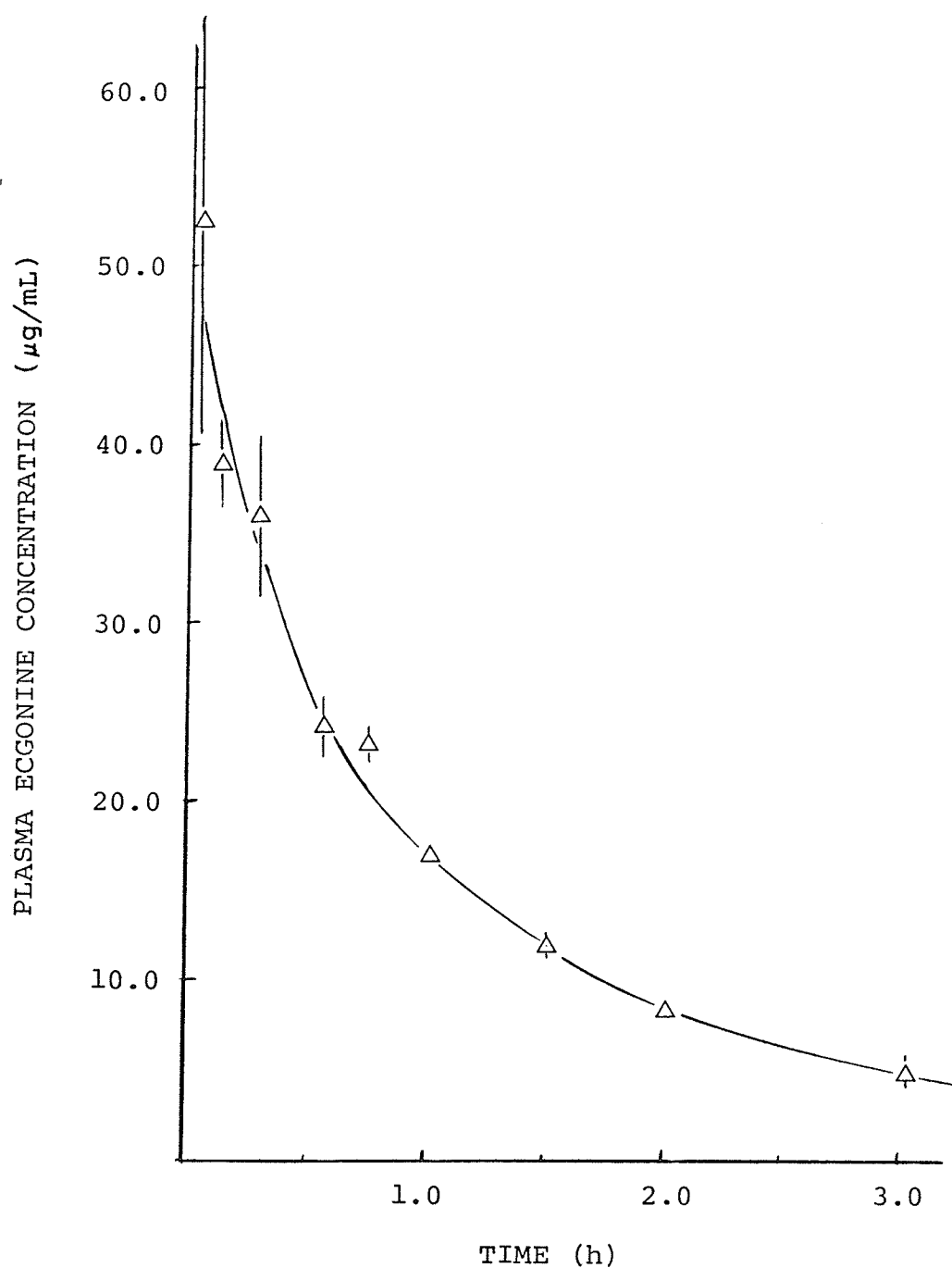
Figure 40:   Theoretical curve of best fit to the plasma
   ecgonine concentration-time data following intravenous
   administration of a single dose of ecgonine, 10 mg/kg, to
   a female Beagle dog.

   Key: (△) observed values.

Table XIII: Amount of Ecgonine Found in the Urine following
Intravenous Administration of a Single Dose of Ecgonine,
10 mg/kg, to a a Female Beagle Dog.

Urine Collection	Volume (mL)	Ecgonine Conc.[a] (μg/mL)	Am't of Ecgonine (mg)
0-48 h	335	36.74	123.1
Rinse	431	4.54	2.0
			125.1

[a]Mean of duplicate assay.

2. INTRAVENOUS BENZOYLECGONINE

A dose of 10 mg benzoylecgonine per kg body weight was administered intravenously to the female Beagle dog, XA59. A series of blood samples was drawn up to 6 h following benzoylecgonine administration. The dog exhibited no observable ill effects which could be attributed to the benzoylecgonine.

A 0-72 h urine sample was collected which had a volume=485 mL, pH=6.05, and specific gravity=1.031. (The tray rinse amounted to 133 mL.)

Plasma Analysis. The concentrations of benzoylecgonine and its hydrolysis product, ecgonine, were determined in each plasma sample according to the extraction/TLC/GC-FID method. Gas chromatograms of control plasma samples did not reveal any peaks which would interfere with the measurement of either benzoylecgonine or ecgonine. The plasma benzoylecgonine concentration-time data are summarized in Table XIV. No ecgonine was detected in any of the plasma samples.

Pharmacokinetic Analysis. A semi-log plot of the natural log of the plasma benzoylecgonine concentration (μg/mL) vs. time (h) was stripped to obtain initial estimates of the coefficients, C_1 and C_2, and the rate constants, λ_1 and λ_2, of the general biexponential equation:

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \qquad (1)$$

Table XIV: Plasma Benzoylecgonine Concentrations following
   Intravenous Administration of a Single Dose of Benzoyl-
   ecgonine, 10 mg/kg, to a Female Beagle Dog.

Time[a] (h)	Concentration[b] (μg/mL)
0.09	24.0 ± 2.2
0.16	19.9 ± 2.9
0.26	19.3 ± 1.7
0.51	16.0 ± 0.9
0.76	16.5 ± 0.8
1.01	14.1 ± 0.3
1.50	13.9 ± 1.1
2.02	11.6 ± 1.9
2.50	11.8 ± 0.3
3.01	9.5 ± 0.6
4.02	9.0 ± 0.8
6.05	6.1 ± 0.8

[a]Mid-point of blood collection period.

[b]$\bar{X}$ ± SD.

The initial estimates of C_1, C_2, λ_1, and λ_2 were used to calculate initial estimates of the pharmacokinetic parameters V_1, k_{12}, k_{21}, and K of Equation 2:

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \qquad (2)$$

which describes a two-compartment open model with rapid IV input. The initial estimates of the several coefficients and pharmacokinetic parameters are given in Table XV.

These initial estimates were used as starting values in determining the final estimates of the pharmacokinetic parameters by least-squares nonlinear regression analysis of the plasma benzoylecgonine concentration-time data. The plasma concentration data was weighted according to $1/y^2$. The final estimates of the pharmacokinetic parameters whcih gave the best-fitting curve are given in Table XV. There was good agreement of the observed plasma benzoylecognine concentrations and the predicted plasma concentrations shown graphically in Figure 41. The test statistics "r^2" and "cor" were 0.988 and 0.991, respectively, indicating good agreement between the observed and the predicted concentration values.

<u>Urine Analysis</u>. The concentrations of benzoylecgonine and ecgonine were determined in the urine collected over 72 hours after intravenous administration of benzoylecgonine. No ecgonine was detected in either the 0-72 h urine sample

Table XV: Initial and Final Estimates of Pharmacokinetic
    Parameters for Benzoylecgonine after Intravenous Adminis-
    tration of a Single Dose, 10 mg/kg, to a Female Beagle
    Dog.

Parameter	Initial Estimate[a]	Final Estimate[b]	± SD
C_1 (μg/mL)	9.300	11.96	5.01
λ_1 (h^{-1})	5.555	7.037	3.402
C_2 (μg/mL)	17.57	17.43	0.66
λ_2 (h^{-1})	0.1765	0.1751	0.0124
V_1 (mL)	5843	5341	941
k_{12} (h^{-1})	1.772	2.680	1.944
k_{21} (h^{-1})	3.694	4.247	1.496
K (h^{-1})	0.2654	0.2903	0.0543

[a]Estimate by graphical analysis.

[b]NONLIN estimate.
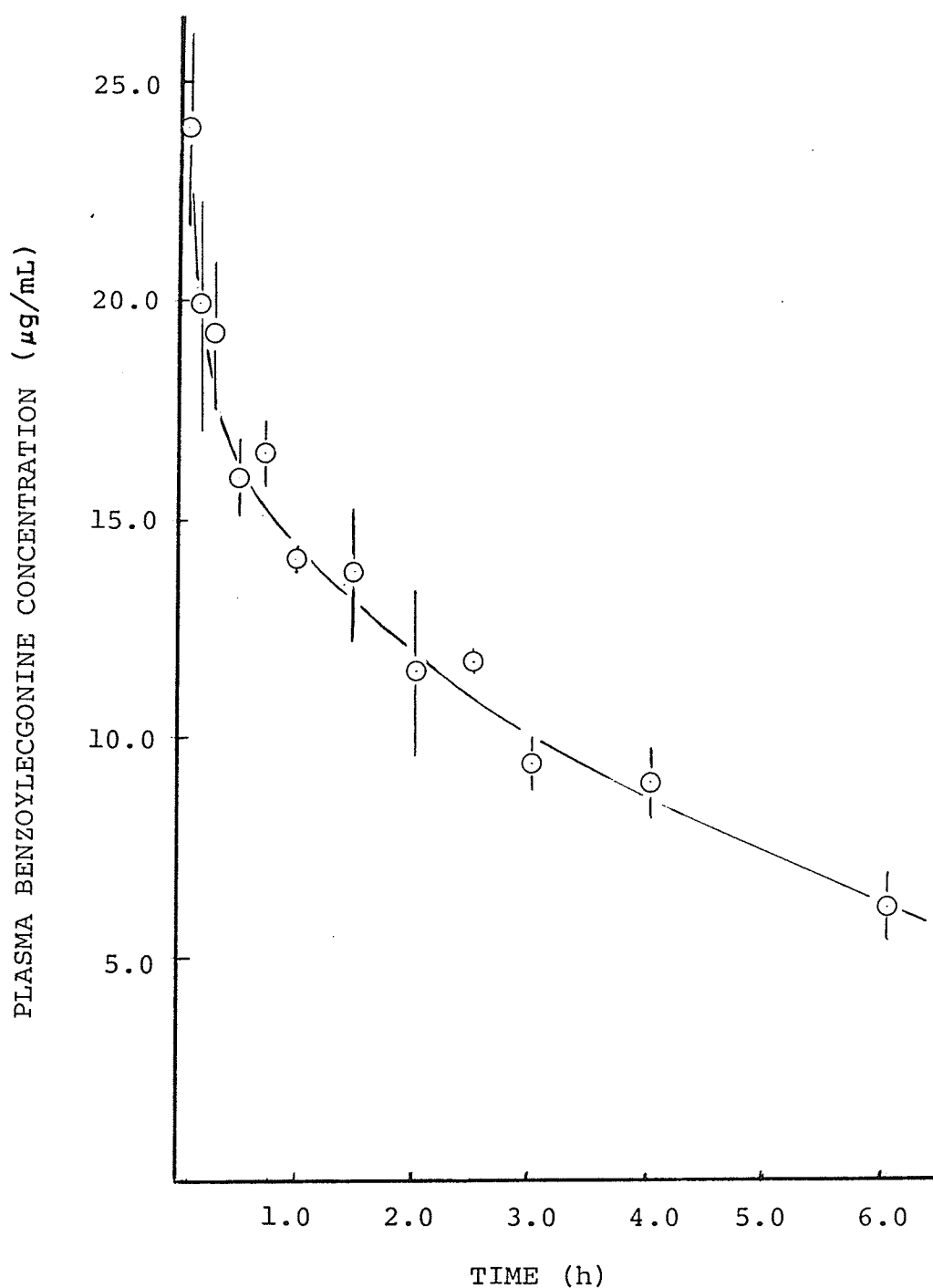
Figure 41:   Theoretical curve of best fit to the plasma
    benzoylecgonine concentration-time data following in-
    travenous administration of benzoylecgonine, 10 mg/kg to
    a female Beagle dog.

    Key: (O) observed values.

or the tray rinse. The concentrations of benzoylecgonine measured are given in Table XVI. A total of 149.8 mg of benzoylecgonine was recovered in the urine representing 95.41% of the dose of benzoylecgonine administered intravenously.

Table XVI: Amount of Benzoylecgonine Found in the Urine
    following Intravenous Administration of a Single Dose of
    Benzoylecgonine, 10 mg/kg, to a Female Beagle Dog.

Urine Collection	Volume (mL)	Benzoyl- ecgonine Conc.[a] (μg/mL)	Am't of Benzoyl- ecgonine (mg)
0-72 hr[b]	485	307.1	148.9
Rinse[b]	133	6.5	0.9
			149.8

[a] Mean of duplicate samples.

[b] No ecgonine present.

3. INTRAVENOUS ECGONINE METHYL ESTER

A dose of ecgonine methyl ester hydrochloride equivalent to 25 mg alkaloid base per kg body weight was administered intravenously to the female Beagle dog, XA59.   A series of blood samples were drawn up to 6 h following administration of ecgonine methyl ester hydrochloride.   Except for the brief discomfort associated with insertion of the in-dwelling venous catheter and injection of the ecgonine methyl ester dose, the dog experienced no observable ill effects.

Two urine collections were made over a 72 hour period following ecgonine methyl ester administration.   The first was a 0-48 h urine collection which had a volume of 487 mL, pH of 6.54, and a specific gravity of 1.042. The accompanying tray rinse had a volume of 355 mL. The second, 48-72 h, urine collection had a volume of 345 mL, pH of 5.70, and a specific gravity of 1.030.   A 225 mL tray rinse was also collected.

Plasma Analysis. The concentrations of ecgonine methyl ester in each plasma sample was determined according to the extraction/GC-FID method.   Gas chromatograms of extracts from control plasma samples did not reveal any peaks which might interfere with the measurement of ecgonine methyl ester.   Plasma ecgonine methyl ester concentration-time data are given in Table XVII.

Table XVII: Plasma Ecgonine Methyl Ester Concentrations
   following Intravenous Administration of a Single Dose of
   Ecgonine Methyl Ester, 25 mg/kg, to a Female Beagle Dog.

Time[a] (h)	Concentration[b] (μg/mL)
0.10	16.0 ± 0.3
0.18	14.2 ± 0.2
0.26	14.5 ± 0.8
0.51	12.8 ± 0.5
0.77	11.6 ± 0.3
1.01	11.1 ± 0.9
1.49	9.6 ± 0.2
2.00	8.0 ± 0.4
2.50	6.9 ± 0.5
3.00	6.1 ± 0.1
4.00	4.3 ± 0.2
6.09	1.8 ± 0.1

[a]Mid-point of collection period.

[b]$\bar{X}$ ± SD.

The ecgonine remaining in the extracted aqueous phases of the 2.00, 2.50, and 3.00 h plasma samples was determined by re-extraction/TLC/GC-FID. The concentrations of ecgonine in these samples were found to be 1.90, 1.85, and 1.47 μg/mL, respectively.

<u>Pharmacokinetic Analysis</u>. A semi-logarithmic plot of the natural log of the plasma ecgonine methyl ester concentration (μg/mL) vs. time (h) was drawn. The ecgonine methyl ester concentrations declined log-linearly (r=0.997) after intravenous administration. The mono-exponential equation:

$$C = [D/V]\exp(-Kt) \tag{3}$$

characterizing a one-compartment open model with rapid IV input was fitted to the data. Initial estimates of the pharmacokinetic parameters V and K obtained directly from the plot are listed in Table XVIII.

Final estimates of the pharmacokinetic parameters were obtained by least-squares nonlinear regression analysis of the plasma ecgonine methyl ester concentration-time data. Curve-fitting according to Equation 3 was accomplished with weighting of the ecgonine methyl ester plasma concentrations according to $1/y^2$. The final estimates of the pharmacokinetic parameters are given in Table XVIII. There was excellent agreement between the observed and predicted plasma ecgonine methyl ester concentrations as can be seen in Fig-

Table XVIII: Initial and Final Estimates of Pharmacokinetic
    Parameters for Ecgonine Methyl Ester after Intravenous
    Administration of a Single Dose, 25 mg/kg, to a Female
    Beagle Dog.

Parameter	Initial Estimate[a]	Final Estimate[b]	± SD
K (h^{-1})	0.3402	0.3445	0.0083
V (mL)	22,310	22,250	472

[a]Estimate by graphical analysis.

[b]NONLIN estimate.
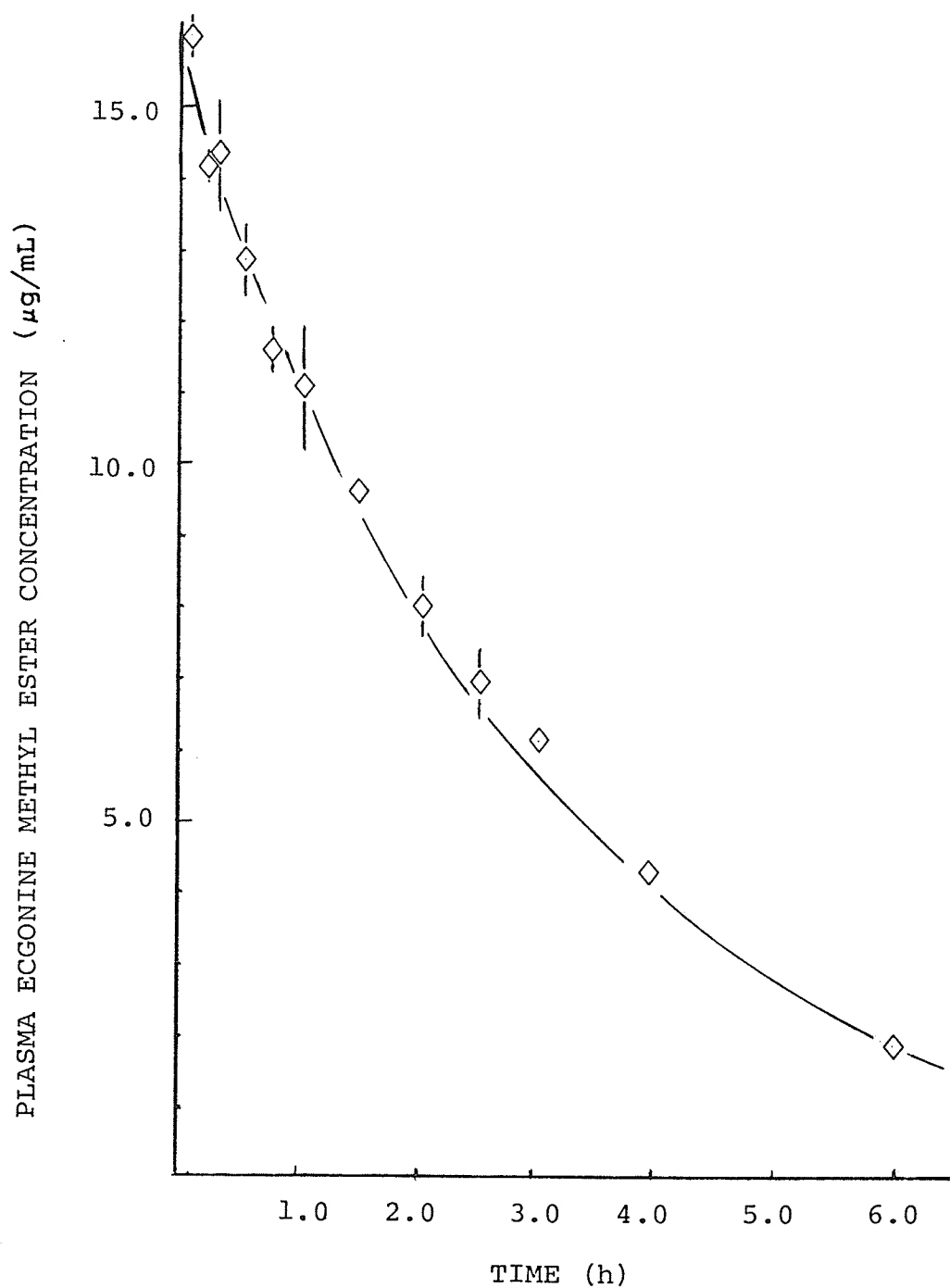
Figure 42:   Theoretical curve of best fit to the plasma
   ecgonine methyl ester concentration-time data following
   intravenous administration of ecgonine methyl ester, 25
   mg/kg, to a female Beagle dog.

   Key: (◇) observed values.

ure 42.   The test statistics "r^2"   and "cor" were 0.997 and
0.996, respectively.

   Urine Analysis.   The concentrations of ecgonine methyl
ester and ecgonine were determined in the urine collections
and rinses.   The amounts of ecgonine methyl ester and ecgo-
nine recovered in the 0-48 h urine sample and tray rinse are
given in Table XIX.   Neither ecgonine methyl ester nor ecgo-
nine was detected in the 48-72 h urine sample or tray rinse.
A total of 67.46 mg of ecgonine methyl ester was recovered
in the urine representing 19.18% of the original ecgonine
methyl ester dose.   A total of 49.18 mg of ecgonine was re-
covered in the urine which represents 15.05% of the ecgonine
methyl ester administered calculated on a molar basis.

Table XIX: Amount of Ecgonine Methyl Ester and Ecgonine
   Found in the Urine following Intravenous Administration
   of a Single Dose of Ecgonine Methyl Ester, 25 mg/kg, to a
   Female Beagle Dog.

| Urine Collection[a] | Volume (mL) | Ecgonine Methyl Ester | | Ecgonine | |
		Conc. (μg/mL)	Am't (mg)	Conc. (μg/mL)	Am't (mg)
0-48 h	487	136.1[b]	66.3	99.1	48.3
Rinse	355	3.3[c]	1.2	2.6	0.9
			67.5		49.2

[a]48-72 h collection, devoid of ecgonine methyl ester and
 ecgonine.

[b]Mean of triplicate assays.

[c]Mean of duplicate assays.

4. INTRAVENOUS COCAINE

A dose of cocaine hydrochloride equivalent to 5 mg cocaine base per kg body weight was administered intravenously to the female Beagle dog, XA59. Blood samples were drawn periodically up to 6 h following cocaine administration. Within a few minutes after injection, the animal exhibited signs of hyperexcitability with muscle tremor and salivation. After approximately 30 minutes the excitability was markedly reduced approaching near normal.

A 0-48 h urine sample was collected with a volume=295 mL, pH=4.51, and specific gravity=1.029. The tray rinse had a volume=96 mL. The 48-96 h urine collection had a volume of 320 mL, pH of 5.58, and specific gravity of 1.036. (The tray rinse amounted to 78 mL.)

Plasma Analysis. The concentration of cocaine in the plasma separated from the blood samples was determined according to the extraction/GC-NPD procedure with n-PEBE as true internal standard. Gas chromatograms of control plasma samples did not reveal any extraneous peaks which might interfere with the measurement of cocaine. The plasma cocaine concentration-time data are given in Table XX.

Pharmacokinetic Analysis. A semi-logarithmic plot of the natural log of the plasma cocaine concentrations (ng/mL) vs. time (h) was resolved into two exponential components by

Table XX: Plasma Cocaine Concentrations following Intrave-
    nous Administration of a Single Dose of Cocaine,   5 mg/kg
    to a Female Beagle Dog.

Time[a] (h)	Concentration[b] (ng/mL)
0.11	2988 ± 198
0.19	2751 ± 333
0.81	436 ± 12
1.00	403 ± 11
1.51	272 ± 50
1.98	185 ± 4
3.00	106 ± 3
4.04	74 ± 4
6.01	32 ± 2

[a]Mid-point of blood collection period.

[b]$\bar{X}$ ± SD.

the method of residuals, used to fit the concentration-time data to the the general biexponential equation:

$$C = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t) \qquad (1)$$

Initial estimates of the coefficients C_1 and C_2, and the rate constants, λ_1 and λ_2, were used to calculate initial estimates of the pharmacokinetic parameters V_1, k_{12}, k_{21}, and K of Equation 2:

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t) \qquad (2)$$

which describes a two-compartment open model with rapid IV input. Initial estimates of the several coefficients and rate constants are given in Table XXI.

Final estimates of the pharmacokinetic parameters were obtained by least-squares nonlinear regression analysis of the plasma cocaine concentration-time data according to Equations 1 and 2. The cocaine concentration data was weighted according to $1/y^2$. The final estimates of the pharmacokinetic parameters which produce the best-fitting curve are given in Table XXI. There was very good agreement between the observed and predicted plasma cocaine concentrations. The test statistics "r^2" and "cor" were 1.000 and 0.993, respectively. The best-fitting theoretical curve is reproduced in Figure 43.

216

Table XXI: Initial and Final Estimates of Pharmacokinetic
    Parameters for Cocaine after Intravenous Administration
    of a Single Dose, 5 mg/kg, to a Female Beagle Dog.

| Parameter	Initial Estimate[a]	Final Estimate[b]	± SD
C_1 (ng/mL)	3122	4310	561
λ_1 (h^{-1})	2.946	4.001	0.527
C_2 (ng/mL)	409.8	450	62
λ_2 (h^{-1})	0.4264	0.4469	0.0363
V_1 (mL)	21,520	15,970	1941
k_{12} (h^{-1})	0.9086	1.381	0.285
k_{21} (h^{-1})	0.7188	0.7830	0.1017
K (h^{-1})	1.745	2.284	0.228

[a]Estimate by graphical analysis.

[b]NONLIN estimate.
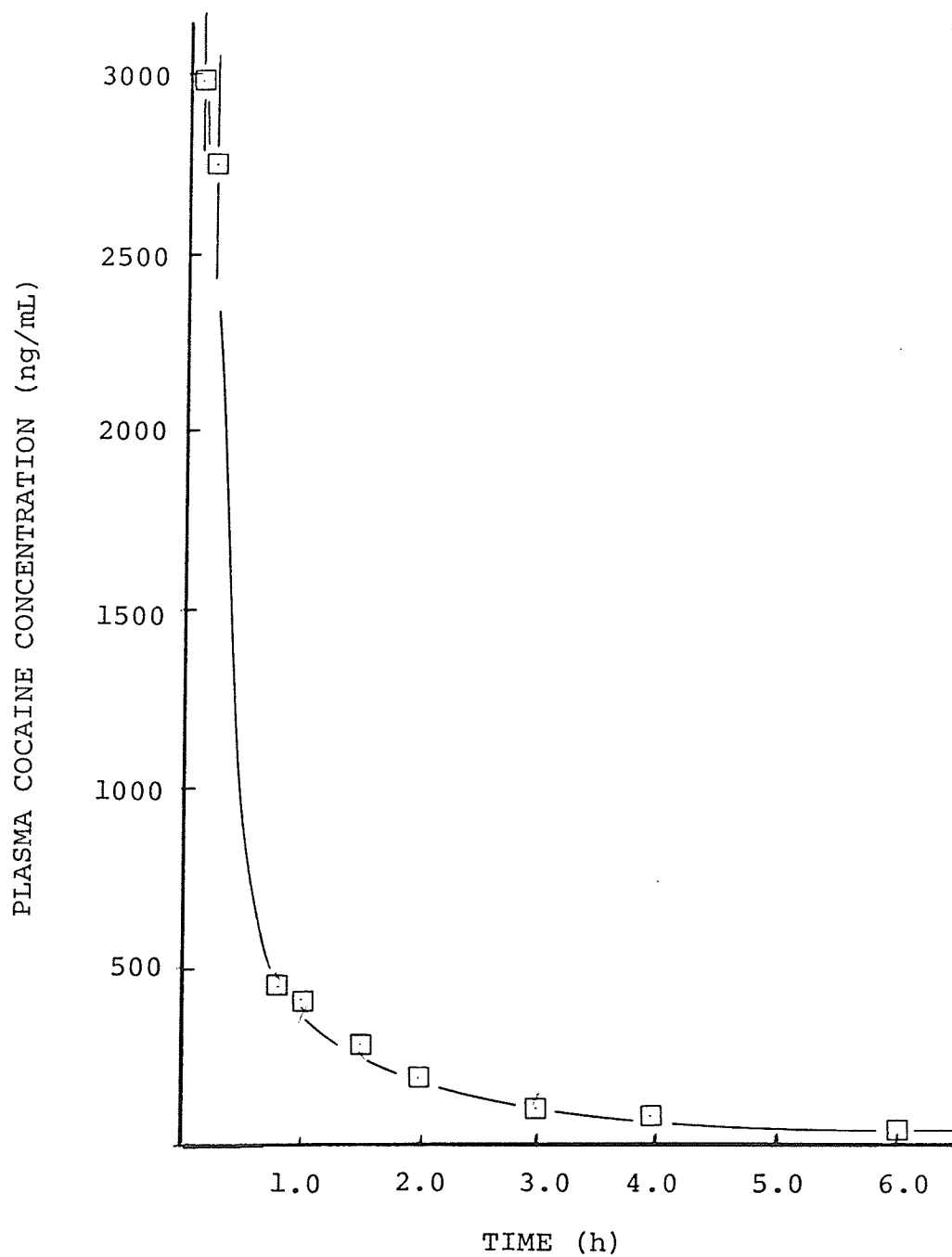
Figure 43: Theoretical curve of best fit to the plasma co-
caine concentration-time data following intravenous ad-
ministration of cocaine, 5 mg/kg, to a female Beagle dog.

Key: (□) observed values.

Urine Analysis. The concentration of cocaine in the two urine collections and rinses was determined according to the extraction/GC-NPD method. The total amount of cocaine recovered in the urine and rinses is given in Table XXII.

The concentrations of ecgonine methyl ester, benzoyl-ecgonine, and ecgonine were also determinied in the 0-48 h urine and rinses. The concentrations of these alkaloids in the 48-96 h urine sample and rinse were too low to be measured. The individual amounts of ecgonine methyl ester, benzoylecgonine, and ecgonine found in the urine over 48 hours are given in Table XXII. A total of 23.7 % of the administered cocaine dose was recovered in the urine as cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine combined when calculated on a molar basis.

Table XXII: Amounts of Individual Ecgonine Alkaloids Found
    in the Urine[a] following Intravenous Administration of a
    Single Dose of Cocaine, 5 mg/kg, to a Female Beagle Dog.

| Compound | Amount Found | | Percent of Cocaine Dose Recovered |
	(mg)	μmol	
Cocaine	0.45	1.48	0.59
Ecgonine Methyl Ester	3.58	17.94	7.16
Benzoylecgonine	7.69	26.60	10.62
Ecgonine	2.48	13.36	5.33
		59.38	23.70

[a]0-48 h urine collection. Only trace amounts of ecgonine
  alkaloids present in 48-96 h collection.

Chapter V

DISCUSSION

V-A.   ANALYTICAL PROCEDURES

1. ANALYSIS OF ECGONINE ALKALOIDS

Commercially available cocaine hydrochloride of high quality was used for the synthesis of benzoylecgonine and ecgonine by relatively simple straightforward procedures. Simple reflux of cocaine (base) in water yielded benzoylecgonine (18), and cocaine (hydrochloride) in 0.75 N HCl yielded ecgonine hydrochloride (19). Both products were obtained in good yields of high quality. The benzoylecgonine crystals when first obtained appeared to have the low variable melting point characteristics of a metastable form, since on exposure to air they reverted to some form with a stable, narrow melting point comparable to its reported literature value.

Ecgonine methyl ester hydrochloride prepared from cocaine hydrochloride contained approximately 5% ecgonine hydrochloride which could not be removed by recrystallization. Also, some ecgonine always formed when an equivalent amount of hydrochloric acid was added to the pure ecgonine methyl ester base and the salt formed, isolated and recrystallized.

The "crude" ecgonine methyl ester hydrochloride, however, was successfully purified by the extraction previously described. Analysis of the resulting ecgonine methyl ester hydrochloride by GC and TLC revealed only a single peak and band, respectively.

Of the two compounds used as internal standards in GC analysis, theophylline and the n-propyl ester of benzoylecgonine (n-PEBE), the latter gave the more reliable, consistent GC results. Its synthesis from benzoylecgonine and anhydrous n-propyl alcohol at reflux temperature with acetyl chloride as catalyst (91) in place of hydrogen chloride gas proceeded much more smoothly giving near quantitative yields of a high quality product. Recrystallized, the n-PEBE gave a single, well-defined GC peak.

Gas Chromatography-FID. The GC-FID method for the quantitation of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine was based on Moore's (47) qualitative method for the analysis of ecgonine and benzoylecgonine present in cocaine samples. N,O-bis-(trimethylsilysl)-acetamide (BSA) was used to prepare trimethylsilyl derivatives of ecgonine and benzoylecgonine which were subsequently separated on a 10% OV-101 column. Hexadecane and tetracosane were used as external reference compounds, chromatographed separately. Roscoe's modification (48) of Moore's method included the addition of theophylline as internal standard prior to derivatization and extended the method to the quan-

titation of ecgonine methyl ester. In the present research the method used by Roscoe was used initially but later modified to improve sensitivity and reproducibility.

N,O-bis-(trimethylsilyl)-acetamide (BSA) was used to prepare trimethylsilyl derivatives of ecgonine methyl ester, benzoylecgonine, ecgonine, and theophylline for chromatography. Cocaine is not derivatized by BSA but is sufficiently soluble in BSA to be analyzed by GC-FID directly. A 10% OV-101 column was quite efficient in separating cocaine, the derivatized ecgonine alkaloids, and the theophylline internal standard.

Following the extraction studies the procedure was modified for all subsequent experiments requiring the quantitation of cocaine and other ecgonine alkaloids. A column with 3% OV-101 as stationary phase on 80/100 mesh Chromosorb W HP was used in place of the 10% OV-101 column. Reducing the stationary phase loading provided a greater than two-fold increase in the response of each compound and better peak shape characteristics (narrower peaks, less tailing). Peaks corresponding to ecgonine methyl ester and ecgonine, however, were not sufficiently separated from the solvent front. Lowering the initial column temperature from 170° to 135° C increased the retention times of ecgonine methyl ester and ecgonine so that the corresponding peaks emerged later than the solvent front. The rate of programming of the column temperature was doubled from 8°/min to 16°/min.

Separation of all peaks was maintained with a further decrease in peak width with the added benefit that analysis time was reduced. The final column temperature, 270°, was held for 2 minutes to ensure all injected materials were eluted from the column. This was especially important in subsequent experiments when extracts of biological fluids were analyzed.

The glass columns packed with 3% OV-101 on 80/100 mesh Chromosorb W HP required extensive conditioning with Silyl-8® at elevated temperatures in order to bring the baseline down to an acceptable level and to attain and maintain optimum response of all alkaloids. A gradual reduction in response and increase in peak tailing with continued use (especially in the case of benzoylecgonine) made it necessary to replace the columns after approximately 3 months. Commercial columns packed with 3% SP-2100 (a methyl silicone stationary phase equivalent to OV-101) on 80/100 Supelcoport proved to be more reliable for the routine analysis of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine. Retention times obtained for all compounds on the 3% SP-2100 column were almost identical to those obtained on the 3% OV-101 column. Optimum responses on the SP-2100 column were achieved after the brief conditioning recommended by the manufacturer and were maintained for a longer period of time (6-12 months). The commercially-packed columns also showed less run-to-run variation than the OV-101 columns.

Theophylline proved to be inferior to n-PEBE as an internal standard for the GC-FID analysis of the ecgonine alkaloids. The theophylline response gradually increased with the first few samples injected at the beginning of each run reaching a constant value only after three or more "priming" injections. On the other hand, consistent responses were achieved immediately on injection of n-PEBE; no priming was required. In addition n-PEBE proved to be a better internal standard in the GC-FID analysis of extracts of biological fluids because the n-PEBE peak appeared at the end of the chromatogram, an area with few interferences.

Due to the lack of a ready commercial source of pure BSA it became necessary to switch to N,O-bis-(trimethyl- silyl)-trifluoroacetamide (BSTFA) as derivatizing agent. Gas chromatograms of samples prepared with BSTFA were consistently cleaner than those pepared with BSA. The use of BSTFA resulted in less contamination of the FID collector.

GC-FID Standard Calibration Curves. GC standards containing known amounts of all four alkaloids: cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine and internal standard (theophylline or n-PEBE) were prepared containing dilute acid (0.6 N HCl in 2-propanol). The acid was added to ensure that each alkaloid was converted to the non-volatile hydrochloride salt prior to being taken to dryness under a stream of inert gas. Acidification also re-

duced the possible complexation of the ecgonine alkaloids with theophylline (48). It was important to remove all traces of methanol and 2-propanol from samples prior to addition of the derivatizing agent because alcohols can react with BSA and BSTFA which could interfere with the analysis.

GC standard calibration curves constructed for cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine with theophylline as internal standard, BSA as derivatizing agent, and analyzed on a 10% OV-101 column were linear over the range of concentrations examined (20-160 ng/μL injected) with correlation coefficients of 0.995-0.999. The GC-FID method was sufficiently sensitive for the accurate measurement of 10-20 ng of each alkaloid. These minimal detectable levels allowed the discrimination of alkaloid peak heights 4-5 mm above baseline noise. Intercepts of the GC standard calibration curves for cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine were 6.2, 2.9, 7.8, and 3.1 ng, respectively. The run-to-run variation was minimal with a coefficient of variation (CV) of replicate samples typically less than 10% at all concentrations. With continuous use and increasing column age, the response to each alkaloid decreased (especially benzoylecgonine) resulting in increased run-to-run variation.

The sensitivity and linearity of the GC-FID method were improved with the use of a 3% SP-2100 column, n-PEBE as internal standard, and BSTFA as derivatizing agent. GC stan-

dard calibration curves were linear over the range of concentrations examined (20-160 ng/μL injected) with correlation coefficients of 0.998-0.999, better than those achieved prior to modification of the method. The intercepts calculated for the reported GC standard calibration curves were also improved (0.1, 4.2, 5.2, and 3.8 ng for cocaine, ecgonine methyl ester, benzoylecgonine and ecgonine, respectively). In addition, run-to-run variations were also reduced with CV's typically less than 5%.

Extraction Studies. The organic solvents tested in the extraction studies of cocaine, ecgonine methyl ester, benzoylecgonine, and ecgonine ranged in polarity from the least polar, cyclohexane, to the most polar, chloroform:methylene chloride:2-propanol. In conducting these experiments, care was taken to minimize the hydrolysis of cocaine, ecgonine methyl ester, and benzoylecgonine. The organic solvent under examination was added to the aqueous buffer before the addition of methanolic solution containing 4 μg of each alkaloid. The tube was immediately shaken to extract the alkaloid minimizing the contact time of the alkaloids with the alkaline aqueous phase. Extraction was completed with a 5 minute shaking on the flatbed shaker.

The extraction studies helped define clearly the extractability of each of the four ecgonine alkaloids present in combination in the same solution. Cocaine was easily extracted into all organic solvents tested. The recovery of

cocaine was 90-100% and was relatively independent of pH on extraction with chloroform, methylene chloride, chloroform:cyclohexane (1:1), methylene chloride:cyclohexane (1:1), or chloroform:methylene chloride:2-propanol (1:1:1). There was a slight decrease in the recovery of cocaine from aqueous buffers pH 10 and 11, which was most likely due to alkaline hydrolysis of the cocaine during extraction. The recovery of cocaine in cyclohexane, the least polar solvent tested, was lower and more pH dependent. Its recovery in cyclohexane increased from 75% at pH 7 to ca. 100% at pH 10.

Ecgonine methyl ester behaved in a manner similar to cocaine. As expected, however, recoveries of ecgonine methyl ester were lower and more dependent on pH and polarity of the extracting solvent. The best recovery of ecgonine methyl ester (ca. 100%) was achieved with chloroform at pH 10. The recovery (ca. 75%) of ecgonine methyl ester extracted into methylene chloride:cyclohexane (1:1) was acceptable and the lower polarity of this solvent mixture made it quite suitable for the extraction of both cocaine and ecgonine methyl ester from biological fluids. The relatively non-polar nature of this solvent mixture made it less likely to extract endogenous substances which would interfere with GC-FID analysis.

The extraction of benzoylecgonine was highly dependent on the polarity of the extracting solvent but relatively independent of pH. The least polar solvents (cyclohexane, me-

thylene chloride:cyclohexane, and chloroform:cyclohexane) were unable to extract benzoylecgonine at any pH. The recovery of benzoylecgonine into chloroform and methylene chloride was approximately 60%. The best recovery of benzoylecgonine (ca. 90%) was obtained with the most polar solvent mixture tested, chloroform:methylene chloride:2-propanol, and was nearly constant over the pH range examined (pH 6-11).

As to be expected from the most amphoteric of the four ecgonine alkaloids, ecgonine was not easily extracted by any of the solvent/pH combinations tested. The extraction of ecgonine required a polar mixed solvent, relatively high alkaline pH, and high electrolyte concentration to "salt-out" the ecgonine from the aqueous phase. Extraction was achieved with chloroform:methylene chloride:2-propanol after saturation of the aqueous phase with solid potassium carbonate.

Thin-layer Chromatography (TLC). Thin-layer chromatographic procedures modified from those described by Munier and Drapier (37) were found to be the most reliable to separate and isolate both ecgonine and benzoylecgonine from other co-extractable ecgonine alkaloids and endogenous substances present in plasma and urine extracts. Initially, the solvent system described by Noirfalise and Mees (32) was tried and subsequently abandoned. This system which is

widely used for the TLC analysis of the ecgonine alkaloids is composed of chloroform:methanol:ammonia (47.5:47.5:5). As a developing solvent it was found to be unsuitable for the present work because of its erratic recoveries. The poor results were due to the high alkalinity of the developing solvent (measured pH 10.8). In a confirmatory experiment cocaine, ecgonine methyl ester, and benzoylecgonine added separately to silica and incubated with this developing solvent were found to undergo spontaneous hydrolysis. Depending on exposure time cocaine was hydrolyzed to ecgonine methyl ester and as far as ecgonine. Similarly treated, ecgonine methyl ester and benzoylecgonine were each hydrolyzed to ecgonine.

Munier and Drapier (37) used highly polar, neutral or acidic TLC solvent systems to effect the separation of the ecgonine alkaloids. One of these solvent systems (methanol:water, 70:30) was adopted for the present work to avoid the hydrolysis problems associated with alkaline developing solvents. The present TLC procedure was more than adequate for the separation and isolation of ecgonine and benzoylecgonine without hydrolysis.

On continued use of the TLC method a number of practical points became apparent. It was found that the actual migration distance from the origin of ecgonine and benzoylecgonine bands visualized on the TLC reference plate was a better indicator of the location of the alkaloid band loca-

tion than Rf values. The solvent front distances for indi-
vidual TLC plates varied according to the relative position
of the plate in the developing tank possibly due to the high
volatility of the developing solvent. These inconsistencies
could have been reduced by saturating the atmosphere of the
developing tank with methanol vapors prior to and during de-
velopment but this would have resulted in decreased resolu-
tion of the ecgonine alkaloid bands (37).

The TLC plates used were pre-run in developing solvent
prior to use in order to move contaminants present in the
silica (binder materials or impurities absorbed from the at-
mosphere) to the top of the plate where they would not in-
terfere with the chromatographic separation. Pre-run plates
were activated at 110° C for 1 hour and kept in a desiccator
over calcium sulfate until used. The best resolution of
ecgonine and benzoylecgonine was achieved when the TLC
plates were kept dry before streaking and development.

Ludy Tenger's reagent (31) was used to visualize ecgo-
nine and benzoylecgonine bands on TLC reference plates.
This reagent allowed the rapid visualization of bands con-
taining as little as 10 μg of ecgonine and benzoylecgonine
and was superior to Dragendorff's reagent (33).

Methanol:water, 80:20, was used to elute ecgonine and
benzoylecgonine from the silica. This mixture allowed the
efficient recovery of ecgonine and benzoylecgonine from the

silica and shorter periods of time (approximately 45 min at 70° under a stream of helium) were required to take 7 mL aliquots of this eluate to dryness than would have been possible if a higher proportion of water was used.

With regard to the recoveries of ecgonine and benzoylecgonine from silica scraped from TLC plates after development, the recovery of benzoylecgonine was high (ca. 85%) and was consistent over the range of streaked loads of 4.0 to 16.0 μg. The recovery of ecgonine was lower and varied with the load of ecgonine streaked. The mean recovery increased from 63% for 4.0 μg to 94% for 16.0 μg. The recoveries of ecgonine and benzoylecgonine were dependent on a number of factors. Non-quantitative transfer on streaking of the prepared test samples, incomplete migration of the alkaloids from the origin, poor localization of the alkaloid bands, incomplete scraping of silica from the TLC plates, incomplete elution of the alkaloids from the silica, and potential interference of silica particles with the derivatization and GC-FID analysis of ecgonine and benzoylecgonine could contribute to reduced recoveries. From the data obtained it would seem that these factors were under control and their effects minimal. The recovery of ecgonine did seem to depend on the original amount streaked suggesting that a constant amount of ecgonine is not eluted from the silica. This was not a problem in the analysis of ecgonine extracted from biological fluids as sufficient ecgonine was recovered to allow accurate quantitation.

There was, however, some variability in the recoveries of the alkaloids (especially ecgonine) following TLC separation and isolation which was most likely attributable to batch differences in the TLC plates used or the conditions under which TLC plates were developed. Care was taken to keep TLC conditions and procedures as uniform as possible.

2. DETERMINATION OF ECGONINE IN WATER, PLASMA, AND URINE

Extraction/TLC/GC-FID Procedure.   An analytical proce-
dure was successfully developed to determine ecgonine in wa-
ter, dog plasma, and urine.   Ecgonine was extracted into
chloroform:methylene chloride:2-propanol (1:1:1) after satu-
ration of the aqueous phase with solid potassium carbonate.
The extraction procedure was a modification of the one used
by Misra et al. (67)   in which chloroform:2-propanol (2:1)
and potassium carbonate were used to extract benzoylecgonine
and ecgonine from rat plasma, urine, and tissue homogenates.
In the present research, it was found that replacing half of
the chloroform with methylene chloride allowed the quantita-
tive extraction of not only ecgonine, but benzoylecgonine,
ecgonine methyl ester, and cocaine as well. This extraction
alone, however, was not suitable for the direct analysis of
the ecgonine alkaloids in plasma and urine.   Large amounts
of co-extractable foreign materials present in the extracts
made derivatization impossible. The problem was resolved to
some extent by initially precipitating the proteins and
washing the protein-free acidified plasma filtrate and urine
samples with ether prior to extraction.   Gas chromatograms
of the resulting extracts, derivatized with BSTFA, still
showed the presence of extractable endogenous substances
which interfered with the analysis of ecgonine and benzoyl-
ecgonine.   TLC was required as an additional clean-up step
to separate and purify the ecgonine- and benzoylecgonine-
containing extracts prior to GC-FID analysis.

Perchloric acid was found most suitable to precipitate any proteins in dog plasma and diluted urine samples. Hydrochloric acid was unsatisfactory and trichloroacetic acid was extracted into the organic solvent which interfered with subsequent sample manipulation. Perchloric acid did not interfere with the extraction because most of it precipitated as potassium perchlorate formed on addition of excess potassium carbonate; very little remained solubilized in the aqueous phase. The volume and concentration of perchloric acid chosen (300 μL of 6.5 N) gave the maximum clean-up of plasma samples. Although little protein was present in diluted urine samples, these received the same acidification with perchloric acid and ether wash to remove acidic and neutral substances.

The amount of potassium carbonate added (4.0 g) gave the maximum recovery of ecgonine from spiked water samples. Extracts were taken to dryness in silanized conical bottom tubes and washed down with distilled ethanol to ensure that as much of the extracted ecgonine as possible was streaked on the TLC plate. The analytical procedure could be interrupted at this point and TLC/GC-FID carried out at a later date with no reduction in the recovery of ecgonine. Alternatively, the procedure could be interrupted following the elution of ecgonine and the eluate refrigerated prior to preparation of samples for GC-FID analysis. Great care was taken to exclude silica particles from the aliquot of eluate

taken for GC-FID analysis. Silica present in the BSTFA of derivatized samples increased baseline noise in the resulting chromatograms.

Spiked Water Samples. Initial experiments carried out with ecgonine-spiked water samples helped to establish baseline recovery data. Gas chromatograms of analyzed water samples were very clean with only a slight increase in baseline noise compared to chromatograms of analyzed ecgonine GC standards. Samples derivatized with BSTFA were stable for several days and could be re-analyzed, if necessary, provided that the tubes were properly sealed to prevent evaporation of the BSTFA or exposure to atmospheric moisture.

The mean recovery of ecgonine from spiked water samples was 65-75% with slightly better recovery at the highest concentration. The recoveries of ecgonine from spiked water samples were reproducible with coefficients of variation less than 5%.

Spiked Plasma Samples. Gas chromatograms of plasma samples spiked with ecgonine and carried through the entire extraction/TLC/GC-FID procedure were almost identical to those of analyzed spiked water samples indicating the efficient removal of interfering endogenous substances. The mean recoveries of ecgonine from spiked plasma samples were 66-74%, almost equal to those obtained for spiked water sam-

ples.   The variability in the recoveries of ecgonine from plasma samples was somewhat greater,   CV's 9-12%,   than the variability in recoveries from spiked water samples.   The cause of this increased variability is not known but may be related to the greater physical or chemical complexity of the samples.

Spiked Urine Samples.   Gas chromatograms obtained from the analysis of spiked urine samples were almost identical to those from spiked water and plasma samples. The mean recoveries of ecgonine from spiked urine, 66-73%, were similar to the recoveries from spiked water and plasma samples (Table V).   The variability in recoveries of ecgonine from spiked urine samples, CV's of 3-11%, was similar to the recoveries of ecgonine from spiked plasma samples.

Experimental (IN VIVO) Plasma and Urine Samples.   The extraction/TLC/GC-FID method was successfully applied to the quantitation of ecgonine in dog plasma and urine following the intravenous administration of ecgonine to the dog in the study of the pharmacokinetics of ecgonine.   Gas chromatograms of analyzed dog plasma and urine samples were similar to chromatograms obtained from spiked plasma and urine samples prepared with plasma and urine from a different animal. The tracings consistently showed well-defined ecgonine and n-PEBE peaks.   There was some increase in baseline noise in individual plasma samples most likely due to the presence of silica particles carried over into the eluate. There was no interference with either the ecgonine or n-PEBE peaks.

A set of ecgonine-spiked plasma samples was included with each run to serve as known standards in the quantitation of ecgonine in experimental in vivo plasma samples. The run-to-run variability (CV's 5-13%) was low enough to allow the pooling of the ecgonine-spiked plasma sample data to produce a single plasma ecgonine concentration calibration curve (Figure 19) which was linear, r=0.990, over the 4-16 μg/mL range of concentrations examined. Experimental samples containing greater than 16 μg/mL ecgonine were diluted prior to analysis with the benefit that cleaner chromatograms were obtained.

The minimal detectable level of ecgonine in the plasma was 1-2 μg/mL. This was sufficient to allow the accurate measurement of plasma ecgonine concentrations up to 3 hours following the intravenous administration of ecgonine, 10 mg/kg, to the dog.

The urine ecgonine concentration calibration curve (Figure 20) prepared from ecgonine-spiked urine samples was almost identical to the plasma calibration curve and was also linear over the same range of concentrations, r=0.996. The extraction/TLC/GC-FID method for urine was sufficiently sensitive for the determination of ecgonine in all experimental urine samples collected during the pharmacokinetic study. With a minimal detectable level of 1-2 μg/mL for the method, in vivo urine samples were diluted 1:50 prior to analysis.

The overall sensitivity of the extration/TLC/GC-FID method was limited by the sensitivity of the flame ionization detector used to quantitate the derivatized ecgonine. The sensitivity could have been increased to some extent by decreasing the attenuation of the electrometer or by decreasing the volume of derivatizing agent used to reconstitute the final extract residue. Each of these approaches was limited by the accompanying increase in baseline noise.

Misra et al. (66) were able to detect 5-1000 ng/mL radiolabelled ecgonine in biological fluids of rats by means of liquid scintillation spectroscopy. Accurate measurement of ecgonine concentrations this low by GC would require a detection system much more sensitive and specific than FID. Gas chromatography-mass spectrometry (GC-MS) would seem particularly well-suited for the quantitation of the trimethylsilyl derivatives of cocaine metabolites (20).

3. DETERMINATION OF BENZOYLECGONINE AND ECGONINE IN WATER,
    PLASMA, AND URINE

Extraction/TLC/GC-FID Procedure. The analytical proce-
dure used for the determination of ecgonine was modified for
the determination of both benzoylecgonine and ecgonine in
dog plasma and urine. After perchloric acid protein-precip-
itation, the ether-washed aqueous phases were cooled in an
ice bath prior to the addition of solid potassium carbonate.
The addition of potassium carbonate generates considerable
heat, but pre-cooling of the samples prevented any signifi-
cant elevation of temperature which otherwise would have in-
creased the chances of spontaneous hydrolysis of benzoyl-
ecgonine. The order of addition of the reagents was also
important. Potassium carbonate was added after the organic
solvent was first added followed by immediate extraction to
reduce the contact time of benzoylecgonine with the alkaline
medium.

The TLC purification of benzoylecgonine was conducted
in the same manner as described for ecgonine. In the case
of samples containing benzoylecgonine and ecgonine, both al-
kaloids were extracted simultaneously, and then separated
and isolated by TLC. Individual bands of silica were re-
moved from each TLC plate and each alkaloid present eluted
separately. It would have been possible to collect a single
combined silica band containing both benzoylecgonine and
ecgonine but the increased amount of silica would have cre-

ated recovery problems in subsequent GC-FID analysis. Other theoretical and practical aspects of the extraction/TLC/GC-FID procedure were similar to those described for the analysis of ecgonine.

Spiked Water Samples. The described extraction/TLC/GC-FID analysis of benzoylecgonine in spiked water samples resulted in clean chromatograms similar to those obtained for benzoylecgonine GC standards. Benzoylecgonine was efficiently and consistently recovered from spiked water samples. The mean recoveries of benzoylecgonine from spiked water samples were 69-80% with CV's less than 5%.

In a separate in vitro experiment benzoylecgonine was found to be quite stable when carried through the entire analytical procedure. Formation of ecgonine was not detected.

Spiked Plasma and Urine Samples. Gas chromatograms from the extraction/TLC/GC-FID anlysis of benzoylecgonine in benzoylecgonine- and ecgonine-spiked plasma and urine samples were almost identical to those of analyzed spiked water samples with only a very slight increase in baseline noise. The recoveries of benzoylecgonine from plasma, 67-70%, and urine, 69-81%, were not significantly different from the recovery of benzoylecgonine from spiked water samples. As was previously seen with ecgonine, there was somewhat more vari-

ability in the recoveries of benzoylecgonine from spiked plasma samples (CV's 5-13%) than was seen for spiked water and urine samples (CV less than 5%).

The recovery of ecgonine from plasma and urine samples spiked with both benzoylecgonine and ecgonine was also determined (Table VII). The recoveries of ecgonine from the spiked plasma and urine were approximately 10 and 30 % lower, respectively, than the recoveries obtained with samples spiked solely with ecgonine. These differences are most likely attributable to run-to-run variation rather than any interaction of the benzoylecgonine and ecgonine. This re-emphasized the necessity of including a set of control spiked samples as references with each analytical run.

Experimental (IN VIVO) Plasma and Urine Samples. The developed extraction/TLC/GC-FID method was successfully used to measure the concentration of benzoylecgonine in experimental samples obtained in the study of the pharmacokinetics of benzoylecgonine in the dog. Gas chromatograms obtained from the analysis of in vivo samples were almost identical to those from spiked plasma and urine samples, being free of any extraneous peaks which might arise from storage or handling. The linearities of the benzoylecgonine concentration calibration curves constructed from data obtained from benzoylecgonine-spiked plasma and urine samples were excellent (r=0.992 and 0.999 for plasma and urine, respectively) allowing the accurate quantitation of benzoylecgonine in the

in vivo samples. The coefficients of variation of replicate samples were typically less than 10%.

The method was sufficiently sensitive with a minimal detectable benzoylecgonine level in the original sample of 1-2 μg/mL to allow the accurate measurement of benzoyl- ecgonine in all pharmacokinetic test samples.   As for ecgo- nine, the overall sensitivity of the method was limited by the sensitivity and specificity of the FID. Quantitation of benzoylecgonine in biological fluids at concentrations as low as 5 ng/mL with GC-MS has been reported (56,59).   The trimethylsilyl derivative of benzoylecgonine is particularly well suited to GC-MS analysis (55)   and it is possible that the developed extraction/TLC method could be used in combi- nation with GC-MS to greatly improve the sensitivity and specificity of the assay.

4. DETERMINATION OF ECGONINE METHYL ESTER AND ECGONINE IN
    WATER, PLASMA,AND URINE

Extraction/TLC/GC-FID Procedures. The analytical pro-
cedures and techniques established for the analysis of ecgo-
nine were found quite compatible with those required for the
determination of ecgonine methyl ester and ecgonine in plas-
ma and urine. Ecgonine methyl ester was first extracted
into methylene chloride:cyclohexane (1:1) at pH 9.5-10, and
the ecgonine remaining in the aqueous phase was extracted
into chloroform:methylene chloride:2-propanol (1:1:1) at pH
11-12. Separation of ecgonine methyl ester and ecgonine was
very efficient.

In the initial steps of the ecgonine methyl ester and
ecgonine analytical procedure, water, plasma, and urine sam-
ples were deproteinized with perchloric acid and washed with
ether. It was found that addition of methanol improved pre-
cipitation of proteins. The deproteinization/ether clean-up
of plasma samples was critical as there was a tendency for
emulsions to form during the extraction with methylene chlo-
ride:cyclohexane if any protein remained in the superna-
tants. Adding the ecgonine methyl ester and ecgonine in
methanolic solution also allowed for simpler and more con-
sistent spiking of samples.

In extracting the ecgonine methyl ester and ecgonine from the deproteinized and ether-washed samples the order of adding solvents and reagents and the method of handling the samples were quite important. The extracting organic solvent was added to the aqueous phase prior to adjustment of pH to 10. Measured volumes of saturated solution of potassium carbonate were added to each tube and then shaken immediately in order to minimize contact time of the ecgonine methyl ester with the now alkaline aqueous phase. An aliquot of the organic phase was analyzed for ecgonine methyl ester. To recover the aqueous phase containing the ecgonine, it was found convenient to freeze it in a dry ice-acetone bath and decant or aspirate off any residual organic solvent. The aqueous phase was kept frozen until extracted for ecgonine. In this manner the possible hydrolysis of unextracted ecgonine methyl ester remaining in the aqueous phase was minimized.

The aliquot of methylene chloride:cyclohexane containing ecgonine methyl ester was taken to dryness at 50° C under a stream of helium after acidification with 100 μL of 0.6 N HCl in 2-propanol. It was extremely important to convert ecgonine methyl ester extracted as the free base to the non-volatile hydrochloride salt. Without acidification more than half of the extracted ecgonine methyl ester was lost on evaporation of the organic solvent at 50° C; at 70° all of the extracted ecgonine methyl ester was lost. The addition

of 100 μL of 0.6 N HCl in 2-propanol ensured maximum recoveries of ecgonine methyl ester and reasonable drying times (ca. 10-15 min) on subsequent evaporation of the organic solvent at 50° under a stream of helium.

The recovery levels of ecgonine methyl ester from spiked water samples were acceptable as measured by GC-FID, but were not consistently reproducible. Approximately 1 in 3 samples showed variable ecgonine methyl ester recovery. Even more troublesome was the fact that the variable recovery occurred randomly with no apparent pattern. A number of possible causes and remedial steps were investigated. A thorough cleaning of all equipment and treatment of tubes with siliconizing fluid or EDTA provided no significant improvement. Likewise, increasing the amount of 0.6 N HCl in 2-propanol added to the organic phase and closely monitoring the evaporation did not prevent the spurious results. It was discovered, however, that on repeated GC-FID analysis of the "bad" samples over several days, the amount of ecgonine methyl ester measured would increase to the point of falling in line with the other samples suggesting that derivatization of ecgonine methyl ester may have proceded more slowly for these samples. Altering the derivatization conditions (temperature and duration) provided no significant improvement. Quite surprisingly, it was discovered that overnight drying of the final extracted ecgonine methyl ester hydrochloride residues over potassium hydroxide in a desiccator

prior to derivatization with BSTFA resulted in extremely low
and erratic recoveries of ecgonine methyl ester. This ob-
servation suggested that complete drying of the ecgonine
methyl ester hydrochloride residue interfered with its deri-
vatization with BSTFA. Possibly, the presence of a trace
amount of moisture (either remaining in the residue or ab-
sorbed from the atmosphere) was necessary to catalyze the
derivatization of ecgonine methyl ester extracted from aque-
ous solution; the exact mechanism, however, remains unknown.
Routine exposure of the ecgonine methyl ester hydrochloride
residues to the atmosphere for 10 minutes prior to the addi-
tion of BSTFA solved the problem of spurious results allow-
ing the precise determination of ecgonine methyl ester in
all samples.

With regard to the extraction/TLC/GC-FID procedure for
determining the ecgonine content of the aqueous phase re-
maining after the extraction of ecgonine methyl ester, pre-
cautions were taken to prevent the in vitro generation of
ecgonine via spontaneous hydrolysis of any residual ecgonine
methyl ester. The aqueous phase was frozen for the short
time required to remove residual methylene chlo-
ride:cyclohexane. The aqueous phase was then allowed to
thaw out but kept cold in an ice bath prior to extraction of
the ecgonine. Pre-cooling prevented any significant eleva-
tion of temperature on addition of solid potassium carbonate
thereby minimizing the hydrolysis of any residual ecgonine

methyl ester in the aqueous phase. Also, any ecgonine methyl ester which may have been extracted along with the ecgonine into chloroform:methylene chloride:2-propanol was lost on evaporation of this solvent mixture (no HCl added) at 70° C under a stream of helium.

Spiked Water Samples. Individual water samples spiked with ecgonine methyl ester and ecgonine were used to establish baseline recovery data. Gas chromatograms of the ecgonine methyl ester and ecgonine fractions were very clean with a slight increase in baseline noise compared to GC standards. There was no interference with the ecgonine methyl ester, ecgonine, or n-PEBE peaks.

The response of ecgonine methyl ester was sufficient to allow the accurate measurement of ecgonine methyl ester at minimal levels of 1-2 μg/mL in spiked water samples. The mean recovery of ecgonine methyl ester from water was ca. 60-70% with a slight increase in recovery with increasing concentration. The reproducibility of the analytical method was excellent with coefficients of variation (CV) less than 8%.

The mean recoveries of 50-60% of the ecgonine remaining in the aqueous phase after the extraction of ecgonine methyl ester were comparable to the recoveries of ecgonine from spiked water samples analyzed directly. As expected there was a slight increase in variability; the CV's were typically less than 10%.

248

As demonstrated in the separate experiment on the stability of ecgonine methyl ester through the entire analytical procedure, the _in vitro_ generation of ecgonine was not detected allowing the procedure to be used for the accurate determination of both ecgonine methyl ester and ecgonine.

Spiked Plasma and Urine Samples. Gas chromatograms of the extracted ecgonine methyl ester fractions of analyzed spiked plasma and urine samples spiked with both ecgonine methyl ester and ecgonine, showed well defined ecgonine methyl ester and n-PEBE peaks with minimal baseline noise. Chromatograms of the ecgonine fractions subsequently extracted from the same samples had increased baseline noise with minimal interference with the ecgonine or n-PEBE peaks.

The percent ecgonine methyl ester recovered from spiked plasma and urine samples was approximately 60-70%, not significantly different from the recoveries found for spiked water samples. As previously observed for ecgonine and benzoylecgonine, the recoveries of ecgonine methyl ester from spiked plasma samples were more variable (CV=3-17%) than the recoveries of ecgonine methyl ester from spiked urine samples (CV=5-10%).

The recoveries of ecgonine from spiked plasma and urine samples were comparable to the recoveries of ecgonine from spiked water samples and similar to recoveries of ecgonine analyzed directly. The coefficients of variation ranged from 5-13% but were typically less than 10%.

In the pharmacokinetic experiment in which a dog received ecgonine methyl ester intravenously, precautions were taken to ensure that collected urine was kept acidic to prevent the alkaline hydrolysis of ecgonine methyl ester to ecgonine. Since, following the administration of ecgonine methyl ester, no urine was voided by the test animal during the first 24 hours, the dog was kept in the metabolic cage for an additional 24 hours when urine was produced. When all the urine samples and rinses for the 0-48 h collection were analyzed much less than 100% of the original dose was recovered with almost half present as ecgonine. (The significance of these results will be discussed in a subsequent section on ecgonine methyl ester pharmacokinetics.)

The possible in situ conversion of ecgonine methyl ester in bladder urine must be taken into consideration. In undiluted, fresh urine (pH 7.57, preserved with thymol) incubated with ecgonine methyl ester at 37° C for 24 hours, it was found that the final concentration of ecgonine methyl ester (C) was 18.86% of its initial ecgonine methyl ester concentration (C_0). If the in vitro hydrolysis of ecgonine methyl ester is assumed to be first order a pseudo-hydroly-

sis rate constant, K_h, can be calculated for this process with equation 4 (see Appendix).

$$K_h = - \ln(C/C_0) \, / \, t \qquad (4)$$

For the hydrolysis of ecgonine methyl ester incubated at 37° in dog urine pH 7.57, where C/C_0 equals 0.1886 at t=24 h, K_h = 0.0695 h^{-1}. Thus the rate of loss of ecgonine methyl ester in urine at body temperature is approximately 7.0% per hour.

Most of the original ecgonine methyl ester (66.63%) was recovered as ecgonine. A small percentage, 14.51%, was unaccounted for. This most likely represents the loss of ecgonine methyl ester and/or ecgonine by extraneous non-hydrolytic processes.

Experimental (IN VIVO) Dog Plasma and Urine Samples.
The gas chromatograms of ecgonine methyl ester and ecgonine in plasma and urine samples from the dog receiving ecgonine methyl ester intravenously were identical to chromatograms from ecgonine methyl ester-spiked plasma samples used as known standards. Gas chromatograms of extracts from the 0-48 h urine collection showed extraneous peaks in both the ecgonine methyl ester and ecgonine fraction. The ecgonine methyl ester extract of each replicate 0-48 h urine sample showed a peak of varying height with a retention time of 4.3 min, the same as that of ecgonine. This peak was not obtained in any of the 48-96 h urine samples nor with any of

the spiked urine samples prepared with urine from an untreated dog. The compound responsible for the peak was not ecgonine since ecgonine is not extracted into methylene chloride:cyclohexane at pH 10. Also, the chromatogram obtained from the GC-FID analysis of the ecgonine extract from the 0-48 h urine revealed a significant peak with a retention time of 5.2 min. The peak, however, was not present in the 48-96 h samples. The identity of the compound giving this peak was not determined.

With a minimal detectable level for ecgonine methyl ester of 1-2 $\mu g/mL$, the analytical method used to determine the concentrations of ecgonine methyl ester in experimental in vivo plasma and urine samples was sufficiently sensitive to allow the accurate measurement of ecgonine methyl ester in 1 mL aliquots of dog plasma obtained up to 4 hours after the intravenous administration of 25 mg/kg ecgonine methyl ester. The last blood sample (t=6.09 h), however, required the analysis of pooled extracts of two 1 mL plasma samples to allow the accurate measurement of ecgonine methyl ester. On the other hand, the concentration of ecgonine methyl ester in the 0-48 h dog urine was diluted 10 times so as to adjust the concentration within the range of the concentration calibration curve. Plasma and urine ecgonine methyl ester concentration calibration curves (Figure 31) were linear, r=0.996 and 0.994, respectively, and virtually identical.

The linearity of the plasma and urine ecgonine concentration curves (Figure 32) were also excellent with r=0.994 and 0.992, respectively. Responses from analyzed ecgonine-spiked plasma and urine samples were similar. The GC-FID method for the analysis of ecgonine was not sufficiently sensitive to accurately measure the concentration of ecgonine in the plasma of the ecgonine methyl ester-treated dog. Ecgonine was detected in pooled plasma samples obtained at 2.00, 2.50, and 3.00 hours after dosing but the observed concentrations fell below the useful range of the plasma ecgonine concentration calibration curve. The GC-FID method, however, was more than adequate for the determination of ecgonine in the urine. Experimental urine samples were diluted 10 times to bring the ecgonine concentrations to within the range of the concentration calibration curve.

The developed analytical procedure represents the first reported method for the quantitation of ecgonine methyl ester in plasma. This method, however, is not sufficiently sensitive to allow the accurate measurement of plasma ecgonine methyl ester concentrations following the administration of cocaine. A GC-MS method with a sensitivity of 0.1 μg/mL ecgonine methyl ester in urine has been recently reported (76). It is possible that the present analytical procedures could be used with little modification to develop a GC-MS assay for ecgonine methyl ester in plasma and urine.

5. DETERMINATION OF COCAINE IN WATER, PLASMA, AND URINE

HP 5880 Multi-Level Calibration Method. A gas chroma-
tographic system employing a nitrogen-phosphorus detector
(GC-NPD) was necessary to provide the required high sensi-
tivity for the accurate quantitation of cocaine in the pres-
ent research. The HP 5880 gas chromatograph with NPD oper-
ated in the multi-level internal calibration mode was quite
compatible with the overall sensitivity requirements of the
present analytical methodology for cocaine. Monitoring the
peak height ratios of the GC cocaine standards to assess the
sensitivity and linearity of the detector revealed the de-
tector response was stable from run to run but after an op-
eration period of several days it became necessary to in-
crease the auxiliary temperature to the NPD ceramic element
to maintain adequate sensitivity and linearity.

The GC column packed with 3% OV-101 on 80/100 mesh
Chromosorb W HP was particularly well suited for the GC-NPD
analysis of cocaine. Flat noise-free baselines were main-
tained with a minimum of conditioning. Cocaine and the in-
ternal standard (n-PEBE), both detected as the free base,
were easily separated. Gas chromatograms of analyzed cali-
bration mixtures showed well-defined cocaine and n-PEBE
peaks with no interferences. Cocaine concentrations as low
as 2.7 ng/2 μL could be measured with a coefficient of vari-
ation ca. 10%. Reproducibility at higher concentrations was
decidedly better (CV's less than 3%). The linearity of the

cocaine GC standard calibration curve was excellent (r=0.999) over the range of concentrations examined, 2.7-63.6 ng/2 μL injected.

Extraction/GC-NPD Procedure. The procedure of Jatlow and Bailey (49) for plasma was used to determine the concentration of cocaine in plasma and was also found to be quite satisfactory for the determination of cocaine in urine. Modifications were made to minimize the potential spontaneous alkaline hydrolysis of cocaine during assay. The organic extracting solvent was added prior to adjustment of the aqueous sample to pH 9.6 with carbonate buffer, and each sample was extracted immediately in order to minimize contact of cocaine with the alkaline medium. Shaking each tube 40 times by hand was sufficient for the efficient extraction of cocaine.

In removing the cocaine-containing organic extract of plasma samples, care was taken to exclude solid material present at the interface which interfered with subsequent GC-NPD analysis. This resulted in less than quantitative recoveries of cocaine when the "modified" internal standard method was used. With the "true" internal standard method the interface could be avoided as n-PEBE was included in the extracting solvent to correct for the variation in volumes of aliquots taken.

Percolation of the final organic extracts through anhydrous sodium sulfate provided a simple means of obtaining dry extracts. The final extracts were taken to dryness at room temperature under a stream of helium in reasonable time, 5-10 min. Elevated temperatures were avoided as cocaine was present as the relatively volatile free base. The use of 50 μL of methanol ensured that all the cocaine residue was dissolved and allowed replicate GC-NPD analysis of 2 μL aliquots.

Spiked Water Samples. The recovery of cocaine from spiked water samples was somewhat dependent on whether the modified or true internal standard method was employed. The overall average recovery of cocaine from spiked water samples based on the "modified" method was 90% with coefficients of variation of recoveries of 0-4%. The 90% figure represents the absolute recovery of cocaine subjected to the entire procedure and includes losses resulting from non-quantitative transfer of the organic extracts and losses associated with samples manipulation. The overall average recovery of cocaine from spiked water samples based on the "true" method (n-PEBE is added during the initial extraction of cocaine) was 100%. Apparently, the behavior of n-PEBE subjected to the entire procedure was similar to that of cocaine making it ideally suited as an internal standard for the extraction/GC-NPD analysis of cocaine. The coefficients of variation for spiked water samples analyzed by the true internal standard method ranged from 1-7%.

Spiked Blood and Plasma Samples.   The recovery   of
cocaine from blank plasma samples spiked with cocaine imme-
diately prior to analysis was 78%.   This is significantly
greater than the 55% mean analytical recovery of cocaine re-
ported by Jatlow and Bailey (49) and may represent increased
extraction of cocaine or decreased loss of cocaine due to
hydrolytic and non-hydrolytic processes.

The recovery of cocaine from spiked plasma samples in
the present research was 7% lower than the 85% recovery of
cocaine from water spiked to approximately the same level
and is most probably due to increased procedural losses as-
sociated with the avoidance of solid material at the inter-
face of the aqueous and organic phases ("modified" internal
standard method used).

The recovery of cocaine from cocaine-spiked plasma sam-
ples stored for four weeks at $-30°$ C was 75%, representing a
loss of of 3% over four weeks which was not statistically
significant ($P<0.05$).   This is in agreement with the find-
ings of Jatlow et al. (87) who demonstrated that 0.14% sodi-
um fluoride added to plasma will completely prevent the hy-
drolysis of cocaine for at least 6 weeks at $-15°$ C. Most of
the cocaine in plasma immediately separated from cocaine-
containing blood, frozen, and stored at $-30°$ C remained un-
changed, ensuring that the cocaine levels found in the deep-
freeze stored samples collected   after   the   intravenous
administration of cocaine represented maximal recoveries.

The recovery of cocaine from the plasma separated from spiked blood samples was 68%, which is 10% lower than the recovery from plasma spiked immediately prior to analysis. The difference was statistically significant ($P<0.05$). It is difficult to attribute this decreased recovery to any specific cause. Possibly, the spiking of blood directly and the greater manipulation of these samples resulted in increased hemolysis which may have contributed to decreased recovery. Alternatively, there may have been some increased loss of cocaine associated with the exposure of cocaine to whole blood. Baselt (89) reported approximately 10% lower recovery of cocaine incubated in whole blood with 0.5% sodium fluoride compared to cocaine incubated in plasma with 0.5% sodium fluoride. This difference, however, appeared only after incubation at 4° C for 21 days. It is unlikely that the brief contact of cocaine with whole blood under conditions used would have resulted in increased breakdown of cocaine. The decreased recovery of cocaine from the plasma obtained from spiked blood samples is more likely a reflection of the relative distribution of cocaine between erythrocytes and plasma. This is in agreement with results obtained by Woods et al. (23) who reported in vitro and in vivo erythrocyte:plasma ratios of 1.2 to 1.4.

With regard to the cocaine-spiked blank plasma samples used to calibrate the gas chromatograph in the analysis of experimental in vivo samples, besides well-defined peaks for

cocaine and n-PEBE, minor peaks with retention times of 3.6, 4.5, 4.7, and 5.3 min were detected. These peaks were present to varying extents in all plasma samples analyzed but did not interfere with the accurate quantitation of cocaine.

Spiked Urine Samples. Spiked urine samples carried through the entire extraction/GC-NPD procedure with n-PEBE added as true internal standard and used to calibrate the gas chromatograph gave chromatograms having well-defined cocaine and n-PEBE peaks with very little baseline noise.

With regard to the possible hydrolysis of cocaine in unacidified urine at 37° C, it was found that after incubation for 24 hours the final concentration of cocaine (C) was 21.38% of its original spiked concentration (C_0). If the in vitro hydrolysis of cocaine is assumed to be a first order reaction, then a pseudo-hydrolysis rate constant, K_h, can be calculated from equation 4 (see Appendix).

$$K_h = -\ln(C/C_0) / t \qquad (4)$$

For the hydrolysis of cocaine incubated for 24 hours in undiluted urine, pH 7.57, $C/C_0 = 0.2138$. K_h as calculated is equal to 0.0643 h^{-1}. Thus, the loss of cocaine in urine at body temperature was approximately 6.4% per hour. This is very similar to the loss of ecgonine methyl ester in urine at body temperature (7.0% per hour) and is not unexpected as non-enzymatic hydrolysis of the same methyl ester function is involved in each case.

Analysis of the incubated test samples for ecgonine methyl ester, benzoylecgonine, and ecgonine revealed that the majority (66%) of the original cocaine was recovered as benzoylecgonine indicating that the non-enzymatic hydrolysis of cocaine to benzoylecgonine in urine is the preferred pathway over the conversion of cocaine to ecgonine methyl ester. Only 3% of the original cocaine was recovered as ecgonine methyl ester, while 16% was recovered as ecgonine. Apparently, most of the ecgonine methyl ester generated from cocaine must have undergone spontaneous hydrolysis to ecgonine as previously demonstrated in a separate experiment with urine incubated with ecgonine methyl ester. These results are in agreement with those reported by Stewart et al. (78) who found benzoylecgonine as the major product of the spontaneous hydrolysis of cocaine incubated in aqueous buffers of moderate alkalinity; very little hydrolysis of benzoylecgonine to ecgonine occurred.

Experimental (IN VIVO) Dog Plasma and Urine Samples. The gas chromatograms from the cocaine analysis of plasma and urine samples obtained from the pharmacokinetic experiment were not appreciably different from those obtained from blank plasma and urine samples spiked with cocaine. Cocaine concentration curves constructed to monitor the analysis of spiked plasma and urine samples were linear over the range of concentrations examined (40-1280 ng/2 mL of diluted plas-

ma or urine). The cocaine concentration calibration curves constructed from spiked plasma and urine samples were identical. The coefficients of variation of replicate determinations ranged between 1 and 5%.

The extraction/GC-NPD method for cocaine with a minimal detectable level of 10-20 ng/mL in plasma and urine was sufficiently sensitive to allow the accurate measurement of the concentration of cocaine in 1 mL or less of plasma obtained up to 4 hours following the intravenous administration of 5 mg/kg cocaine. Two milliliter samples of plasma collected 6.01 h after cocaine administration, however, were required for accurate analysis. The method was sufficiently sensitive to allow the accurate measurement of cocaine in urine collected up to 48 hours after cocaine administration. The urine had to be diluted prior to analysis to bring the concentration within the range of concentrations of spiked urine reference samples.

V-B. PHARMACOKINETICS

1. INTRAVENOUS ECGONINE

Pharmacokinetic Analysis. The analytical procedures developed for the determination of ecgonine in plasma and urine were successfully applied to elaborating the pharmacokinetics of ecgonine following intravenous administration of a single dose of ecgonine, 10 mg/kg, to a mature, female Beagle dog. The plasma ecgonine concentration-time data obtained are given in Table VIII. The plasma ecgonine concentrations rapidly declined from a peak of 53.1 μg/mL at 0.05 h to 4.9 μg/mL 3.03 h after the intravenous administration of ecgonine.

The disposition of ecgonine in the mature dog following rapid intravenous injection was best described by a two-compartment open model. Nonlinear regression analysis of the observed plasma ecgonine concentration-time data by means of the iterative computer program NONLIN (94) provided the best estimates of the pharmacokinetic parameters describing a two-compartment model. An inverse squared weighting of the observed plasma ecgonine concentration values (y values) gave the best fit of predicted to observed values with fewer iterations. Weighting is necessary when observed values in y do not have the same variance (95).

There was good agreement between the predicted ecgonine plasma concentrations and observed plasma concentrations

(Figure 35) as indicated by NONLIN test statistics: $r^2=0.998$ and cor=0.990. The final parameter values obtained from this fitting were the best estimates of the individual pharmacokinetic parameters describing the disposition of ecgonine in the test dog (Table IX). Some measure of the precision of the final parameter estimates is given by the standard deviations as calculated along with the fitting. These SD's are estimates approaching the true SD's (σ) only as the number of data points approaches infinity (95). Large values for SD relative to the estimated parameter are not necessarily indicative of poor data or a poor fit. A large SD indicates that any parameter estimate over a large range will give an equally good fit to the data (95).

Large standard deviations were obtained for the pharmacokinetic rate constants associated with the distribution of ecgonine following intravenous administration i.e. λ_1, k_{12}, and k_{21}. These rate constants, however, were determined for the initial steep part of the plasma ecgonine concentration curve where a small change in time is associated with a large change in plasma ecgonine concentration. Thus, large standard deviations in the final estimates of the distributional rate constants are to be expected. Fortuitously, distribution has much less effect on the overall plasma ecgonine profile than does elimination.

Following intravenous injection in the dog, ecgonine is rapidly distributed and eliminated. The rate constant asso-

ciated with the distribution phase, λ_1, was found to be 2.410 h^{-1}, corresponding to a distributional half-life of 17.3 minutes. The transfer of ecgonine from the central to the peripheral compartment was relatively slow, $k_{12}=0.5718$ h^{-1} while the return of ecgonine from the peripheral compartment to the central compartment was significantly faster, $k_{21}=1.443$ h^{-1}; approximately 2.5 times faster. Ecgonine did not accumulate in the peripheral compartment but returns to the central compartment from which it is rapidly eliminated, $K=0.9029$ h^{-1}. The distributional and elimination parameters are in keeping with the highly polar nature of the ecgonine moiety.

The final estimate of the apparent volume of the central compartment, V_1, was found to be 3001 mL which was equivalent to 0.1911 L/kg. This small volume indicates that ecgonine was confined to the extracellular fluids and did not readily cross membrane barriers to bind with extravascular tissues. Again, this is not unexpected given the highly polar, amphoteric nature of ecgonine. An apparent volume can be calculated for the peripheral compartment from the volume of the central compartment and the transfer rate constants, i.e. $V_2=V_1(k_{12}/_{21})$ (96) and was found to be 1189 mL for ecgonine. This volume does not represent a true anatomic volume but its magnitude is indicative of the limited distribution of ecgonine in the peripheral tissues of the dog.

The hybrid rate constant, λ_2, which represents the elimination of ecgonine from the entire body at a time after equilibrium between the central and peripheral compartments was presently found to be 0.5578 h^{-1} in the dog corresponding to an elimination half-life of 1.24 h for ecgonine in the dog. This is similar in magnitude to the biological half-life of approximately 0.6 h in the rat calculated from the plasma ecgonine concentrations data reported by Misra et al. (66) following intravenous administration of 10 mg/kg radiolabelled ecgonine.

An estimate of the elimination rate constant for ecgonine from the central compartment, K, was also determined and found to be 0.9029 h^{-1}. This is greater than λ_2 as it is not influenced by distribution between the central and peripheral compartments. Total body clearance, Cl_T , of ecgonine, calculated as the product of the rate of elimination from the central compartment and the volume of the central compartment, $Cl_T = KV_1$ (96), was 45.16 mL/min.

Urinary Exretion of Ecgonine. Apparently, ecgonine was not metabolized in the dog. Most (80%) of the administered ecgonine dose was recovered in the 0-48 h urine collection. Feces were not analyzed but most likely contained some of the remaining ecgonine not accounted for. (A small percentage of the administered ecgonine was present in the small volume of urine (ca. 10 mL) voided and inadvertently lost in the handling of the test animal.) This finding is in agree-

ment with those of Misra et al. (66) who found 90.6 and 8.8 % of the ecgonine administered intravenously to rats was excreted in 96 hours in the urine and feces, respectively.

As ecgonine was eliminated from the dog primarily via urinary excretion the total body clearance of ecgonine provides an estimate of the renal clearance of ecgonine. The value calculated for Cl_T , 45.16 mL/min, is significantly lower than the glomerular filtration rate of the dog, 84 mL/min (97). The difference may suggest some measure of tubular reabsorption of ecgonine or binding to plasma proteins (96,98).

2. INTRAVENOUS BENZOYLECGONINE

Pharmacokinetic Analysis.   The analytical   procedures
developed for the determination of benzoylecgonine in plasma
were successfully applied to elaborating the pharmacokinet-
ics of benzoylecgonine following intravenous administration
of a single dose, 10 mg/kg, to a mature, female Beagle dog.
A 0-72 hour urine sample was collected and the amount of
benzoylecgonine excreted in the urine determined.   The plas-
ma benzoylecgonine concentration-time data are given in Ta-
ble XI.   Plasma benzoylecgonine declined from a peak of 24.0
μg/mL 5 at minutes after intravenous administration to 6.1
μg/mL 6 hours after the dose of benzoylecgonine.   No ecgo-
nine was detected in any of the plasma samples indicating
that the in vivo conversion of benzoylecgonine to ecgonine
did not occur or proceeds at an extremely slow rate.

According to the plasma concentration-time data the
disposition of benzoylecgonine in the mature dog was best
described by a two-compartment open model.   Final estimates
of the pharmacokinetic parameters relevant to this model
which were obtained by least-squares nonlinear regression
analysis of the plasma benzoylecgonine concentration-time
data are given in Table XII.   Good agreement was obtained
between the observed and predicted plasma benzoylecgonine
concentrations (Figure 36, r^2=0.988, cor=0.991).

Following intravenous administration benzoylecgonine is very rapidly distributed in the dog. The best estimate of the rate constant associated with the distribution phase, λ_1, was 7.037 h^{-1}, almost three times the value of λ_1 determined for ecgonine in the same animal. The distributional half-life for benzoylecgonine was 5.9 min indicating very rapid distribution of benzoylecgonine in the dog. The individual rate constants associated with the transfer of benzoylecgonine between the central and peripheral compartments, k_{12} and k_{21}, were 2.680 and 4.246 h^{-1}, respectively. Benzoylecgonine, therefore, would not be expected to accumulate in the peripheral compartment as k_{21} is greater than k_{12} as was the case with ecgonine.

The volume of the central compartment, V_1, was 5341 mL, equivalent to 0.3402 L/kg. The volume of the peripheral compartment, V_2, calculated as before was 3371 mL. The apparent volumes of each of these compartments is larger than the corresponding volumes obtained with ecgonine indicating that the extent as well as the rate of distribution of benzoylecgonine in the dog is greater than that of ecgonine.

The elimination of benzoylecgonine, however, was much slower than that of ecgonine in the same animal. The hybrid rate constant, λ_2, associated with the elimination of benzoylecgonine was 0.1751 h^{-1} giving a terminal half-life of 3.96 h. This is greater than three times the elimination half-life of ecgonine in the same animal. The slower elimi-

nation of benzoylecgonine is also reflected in the estimate of the rate constant for the elimination of benzoylecgonine from the central compartment, $K=0.2903$ h^{-1}, which is less than one-third that obtained for ecgonine. The total body clearance, Cl_T , for benzoylecgonine was 25.84 mL/min, approximately 60% of that of ecgonine in the same dog.

The elimination of benzoylecgonine in the one dog tested was considerably slower than that reported for rats. Misra et al. (67) found a biological half-life of 0.8 h in rats given 10 mg/kg radiolabelled benzoylecgonine intravenously.

The elimination half-life obtained for benzoylecgonine in the dog tested, however, is similar to benzoylecgonine half-lives following administration of cocaine in human subjects. Johns et al. (25) found the mean half-life of benzoylecgonine in the serum following intranasal application of 200 mg cocaine was 5.5 hours. The half-lives of benzoylecgonine following nasal inhalation of cocaine hydrochloride, 1.5 mg/kg, calculated from the data of Hamilton et al. (74) averaged 7 hours. Ambre et al. (84) found the elimination half-life of benzoylecgonine averaged 5.1 h following intravenous and intranasal cocaine administration. These values are very similar to the estimated elimination half-life of benzoylecgonine in the single dog tested in the present study. Much more data on the disposition of benzoylecgonine in the dog are needed before a fair comparison can be made.

Urinary Excretion of Benzoylecgonine.   Benzoylecgonine was not metabolized in the dog since 95.41% of the dose administered was recovered in the urine within 72 hours.   The remaining 5% was most likely excreted in the feces. This is a very significant finding since it demonstrates that the conversion of benzoylecgonine to ecgonine via enzymatic or non-enzymatic hydrolysis did not occur.   This is in agreement with the finding of Stewart et al. (79).   They found that plasma cholinesterase was able to hydrolyze benzoylecgonine in vitro only to a very limited extent and concluded that plasma cholinesterase had a very low affinity for benzoyecgonine.   In previous work, the same workers found that the conversion of benzoylecgonine to ecgonine via spontaneous alkaline hydrolysis did not occur readily (78).

Misra et al. (66), however, detected ecgonine in the urine collected from rats after the intravenous administration of radiolabelled benzoylecgonine but no quantities were reported.   These workers carried out their analysis which included an extraction at pH 11-12 followed by TLC with ethyl acetate:methanol:concentrated ammonia (15:4:1).   Because of the strong alkaline conditions the possibility of hydrolysis of benzoylecgonine to ecgonine during their analysis cannot be ruled out.

In the present work, the recovery of 95% of the administered benzoylecgonine gives support to the suitability of the analytical method developed for the present study on the

disposition of benzoylecgonine in the dog. More important-
ly, the elimination of benzoylecgonine in the dog was found
to occur exclusively via excretion in the urine. There was
no _in_ _vivo_ hydrolysis of benzoylecgonine to ecgonine.

3. INTRAVENOUS ECGONINE METHYL ESTER

Pharmacokinetic Analysis.   The disposition of ecgonine
methyl ester following the intravenous administration of a
single dose, 25 mg/kg, was studied in the same dog which had
previously received ecgonine and benzoylecgonine.   In a pre-
vious preliminary experiment 10 mg/kg ecgonine methyl ester
was administered intravenously but the resulting plasma lev-
els were too low to measure accurately.   Increasing the dose
of ecgonine methyl ester to 25 mg/kg in a second experiment
resulted in plasma levels of ecgonine methyl ester which
could be accurately measured and produced no ill effects.
After intravenous administration, plasma ecgonine methyl es-
ter concentrations declined from a peak value of 16.0 μg/mL
6 minutes after injection to 1.8 μg/mL 6 hours later (Table
XIV).

Plasma samples were also analyzed for ecgonine content.
Ecgonine was detected in the plasma 2.0, 2.5, and 3.0 hours
following ecgonine methyl ester administration but the con-
centrations were too low to measure accurately (1.90, 1.85,
and 1.47 μg/mL, respectively).

In contrast to the findings for ecgonine and benzoyl-
ecgonine, the disposition of ecgonine methyl ester in the
mature female Beagle dog was best described by a one-com-
partment open model with rapid intravenous input.   Accord-
ingly, the ecgonine methyl ester was rapidly distributed

throughout the body via the circulatory system and rapidly equilibrated with body tissues (96). The initial distribution phase observed with injected ecgonine and benzoylecgonine did not occur with ecgonine methyl ester. Estimates of the pharmacokinetic parameters, K and V, relevant to a 1-compartment model were obtained by least-squares nonlinear regression analysis of the plasma ecgonine methyl ester concentration-time data with the computer program NONLIN. There was excellent agreement between observed plasma ecgonine methyl ester concentrations and plasma levels predicted with the estimated pharmacokinetic parameters (Figure 37). Standard deviations associated with the NONLIN generated final estimates were small (Table XV).

Ecgonine methyl ester was rapidly eliminated in the dog, about 34% per hour ($K=0.3445$ h^{-1}). For a 1-compartment model, K, the rate constant for elimination of drug from the central compartment also represents the elimination of drug from the entire body and is analagous to λ_2 for a 2-compartment model. The elimination half-life of ecgonine methyl ester in the dog was found to be 2.01 h, which was almost twice that of ecgonine but only half that of benzoylecgonine in the same animal. Ecgonine methyl ester was eliminated from the dog more rapidly than benzoylecgonine but not as rapidly as ecgonine.

The apparent volume of distribution of ecgonine methyl ester in the dog was very large, $V=22,250$ mL equivalent to

1.578 L/kg. This large volume is indicative of extensive distribution of ecgonine methyl ester throughout body tissues. This is not unexpected since ecgonine methyl ester is relatively nonpolar, similar to cocaine which is extensively distributed in the dog (35). Equilibration of ecgonine methyl ester within this large apparent volume of distribution occurred so rapidly after injection that a distribution phase was not evident in the plasma profile.

Total body clearance of ecgonine methyl ester, Cl_T, calculated as the product of K and V was 127.8 mL/min, approximately 3-5 times greater than the clearances of ecgonine and benzoylecgonine in the same dog. This clearance represents the total of all elimination processes including metabolism and excretion. Ecgonine was detected in the plasma providing proof for the in vivo conversion of ecgonine methyl ester to ecgonine. It is not certain whether this is a result of enzymatic or non-enzymatic hydrolysis of the ecgonine methyl ester. The conversion of ecgonine methyl ester to ecgonine represents the hydrolytic loss of the same methyl group as does the conversion of cocaine to benzoylecgonine. The latter conversion has been shown to be mediated via non-enzymatic hydrolysis and it has been suggested that the spontaneous hydrolysis of ecgonine methyl ester to ecgonine should also occur quite slowly at physiological pH (78).

<u>Urinary Excretion of Ecgonine Methyl Ester</u>. Hydrolysis of ecgonine methyl ester was confirmed by the presence of ecgonine in the urine collected 48 hours following adminis-tration of ecgonine methyl ester. Totals of 67.46 mg of ecgonine methyl ester and 49.18 mg ecgonine were found in the urine following intravenous administration of 352.5 mg of ecgonine methyl ester. These amounts represent 19.18 and 15.05 % of the dose calculated on molar basis recovered as ecgonine methyl ester and ecgonine, respectively.

The ecgonine methyl ester and ecgonine found in the urine accounted for only 34% of the ecgonine methyl ester administered. This finding suggests that some process(es) other than hydrolysis to ecgonine or excretion in the urine is responsible for the elimination of ecgonine methyl ester in the dog. It is possible that some ecgonine methyl ester and/or ecgonine may have been excreted in the bile and feces but excretion via these routes would likely account for only a small fraction of the total ecgonine methyl ester elimi-nated. The possibility also exists that ecgonine methyl es-ter may undergo biotransformation via pathway(s) other than hydrolysis.

One possible pathway for the biotransformation of ecgo-nine methyl ester is N-demethylation. Cocaine is N-demethy-lated to norcocaine in rats [81], dogs [35], monkeys [36,82], and man [75], but in comparison to the hydrolysis pathway this represents a minor pathway. It is not certain

whether or not any of the hydrolysis products of cocaine are N-demethylated. Inaba et al. (75) discussed unpublished results indicating that "the metabolites of cocaine" were N-demethylated in isolated rat hepatocytes but gave no further details.

In the present study chromatograms obtained from the analysis of ecgonine methyl ester and ecgonine (Figure 27) in the 0-48 h urine collection did contain peaks other than those expected for ecgonine methyl ester, ecgonine, and the internal standard n-PEBE. The extraneous peaks were not identified. It is possible that these peaks may represent some novel metabolites of ecgonine methyl ester which may be important not only in delineating the disposition of ecgonine methyl ester administered as such, but also in the disposition of the ecgonine methyl ester present in the plasma following the administration of cocaine. Much more research is required before any conclusions can be made.

It was hoped that the relative amounts of ecgonine methyl ester and ecgonine found in the urine would provide some information on the relative importance of the excretion of ecgonine methyl ester in the urine and its in vivo hydrolysis to ecgonine on the overall elimination of ecgonine methyl ester in the dog. Precautions were taken that urine was acidified during collection to ensure that any ecgonine recovered in the urine resulted from true in vivo hydrolysis of ecgonine methyl ester. The experimental animal, however,

did not urinate during the first 24 hours after ecgonine methyl ester administration. It is possible that some ecgonine methyl ester in any urine retained in the bladder could have have undergone spontaneous hydrolysis especially if an alkaline urine was produced.

In this connection the in vitro experiment conducted to assess the stability of ecgonine methyl ester in unacidified urine incubated at 37° C was particularly important. After incubation in undiluted urine pH 7.57 at 37° for 24 hours only 19% of the originally added ecgonine methyl ester remained unchanged. The calculated pseudo-hydrolysis rate constant, K_h, was relatively small in magnitude, 0.0695 h^{-1}, indicating that in urine at body temperature the hydrolytic conversion of ecgonine methyl ester to ecgonine proceeds at only a modest rate. In the pharmacokinetic dog experiment the length of time that ecgonine methyl ester excreted in the urine was retained in the bladder was unknown, and it is therefore impossible to estimate how much of the ecgonine recovered in the urine resulted from the hydrolysis of ecgonine methyl ester in the urine retained in the bladder and how much ecgonine was produced in the plasma. In future studies of the disposition of ecgonine methyl ester it would be necessary to provide for continuous urine collection by means of an open in-dwelling urethral catheter followed by immediate acidification of the collected urine. These precautions will help detect any in vivo conversion of ecgonine methyl ester to ecgonine.

4. INTRAVENOUS COCAINE

Pharmacokinetic Analysis. The pharmacokinetic disposi-
tion of cocaine following a single intravenous dose, 5 mg/
kg, was examined in the same dog which had received the
three other ecgonine alkaloids in previous experiments. The
concentrations of cocaine in all plasma samples collected
over the 6 hour period of observation are given in Table
XVII. Plasma cocaine concentration declined rapidly from an
initial level of 2988 ng/mL at 7 minutes after injection to
32 ng/mL at 6 hours after cocaine administration.

The disposition of cocaine following rapid intravenous
injection was best described by a two-compartment open mod-
el. The decline in plasma cocaine concentration was bipha-
sic with a brief initial distribution phase followed by a
somewhat sustained elimination phase. Estimates of the
pharmacokinetic parameters associated with the disposition
of cocaine in the dog obtained by least-squares nonlinear
regression analysis of the plasma cocaine concentration-time
data are presented in Table XVIII. Agreement between ob-
served and predicted plasma cocaine concentration was excel-
lent with $r^2=1.000$ and cor=0.993 (Figure 36).

The best estimate of the first-order rate constant as-
sociated with the distribution of cocaine following intrave-
nous administration, λ_1, was 4.001 h^{-1}, corresponding to a
distribution half-life of 10.4 minutes. The distribution of

cocaine was faster than that observed for ecgonine but considerably slower than that for benzoylecgonine in the same animal. Cocaine was unique, however, in being the only compound tested to exhibit significant accumulation in the peripheral compartment. The rate constant associated with transfer of cocaine from the central to peripheral compartment, $k_{12}=1.381$ h^{-1}, was significantly greater that the peripheral to central transfer rate constant, $k_{21}=0.7830$ h^{-1}. The slow return of cocaine from the peripheral compartment is also reflected in the apparent volumes of each compartment. The apparent volume of the central compartment estimated directly from the plasma cocaine concentration-time data was 15,970 mL, equivalent to 1.051 L/kg. The apparent volume of the peripheral compartment calculated for cocaine, 28,170 mL, was significantly larger than the volume of the central compartment. This very large compartment kinetically separate from the central compartment is indicative of significant accumulation of cocaine in the peripheral tissues. Cocaine has high lipid solubility and has been shown to possess a marked affinity for many lipid-rich tissues (34,35). Cocaine was sequestered in the fat tissue of chronically-treated rats for prolonged periods (34). In acutely- and chronically-treated dogs cocaine remained in the fat tissue for much shorter periods of time (35).

As expected the apparent volume of distribution of cocaine in the dog in the present study was much greater than

the volumes of distribution obtained in the same dog for ecgonine and benzoylecgonine, which can be explained by the much greater lipid solubility of cocaine compared to that of the two highly polar metabolites. Surprisingly, the apparent volume of distribution for ecgonine methyl ester was also very large, V=22,250 mL. The profile of plasma ecgonine methyl ester concentrations following its administration did not exhibit a distributional phase indicating that ecgonine methyl ester is distributed throughout this large volume almost instantaneously and leaves all tissues at the same rate. The slight increase in polarity of ecgonine methyl ester compared to cocaine may be responsible for its rapid return to and elimination from the plasma.

Following distribution, cocaine was rapidly eliminated from the plasma of the test animal with $\lambda_2 = 0.4469$ h^{-1}, corresponding to an elimination half-life of 1.55 hours. This is similar to the plasma cocaine half-life of 1.2 h found by Misra et al. (35) for dogs treated intravenously with 5 mg/kg cocaine.

The rate constant specific for the elimination of cocaine from the central compartment, K, was 2.284 h^{-1} which, taken together with the volume of the central compartment, would indicate that cocaine is very rapidly cleared from the central compartment; total body clearance (Cl_T) was equal to 607.9 mL/min in the dog. The rapid elimination of cocaine from the central compartment occurs primarily via metabolism

as very little unchanged cocaine was found excreted in the urine.

In the present research it was not possible to estimate the rates of concurrent competing pathways for the in vivo hydrolysis of cocaine. This would require the accurate measurement of the concentrations of both ecgonine methyl ester and benzoylecgonine in the same plasma samples following the administration of cocaine. The GC-NPD method was developed for the determination of cocaine in the plasma; however, the plasma concentrations of ecgonine methyl ester, benzoylecgonine, and ecgonine in the plasma following cocaine administration could not be determined by this method. Benzoylecgonine and ecgonine could not be extracted under the conditions employed for cocaine (heptane:iso-amyl alcohol, pH 9.6). Ecgonine methyl ester could be extracted only to a very limited extent (ca. 13% was recovered from spiked water samples as determined by GC-FID). An attempt was made to adapt the extraction procedures used to determine ecgonine methyl ester in the plasma with GC-FID to GC-NPD analysis but proved unsuccessful in detecting underivatized ecgonine methyl ester. A number of derivatization schemes commonly used for the derivatization of alcohols (99) were tried but also proved unsuccessful. It is possible that in the future the procedures developed for the extraction of the hydrolysis products of cocaine might be coupled to a technique more sensitive than GC-FID and allow the determi-

nation of the ecgonine alkaloids present in the plasma fol-
lowing the administration of cocaine.   Gas chromatography-
mass spectrometry (GC-MS) appears particularly well suited
for the determination of cocaine and its derivatized metabo-
lites.

Urinary Excretion of Cocaine and Metabolites.   In view
of the inability to measure plasma levels of cocaine metabo-
lites it was hoped that the results from the analysis of
urine collected following intravenous cocaine would shed
some light on the in vivo metabolism of cocaine in the dog.

The results of the urinary excretion data, however,
must be evaluated in the light of results obtained from the
in vitro experiment carried out to assess the stability of
cocaine in unacidified urine.   The experiment was designed
to test the possibility that some cocaine excreted in the
urine may undergo spontaneous hydrolysis while being re-
tained in the bladder.   It was found that after incubation
in urine pH 7.57 at 37° C for 24 hours only 21% of the orig-
inally added cocaine remained.   Benzoylecgonine was the ma-
jor product, 66% being recovered as benzoylecgonine.   Ecgo-
nine methyl ester and ecgonine represented minor hydrolysis
products, corresponding to 3 and 16 % of the original co-
caine, respectively.

Following the intravenous administration of 76 mg of
cocaine to the test dog only 0.59% was recovered in the

urine as unchanged cocaine within 96 hours. This is an under-estimation of the true urinary excretion of unchanged cocaine since some of the cocaine excreted in the urine would have undergone slow, non-enzymatic hydrolysis while in the bladder similar to that demonstrated in the in vitro experiment. The estimate of cocaine excreted is, however, similar in magnitude to the 0.9-5.0% values reported by Misra et al. (35).

Benzoylecgonine amounting to 10.6% of the cocaine dose, was recovered in the urine 48 hours after the administration of cocaine. The results of the intravenous benzoylecgonine study indicated that benzoylecgonine is not metabolized in the dog but excreted unchanged in the urine. Therefore, the amount of benzoylecgonine occurring in the urine following cocaine administration represents the conversion of cocaine to benzoylecgonine via non-enzymatic hydrolysis. It is not possible to calculate how much of this benzoylecgonine originated from the spontaneous hydrolysis of excreted unchanged cocaine while being retained in the bladder. The frequent collection of urine with an in-dwelling catheter followed by acid-stabilization of the urine would allow an accurate determination of the hydrolysis in vivo of cocaine to benzoylecgonine in the plasma as well as an accurate estimate of the excretion of unchanged cocaine in the urine. The present findings do indicate that any spontaneous hydrolysis of cocaine to benzoylecgonine which may occur in vivo plays only a minor role in the elimination of cocaine in the dog.

In the present dog study, in addition to the excretion of 10.6% of the administered cocaine as benzoylecgonine, a total of 12.5% of the dose was recovered in the 0-48 h urine sample as ecgonine methyl ester and ecgonine (7.2 and 5.3%, respectively). The ecgonine recovered in the urine originated from the _in vivo_ hydrolysis of ecgonine methyl ester both in the plasma and in the urine retained in the bladder. The total amount of ecgonine methyl ester and ecgonine recovered in the urine probably represents only a fraction of the ecgonine methyl ester generated in the plasma. Only one-third of the ecgonine methyl ester administered to the same dog in a previous separate experiment was recovered in the urine as ecgonine methyl ester and ecgonine. The same would be expected to be true for ecgonine methyl ester produced from the metabolism of cocaine. This would mean that as much as 37.5% of the administered cocaine dose may have been converted via enzymatic hydrolysis of cocaine to ecgonine methyl ester. The hydrolysis of cocaine to ecgonine methyl ester would appear to be preferred over the formation of benzoylecgonine _in vivo_ in keeping with recently reported research (76) indicating that the enzymatic hydrolysis of cocaine to ecgonine methyl ester is the important pathway for cocaine metabolism.

In the present study with the Beagle dog de-esterification accounted for less than half of the elimination of cocaine from the plasma with more than half of the adminis-

tered cocaine remaining unaccounted for. Misra et al. (35) administered 5 mg/kg cocaine radiolabelled in the tropane ring to two dogs and recovered 46.2% of the dose as total radioactivity excreted in the urine in 48 hours. An additional 13.3% of the dose was excreted in the feces during the same time period. The measured radioactivity represented the excretion of unchanged cocaine and all radioalabelled metabolites. Analysis of urine collected showed that unchanged cocaine and its hydrolysis products: ecgonine methyl ester, benzoylecgonine and ecgonine constituted 72% of the urinary excretion products. The N-demethylated metabolites of cocaine: norcocaine, benzoylnorecgonine, and norecgonine accounted for 18% of the urinary metabolites excreted. The remaining metabolites (5%) were not identified.

In the present intravenous cocaine study only 22.1% of the administered cocaine was recovered in the urine as the de-esterified metabolites: ecgonine methyl ester, benzoylecgonine, and ecgonine. Fecal excretion of these metabolites was not examined but would not be expected to be significant. The N-demethylation of cocaine considered a minor pathway for the metabolism of cocaine in man (75) was not assessed. Nevertheless, in the present research a number of significant observations were made and along with the findings of the pharmacokinetic studies carried out with the individual de-esterified metabolites allows some speculation in formulating an integrated picture of the _in vivo_ hydrolysis of cocaine in the dog.

Cocaine is rapidly distributed and metabolized in the dog. Cocaine is hydrolyzed _in_ _vivo_ to form not only benzoylecgonine but also ecgonine methyl ester. The benzoylecgonine generated is rapidly distributed throughout body tissues. The benzoylecgonine, however, is not hydrolyzed further to ecgonine but is eliminated from the plasma unchanged at a slower rate than the other hydrolysis products. Benzoylecgonine is eliminated almost exclusively through renal excretion. The ecgonine methyl ester is formed _in_ _vivo_ presumably via enzymatic hydrolysis mediated by cholinesterase and its formation is favored over that of benzoylecgonine. Ecgonine methyl ester in the plasma is in turn hydrolyzed presumably non-enzymatically to ecgonine, with both ecgonine methyl ester and ecgonine being excreted in the urine. Most of the remaining ecgonine methyl ester is rapidly eliminated via metabolic or excretory processes as yet unidentified. Only a small fraction of the administered cocaine is excreted in the urine unchanged. Future research is needed to more clearly define pharmacokinetically the routes of cocaine metabolism other than hydrolysis, and their integration in the overall disposition of cocaine.

Chapter VI

SUMMARY AND CONCLUSIONS

Analytical methods were developed for the determination
of cocaine and its hydrolysis products: ecgonine methyl es-
ter, benzoylecgonine, and ecgonine in plasma and urine and
applied to elaborating the pharmacokinetic dispostion of co-
caine and its metabolites in the mature dog.

1. An extraction/TLC/GC-FID procedure for the determina-
tion of ecgonine in plasma and urine above the minimal
detectable limit of 1-2 $\mu g/mL$ was used to elaborate the
pharmacokinetics of ecgonine following intravenous ad-
ministration of 10 mg/kg to a mature female Beagle dog.

2. The disposition of ecgonine in the mature dog was best
described by a two-compartment open model. Ecgonine
was rapidly distributed and eliminated with a terminal
half-life of 1.2 h.

3. Ecgonine was eliminated primarily via urinary excre-
tion; 80% of the administered ecgonine dose was recov-
ered in the urine 48 hours after intravenous adminis-
tration.

4. An extraction/TLC/GC-FID method for the determination
of both benzoylecgonine and ecgonine in plasma and

urine above the minimal detectable limit of 1-2 μg/mL was used to elaborate the pharmacokinetics of benzoylecgonine following intravenous administration of 10 mg/kg to the same mature dog.

5. Like ecgonine, the disposition of benzoylecgonine was best described by a two-compartment open model. The distribution of benzoylecgonine was very rapid, at a rate almost three times that for ecgonine. The elimination of benzoylecgonine, however, was much slower than that of ecgonine in the same dog. The elimination half-life of benzoylecgonine was 4.0 h.

6. Benzoylecgonine was eliminated almost entirely via urinary excretion with 95% of the administered benzoylecgonine recovered in the urine within 72 hours. Hydrolysis of benzoylecgonine in vivo did not occur; no ecgonine was detected in plasma or urine.

7. Extraction/TLC/GC-FID procedures developed for the determination of ecgonine methyl ester and ecgonine in plasma and urine above the minimal detectable level of 1-2 μg/mL were employed in the investigation of the disposition of ecgonine methyl ester following intravenous administration of 25 mg/kg to the same mature dog.

8. In contrast to the other alkaloids tested, the disposition of ecgonine methyl ester was best described by a one-compartment open model. Ecgonine methyl ester was eliminated at a rate intermediate to those of ecgonine and benzoylecgonine. The elimination half-life of ecgonine methyl ester was 2.0 h.

9. Ecgonine, the product of the <u>in</u> <u>vivo</u> hydrolysis of ecgonine methyl ester was detected in plasma 2-3 hours following ecgonine methyl ester administration.

10. Ecgonine methyl ester and ecgonine were found in the urine following intravenous ecgonine methyl ester administration to the mature dog accounting for only 19 and 15 % of the administered ecgonine methyl ester dose, respectively. Presumably, ecgonine methyl ester is eliminated by route(s) other than hydrolysis and urinary excretion in the dog.

11. The spontaneous hydrolysis of ecgonine methyl ester incubated in unacidified urine at body temperature to form ecgonine was observed in a separate <u>in</u> <u>vitro</u> experiment.

12. An extraction/GC-NPD method was employed for the determination of cocaine above the minimal detectable limit of 10-20 ng/mL in plasma and urine following the intravenous administration of 5 mg/kg cocaine to the mature dog. The amounts of ecgonine methyl ester, benzoylecgonine, and ecgonine excreted in the urine following intravenous cocaine were determined by extraction/TLC/GC-FID.

13. The disposition of cocaine following intravenous administration was best described by a two-compartment open model. Cocaine was rapidly distributed and was unique in being the only alkaloid tested to exhibit significant accumulation in the peripheral compartment. Co-

caine was rapidly eliminated with a terminal half-life of 1.6 hours.

14. Benzoylecgonine, ecgonine methyl ester, ecgonine, and cocaine were recovered in the urine following intravenous cocaine administration to the mature dog. Taken together, unchanged cocaine and the products of its _in vivo_ hydrolysis excreted in the urine accounted for less than half of the administered cocaine dose suggesting that in the dog the elimination of cocaine via process(es) other than de-esterification and excretion of the products in the urine may be significant.

15. The spontaneous hydrolysis of cocaine in unacidified urine at body temperature to benzoylecgonine, ecgonine, and ecgonine methyl ester was observed in a separate _in vitro_ experiment. Benzoylecgonine was the major product accounting for 66% of the originally added cocaine.

16. Much more research on the biological fate of cocaine and its metabolic congeners is required before its pharmacokinetics in man and laboratory animals can be more clearly and completely defined.

REFERENCES

1. Plowman, T., "Botanical Perspectives on Coca," J. Psychedelic Drugs 11(1,2), 103-17, 1979.

2. Holmstedt, B., Jaatma, E., Leardri, K., and Plowman, T., "Determination of Cocaine in Some South American Species of _Erythroxylum_ using Mass Fragmentography," Phytochemistry 16, 1753-5, 1977.

3. Henry, Thomas Anderson, "The Plant Alkaloids," 4th ed., Blakiston Co., Toronto, 1949; pp. 92-105.

4. Van Dyke, C., and Byck, R., "Cocaine," Sci. Am. 246(3), 128-41, 1982.

5. Aldrich, M. R., and Barker, R. W., "Historical Aspects of Cocaine Use and Abuse," in "Cocaine: Chemical, Biological, Clinical, Social and Treatment Aspects," Mulé, S. J., ed., CRC Press, Cleveland, 1976; pp. 3-10.

6. Gay, G. R., Inaba, D. S., Sheppard, C. W., Newmayer, J. A., and Rappelt, R. T., "Cocaine: History, Epidemology, Human Pharmacology, and Treatment. A Perspective on a New Debut for an Old Girl," Clin. Toxicol. 8(2), 149-78, 1975.

7. Nuranjo, P., "Social Function of Coca in Pre-Columbian America," J. Ethnopharmacol. 3(2,3), 161-73, 1981.

8. Mortimer, W. G., "History of Coca 'The Divine Plant of the Incas'," And/Or Press, San Francisco, 1974.

9. Holmstedt, B., and Fredga, A., "Sundry Episodes in the History of Coca and Cocaine," J. Ethnopharmacol. 3(2,3), 113-48, 1981.

10. Pearman, K., "Cocaine: A Review," J. Laryngol. Otol. 93, 1191-9, 1979.

11. Grinspoon, L., and Bakalar, J. B., "Coca and Cocaine as Medicines: An Historical Review," J. Ethnopharmacol. 3(2,3), 149-60, 1981.

12. Knox, R. A., "Powerfully Addictive Cocaine," Winnipeg Free Press, Thursday December 27, 1984; p. 23.

13. Archer, S., and Hawks, R. L., "The Chemistry of Cocaine and its Derivatives," in "Cocaine: Chemical, Biological, Clinical, Social and Treatment Aspects," Mulé, S. J., ed., CRC Press, Cleveland, 1976; pp. 15-32.

14. Bastos, M. L., and Hoffman, D. B., "Detection and Identification of Cocaine, its Metabolites, and its Derivatives," in "Cocaine: Chemical, Biological, Clinical, Social and Treatment Aspects," Mulé, S. J., ed., CRC Press, Cleveland, 1976; pp. 35-58.

15. "Beilsteins Handbuch der Organischen Chemie," Springer-Verlag, Berlin, Band XXII, System No. 3326, 195-205, 1935.

16. "The Merck Index," 10th ed., Merck and Co. Inc., Rahway, 1983.

17. Perrin, D. D., ed., "Dissociation Constants of Organic Bases in Aqueous Solution," Butterworths, London, 1965; pp. 342-5.

18. Findlay, S. P., "The Three-Dimensional Structure of the Cocaines. Part I. Cocaine and Pseudococaine," J. Am. Chem. Soc. 76(11), 2855-62, 1954.

19. Bell, M. R., and Archer, S., "L(+)-2-Tropinone," J. Am. Chem. Soc. 82(16), 4642-4, 1960.

20. Jatlow, P., "Analysis of Cocaine and its Metabolites in Biological Fluids," in "Cocaine: Chemical, Biological, Clinical, Social and Treatment Aspects," Mulé, S. J., ed., CRC Press, Cleveland, 1976; pp. 61-9.

21. Lindgren, J.-E., "Guide to the Analysis of Cocaine and its Metabolites in Biological Material," J. Ethnophar-macol. 3(2,3), 337-51, 1981.

22. Woods, L. A., Cochin, J., Fornfeld, E. J., McMahon, F. G., and Seevers, M. H., "The Estimation of Amines in Biological Materials with Critical Data for Cocaine and Mescaline," J. Pharm. Exp. Ther. 101(2), 188-99, 1951.

23. Woods, L. A., McMahon, F. G., and Seevers, M. H., "Distribution and Metabolism of Cocaine in the Dog and Rabbit," J. Pharm. Exp. Ther. 101(2), 200-4, 1951.

24. Kaul, B., Millian, S. J., and Davidow, B., "The Development of a Radioimmunoassay for Detection of Cocaine Metabolites," J. Pharm. Exp. Ther. 199(1), 171-8, 1976.

25. Johns, M. E., Berman, A. R., Price, J. C., Pillsbury, R. C., and Henderson, R. L., "Metabolism of Intranasally Applied Cocaine," Ann. Otol. Rhinol. Laryngol. 86, 342-7, 1977.

26. Robinson, K., and Smith, R. N., "Radioimmunoassay of Benzoylecgonine in Samples of Forensic Interest," J. Pharm. Pharmacol. 36(3), 157-62, 1984.

27. Bastos, M. L., Jukofsky, D., and Mulé, S. J., "Routine Identification of Cocaine Metabolites in Human Urine," J. Chromatogr. 89, 335-42, 1974.

28. Valanju, N. N., Baden, M. M., Valanju, S. N., Mulligan, D., and Verma, S. K., "Detection of Biotransformed Cocaine in Urine from Drug Abuser," J. Chromatogr. 81, 170-3, 1973.

29. Van Dyke, C., Byck, R., Barash, P. G., and Jatlow, P., "Urinary Excretion of Immunologically Reactive Metabolite(s) after Intranasal Administration of Cocaine as Followed by Enzyme Immunoassay," Clin. Chem. 23(2), 241-4, 1977.

30. Decato, L., Liu, C.-T., and Adler, F. L., "Immunologic Studies on Drug Addiction: III. Antibodies Reactive with Cocaine Metabolites and their Use for Drug Detection," J. Immunol. Methods 18, 201-13, 1977.

31. Misra, A. L., Pontani, R. B., and Mulé, S. J., "Separation of Cocaine, Some of Its Metabolites and Congeners on Glass Fibre Sheets," J. Chromatogr. 81, 167-9, 1973.

32. Noirfalise, A., and Mees, G., "Chromatographie sur Couche Mince de Quelques Alcaloides et Bases Aminées," J. Chromatogr. 31, 594-7, 1967.

33. Wallace, J. E., Hamilton, H. E., Schwertner, H., King, D. E., McNay, J. L., and Blum, K., "Thin-layer Chroma-

tographic Analysis of Cocaine and Benzoylecgonine in Urine," J. Chromatogr. 114, 433-41, 1975.

34. Nayak, P. K., Misra, A. L., and Mulé, S. J., "Physiological Disposition and Biotransformation of [^{3}H]-Cocaine in Acutely and Chronically Treated Rats," J. Pharm. Exp. Ther. 196(3), 556-69, 1976.

35. Misra, A. L., Patel, M. N., Alluri, V. R., Mulé, S. J., and Nayak, P. K., "Disposition and Metabolism of [^{3}H]-Cocaine in Acutely and Chronically Treated Dogs," Xenobiotica 6, 537-52, 1976.

36. Misra, A. L., Giri, V. V., Patel, M. N., Alluri, V. R., and Mulé, S. J., "Disposition and Metabolism of [^{3}H]-Cocaine in Acutely and Chronically Treated Monkeys," Drug Alcohol Depend. 2, 261-72, 1977.

37. Munier, R. L., and Drapier, A. M., "Nouvelle Méthodes de Séparation et Comportement des Alcaloides en Chromatographie en Millieux Très Polaires, Neutres, ou Acides, sur Gels de Silice non Modifié (1ère partie)," Chromatographia 10(5), 226-32, 1977.

38. Munier, R. L., and Drapier, A. M., "Nouvelle Méthodes de Séparation et Comportement des Alcaloides en Chromatographie en Millieux Très Polaires, Neutres, ou Acides, sur Gels de Silice non Modifié (2ième partie)," Chromatographia 10(6), 290-7, 1977.

39. Jatlow, P. I., Van Dyke, C., Barash, P., and Byck, R., "Measurement of Benzoylecgonine and Cocaine in Urine, Separation of Various Cocaine Metabolites Using Re-

versed-Phase High-Performance Liquid Chromatography,"
J. Chromatogr. 152, 115-21, 1978.

40. Evans, M. A., and Moriarity, T., "Analysis of Cocaine
and Cocaine Metabolites by High Pressure Liquid Chroma-
tography," J. Anal. Toxicol. 4, 19-22, 1980.

41. Masoud, A. N., and Krupski, D. N., "High-Performance
Liquid Chromatographic Analysis of Cocaine in Human
Plasma," J. Anal. Toxicol. 4, 305-10, 1980.

42. Fish, F., and Wilson, W. D. C., "Gas Chromatographic
Determination of Morphine and Cocaine in Urine," J.
Chromatogr. 40, 164-8, 1969.

43. Wallace, J. E., Hamilton, H. E., Bason, D. J., Schwert-
ner, H. A., and Harris, S. C., "Gas-Liquid Chromato-
graphic Determination of Cocaine and Benzoylecgonine in
Urine," Anal. Chem. 48(1), 34-8, 1976.

44. Jain, N. C., Chinn, D. M., Budd, R. D., Sneath, T. S.,
and Leung, W. J., "Simultaneous Determination of Co-
caine and Benzoylecgonine in Urine by Gas Chromatogra-
phy with On-Column Alkylation," J. Forensic Sci. 22(1),
7-16, 1977.

45. Von Minden, D. L., and D'Amato, N. A., "Simultaneous
Determination of Cocaine and Benzoylecgonine in Urine
by Gas-Liquid Chromatography," Anal. Chem. 49(13),
1974-7, 1977.

46. Kogan, M. J., Verebey, K. G., DePace, A. C., Resnick,
R. B., and Mulé, S. J., "Quantitative Determination of
Benzoylecgonine and Cocaine in Human Biofluids by Gas-

Liquid Chromatography," Anal. Chem. 49(13), 1965-9,
1977.

47. Moore, J. M., "Gas Chromatographic Detection of Ecgo-
nine and Benzoylecgonine in Cocaine," J. Chromatogr.
101, 215-18, 1974.

48. Roscoe, R. M. H., "A Study of the Hydrolysis In Vitro
and Certain Biological Effects of Some Ecgonine Alka-
loids Found in Erythroxylon Coca," Ph.D. thesis, Uni-
versity of Manitoba, 1979.

49. Jatlow, P. I., and Bailey, D. N., "Gas Chromatographic
Analysis for Cocaine in Human Plasma, with Use of a Ni-
trogen Detector," Clin. Chem. 21(13), 1918-21, 1975.

50. Byck, R., Jatlow, P., Barash, P., Van Dyke, C., "Co-
caine: Blood Concentration and Physiological Effect
After Intranasal Application in Man," Adv. Behav. Biol.
21, 629-45, 1977.

51. Dvorchik, B. H., Miller, S. H., and Graham, W. P., "Gas
Chromatographic Determination of Cocaine in Whole Blood
and Plasma Using a Nitrogen-sensitive Flame Ionization
Detector," J. Chromatogr. 135, 141-8, 1977.

52. Jacob III, P., Elias-Baker, B. A., Jones, R. T., and
Benowitz, N. L., "Determination of Cocaine in Plasma by
Automated Gas Chromatography," J. Chromatogr. 306,
173-81, 1984.

53. Blake, J. W., Noonan, J. S., and Murdick, P. W., "Rapid
Sensitive Gas-Liquid Chromatographic Screening Proce-
dure for Cocaine," Anal. Chem. 46(2), 288-9, 1974.

54. Javaid, J. I., Dekirmenjian, H., Davis, J. M., and Schuster, C. R., "Determination of Cocaine in Human Urine, Plasma and Red Blood Cells by Gas-Liquid Chromatography," J. Chromatogr. 152, 105-13, 1978.

55. Leighty, E. G., and Fentiman Jr., A. F., "Metabolism of Cocaine to Norcocaine and Benzoyl Ecgonine by an In Vitro Microsomal Enzyme System," Res. Commun. Chem. Pathol. Pharmacol. 8(1), 65-73, 1974.

56. Jindal, S. P., and Vestergaard, P., "Quantitation of Cocaine and its Principal Metabolite Benzoylecgonine, by GLC-Mass Spectrometry Using Stable Isotope Labeled Analogs as Internal Standards," J. Pharm. Sci. 67(6), 811-4, 1978.

57. Jindal, S. P., Lutz, T., and Vestergaard, P., "Mass Spectrometric Determination of Cocaine and its Biologically Active Metabolite Norcocaine in Human Urine," Biomed. Mass. Spectrom. 5(12), 658-63, 1978.

58. Rafla, F. K., and Epstein, R. L., "Identification of Cocaine and Its Metabolites in Human Urine in the Presence of Ethyl Alcohol," J. Anal. Toxicol. 3, 59-63, 1979.

59. Chinn, P. M., Crouch, D. J., Peat, M. A., Finkle, B. S., and Jennison, T. A., "Gas Chromatography-Chemical Ionization Mass Spectrometry of Cocaine and Its Metabolites in Biological Fluids," J. Anal. Toxicol. 4, 37-42, 1980.

60. Goodman, L. S., and Gilman, A., eds. "The Pharmacological Basis of Therapeutics," 5th ed., MacMillan, New York, 1975; pp. 379-403.

61. Campbell, D., and Adriani, J., "Absorption of Local Anesthetics," J. Am. Med. Assoc. 168(7), 873,7, 1958.

62. Van Dyke, C., Barash, P. G., Jatlow, P., and Byck, R., "Cocaine: Plasma, Concentrations after Intranasal Application in Man," Science 191, 859-61, 1976.

63. Wilkinson, P., Van Dyke, C., Jatlow, P., Barash, P., and Byck, R., "Intranasal and Oral Cocaine Kinetics," Clin. Pharmacol. Ther. 27(3), 386-94, 1980.

64. Van Dyke, C., Jatlow, P., Ungerer, J., Barash, P. G., and Byck, R., "Oral Cocaine: Plasma Concentrations and Central Effects," Science 200, 211-3, 1978.

65. Holmstedt, B., Lindgren, J.-E., Rivier, L., and Plowman, T., "Cocaine in Blood of Coca Chewers," J. Ethnopharmacol. 1, 69-78, 1979.

66. Misra, A. L., Vadlamani, N. L., Bloch, R., Nayak, P. K., and Mulé, S. J., "Physiologic Disposition and Metabolism of [^{3}H]ecgonine (Cocaine Metabolite) in the Rat," Res. Commun. Chem. Pathol. Pharmacol. 8(1), 55-63, 1974.

67. Misra, A. L., Nayak, P. K., Bloch, R., and Mulé. S. J., "Estimation and Disposition of [^{3}H]benzoylecgonine and Pharmacological Activity of Some Cocaine Metabolites," J. Pharm. Pharmacol. 27, 784-6, 1975.

68. Misra, A. L., Giri, V. V., Patel, M. N., Alluri, V. R., Pontani, R. B., and Mulé, S. J., "Disposition of [^{3}H]benzoylnorecgonine (Cocaine Metabolite) in the Rat," Res. Commun. Chem. Pathol. Pharmacol. 13(4), 579-84, 1976.

69. Smith, R. M., Poquette, M. A., and Smith, P. J., "Hydroxymethoxybenzoylmethylecgonines: New Metabolites of Cocaine from Human Urine," J. Anal. Toxicol. 8, 29-34, 1984.

70. Smith, R. M., "Arylhydroxy Metabolites of Cocaine in the Urine of Cocaine Users," J. Anal. Toxicol. 8, 35-37, 1984.

71. Smith, R. M., "Ethyl Esters of Arylhydroxy- and Arylhydroxymethoxycocaines in Urines of Simultaneous Cocaine and Ethanol Users," J. Anal. Toxicol. 8, 38-42, 1984.

72. Valenzuela-Ortiz, R., "Distribution and Metabolism of Cocaine in the Rat," An. Fac. Quim. Farm. Univ. Chile 18, 15-19, 1966.

73. Fish, F., and Wilson, W. D. C., "Excretion of Cocaine and its Metabolites in Man," J. Pharm. Pharmacol. 21 Suppl., 135s-138s, 1969.

74. Hamilton, H. E., Wallace, J. E., Shimek Jr., E. L., Land, P., Harris, S. C., Christenson, J. G., "Cocaine and Benzoylecgonine Excretion in Humans," J. Forensic Sci. 22, 697-707, 1977.

75. Inaba, T., Stewart, D. J., and Kalow, W., "Metabolism of Cocaine in Man," Clin. Pharmacol. Ther. 23(5), 547-52, 1978.

76. Ambre, J. J., Ruo, T.-I., Smith, G. L., Backes, D., Smith, C. M., "Ecgonine Methyl Ester, a Major Metabolites of Cocaine," J. Anal. Toxicol. 6, 26-9, 1982.

77. Taylor, D., Estevez, V. S., Englert, L. F., and Ho, B. T., "Hydrolysis of Carbon-labeled Cocaine in Human Serum," Res. Commun. Chem. Pathol. Pharmacol. 14(2), 249-57, 1976.

78. Stewart, D. J., Inaba, T., Tang, B. K., and Kalow, W., "Hydrolysis of Cocaine in Human Plasma by Cholinesterase," Life Sci. 20, 1557-64, 1977.

79. Stewart, D. J., Inaba, T., Lucassen, M., and Kalow, W., "Cocaine Metabolism: Cocaine and Norcocaine Hydrolysis by Liver and Serum Esterases," Clin. Pharmacol. Ther. 25(4), 464-8, 1979.

80. Just, W. W., and Hoyer, J., "Local Anesthetic Potency of Norcocaine, a Metabolite of Cocaine," Experientia 33(1), 70-1, 1977.

81. Misra, A. L., Nayak, P. K., Patel, M. N., Vadlamani, N. L., and Mulé, S. J., "Identification of Norcocaine as a Metabolite of [^{3}H]-Cocaine in Rat Brain," Experientia 30(11), 1312-4, 1974.

82. Hawks, R. L., Kopin, I. J., Colburn, R. W., and Thoa, N. B., "Norcocaine: a Pharmacologically Active Metabolite of Cocaine found in Brain," Life Sci. 15, 2189-95, 1974.

83. Estevez, V. S., Ho, B. T., and Englert, L. F., "Inhibition of the Metabolism of Cocaine by SKF-525A," Res. Commun. Chem. Pathol. Pharmacol. 17(1), 179,82, 1977.

84. Ambre, J., Fischman, M., and Ruo, T.-I., "Urinary Excretion of Ecgonine Methyl Ester, a Major Metabolite of Cocaine in Humans," J. Anal. Toxicol. 8, 23-5, 1984.

85. Javaid, J. I., Fischman, M. W., Schuster, C. R., Dekirmenjian, H., and Davis, J. M., "Cocaine Plasma Concentration: Relation to Physiological and Subjective Effects in Humans," Science 202, 277-8, 1978.

86. Barnett, G., Hawks, R., and Resnick, R., "Cocaine Pharmacokinetics in Humans," J. Ethnopharmacol. 3(2,3), 353-66, 1981.

87. Jatlow, P., Barash, P. L., Van Dyke, C., and Byck, R., "Impaired Hydrolysis of Cocaine in Plasma from Succinylcholine Sensitive Individuals," Clin. Res. (abstr.), 24, 255A, 1976.

88. Liu, Y., Budd, R. D., and Griesemer, E. C., "Study of the Stability of Cocaine and Benzoylecgonine, its Mayor Metabolite, in Blood Samples," J. Chromatogr. 248, 318-20, 1982.

89. Baselt, R. C., "Stability of Cocaine in Biological Fluids," J. Chromatogr. 268, 502-5, 1983.

90. Catravas, J. D., Waters, I. W., Walz, M. A., and Davis, W. M., "Acute Cocaine Intoxication in the Conscious Dog: Pathophysiology Profile of Acute Lethality," Arch. Int. Pharmacodyn. 235, 328-40, 1978.

91. Riegel, B., Moffett, R. B., and McIntosh, A. V., "3,12-Diacetoxy-bisnor-Cholanyldiphenylethylene," in Horning, E. C., ed., "Organic Syntheses Collective Vol-

ume 3," John Wiley and Sons, Inc., London, 1955; pp. 237-9.

92. Long, Cyril, ed. "Biochemists' Handbook," E and F. N. Spon Ltd., London, 1961; pp. 32, 36.

93. "5880A Gas Chromatograph Technical Manual, Volume 5: Integration and Methods," Hewlett-Packard, Avondale, August 1979.

94. Metzler, C. M., Elfring, G. L., and McEwen, A. J., "A User's Manual for NONLIN and Associated Programs," The Upjohn Co., Kalamazoo, 1974.

95. Boxenbaum, H. G., Riegelman, S., and Elashoff, R. M., "Statistical Estimations in Pharmacokinetics," J. Pharmacokinet. Biopharm. 2(2), 123-48, 1974.

96. Shargel, L., and Yu, A. B. C., "Applied Biopharmaceutics and Pharmacokinetics," 2nd ed., Appleton-Centruy Crofts, Norwalk, 1985.

97. Holliday, M. A., and Egan, T. J., "Renal Function in Man, Dog and Rat," Nature 193(4817), 748-50, 1962.

98. Gibaldi, M., and Perrier, D., "Pharmacokinetics," 2nd ed., Marcel Dekker, Inc., New York, 1982.

99. Knapp, D. R., "Handbook of Analytical Derivatization Reactions," John Wiley and Sons, New York, 1979; pp. 30-39.

APPENDIX

The pharmacokinetics of the disposition of cocaine and each of its metabolic congeners was characterized in the mature female Beagle dog after receiving a single intravenous dose of each alkaloid. While the the one-compartment model appeared to adequately describe the pharmacokinetics of ecgonine methyl ester, the two-compartment model was necessary for cocaine, benzoylecgonine, and ecgonine.

One-Compartment Model. The following scheme represents the one-compartment open model with intravenous input as applied to ecgonine methyl ester following a single bolus intravenous injection:
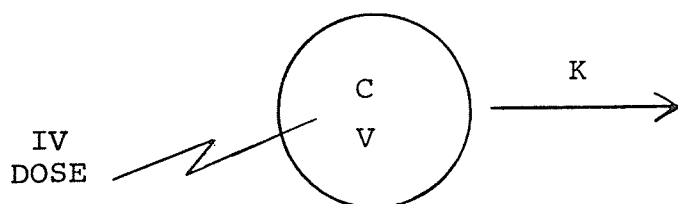
The body is depicted as a single, kinetically homogeneous compartment with a volume, V, throughout which the drug is almost instantaneously distributed following a rapid intravenous injection. Elimination of the drug from the body occurring via biotransformation and excretion is a first-or-

der process represented by K, the elimination rate constant. The rate of change of the concentration of drug, C, in the single compartment is given by the differential equation:

$$dC/dt = -KC$$

which can be integrated to give the concentration of drug, C, in the body at any time, t, following intravenous injection:

$$C = C_0 exp(-Kt)$$

Replacing C_0, the theoretical concentration of the drug in the body immediately after rapid intravenous injection, with D/V where D is the administered dose, the equation becomes:

$$C = D/V[exp(-Kt)]$$

which can be rewritten in natural log form:

$$lnC = ln(D/V) - Kt$$

Thus a plot of the natural log of the plasma drug concentration vs. time will give a straight line with slope equal to -K and y-intercept equal to ln(D/V), whereby one can obtain initial graphical estimates of the pharmacokinetic parameters K and V. Final estimates of the parameters K and V can be obtained by nonlinear regression analysis of the plasma drug concentration-time data with the graphical estimates as starting values.

Two-Compartment Model. The two-compartment model with instantaneous intravenous input is depicted as follows:
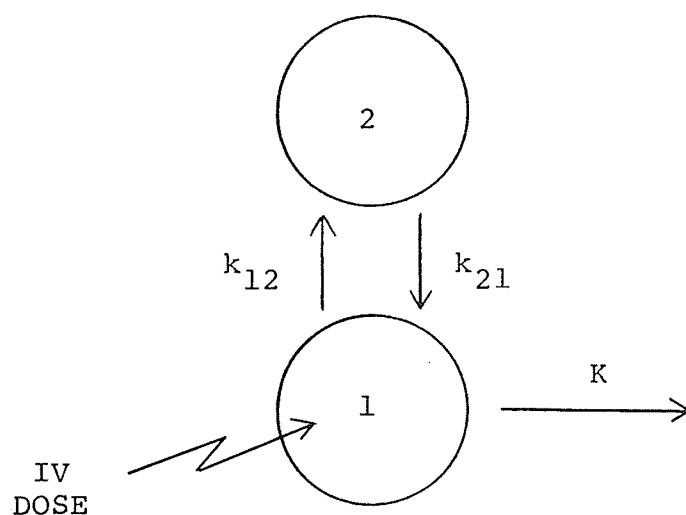
in which the body is divided in two kinetically separate compartments, the central and peripheral compartments with volumes V_1 and V_2, respectively. The central compartment represents the blood and the peripheral compartment represents tissues kinetically distinct from the central compartment, into which drug distributes more slowly. The first-order rate constants k_{12} and k_{21} are associated with the transfer of drug between compartments, k_{12} is the rate constant for the transfer of drug from the central to the peripheral compartment and k_{21} is the rate constant for the transfer of drug from the peripheral to the central compartment. The drug is assumed to be solely eliminated from the central compartment with K as the first-order elimination rate constant. Elimination of drug from the central compartment is assumed to occur via biotransformation and excretion processes.

The integrated equation giving the concentration of drug in the central compartment, C, at any time, t, following intravenous administration is (96,97):

$$C = [D(k_{21}-\lambda_1)/V_1(\lambda_2-\lambda_1)] \exp(-\lambda_1 t)$$
$$+ [D(k_{21}-\lambda_2)/V_1(\lambda_1-\lambda_2)] \exp(-\lambda_2 t)$$

where D is the dose of drug administered intravenously, and λ_1 and λ_2 are defined as hybrid first-order rate constants for the distribution phase and elimination phase, respectively. The hybrid first-order rate constants λ_1 and λ_2 are related to k_{12}, k_{21}, and K as follows:

$$\lambda_1 + \lambda_2 = k_{12} + k_{21} + K$$
$$\lambda_1\lambda_2 = k_{21}K$$

The expanded equation for the concentration of drug in the central compartment can be rewritten as the general biexponential equation:

$$C = C_1\exp(-\lambda_1 t) + C_2\exp(-\lambda_2 t)$$

Initial estimates of the coefficients C_1 and C_2 and the rate constants λ_1 and λ_2 can be obtained by the method of residuals from a plot of the natural logarithm plasma drug concentration vs. time data (96,97). Final estimates of the coefficients C_1 and C_2 and rate constants λ_1 and λ_2 can be obtained by nonlinear least-squares regression analysis of the plasma drug data.

Initial estimates of V_1, k_{21}, K, and k_{12} were obtained from the following equations:

$$V_1 = D/(C_1 + C_2)$$

$$k_{21} = (C_1\lambda_2 + C_2\lambda_1)/(C_1 + C_2)$$

$$K = \lambda_1\lambda_2/k_{21}$$

$$k_{12} = \lambda_1 + \lambda_2 - k_{21} - K$$

Starting with these initial estimates together with the plasma concentration data, final estimates can be obtained by least-squares nonlinear regression analysis as carried out by the NONLIN computer program.

Hydrolysis of Ecgonine Methyl Ester and Cocaine in Urine. The non-enzymatic hydrolysis of ecgonine methyl ester and of cocaine in unacidified urine in vitro was investigated in separate experiments. Since the hydrolysis of the two alkaloids is assumed to proceed by way of a first-order process, the following differential equation describes the change in concentration over time:

$$dC/dt = -K_h C$$

where C is the concentration of alkaloid remaining at the end of the incubation time, t and K_h is the first-order pseudo-hydrolysis rate constant. Integration of the equation gives:

$$C = C_0 \exp(-K_h t)$$

where C_0 is the initial concentration of alkaloid in the incubation medium (e.g. urine) at t=0. Re-arranging the equation and taking the natural log of both sides gives:

$$K_h = \ln(C_0/C)/t$$

which was used to calculate a pseudo-hydrolysis rate constant for ecgonine methyl ester and for cocaine incubated in unacidified urine at body temperature.