THE PHARMACOKINETICS AND METABOLISM OF COCAINE IN MATERNAL AND FETAL GUINEA PIGS

by

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This thesis is dedicated to my grandfather,

Holger "Sandy" Sandberg

who truly knows the importance of education, and to my parents,

Gary and Janice Sandberg

who have always encouraged me to do my best.

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Abstract

The pharmacokinetics and metabolism of cocaine were determined in late gestation maternal and fetal Dunkin-Hartley guinea pigs. A high-performance liquid-chromatography microassay was developed to simultaneously determine cocaine and its active metabolites benzoylecgonine, benzoylnorecgonine, and norcocaine. Following solid phase extraction, chromatographic separation was performed on a reverse-phase column with detection at 233 nm. Using 80 μl samples, the detection limit was 18 ng/ml for benzoylecgonine and benzoylnorecgonine and 35 ng/ml for cocaine and norcocaine.

This assay was used to determine the pharmacokinetics of cocaine after a single intravenous dose of 2-12 mg/kg or 2-20 mg/kg given subcutaneously. Total body clearance of cocaine was not dose dependent and the average ± S.D. was 59 ± 16 ml/min/kg over the i.v. dose range studied. Volume of distribution, mean resident time and elimination half-life were dose-dependent over this dose range with changes occurring between the 2 and 4 mg/kg dose of cocaine. Volume of distribution was 2.1 and 3.9 l/kg, mean resident time was 42 and 57 min, and elimination half-life was 34 and 49 min at the 2 and 4 mg/kg dose, respectively. Clearance and volume of distribution after 2 to 20 mg/kg subcutaneous cocaine administration were similar to values after intravenous administration, but the mean resident time was significantly greater due to delayed absorption. Benzoylecgonine and benzoylnorecgonine were major and persisting metabolites in maternal plasma. Norcocaine was seen transiently in plasma at low concentrations for doses of 4 mg/kg or higher.

To examine cocaine and metabolite distribution in the dam and fetus after chronic cocaine exposure, time-bred pregnant guinea pigs were given 10 daily 6 mg/kg subcutaneous injections from day 50 of gestation (term=70 days). One hour after the last injection, plasma, brain, urine and amniotic fluid samples were taken. There was no difference between maternal and fetal plasma cocaine concentrations. Benzoylecgonine was significantly higher in maternal plasma and benzoylnorecgonine was significantly higher in fetal plasma. Cocaine brain-to plasma ratios were similar in the dam and fetus and maternal urine contained very little parent drug. Cocaine accumulated in amniotic fluid 3-4 times fetal plasma concentrations, and benzoylnorecgonine in amniotic fluid was two-fold greater than in fetal plasma. The *in vitro* half-life of cocaine in amniotic fluid was 30 times longer than plasma elimination half-life *in vivo*. The high concentration and slow degradation of cocaine in amniotic fluid serve as a reservoir for prolonged fetal cocaine exposure.

The degradation half-life of cocaine *in vitro* in maternal and fetal guinea pig plasma was 10 times longer than the plasma *in vivo* elimination half-life. This indicated that plasma esterases play a minimal role in cocaine biotransformation and that hepatic biotransformation is much more important for the clearance of cocaine. To determine whether metabolites seen in fetal plasma could be made by the fetus or were solely maternally derived, fetal and maternal hepatic microsomes were incubated with cocaine and the appearance of metabolites was measured. Preliminary studies indicate that the late gestation guinea pig fetus (60 days) was capable of forming norcocaine but at much slower rates than the dam.

The *in vitro* and *in vivo* studies together suggest that the rate of cocaine elimination is primarily governed by N-demethylation and hydrolysis and that the hydrolysis of cocaine to benzoylecgonine is largely a nonenzymatic process.

Introduction

I. Cocaine use in pregnancy

Cocaine is used in approximately 3 to 15% of pregnancies in the United States and is common to all races and socioeconomic backgrounds (Streissguth et al., 1991; Chasnoff et al., 1990). In addition to the problems seen in the general population after cocaine use, such as tachycardia and myocardial infarction (Isner et al., 1986), the use of cocaine by pregnant women has been associated with such serious conditions such as abruptio placentae, or premature separation of the placenta from the uterine wall (Acker et al., 1983), premature labor and delivery, and a higher incidence of stillbirths and spontaneous abortions (Chasnoff et al., 1985; Bingol et al., 1987; Streissguth et al., 1991). There is also a suggestion from a study in sheep that pregnancy itself increases the risk of cardiovascular toxicity from cocaine (Woods and Plessinger, 1990). A decrease in uterine blood flow has been demonstrated in sheep after maternal exposure to cocaine with subsequent fetal hypoxemia (Woods et al., 1987). The developing fetus is not only affected by maternal morbidity, but since cocaine crosses the placenta, it is also subject to the direct actions of cocaine. There has been a case report of an in utero cerebral infarction after maternal cocaine exposure (Chasnoff et al., 1986) as well as intrauterine growth retardation (Bingol et al., 1987), and skeletal and urogenital malformations (Chasnoff et al., 1985; Bingol et al., 1987, Hoyme, et al., 1990).

Once a cocaine baby is born, there is another host of problems related to

the intrauterine drug exposure. Chasnoff et al. (1985) have described behavioral deficits in infants exposed prenatally to cocaine. The infants were tremulous, difficult to console, had depression of interactive behavior and difficulty in responding to environmental stimuli. One of three infants prenatally exposed to cocaine in another study had abnormal ventilatory patterns and it was suggested that this may predispose these infants to sudden infant death syndrome (SIDS) (Ward et al., 1986). Abnormal pulmonary ventilation has also been demonstrated in neonatal guinea pigs after chronic cocaine exposure *in utero* (Olsen and Weil, 1992). However, the link between intrauterine cocaine exposure and SIDS has not been as firmly established as that for maternal smoking (Kandall and Gaines, 1991). In a prospective study by Bauchner et al. (1988), the incidence of SIDS in the cocaine-exposed population was no different from controls.

Given the current usage of cocaine in pregnancy and the deleterious physical and psychological outcomes associated with its use, basic research into the pharmacokinetics and pharmacodynamics of cocaine in pregnancy are certainly warranted. The goals of this thesis were fourfold: **1)** To develop a microassay to measure cocaine and metabolites norcocaine, benzoylecgonine and benzoylnorecgonine in small sample volumes; **2)** to determine the pharmacokinetics of cocaine and the time course of cocaine metabolites in the pregnant guinea pig after single cocaine doses; **3)** to determine the distribution of cocaine and metabolites in the maternal/fetal unit after chronic dosing of the dam; and **4)** to examine *in vitro* cocaine metabolism in maternal and fetal hepatic

microsome preparations. The information gained from this work will and has already been the basis for gaining more insight into the functional teratology of cocaine (Olsen and Weil, 1992).

The first manuscript in this thesis describes a high-performance liquid-chromatography assay for the measurement of cocaine and its major metabolites (Sandberg and Olsen, 1990). This assay has provided the major tool for the drug analysis in further studies. The second paper examines the pharmacokinetics of different doses and routes of administration of cocaine in the pregnant guinea pig (Sandberg and Olsen, 1991). Because of the potential for metabolite accumulation as noted in the pharmacokinetics paper, the disposition of cocaine and metabolites after chronic administration was evaluated in the third paper (Sandberg and Olsen, 1992). These three papers are printed in peer reviewed journals. In the section on ADDITIONAL STUDIES, preliminary work describes the *in vitro* production of cocaine metabolites in maternal and fetal liver microsomal preparations. The topic of each paper is well detailed in individual introductions, so will not be restated in this introduction. This introduction will be used to examine in detail topics important for the interpretation of this body of work as a whole. A discussion at the end of the three manuscripts and ADDITIONAL STUDIES will attempt to make some unifying comments on the results of the papers and suggest the direction for further research.

ii. Animal model - the guinea pig

The work described here has been done using the guinea pig as an animal

model for pregnancy. Not only was this done for ethical reasons, but using a well chosen animal model allows far greater control of variables such as polydrug-abuse, nutritional status and social interactions that cannot be adequately controlled in human studies (Streissguth, et al., 1991). To dissect out the actions of cocaine alone, these variables must be eliminated. The use of other drugs such as alcohol and nicotine are associated with similar effects as seen with cocaine on the developing fetus such as low birth weight and prematurity (Ouellette et al., 1977; Meyer et al., 1976) and co-abuse with these drugs makes it difficult to determine which drug is responsible for what effect in the human population.

The guinea pig was originally chosen for these studies for two reasons. First, the guinea pig has a hemomonochorial placenta which is similar to the human placenta in structural and permeability characteristics (Faber and Thornburg, 1983; Willis et al., 1986, Olsen et al., 1989). This is an important consideration when examining the distribution of hydrophilic substances such as cocaine metabolites benzoylecgonine and benzoylnorecgonine, between maternal and fetal circulation. Olsen et al. (1988, 1989) have shown that the distribution of morphine's polar metabolites is different across placentas in species with different placental tissue layers. The second reason the guinea pig was chosen was because many of the critical periods of neurological development occur in utero for the guinea pig as in the human (Dawes, 1968), and unlike many other species such as rats, mice, dogs and cats. This provides an opportunity to model the time course of cocaine exposure during critical periods of development that might be

missed with prenatal exposure in other species. Although this point is not as important for the pharmacokinetic studies as it is for dynamic studies, the pharmacokinetics and pharmacodynamics of cocaine are entwined.

III. High-performance liquid-chromatography analysis

Radioimmunoassays are available commercially for qualitative determination of cocaine and one of its metabolites, benzoylecgonine. These assays are often used in toxicological screens but due to cross-reactivity and low sensitivity, positive samples are usually confirmed by the more costly method of gas chromatography-mass spectrometry (GCMS). Less expensive than GCMS and much more specific and quantitative than radioimmunoassays, high-performance liquid-chromatography is commonly used for the determination of polar, non-volatile compounds. The absorption of light around 230 nm by cocaine and metabolites benzoylecgonine, benzoylnorecgonine and norcocaine allows ultraviolet detection of these compounds. When this research was begun, there were various methods of measuring cocaine and metabolites by high-performance liquid chromatography (Table 1). However, no one assay adequately provided the sensitivity and specificity needed to analyze pharmacokinetic data from small animals. Therefore an assay was developed to measure cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine from biological fluids.

IV. Noncompartmental pharmacokinetic analysis

There are two major methods of quantifying pharmacokinetic information: noncompartmental and compartmental analysis. The pharmacokinetic data were

Table 1. Existing high-performance liquid-chromatography assays for the determination of cocaine and metabolites.

Detectable Drugs	*Recovery (%)*	*Sample Volume*	*Sensitivity*	*Variation (%)*	*Reference*
cocaine	70-90	2 ml	1 ng/ml	11	Khan et al., 1987
cocaine benzoylecgonine	84 80	5 ml	100 ng/ml	2.8 7	Jatlow et al., 1978
cocaine benzoylecgonine norcocaine	100 82 100	0.5 ml	1000 ng/ml	2-4	Evans and Morarity, 1980
cocaine norcocaine N-hydroxy-norcocaine	? ? ?	20 µl from tissue homogenate	1000 ng/ml	?	Benuck et al., 1988
cocaine benzoylecgonine benzoylnorecgonine norcocaine	78 53 ? ?	100 µl	200 ng/ml	?	Benuck et al., 1987

analyzed in Manuscript #2 by noncompartmental analysis with the exception of elimination half-life which was calculated from the terminal portion of the plasma drug concentration decay curve. Formulae for noncompartmental kinetics are based on the area under the time concentration curve (AUC) and the first moment (or first derivative) of that curve (AUMC). As described in Manuscript #2 and in the kinetic proofs in Appendix B, the calculation of clearance, volume of distribution and mean residence time are based on these two areas. The benefit of noncompartmental analysis is that fewer mathematical assumptions are made in the calculations than in compartmental analysis (e.g. determining if the terminal portion of the plasma drug concentration decay curve has been reached) and it is not model dependent (Gibaldi and Perrier, 1982). Compartmental analysis is based on determining the number of compartments or number of exponentials fit to the plasma elimination curve. Elimination half-life was determined by compartmental analysis and was calculated in this study for comparison with other work since published data in the field are a combination of noncompartmental and compartmental analysis (Jeffcoat et al., 1989; Wilkinson et al., 1980).

V. Physical properties of cocaine

Cocaine or benzoylmethylecgonine ($C_{17}H_{21}NO_4$) (Fig. 1) is derived from the plant *Erythroxylon coca* indigenous to areas of South America. The hydrochloride is used medically as a local anesthetic for bronchoscopy, and ear, nose, and throat procedures. As an illegal recreational drug, cocaine hydrochloride is inhaled ("snorted"), injected intravenously and taken orally. Cocaine hydrochloride

Figure 1. Structures of cocaine and major metabolites.

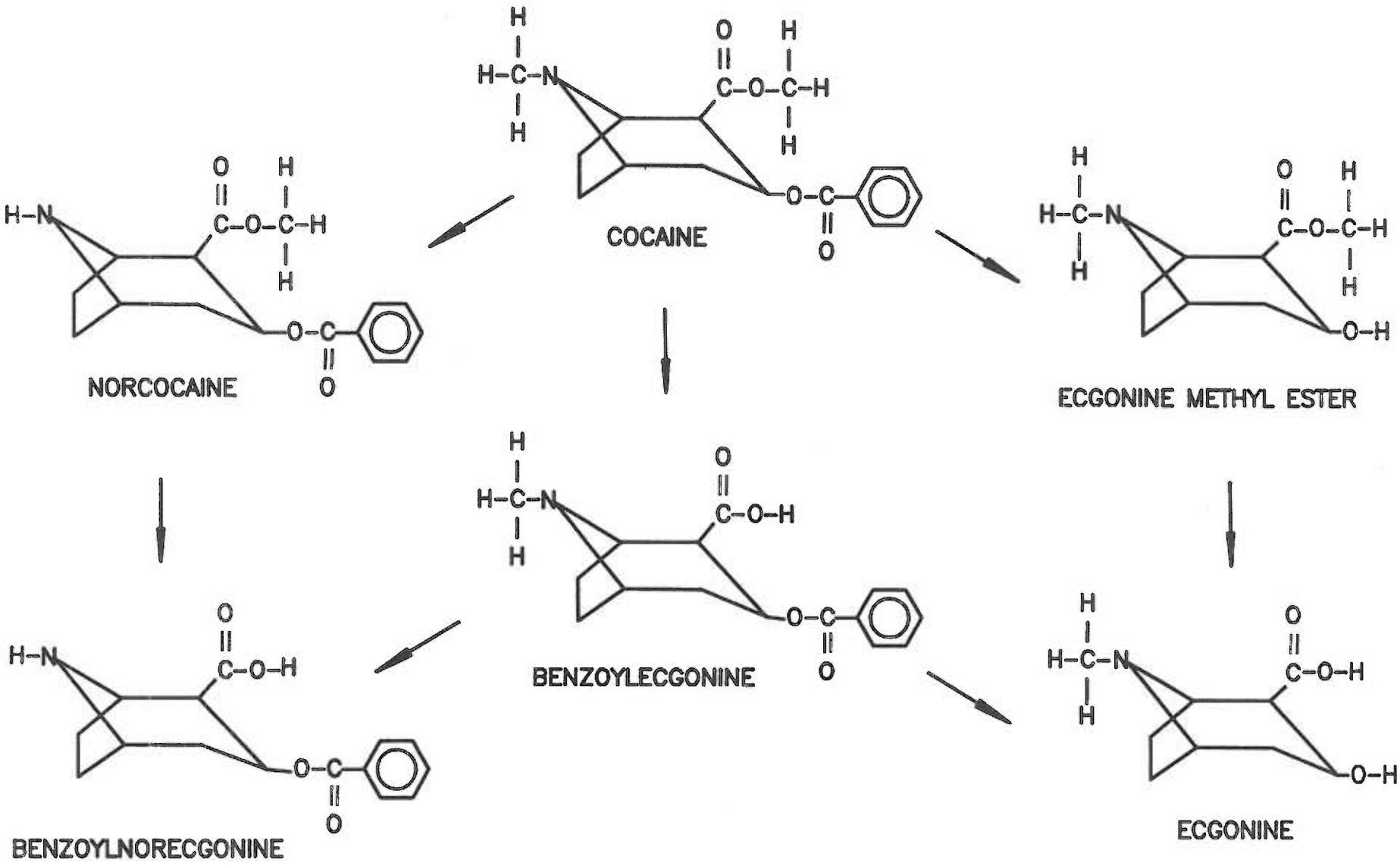
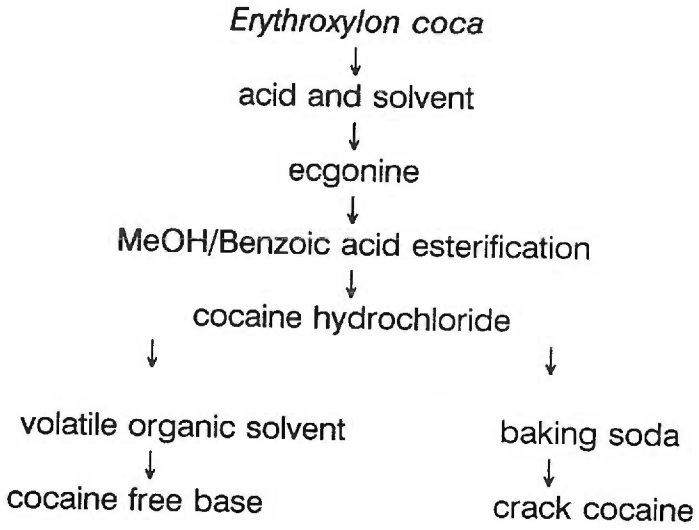

COCAINE
NORCOCAINE
ECGONINE METHYL ESTER
BENZOYLECGONINE
BENZOYLNORECGONINE
ECGONINE

Figure 2. Formation of "crack" and free base cocaine.



decomposes upon heating, so it is the carbonate or "crack" and free base forms that are smoked (Fig 2.). The free base volatilizes at temperatures above 90°C (Budavari, 1989) and produces an intense "high" (Perez-Reyes, et al., 1982).

VI. Cocaine pharmacology

(A) General cocaine pharmacokinetics

The pharmacokinetics of cocaine have been fairly well described in man for a variety of routes of administration (Table 2). All of the studies listed in Table 2 were done on male volunteers. The only study found including women (nonpregnant) was by Hamilton et al. (1977) and it only quantified urine excretion of cocaine and benzoylecgonine after intranasal cocaine application. With the exception of the study by Kogan et al. (1977), the studies shown in Table 2 specifically state that the men were recreational cocaine users. Two of the three volunteers in Kogan's study were on methadone maintenance at the time of the cocaine study. The past drug history of the study subjects as well as the fact that they were all male should be kept in mind when examining the human pharmacokinetic data. Co-abuse with known hepatic enzyme inducers such as phenobarbital or alcohol could affect a drug's rate of elimination (Gonzalez, 1989). It should also be noted that blood volume is increased 50% in late gestation and could affect cocaine's volume of distribution in pregnant women (Pritchard, 1965).

The bioavailability of cocaine from the routes as shown in Table 2 are variable and depend on the route. Using intravenous data from other studies, Wilkinson et al. (1980) estimated bioavailability of cocaine to range from 16 to 60%

Table 2. Pharmacokinetics of cocaine in humans after intravenous (i.v.), intranasal (i.n.), oral (p.o) or smoke inhalation (s.i.) administration. (Absorption half-life ($T^1/_{2_a}$); elimination half-life ($T^1/_{2_e}$); total body clearance (Cl_t); volume of distribution (Vd)).

Route	*Dose (mg/kg)*	*Peak [CONC] (ng/ml)*	*$T^1/_{2_a}$ (min)*	*$T^1/_{2_e}$ (min)*	*Cl_t (ml/min/kg)*	*Vd (l/kg)*	*n*	*Reference*
i.v.	1-3	682-3868[c]	--	40-83[*]	10-333[*]	1.2-1.9	4[a]	Barnett et al., 1981
	0.2-0.3	180[c]	--	78	23	2.7	4	Jeffcoat et al., 1989
	1.0-1.85	1080-1820[c]	--	--	16[b]	1.2[b]	3	Kogan et al., 1977
i.n.	1.1-1.5	220	11.7	78	--	--	6	Jeffcoat et al., 1989
	0.2-2.0	12-170	--	75	--	--	7[a]	Wilkinson et al., 1980
p.o.	2,3	242	--	48	--	--	6[a]	Wilkinson et al., 1980
s.i.	0.4-0.6	203	1.1	69	--	--	6	Jeffcoat et al., 1989

[*] - dose dependent
[a] - subjects received more than one dose
[b] - calculated by J.A.S. from published data, see Appendix D
[c] - cocaine (COC) concentrations 5 min after i.v. injection

after intranasal and oral administration. Barnett et al. (1981) calculated the bioavailability of cocaine to be 60% after intranasal administration in a study by Javid et al. (1978). Bioavailability after intranasal administration in a study by Jeffcoat et al. (1989) was 80%. After smoking, bioavailability was reported to be 32 to 77% by Jeffcoat et al. (1989) and 32% by Perez-Reyes et al. (1982).

For non-intravenous routes of administration (Table 2), peak plasma levels occur the fastest after smoking - within 5 minutes (Jeffcoat et al., 1989). Peak levels occurred at 40-60 minutes after intranasal administration, depending on the study (Jeffcoat et al., 1989; Wilkinson et al., 1980). It also takes approximately an hour for peak levels to occur after oral administration (Wilkinson et al., 1980).

Protein binding of cocaine in humans has been determined in two studies. The unbound portion of cocaine in the blood is able to cross membranes including the placenta and glomerulus. At concentrations ranging from 0.1 to 500 μg/ml, the average free fraction of cocaine was 8.3% (91.7% bound) in serum although the free fraction showed dose dependence increasing with cocaine concentrations above 5 μg/ml (Edwards and Bowles, 1988). Cocaine was found to bind primarily albumin and α_1-acid-glycoprotein. In a separate study by Jeffcoat et al. (1989), the binding of cocaine to human serum albumin (11-14%) and α_1-acid glycoprotein (24-34%) was also concentration dependent but the total percent bound was lower than the study by Edwards and Bowles. This may be due to the fact that Jeffcoat et al. (1989) used purified proteins instead of whole serum and binding to other proteins was ignored.

Edwards and Bowles (1988) also examined the binding of some of the metabolites. Addition of benzoylecgonine or ecgonine to serum did not affect binding of cocaine, but addition of norcocaine at a concentration of 10 μg/ml increased cocaine free fraction by 35%. The significance of the norcocaine binding study is questionable since norcocaine concentrations, when reported, are typically 1000-fold lower than this study.

Relatively small amounts of unchanged cocaine are recovered in the urine. Reports range as high as 9% (Fish and Wilson, 1969) down to less than 1% and appear to be independent of the route of administration (Jeffcoat et al., 1989). Fish and Wilson showed that the amount of cocaine in urine was pH dependent with the highest percent of cocaine recovered (9%) in acidified urine. This is probably due to ion trapping of cocaine (a weak base with a pK_a of 8.6). What is excreted in the urine is a combination of the hydrolysis products benzoylecgonine (36-54%), ecgonine methyl ester (19-49%) and ecgonine (3%) (Jeffcoat et al., 1989; Inaba et al., 1978). The excretion of benzoylecgonine in the study by Fish and Wilson was not shown to be pH dependent. The minor elimination of unchanged cocaine in the urine suggests biotransformation plays an important role in the overall clearance of cocaine from the body.

(B) Pharmacokinetics in pregnancy

Very little is known about the pharmacokinetics of cocaine during human pregnancy. In an abstract by Chasnoff and Lewis (1988), urine from pregnant women revealed substantial quantities of benzoylecgonine (64 μg/ml) and

benzoylnorecgonine (20 μg/ml) with almost 500-fold lower amounts of norcocaine (95 ng/ml). Unfortunately no information on the women, including the doses, routes of administration, time from last dose or duration of pregnancy, were given. Maternal and fetal tissue levels of cocaine were determined following a cocaine overdose in a woman (Mittleman, et al., 1989). Maternal brain to blood and kidney to blood ratios were 2.1:1 and 1.1:1, respectively and fetal blood cocaine levels were nine-times lower than maternal levels. It was suggested that this was due either to limitation of blood flow to the placenta by cocaine's vasoconstrictive actions, or that death had occurred before cocaine reached an equilibrium between maternal and fetal compartments. Fetal brain levels were three times higher than fetal blood cocaine levels.

Disposition of cocaine in pregnancy has also been examined in rats. After chronic subcutaneous cocaine administration, maternal brain and plasma concentrations were approximately 2-3 times greater than in the fetal plasma and brain tissue (Spear et al., 1989). However, fetal brain benzoylecgonine levels were greater than in the dam two hours after a dose of 20 or 40 mg/kg even though plasma levels were not. Benzoylecgonine was the only metabolite analyzed in this study. After a single intraperitoneal injection of 30 mg/kg, the elimination half-life was found to be 46 and 55 min in maternal and fetal rat plasma, respectively (DeVane et al., 1989). Norcocaine was the only metabolite analyzed in this study and it was seen in low concentrations (levels not reported). It should be noted that in a study by Clarke et al. (1985) ethanol dosing of pregnant rats by the

intraperitoneal route produced high concentrations of ethanol in the amniotic fluid as compared with oral administration. This was thought to be due to transfer of ethanol from the peritoneal space, across the uterus and chorioamniotic membranes and into amniotic fluid. Therefore, for lipid soluble compounds such as ethanol and cocaine, intraperitoneal dosing may not be appropriate.

The pharmacokinetics of cocaine in pregnant sheep are different than that in rodents or man. Following intravenous boluses of 0.5-4.0 mg/kg in the ewe, the elimination half-life in both maternal and fetal plasma was 4-5 min (DeVane et al., 1991). This is substantially faster than in other animal or human studies. The rapid and substantial appearance of ecgonine methyl ester and benzoylecgonine indicate the esterases in the pregnant sheep are more active in the clearance of cocaine than in other species (see section on BIOTRANSFORMATION below).

(C) Biotransformation

There are two major routes of biotransformation for cocaine. Hydrolysis of the methyl or benzoyl ester bond results in formation of benzoylecgonine or ecgonine methyl ester, respectively (Fig. 1). Further hydrolysis of the remaining ester bond results in ecgonine. The second route of biotransformation is the demethylation of the nitrogen group to produce norcocaine. Benzoylnorecgonine is then formed by the hydrolysis of the methyl ester bond on norcocaine. A variety of other minor metabolites have been found in various tissues and species, but little work besides identification has been done on them at this time (Table 3).

The hydrolysis products are produced from both enzymatic and

Table 3. Metabolites of cocaine.

Metabolite	*Tissue (species)*	*Comments*	*Reference*
Benzoylecgonine	urine (human)	after intravenous, intranasal, and smoking use	Jeffcoat et al., 1989
Ecgonine			
Ecgonine Methyl Ester			
Benzoylnorecgonine	brain (rat) urine (human)	-- in pregnant women	Misra et al., 1974 Chasnoff and Lewis, 1988
Norcocaine	brain (rat)	--	Misra et al., 1974
Ethylcocaine	blood,brain (human)	found with co-use with ethanol	Hearn, et al., 1991
Methylecgonidine	urine (human)	pyrolysis product, may differentiate smoking from other routes	Jacob, et al, 1990
ecgonidine	urine (human)	--	Zhang and Foltz, 1990
norecgonidine methylester			
m-hydroxy-benzoylecgonine			
ecgonidine methylester			
p-hydroxy-cocaine			
m-hydroxycocaine			
N-hydroxynorcocaine	brain, plasma, liver (mouse)	inducible, hepatotoxic	Benuck, et al, 1988

nonenzymatic processes. The enzymatic hydrolysis of cocaine to ecgonine methyl ester has historically been attributed to plasma cholinesterases (Stewart et al, 1977; Baselt, 1983). Early *in vitro* work by Stewart, et al. (1977) demonstrated that benzoic acid (an indirect measurement of ecgonine methyl ester formation) was produced by plasma cholinesterases. After 30 min, 15.4% of cocaine was liberated as benzoic acid and hydrolysis could be completely blocked with eserine and diisopropylfluorophosphate and partially with sodium fluoride. Purified human plasma cholinesterase also produced benzoic acid at similar rates to whole plasma in this study. Human placental esterases have been shown to be responsible for some cocaine degradation although only disappearance of cocaine was measured and not appearance of any metabolites (Roe et al., 1990). Addition of an anticholinesterase with these placental microsomes reduced cocaine disappearance at 135 min from 20% to 10%. A recent study has directly measured ecgonine methyl ester formation by gas chromatography after incubation with purified pseudocholinesterase and carboxylesterase (Isenschmid, et al., 1989). The purified esterase found in red blood cells, acetylcholinesterase, was not found to metabolize cocaine in that study. The involvement of psuedocholinesterases in the degradation of cocaine could have an important clinical implication. It has been suggested from an *in vitro* study that people with low dibucaine numbers (an indication of plasma cholinesterase activity) may have slower clearance of cocaine due to decreased hydrolysis (Jatlow et al., 1979). Only 40% of cocaine remained after two hours when incubated in plasma from 10 volunteers with normal

dibucaine numbers, but over 90% remained in incubations of plasma from six patients with low dibucaine numbers. Lower cholinesterase activity has been associated with an increase in the N-demethylation pathway of cocaine degradation (Inaba et al., 1978). Cumulative excretion of $^{14}CO_2$ after ingestion of [N-$^{14}CH_3$] cocaine was three times greater in one patient with low cholinesterase activity (dibucaine number not given) as compared to a normal subject. However, as will be discussed in DISCUSSION, the overall contribution of plasma esterases to the total biotransformation of cocaine, at least in the pregnant guinea pig, is minimal.

The literature is divided on the mechanism for benzoylecgonine formation. Some data suggest benzoylecgonine is formed entirely by nonenzymatic hydrolysis. Cocaine was incubated in fresh human serum and in the S9 fraction of frozen human liver tissue by Stewart et al. (1979). It was reported that benzoylecgonine was formed nonenzymatically in both tissues but the only data shown was the percent of cocaine that was converted to benzoylecgonine in buffer for 24 hr. In a study by Isenschmid et al. (1989), benzoylecgonine concentrations produced over 4 hours from incubations with excess carboxylesterase, acetylcholinesterase or pseudocholinesterase were no different from buffered saline control. However, Matsubara et al. (1984) showed pretreatment with the carboxylesterase inhibitor tri-o-tolylphosphate in dogs did inhibit approximately 60% of the production of benzoylecgonine *in vivo* without a loss of plasma cholinesterase activity.

Recent work by Dean et al. (1991) appears to answer the question of whether benzoylecgonine is produced nonenzymatically or enzymatically. Two nonspecific acetyl esterases (1 and 2) were isolated from human liver. After incubation of 24.5 μM cocaine with esterase 1, four-times the level of benzoylecgonine was produced after three hours than when cocaine was incubated with buffer. The formation of benzoylecgonine with this enzyme was inhibited by 40 mM sodium fluoride. Eserine incompletely blocked the formation, suggestive of a carboxylesterase and not a cholinesterase (Augustinsson, 1961). In the presence of ethanol, esterase 1 produced ethylcocaine. Esterase 2 was responsible for the enzymatic production of ecgonine methyl ester only and was completely blocked by eserine. The results of this study as well as that by Matsubara et al. (1984) as described above, indicate benzoylecgonine and ecgonine methyl ester are formed by different esterases and benzoylecgonine is formed enzymatically by a hepatic carboxylesterase. The apparent contradiction between these studies and that by Isenschmid et al. (1989) is that an isoenzyme may have been used in this latter work that is not active in producing benzoylecgonine.

It is clear by all the studies mentioned above that nonenzymatic hydrolysis of cocaine does produce some benzoylecgonine in both *in vivo* and *in vitro* studies. The extent to which this process occurs is also dependent upon temperature and pH. The lower the temperature and pH, the more stable the alkyl ester bond is to hydrolysis (Baselt, 1983, Garrett and Seyda, 1983; Murray and Al-

Shora, 1978). The relative importance of the nonenzymatic production of benzoylecgonine to the overall biotransformation of cocaine will be addressed in the DISCUSSION AND CONCLUSIONS at the end of this thesis.

Norcocaine is formed by the N-demethylation of cocaine in the mixed function oxidase system. The production of norcocaine has been demonstrated in rat liver microsomes (Leighty and Fentiman, Jr., 1974;Benuck et al., 1989), rat hepatocyte cultures (Stewart et al., 1978) and recently in human liver microsomes (Roberts et al., 1991). The mixed function oxidase system is made up of multiple families of enzymes (Gonzalez, 1989) and it is possible that more than one P450 enzyme is involved with the production of norcocaine. In rat hepatocytes, similar kinetics and competitive inhibition of metabolism with cocaine and aminopyrine suggest N-demethylation of the two drugs may be the result of the same isoenzyme(s) (Stewart et al., 1978). Recent work has shown that cocaine binds to P450IIBI and is involved with the production of the hepatotoxic metabolite N-hydroxynorcocaine in rat hepatocytes (Boelsterli et al., 1992) and LeDuc et al. (1992) have shown P450IIIA is responsible for at least 50% of norcocaine produced in microsomes from a human lymphoblastoid cell line.

N-demethylation seems to be relatively minor in comparison to the hydrolysis pathways since fairly low concentrations of norcocaine are seen in biological tissue. In a study by Inaba et al. (1978) excretion of radioactive CO_2 was followed for 5 hours as a measure of N-demethylation of [N-$^{14}CH_3$]cocaine by two male volunteers. It was found that less than 7% of the administered dose was

recovered by radioactive CO_2 production. Using the same techniques in rats, *in vivo* radioactive CO_2 measurements indicated 14% of a 30 mg/kg dose of cocaine was N-demethylated (Stewart et al., 1978). Chasnoff and Lewis (1988) reported that norcocaine levels were a thousand fold less than those of benzoylecgonine and benzoylnorecgonine in the urine of pregnant cocaine-using women. There also seems to be an inverse relationship between the hydrolysis and N-demethylation pathways of cocaine biotransformation since an increased N-demethylation may be seen in conjunction with reduced activity of the cholinesterase system (Inaba et al, 1978).

(D) Mechanisms of action

1. Cocaine

There are two main actions of cocaine. High concentrations of cocaine act as a local anesthetic by blocking voltage-gated sodium channels. At 10^{-4} M, cocaine completely blocked veratridine-stimulated sodium channel uptake of $^{22}Na^{+}$ into rat brain homogenates (Matthews and Collins, 1983). The local anesthetic effect was demonstrated as a reduction of maximum rate of rise of the action potential in isolated ganglion cells of *Aplysia*. The local anesthetic properties of cocaine may be responsible for the cardiodepressant effects such as a decrease in sinus node rate seen in rabbit heart tissue (Przywara and Dambach, 1989).

The developing fetus may also be susceptible to the local anesthetic effects of cocaine. Flexner and Flexner (1949) suggest that the guinea pig brain becomes selectively permeable to sodium ions compared to chloride ions on approximately

day 45 of gestation. Exposure to a sodium channel blocker at this time may disrupt development of ion selectivity in the central nervous system and alter electrophysiological events necessary to normal neurological development.

The second major action of cocaine is to inhibit uptake of neuronal monoamine neurotransmitters by binding to dopamine, norepinephrine and serotonin transporters (Ritz et al., 1990). Hawks et al. (1975) demonstrated 10^{-4} M cocaine could inhibit 97% of H^3-norepinephrine uptake into monkey brain synaptosomes. *In vivo* brain concentrations are generally 10^{-5} M or lower (Hawks et al., 1975; Spear et al., 1989; Sandberg and Olsen, 1992) and at this concentration, 70% of H^3-norepinephrine uptake was inhibited. Norepinephrine reuptake blockade was consistent with prolongation of inhibitory post synaptic potentials and current in guinea pig locus coeruleus neurons and submucous plexus neurons (Suprenant and Williams, 1987). Recently, two groups have cloned and expressed a cocaine-sensitive dopamine transporter, and it is thought that transporters such as these make up "cocaine receptors" in the brain (Shimada et al., 1991; Kilty, et al., 1991). A cocaine-sensitive norepinephrine transporter has also been cloned and expressed (Pacholczyle et al., 1991).

Cocaine's interaction with the monoaminergic system is responsible for many of its actions seen *in vivo*. Its addictive properties have been attributed to cocaine's binding to dopamine transporters (Ritz et al., 1987) and subsequent alterations in extracellular dopamine concentrations in the dopaminergic system such as in limbic and subcortical areas (Di Chiara and Imperato, 1988). Cardiotoxic

events such as myocardial infarction and fibrillation were attributed to cocaine induced increase in catecholamine concentrations (Billman, 1990).

Alpha$_1$ adrenergic stimulation has been demonstrated by Dolkart et al. (1990) to be the mechanism by which cocaine causes maternal and fetal sheep hypertension. However, cocaine still caused total uterine blood flow to decrease by 44% and vascular resistance to increase 59% after an infusion of the α_1 receptor blocker, phenoxybenzamine. The authors suggest that the pressor response in the uterine vasculature may be due to not only effects of norepinephrine, but other neurotransmitters such as serotonin and dopamine which are increased after cocaine administration.

It has been suggested that the ability of cocaine to cause vasoconstriction through its sympathomimetic actions could be responsible for vasculature disruptions in the fetus (MacGregor et al., 1987). Many of the structural abnormalities seen in cocaine babies such as limb reduction defects, intestinal atresia or infarction could be explained by vascular disruptions during development (Hoyme et al., 1990).

Cocaine has been shown to be a competitive antagonist at muscarinic receptors (Sharkey et al., 1988). However, the antimuscarinic effects of cocaine may only be important at extremely high doses since the K_i (18.8 μM) was 0.6-2 times plasma levels seen after intravenous administration (Barnett et al. 1981), 27 times than after nasal insufflation and 36 times than after smoking (Jeffcoat et al., 1989).

2. Metabolites

The mechanisms of action for the metabolites are generally less well known than those for cocaine. As shown in Table 4, the metabolites do share many of the activities of cocaine to varying degrees.

As with cocaine, norcocaine is a potent local anesthetic. Just and Hoyer (1977) showed in isolated *Aplysia* ganglion cells that norcocaine is a more effective local anesthetic than cocaine and suggested this may be due to differences in ionization and/or lipophilicity. In a later study by Matthews and Collins (1983), norcocaine competitively inhibited the veratridine stimulated $^{22}Na^{+}$ uptake in rat membrane homogenates over the concentration range of 10^{-4} to 10^{-7} M and was approximately three times more potent than cocaine. The inhibitory action of norcocaine at the sodium channel is consistent with its relative local anesthetic potency compared to cocaine. The hydrolysis products showed very little inhibition of uptake, which may be the result of decreased lipophilicity.

Until recently, norcocaine has been thought to be the only biologically active metabolite of cocaine. Although Misra et al. (1975) showed in the mid 1970's that benzoylecgonine and benzoylnorecgonine could cause seizures when injected intracisternally, this information was largely ignored until confirmed by Konkol et al. (1992). At concentrations of benzoylecgonine seen in the autopsied brains of human cocaine users and lower than those seen in an accumulation study in fetal rats (Spear et al., 1989), Konkol's group showed a dose dependent incidence of seizures in neonatal rats injected intracerebroventricular with benzoylecgonine.

Table 4. Relative potencies of cocaine and metabolites.

Drug	*Local Anesthetic*	*Norepinephrine Re-uptake Blocker*	*Seizures*	*Ca^{2+} Binding*	*Vasoconstriction*
Cocaine	1	1	1	>BE, BN	1
Benzoylecgonine (BE)	--	0.1	50 x	BE=BN	3
Benzoylnorecgonine (BN)	--	0.3	500 x	BE=BN	--
Norcocaine	1.1-1.4	1	--	<BE,BN	--
Reference	Just & Hoyer, 1977	Hawks et al., 1975	Konkol, personal communication	Misra & Mule, 1975	Madden & Powers, 1990

Some other work of Misra and Mulé (1975) showed that most of the metabolites and particularly benzoylecgonine and benzoylnorecgonine form complexes with calcium. Whether this has any biological significance is not known at this time, but the potential for disruption of calcium regulated events such as second messenger systems, neurotransmitter release and muscle contraction by cocaine or its metabolites is worth investigation.

Benzoylecgonine and ecgonine (10^{-5} M) have also been recently shown to cause vasoconstriction of an *in vitro* cat middle cerebral artery preparation (Madden and Powers, 1990). At equimolar concentrations, benzoylecgonine caused a decrease in vessel diameter intermediate between serotonin and norepinephrine but greater than cocaine. These effects were demonstrated at concentrations of benzoylecgonine (10^{-6} to 10^{-7} M) that have been shown to occur *in vivo* in various tissues (Spear et al., 1989; Konkol et al., 1992; Sandberg and Olsen, 1991, 1992). Reserpine attenuated the vasoconstrictive effects of benzoylecgonine, ecgonine and cocaine which suggests that the metabolites as well as cocaine interact with the adrenergic system to elicit the vasoconstrictive response. However, the mechanism of the vasoconstrictive action may not be the same as for cocaine since benzoylecgonine inhibited only 11% of ^{3}H-norepinephrine uptake in synaptosomes produced by cocaine (Hawks et al., 1975).

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Manuscript I

(J. Chromatogr. 525:113-121, 1990)

Title: Microassay for the Simultaneous Determination of Cocaine, Norcocaine, Benzoylecgonine and Benzoylnorecgonine by High-Performance Liquid Chromatography

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SUMMARY

An improved method for the simultaneous determination of cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine using reversed-phase high-performance liquid chromatography with ultraviolet detection is described. Following solid-phase extraction, chromatography was performed using a column containing an octadecylsilica-coated packing, eluted with 6% acetonitrile in phosphate buffer, pH 2.1, and detected at 233 nm. Using 80 μl samples, the detection limit is 18 ng/ml for benzoylecgonine and benzoylnorecgonine and 35 ng/ml for cocaine and norcocaine. The coefficients of variation range from 3.5% (benzoylecgonine) to 7.0% (norcocaine). The procedure has been applied to samples of guinea pig plasma, urine and amniotic fluid and human urine.

INTRODUCTION

Cocaine (COC) use among women of childbearing age has been a significant problem in recent years and the number of clinical reports suggesting increased morbidity and mortality of the infants of these substance abusing mothers has increased [1,2]. Detrimental effects to the fetus could be caused by the actions of COC itself and/or metabolites such as norcocaine (NOR), benzoylnorecgonine (BN) and benzoylecgonine (BE) [3]. BE is a major urinary metabolite [4] often used in toxicology screens to confirm COC abuse [5]. Animal studies have demonstrated that COC is also demethylated to NOR [6], a metabolite with adrenergic [3] and local anesthetic properties similar to COC [7]. The extent to which these metabolites are formed in the fetus is not known. In this laboratory, the guinea pig is used as an animal model to determine the pharmacokinetics of COC and its metabolites in pregnancy. The guinea pig is an appropriate model since its placental structure and permeability characteristics are similar to those of the human placenta [8].

Reversed-phase high-performance liquid chromatography (HPLC) is commonly used for the determination of polar, non-volatile compounds such as BE and related substances. Various assays exist that can detect COC [9], COC, BE and NOR [10] and COC, BE, NOR and BN [11,12]. However, most methods either require sample sizes too large to be compatible with repeated sampling from a small animal [11] or do not demonstrate sensitivity low enough to fully delineate the elimination curve of COC and metabolites in the guinea pig [12]. In addition,

these methods use a solvent extraction procedure which is less convenient that the solid-phase extraction procedure proposed in this paper. The solid-phase method is similar to that used in this laboratory for the determination of morphine and metabolites morphine-3- and -6-glucuronides [13].

The assay described in this study was developed for the simultaneous determination of COC, NOR, BE and BN following solid-phase extraction of microsamples obtained from maternal and fetal guinea pigs. The solid-phase procedure was compared to solvent extraction. Validation of the assay for COC and BE was done by gas chromatographic-mass spectrometric (GC-MS) analysis.

EXPERIMENTAL

Chemicals

COC•HCL, NOR, BE, BN-HCl, methamphetamine•HCl, amphetamine•HCl, morphine, morphine-3-glucuronide, ecgonine methyl ester (EME) and ecgonine were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the National Institute of Drug Abuse (Bethesda, MD, U.S.A.). The internal standard lidocaine (LID) was purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (50 ng/μl) were made by dissolving COC, BE, BN and LID in water and NOR in ethanol. Aliquots of 1 ml were stored at -15°C until used. Methanol, water and acetonitrile (Mallinckrodt, Paris, KY, U.S.A.) were HPLC grade. Tetrabutylammonium hydroxide (TBA-OH, Aldrich, Milwaukee, WI, U.S.A.) and all other chemicals were reagent grade.

Liquid chromatography

Mobile phase (100 μl) containing drugs was injected into a Model 334 liquid chromatographic system (Beckman Instruments, Berkeley, CA, U.S.A.) in line with a 100 mm x 3.2 mm I.D., 3 μm particle size, reversed-phase cartridge column (Velosep ODS, Applied Biosystems, Santa Clara, CA, U.S.A.) and a Model 783A programmable absorbance detector (Applied Biosystems, Ramsey, NJ, U.S.A.) set at 233 nm and 0.002 absorbance units full scale (a.u.f.s.). The detector response was integrated with a Model 5640A integrator (Hewlett-Packard, Los Angeles, CA, U.S.A.). The mobile phase consisted of a 0.01 M phosphate buffer, pH 2.1, and

0.0002 M TBA-OH with 6% (v/v) acetonitrile. The mobile phase was filtered through a 0.22 μm membrane filter (Rainin, Woburn, MA, U.S.A.) and run at a flow-rate of 1.5 ml/min.

Sample extraction

Solvent extraction. A 80 μl sample (plasma, urine or amniotic fluid) was added to 720 μl of 0.01 M phosphate buffer, pH 9.5, containing 450 ng of LID as the internal standard. A 5 ml mixture of chloroform-isopropanol (95:5, v/v) was added, vortex-mixed for 30 s and then centrifuged at 1700 g for 20 min. The aqueous phase was discarded and 4 ml of the organic phase were dried at 45°C under nitrogen. The sample was reconstituted in 300 μl of mobile phase for injection into the HPLC system.

Solid-phase extraction. A 80 μl sample was mixed with 720 μl of 0.5 M ammonium sulfate, pH 8.7, containing 450 ng LID as the internal standard, and injected through a Chromprep PRP-1 80 μl cartridge (Hamilton, Reno, NV, U.S.A.) at an average rate of 1.5 ml/min. The cartridge was then washed with 2 ml of 0.01 M ammonium sulfate, pH 8.7, and 1 ml of 0.01 M phosphate, pH 2.0, followed by 80 μl of water and 250 μl of diethyl ether. The cartridge deadspace was cleared of diethyl ether by injecting air. The drugs were eluted with 300 μl of methanol and the eluate was dried at 45°C under nitrogen. Samples were reconstituted in 300 μl of mobile phase. Siliconized glassware was used throughout the extraction and reconstitution procedure.

Gas chromatography-mass spectrometry

Samples were analyzed by the Toxicology Department of The Oregon Health Sciences University with a method based on the work of Griesemer et al. [14] and Clark and Hajar [15]. The method has been modified to use the deuterated compounds $[^2H_3]$COC and $[^2H_3]$BE as internal standards instead of SKF 525-A. BE was derivatized to ethylbenzoylecgonine. EME can also be determined by this method. Sample preparation and GC-MS analysis was performed as described previously [15]. Samples were triply extracted progressing from basic to acidic to basic pH in a solvent extraction method. The following ions (m/z) were monitored in the GC-MS selected-ion monitoring mode: m/z 303 for COC; m/z 306 for $[^2H_3]$COC; m/z 361 for BE; m/z 364 for $[^2H_3]$BE; m/z 96 for EME. Quantitation was based on ion peak-area ratios for the undeuterated and deuterated fragments.

Animals

Experiments were carried out using pregnant Dunkin-Hartley guinea pigs (Charles River, Wilmington, MA, U.S.A.; Simonsen Labs, Gilroy, CA, U.S.A.) in the last half of gestation (term is 65 days). Animals were housed indoors with controlled light cycles, continuous ad libitum food and water and daily health checks. The Animal Care Department is fully accredited by the American Association for accreditation of Laboratory Animal Care. The Department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health. Maternal samples were obtained by the method of

Olsen et al. [16] with the following changes: isoflurane replaced halothane as the anesthetic and an external jugular vein catheter was introduced as well as a carotid artery catheter. Dams were allowed to recover from surgery at least 24 h before experiments were performed. Samples of 1 ml were drawn into heparinized disposable syringes and placed in polypropylene microfuge tubes containing 2.5 mg/ml sodium fluoride. After centrifugation at 5°C, the plasma was removed and stored at -15°C until assayed.

RESULTS AND DISCUSSION

Several published methods have been adapted to develop a microassay for the simultaneous determination of COC and metabolites. The mobile phase was a modification of that of Svensson [17]. Several mobile phase modifiers have been tested and the one used by Garret and Seyda [18], TBA-OH, was selected for use because it resulted in the best separation of drug peaks. Three compounds, LID, m-toluic acid, and the ethyl ester of benzoylecgonine (ETBE) were evaluated as internal standards. ETBE was synthesized as described by Jatlow et al. [11] and purity was verified by GC-MS. Of the three, LID was chosen as the internal standard because of its short retention time.

Fig. 1 shows the separation of COC, BE, BN, NOR and LID under the chromatographic conditions described. Extractions were originally performed by the solvent method but this proved unsatisfactory for the small sample size due to relatively poor recoveries (average 50%), high coefficients of variation (average 15%) and decreased sensitivities (varying from 35 ng for BE to 300 ng for NOR). A solid-phase extraction method similar to one used in this laboratory for morphine and its glucuronides [13] was modified to purify COC and the three metabolites from biological fluids. Because the solid-phase method gave much higher recoveries and increased sensitivity compared to the solvent method (Table I), all subsequent extractions were done using the solid-phase method. Considering the 80 μl sample size and dilutions inherent within the assay, the sensitivity of the assay was 18 ng for BE and BN and 35 ng for COC and NOR, while the lowest

quantifiable levels for BE and BN were 35 ng/ml and 75 ng/ml for COC and NOR. The coefficient of variation within an assay was less than 7% for COC and the metabolites tested (Table I). The assay is also specific for COC, NOR, BN and BE. Relative retention times for other drugs compared to COC are listed in Table II.

Fig. 1 shows chromatograms of extracted guinea pig control plasma and plasma with 15 ng of COC, BE, BN and NOR and 150 ng of LID. There are no interfering peaks eluting with COC, BN, NOR or LID. Occasionally, a small plasma peak coelutes with BE but because it is relatively small, its area can be subtracted from that of the samples. Various solvents were tested for their ability to remove interfering peaks from the solid-phase extraction. These included hexane, heptane, chloroform-isopropanol (95:5), chloroform-ethanol (80:20) and diethyl ether. The method presented proved to be the most effective at cleaning the samples. The peak eluting at 4.5 min is a constituent from the Chromprep cartridge which elutes with the sample. However, it does not interfere with the quantitation of BN or BE.

Standard curves were obtained by analysis of plasma combined with a range of drug concentration (35-2260 ng/ml) (Fig. 2). The ratios of the area under the curve (AUC) of COC, BE, BN and NOR to LID, multiplied by 100, versus the drug concentrations gave standard curves, determined by linear regression analysis, with an average *r* value of 0.998. Samples too concentrated to fit on the standard curve were diluted before extraction.

The HPLC method was validated for COC and BE by GC-MS. Aliquots of guinea pig plasma with 280-1130 ng/ml COC and BE were analyzed by GC-MS. By this

method, the average error from the known concentrations was 13 and 8% for COC and BE, respectively. By HPLC analysis, the error for plasma samples with 560 ng/ml COC, NOR, BN and BE ranged from 1 to 4 % (Table I). The larger error with GC-MS analysis found for COC and BE is probably due to the fact that the analysis was done on 0.5 ml samples in an assay that was designed to use 5 ml. The two methods have been compared using unknown guinea pig samples and the agreement is good with an average difference of less than 10%. It should be noted that no EME was detected in guinea pig plasma by GC-MS. From Table I, Figs. 1 and 2 and the GC-MS work it is evident that the solid-phase extraction method followed by HPLC analysis can be used to accurately quantitate COC and metabolite concentrations in very small (80 μl) samples.

The solid-phase extraction and HPLC methods described here have been successfully used to measure COC and metabolite concentrations in maternal and fetal guinea pig plasma, urine and amniotic fluid samples as well as in human urine samples. Fig. 3 shows plasma concentrations of COC and BE in a maternal guinea pig after a 2 mg/kg intravenous injection. COC is no longer detectable in plasma approximately 1.5 h after COC administration and BE is still quantifiable at 7 h after injection when sampling ended. Because the elimination half-life of BE is twelve times longer than COC, BE would accumulate in plasma with repeated COC injections.

In conclusion, the HPLC microassay described here provides an accurate, sensitive way to simultaneously measure COC and metabolites BE, BN and NOR

from plasma, urine and amniotic fluid. The assay is an improvement over standard solvent extractions and has been validated by GC-MS for COC and BE.

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TABLE 1

COMPARISON OF LIDOCAINE, COCAINE, BENZOYLECGONINE, BENZOYLNORECGONINE AND NORCOCAINE RECOVERY, ACCURACY AND COEFFICIENT OF VARIATION FOR SOLVENT AND SOLID-PHASE EXTRACTION METHODS

For the solvent extraction method, 80 μl samples in a basic phosphate buffer were extracted with 95:5 chloroform-isopropanol. Benzoylnorecgonine was not studied with this method. In the solid-phase method, 80 μl samples in a basic ammonium sulfate buffer were injected onto a Chromprep cartridge, washed with acidic phosphate buffer, water and diethyl ether and eluted with methanol. For all solvent extraction values n=6 and for solid-phase values n=15, except for cocaine n=12. Accuracy is expressed as the percentage error of the difference between expected and observed values.

Drug	Recovery (%)		Accuracy (%)		Coefficient of variation (%)	
	Solvent	Solid	Solvent	Solid	Solvent	Solid
Lidocaine	59	92	-	-	9.7	4.4
Cocaine	57	86	33	3.8	16	6.0
Benzoylecgonine	66	91	46	2.1	14	3.5
Benzoylnorecgonine	-	92	-	0.6	-	5.6
Norcocaine	21	85	14	4.0	15	7.0

TABLE 2

RELATIVE RETENTION TIMES OF VARIOUS SUBSTANCES COMPARED TO COCAINE

Non-extracted samples in mobile phase were injected and the chromatogram recorded for 30 min. A 30 ng amount of each of NOR, COC, BN and BE were injected. Because of decreased sensitivity, 150 ng of LID and 250 ng of the remaining drugs were injected. No peaks were seen during the 30 min for morphine, morphine-3-glucuronide, ecgonine methyl ester and ecgonine. Retention time relative to cocaine is listed.

Substance	Relative retention time
Norcocaine	1.41
Cocaine	1.00
Benzoylnorecgonine	0.65
Benzoylecgonine	0.62
Lidocaine	0.29
Methamphetamine	0.21
Amphetamine	0.17
Morphine	-
Morphine-3-glucuronide	-
Ecgonine methyl ester	-
Ecgonine	-

Figure 1. Chromatograms of (A) mobile phase, (B) mobile phase with 15 ng each of BE, BN, COC and NOR and 150 ng of LID, (C) extracted undiluted guinea pig plasma and (D) guinea pig plasma with 15 ng each of BE, BN, COC and NOR and 150 ng of LID. All extractions were done by the solid-phase method. Extraction volume was 80 μl and injection volume was 100 μl.

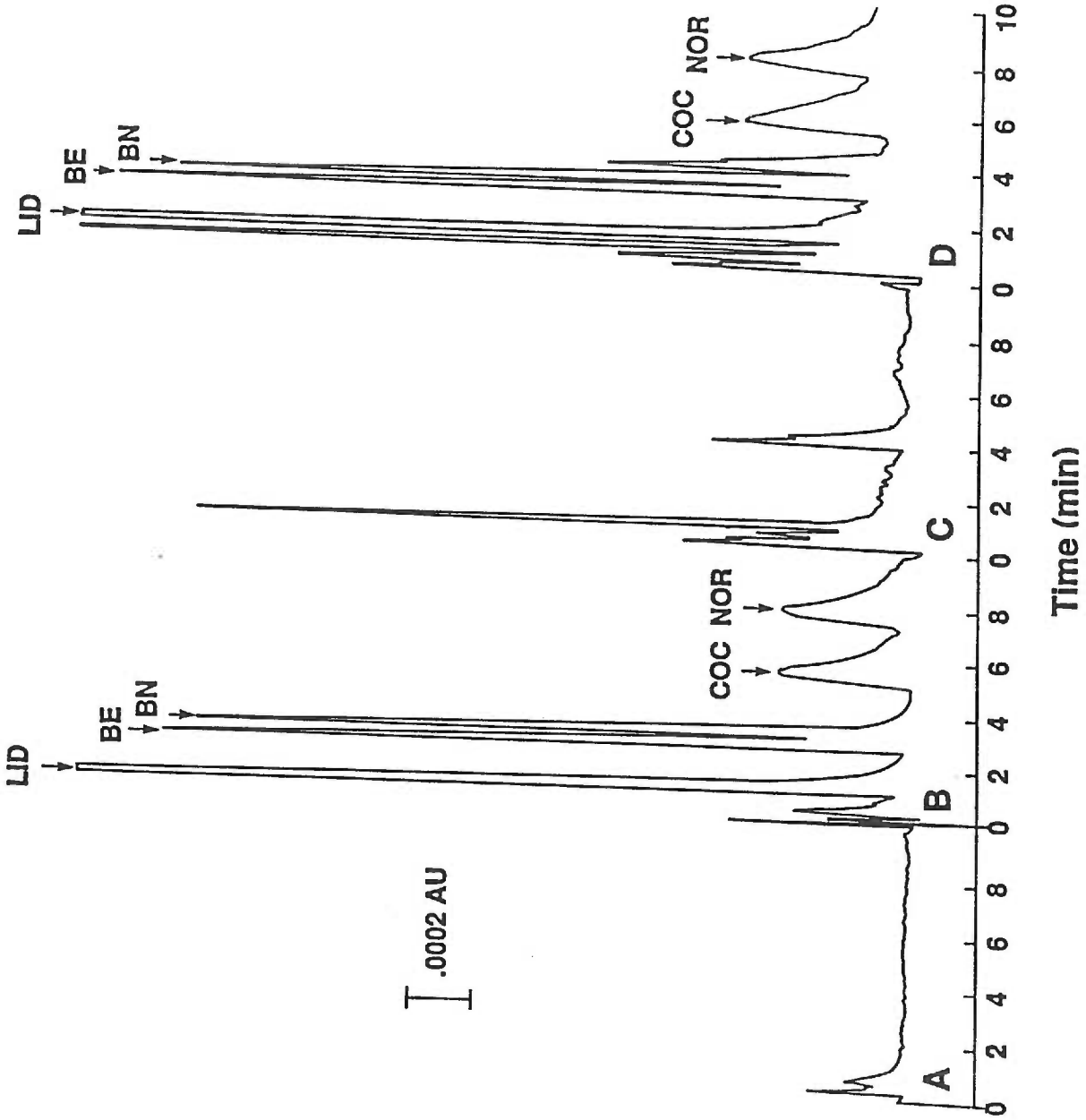

LID
BE
BN
COC
NOR
A
B
C
D
.0002 AU
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 10
Time (min)

Figure 2. Standard curves for COC, BE, BN and NOR. Guinea pig plasma (80 μl) was extracted by the solid-phase method. The extract was dried under nitrogen and reconstituted in 300 μl of mobile phase. The ratios of the AUC for each drug (COC, BE, BN and NOR) to the AUC of the internal standard (LID) multiplied by 100 are plotted as functions of the amount of COC, BE, BN or NOR in the injection volume of 100 μl.

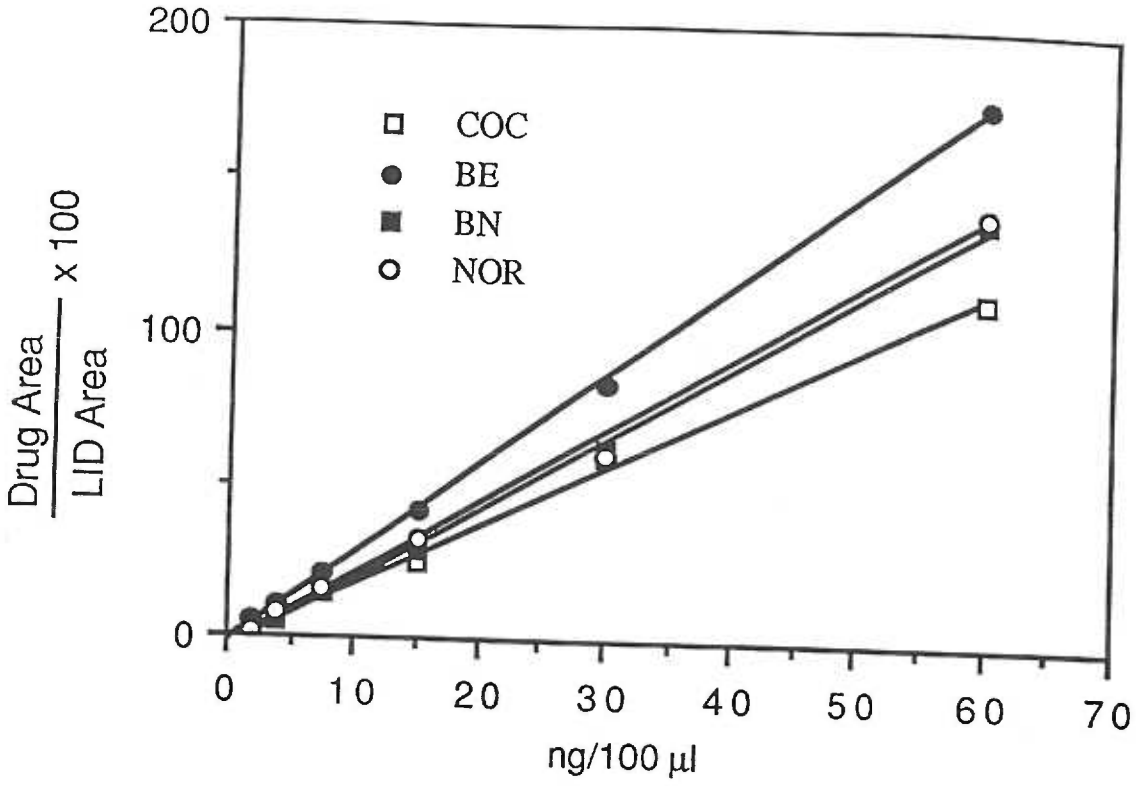
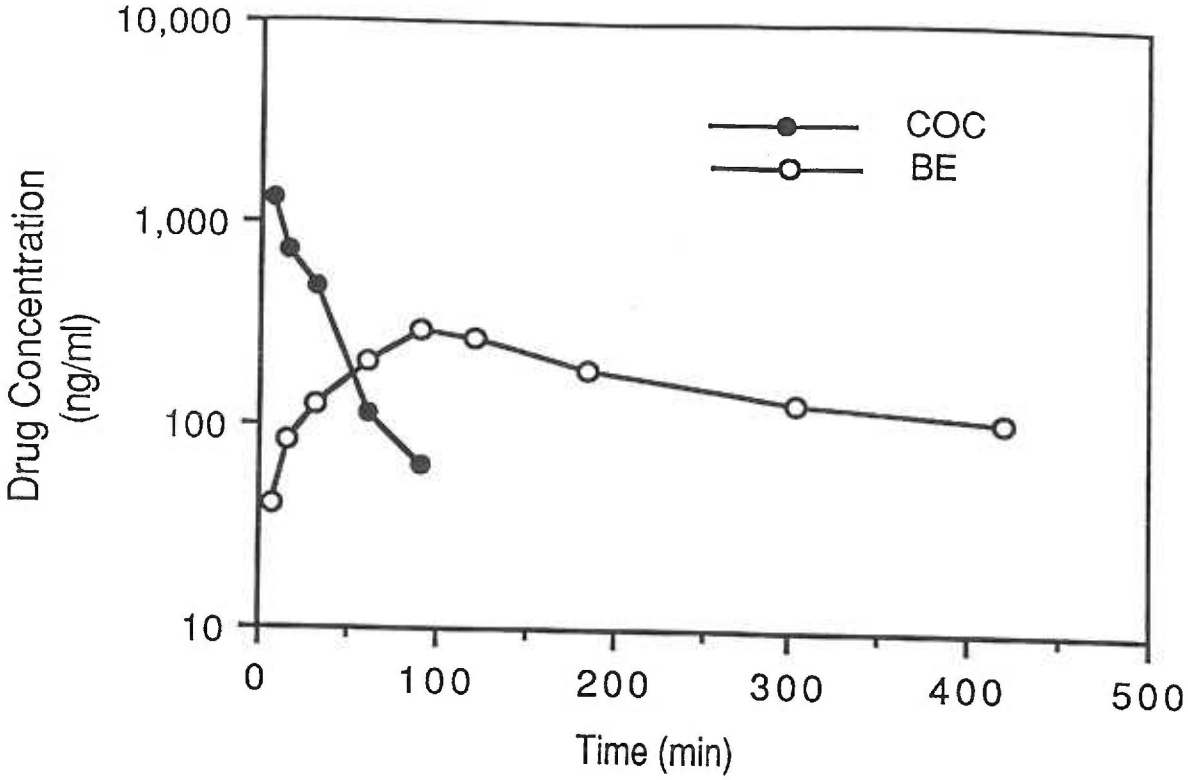

200
100
0
COC
BE
BN
NOR
Drug Area / LID Area x 100
0
10
20
30
40
50
60
70
ng/100 µl

Figure 3. Plasma concentrations of COC and BE in a material guinea pig after a 2 mg/kg intravenous dose of COC. Arterial samples were taken at 7, 16, 31, 61, 92, 121, 184, 304 and 419 min after the COC injection. COC was no longer detectable after 92 min while BE was still quantifiable at 419 min when sampling ended. The elimination half-life of COC and BE were 20 and 239 min, respectively.


10,000
1,000
100
10
Drug Concentration (ng/ml)
COC
BE
0
100
200
300
400
500
Time (min)

Manuscript II

(J. Pharmacol. Exp. Ther. 258:477-482, 1991)

Title: Cocaine pharmacokinetics in the pregnant guinea pig.

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Abbreviations:

COC	cocaine
BE	benzoylecgonine
BN	benzoylnorecgonine
NOR	norcocaine
LID	lidocaine
HPLC	high-performance liquid-chromatography
Ke	terminal elimination rate constant
f	fraction of dose absorbed
AUC	area under the concentration-time curve
AUMC	area under the first moment of the concentration-time curve
Vdss	volume of distribution at steady state
MAT	mean absorption time
MRT	mean resident time
T½	half-life
Cl	clearance
Cp	plasma concentration

ABSTRACT

The pharmacokinetics of cocaine (COC) were studied during late gestation in guinea pigs. Clearance (Cl) was not dose-dependent and the average ± S.D. was 59 ± 16 ml/min/kg over the i.v. dose range of 2-12 mg/kg. Volume of distribution at steady state (Vdss), mean resident time (MRT) and elimination half-life (T½) were dose dependent over this dose range with changes occuring between the 2 and the 4 mg/kg dose of COC. Vdss was 2.1 and 3.9 l/kg, MRT was 42 and 58 min, and elimination T½ was 34 and 50 min at the 2 and 4 mg/kg dose respectively. Cl and Vdss values following 2 to 20 mg/kg of COC s.c. were similar to those after i.v. administration while MRT was significantly greater as a result of delayed absorption. Absorption of COC s.c. was nearly complete (84%) and had a T½ of 51 min. Benzoylecgonine (BE) and benzoylnorecgonine (BN) were major and persistent metabolites of COC. Norcocaine (NOR) was present after COC doses of 4 mg/kg or higher but could only be detected during the first 2 hr.

Increased use of COC in recent years by pregnant women has crossed all socioeconomic barriers (Chasnoff *et al.*, 1990). Besides prenatal complications, this has resulted in offspring with congenital abnormalities, low birth weight, and respiratory abnormalities (Little *et al.*, 1989; Chasnoff *et al.*, 1989; Ward *et al.*, 1986). Human studies on the effect of COC use in pregnancy have relied on patient drug and prenatal histories for information. Consequently, many variables such as drug dose and purity, nutritional status and poly-drug abuse cannot be accurately ascertained. The development of pregnant and fetal animal models to conduct controlled studies on the effects of prenatal COC exposure is needed.

The guinea pig has been chosen by this laboratory as a model to study COC pharmacokinetics and pharmacodynamics in pregnancy for several reasons. The guinea pig is similar to humans in that it has a hemomonochorial placenta with similar permeability characteristics to the human placenta, unlike other laboratory animals such as mice, rats and rabbits (Faber and Thornburg, 1983). The neonatal guinea pig is neurologically mature at birth (Dawes, 1968). Finally the guinea pig has a gestation length (70 days) which is convenient for developmental studies.

The purpose of this study was to determine and compare the i.v. and s.c. pharmacokinetics of COC and the appearance and duration of COC metabolites in the late gestational guinea pig. A sensitive and specific high-performance liquid-chromatography (HPLC) assay was used to determine

plasma concentrations of COC, BE, BN and NOR (Sandberg and Olsen, 1990). Doses of COC were chosen to approximate plasma levels of human abusers (Barnett *et al.*, 1981) and to encompass doses to be used in guinea pigs for pharmacodynamic studies in this laboratory.

METHODS

Chemicals. COC•HCL, NOR, BE, and BN•HCL were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute on Drug Abuse (Bethesda, MD). COC•HCL and lidocaine (LID) were purchased from Sigma (St. Louis, MO). COC solution was prepared in normal saline just prior to use, however, frozen solutions (-15°C) of COC were stable with no decrease in COC or increase in BE for at least 4 months. For i.v. injections, the solution was sterilized by injection through a 22 μm Millex-GS filter (Millipore Corp., Bedford, MA). Aliquots of these solutions were analyzed by HPLC and all drug concentrations confirmed.

Animals. Twenty-five time-bred late gestational (mean 51 days, term 70 days) Dunkin-Hartley guinea pigs were studied (Charles River, Wilmington, MA; Simonsen Labs, Gilroy CA). Twenty animals were entered in the i.v. protocol, 4 in the s.c. and 2 in the blood/plasma study. One animal was used in both i.v. and s.c. protocols. Animals were housed indoors with controlled light cycles, continuous *ad libitum* food and water, and daily health checks. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health.

Drug injection and blood sampling. Blood samples from the pregnant dam were obtained by the method of Olsen *et al*. (1989) with the following changes: isoflurane replaced halothane as the anesthetic and an external jugular vein catheter was introduced as well as a carotid artery catheter. Dams were allowed to recover from surgery at least 24 hours before experiments were performed.

A blood sample was taken from the arterial catheter for a control before any experiment was begun. Animals were used only once with the exception of animal 8, who received a 4 mg/kg i.v. injection followed 2 days later by a 4 mg/kg s.c. injection. COC and metabolites could not be measured in a plasma blank taken before the second injection. COC was injected either as an i.v. dose of 2, 4, 6 or 12 mg/kg into the jugular vein over a period of 1 minute or a s.c. dose of 2, 4, 6 or 20 mg/kg injected into the shaved scruff of the neck. The i.v. dose was followed by a 1 ml saline flush or 3 times the catheter dead space. This was considered time 0. Blood samples were drawn from the arterial catheter before and at 1, 5, 15 and 30 min and 1, 1.5, 2, 3, 5 and 8 hr after injection for both the i.v. and s.c. protocols. Samples (1 ml) were drawn into heparinized disposable syringes and placed in polypropylene microfuge tubes containing 2.5 mg/ml sodium fluoride. After centrifugation at 5°C, the plasma was removed and stored at -15°C until assayed. Frozen plasma samples showed no degradation of COC or metabolites after 1 month of storage.

Blood/Plasma COC ratios. Blood was obtained from 2 late gestation guinea

pigs by cardiac puncture and used immediately. COC stock in 2 M NaH_2PO_4 buffer, pH 7.35 was added to blood resulting in a 1:10 dilution. Final COC concentrations were 200, 800 and 1500 ng/ml. These were chosen to approximate plasma concentrations seen in the pregnant guinea pig in the pharmacokinetic studies. Aliquots were incubated at guinea pig body temperature, 39°C (Hill, 1980). Preliminary incubations were done for 1, 5 and 20 min. Equilibrium was found to be complete by 5 min and results are expressed for 5 min incubations. Whole blood and plasma COC concentrations were analyzed by HPLC.

HPLC analysis of samples. Guinea pig plasma samples were analyzed for COC and metabolites BE, BN and NOR according to the method of Sandberg and Olsen (1990). Briefly, samples were prepared by a solid-phase extraction procedure with LID as the internal standard and immediately assayed by HPLC. This method allows for the simultaneous determination of COC, BE, BN and NOR. Since the publication of this method, it has been determined that benzoic acid (a breakdown product liberated in the formation of ecgonine methyl ester and ecgonine) can be detected under these conditions with a relative retention time to COC of 1.9 but does not extract in the solid phase extraction procedure. Recently, the Applied Biosystems Velosep ODS column (Santa Clara, CA) recommended for this method has had low and inconsistent silanol deactivation. Therefore the Rainin Microsorb C18 column (Woburn, MA), 100

mm x 4.6 mm I.D., 3 μm particle size, has also been used with the following changes to the assay conditions: the mobile phase consisted of 0.01 M phosphate buffer, pH = 2.0, 0.0002 M tetrabutylammonium hydroxide and 12% (v/v) acetonitrile. Flow rate was 1.75 ml/min. Samples were assayed in duplicate and values averaged.

Data analysis. The formulas used for the noncompartmental pharmacokinetic analysis of the COC single dose injections were as follows (Gibaldi and Perrier, 1982; Perrier and Mayersohn, 1982):

$$f = \frac{AUC_{s.c.}}{AUC_{i.v.}} \times \frac{dose_{i.v.}}{dose_{s.c.}} \quad (1)$$

$$Cl = \frac{f \times dose}{AUC} \quad (2)$$

$$Vdss = \frac{f \times dose}{AUC}\left[\frac{AUMC}{AUC} - MAT\right] \quad (3)$$

$$MAT = MRT_{s.c.} - MRT_{i.v.} \quad (4)$$

$$MRT = \frac{AUMC}{AUC} \quad (5)$$

$$\text{Elimination T1/2} = \frac{.693}{Ke} \quad (6)$$

$$\text{Absorption T1/2} = .693 \times \text{MAT} \quad (7)$$

The elimination rate constant (Ke) was determined by non-linear regression analysis of the terminal portion of the decay curve. The area under concentration-time curve (AUC), the first moment of that curve (AUMC), Cl, Vdss, MRT and the elimination T½ were determined using the HOTSHOT program (Assist/One, Grants Pass, OR). This program uses both the logarithmic and linear trapezoid rules to determine AUC and AUMC from 0 to ∞ as described in Olsen *et al.* (1988). Values reported used the logarithmic trapezoid method. Calculating AUC and AUMC with the linear trapezoid method changed areas by only 3% and 2%, respectively. When calculating Cl and Vdss after i.v. administration, the fraction of the dose absorbed (f) equals one and the MAT is zero.

Statistical analysis. One-way ANOVA was used to test for differences between doses and post hoc analysis was done by Least Significant Differences comparisons (NWA Statpak software, Portland, OR). Non-linear and linear regression was done with the statistical package for the Hewlett-Packard 41C calculator and volume 1 of the statistical library for the Hewlett-Packard 9815A programmable calculator (Hewlett-Packard, Palo Alto, CA). The unpaired t-test was also done using the Hewlett-Packard 9815A programmable calculator and

volume 1 of their statistical library. P values less than 0.05 were considered significant (Snedecor and Cochran, 1980).

RESULTS

Plasma concentrations (Cp) of COC and metabolites following a single i.v. bolus of 6 or 2 mg/kg COC are shown in Fig. 1A and 1B respectively. COC could not be measured in plasma after 3 hrs whereas BE was detectable from 1 min through 8 hr in all animals and at all doses. BN usually appeared later than BE and was first detectable from 1 to 90 min after injection and remained through the 8 hr sampling period. NOR was only seen in doses of 4 mg/kg or higher and could only be detected between 1 min and 2 hr. For all i.v. doses, peak Cp BE ranged from 91-294 ng/ml and occurred at 1-300 min; peak Cp BN ranged from 32-176 ng/ml at 2-8 hr; and peak Cp NOR ranged from 39-108 ng/ml at 1-30 min. The concentration-time profile of COC and metabolites after s.c. injection of 6 or 2 mg/kg in different animals was similar except for the longer duration of COC in plasma (Fig. 2A and 2B). For all s.c. doses, peak Cp BE ranged from 88-1008 ng/ml and occurred at 90-300 min; peak Cp BN ranged from 41-315 ng/ml at 5 hr; and peak Cp NOR ranged from 36-147 ng/ml at 15-90 min.

The pharmacokinetic parameters for the i.v. bolus injection of COC after doses of 2, 4, 6 and 12 mg/kg are shown in Table 1. Administration of the two highest doses of COC was discontinued after 3 of these 4 animals developed seizures within 1 min, lasting 5-10 min, and characterized by opisthotonos. Breathing continued throughout seizures in all these animals. $AUC_{i.v.}$ was proportional to dose by linear regression ($AUC_{i.v.}$=13722 Dose + 12342,

$F_{1,18}$=115.39, r^2=0.865, P < 0.005). Cp at 5 min ranged from 617-4780 ng/ml, and was also dose-dependent (Cp=399 dose + 15, $F_{1,17}$=140.14, r^2=0.892, P < 0.005). Cl was not dose dependent and the average ± S.D. was 59 ± 16 ml/min/kg. By one-way ANOVA there was a significant dose effect for Vdss ($F_{3,16}$=4.81, P < .05), elimination T½ ($F_{3,16}$=3.55, P < .05) and MRT ($F_{3,16}$=3.75, P < .05) with changes occurring between the 2 and 4 mg/kg of COC. Mean Vdss was 2.1 and 3.9 l/kg, T½ was 34 and 50 min and MRT was 42 and 58 min at the 2 and 4 mg/kg dose respectively. The elimination T½ for BE, estimated after COC could no longer be detected and after peak Cp BE was reached, was approximately 7 hr (harmonic mean n=10).

The pharmacokinetics of s.c. COC were similar to that after i.v. administration. Mean values ± S.D. after s.c. for all doses were: (n=4), Cl 53 ± 14 ml/min/kg; Vdss 2.9 ± 2.4 and MRT 125 ± 30 min. No seizures were observed in these animals. $AUC_{s.c.}$ was proportional to dose by linear regression ($AUC_{s.c.}$=22150 Dose - 25308, $F_{1,2}$=7,429.14, r^2=0.999, P < 0.005). Cp at 5 min ranged from 60-4262 ng/ml, and was dose dependent (Cp=236 dose - 413, $F_{1,2}$=146.23, r^2=0.987, P < 0.01). Although Cl and Vdss in the s.c. studies were similar to those after i.v. administration there was a significant difference between i.v. and s.c. mean values for MRT (t_{22} = 8.81, P < .001) with no overlap in the individual values. Animal 8 received both i.v. and s.c. 4 mg/kg injections, and the AUC for the two routes were nearly equal with a s.c. to i.v. AUC ratio of 0.95. This indicated complete absorption by the s.c. route in this

animal. All animals at the same dose had nearly complete absorption with an average f of 0.84. The average s.c. to i.v. AUMC ratio compared at the same dose was 1.9. As calculated from equation (8), the average COC absorption T½ for all doses was 51 min.

The average COC blood/plasma ratio for two late gestation pregnant guinea pigs was 1.11 at 200 ng/ml, .96 at 800 ng/ml and .98 at 1500 ng/ml.

DISCUSSION

Currently there are no detailed published COC pharmacokinetic data obtained during pregnancy in any species and little information exists for nonpregnant females. In humans, most pharmacokinetic data are from male volunteers (Barnett *et al.*, 1981; Wilkinson *et al.*, 1980, Jeffcoat *et al.*, 1989). The Wilkinson *et al.* (1980) study examined intranasal and oral COC kinetics while Barnett *et al.* (1981) examined i.v. kinetics. In the Barnett *et al.* study (1981), when 1-3 mg/kg COC was given i.v., 5 min Cp ranged from 682 - 3868 ng/ml, and elimination T½, which was dose dependent, ranged from 40 to 82 min. Similar 5 min Cp (617 - 4780 ng/ml) were seen in pregnant guinea pigs at i.v. doses of 2 - 12 mg/kg, and were observed to be a linear function of dose. The elimination T½ for the pregnant guinea pigs was also dose dependent in that there was a significant increase in T½ when 2 mg/kg was doubled and the T½ remained elevated at higher doses. The range in elimination T½ of 20 - 67 min was similar to that in human volunteers.

COC Cl in the pregnant guinea pig, unlike the elimination T½, was not dose dependent. In Barnett's study the average COC Cl was 20 ml/min/kg which was one-third of that calculated after i.v. administration in the pregnant guinea pig. The major contributors to the total body Cl of COC in the pregnant animal are most likely hepatic, placental and renal Cl; however, the extent of each contribution is not known. The estimated blood flow in pregnant guinea pigs of comparable gestation is 59 ml/min/kg for the liver (hepatic artery plus

portal venous flow), 14 ml/min/kg for the placenta, and 29 ml/min/kg for the kidney (Peeters *et al.*, 1980). These values represent the maximum possible Cl by each organ. Since the COC Cl determined in this study is almost identical to total hepatic blood flow and since both COC Cl and hepatic blood flow values are much larger than placental or renal blood flow, it is reasonable to assume that the majority of total COC Cl is accounted for by hepatic Cl. Furthermore, the large amounts of metabolites due to hydrolysis and N-demethylation which appear in plasma soon after injection of COC suggest a major portion of total COC Cl may be accounted for by hepatic Cl. Preliminary evidence suggest that the hydrolysis of COC in plasma is several times slower than in the whole animal which also suggests metabolism occurs predominantly in the liver (unpublished observations). Some of the Cl of COC must be accounted for by placental transfer because the drug is lipid soluble (Hansch *et al.*, 1987), and because it appears in the fetus after maternal injection (unpublished observations). In addition, a small amount of the drug could be metabolized by the placenta (Roe *et al.*, 1990). Renal metabolism or elimination could contribute to the Cl of COC. Cytochrome P450 oxidases are present in extrahepatic tissues including the kidney (Alvares and Pratt, 1990), but the capacity for metabolism is less than that in the liver. The free fraction of COC (42%, unpublished observations) would be available for glomerular filtration and some of the filtered COC would be reabsorbed in the renal tubule depending on urine pH. Extensive renal secretion of COC has not been reported, therefore

the total renal Cl of COC would probably be less than renal blood flow.

The blood to plasma ratio of cocaine was determined at three drug concentrations. Since the values were essentially unity, the ratio does not affect the discussion of organ blood flows and clearances. Blood to plasma ratios have recently been measured in human volunteers and the ratios were found to be similar to our results (Jeffcoat *et al.*, 1989).

Although the Cl of BE and BN were not determined, the sustained high levels of these metabolites suggest that their Cl is considerably slower than that for COC. Because of their polarity, it is likely that the metabolites are filtered by the glomerulus but not reabsorbed in the renal tubule, and that their Vdss is much less than that for COC. BE could also be cleared slowly by hepatic conversion to BN.

Vdss in this study was dose-dependent. There are several possible expanations for this trend. First, protein binding of COC might decrease with increasing dose because of saturation of binding sites. This would allow less drug to be retained in the vasculature system and result in a higher apparent Vdss. This is likely only at plasma concentrations greater than 1500 mg/ml (which were achieved after doses of 4 mg/kg higher) since binding data from our laboratory indicated a constant percent bound for the COC in the concentration range of 200 to 1500 ng/ml (unpublished observations). Second, hemodynamic changes could also be responsible for the dose dependence of Vdss. COC has been shown to cause dose dependent increases in

hypertension (Pitts *et al*, 1987; Woods and Plessinger, 1990). An increase in hydrostatic pressure could cause hemoconcentration, as suggested by fetal lamb studies with angiotensin I (Anderson and Binder, 1989), by pushing fluid and COC out of the vasculature and into the periphery before significant metabolism of COC could take place either in plasma or the liver. The result would be an increase in Vdss with increasing dose. Finally, a decrease in metabolism due to saturation of enzymes could result in an increase in Vdss. Since T½ and MRT would be increased, COC levels would be elevated for a longer period and distribution to tissues of high capacity but low blood flow could occur. This is possible because the rate of transfer of highly lipid soluble compounds across membranes is flow dependent. However, saturation of enzymes is not probable because the Cl of COC is not dose dependent.

In human male subjects, the Vdss of COC was always less than 2 liter/kg, whereas Vdss in the pregnant guinea pig ranged from 1.4 to 6.0 liter/kg. The larger Vdss in the pregnant guinea pig may be a species difference but is probably due in part to increased extracellular fluids, including amniotic fluid and fetal water, and the increased blood volume in pregnancy. In guinea pigs and humans there can be as much as a 50% increase in blood volume late in pregnancy (Hart *et al*., 1985; Pritchard, 1965).

The pharmacokinetics of COC after s.c. injections were also determined because this route is more convenient and less invasive than the i.v. injection. It is the route that will be used for chronic studies planned in this laboratory and

it has been used for pharmacodynamic studies in other laboratories (Spear *et al.*, 1989). The average s.c. to i.v. AUC ratio for all animals compared at the same dose was 0.84. This indicates that COC absorption was nearly complete at the s.c. injection site. In contrast to the AUC ratio, the s.c. to i.v. AUMC ratio for all animals calculated at the same dose was 1.9. The AUMC ratio suggests absorption is delayed, as would be expected after s.c. administration. One mechanism that might account for the delay is that after s.c. administration COC may inhibit its own absorption by vasoconstriction (Strauss and LaCandia, 1989).

A major metabolite in pregnant guinea pigs and humans (Ambre, 1989) is BE. The half-life of BE in pregnant guinea pigs, estimated to be about 7 hr, was longer than that for humans, 4.5 hr (Ambre and Connelly, 1990). The other major metabolite in guinea pigs was BN. It is of interest that BN in high concentrations has recently been found in the urine of pregnant human COC users (Chasnoff and Lewis, 1988). This metabolite has been detected but not quantitated in mice (Benuck *et al.*, 1987), and has not been previously reported in guinea pigs. The appearance of large amounts of BN in the pregnant guinea pig and human may be related to the altered physiological state of pregnancy. In humans, serum esterase activity is moderately decreased during pregnancy (Shnider, 1965) and it would be reasonable to expect that the metabolite profile would reflect this with an increase in liver derived metabolites such as NOR and BN. Although the pathway of BN formation has not been experimentally

described, BN could be formed by the de-carboxylation of NOR or the N-demethylation of BE. Low and transient levels of NOR were seen, but only at the higher doses in this study. Similarly, in human COC users NOR is present in the urine of pregnant subjects at concentrations a thousand-fold less than BE and BN.

Formation of these metabolites could have detrimental effects on both mother and fetus. BE and BN have been demonstrated to form complexes with Ca^{2+} (Misra and Mulé, 1975) and could potentially interfere with Ca^{2+} regulated events. Intracisternal injections of BE have been shown to produce seizures in mice (Erickson *et al.*, 1990) and rats (Misra *et al.*, 1975) and *in vitro* studies of BE indicate it causes vasoconstriction of cat cerebral arteries (Powers and Madden, 1990). BN might also cause cerebral artery vasoconstriction since structure-activity relationships indicated that the benzoylester at carbon 3 is the necessary group for this action (Powers and Madden, 1990). NOR is a more potent local anesthetic than COC. It can block Na^+ channels (Just and Hoyer, 1977) and is essentially as active as COC in inhibiting uptake of norepinephrine from rat synaptosomes (Hawks *et al.*, 1975). Metabolism of NOR has been implicated in liver toxicity (Kloss *et al.*, 1984).

Placental structure and permeability characteristics of the guinea pig, as well as the profile of COC metabolites make this species an appropriate model for evaluation of COC pharmacokinetics and pharmacodynamics in the maternal-fetal unit. The Vdss of COC, which affects T½ and MRT, was dose

dependent at the doses studied after i.v. administration, but Cl was not. Although COC absorption is delayed after s.c. administration, it is nearly complete and results in similar COC exposure. The major metabolites of COC are BE and BN and their long duration in plasma following a single dose of COC in the pregnant animal indicates that accumulation of metabolites is likely after chronic COC dosing. Since the metabolites have biological activity their accumulation may prove toxic to the maternal-fetal unit.

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FOOTNOTES

1. This work was supported in part by National Institute on Drug Abuse Grant DA04905 and an American Heart Association, Oregon Affiliate, Grant-in-Aid.

2. A preliminary report of some of these results was presented at the 74th annual meeting of the Federation of American Societies of Experimental Biology, April, 1990 (The FASEB Journal 4: A874, 1990).

Table 1: Intravenous cocaine pharmacokinetics in pregnant guinea pigs. (mean ± S.D.)

Dose (mg/kg)	n	Cl (ml/min/kg)	Vdss* (l/kg)	MRT* (min)	T½* (min)
2	10	53 ± 14	2.1 ± 0.5†	42 ± 10†	31 ± 9[a,†,‡]
4	6	67 ± 18	3.9 ± 1.1	57 ± 7.4	49 ± 9[a]
6	3	59 ± 13	3.4 ± 1.7	55 ± 14	48 ± 11[a]
12	1	70	3.4	49	44

[a]Mean and S.D. values for elimination T½ are the harmonic mean and pseudo-standard deviation (Lam *et al.*, J. Pharm. Sci. 74:229-231, 1985.)

*Significant dose effect by one-way ANOVA, $P < .05$; †Significant difference between 2 and 4 mg/kg as indicated by post-hoc analysis, $P < .05$; ‡Significant difference between 2 and 6 mg/kg as indicated by post-hoc analysis, $P < .05$.

Figure 1. Plasma concentrations of COC, BE, BN and NOR in pregnant guinea pigs after (A) 6 mg/kg, i.v. OR (B) 2 mg/kg, i.v. Arterial samples were taken at 1, 5, 15, 30, 60, 90, 120, 180, 300 and 480 min after the COC injection.

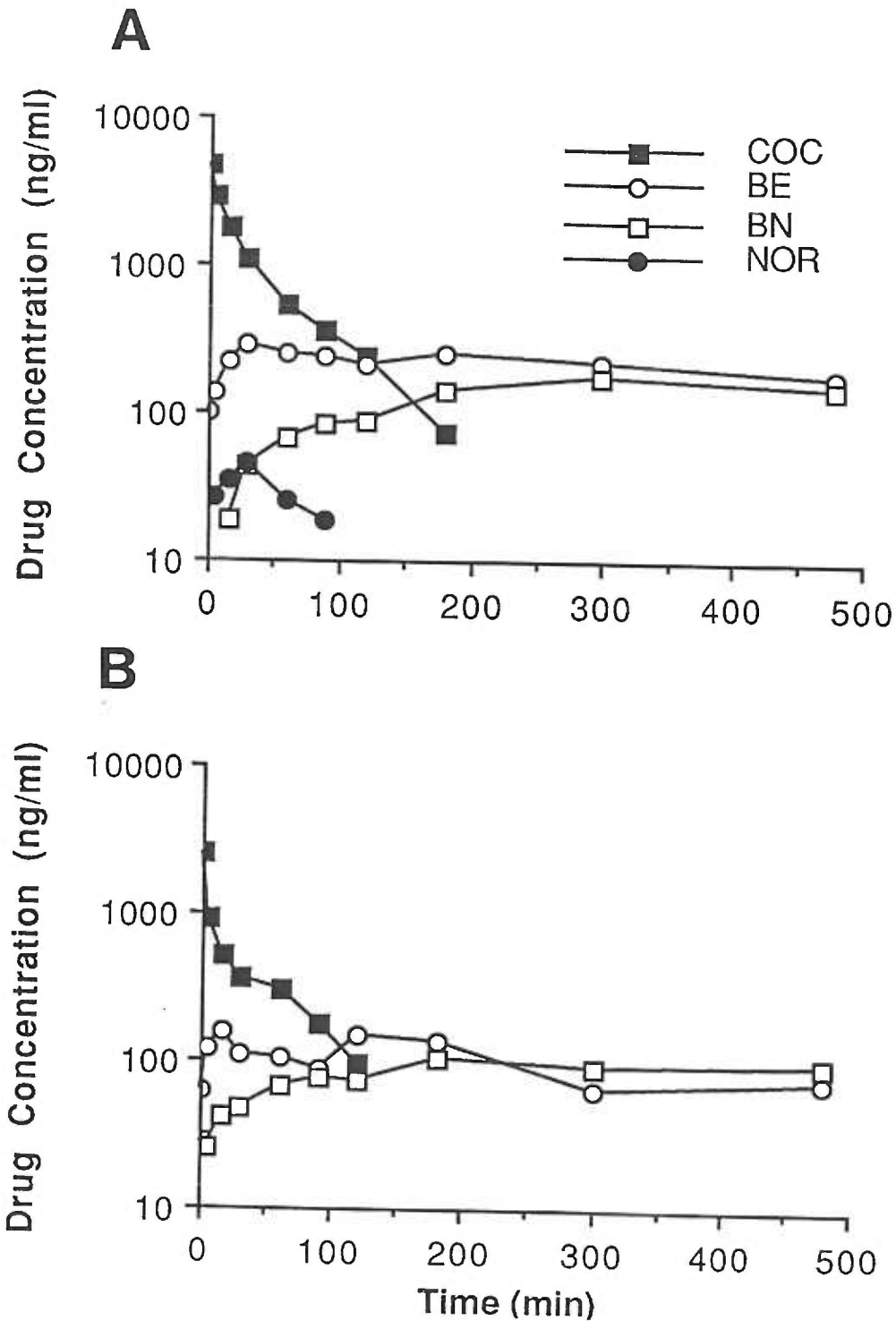
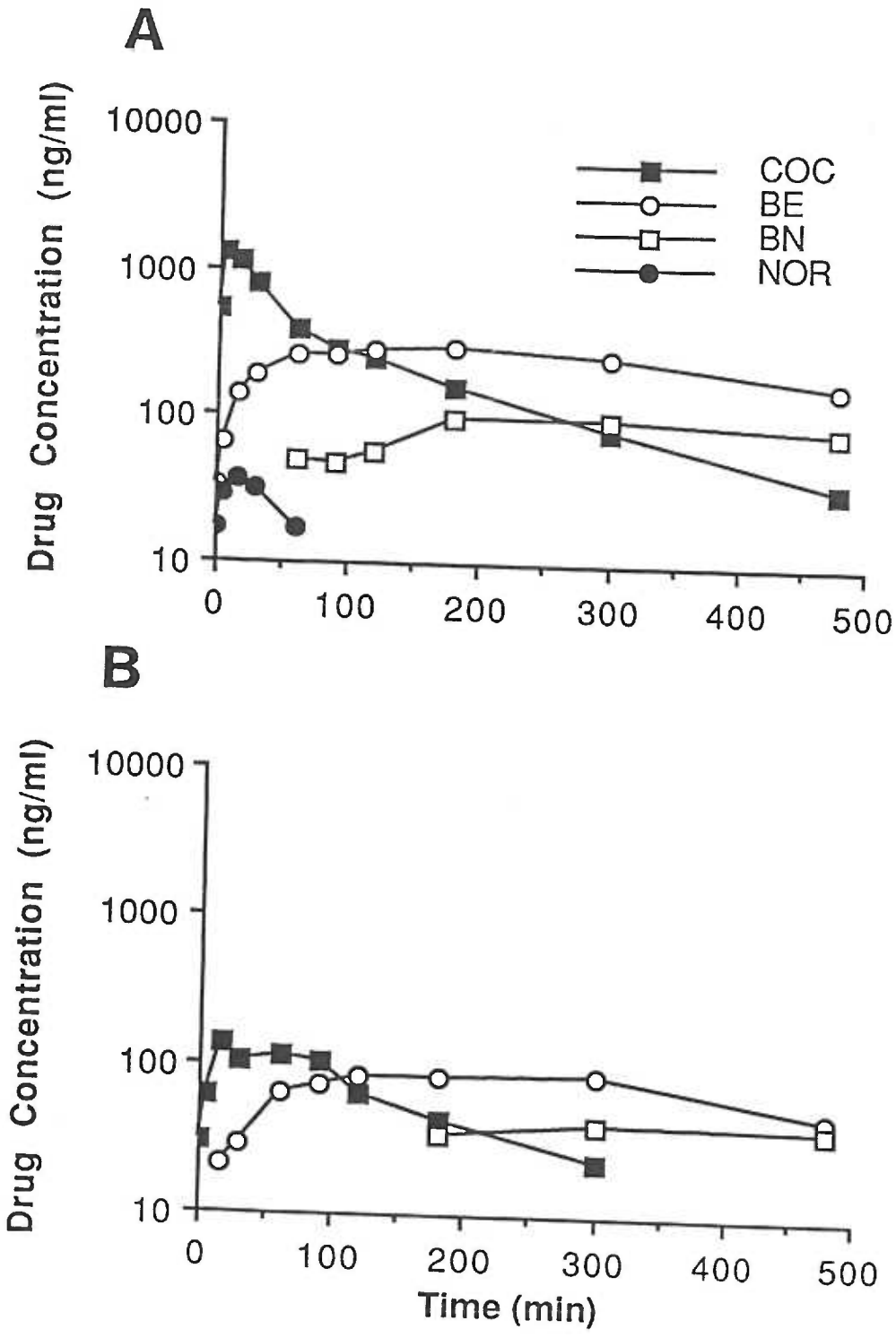

A
B
Drug Concentration (ng/ml)
10000
1000
100
10
0
100
200
300
400
500
COC
BE
BN
NOR
Time (min)

Figure 2. Plasma concentrations of COC, BE, BN and NOR in pregnant guinea pigs after (A) 6 mg/kg, s.c. OR (B) 2 mg/kg, s.c. Arterial samples were taken at 1, 5, 15, 30, 60, 90, 120, 180, 300 and 480 min after the COC injection.


A
Drug Concentration (ng/ml)
10000
1000
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0
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COC
BE
BN
NOR
B
Drug Concentration (ng/ml)
10000
1000
100
10
0
100
200
300
400
500
Time (min)

INDEX TERMS

Cocaine, benzoylecgonine, benzoylnorecgonine, norcocaine, pregnant, guinea pig, absorption, pharmacokinetics, subcutaneous, intravenous, metabolism

Manuscript III

(J. Pharmacol. Exp. Ther. 260:587-591, 1992)

Title: Cocaine and metabolite concentrations in the fetal guinea pig after chronic maternal cocaine administration.

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RUNNING TITLE PAGE

Running title: Chronic Fetal Cocaine Concentrations

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Abbreviations:

COC	cocaine
BE	benzoylecgonine
BN	benzoylnorecgonine
NOR	norcocaine
LID	lidocaine
HPLC	high-performance liquid-chromatography
ANOVA	analysis of variance
AAG	α_1-acid glycoprotein
Cp	plasma concentration

ABSTRACT

To determine the disposition of cocaine (COC) and metabolites after chronic COC exposure in the late-gestation guinea pig, 6 time-bred Dunkin-Hartley guinea pigs were given 10 daily 6 mg/kg COC s.c. injections from day 50 of gestation. Maternal blood and urine, fetal cord blood, brain and amniotic fluid were collected one hour after the last injection. There was no difference between maternal and fetal plasma COC concentrations. This may be due to the combined effect of lower protein binding and ion trapping of COC in the fetus. Benzoylecgonine (BE) was higher in maternal plasma but benzoylnorecgonine (BN) was higher in fetal plasma. COC brain to plasma ratios were similar in the dam and fetus. BE was the only metabolite that could be detected in the brain but levels were too low to quantitate. COC accumulated 3-4 times plasma concentrations in the amniotic fluid and was directly proportional to fetal plasma COC concentrations. BN in amniotic fluid accumulated to 2 times fetal plasma levels. The in vitro half-life of COC in amniotic fluid was 30 times longer than plasma elimination half-life in vivo. The high level and long duration of COC in amniotic fluid serve as a reservoir for prolonged fetal COC exposure.

The dramatic increase in COC use in recent years has led to many social problems including COC use among women of childbearing age. Women addicted to COC often continue their drug habit during pregnancy resulting in prenatal complications and offspring with congenital abnormalities, low birth weight and respiratory abnormalities (Little, et al., 1989; Chasnoff et al., 1989; Ward et al., 1986).

This laboratory has recently shown that the metabolite profile of COC in the guinea pig (Sandberg and Olsen, 1991) is similar to that reported in human pregnancy (Chasnoff and Lewis, 1988). The major metabolites in the pregnant guinea pig are BE and BN and their long duration in plasma following a single dose of COC indicates that accumulation of metabolites is likely after chronic dosing. Since the metabolites of COC have biological activity (Erickson, et al., 1990), their accumulation may prove toxic to the maternal-fetal unit.

The purpose of this study was to determine the disposition of COC and metabolites in the guinea pig fetus after chronic *in utero* exposure to COC. Since drug effects are often dependent on the amount of drug that is free or unbound in the serum, the binding of COC to serum proteins in maternal and fetal guinea pigs was compared. To estimate the contribution of some types of extrahepatic degradation to *in vivo* metabolism, *in vitro* studies were done in plasma and amniotic fluid. A sensitive and specific high-performance liquid-chromatography (HPLC) assay was used to determine amniotic fluid, brain and plasma COC, BE, BN and NOR (Sandberg and Olsen, 1990).

METHODS

Chemicals. COC•HCL, NOR , BE and BN•HCL were obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute on Drug Abuse (Bethesda, MD). COC•HCL and lidocaine (LID) were purchased from Sigma (St. Louis, MO). COC solution was prepared in normal saline at a concentration of 6 mg/ml and frozen at -15°C. Frozen solutions of COC were stable with no decrease in COC or increase in BE for at least 4 months although only solutions less than one month old were used in this study. Aliquots of these solutions were analyzed by HPLC and drug concentrations confirmed.

Animals. Six time-bred late-gestation (50 ± 2 days, mean ± S.D.) Dunkin-Hartley guinea pigs were used for the in vivo distribution study, 8 for the in vitro protein binding determinations and one for the in vitro degradation study (Simonsen Labs, Gilroy, CA). Animals were housed indoors with controlled light cycles, continuous *ad libitum* food and water, and daily health checks. The Animal Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health.

Accumulation study. From gestation days 50 to 59, the dam was injected s.c. once daily with 6 mg/kg COC into the shaved scruff of the neck. The dam was anesthetized 45 min after the last injection with isoflurane and nitrous oxide

balanced with oxygen. The one hour sampling time was selected for two reasons. First, that allowed ample time to reach equilibration in the maternal-fetal unit as shown by the theoretical calculations of Pratt, Fig. 3-32, (1990). Second, this allowed time for metabolite formation as suggested by single dose studies of COC in maternal guinea pigs (Sandberg and Olsen, 1991). Starting one hour after the injection, amniotic fluid was sampled by placing syringes between each fetus and amniotic membranes. Cord blood was drawn from the umbilical vein into heparinized syringes. Brain tissue was removed and placed in .01 M NaH_2PO_4, pH 2 (1.2 ml buffer/g) and 4% NaF (154 μl/g). The volumes of buffer and NaF were based on those used by Spear et al. (1989). All brain COC concentrations were calculated based on brain wet weight. Maternal blood was obtained by cardiac puncture and urine samples were taken if possible. All blood, amniotic fluid, and urine samples were placed in polypropylene microfuge tubes containing 2.5 mg/ml sodium fluoride. After centrifugation at 5°C, the plasma was removed and all samples stored at -15°C until assayed by HPLC. Frozen plasma samples showed no degradation of COC or metabolites after one month of storage.

In vitro serum protein binding. Blood samples were obtained, as described above from animals not treated with COC. Fetal blood of litter mates was pooled. The extent of serum protein binding was determined by an ultrafiltration method (Edwards and Bowles, 1988). Protein binding was not done on serum from the in vivo studies because of sample size limitations. COC in 5 ml 0.5 M NaH_2PO_4, pH

7.35 was added to serum resulting in 200, 800 or 1500 ng/ml COC and a 10% dilution of the serum. Prefiltrate samples were taken before centrifugation in ultrafiltration cones (Centrifree, Amicon Division, S.R. Grace & Co., Danvers, MA) at 935 g for 8 min. Ultrafiltrate and prefiltrate samples were stored in microfuge tubes with 2.5 mg/ml sodium fluoride and frozen at -15°C until assayed.

Serum protein analysis. Serum samples were assayed for total protein by the Biuret method (Kingsley, 1942), albumin by the bromcresol green method (Peters et al, 1982), and protein electrophoresis separations were performed with Beckman High Resolution agarose gel and stained with Paragon Blue (Beckman Instruments, Inc., Brea, CA) in the Clinical Chemistry section of the Clinical Pathology Department, OHSU. α_1-acid glycoprotein (AAG) analysis was performed by the Immunology-Serology Department of Associated Regional and University Pathologists, Inc. (Salt Lake City, UT) utilizing a N-glycoprotein kit with a Behring nephelometer (Behring Diagnostics, Inc., Somerville, NJ).

In vitro COC degradation. Fetal blood, amniotic fluid and maternal blood were collected from two pregnant guinea pigs. Fetal blood and amniotic fluid were pooled for each litter. COC in 0.5 M phosphate buffer (0.097 M KH_2PO_4 and 0.403 M Na_2HPO_4) was added to plasma, amniotic fluid and saline resulting in a 1:10 dilution. Final COC concentrations were 1500 ng/ml and maternal plasma pH with buffer was 7.36. Incubation mixtures were gassed with 5% CO_2 after each sample

and incubated at 39° C. Plasma samples were taken at 0, .5, 1, 2, 4, 6, 12, 18, 24, and 30 hr. Amniotic fluid samples were taken at 0, .5, 1, 2, 4, 6, 12, 30 and 48 hr. Saline samples were taken at 0, 1, 2, 4, 6, 24, 30, 48, 72 and 96 hr.

HPLC analysis of samples. Guinea pig plasma, brain and amniotic fluid samples were analyzed for COC and metabolites BE, BN and NOR according to the method of Sandberg and Olsen (1990). Briefly, samples were prepared by a solid-phase extraction procedure on a 50 mg C-18 Bond Elut column (Varian, Harbor City, CA) with LID as the internal standard and immediately assayed by HPLC. The ether wash was doubled to 500 μL for the brain samples. This method allows for the simultaneous determination of COC, BE, BN and NOR. The lower limit of quantitation in brain tissue was 150 ng/g for cocaine and metabolites. Samples were assayed in duplicate and values averaged.

Gas chromatography - mass spectrometry. Urine samples were analyzed by the Toxicology Department of the Oregon Health Sciences University by gas chromatography - mass spectrometry as described in Sandberg and Olsen (1990). This method can determine COC, BE and ecgonine methyl ester. Since this method does not measure BN, urine BN concentrations were measured by HPLC.

Data analysis. Fetal blood was pooled for the in vitro serum protein binding and degradation studies. For the accumulation study, fetal blood from individuals was

analyzed and the means reported are the means of all fetuses and not means of litters. Statistical analysis was done, however, on both the data from individuals as well as litters and when the results were significant, they were significant by either analysis.

Statistical analysis. One-way ANOVA was used to test for drug concentration differences between maternal and fetal tissues and when significant, post hoc analysis was done by Newman-Keuls' Multiple-Range Test (NWA Statpak software, Portland, OR). Two-way ANOVA with repeated measures was used to test for differences in protein binding. Factor A was defined as age with two levels (maternal or fetal) and factor B was defined as total COC concentration in serum with three levels (200, 800 or 1500 ng/ml). One-way ANOVA was used to test for differences in the serum protein concentrations between fetal and maternal samples. Linear regression to determine maternal and fetal drug concentration correlations was done with volume 1 of the statistical library for the Hewlett-Packard 9815A programmable calculator (Hewlett-Packard, Palo Alto, CA). Any p values less than .05 were considered significant (Snedecor and Cochran, 1980).

RESULTS

Accumulation study. Maternal and fetal COC and metabolite concentrations can be seen in Fig. 1 and Table 1. Urine drug concentrations for five dams (mean ± S.E., μg/ml) were: BE 3.01 ± .25, BN 2.8 ± 1.6, and ecgonine methyl ester 2.3 ± .72. COC was found in only two of the five urine samples with mean .91 μg/ml. In plasma, NOR was seen only sporadically and in low amounts. COC was significantly higher in amniotic fluid than maternal and fetal plasma; however, there was no significant difference between maternal and fetal plasma COC concentrations. BE was significantly lower in fetal plasma and amniotic fluid than maternal plasma, but was not different between fetal plasma and amniotic fluid. BN was significantly higher in fetal plasma than maternal plasma and higher in amniotic fluid than either fetal or maternal plasma. There was a linear correlation between maternal BN plasma concentration (Cp) and fetal BN Cp averaged for each litter (fetal Cp=1.38 maternal Cp + 41, $F_{1,4}$=8.67, r^2=.68, $p<.05$) and fetal COC Cp and amniotic fluid COC concentrations (amniotic fluid=2.74 fetal Cp + 183, $F_{1,4}$=15.62, r^2=.80, $p<.05$). There was no correlation between fetal BN Cp and amniotic fluid BN concentrations.

There was no significant difference between maternal and fetal brain COC concentrations (Table 1). The mean ± S.E. COC brain to plasma ratios were 1.99 ± .13 in the dam and 1.88 ± .09 in the fetus. There was no difference between maternal and fetal COC brain to plasma ratios. BE could only be detected in two maternal brain samples but concentrations were too low to quantitate.

Serum protein binding. The mean COC free fraction ± S.E. in fetal serum was .66 ± .05 and in maternal serum was .42 ± .04. There was a significant difference in COC binding between maternal and fetal serum as determined by two-way ANOVA ($F_{1,12}$=7.07, p<.02). Binding was not dose-dependent.

Total protein, albumin, α-1 fraction and AAG concentrations are in Table 2. The fetal albumin concentration was 30% higher than the maternal concentration by one-way ANOVA ($F_{1,9}$=7.36, p<.02). Fetal AAG concentration was always greater than maternal AAG. There was no correlation between total protein, albumin, α-1 fraction or AAG concentration and the free fraction of COC in either maternal or fetal serum samples.

In vitro COC degradation. COC disappearance half-life in maternal and fetal plasma was 8.7 and 8.3 hr, respectively. The disappearance half-life in amniotic fluid was 17 hr and in buffer 28 hr. 90% of the disappearance of COC in vitro in maternal plasma at 4 hr could be accounted for by the appearance of BE.

DISCUSSION

It was unexpected that COC concentrations in pregnant guinea pig and fetal plasma were the same in this study since previous work by Spear et al., (1989) in the rat and our protein binding experiments in the guinea pig suggested otherwise. After chronic s.c. COC administration in the pregnant rat, fetal COC plasma concentrations were 1/2 to 1/3 of those of the dams (Spear et al., 1989). These authors suggested that this could be due to COC-induced placental vasoconstriction and subsequent decrease in placental blood flow. However, the lower concentrations in the fetal rat could be explained by differences in protein binding. In the guinea pig, the free fraction of COC in the fetus was 50% higher than in the dam. It would be expected that once the free drug was in equilibrium across the placenta, total plasma concentrations would be lower in the fetus. If the protein binding of COC in the rat fetus is lower than maternal binding then the difference may explain the lower COC levels in the rat fetus. Since total concentrations were the same, and not lower, in the guinea pig fetus compared to the dam, another physical chemical interaction may be important.

It has been demonstrated that fetal blood pH is more acidic than maternal blood (Carter and Grønlund, 1982;Brace and Moore, 1991). Because COC is a weak base with a pk_a of 8.6, more of the drug would be ionized in fetal blood than maternal blood. Estimating fetal and maternal blood pHs to be 7.2 and 7.4, respectively, the Henderson-Hasselbalch equation can be used to determine ionized to unionized ratios of 25:1 for the fetus and 15.8:1 in the dam. If the

average unbound COC concentrations are calculated to be 215 ng/ml for the fetus and 129 ng/ml for the dam, then the unionized and unbound COC concentrations would be 8.5 ng/ml and 8.1 ng/ml for the fetus and dam, respectively. The combined effects of protein binding and ion trapping of COC in the fetus may thus explain the equal total COC concentrations in guinea pig dams and fetuses.

The maternal and fetal guinea pig total protein and albumin concentrations in this study (Table 1) were at the low end or below the range for adult guinea pigs (50-68 g/L total protein; 21-39 g/L albumin) (Hill, 1980). This is probably due to hemodilution since in guinea pigs and humans, there can be as much as a 50% increase in blood volume late in pregnancy. (Hart, et al., 1985; Pritchard, 1965). Our study did not attempt to determine the proteins to which COC binds to in the guinea pig. In nonpregnant humans, COC appears to bind to both albumin and alpha-1-acid glycoprotein (AAG) (Edwards and Bowles, 1988). AAG may be less important for COC binding in the maternal-fetal guinea pig unit since AAG could not be detected in the dam and only low levels were found in the fetus. To our knowledge, AAG concentrations have not been reported in the guinea pig for pregnant or nonpregnant states.

As in maternal and fetal rats (Spear et al., 1989), COC brain to plasma concentration ratios were >1 in both maternal and fetal guinea pigs. COC would be expected to pass easily through the blood brain barrier based on its lipid solubility (Hansch et al., 1987). Unlike Spear et al. (1989), significant levels of BE were not found in guinea pig brain tissue. This may be due to the fact that the

lower limit of quantitation in our assay of brain tissue was close to the concentrations seen in Spear's study.

COC accumulated to significant levels in amniotic fluid after chronic COC administration in the fetal guinea pig. This may be a result of ion trapping since amniotic fluid is generally more acidic than fetal blood (Seeds and Hellegers, 1968). The accumulation of COC may cause further exposure of the fetus to COC since the guinea pig fetus swallows amniotic fluid (Becker et al., 1940) and COC could be absorbed through the gut wall into fetal blood. It is interesting to note that ethanol, another lipid soluble drug, and its metabolites have also been found to accumulate in guinea pig amniotic fluid (Clarke et al., 1986).

As would be expected, maternal urine levels of BE were much higher than those in maternal plasma. COC was lower in urine and quite variable among the dams. The average urine COC to BE ratio, .3, was similar to those seen in humans (Ambre, 1985). BN was found in all five urine samples but was quite variable. This is not unexpected since plasma levels of BN do not peak for several hours after a single injection (Sandberg and Olsen, 1991).

It has been suggested that COC may be degraded nonenzymatically to BE (Stewart et al., 1979). To explore this idea and to determine the contribution of extra hepatic degradation to *in vivo* metabolism of COC, *in vitro* studies were done. While the *in vitro* COC disappearance half-life in maternal and fetal plasma was 8.5 hr, or 10 times longer than *in vivo* (Sandberg and Olsen, 1991), it was still not as long as that in buffer, 28 hr. The disappearance half-life of COC in amniotic

fluid, 17 hr, was intermediate between that in buffer and plasma. BE was the only metabolite detected *in vitro* and 90% of the disappearance of COC *in vitro* could be accounted for by the appearance of BE in maternal plasma at 4 hr. The difference between *in vivo* and *in vitro* data suggests BE is formed enzymatically in guinea pig plasma, but that hepatic esterases are more important for the degradation of COC. It has recently been shown that a partially purified NaF-inhibitable esterase in human liver catalyzes the hydrolysis of COC to BE (Dean et al., 1991). Because COC accumulated on the average up to 3 times plasma levels in amniotic fluid, the extremely long half-life in amniotic fluid may cause prolonged fetal drug exposure due to swallowing of the fluid.

As in the maternal guinea pig, BE and BN are predominant metabolites in the guinea pigs fetus. However, BN is the major metabolite in the fetus and BE is the major metabolite in the dam. BN was not reported in fetal rats (Spear et al., 1989) but has been detected in mice (Benuck et al., 1987). High concentrations of BN have recently been found in urine of pregnant human COC users (Chasnoff and Lewis, 1988). The three possible sources of origin of the metabolites found in the fetus are: **(1)** the metabolites are made in the dam and cross the placenta into the fetus; **(2)** the placenta forms some BE because COC has been shown to degrade in placental microsomes (Roe et al., 1990); and **(3)** the fetus forms the metabolites. Since BE and BN are more polar than COC and NOR, they would be expected to cross the placenta more slowly. In preliminary studies, we have found maternal plasma BE concentrations to be 3.5 to 4 times higher than in the fetus

30 min after a single i.v. dose of BE (unpublished observations). Since maternal BE levels after chronic COC exposure in this study are approximately 3 times fetal levels, the majority of BE in the fetus may be from maternal plasma. Unlike BE, the majority of BN may be formed in the guinea pig fetus since it appears to accumulate in fetal plasma and amniotic fluid. Like humans and unlike mice, rats and rabbits, the guinea pig has a hemomonochorial placenta with similar permeability characteristics to the human placenta (Faber and Thornburg, 1983). COC metabolites with low permeability in the guinea pig placenta would be expected to have similar permeability in the human placenta. To determine the extent to which placental and fetal COC metabolism contribute to fetal guinea pig plasma metabolite concentrations, *in vitro* studies need to be done.

It is not known at this time whether the adverse effects of COC in the fetus are due to COC itself or to a combination of COC and its metabolites. Intracisternal injections of BE have been shown to cause seizures in rats (Misra et al., 1975; Erickson et al., 1990) and recently in rat pups, at brain BE concentrations found at autopsies of cocaine abusers (Konkol et al., in press). In preliminary studies, BN was found to be a more potent convulsant than BE when injected intracisternally in rats (Konkol, R.J., personal communication). Both BE and BN form complexes with Ca^{2+} (Misra and Mulé, 1975) and could interfere with Ca^{2+} regulated events. In vitro studies of BE indicate it causes vasoconstriction of cat cerebral arteries (Madden and Powers, 1990) and structure-activity relationships indicate that BN might also cause vasoconstriction. NOR blocks Na^{+} channels (Just and Hoyer,

1977) and inhibits norepinephrine uptake (Hawks et al., 1975). The apparently short half-life of NOR in the guinea pig (Sandberg and Olsen, 1991) probably explains the lack of significant levels of NOR in the current study. NOR may be eliminated by de-esterification to BN.

The placental structure and metabolite profile of the guinea pig make this species an appropriate model for the evaluation of COC pharmacology in the maternal-fetal unit. The high concentration and stability of COC in amniotic fluid may provide a source of drug exposure to the fetus long after maternal plasma levels have declined. Unlike the dam, BN is the major metabolite in the fetal guinea pig and also accumulates in amniotic fluid. The appearance of large amounts of metabolites in the guinea pig fetus after chronic COC exposure may also contribute significantly to the overall effects of prenatal COC exposure.

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FOOTNOTES

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2. A preliminary report of some of these results was presented at the 75th annual meeting of the Federation of American Societies of Experimental Biology, April, 1991 (The FASEB Journal 5: A1237, 1991) and will be presented at the 1991 meeting for the American Society for Pharmacology and Experimental Therapeutics, August, 1991.

Table 1. Cocaine and metabolite concentrations in maternal and fetal guinea pig plasma, amniotic fluid (ng/ml) and brain tissue (ng/g) after 10 daily s.c. injections of 6 mg/kg cocaine in the dam. (mean ± SEM)

	Maternal Plasma	Fetal Plasma	Amniotic Fluid	Maternal Brain	Fetal Brain
n	6	25	25	6	25
COC	306 ± 16	326 ± 18	1062 ± 62**	731 ± 119	587 ± 23
BE	251 ± 18	91 ± 3*	72 ± 7+	-	-
BN	47 ± 10	106 ± 8*	248 ± 17**	-	-

*Significant difference between fetal and maternal plasma by post-hoc analysis, $p < .05$.

**Significant different between amniotic fluid and maternal plasma, and amniotic fluid and fetal plasma by post-hoc analysis, $p < .01$.

+Significant difference between amniotic fluid and maternal plasma by post-hoc analysis, $p < .01$.

Table 2. Total protein, albumin, α-1 fraction and AAG concentrations in maternal and pooled fetal serum.

	Maternal Samples				Fetal Samples			
	TP g/L	Albumin g/L	α-1 fraction g/L	AAG g/L	TP g/L	Albumin g/L	α-1 fraction g/L	AAG g/L
mean	33	17	2.5	--[*]	35	22[†]	1.6	0.11
S.D.	5	3	0.8	--	4	2	0.7	0.05
n	6	6	6	7	5	5	5	5

TP = Total proteins

[*]All maternal values were <0.05 g/L, the lower limit of detection.

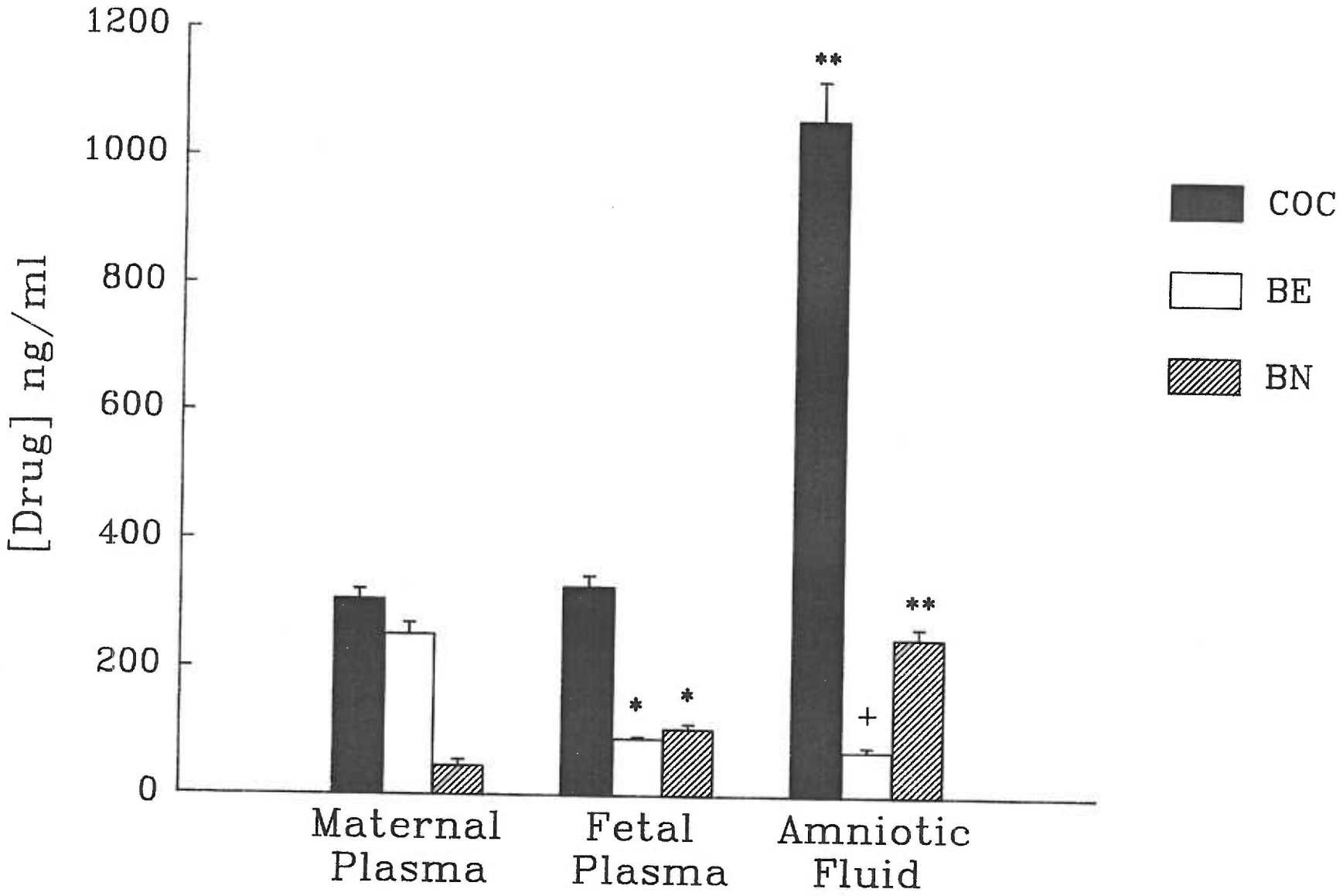
[†]Significant age effect (fetal versus maternal) by one-way ANOVA, $p<.02$, $F_{1,9}=7.36$.

Figure 1. Comparison of COC, BE and BN concentrations in maternal and fetal guinea pig plasma and amniotic fluid (mean ± S.E.).

*Significant difference between fetal and maternal plasma by post-hoc analysis, $p<.05$.

**Significant difference between amniotic fluid and maternal plasma, and amniotic fluid and fetal plasma by post-hoc analysis, $p<.01$.

+Significant difference between amniotic fluid and maternal plasma by post-hoc analysis, $p<.01$.


1200
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COC
BE
BN
Maternal Plasma
Fetal Plasma
Amniotic Fluid

INDEX TERMS

Cocaine, benzoylecgonine, benzoylnorecgonine, norcocaine, pregnancy, guinea pig, fetus, amniotic fluid, protein binding, distribution, brain

ADDITIONAL STUDIES

In vitro biotransformation of cocaine in hepatic microsomes from maternal and fetal guinea pigs:

Methods

Microsomal preparation. Dams were given 6 mg/kg cocaine (5 dams) or saline (5 dams) by daily subcutaneous injection on gestation days 50 through 59. Microsomes were made from the livers of the dams and fetuses by a modification of the methods of Williams, et al. (1984). Briefly, animals were sacrificed by CO_2 asphyxiation 24 hr after the last injection. Livers were quickly removed and placed in 4 volumes of ice cold homogenizing buffer [10 mM potassium phosphate, pH 7.5; 0.15 M KCL; 1 mM EDTA; 0.1 mM phenylmethylsufonyl fluoride (PMSF); and 0.02 mM butylated hydroxytoluene (BHT)] and homogenized. The homogenate was centrifuged at 10,000 g for 30 min at 4°C and the supernatant was centrifuged at 100,000 g for 60 min at 4°C. The resulting pellet was washed by resuspension in a wash buffer (0.1 M potassium pyrophosphate, pH 7.5; 1 mM EDTA; 0.1 mM PMSF; and 0.02 mM BHT) and ultracentrifugation was repeated as above. The final pellet was resuspended in a minimum volume of storage buffer (0.1 M potassium phosphate, pH 7.25; 30% glycerol; 1 mM EDTA; 1 mM dithiothreitol; and 0.02 mM BHT) and stored at -70°C until use. Protein was determined according to the method of Lowry et al. (1951). P450 concentrations were determined by the method of Omura and Sato (1964, 1967).

Reconstitution. Reactions were carried out in open containers with 1 ml volumes of 50 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 5 mM $MgCl_2$, 0.2 M isocitrate and 0.1 mg isocitrate dehydrogenase and 0.01 M NADP. Protein was added to the buffer and preincubated for 3 min. The reaction was started with the addition of cocaine. Samples from maternal hepatic microsomes were removed at 0, 4, 8, 12 and 20 min and immediately placed on ice. Samples were frozen at -20°C until analyzed for metabolites by HPLC (Sandberg and Olsen, 1990). Four control experiments were run simultaneously: **1)** microsomes heated at 90°C for 2 min; **2)** addition of 2.5 mg/ml sodium fluoride; **3)** addition of 1 mM SKF-525A; **4)** or omission of NADP.

Results and Discussion

In preliminary studies, the average hepatic cytochrome P450 enzyme concentration (nmol/mg protein ± S.D.) in 5 dams treated with saline and their fetuses at 60 days gestational age were 1.14 ± .12 and 0.17 ± 0.07, respectively, which agrees closely with previous work in the guinea pig (Card and Brien, 1989).

Figure 1 shows the appearance of cocaine metabolites, norcocaine, benzoylnorecgonine and benzoylecgonine after incubation of cocaine in maternal guinea pig hepatic microsomes. The apparent rate of formation for norcocaine was much faster than for benzoylnorecgonine or benzoylecgonine. The complete inhibition of norcocaine production by either heating the protein, omitting cytochrome P450 co-factor NADP or adding the cytochrome P450 inhibitor SKF-525A, all support that cocaine is N-demethylated by a cytochrome P450 in the

pregnant guinea pig. In this preliminary study, the specific activity (nmol/mg/min) could not be determined for norcocaine due to the fact that the maximum rate of formation had already been reached at the earliest quantifiable sample of 4 min. Future studies will attempt to determine this rate and show linearity for microsome protein concentration and time.

In an effort to determine if cocaine itself acts as a cytochrome P450 inducer, five dams were pretreated with cocaine before livers were harvested. Cytochrome P450 concentrations were no different from controls as determined by one-way ANOVA. Although a comparison of initial rates cannot be made in this preliminary study, the amount of norcocaine produced in microsomes from the cocaine pretreated animal was 65% higher at 20 min than the saline treated animal (Fig. 2). This suggests that although overall P450 levels are not increased, cocaine pretreatment may induce a particular isoenzyme(s).

Microsomes from non-treated guinea pig fetuses were capable of forming norcocaine, benzoylnorecgonine and benzoylecgonine (Fig. 3). Therefore, the metabolites seen in fetal tissues in Manuscript #3 could be fetally derived. The apparent formation rate of norcocaine is much lower than that observed in maternal microsomes.

Benzoylnorecgonine was seen only in incubations where norcocaine was present. To determine if benzoylnorecgonine was formed by N-demethylation of benzoylecgonine or hydrolysis of norcocaine, benzoylecgonine and norcocaine were incubated with microsomes in the absence of cocaine. No

benzoylnorecgonine was measured up to 120 min after incubation of benzoylecgonine. However, benzoylnorecgonine was produced by *in vitro* hydrolysis of norcocaine (Fig. 4) and only 20% could be inhibited at 90 min with the addition of 60 mM (2.5 mg/ml) sodium fluoride. This indicates that like the hydrolysis of cocaine to benzoylecgonine (Fig. 5) the hydrolysis of norcocaine to benzoylnorecgonine is also largely nonenzymatic.

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Figure 1 Appearance of norcocaine, benzoylnorecgonine and benzoylecgonine after 0.1 mM cocaine was incubated with 0.5 mg/ml maternal guinea pig #9164 (saline treated) hepatic microsomes for 20 min.

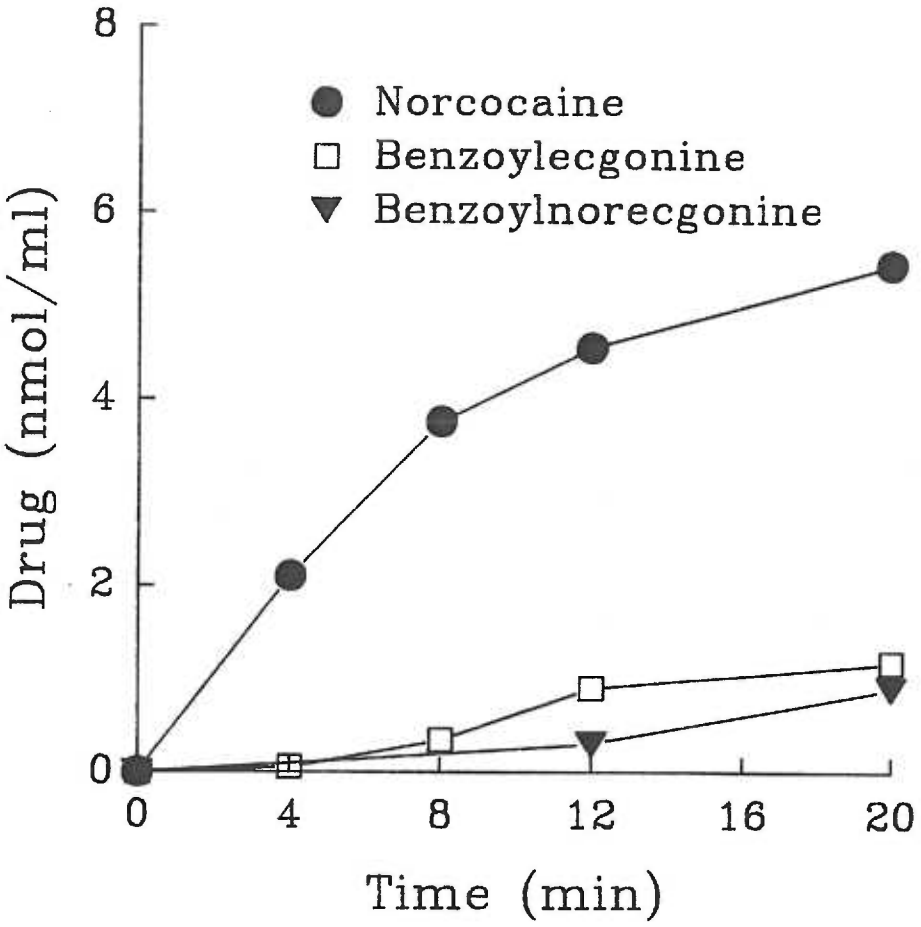

Norcocaine
Benzoylecgonine
Benzoylnorecgonine
Drug (nmol/ml)
0
2
4
6
8
0
4
8
12
16
20
Time (min)

Figure 2 Appearance of norcocaine after 0.1 mM cocaine was incubated with 0.5 mg/ml saline pre-treated (#9164) and cocaine pre-treated (#9147) maternal guinea pig hepatic microsomes for 20 min.

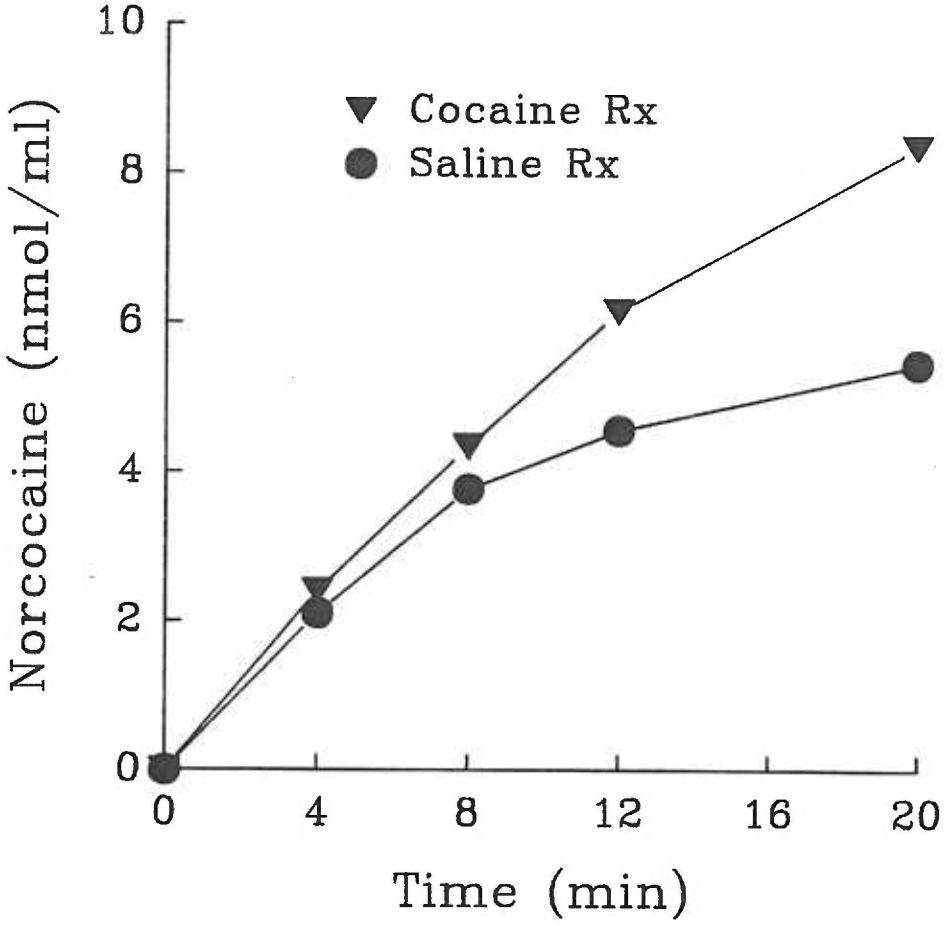

Norcocaine (nmol/ml)
10
8
6
4
2
0
Cocaine Rx
Saline Rx
0
4
8
12
16
20
Time (min)

Figure 3 Appearance of norcocaine after 0.1 mM cocaine was incubated with 4 mg/ml fetal guinea pig hepatic microsomes for 60 min, N=5.

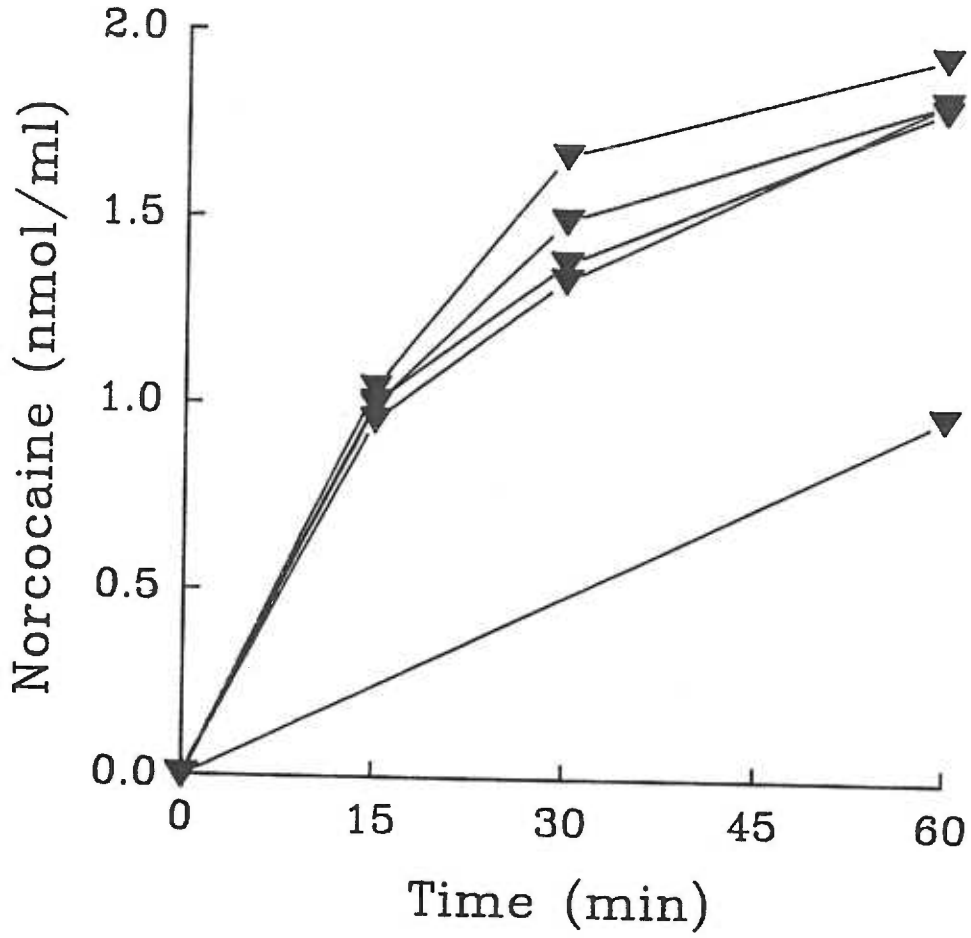

2.0
1.5
1.0
0.5
0.0
Norcocaine (nmol/ml)
0
15
30
45
60
Time (min)

Figure 4 Appearance of benzoylnorecgonine after incubation of 16 μM norcocaine with and without 2.5 mg/ml sodium fluoride in 0.5 mg/ml maternal guinea pig hepatic microsomes for 90 min.

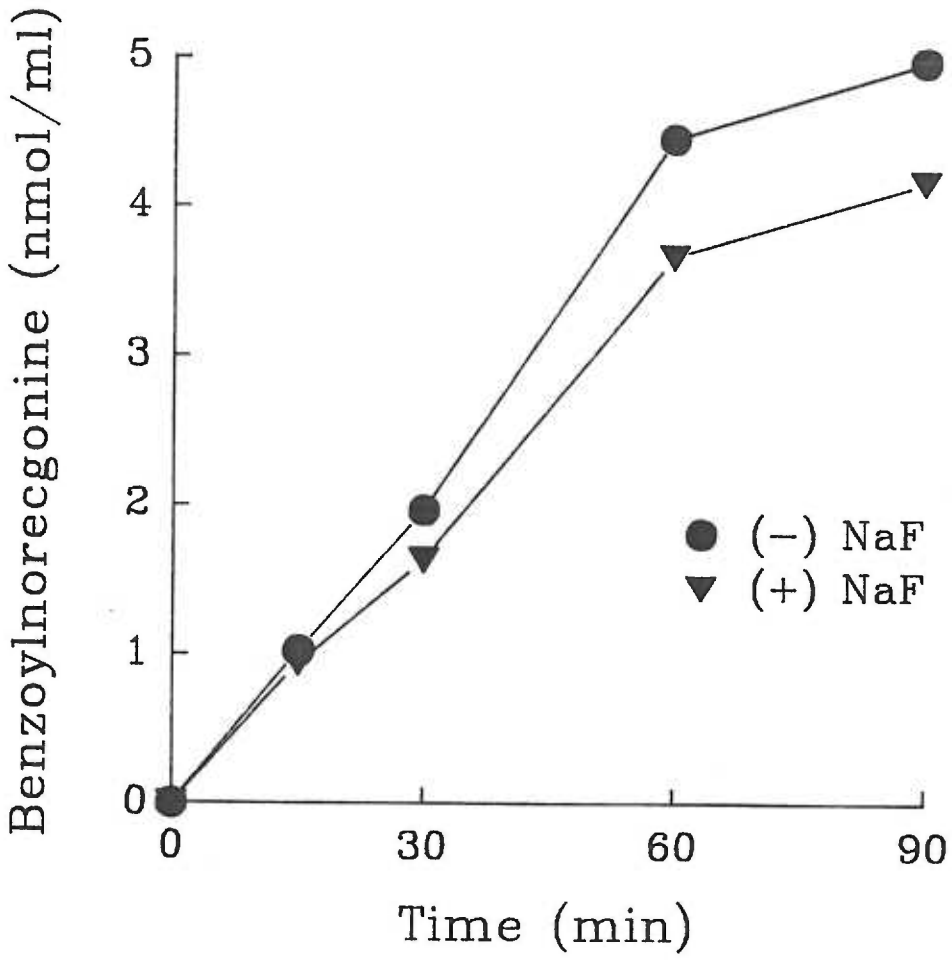
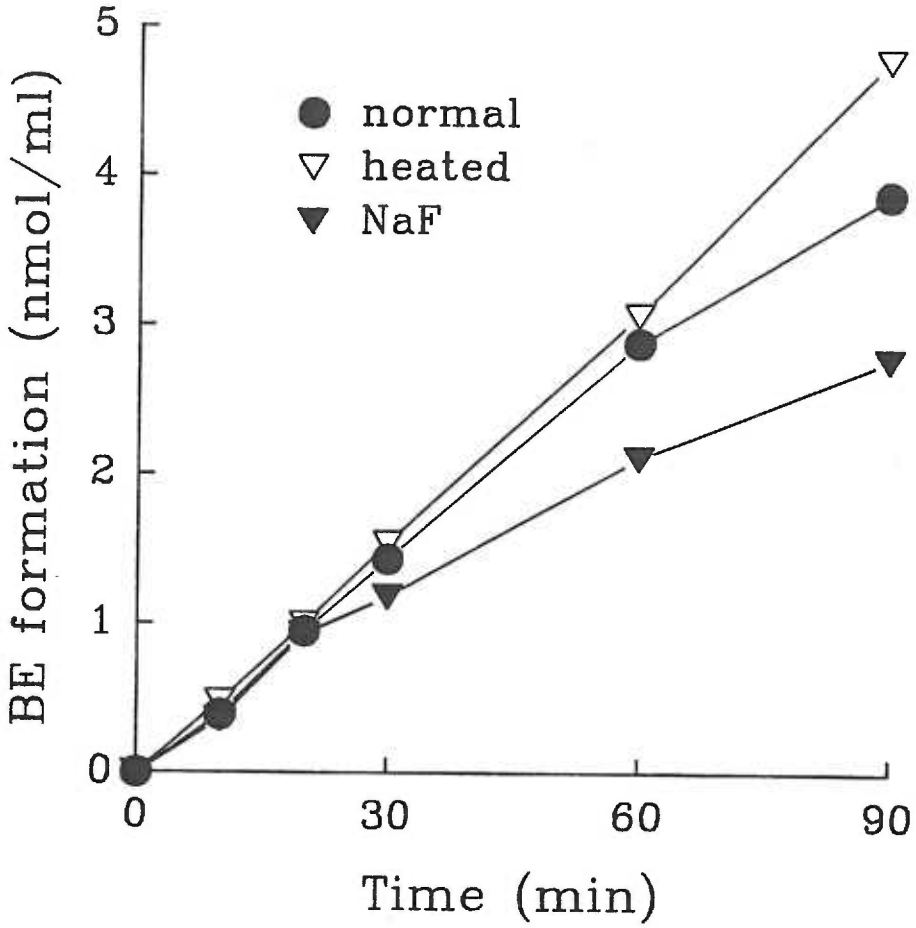

Benzoylnorecgonine (nmol/ml)
5
4
3
2
1
0
0
30
60
90
Time (min)
(−) NaF
(+) NaF

Figure 5 Formation of benzoylecgonine (BE) after incubation of 0.1 mM cocaine with and without 2.5 mg/ml sodium fluoride, or heated protein in 2 mg/ml maternal guinea pig hepatic microsomes for 90 min.


5
4
3
2
1
0
BE formation (nmol/ml)
normal
heated
NaF
0
30
60
90
Time (min)

DISCUSSION and CONCLUSIONS

This research began with the development of an assay to measure cocaine and its metabolites in biological fluids. Although other assays existed in the scientific literature at the time (Jatlow et al., 1978; Benuck et al., 1988; Khan, et al., 1987; Evans and Morarity, 1980), no single assay was adequate for the examination of small samples, sensitive enough for in-depth pharmacokinetic analysis and specific for measurement of the three active metabolites of cocaine: benzoylecgonine, benzoylnorecgonine and norcocaine. Therefore, to meet these needs, a high-performance liquid-chromatography microassay was developed as described in Manuscript #1. This specific, sensitive, and reproducible assay allows for the simultaneous determination of cocaine and its active metabolites from 80 μl samples with detection limits of 18 ng/ml for benzoylecgonine and benzoylnorecgonine and 35 ng/ml for cocaine and norcocaine. The solid phase extraction method improved recovery compared to a solvent extraction method. Recovery increased to 85-92% from 21-66% for all compounds. A later improvement was made, as noted in Manuscript #3, by switching from Chromprep PRP-1 80-μl cartridges (Hamilton, Reno, NV) to Bond Elut C-18 columns (Varian, Harbor City, CA) for the solid phase extraction. The Bond Elut columns improved sample clean-up, allowed for multi-sample preparation and were necessary for examination of brain tissue samples. This assay has been used for the determination of cocaine, benzoylecgonine, benzoylnorecgonine, and norcocaine in maternal and fetal guinea pig plasma, amniotic fluid, urine, brain tissue and

hepatic microsomes as described in Manuscripts #2 and #3 and ADDITIONAL STUDIES.

The pharmacokinetics of cocaine were ascertained in an animal model since there are no detailed pharmacokinetic data profiles described in pregnancy for any species. The guinea pig was chosen as a pregnant animal model of human pregnancy because its hemomonochorial placenta is structurally and functionally similar to humans (Faber and Thornburg, 1983) and the neonate is appropriate for cocaine pharmacodynamic studies (Olsen and Weil, 1992).

Cocaine doses of 2-12 mg/kg for intravenous injections and 2-20 mg/kg for subcutaneous administration were selected in order to give plasma levels that approximate those described in human studies (Barnett et al., 1981) without causing serious or overt toxicity in the guinea pig. These doses were significantly lower than those used in other rodent studies (Spear et al., 1989) but achieved desired plasma levels. The assumption was made that similar plasma levels in the guinea pig and human would cause similar pharmacological effects. Although this was not rigorously tested in this work, the observation of seizures in animals receiving the highest intravenous doses as well as the cardiovascular changes seen in one animal (Appendix E) suggest that this was an appropriate assumption and that a suitable dose range was used in the guinea pig.

Elimination half-life of cocaine in the pregnant guinea pig after intravenous administration (34-49 min) was very similar to values published for human volunteers (40-83 min). The elimination half-life was dose-dependent in the guinea

pig as has been reported in some human studies (Barnett et al., 1981; Jeffcoat et al., 1989). Since elimination is directly proportional to volume of distribution and inversely proportional to clearance, it might be expected that these parameters would also be similar in the guinea pig and human since the elimination half-lives were similar. However, both volume of distribution and clearance values were larger in the pregnant guinea pig studies than in the human studies (Barnett et al., 1981; Jeffcoat et al., 1989; Kogan et al., 1977). This might be explained by interspecies variation, however, some of the differences may also be attributed to the condition of pregnancy. The most appropriate comparison of course would be between these guinea pig data and data from pregnant humans, but cannot be done due to the lack of pharmacokinetic data in pregnant humans.

Volume of distribution of a drug is the theoretical volume of fluid in which a drug appears to be dissolved and can be affected by interactions such as plasma protein binding, drug ionization, blood flow and extracellular fluid volume (Benet et al., 1990). In humans as well as guinea pigs, there is as much as a 50% increase in blood volume late in gestation which would contribute significantly to an increased volume of distribution (Hart et al., 1985; Pritchard, 1965). Since Manuscript #3 as well as other investigators have shown that cocaine does cross the placenta of various species (Spear et al., 1989; Mittleman et al., 1989), the fetal compartment could provide another source of drug concentration away from maternal blood especially if there were ion trapping in the fetal compartment. The examination of cocaine plasma protein binding and ionization in the pregnant and

fetal guinea pig in Manuscript #3 supports this idea and provides a mechanism by which this increased volume of distribution might occur.

When considering the distribution of a drug in the fetal compartment, it is the unbound, unionized drug that is in equilibrium across the placenta and other membranes. Since the free fraction of cocaine in the guinea pig fetus is 50% higher than in the dam, it would be expected that fetal plasma levels would be lower than maternal levels. However, in Manuscript #3, plasma levels taken one hour after the last cocaine dose were equal in the fetus and dam indicating that protein binding may not be the only factor influencing fetal cocaine distribution. Bingol et al. (1987) suggested that theoretically, cocaine concentrations would be higher in the fetus due to lower fetal blood pH. However, they provided no data to support this hypothesis and concluded that cocaine is lower in fetal tissue due to vasoconstriction.

Cocaine is a weak base with a pK_a of 8.6 and ionization increases as pH values go below 8.6. Based on published values for maternal and fetal guinea pig plasma pH's in late pregnancy during anesthesia, the unionized to ionized ratio of cocaine in fetal and maternal plasma were calculated using the Henderson-Hasslebalch equation (Manuscript 3). Although the cocaine levels were not at steady-state, the use of this equation does give an approximation of drug ionization in the animals. Using the protein binding data and the ionization calculations, it was shown that the unbound, unionized cocaine concentrations were equal in maternal and fetal guinea pig plasma one hour after a maternal

cocaine injection. This demonstrated that the combined influence of cocaine plasma protein binding and ionization accounted for the distribution of cocaine in maternal and fetal plasma as shown in Manuscript #3.

Seeds and Hellegers (1968) demonstrated that human amniotic fluid pH decreases from 7.23 at 10-23 weeks gestation to 7.17 at 25-31 weeks of gestation. Preliminary studies in this laboratory indicate that guinea pig amniotic fluid was approximately 0.6 pH units lower than fetal blood and that fetal blood was 0.2 units lower than maternal blood. Based on ionization, cocaine would be expected to be found in higher quantities in the more acidic amniotic fluid than fetal plasma. This was demonstrated in Manuscript #3, where cocaine was found to accumulate in amniotic fluid 3 to 4 times fetal plasma concentrations after chronic cocaine administration in the late gestation guinea pig. Roberts et al. (1989) suggested the distribution of basic drugs in the maternal and fetal unit is due to a combination of ionization as well as other factors such as tissue binding. The interaction of protein binding and degree of ionization in the guinea pig fetus and amniotic fluid therefore provides a plausible explanation for the significant distribution of cocaine into the fetal compartment.

Total body clearance of cocaine in the pregnant guinea pig was 2 to 6 times the values reported in human studies (Barnett et al., 1981; Jeffcoat et al., 1989; Kogan et al., 1977). Cocaine clearance in the pregnant guinea pig was probably a combination of placental, renal and hepatic elimination. The maximum possible clearance of cocaine by the placenta would be up to 14 ml/min/kg since that is the

maximum placental blood flow in mid to late gestation guinea pigs (Peeters et al., 1980). The maximum possible contribution of maternal renal clearance of cocaine would be equal to the renal blood flow of 29 ml/min/kg in the late gestation guinea pig (Peeters et al., 1980). However, in reality, renal clearance of cocaine is probably much less than total renal blood flow due to plasma protein binding and renal tubular reabsorption. In a study by Kogan et al. (1977), human renal clearance accounted for 2.4% or less of total cocaine clearance (calculations of clearance based on data presented in this paper may be found in Appendix D). In the pregnant guinea pig, only the free fraction of cocaine (42%) would be available for glomerular filtration and the majority of the filtered cocaine would be reabsorbed due to the normal alkaline urine, pH 9, found in guinea pigs (Ganaway and Allen, 1971). In Manuscript #3, cocaine could only be found in urine from two of five dams and the concentration was at least a thousand fold less than the concentrations of cocaine metabolites. Therefore, the combination of placental and renal clearance can account for only a modest portion of total cocaine clearance in the pregnant guinea pig.

The other major route of maternal clearance would be through hepatic metabolism. Blood flow to the liver in the late gestation guinea pig is approximately 59 ml/min/kg, which is no different from the nonpregnant animal (Peeters et al., 1980). The average cocaine clearance measured in the pregnant guinea pig, 59 ml/min/kg, was identical to the blood flow to the liver and both were larger than total placental and renal blood flow combined. Due to the large amounts of

metabolites found in both plasma and urine, the modest clearance by the placenta and kidneys, and the minimal biotransformation of cocaine seen in plasma *in vitro*, the liver has to be the major contributor to total body clearance of cocaine in the pregnant guinea pig.

In addition to the pharmacokinetics of cocaine, another goal of this work was to describe the time course of cocaine metabolites found in pregnant guinea pig plasma. Low concentrations of norcocaine were only seen within the first hour after single doses of 4 mg/kg of cocaine or higher. Norcocaine has been detected in pregnant and fetal rat tissue after a single cocaine dose of 30 mg/kg late in gestation (DeVane et al., 1989). Levels of norcocaine were always 20% or less than cocaine concentrations in this rat study and no other metabolites were specifically quantitated.

Both benzoylecgonine and benzoylnorecgonine were seen at all cocaine doses as shown in Manuscript #2 and were major and persisting metabolites. Benzoylecgonine has been described as a major metabolite in humans (Hamilton et al., 1977). Benzoylecgonine has been shown to have an elimination half-life of 4.5 hr in humans (Ambre and Connelly, 1990) which is almost 5 times that of cocaine elimination. The elimination half-life of benzoylecgonine after a single intravenous dose of cocaine in the pregnant guinea pig was approximately 7 hr which was also considerably longer than that for cocaine. However, with the exception of an abstract by Chasnoff and Lewis (1988), benzoylnorecgonine has not been reported in humans. It is interesting to note that this is an abstract

describing metabolites from the urine of cocaine using pregnant women and benzoylecgonine and benzoylnorecgonine were found at approximately micromolar concentrations and the norcocaine concentration was a thousand-fold less. Unfortunately, information such as cocaine dose or when the urine sample was taken in relation to the dose was unknown. Benzoylnorecgonine has been detected in mice after intraperitoneal cocaine administration, although amounts were not quantified (Benuck et al., 1987).

The metabolite profile in the pregnant guinea pig after chronic cocaine dosing correlated well with the single dose studies. Benzoylecgonine and benzoylnorecgonine concentrations were similar to those seen in the single dose subcutaneous 6 mg/kg injection study. Norcocaine was not detected in samples from the chronic study, but this also agrees with the single dose study since samples in the chronic study were taken an hour after injection at a time in which norcocaine could not be detected in all single dose studies.

The metabolite profile in the fetus was unexpected. Benzoylnorecgonine was significantly higher in the fetus than in the dam. This suggests either ion trapping or fetal production of this metabolite. Chasnoff (1988) has suggested that norcocaine is a major metabolite in the fetus and may last up to four days in the neonate after last exposure, but has not published any data to support this. An obvious question raised by the metabolite profile seen in the fetus after chronic maternal cocaine dosing, is where are the metabolites being formed?

One possibility is that they are all maternally derived and cross the placenta

into the fetus. This is plausible for benzoylecgonine for several reasons. First, benzoylecgonine levels are 2-3 times greater in the dam and may enter the fetal compartment in a concentration driven manner. Also, in two preliminary studies, when benzoylecgonine was injected into the pregnant guinea pig, fetal levels were 25% of maternal levels 30 min after the injection (Appendix A). Therefore benzoylecgonine does cross the guinea pig placenta and could account for the appearance of this metabolite in fetal plasma. Since benzoylnorecgonine is similar in structure and molecular weight, it should be able to cross the placenta. However, since benzoylnorecgonine levels are twice as high in the fetus as compared to the dam, this metabolite may be the product of fetal cocaine biotransformation via norcocaine hydrolysis.

Another explanation for the appearance of metabolites in the fetus is that the late gestation guinea pig fetus is capable of forming the metabolites. To examine the hypothesis that the fetus can metabolize cocaine, as well as to examine maternal hepatic biotransformation of cocaine, *in vitro* hepatic metabolism studies with maternal and fetal tissue as described in the section on ADDITIONAL STUDIES were undertaken. Only preliminary data are available at this time, but they demonstrate that both maternal and fetal guinea pig hepatic microsomes are capable of cytochrome P450-mediated cocaine N-demethylation. Metabolism was demonstrated in microsomes from cocaine and saline treated dams and fetuses. Apparent specific activity in all fetal microsomes was much lower than in the dam, as indicated by the increased protein concentrations and lengthened incubation

time needed to detect norcocaine. It is interesting to note in the *in vitro* metabolite studies, the initial rate of norcocaine production in hepatic microsomes from saline and cocaine treated dams appear similar. The rate could not be quantified in the preliminary study since at the earliest time samples that could be analyzed by HPLC (4 min), the rate had already reached its maximum. However, the extent of norcocaine production seems to be 65% greater after 20 min in the cocaine treated animals. The percentage of cocaine degraded at any one time point is also greater in the cocaine treated animals and correlates with the difference in norcocaine productions.

The *in vitro* production of norcocaine correlates well with that seen *in vivo* in the pregnant guinea pig. After a single intravenous or subcutaneous injection, norcocaine appeared in the first sample at 1 min post injection, and could be detected only transiently compared with other metabolites. Both the *in vivo* and *in vitro* data suggest that the maximum rate of metabolism occurs very early after cocaine reaches the cytochrome P450 enzymes.

Benzoylnorecgonine seen *in vivo* is formed by the hydrolysis of norcocaine. This is supported by our *in vitro* work as well as others (Nayak et al., 1976). The majority of this hydrolysis is nonenzymatic as demonstrated by only a 20% decrease in formation at 90 min when norcocaine was incubated in microsomes in the presence of the esterase inhibitor sodium fluoride. It was suggested in Manuscript #2 that benzoylecgonine could theoretically be N-demethylated to produce benzoylnorecgonine. However, no benzoylnorecgonine was produced

when benzoylecgonine was incubated in pregnant guinea pig microsomes and Nayak et al. (1976) also were unable to show conversion of benzoylecgonine to benzoylnorecgonine in rat microsomes. Therefore, the appearance of benzoylnorecgonine in maternal and fetal guinea pig plasma and amniotic fluid is an indication of the prior production of norcocaine, even when norcocaine itself cannot be detected.

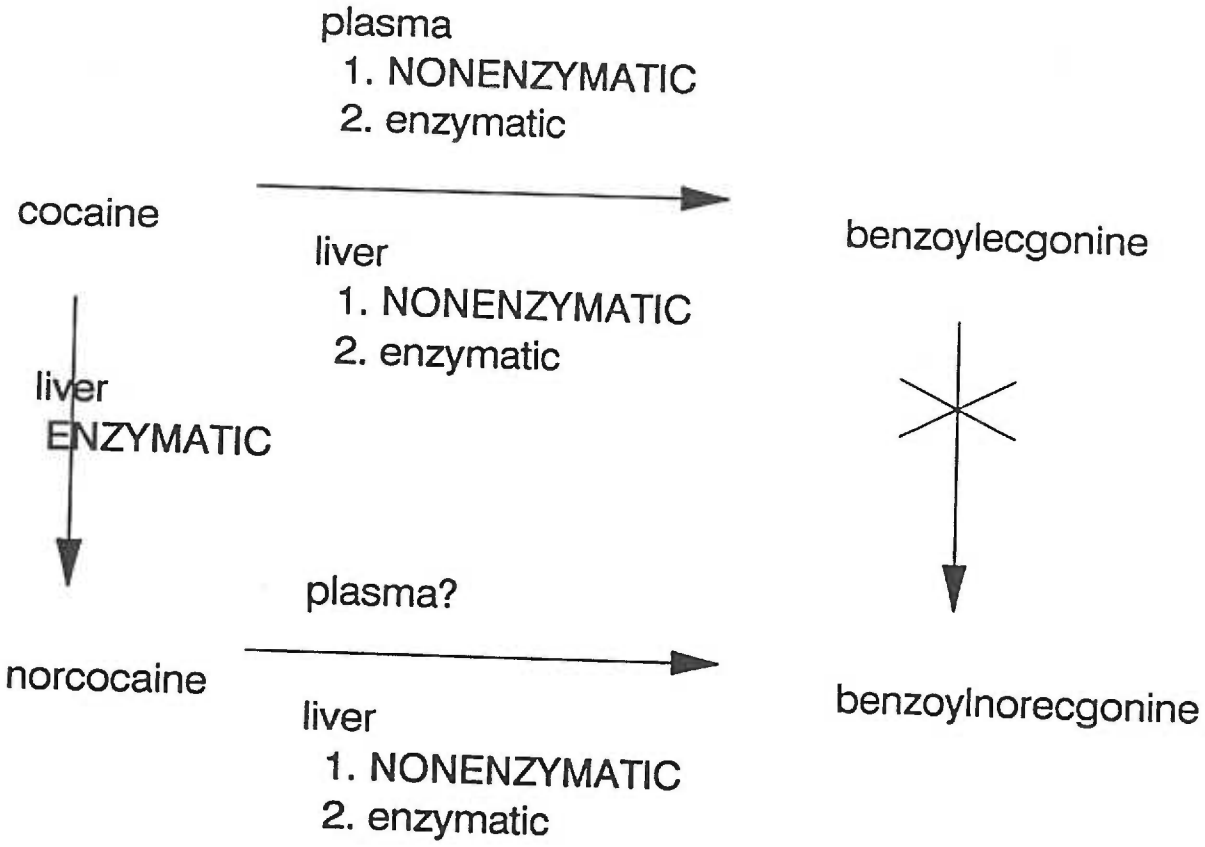
An initial attempt was made using several *in vitro* studies to determine the relative contributions of enzymatic versus nonenzymatic formation of benzoylecgonine *in vivo*. In one *in vitro* plasma incubation, the appearance of benzoylecgonine from 0 to 4 hours was nearly equal in buffer and maternal guinea pig plasma at physiological pH and temperature (Appendix C). After 4 hours, appearance of benzoylecgonine was 12-29% greater in maternal plasma than buffer. This indicates that benzoylecgonine can be formed enzymatically to a limited extent in maternal guinea pig plasma. A similar lack of significant enzymatic conversion of cocaine to benzoylecgonine was also demonstrated when cocaine was incubated with pregnant guinea pig hepatic microsomes.The similar rates of benzoylecgonine appearance in normal and heat treated guinea pig hepatic microsomes also suggests that the majority of benzoylecgonine is produced nonenzymatically in the pregnant guinea pig liver. However, because of (1) the minimal contribution of enzymatic hydrolysis to benzoylecgonine formation in the first four hours in plasma when 99% of cocaine is cleared *in vivo* and of (2) the lack of significant enzymatic formation of benzoylecgonine in microsomes, the

majority of benzoylecgonine seen *in vivo* is probably due to nonenzymatic hydrolysis. Both the *in vitro* plasma and liver microsome studies were preliminary and should be repeated with appropriate controls such as heated protein and the addition of enzyme inhibitors.

As described by the preceding paragraphs, the biotransformation of cocaine is complex. Figure 1 shows the possible routes as determined by this work and emphasizes where enzymatic and nonenzymatic processes occur. The formation of benzoylecgonine and benzoylnorecgonine from cocaine and norcocaine, respectively, occur mostly through nonenzymatic hydrolysis. Interestingly, both metabolites are formed by the hydrolysis of the same alkyl ester bond. Formation of norcocaine and benzoylnorecgonine from cocaine and benzoylecgonine, respectively, would also be the result of N-demethylation of the same nitrogen. However, only norcocaine is formed by N-demethylation and benzoylnorecgonine is formed by hydrolysis of norcocaine. This suggests that benzoylecgonine cannot reach the membrane-bound P450 enzyme due to increased polarity as compared to cocaine and/or that benzoylecgonine does not fit the active site of the P450 enzyme for subsequent N-demethylation.

Regardless of the site of formation, the appearance of cocaine metabolites in fetal plasma and amniotic fluid is not just of academic interest. As was discussed thoroughly in the introduction as well as in Manuscripts #2 and #3, the metabolites norcocaine, benzoylecgonine and benzoylnorecgonine possess biological activity. The capability to convert cocaine into these compounds may not

Fig. 1 Routes of cocaine biotransformation in the pregnant guinea pig. The major type of reaction (enzymatic vs. nonenzymatic) is in capital letters.



"protect" the fetus from deleterious effects such as suggested by Roe et al., (1990). The seizures produced by intracerebroventricular administration of benzoylecgonine (Konkol et al., 1992) and benzoylnorecgonine (Konkol, personal communication) in neonatal rats demonstrate that these metabolites of cocaine could be responsible for some of the stimulatory behaviors seen in the cocaine exposed human neonate or fetus such as seizures (Chasnoff et al., 1989). Six out of 75 prenatally cocaine exposed infants in a study by Chasnoff et al. (1989) developed seizures after birth. Although these infants tested positive for cocaine or metabolites at birth, the seizures did not always occur when cocaine was present. Unfortunately, age at time of seizure was not indicated. In addition to cocaine or withdrawal of cocaine, the presence of metabolites with long elimination half-lives and biological activity could cause stimulation of neonatal pulmonary ventilation as suggested by Olsen and Weil (1992).

In summary, this thesis has shown the following: **1)** cocaine and metabolites benzoylecgonine, benzoylnorecgonine and norcocaine can be measured in maternal and fetal guinea pigs with a high-performance liquid-chromatography microassay; **2)** elimination half-life and plasma concentrations of cocaine after the intravenous and subcutaneous doses administered are similar to that described in human studies, however, clearance and volume of distribution were greater in the pregnant guinea pig; **3)** the metabolite profile in the pregnant guinea pig is similar to the one preliminary report of metabolites in urine from pregnant human users, and in that regard is a good model for cocaine abuse during human

pregnancy; **4)** cocaine and benzoylecgonine cross the guinea pig placenta after maternal exposure; **5)** cocaine plasma concentrations in the fetus are related to protein binding as well as maternal drug concentration and degree of ionization; **6)** benzoylnorecgonine accumulates in fetal plasma, and both cocaine and benzoylnorecgonine accumulate in amniotic fluid after chronic maternal cocaine administration, and therefore amniotic fluid provides a reservoir of these compounds; **7)** the appearance of benzoylnorecgonine is an indication of production of norcocaine and both maternal and fetal guinea pig livers are capable of forming these metabolites; **8)** plasma hydrolysis of cocaine to benzoylecgonine is largely nonenzymatic, and plays no significant role in the overall degradation of cocaine.

Future work in the area of cocaine in developmental pharmacology should focus on both *in vitro* and *in vivo* studies. Obviously the *in vitro* data shown in ADDITIONAL STUDIES were preliminary and this laboratory continues the biochemical examination of the rate and extent of norcocaine, benzoylnorecgonine and benzoylecgonine production in late gestation guinea pigs. A control group of saline treated animals will be compared with cocaine treated animals. Future goals will include the determination of a dose response relationship for the induction of norcocaine metabolism in cocaine treated animals. The role of these metabolites is just beginning to be understood (Konkol et al., 1992), and *in vivo* behavioral work as well as *in vitro* examination of the mechanisms of actions of these compounds needs further elucidation.

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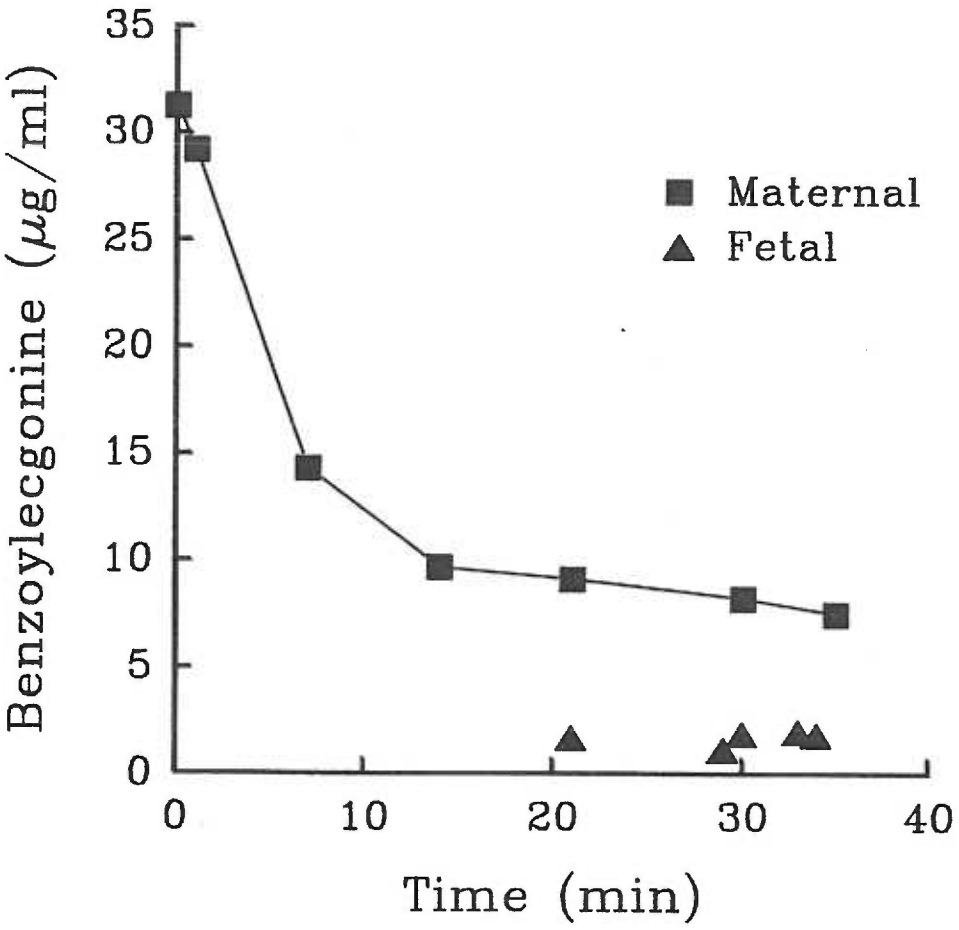
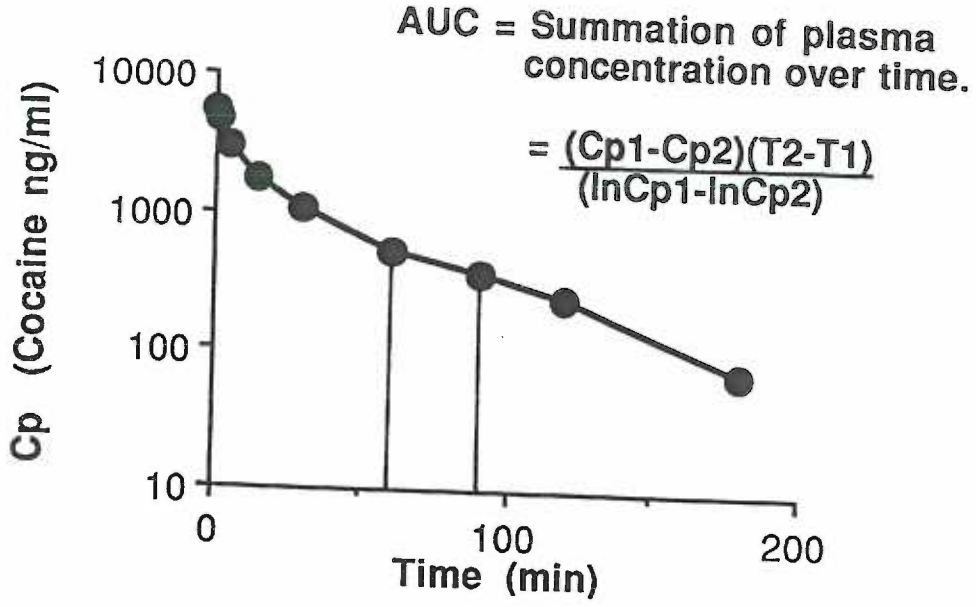
Appendix A

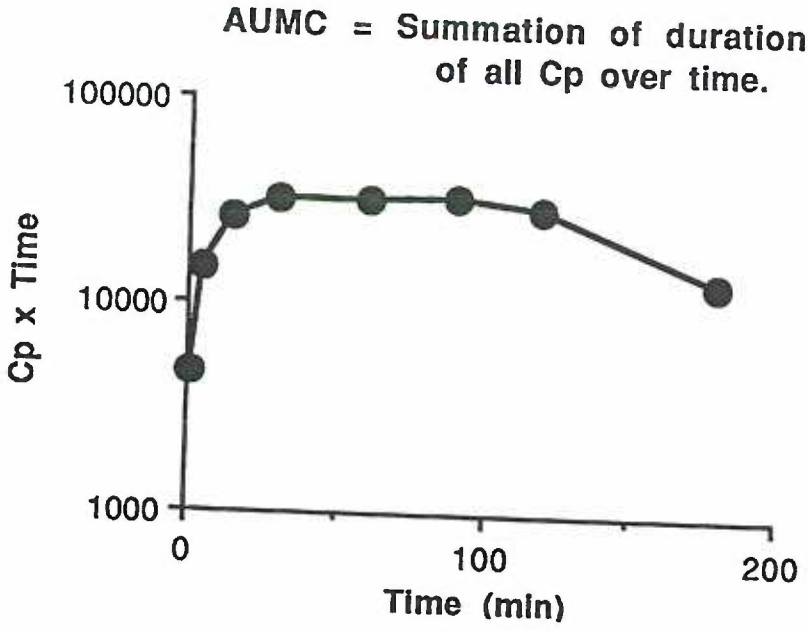
Appearance of benzoylecgonine in fetal plasma 30 min after a maternal intravenous injection of 9 mg/kg benzoylecgonine. The distribution phase of benzoylecgonine was very rapid compared to the elimination phase in maternal plasma. The average fetal concentration was 25% of the maternal concentration.


Benzoylecgonine (μg/ml)
35
30
25
20
15
10
5
0
0
10
20
30
40
Time (min)
Maternal
Fetal

Appendix B

Equations and proofs for noncompartmental pharmacokinetic analysis to supplement those in Manuscript #2.


AUC = Summation of plasma concentration over time.
= (Cp1-Cp2)(T2-T1) / (lnCp1-lnCp2)
Cp (Cocaine ng/ml)
10000
1000
100
10
0
100
200
Time (min)


AUMC = Summation of duration of all Cp over time.
100000
10000
1000
Cp x Time
0
100
200
Time (min)

The fraction (f) of the dose (D) that is absorbed after nonintravenous administration. (Subcutaneous administration is shown.)

$$f = \frac{AUC_{s.c.}}{AUC_{i.v.}} \times \frac{D_{i.v.}}{D_{s.c.}}$$

Clearance (Cl) = volume of plasma from which a substance is completely removed per unit time. (It also is the amount of the distribution volume of drug cleared per unit time.)

$$Cl = \frac{\text{rate eliminated}}{\text{average plasma concentration}}$$

$$\text{Average plasma concentration} = \overline{C}_p = \frac{AUC}{\text{time}}$$

$$\text{rate eliminated} = \frac{\text{dose}}{\text{time}}$$

$$Cl = \frac{\frac{\text{dose}}{\text{time}}}{\frac{AUC}{\text{time}}} = \frac{\text{dose}}{AUC}$$

Mean residence time (MRT) = average time

drug remains in the pool without being irreversibly removed.

$$MRT = t = \frac{\overline{C}_p t}{\overline{C}_p} = \frac{\frac{AUMC}{time}}{\frac{AUC}{time}} = \frac{AUMC}{AUC}$$

$$\text{mean absorption time} = MAT = MRT_{s.c.} - MRT_{i.v.}$$

$$\text{apparent first order absorption rate constant} = k_a = \frac{1}{MAT}$$

$$\text{absorption } T\frac{1}{2} = \frac{.693}{k_a}$$

Volume of distribution (Vd) = the fluid volume in which the drug appears to be dissolved.

$$Vd = \frac{\text{amount of drug in body}}{\overline{C}_p}$$

Volume of distribution at steady state = Vdss = Cl x MRT,

i.e. Vd_{ss} = the amount of the distribution volume cleared of drug per unit time multiplied by average time of the drug in the body.

$$\text{if } Cl = \frac{dose}{AUC} \text{ and } MRT = \frac{AUMC}{AUC}$$

$$\text{then } Vdss = \frac{dose}{AUC} \times \frac{AUMC}{AUC} = \frac{dose \times AUMC}{AUC^2}$$

$$Vdss_{i.v.} = Vdss_{s.c.}$$

$$\frac{dose}{AUC_{i.v.}}\left[\frac{AUMC_{i.v.}}{AUC_{i.v.}}\right] = \frac{f \times dose}{AUC_{s.c.}}\left[\frac{AUMC_{s.c.}}{AUC_{s.c.}} - \frac{1}{k_a}\right]$$

$$= \frac{\left(\frac{AUC_{s.c.}}{AUC_{i.v.}}\right) dose}{AUC_{s.c.}}\left[\frac{AUMC_{s.c.}}{AUC_{s.c.}} - \frac{1}{k_a}\right]$$

$$= \frac{dose}{AUC_{i.v.}}\left[\frac{AUMC_{s.c.}}{AUC_{s.c.}} - MAT\right]$$

$$= \frac{dose}{AUC_{i.v.}}\left[\frac{AUMC_{s.c.}}{AUC_{s.c.}} - (MRT_{s.c.} - MRT_{i.v.})\right]$$

$$= \frac{dose}{AUC_{i.v.}}\left[\frac{AUMC_{s.c.}}{AUC_{s.c.}} - \frac{AUMC_{s.c.}}{AUC_{s.c.}} + \frac{AUMC_{i.v.}}{AUC_{i.v.}}\right]$$

$$= \frac{dose}{AUC_{i.v.}}\left[\frac{AUMC_{i.v.}}{AUC_{i.v.}}\right]$$

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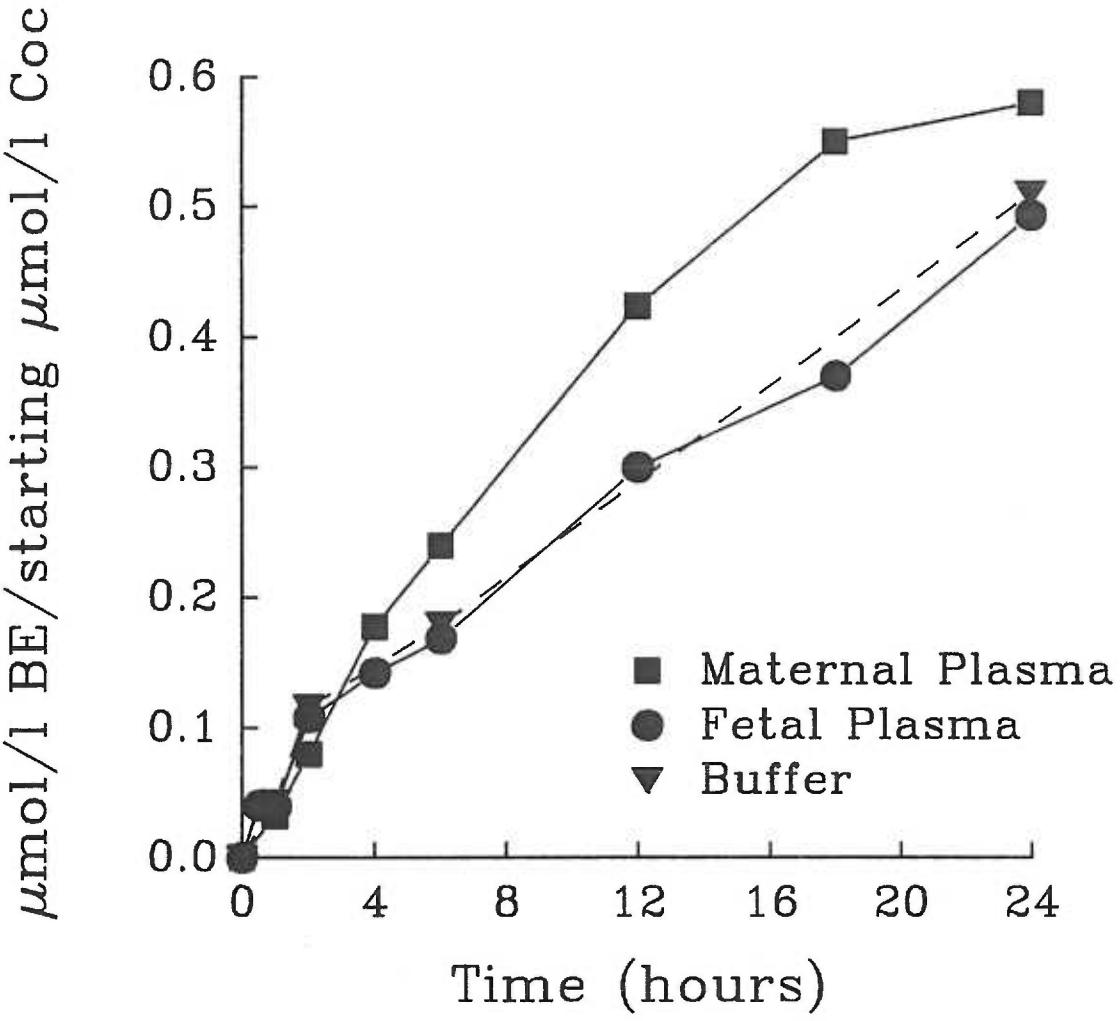
Appendix C

In vitro formation of benzoylecgonine from cocaine in buffer, and maternal and fetal guinea pig plasma. Samples were incubated at 39°C (guinea pig body temperature). The rate of benzoylecgonine formation was similar in the three fluids for the first 3 hours, after which the rate was greatest in maternal plasma.


μmol/l BE/starting μmol/l Coc
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
4
8
12
16
20
24
Time (hours)
Maternal Plasma
Fetal Plasma
Buffer

Appendix D

Example of calculations (by J.A. Sandberg) used to determine renal clearance (Cl_r) in humans from published data (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-chromatography. Anal. Chem. 49: 1965-1969, 1977).

For patient A.M.:

1. The cumulative amount of cocaine in urine samples was determined by multiplying urine cocaine concentration by urine volume in Table III of Kogan et al., 1977.

   0 - 2 hr

   12.2 μg/ml x 92 ml = 1122.40 μg

   2 - 4 hr

   3.7 μg/ml x 175 ml = 647.50 μg

   4 - 6 hr

   2.4 μg/ml x 255 ml = 612.00 μg

   2381.90 μg = 2381900 ng

2. Calculation of renal clearance (Cl_r) was done using the equation from Wilkinson, R.G.: Clearance approaches in pharmacology. Pharmacol. Rev. 39: 1-47, 1987.

$$Cl_r = \frac{A_{e,0-6}}{AUC_{0\rightarrow 6}}$$

where $Ae_{,0-6}$ is the cumulative amount of drug in urine from 0-6 hours and $AUC_{0\rightarrow 6}$ was calculated by noncompartmental analysis with the Hotshot program (Assist/One, Grants Pass, OR) by J.A. Sandberg as described in Manuscript #2.

$$Cl_r = \frac{A_{e_{0-6}}}{AUC_{0\rightarrow 6}} = \frac{2381900 \text{ ng}}{126989 \text{ ng/ml/min}} = 18.76 \text{ ml/min}$$

3. The contribution of renal clearance to total body clearance of cocaine is:

$$\frac{Cl_r}{Cl_T*} = \frac{18.76 \text{ ml/min}}{787.47 \text{ ml/min}} = .024 \times 100 = 2.4\%$$

*Cl_T calculated from the Kogan et al. (1977) data with noncompartmental analysis using the Hotshot program as described in Manuscript #2.

Appendix E

Cardiovascular effects of intravenously administered cocaine and benzoylnorecgonine in a day one postpartum guinea pig. The dam's arterial catheter was connected to a physiological pressure transducer (Gould Statham, Oxnard, CA) attached to a Beckman R611 eight channel polygraph (Sensor Medics Co., Anaheim, CA) to record heart rate and blood pressure.

Drug Dose	*Pre, Post or % Change*	*Heart Rate (bpm)*	*Average Blood Pressure (mm Hg)*	*Time to maximum response after injection (min)*	*Time to baseline (min)*
Cocaine					
2 mg/kg	pre	256	55		
	post	184	69		
	% change	-28%	+20%	4	20
6 mg/kg	pre	237	55		
	post	168	86.5		
	% change	-29%	+57%	12.5	55
Benzoylnorecgonine					
6 mg/kg	pre	240	61		
	post	274	70		
	% change	+14	+15	0.1	2