

**Title: Gestation time-dependent pharmacokinetics of intravenous (+)-methamphetamine in rats**

**Authors:** Sarah White, Elizabeth Laurenzana, Howard Hendrickson, W. Brooks Gentry and S. Michael Owens

**Author Affiliations:**

Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA (S.W., E.L., W.B.G., S.M.O.)

Department of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, AR 72205 (H.H.)

Department of Anesthesiology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205 (W.B.G.)

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**b.) Corresponding Author:**

S. Michael Owens, PhD

Department of Pharmacology and Toxicology

University of Arkansas for Medical Sciences

4301 W. Markham, #611

Little Rock, AR 72205, USA

mowens@uams.edu

Phone: (501) 686-5487

Fax: (501) 526-4618

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ANOVA, analysis of variance; AMP, (+)-amphetamine;  $AUC_0^\infty$ , area under the serum concentration-versus-time curve from time zero to infinity;  $Cl_{NR}$ , nonrenal clearance;  $Cl_R$ , renal clearance;  $Cl_T$ , total body clearance;  $Cl/F$ , systemic clearance corrected for bioavailability;  $f_m$ , fraction of parent drug converted to a metabolite;  $f_u$ , fraction of unchanged parent drug in urine; GD, gestational day;  $\lambda_z$ , terminal elimination rate constant; LC, liquid chromatography; LLE, liquid-liquid phase extraction; METH, (+)-methamphetamine; MRT, mean residence time; PND,

postnatal day; SPE, solid phase extraction;  $t_{1/2\lambda_z}$ , terminal elimination half-life;  $V_d$ , volume of distribution;  $V_{d_{ss}}$ , volume of distribution at steady-state.

## Abstract

We tested the hypothesis that changes in (+)-methamphetamine (METH) disposition occur during late rat pregnancy that could be causes of increased vulnerability to METH effects. The disposition of single 1 mg/kg iv METH doses were administered during early (gestation day 7, GD7) and late (GD21) gestation. Results showed gestation time-dependent pharmacokinetics, characterized by a significantly higher area under the METH serum concentration versus time curve and a lower clearance on GD21 ( $p < 0.05$ ; total, renal and non-renal clearance). The terminal elimination half-life ( $t_{1/2\lambda z}$ ) of METH and (+)-amphetamine (AMP, a pharmacologically active metabolite of METH) were not different on GD7, but by GD21 AMP  $t_{1/2\lambda z}$  was 37% longer than METH  $t_{1/2\lambda z}$  ( $p < 0.05$ ). To identify the mechanism for AMP metabolite changes, iv AMP pharmacokinetics on GD21 were compared to AMP metabolite pharmacokinetics following iv METH. The iv AMP  $t_{1/2\lambda z}$  was significantly shorter than metabolite AMP  $t_{1/2\lambda z}$  ( $p < 0.05$ ), which suggested AMP metabolite formation (not elimination) was an elimination rate-limiting process. To understand the medical consequence of METH use during late-stage pregnancy, timed-pregnant rats received an iv-dose of saline or METH (1, 3 or 5.6 mg/kg) on GD21, 0-2 days antepartum. Even though one rat died and another had stillbirths at term following the 5.6 mg/kg dose, the pharmacokinetic values for all of the other animals were not significantly different. In conclusion, late-gestational clearance reductions lengthen METH exposure time possibly increasing susceptibility to adverse effects, including death.

## Introduction

(+)-Methamphetamine (METH) abuse has evolved over the last few decades to include an expanding population of users (Anglin et al., 2000). A particularly troubling trend is the rise in female users, who now account for ~45% of METH abusers (Cohen et al., 2007). Since the majority of these women are of child-bearing age and METH use impairs decision-making (Kalechstein et al., 2003), it should not be surprising that there is also an increased use during pregnancy (Arria et al., 2006). In a study of 72 self-reported female METH users in the Infant, Development, Environment and Lifestyle study, Smith et al. (2008) report 83% of the women use the drug during the first trimester and 42% during the third trimester. The mothers' choice to use METH places the fetus at risk for serious adverse effects including developmental problems, accidents and addiction.

The powerful force of METH addiction can lead to a quick progression from infrequent use of low doses to "binge" use, which is characterized by shorter time intervals between escalating METH doses (Cho and Melega, 2002). During these periods of binge use, a substantial METH body burden can accumulate since the time interval between each new dose is short relative to the terminal elimination half-life ( $t_{1/2\lambda z}$ ) of METH (12 h in humans; Cook et al., 1993). To add to the problem, METH metabolism leads to the formation of a pharmacologically active metabolite, (+)-amphetamine (AMP), which could also accumulate. When METH is used during pregnancy, the fetus is also vulnerable to METH-induced effects (Szeto, 1993). In an animal model of METH use, Burchfield et al. (1991) report METH crosses the placenta in  $\leq 30$  s after maternal METH administration in near-term pregnant sheep. Assuming similar rapid METH penetration into the fetus occurs in humans, METH exposure to mother and fetus could result in an increased risk of toxicity and death.

Pregnancy likely alters the degree of maternal and fetal exposure to METH through pregnancy-induced physiological changes (Loebstein et al., 1997). Some of these pregnancy-related maternal physiological changes include increased plasma volume, decreased plasma protein binding, delays in gastric emptying time (Mattison et al., 1991; Loebstein et al., 1997), and alterations in metabolic enzyme activity (Loebstein et al., 1997). These physiological changes evolve throughout pregnancy and can be organ-specific. For instance, renal blood flow in rats increases during mid-gestation but returns to non-pregnant levels by late-gestation (rat gestation is 21-23 days; Reckelhoff et al., 1992).

Studies from our laboratory in pregnant rats demonstrate that METH systemic clearance (corrected for bioavailability; Cl/F) significantly decreases near the end of pregnancy on gestational day 20 (GD20) and GD21 (just prior to delivery). In these prior studies, pregnant Sprague-Dawley rats received steady-state infusions of METH at 5.6, 10 or 13.2 mg/kg/day (White et al., 2009). The infusion of METH to steady-state levels provided a means to accurately determine METH (and AMP) partitioning and equilibration across the maternal and fetal blood-brain barrier and placental-blood barrier. However, most human METH users prefer more rapid routes of administration like smoking, snorting and injecting (Rawson and Condon, 2007) to significantly enhance the “rush” of pharmacological effects.

Since studies of the high and dangerous METH doses used by addicted humans (e.g., 1 g/day; Cho et al., 1990; EROWID, 2010) are not possible in pregnant or non-pregnant humans, animal models such as the rat must be used to help predict human adverse effects, including the potential for toxicity. Although the pregnant rat is not a perfect model for human pregnancy, the rat and human exhibit many comparable pregnancy-induced physiological changes (Dowell and Kauer, 1997; Conrad, 1987; Mattison et al., 1991) and have many placental similarities (DeSesso, 1997).

We tested the hypothesis that changes in METH (and AMP) disposition following iv METH administration occur during late-stage rat pregnancy. To assess the possibility of gestation time-dependent changes, pregnant Sprague- Dawley rats were treated with 1 mg/kg METH on GD7 (during early pregnancy) and 1 mg/kg METH or AMP on GD21 (late in pregnancy). To determine if there were acute METH dose-dependent toxicological changes, we studied iv-bolus doses of METH (1, 3 or 5.6 mg/kg) administered to gravid rats on GD21. Even though rats at the 5.6 mg/kg dose died, the pharmacokinetic values for the surviving animal groups were not significantly different. Results also showed there were late gestational-stage reductions in METH clearances that lengthened METH exposure time and thus, increased the susceptibility to adverse effects, including the potential for lethality.

## Materials and Methods

**Drugs and Reagents.** (+)-Methamphetamine HCl, (+)-amphetamine sulfate, (±)-4-hydroxy-methamphetamine hydrochloride and (±)-4-hydroxy-amphetamine hydrobromide were obtained from the National Institute of Drug Abuse (Rockville, MD). All drug concentrations were calculated as the free base form and prepared in sterile saline. (±)-Methamphetamine-d<sub>5</sub> and (±)-amphetamine-d<sub>11</sub> were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used in these studies were purchased from Fisher Scientific Co. (Fairlawn, NJ), unless specified otherwise.

**Animals.** Timed-pregnant, female Sprague-Dawley rats with dual indwelling polyurethane jugular venous catheters (0.025" inner × 0.04" outer diameter) arrived on GD3 from Charles River Laboratories (Raleigh, NC). The catheters were imbedded in the sc space between the scapulae. One week after arrival, the animals were anesthetized with isoflurane and their catheters were externalized. Catheters were flushed three times a week with 200 µl saline followed by 50 µl heparin/glycerol (500 U) to maintain patency. Rats were housed individually in an animal care facility with a 12 hour light/dark cycle (6:00 AM-6:00 PM) and an average 22°C ambient temperature. They received food (8640 Rodent Diet with 22% crude protein/5% crude fat, Harlan Teklad, Madison, WI) and water *ad libitum*. Animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. They were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (Little Rock, AR) prior to beginning the experiments.

**Effect of Dose on GD21 Pharmacokinetics.** On GD19, a pre-drug blood sample (80 µl) was collected to determine baseline hematocrit levels. On GD21, the gravid animals were treated with an iv-bolus dose of saline or METH (1, 3 or 5.6 mg/kg; n=5 per dose) over 15 s via



the right jugular venous catheter. The catheter was then flushed with 200  $\mu$ l of saline. Blood was drawn at pre-determined times of 1, 5, 15, 30, 60, 120, 210, 300, 390 and 510 min after METH administration. This sampling period was based on previous experiments from our laboratory where the METH  $t_{1/2\lambda z}$  was determined to be ~70 min in male and non-pregnant female rats dosed with 1 or 3 mg/kg iv METH (Milesi-Hallé et al., 2005), thus >99% of METH would be cleared by 510 min. Blood sample volumes consisted of 50  $\mu$ l for earlier time-points (1-210 min) and 100  $\mu$ l for later time-points; however, an additional 30  $\mu$ l was collected at 30 and 510 min for determination of hematocrits. Sterile saline was administered to the animals in volumes equal to the volume of blood collected after each time-point to prevent hypovolemia. Total volume of blood collected from each animal during the pharmacokinetic study was no more than 5% of the rat's total blood volume, based on the animal's weight on GD21 (320-446 g). Serum was collected following centrifugation and stored at -80°C until analyzed.

In addition to the experimental saline-treated control group, two further control groups were used for monitoring procedural and health stress. A non-catheterized group was used to control for procedural stresses (i.e., catheter implantation and externalization; anesthesia). No drugs were administered and no blood was collected for this group. A third control group served to control for potential changes in the health of the experimental animals due to procedures (e.g., effects of blood loss from sampling) and handling. These rats were exposed to the same procedures as the saline group, with the exception of blood sampling for pharmacokinetic analysis. Minimal volumes of blood were drawn (30  $\mu$ l) to monitor hematocrit levels at times that coincided with blood samples drawn from the treatment groups. As mentioned previously, these time-points included GD19 and GD21 (30 and 510 min), as well as postnatal day 1 (PND1), PND4 and PND7.

**Urinary Excretion and Pharmacokinetic Studies on GD7 and GD21.** Pregnant catheterized rats received a 15 s iv-bolus injection of 1 mg/kg METH on GD7 or GD21, or a dose of AMP (1.1 mg/kg) equimolar to 1 mg/kg METH on GD21 (n=5 per group). After drug

administration and the first blood collection at 5 min, the animals were placed in metabolism cages (Nalgene, Nalge Nunc International, Rochester, NY) where they had free access to food and water. Animals were removed briefly from their cages for blood sampling. Sampling times, volumes and processing were the same as in the previous experiment.

Animals remained in the metabolism cages until the final urine samples were collected (24 hr after drug administration). For the first hour of the pharmacokinetic experiment, each urine void was quickly collected. After the first hour, urine samples were collected when volumes were >500  $\mu$ l. Urine pH, appearance, volume and time of collection were noted at each collection. After centrifugation, urine was stored at -80°C until analysis.

**Myoglobin Detection in Urine and Serum on GD7 and GD21.** The urine collected during the pharmacokinetic experiments on GD7 and GD21 was darker than normal or red-colored on some occasions for all of the animals, which suggested the possibility of hemoglobin in the urine. The presence of hemoglobin was screened for by measurements with urine test strips (Multistix 10 SG Reagent Strips for Urinalysis, Bayer, Pittsburg, PA); however, these test strips do not differentiate between the presence of myoglobin or free hemoglobin. To determine which was present, the solubility of the proteins was assessed by ammonium sulfate precipitation. Hemoglobin precipitates out of the urine in the presence of an 80% saturation with ammonium sulfate, while myoglobin precipitates only after 100% saturation (Hamilton et al., 1989). Ammonium sulfate precipitation (80% saturation) was performed by adding 0.56 g of ammonium sulfate to a 1 ml specimen of fresh un-centrifuged urine. All crystals were dissolved by mixing the solution for five min, followed by centrifugation at 2,300 rpm for three min. The supernatant from the solution of urine was again tested with a Multistix SG10 test strip and if still positive, the test was reported as positive for myoglobin in the urine. If negative, it was reported as positive for hemoglobin in the urine. Myoglobin from equine skeletal muscle was used as a positive control (BioChemika, Sigma-Aldrich, St. Louis, MO).

**Quantitation of METH and AMP Concentrations.** Determination of METH and AMP concentration in rat serum was based on the liquid-liquid phase extraction (LLE) method developed by Hendrickson et al. (2006). Briefly, 25 or 50  $\mu\text{l}$  of each serum sample was diluted to 100  $\mu\text{l}$  using drug-free normal rat serum (Pel-Freez Biologicals, Rogers, AR). Calibration (0.3-1000 ng/ml) and quality control (3-800 ng/ml) drug standards were also prepared using drug-free normal rat serum. Internal standards (10  $\mu\text{l}$ ), consisting of a mixture of 100  $\mu\text{l}$  of ( $\pm$ )-methamphetamine- $\text{d}_5$  and ( $\pm$ )-amphetamine- $\text{d}_{11}$ , were added to all samples and standards. Proteins were precipitated with the addition of ice-cold trichloroacetic acid (100  $\mu\text{l}$  of 20% w/v). The samples were vortex-mixed, rotated and centrifuged. The supernatant was then collected and filtered.

Analysis of METH and AMP concentrations in urine was accomplished using solid-phase extraction (Hendrickson et al., 2004). To summarize, urine samples were centrifuged at 10,000 rpm for five min. Since the METH and AMP concentrations were expected to be very high, the supernatant was diluted 100-fold in drug-free normal rat serum. Internal standard (10  $\mu\text{l}$ ) was added to diluted samples (100  $\mu\text{l}$ ), along with calibration and quality control standards. A 100  $\mu\text{l}$  aliquot of 8 M Guanidine-HCl and 200  $\mu\text{l}$  of 10% w/v  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  were added to each sample to denature and precipitate the proteins, followed by vortexing and centrifugation. The supernatants were loaded and washed using conditioned Oasis HLB (1 ml  $\times$  30 mg) SPE Columns (Waters Corp., Milford, MA). Analytes were eluted with 1 ml of acetonitrile containing 0.1% (v/v) acetic acid. The eluent was evaporated using  $\text{N}_2$  vaporizer at 38°C for ~20 min. The residue was resuspended in 100  $\mu\text{l}$  of 5 mM ammonium acetate, pH 3.7 containing 5% (v/v) acetonitrile.

The drug containing solutions from LLE were injected (20  $\mu\text{l}$ ) onto a 3  $\mu\text{m}$  BDS Hypersil C8 column (Keystone Thermo-Electron, Belafonte, PA) and analytically separated using a binary linear gradient (Hendrickson et al., 2006). The flow rate was 0.3 ml/min. A Waters Alliance 2695 LC (liquid chromatography) coupled to a Waters/Micromass Quattro LC triple

quadrupole mass spectrometer (Waters Corp., Milford, MA) with an electrospray interface and a Mark II source in the positive ion mode was used for analyzing part of the serum concentrations following 1, 3 or 5.6 mg/kg METH. Retention times were  $8.22 \pm 0.04$  and  $7.95 \pm 0.04$  min for METH and AMP, respectively.

A SIL-HTa autosampler (Shimadzu, Columbia, MD) coupled to a Waters/Micromass Quattro Premiere triple quadrupole mass spectrometer with an electrospray interface in the positive ion mode was used for the remaining serum and urine samples. Samples (20  $\mu$ l) were injected onto a 3  $\mu$ m BDS Hypersil C8 column and the column was maintained at 40°C using a CTO-10A column oven (Shimadzu, Columbia, MD). The analytes were separated using a binary gradient with a flow rate of 0.3 ml/min through two LC 10-ADVP pumps (Shimadzu, Columbia, MD). Solvent A was 5 mM ammonium acetate buffer (pH 3.7) with 5% (v/v) acetonitrile. Solvent B was 5 mM ammonium acetate buffer (pH 3.7) with 95% (v/v) acetonitrile. The initial conditions consisted of 100% solvent A for the first 2.5 min after the sample was injected. Subsequent increases of solvent B to 65% were achieved over the next 5 min and held for 2.5 min. Solvent B was reduced back to 0% over the next 2 min. The first 4 min of the chromatographic run were directed to waste, followed by a run time of 11 min. The retention times for LLE method were  $7.86 \pm 0.06$  min for AMP and  $8.28 \pm 0.2$  min for METH. The retention times were longer for SPE method,  $8.09 \pm 0.06$  and  $8.37 \pm 0.06$  min for AMP and METH, respectively. Both systems were controlled using the computer software Masslynx 4.1 (Waters, Milford, MA). No attempt was made to determine the concentrations of Phase II metabolites.

**Pharmacokinetic Analysis.** To determine the pharmacokinetic parameters of METH and AMP, log concentration-versus-time curves for each animal were analyzed by model-dependent and model-independent methods using WinNonLin (Pharsight Corporation, Mountain View, CA). For model-dependent analyses of METH, bi- and tri-exponential curves were fit successively to the individual data sets using no weighting,  $1/y$  and  $1/y^2$  weighting functions, where  $y$  was the predicted concentration. The best-fit line was chosen by statistical analysis,

visual inspection, analysis of the residuals, and examination of the coefficients of variation for each parameter.

For the model-independent analyses, pharmacokinetic parameters were derived from the log concentration-versus-time graph of METH and AMP concentrations in the terminal elimination phase with a first-order input function and no weighting,  $1/y$  and  $1/y^2$  weighting functions. The best-fit line was determined visually and statistically based on the best-fit line to the concentration-time data points. Pharmacokinetic parameters from the chosen model-dependent analysis of each animal were compared to the parameters determined from the model-independent analysis to assure validity of both methods.

METH pharmacokinetic parameters were determined using the following equations:

$$\text{Dose} = \text{Cl}_T \times \text{AUC}$$

$$\text{Vd} = \text{Cl}_T \times \lambda_z$$

$$t_{1/2\lambda_z} = 0.693 / \lambda_z$$

$$\text{Cl}_T = \text{iv-dose} / \text{AUC}_0^\infty$$

$$\text{MRT} = 1 / \lambda_z$$

$$\text{Cl}_R = f_u \times \text{Cl}_T$$

$$\text{Vd}_{ss} = \text{MRT} \times \text{Cl}_T$$

$$\text{Cl}_{NR} = \text{Cl}_T - \text{Cl}_R$$

where  $\lambda_z$  is terminal elimination rate-constant, MRT is mean residence time,  $\text{Vd}_{ss}$  is volume of distribution at steady-state, Vd is the apparent volume of distribution,  $\text{AUC}_0^\infty$  is area under the serum concentration-versus-time curve from time zero to infinity,  $\text{Cl}_R$  is renal clearance,  $f_u$  is fraction of unchanged METH in the urine and  $\text{Cl}_{NR}$  is the nonrenal clearance.

AMP pharmacokinetic parameters consisted of  $\text{AUC}_0^\infty$ ,  $t_{1/2\lambda_z}$ , maximum AMP serum concentrations ( $C_{max}$ ), time to reach maximum serum AMP concentrations ( $T_{max}$ ) and fraction of parent drug converted to a metabolite ( $f_m$ ). The calculation used to determine  $f_m$  is given by  $f_m = [(\text{AUC}_m)_p / D_p] \times [D_m / (\text{AUC}_m)_m]$  where  $(\text{AUC}_m)_p$  refers to the total AUC of metabolite (AMP) after a single iv-dose of the parent drug (METH),  $(\text{AUC}_m)_m$  is the total AUC of AMP after a single iv-dose of the metabolite (AMP),  $D_p$  is the dose of the parent drug and  $D_m$  is an equimolar dose of the metabolite.

**General Experimental Observations.** Once the animals completed the pharmacokinetic experiment (GD21-GD22), they were returned to their home cages and monitored 4-5 times a day to determine time of parturition. This time-point was then designated as PND0. Beginning on the morning of PND1 at approximately 9:00 A.M., postnatal observations (five min duration) were performed at the animal's home cage with minimal disruptions to behavior and were not blinded. These observations consisted of monitoring non-maternal activities (i.e., eating, drinking, grooming and sleeping) and maternal activities (i.e., grooming pups, carrying pups and nursing). Activity ratings were scored based on the absence or presence of a particular behavior. Subsequently, daily measurements of litter size and weight were determined. Postnatal maternal observations were conducted, as described previously, at approximately 3:00 P.M. Dam and litter measurements continued daily from PND1 through PND7. As mentioned previously, hematocrits were monitored in dams on PND1, PND4 and PND7 in all catheterized dams (following litter size and weight measurements). Once the last postnatal observation was complete on the afternoon of PND7, all dams and litters were humanely sacrificed by decapitation under isoflurane anesthesia.

**Statistical Analysis.** All values are represented as the means  $\pm$  SD; however, harmonic means and "pseudo" SD values were calculated for the  $t_{1/2\lambda z}$  values (Lam et al., 1985). A Grubbs' test was used to detect any outliers among the METH concentrations at 510 min following 1, 3 or 5.6 mg/kg iv METH. A one-way analysis of variance (ANOVA) with dose as the main factor was used to test for any significant differences for each pharmacokinetic parameter of the dose-range finding pharmacokinetics studies conducted on GD21. For litter weight and size data, as well as hematocrit measurements in pregnant rats, a two-way repeated measures ANOVA was performed with one within factor (GD or PND) and one between factor (dose). To determine if statistically significant differences exist in the average maternal activity ratings, a two-way ANOVA was used with one within factor (activity rate) and one between factor (dose). This statistical test was also conducted to detect any significant differences among METH and

its metabolite AMP  $t_{1/2\lambda z}$  on GD7 and GD21. If any of the parameters were found to be significant, a Student-Newman-Keuls post-hoc test was conducted. In addition, a  $t$  test was performed on each pharmacokinetic parameter of METH and AMP following an iv-bolus dose of 1 mg/kg METH on GD7 or GD21. Finally, statistical comparisons were made using a  $t$  test for METH and AMP pharmacokinetic parameters following an iv-bolus dose of 1 mg/kg METH or an equimolar dose of AMP on GD21. ANOVA and  $t$  tests were conducted with SigmaStat version 2.03 software (Jandel Scientific, San Rafael, CA). For all analyses, a value of  $p < 0.05$  was considered to be statistically significant.

## Results

**Effect of Gestation Time on METH and AMP Pharmacokinetics.** To determine if gestation time affects METH pharmacokinetic properties, we first studied the pharmacokinetics of a 1 mg/kg iv METH dose on GD7 and GD21, as representative of time points for early and late pregnancy. Comparisons of METH pharmacokinetic values derived from model-independent and model-dependent analyses of these data were very similar, with less than a 9% difference between pharmacokinetic parameters (data not shown). Model-dependent pharmacokinetic models can be complex and sometimes difficult to interpret, especially in pregnancy (Krauer and Krauer, 1977); therefore, we chose to use the pharmacokinetic values from the non-compartmental analysis. In most of the pharmacokinetic concentration versus time figures, we present data from the single animal that best represents the average values of all animals in their treatment groups. Average data and the associated variance for all the animals in each study are shown in the tables.

Representative average METH and AMP serum concentration-versus-time curves from GD7 and GD21 following a 1 mg/kg dose of METH are shown in Figure 1. A bi-exponential decline in concentrations, similar to GD21, was seen for METH on GD7; however, the terminal elimination phase for GD7 declined more rapidly. Consequently, the AUC was significantly smaller ( $p < 0.05$ ) on GD7 than GD21. These values, along with the other average METH and AMP pharmacokinetic parameters, are summarized in Table 1.

The GD21 values for  $Cl_T$ ,  $Cl_{NR}$  and  $Cl_R$  were significantly decreased ( $p < 0.05$ ) compared to values on GD7 (Table 1). The cumulative percentage of METH and AMP eliminated unchanged in the urine was not affected by the gestation stage (Table 1, Figure 2). METH  $t_{1/2\lambda z}$  was significantly prolonged (by 26%) on GD21, which resulted from a significantly decreased



$Cl_T$  without a change in the  $V_d$ . AMP metabolite  $t_{1/2\lambda z}$  was also significantly longer on GD21 compared to GD7 values (99% longer, Table 1 and Figure 1).

**Rate-limiting step of AMP elimination on GD21.** To help determine if AMP metabolite disposition was formation or elimination rate-limited, an equimolar dose of AMP was administered to a separate group of animals on GD21. As shown in Figure 3, the serum concentration versus time profiles of iv AMP and iv METH were virtually superimposable, and pharmacokinetic parameters calculated for iv AMP (Table 2) and iv METH (Table 1) were not significantly different. Furthermore, after administering AMP to pregnant rats on GD21,  $f_m$  was calculated as  $0.32 \pm 0.06$ . This was not different from the AMP/METH AUC molar ratio determined on GD21 ( $0.31 \pm 0.03$ ; Table 1) after METH administration.

Because the  $T_{max}$  values for the AMP metabolite was significantly later on GD21 than GD7 ( $p < 0.05$ , Table 1), the AMP metabolite formation rate was slower on GD21 (Figure 1). In addition, AMP concentrations on GD21 declined significantly more slowly than on GD7, which resulted in a 64% longer AMP metabolite  $t_{1/2\lambda z}$  compared to METH. The combined effect of a slower formation and longer AMP  $t_{1/2\lambda z}$  resulted in significant accumulation of AMP metabolite on GD21. This is best revealed by the 48% increase in the molar ratio of AMP/METH AUC on GD21 versus GD7.

Considered together, this series of experiments showed the rate-limiting step for AMP systemic elimination was AMP formation, and not the AMP elimination rate constant.

#### **Effect of METH Dose on METH and AMP Pharmacokinetics on GD21.**

Representative rat METH and AMP serum concentration versus time curves after an iv-bolus dose of 1, 3 or 5.6 mg/kg METH are shown in Figure 4 ( $n=5$  per group). A total of 7 rats were used for the 5.6 mg/kg METH dose because two of the seven developed METH-induced toxicities, which resulted in lethality. The METH and AMP serum concentrations in these two rats are shown in Figure 5. One rat (along with the pups) died ~30 min after completion of blood

sampling; the other rat survived but the pups delivered as stillbirths on GD25. The data from these two animals are not included in the detailed report of these studies in Table 3.

When the first death occurred unexpectedly during the study of the third rat in this dose group, we considered the possibility that this animal's outcome was an experimental outlier. However, when we continued to study additional rats to obtain a total of five animals in this group, we discovered another animal with toxicity in the form of stillborn pups. Since these experiments were not designed to determine a METH LD<sub>50</sub> value in pregnant rats, we did not continue to study more rats after we successfully completed a series of five animals without deaths.

However, later determination of the METH and AMP concentrations versus time curves in these rats (Figure 5), especially after about 120 min, revealed the  $t_{1/2\lambda z}$  for the animal that delivered stillbirths was significantly prolonged at 249 min for METH and 484 min for AMP. Since the METH and AMP concentrations were higher at 510 min than 390 min for the pregnant rat that died, meaningful pharmacokinetic analysis could not be accomplished. Nonetheless, to show the magnitude of METH and AMP concentrations of the animals, the average METH and AMP  $AUC_0^{510\text{min}}$  of the five animals were 1376 ng-h/ml and 507 ng-h/ml, respectively. In comparison, the METH and AMP  $AUC_0^{510\text{min}}$  of the animal that delivered stillbirths was 1978 ng-h/ml and 794 ng-h/ml and of the animal that died, 2380 ng-h/ml and 1111 ng-h/ml, respectively.

The remaining five animals administered 5.6 mg/kg METH completed the studies without serious complications; however they did exhibit temporary chromodacryorrhea and stereotypic behavior like excessive sniffing and licking. The average serum pharmacokinetic parameters for METH and AMP after the 1, 3 or 5.6 mg/kg METH doses (n=5 per group) are presented in Table 3. There were no significant differences in the pharmacokinetic values determined for METH and AMP in the total of 15 rats studied at these three doses (after excluding the two animals from the 5.6 mg/kg dose). This suggested dose-independent pharmacokinetics in 15 out of 17 rats.

The results from these iv bolus doses of METH in the current studies are consistent with the values for Cl/F in pregnant rats receiving continuous sc-infusions of METH (5.6, 10 or 13.2 mg/kg/day) from GD7 to GD21 (White et al., 2009). Cl/F values corrected for fraction of the dose absorbed following sc-infusions were similar from GD8 to GD17 (ranged from  $41 \pm 12$  to  $59 \pm 16$  ml/min/kg;  $p \geq 0.05$ ). However, these values on GD20 and GD21 (ranged from  $25 \pm 4$  to  $36 \pm 9$  ml/min/kg) were significantly decreased versus the values on GD8, GD10 and GD13. Thus, regardless of the route of METH administration (iv or sc-infusion), there are late-gestational reductions in METH clearance.

**Detection of Hemoglobin and Protein in Urine on GD7 and GD21.** Each animal treated with 1 mg/kg METH on both GD7 and GD21 had noticeably dark urine at times (three were found to have light red colored urine), suggesting METH-induced hematuria. Dipstick testing was conducted, which confirmed the presence of heme in the urine, as well as protein. Results from ammonium sulfate precipitation tests showed that urine was negative for myoglobin, suggesting rhabdomyolysis was not the cause. Saline-treated control pregnant rats (n=2) experienced the same experimental conditions as METH-treated rats, and heme and protein were also detected in the urine of these animals. In addition, no differences were found in urine pH (ranged from 5.5-9) or in average total urine volume (METH administration on GD7:  $18.4 \pm 4.8$  ml; METH administration on GD21:  $12.1 \pm 3.7$  ml; AMP administration on GD21:  $16.7 \pm 3.7$  ml), respectively.

**General Observations during Parturition and Postnatal.** All of the animals, *with the exception of the two animals treated with 5.6 mg/kg*, delivered within the normal time-frame (GD22-GD23; data not shown). Average litter size and pup weight on PND1 are shown in Table 4. No significant differences were found between groups.

Maternal activity ratings (nursing, caring for self, and caring for pups) were determined for animals in the three METH dose groups, as well as the experimental control group (saline-treated), the health control group (saline-treated; hematocrits taken only), and the procedural

control group (non-catheterized; no drug treatment) (data not shown). Activity ratings were scored in the animal's home cage and based on the presence or absence of an activity for 5 min observations at 9:00 A.M. and 3:00 P.M. from PND1 to PND7. There were no significant differences found for the average activity ratings of the combined data collected from PND1 to PND7.

Finally, hematocrit values were not significantly different between study animals with blood sampling and study animals in which blood was only collected for determination of hematocrits (data not shown). This comparison was made from GD19 to the end of the study on PND7. Average hematocrits, excluding the two rats with lethal events, were  $38 \pm 2\%$  on GD19 (prior to drug administration),  $35 \pm 2\%$  on GD21 (30 min),  $35 \pm 4\%$  on GD21 (510 min),  $32 \pm 2\%$  on GD22,  $35 \pm 2\%$  on PND1,  $40 \pm 3\%$  on PND4, and  $41 \pm 3\%$  on PND7. By PND7, the hematocrits of the animals had returned to levels of non-pregnant female Sprague-Dawley rats (41%; Giknis, 2006).

## Discussion

We wanted to understand the possible mechanism(s) for increased vulnerability to METH use for pregnant dams and pups. To accomplish this goal, we studied effects of METH doses during early (GD7) and late (GD21) pregnancy. We found METH  $Cl_T$  was significantly decreased and  $t_{1/2\lambda z}$  was increased in late stage pregnancy (Table 1, Figure 1), without any change in  $V_d$ . This occurred in spite of the fact that the pharmacokinetic properties were found to be METH dose-independent on GD21 (Table 3).

The three METH doses were chosen for study because low and middle doses produce only moderate (1 mg/kg) to high (3 mg/kg) pharmacological effects (e.g., locomotor activity) without any overt toxicity in male and non-pregnant female Sprague-Dawley rats (Milesi-Hallé et al., 2005; Milesi-Hallé et al., 2007). Higher METH iv-dose of 5.6 mg/kg in drug naïve male rats, produce intense focused stereotypy and severe chromodacryorrhea, which are overt signs of METH toxicity (Byrnes-Blake et al., 2003). IV doses of 10 mg/kg are sometimes lethal. Thus, studying METH doses at the threshold of toxicity (e.g.,  $\leq 3$  mg/kg) gave us the best chance to discover changes at the interface between pharmacological and toxicological events. This also represented a model of human use in which binge use of METH by addicts is often at the boundary between a desired pharmacological high and serious toxicity.

In the current studies, similar effects were observed in pregnant rats administered 5.6 mg/kg METH as in male rats treated with 10 mg/kg METH (Byrnes-Blake et al., 2003). Indeed, deaths occurred in two of seven animals administered 5.6 mg/kg METH (one dam died on GD21 shortly after the last blood sample; the other delivered stillbirths on GD25; Fig. 5). This finding appears to suggest that late-stage pregnant rats were more susceptible to harmful METH effects than male rats. While the exact cause of death on GD21 was not determined, METH intoxication in humans causes shock, hyperthermia and organ failure (Lan et al., 1998). It

is likely that one or more of these METH-induced complications contributed to the rise in METH and AMP concentrations found at 510 min in the pregnant rat that died (Figure 5). No attempt was made to increase the number of study animals or METH doses to determine a METH LD<sub>50</sub>.

Interestingly, the other five animals in the 5.6 mg/kg dose group not only recovered quickly from the METH-induced chromodacryorrhea and stereotypy, they displayed pharmacokinetic profiles that were not significantly different from animals treated with lower doses (Table 3). In related studies, White et al. (2009) found pregnant rats chronically infused with 17.8 mg/kg/day sc METH from GD7-GD21 died by GD11, yet animals infused with 13.2 mg/kg/day became tolerant to the METH-induced effects by mid-gestation. These and the current studies suggest that chronic and acute METH administration at high doses results in a steep dose-response lethality curve.

Since METH pharmacokinetics were dose-independent at 1, 3 and 5.6 mg/kg on GD21, we used 1 mg/kg METH dose to examine the effect of gestational time on METH and AMP pharmacokinetics. GD7 was chosen as the early time point for comparison with the GD21 late time point because GD7 is after implantation (GD5-6) but before early differentiation (GD10) (DeSesso, 1997). In addition, the placenta-blood barrier is not mature until GD12 (de Rijk et al., 2002). These developmental events were considered important in our choice of an early time point for the studies, because placental permeability, along with the rate of maternal drug elimination, are important determinants of drug disposition in pregnancy (Szeto, 1993).

The pharmacokinetic properties were dependent on gestation time, with both METH and AMP elimination occurring more slowly as pregnancy advanced (Fig. 1; Table 1). The main mechanism for decreased elimination was a 28% reduction in Cl<sub>NR</sub>. Although Cl<sub>R</sub> decreased by 50% on GD21 (6 vs. 3 ml/min/kg), this route of elimination contributed only 8-11% of the Cl<sub>T</sub> on GD7 and GD21. These gestation time-dependent clearance reductions could arise from pregnancy-induced physiological changes in plasma protein binding and hemodynamics. However, METH serum protein binding is unaltered throughout pregnancy (~10%; White et al.,

2009). Since glomerular filtration rate and renal blood flow increase during early- and mid-gestation and decline prior to delivery (Reckelhoff et al., 1992), renal hemodynamic changes likely contribute to the reduction in  $Cl_R$  seen on GD21.

It is unlikely that urine volume or pH contributed to METH  $Cl_R$  changes, since these physiological factors were not significantly different between GD7 and GD21. The late-gestational reduction in  $Cl_R$  was also not a result of proteinuria or hematuria, since proteinuria and hematuria were detected on both GD7 and GD21. The hematuria could also be due to mechanical stress, along with administration of low doses of heparin used to maintain long-term catheter patency (Schuster and Lewis, 1987).

Hormonal fluctuations during pregnancy can impact transporters and enzymes. The organic cation transporter 3 is involved in the transport of METH across the brush-border membrane in rat kidneys and is inhibited by estradiol (Wu et al., 1998). Before delivery, estradiol concentrations increase compared to nulliparous and early-stage pregnant rats (Garland et al., 1987). Thus, competitive inhibition by estradiol in late stage pregnancy could lead to less binding of METH to the transporters and reduced METH  $Cl_R$ .

A possible mechanism for reduced  $Cl_{NR}$  is decreased metabolic capacity. Neale and Parke (1973) report a 25% reduction in CYP450 in late-gestational rats (GD19-GD20) relative to virgins. Ward and Pollack (1996) find *in vitro* metabolism of methanol in pregnant Sprague-Dawley rat (GD20) liver homogenates is significantly lower than non-pregnant rats, with no change in  $K_m$  values.

When a metabolite like AMP is eliminated more slowly than the parent drug; the rate-limiting step is the metabolite's elimination. Since AMP  $t_{1/2\lambda z}$  was significantly longer than METH  $t_{1/2\lambda z}$  on GD21 (Table 1), it appeared that the decline in AMP serum concentrations was elimination rate-limited. Yet, the significantly longer AMP  $T_{max}$  on GD21 compared to GD7 suggested AMP metabolite was formation rate-limited. To determine the correct mechanism for the change on GD21, an equimolar dose of iv AMP was administered to pregnant rats to allow

comparison of iv AMP and AMP metabolite kinetics. The results showed iv AMP declined at the same rate as iv METH, and not at the same rate as AMP metabolite (Fig. 3; Table 2). Therefore, AMP metabolite disposition is formation rate-limited on GD7 and GD21.

Gestation stage-dependent pharmacokinetic patterns in the present study were consistent with previous studies using pregnant rats chronically infused with METH (White et al., 2009). Yet, METH pharmacokinetics in pregnant rats are different from non-pregnant rats Milesi-Hallé et al. (2005), since dose-dependent pharmacokinetics of METH and AMP in non-pregnant females are found at iv doses of 1 or 3 mg/kg METH.

The potential for enhanced exposure during late-stage pregnancy prompted us to examine the health and pharmacological effects of METH on dams and pups at parturition and postnatally. Contrary to reports citing that prenatal METH use can increase the risk of premature delivery (Eriksson et al., 1978), a significant difference in parturition time compared to control dams was not found. Indeed, one rat treated with 5.6 mg/kg METH delivered stillbirths 2-4 days after normal parturition time. Slamberova et al. (2005) also observed the length of gestation was longest (GD23-GD24) in rats treated with 5 mg/kg METH sc throughout gestation.

In addition to increased risk of delivering prematurely, female METH users neglect and abuse their children (Connell-Carrick, 2007). Animal research aimed at understanding maternal behavior show rats treated with METH, cocaine or opiates throughout gestation display attenuations in maternal behaviors, like nursing, grooming pups and nest-building (Johns et al., 1994; Stafisso-Sandoz et al., 1998; Slamberova et al., 2005). We observed no significant differences in maternal behavior. Possible weaknesses of our experimental design include the limited range of maternal behavior monitored and the use of only a single treatment of METH; however, a single dose of cocaine administered on PND0 disrupts the onset of maternal behavior on PND1 (Johns et al., 1994).

These studies show changes in METH clearance during late-stage pregnancy are a possible cause of increased vulnerability to METH effects, if they exist, especially in human



MEH addicts that frequently “binge” (Cho and Melega, 2002). This can lead to substantial accumulation if the pregnant user does not increase the length of time between doses, as during non-pregnancy. The gestational-time dependent differences consisted of significant decreases in METH clearance and prolongation of  $t_{1/2\lambda z}$  on GD21 compared to GD7. The mechanism of organ clearance changes were attributed to significant reductions of  $Cl_{NR}$  and  $Cl_R$  as pregnancy advanced. While the  $Cl_R$  was a minor elimination process in rats (8-11%), in humans it is a major mechanism of METH clearance with 37-45% of the METH dose excreted unchanged in urine (Cook et al., 1993). These METH clearance reductions in late-stage pregnant rats lengthen METH exposure time and thus, increase the susceptibility to adverse effects, including the potential for maternal and fetal lethality.

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## Authorship Contributions

### Authorship Responsibilities:

*Participated in research design:* White, Laurenzana, Hendrickson, Gentry and Owens

*Conducted experiments:* White, Hendrickson and Laurenzana

*Contributed new reagents or analytic tools:* Hendrickson

*Performed data analysis:* White

*Wrote or contributed to the writing of the manuscript:* White, Laurenzana, Hendrickson, Gentry and Owens

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## Footnotes

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S.M.O. and W.B.G. have financial interests in and serve as Chief Scientific Officer and Chief Medical Officer of InterveXion Therapeutics LLC (Little Rock, AR), a pharmaceutical biotechnology company focused on treating human drug addiction with antibody-based therapy.

### **Requests to receive reprints should be sent to:**

S. Michael Owens, PhD  
Department of Pharmacology and Toxicology  
University of Arkansas for Medical Sciences  
4301 W. Markham, #611  
Little Rock, AR 72205, USA  
mowens@uams.edu

Sarah White's current address is Mayo Vaccine Research Group, Gu-695, 200 First Street SW, Rochester, MN 55905. Elizabeth Laurenzana's current address is Department of Veterinary and Biomedical Sciences, Center for Molecular Toxicology and Carcinogenesis, Pennsylvania State University, University Park, PA 16802



## Legend for Figures

**Figure 1.** Serum concentration-versus-time profile of METH and AMP after iv-bolus dose of METH at 1 mg/kg in pregnant female Sprague-Dawley rats on GD7 or GD21. Semi-logarithmic graphs are representative of one animal from each group (n=5 per dose group) that portrays the mean pharmacokinetic values. The best-fit line for the serum concentration-versus-time profile of METH (solid line) and AMP (dashed line) were determined by a non-compartmental model in the terminal elimination phase.

**Figure 2.** Representative plot of the cumulative percentage of METH (●) and AMP (○) excreted unchanged in the urine of a pregnant rat on GD7 (top panel) and GD21 (lower panel) after iv METH dose of 1 mg/kg on GD7 and GD21. Urine was collected up to 24 hr after drug treatment.

**Figure 3.** Serum concentration-versus-time curve of AMP from one animal representative of the average pharmacokinetics after an iv-bolus dose of 1 mg/kg AMP to pregnant rats on GD21, as well as the serum concentration-versus-time curves of METH and its metabolite AMP (from Fig. 4) after a 1 mg/kg METH dose on GD21. The best-fit line for the serum concentration-versus-time profile of iv METH and iv AMP (solid line) and AMP metabolite (dashed line) were determined by a non-compartmental model in the terminal elimination phase.

**Figure 4.** Serum concentration-versus-time profile of METH and AMP after iv-bolus dose of METH at 1, 3 or 5.6 mg/kg in pregnant female Sprague-Dawley rats on GD21. Semi-logarithmic graphs were representative of one animal from each group (n=5 per dose group) that portrays the mean pharmacokinetic values. The best-fit line for the serum concentration-versus-time profile of METH (solid line) and AMP (dashed line) were determined by a non-compartmental model in the terminal elimination phase.

**Figure 5.** Serum concentration-versus-time graph of METH and AMP after iv-bolus METH (5.6 mg/kg) administration on GD21 to pregnant rats that developed lethal toxicity after METH administration. The top panel shows the METH and AMP concentrations for a pregnant rat that died approximately 9 hr after METH administration. The bottom panel shows the METH and AMP concentrations for a pregnant rat that delivered stillbirths on GD25. The best-fit line for the serum concentration-versus-time profile of METH (solid line) and AMP (dashed line) were determined by a non-compartmental model in the terminal elimination phase. These METH and AMP concentrations were compared to the concentrations for the pregnant rat representative of the mean pharmacokinetic values (n=5) of this dose group shown previously in Figure 4 (bottom panel). However, data from these two rats were not included with the surviving, apparently normal rats (n=5) in the pharmacokinetic data analysis in Table 3.

**Table 1.** METH and AMP metabolite pharmacokinetics in timed-pregnant Sprague-Dawley rats following an iv-bolus dose of 1 mg/kg METH on GD7 or GD21.

METH	GD7	GD21
AUC (ng-min/ml)	17997 ± 1489	24445 ± 4477 <sup>c</sup>
t <sub>1/2λz</sub> (min)	91 ± 16 <sup>a</sup>	115 ± 6 <sup>a,c</sup>
MRT (min)	95 ± 3	157 ± 19 <sup>c</sup>
Vd (L/kg)	7 ± 1	7 ± 1
Vd <sub>ss</sub> (L/kg)	5 ± 0.3	6 ± 1
Cl <sub>T</sub> (ml/min/kg)	55 ± 5	39 ± 6 <sup>c</sup>
Cl <sub>R</sub> (ml/min/kg)	6 ± 1	3 ± 1 <sup>c</sup>
Cl <sub>NR</sub> (ml/min/kg)	50 ± 5	36 ± 5 <sup>c</sup>
METH dose in urine (%)	10 ± 3	8 ± 2
AMP metabolite		
AUC (ng-min/ml)	3459 ± 926	7025 ± 1625 <sup>c</sup>
t <sub>1/2λz</sub> (min)	95 ± 12 <sup>a</sup>	189 ± 11 <sup>a,c,d</sup>
T <sub>max</sub> (min)	72 ± 27	156 ± 49 <sup>c</sup>
C <sub>max</sub> (ng/ml)	16 ± 4	22 ± 4 <sup>c</sup>
AMP dose in urine (%)	2 ± 0.4	3 ± 0.6
Molar ratio of AMP/METH AUC <sup>b</sup>	0.21 ± 0.05	0.31 ± 0.03 <sup>c</sup>

Pharmacokinetic parameters were determined from model-independent analyses of the METH serum concentration versus time profiles.

<sup>a</sup>Harmonic mean and “pseudo” SD (Lam et al., 1985).

<sup>b</sup>The molar ratio of AMP/METH AUC was achieved by dividing the nmol/ml/hr AMP AUC value by the nmol/ml/hr METH AUC value.

<sup>c</sup>  $p < 0.05$  when compared to GD7 values.

<sup>d</sup>  $p < 0.05$  when compared to METH values on GD21.

**Table 2.** IV AMP pharmacokinetics in timed-pregnant Sprague-Dawley rats following an iv-bolus dose of 1 mg/kg AMP on GD21.

Pharmacokinetic Parameter	
(iv AMP)	GD21
AMP AUC (ng-min/ml)	23952 ± 2909
$t_{1/2\lambda z}$ (min)	108 ± 8 <sup>a</sup>
MRT (min)	141 ± 17
Vd (L/kg)	6 ± 1
Vd <sub>ss</sub> (L/kg)	6 ± 0.4
Cl <sub>T</sub> (ml/min/kg)	41 ± 6
Cl <sub>R</sub> (ml/min/kg)	3 ± 1
Cl <sub>NR</sub> (ml/min/kg)	38 ± 6
AMP dose in urine (%)	7 ± 2

Pharmacokinetic parameters were determined from model-independent analyses of the AMP serum concentration-versus-time profiles.

<sup>a</sup>Harmonic mean and “pseudo” SD (Lam et al., 1985).

**Table 3.** METH and AMP metabolite pharmacokinetics in timed-pregnant Sprague-Dawley rats following an iv-bolus dose of 1, 3 or 5.6 mg/kg METH on GD21.

<b>METH</b>			
	1 mg/kg	3 mg/kg	5.6 mg/kg
AUC (normalized for dose) (ng-min/ml)/(mg/kg)	22565 ± 5278	21832 ± 1163	24756 ± 3212
$t_{1/2\lambda z}$ (min)	110 ± 17 <sup>a</sup>	117 ± 5 <sup>a</sup>	109 ± 19 <sup>a</sup>
MRT (min)	131 ± 14	142 ± 8	127 ± 15
Vd (L/kg)	7 ± 2	7 ± 1	6 ± 2
Vd <sub>ss</sub> (L/kg)	6 ± 1	6 ± 1	5 ± 1
Cl <sub>T</sub> (ml/min/kg)	44 ± 10	44 ± 2	38 ± 6
<b>AMP</b>			
AUC (normalized for dose) [(ng-min/ml)/(mg/kg)]	5369 ± 1532	5370 ± 881	5768 ± 1282
$t_{1/2\lambda z}$ (min)	170 ± 31 <sup>a,c</sup>	180 ± 10 <sup>a,c</sup>	151 ± 17 <sup>a,c</sup>
T <sub>max</sub> (min)	108 ± 27	120 ± 1	108 ± 27
C <sub>max</sub> (ng/ml)	17 ± 3	51 ± 8	129 ± 32
Molar ratio of AMP/METH AUC <sup>b</sup>	0.26 ± 0.02	0.27 ± 0.03	0.26 ± 0.08

Pharmacokinetic parameters were determined from model-independent analyses of the METH and AMP serum concentration-versus-time profiles.

<sup>a</sup> Harmonic mean and “pseudo” SD (Lam et al., 1985).

<sup>b</sup> The molar ratio of AMP/METH AUC was achieved by dividing the nmol/ml/hr AMP AUC value by the nmol/ml/hr METH AUC value.

<sup>c</sup>  $p < 0.05$  vs. METH  $t_{1/2\lambda z}$ .

**Table 4.** Average litter size and pup weight on PND1 for pups exposed prenatally to an iv-bolus dose of saline or METH (1, 3 or 5.6 mg/kg) on GD21 or no treatment (control rats non-catheterized and without METH dosing).

	Average Litter Size	Average Pup Weight (g)
Non-catheterized	13 ± 3	6.9 ± 0.8
Saline-Hematocrit Only	12 ± 2	6.4 ± 0.3
Saline	12 ± 2	6.5 ± 0.6
1 mg/kg METH	12 ± 1	7.1 ± 0.5
3 mg/kg METH	13 ± 1	6.4 ± 0.6
5.6 mg/kg METH	10 ± 2	6.4 ± 0.5

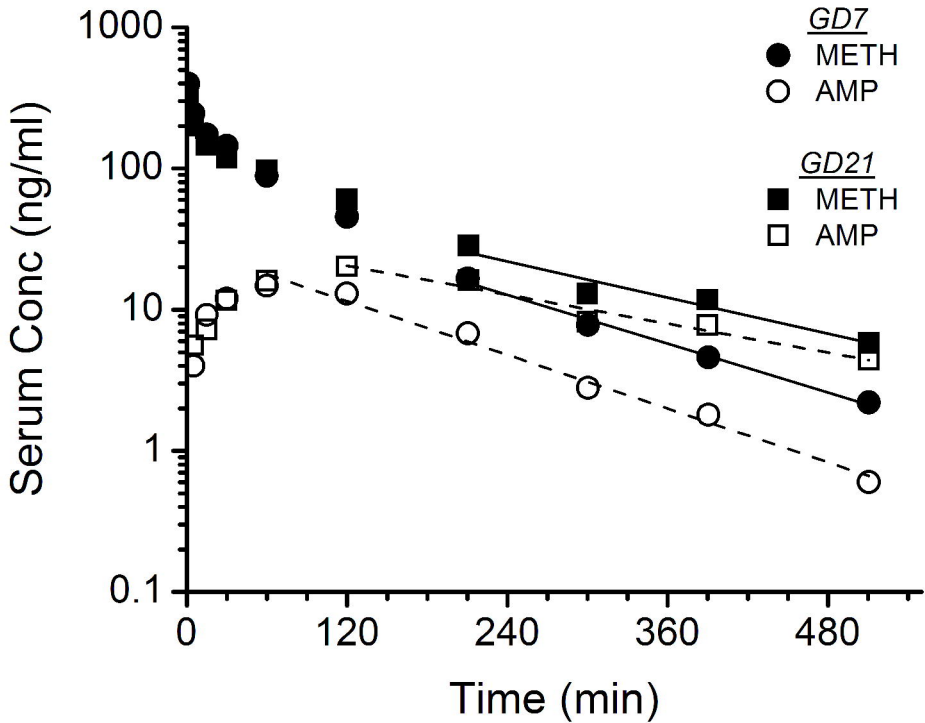


Figure 1



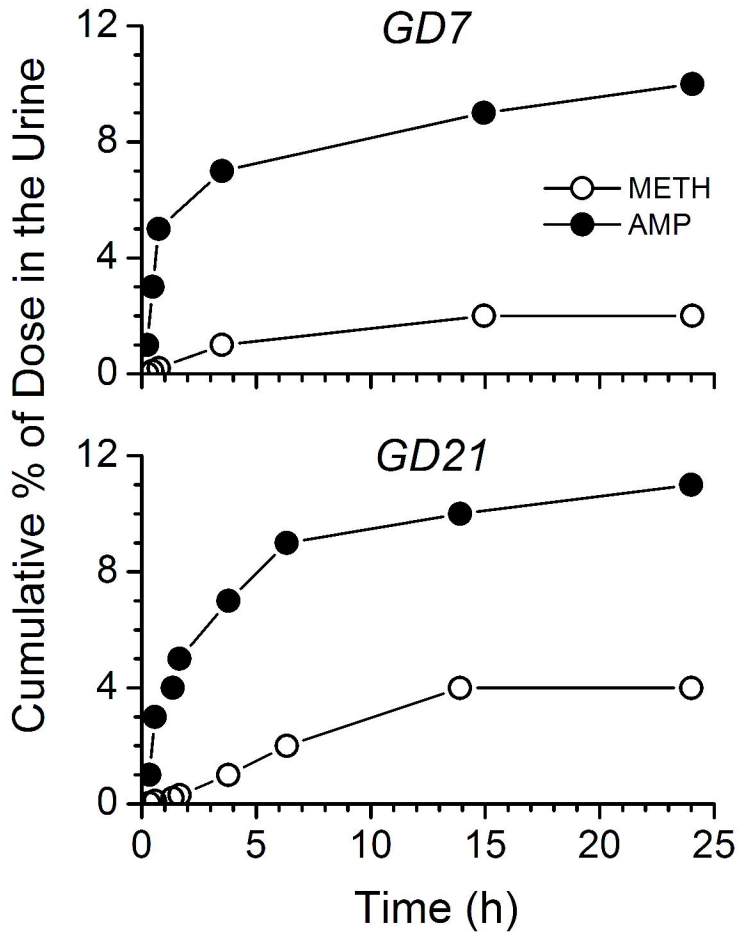


Figure 2

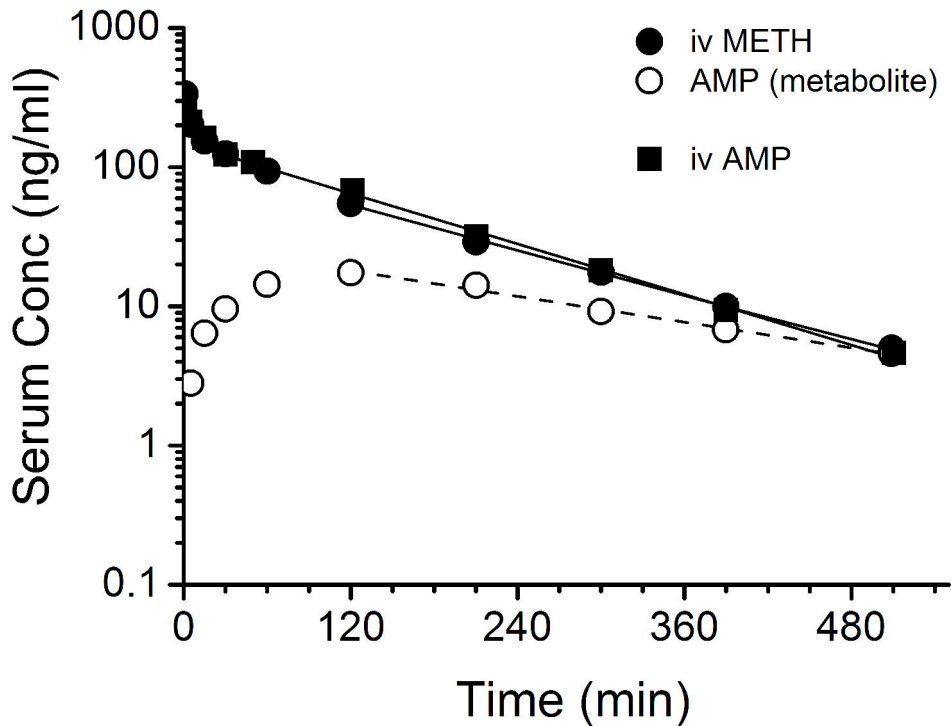


Figure 3

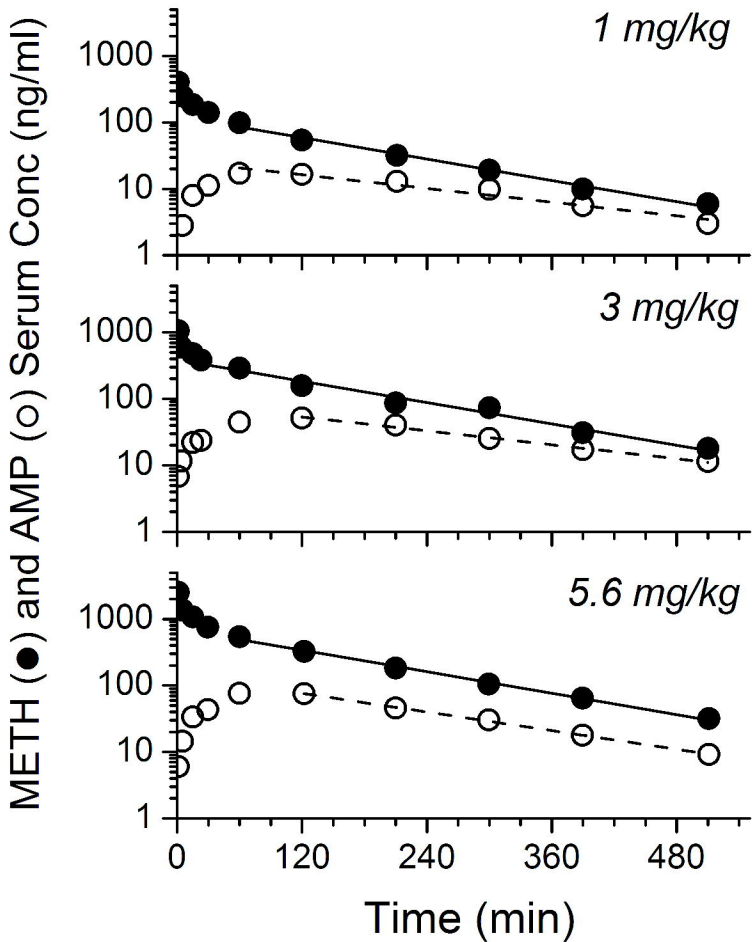


Figure 4

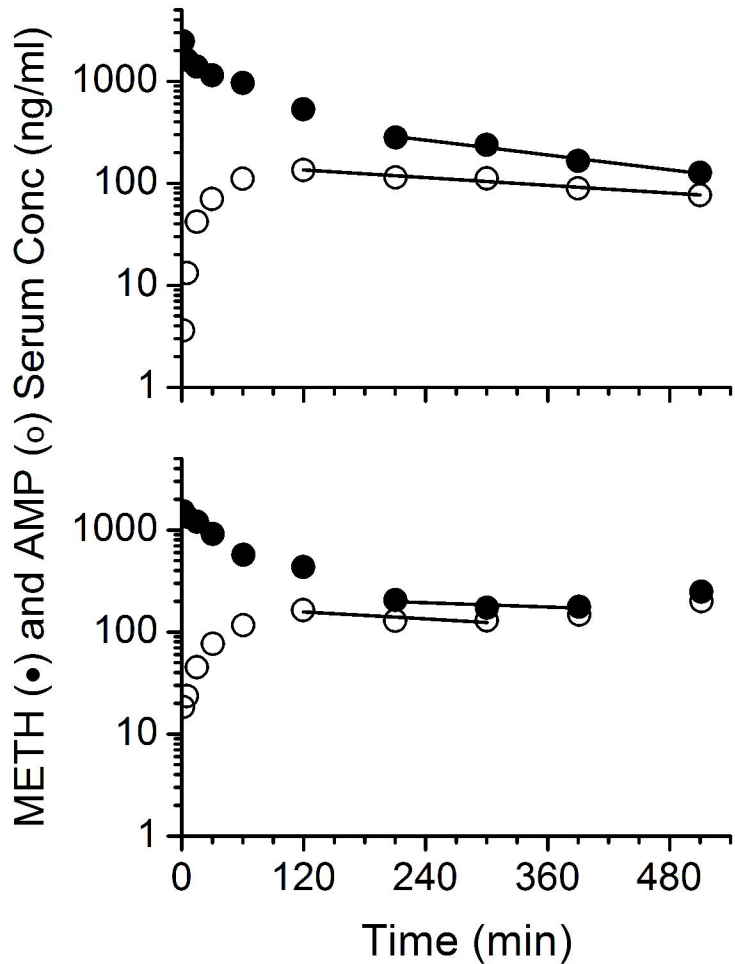


Figure 5