Nonlinear pharmacokinetics of (±)3,4-methylenedioxymethamphetamine (MDMA) and its pharmacodynamic consequences in the rat

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Non-standard abbreviations:

5-HT: serotonin

AUC: area-under-the-curve

C_{max}: maximum concentration

COMT: cathecol-o-methyltransferase

HHMA: (±)-3,4-dihydroxymethamphetamine

HMMA: (±)-4-hydroxy-3-methoxymethamphetamine

LC-MS/MS: liquid chromatography tandem mass spectrometry

MDA: (±)-3,4-methylenedioxyamphetamine

MDMA: (±)-3,4-methylenedioxymethamphetamine

MRM: multiple reaction monitoring

OH-MAMP: 4-hydroxymethamphetamine

QC: quality control

t_{1/2}: elimination half-life

T_{max}: time of maximum concentration
Abstract

3,4-Methylenedioxymethamphetamine (MDMA) is a widely abused illicit drug that can cause severe and even fatal adverse effects. However, interest remains for its possible clinical applications in post-traumatic stress disorder and anxiety treatment. Preclinical studies to determine MDMA’s safety are needed. We evaluated MDMA pharmacokinetics and metabolism in male rats receiving 2.5, 5 and 10 mg/kg subcutaneous MDMA, and the associated pharmacodynamic consequences. Blood was collected via jugular catheter at 0, 0.5, 1, 2, 4, 6, 8, 16 and 24 h, with simultaneous serotonin (5-HT) behavioral syndrome and core temperature monitoring. Plasma specimens were analyzed for MDMA and metabolites (±)-3,4 dihydroxymethamphetamine (HHMA), (±)-4-hydroxy-3-methoxymethamphetamine (HMMA) and (±)-3,4-methylenedioxyamphetamine (MDA) by liquid chromatography-tandem mass spectrometry. After 2.5 mg/kg MDMA, mean maximum MDMA concentration was 164±47.1 ng/ml, HHMA and HMMA were major metabolites, and <20% MDMA was metabolized to MDA. After 5 and 10 mg/kg doses, MDMA areas-under-the-curve (AUC) were 3- and 10-fold greater than those after 2.5 mg/kg; HHMA and HMMA AUC values were relatively constant across doses, while MDA AUC values were greater than dose proportional. Our data provide decisive in vivo evidence that MDMA and MDA display nonlinear accumulation via metabolic auto-inhibition in the rat. Importantly, 5-HT syndrome severity correlated with MDMA concentrations (r=0.8083, p<0.0001), while core temperature correlated with MDA concentrations (r=0.7595, p<0.0001), suggesting MDMA’s behavioral and hyperthermic effects may involve
distinct mechanisms. Given key similarities between MDMA pharmacokinetics in rats and humans, data from rats can be useful when provided at clinically-relevant doses.
Introduction

3,4-Methylenedioxymethamphetamine (MDMA) was originally synthesized by Merck & Co. in 1912. The drug was largely forgotten until the late 1970’s when Shulgin and Nichols reported its unique ‘entactogen’ properties that include euphoria, friendliness, and increased emotional closeness with others (Shulgin AT, 1978). Widespread MDMA misuse during the 1980’s prompted placement of the drug into Schedule I control, banning its sale and use in the United States. Today, MDMA remains a popular illicit drug with estimated worldwide prevalence of 0.2-0.6% of the population aged 15-64 (10.5-28 million users) (UN, 2012). MDMA abuse can be associated with severe or even fatal adverse effects in humans (Walubo and Seger, 1999; Logan, 2001), while drug administration to laboratory animals causes long-term deficits in brain serotonin (5-HT) neurons (Ricaurte et al., 2000). Despite the potential for such risks, there is increasing interest in MDMA as an adjunctive treatment for post-traumatic stress disorder (Mithoefer et al., 2011) and anxiety-related problems (www.clinicaltrials.gov). For these reasons, it is prudent for preclinical investigators to examine MDMA effects at clinically relevant doses and relate these findings to adverse effects at higher doses to establish the margin of safety in animal models.

MDMA exerts its pharmacological effects by interacting with plasma membrane transporter proteins expressed on neurons, causing release of neurotransmitters by reverse transport (Baumann et al., 2007; Verrico et al., 2007). MDMA’s in vivo pharmacology in humans and animals is complicated by extensive hepatic metabolism via two pathways (Maurer et al., 2000; de la Torre et al., 2004). In the major pathway, MDMA is O-demethylenated by cytochrome P450 (CYP) CYP2D6, 1A2, 2B6 and 3A4 in
humans and by CYP2D1 and 3A2 in rats to form (±)-3,4-dihydroxymethamphetamine (HHMA), which is O-methylated to generate (±)-4-hydroxy-3-methoxymethamphetamine (HMMA) by catechol-o-methyl transferase (COMT). In the minor pathway, MDMA is N-demethylated by CYP2B6, 1A2 and 2D6 in humans and CYP1A2 and 2D1 in rats to form (±)-3,4-methylenedioxyamphetamine (MDA). MDA is O-demethylenated by the same enzymes that act on MDMA, with subsequent metabolism by COMT. The hydroxylated metabolites of MDMA are excreted in urine as glucuronide and sulfate conjugates. The main differences between human and rat MDMA metabolism are the specific CYP isoforms involved, shorter MDMA and metabolite half-lives in rats, a tendency for rats to form more MDA than humans and that glucuronide conjugated metabolites predominate in rats (Maurer et al., 2000; de la Torre et al., 2004; Shima et al., 2008).

A clearer understanding of MDMA pharmacodynamics and pharmacokinetics in animals and humans is needed to extrapolate data between species. A wealth of pharmacodynamic data shows that neurochemical, endocrine and behavioral effects of MDMA occur at similar doses in rats and humans (Schechter, 1988; Johanson et al., 2006; Baumann et al., 2007; Kolbrich et al., 2008a). However, few studies have examined MDMA pharmacokinetics in rats given doses <10 mg/kg comparable to those taken by humans (Chu et al., 1996; Starr et al., 2008; Baumann et al., 2009), and just one assessed the relationship between pharmacodynamics and pharmacokinetics (Chu et al., 1996). Most MDMA pharmacokinetic studies in rats investigated doses of 10mg/kg or higher (Valtier et al., 2007; Meyer et al., 2008; Upreti and Eddington, 2008; Mueller et al., 2009). In two rat studies employing doses <10 mg/kg, evidence for
MDMA nonlinear accumulation was reported (Chu et al., 1996; Baumann et al., 2009), similar to the human situation (Mas et al., 1999; de la Torre et al., 2000; Kolbrich et al., 2008b). By contrast, Green et al (Green et al., 2009) and Hirt et al (Hirt et al., 2010) suggested linear MDMA kinetics in rat.

Given the discrepancies in data regarding the occurrence of nonlinear MDMA pharmacokinetics in rodent models, we sought to evaluate this phenomenon and examine its possible pharmacodynamic consequences. We report plasma concentrations of MDMA, HHMA, HMMA and MDA in male rats receiving incremental subcutaneous (sc) MDMA (2.5, 5 and 10 mg/kg). Rats were fitted with indwelling catheters so repeated blood specimens could be collected, while simultaneously measuring 5-HT behavioral syndrome and core body temperature. Our findings reveal that increasing MDMA doses administered to rats produce greater than expected area-under-the curve (AUC) values for MDMA and MDA, while AUC for HHMA and HMMA were lower than expected. These results provide compelling in vivo evidence that MDMA displays nonlinear accumulation in rats via inhibition of metabolism, similar to the effects in humans. 5-HT syndrome severity correlated with MDMA plasma concentrations, but core temperature correlated with MDA, showing distinct contributions of MDMA and its metabolite MDA to the overall pharmacology of administered MDMA.
Materials and methods

Drugs and reagents

Racemic MDMA HCl was received from the National Institute on Drug Abuse, Drug Supply Program (Rockville, MD, USA). MDMA solutions for injection into rats were prepared in sterile 0.9% NaCl (saline) immediately before administration. Reagents for liquid chromatography tandem mass spectrometry (LC-MSMS) assays were analytical grade. Analyte standards at 1mg/ml in methanol were purchased from Cerilliant (Round Rock, TX, USA) and from Lipomed (Cambridge, MA, USA).

Animals and surgery

Male Sprague-Dawley rats weighing 250-300 g were double-housed (lights on: 7:00 AM- 7:00 PM) under controlled temperature (22±2°C) and humidity (45±5%) with free access to food and water. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Vivarium facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and study procedures were approved by the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Committee. After two weeks of acclimation to the vivarium facilities, rats were anesthetized with pentobarbital sodium (60 mg/kg, ip) and indwelling catheters made of Silastic tubing (Dow Corning, Midland, MI, USA) were implanted into the jugular vein as described previously (Baumann et al., 2008). In brief, the proximal end of the catheter was inserted into the right jugular vein and advanced to the atrium, with the distal end exteriorized on the nape and plugged with a metal stylet.
Rats were single-housed post-operatively and allowed one week to recover from surgery.

**Blood sampling procedures**

On the morning of an experiment, rats were moved to the testing room in their home cages and given 1 h to acclimate to surroundings. Feeding trays were removed, and wire lids were placed atop cages. Polyethylene extension tubes (30 cm) were filled with sterile saline, connected to intravenous catheters, and threaded outside the cages. Catheters were flushed with 0.3 ml of 48 IU/ml heparin saline to facilitate blood withdrawal. Groups of 6 to 7 rats received 2.5, 5 or 10 mg/kg MDMA in a volume of 1 ml/kg by the sc route; injections were administered posterior to the shoulder blades on the back. Blood specimens (0.2 ml) were withdrawn before (t=0) and at 0.5, 1, 2, 4, 6, 8, 16 and 24 h after treatments. Blood was collected into 1 ml disposable tuberculin syringes, transferred to 1.5 ml plastic tubes on ice, and spun for 10 min at 1,500 rpm; plasma was decanted and stored at -80°C. An equal volume of sterile saline was infused after each blood withdrawal to maintain volume and osmotic homeostasis.

**5-HT behavioral syndrome and core temperature evaluation**

The occurrence of the 5-HT behavioral syndrome and changes in core temperature were evaluated on the same schedule as specimen collection, with behaviors observed immediately before blood withdrawal and core temperatures measured after. Specific measured symptoms of 5-HT syndrome included flat body posture, forepaw treading, ambulation and head weaving. Before each blood withdrawal, rats were observed for 1 min, and the different elements were scored on a graded scale where 0=absent, 1=equivocal, 2=present, and 3=intense or continuous. Rats were given a single score at
each time point that consisted of the summed scores for all behaviors (i.e., highest possible score of 12). Core temperatures were measured immediately after blood sampling by gently inserting a RET-2 temperature probe (Physitemp Instruments Inc., Clifton, NJ, USA) into the colon.

**MDMA and metabolite plasma analysis**

MDMA, HHMA, HMMA and MDA were measured concurrently in 0.1 ml plasma specimens, with a liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure based on the method described by Mueller et al (Mueller et al., 2007). Eleven calibrators were prepared in blank plasma to yield final concentrations of 5 to 1,500 ng/ml. In addition, three quality control samples (QCs) at 15, 135 and 1,350 ng/ml were prepared in blank plasma. Each calibrator, QC and plasma specimen was spiked with 100 µl internal standard stock solution (50 ng/ml MDMA-d₅, MDA-d₅ and 4-hydroxymethamphetamine, OH-MAMP), and with 20 µl sodium metabisulfite (250 mM) and 10 µl EDTA (250 mM). To hydrolyze samples, 10 µl glucuronidase (*Helix pomatia*, Type HP-2) was added to each tube before incubation at 50°C for 90 min. Samples were cooled at room temperature and 20 µl 4-methylcatechol at 1 g/l was added. Plasma proteins were precipitated with 10 µl perchloric acid. After mixing and centrifugation at 15,000xg at 4°C for 10 min, supernatants (125 µl) were transferred to autosampler vials and 5µl injected into the LC-MSMS.

LC-MSMS analysis was performed with a Shimadzu liquid chromatography system (Shimadzu Corporation, Columbia, MD, USA) interfaced to a 3200 QTrap (AB Sciex, Foster City, CA, USA) with a Turbo V electrospray source. The Shimadzu system consisted of LC-20AD XR pumps, DGU-20A3 degasser, SIL-20AD XR autosampler and
CTO-10AC column oven. Chromatographic separation was performed with a Synergi Polar-RP 100A, 100x2 mm, 4 µm, with a 4x2 mm identically packed guard column (Phenomenex, Torrance, CA, USA) and gradient elution included mobile phase A (4 mM ammonium formate pH 3.4 with 1% formic acid) and mobile phase B (acetonitrile) at a flow rate of 0.5 ml/min. The initial mixture (60A:40B) was maintained for 3.7 min, mobile phase B was increased to 90% at 5 min and held for 2.5 min. The mixture returned to initial conditions at 8 min, followed by 3 min equilibration. The total run time was 11 min. Mass spectrometric data were acquired in positive electrospray ionization mode with the following source parameters: IonSpray voltage 3,000V; temperature 500°C; curtain gas 25; ion source gas1 50 and ion source gas2 75. Data were recorded in multiple reaction monitoring mode (MRM). The following transitions were monitored (quantification transition in bold): 194.2->163.2 and 194.2->105.1 for MDMA; 180.1->132.9 and 180.1->105 for MDA; 182.1->151 and 182.1->122.9 for HHMA; 196.2->165.1 and 196.2->105.2 for HMMA; 199.1->165.1 and 199.1->107.2 for MDMA-d5; 185.1->110.1 and 185.1->138.2 for MDA-d5, and 166.1->107 and 166.1->135.1 for OH-MAMP. The ratio of these transitions also was required to be within ±20% of the average ratio of calibrators. Linearity range with 1/x weighting was from 5 to 1,500 ng/ml. The lower limit of quantification was 5 ng/ml, and the limit of detection was 1 ng/ml for HMMA and HHMA and 2.5 ng/ml for MDMA and MDA. Assay accuracy at low, medium and high QCs was 95.8-107.1% (n=10) and imprecision 1.6-10.2% (n=10). Extraction efficiency was 89-114% at three QC concentrations for all compounds (n=5), and no matrix effect was observed for any compound in any QC (n=5), except ion enhancement for low MDA QC.
(30.1%). Samples were stable after 72 h on the autosampler, and there was no
evidence of carryover in negative injections after a sample spiked at 3,000 ng/ml.

Data analysis and statistics

Pharmacokinetic and pharmacodynamic data were tabulated, analyzed and graphically
depicted using GraphPad Prism (version 5.02; GraphPad Software, Inc., San Diego,
CA, USA). Plasma pharmacokinetic data were further analyzed using WinNonlin
(version 5.2; Pharsight, Mountain View, CA, USA) to determine noncompartmental
pharmacokinetic constants including maximum concentration (C<sub>max</sub>), time of maximum
concentration (T<sub>max</sub>), area-under-the-curve (AUC), and elimination half-life (t<sub>1/2</sub>). At least
three data points on the descending linear limb of the time-concentration curve were
employed to calculate t<sub>1/2</sub> values. To evaluate possible nonlinearity for plasma analyte
concentrations, AUCs following 2.5 mg/kg were multiplied by two and four to calculate
expected AUC values for 5 and 10 mg/kg doses, respectively. The expected values
were compared to observed results at each MDMA dose. Expected versus observed
AUC data were compared at each dose by unpaired t-tests. Behavioral and temperature
data were evaluated by two-way analysis of variance (ANOVA) (treatment x time),
followed by Bonferroni post hoc test. In order to examine potential relationships
between pharmacokinetic and pharmacodynamics endpoints, Pearson’s correlation
coefficients were calculated using data from individual rats treated with 10 mg/kg
MDMA. Specifically, coefficients were generated for MDMA and MDA versus syndrome
score and temperature. P<0.05 was considered the minimal criterion for statistical
significance.
Results

Pharmacokinetics of MDMA, MDA, HHMA and HMMA

Figure 1 shows the time-concentration profiles for plasma MDMA and its N-demethylated metabolite MDA after sc administration of 2.5, 5 and 10 mg/kg MDMA. Pharmacokinetic constants derived from the MDMA curves are summarized in Table 1. MDMA $C_{\text{max}}$ was 164.1±47.1 ng/ml after 2.5 mg/kg MDMA, 2-fold higher after 5 mg/kg MDMA, and 5-fold higher after 10 mg/kg MDMA. $T_{\text{max}}$ was 0.6±0.2 h after 2.5 mg/kg, 0.9±0.6 h after 5 mg/kg, and 1.1±0.4 after 10 mg/kg. MDA plasma concentrations were much lower than MDMA after all administered doses, and MDA had a longer half-life than MDMA. MDA $C_{\text{max}}$ was 15.4±3.7 ng/ml after 2.5 mg/kg MDMA, and increased 3.7- and 12.6-fold after 5 and 10 mg/kg MDMA, respectively. With increasing doses of MDMA, the ratio of MDA:MDMA rose markedly such that MDA AUC was 17% of MDMA AUC after 2.5 mg/kg, 30% after 5 mg/kg and 40% after 10 mg/kg.

As depicted in Figure 2, the observed AUC values for MDMA after 5 and 10 mg/kg were greater than dose proportional, based on linear extrapolation from the 2.5 mg/kg dose. In the case of linear pharmacokinetics, AUC should increase 2-fold when the dose is increased from 2.5 to 5 mg/kg, and 4-fold when the dose is increased from 2.5 to 10 mg/kg. However, we found that MDMA AUC after 5 and 10 mg/kg were 3- and 10-fold greater than AUC after 2.5 mg/kg. The observed AUC values for MDMA were significantly greater than the expected AUC at 5 mg/kg ($t=4.507$, df=10; $p<0.01$) and 10 mg/kg ($t=8.332$, df=12; $p<0.0001$). The augmented AUCs, especially at 10 mg/kg, indicate that MDMA displays nonlinear kinetics at high doses in rats. MDMA $t_{1/2}$ was about 1 h after 2.5 and 5 mg/kg doses, but close to 2 h after 10 mg/kg, demonstrating
prolonged MDMA elimination times at high doses. A similar situation was observed for MDA AUC values, where observed AUC was significantly greater than expected after 5 mg/kg (t=4.557, df=11; p<0.001) and 10 mg/kg (t=9.789, df=12; p<0.0001).

Figure 3 illustrates time-concentration profiles for plasma HHMA and HMMA in rats receiving 2.5, 5 and 10 mg/kg MDMA. Pharmacokinetic constants are summarized in Table 1. HHMA Cmax was 61.1±11.2 ng/ml after 2.5 mg/kg MDMA, 93.4±29.9 ng/ml after 5 mg/kg MDMA, and 102.7±35.1 ng/ml after 10 mg/kg MDMA. HMMA Cmax values were 159.1±46.9, 212±74.6, and 276±100.6 ng/ml after 2.5, 5, and 10 mg/kg MDMA, respectively. Cmax only increased 1.3- and 1.7-fold for HHMA and HMMA as dose of MDMA increased 4-fold from 2.5 to 10 mg/kg. The concentration of both metabolites increased slowly over time, reaching Tmax at 4 h for HHMA and 5 h for HMMA. After the 2.5 mg/kg dose of MDMA, HHMA and HMMA AUC values were higher than those for MDMA. However, after the 10 mg/kg dose of MDMA, HHMA and HMMA AUC were lower than MDMA AUC.

As depicted in Figure 4, the observed AUC values for HHMA and HMMA were much lower than expected as MDMA dose increased. More specifically, observed AUC for these metabolites after 5 and 10 mg/kg MDMA were less than 2-fold above those after 2.5 mg/kg MDMA. The observed AUC for HHMA was significantly lower than expected at 5 mg/kg (t=2.534, df=11; p<0.02) and 10 mg/kg (t=6.642, df=12; p<0.0001), and similar data were found for HMMA. Collectively, the metabolite data indicate an impaired ability to form HHMA after high-dose MDMA, which could underlie the elevated MDMA concentrations and longer MDMA t½ seen at the 10 mg/kg drug dose.

Pharmacodynamic effects
Figure 5 shows the time-effect curves for 5-HT behavioral syndrome and core body temperature for rats that received 2.5, 5 and 10 mg/kg MDMA. The occurrence of 5-HT syndrome was significantly affected by MDMA dose ($F_{3,207}=35.72, p<0.0001$), and there was significant dose x time interaction ($F_{24,207}=5.92, p<0.0001$). Syndrome scores were significantly greater than those of saline-treated controls at 0.5h after 2.5 mg/kg MDMA ($p<0.05$), and at 0.5 and 1 h after 5 mg/kg MDMA ($p<0.05$). The most robust effects were observed after 10 mg/kg, where 5-HT syndrome was significantly elevated above control levels for 4 h post-injection ($p<0.05$). It is noteworthy that the time course for behavioral activation seemed to track with plasma MDMA concentration (compare Figures 1 and 5). Core temperature was significantly influenced by MDMA dose ($F_{3,207}=44.59, p<0.0001$) and there was a significant dose x time interaction ($F_{24,207}=4.602, p<0.001$). The 2.5 mg/kg MDMA dose did not affect temperature, but 5 mg/kg produced significant hyperthermia after 4 and 6 h ($p<0.05$). At the 10 mg/kg MDMA dose, core temperature was elevated above control levels at 2, 4, 6 and 8 h post-injection. The time course of hyperthermic effects displayed a slow onset, and appeared to track with plasma MDA concentrations (compare Figures 1 and 5).

The design of our experiments (i.e., repeated blood sampling from freely-behaving rats) allowed us to evaluate potential relationships between pharmacokinetic and pharmacodynamic endpoints for individual subjects. We chose to examine correlations between plasma concentrations of MDMA or MDA with behavior and temperature at the 10 mg/kg dose because this treatment produced robust pharmacodynamic effects in all subjects. Figure 6 shows that plasma MDMA was significantly correlated with 5-HT syndrome (Pearson’s $r=0.8083, p<0.0001$) but not
temperature. By contrast, Figure 7 reveals that plasma MDA was significantly correlated with temperature \((r=0.7595, p<0.0001)\) but not 5-HT syndrome. These findings suggest behavioral and hyperthermic effects of MDMA may involve distinct mechanisms.
Discussion

We examined MDMA pharmacokinetics and metabolism in rats given increasing MDMA doses, and assessed the relationship between pharmacokinetics and pharmacodynamic effects. Injections were administered sc because this route engenders the slowest possible MDMA kinetics in rats, and is most similar to the po route in humans (Baumann et al., 2009). Additionally, the bulk of MDMA neurotoxicity studies in rats employed the sc route, making our findings directly comparable to the literature (Ricaurte et al., 2000). Few investigations in rats examined MDMA pharmacokinetics after doses similar to those taken by humans (i.e., 1-3 mg/kg), and only a handful of studies assessed the effects of dose on MDMA pharmacokinetic and pharmacodynamic parameters (Chu et al., 1996; Baumann et al., 2009).

There are discrepancies in whether or not MDMA follows non-linear pharmacokinetics in rats as documented in humans, monkeys and mice (de la Torre et al., 2000; Kolbrich et al., 2008b; Mueller et al., 2008; Scheidweiler et al., 2011). Green et al (Green et al., 2009; Green et al., 2012) compared literature values for plasma MDMA concentrations following high-dose MDMA administration in rats (i.e., 5-20mg/kg via ip, sc or po routes) to those produced by low-dose administration in humans (i.e., 0.5-2mg/kg, po). From this comparison, the authors concluded MDMA doses in rats must be 4-fold higher than human doses to achieve similar peak plasma MDMA concentrations and that MDMA pharmacokinetics are linear in rats. Since there are published findings contradicting both of these conclusions (Chu et al., 1996; Delaforge et al., 1999; Starr et al., 2008; Baumann et al., 2009), we sought to investigate these issues in more detail.
After sc administration of 2.5 mg/kg MDMA to rats, we found that MDMA $C_{\text{max}}$ was 164 ng/ml, a concentration similar to those reported for human males receiving 1.3-1.6 mg/kg po MDMA in laboratory settings (de la Torre et al., 2000; Kolbrich et al., 2008b). Starr et al. (Starr et al., 2008) reported plasma MDMA $C_{\text{max}}$ of 300 ng/ml in male Long-Evans rats given ip injections of 3 mg/kg MDMA, and this concentration is in line with the findings reported here and elsewhere (Baumann et al., 2009). The similarities between MDMA $C_{\text{max}}$ in rats and humans support the shared sensitivity of both species to the same drug doses in vivo (Schechter, 1989; Johanson et al., 2006; Baumann et al., 2007; Kolbrich et al., 2008a). On the other hand, MDMA $t_{1/2}$ in rat plasma after a low dose (2.5 mg/kg) is 1 h, a much shorter interval than 8-9 h observed in humans receiving oral doses (Mas et al., 1999; de la Torre et al., 2000; Kolbrich et al., 2008b). Due to rapid MDMA clearance in rats, repeated sc injections of low-dose MDMA in this species might be an acceptable model for po doses administered to humans, but this hypothesis needs to be tested. In any case, the present findings do not support the notion that rats must receive 4-fold higher doses of MDMA to achieve peak plasma concentrations similar to those observed in humans (Green et al., 2009).

Previous studies showed that HHMA and HMMA are formed in vivo after MDMA administration in both rats and humans (Lim et al., 1992; Segura et al., 2001; de la Torre et al., 2004; Valtier et al., 2007; Goni-Allo et al., 2008; Kolbrich et al., 2008b; Baumann et al., 2009; Mueller et al., 2009), but there is uncertainty about whether these hydroxylated metabolites represent major MDMA metabolites in rodents. Here we observed that AUC for HHMA and HMMA were always greater than AUC for MDMA in rats given 2.5 mg/kg, confirming that $O$-demethylenation is the predominant pathway for
biotransformation in rats given clinically-relevant MDMA doses. Following 2.5 mg/kg
MDMA, MDA $C_{\text{max}}$ was only 15 ng/ml and its AUC represented only 17% of MDMA
AUC, documenting that $N$-demethylation is a secondary metabolic pathway in rats
treated with low-dose MDMA. Nevertheless, humans typically convert less than 10% of
MDMA to MDA (de la Torre et al., 2004), so our results support the contention that rats
produce more MDA than humans, even at low MDMA doses. MDA is an important
bioactive metabolite of MDMA that acts as a potent monoamine releaser and agonist at
5-HT$_{2B}$ receptors (Baumann and Rothman, 2009).

The first published report of nonlinear MDMA pharmacokinetics in rats appeared
in 1996 (Chu et al., 1996). Chu et al. found that sc administration of 5-40 mg/kg (+)-
MDMA produced elevations in plasma and brain MDMA concentrations that were in far
greater proportionally than increases in administered dose, suggesting nonlinear drug
accumulation. No putative mechanism to explain the observed nonlinearity was
proposed in that initial report. In our previous study in rats, we showed that MDMA AUC
after 10 mg/kg was 10-fold greater than AUC after 2 mg/kg, while HMMA AUC did not
differ. Those data provided circumstantial evidence for inhibition of MDMA metabolism,
but the most direct $O$-demethylenated metabolite HHMA was not quantified in previous
work. In the current experiments, we validated a method by LC-MS/MS (Mueller et al,
2007) to determine concentrations of MDMA, HHMA, HMMA and MDA in small volume
plasma specimens from rats. Our data showed that AUC for HHMA and HMMA
increased less than 2-fold as MDMA dose increased from 2.5 to 10 mg/kg. In the same
rats, AUC for MDMA increased 10-fold. Collectively, these findings provide decisive $in$
vivo evidence that MDMA displays nonlinear pharmacokinetics in the rat, most likely
due to metabolic inhibition. If MDMA pharmacokinetics were linear, we should have observed AUC increases proportional to dose. We also observed evidence for robust nonlinear accumulation of MDA. As MDMA doses increased from 2.5 to 10 mg/kg, MDA AUC increased 24-fold. MDMA is O-demethylenated to form HHMA mainly by CYP2D6 in humans, and interaction of the drug with the enzyme causes irreversible inhibition of activity, thereby blocking the major pathway of MDMA biotransformation (Wu et al., 1997; de la Torre and Farre, 2004). At molecular level, MDMA is capable of forming an inhibitory metabolite complex with human CYP2D6, and with the rat homologue CYP2D1 (Delaforge et al., 1999; Heydari et al., 2004), suggesting a common mechanism may underlie sustained metabolic inhibition in humans and rats. In fact, it is well established that the methylenedioxy moiety found in the structure of MDMA and MDA can inhibit various enzymes, including rat CYP isoforms (Brady et al., 1986; Murray, 1997). It would be interesting to determine whether rats pretreated with high-dose MDMA have an impaired ability to O-demethylenate subsequent MDMA doses, as has been shown in humans (Farre et al., 2004).

In the present study, we evaluated two established MDMA pharmacodynamic measures, the 5-HT behavioral syndrome and core temperature, and the relationship between these pharmacodynamic effects and MDMA pharmacokinetics. Interestingly, neither of these endpoints was markedly altered by the 2.5 mg/kg dose of MDMA, a dose that generated clinically relevant amounts of MDMA in plasma. Previous studies showed that sc doses of 1-3 mg/kg MDMA induce large elevations in extracellular 5-HT in the brain (Baumann et al., 2008; Starr et al., 2008), yet these doses do not induce robust non-contingent behaviors or hyperthermia. By contrast, the 10 mg/kg dose of
MDMA produced large and sustained increases in 5-HT syndrome and core temperature. We observed differential relationships between plasma MDMA, plasma MDA, and the pharmacodynamic endpoints after administration of high-dose MDMA. Specifically, plasma MDMA concentrations were positively correlated with 5-HT syndrome, whereas plasma MDA concentrations were positively correlated with core temperature. The correlative data indicate that the biological mechanisms underlying these two responses are distinct. Rodisiri et al (Rodisiri et al., 2011) reported that the change in locomotor activity and the magnitude of the hyperthermia appeared to be unrelated both to each other and to the magnitude of MDMA induced 5-HT release. Previous studies suggested that MDMA hyperthermia may result from increase release of dopamine rather than 5-HT (Mechan et al., 2002). Our findings point to the nonlinear accumulation of MDA as a critical variable in mediating hyperthermia induced by high-dose MDMA administration. Future studies should be carried out to address this hypothesis in more detail.

To summarize, we demonstrated that sc administration of 2.5 mg/kg MDMA to rats produces peak plasma MDMA concentrations comparable to those observed in humans receiving recreational doses. After this low dose in the rat, HHMA and HMMA AUC exceeded that of MDMA, implicating HHMA and HMMA as major MDMA metabolites under these conditions. After high-dose MDMA administration, MDMA AUC exceeds that of HHMA and HMMA because plasma MDMA concentrations increase nonlinearly in conjunction with an impaired ability to form HHMA due to metabolic inhibition. As MDMA dose increases above 2.5 mg/kg, a larger percentage of the administered dose is shunted to the N-demethylation pathway resulting in greatly
enhanced formation of MDA. MDMA pharmacokinetics correlated with 5-HT syndrome severity while MDA correlated with core temperature, suggesting nonlinear accumulation of MDA could be a critical factor contributing to dangerous hyperthermia. Our findings indicate that MDMA studies in rats can generate clinically meaningful results if the dose and route of administration are carefully chosen.
Authorship Contributions

Participated in research design: Baumann, Scheidweiler, Rothman, Huestis

Conducted experiments: Concheiro, Baumann, Scheidweiler, Marrone

Contributed new reagents or analytical tools: Concheiro, Scheidweiler

Performed data analysis: Concheiro, Baumann, Scheidweiler, Huestis

Wrote or contributed to the writing of the manuscript: Concheiro, Bauman, Rothman, Scheidweiler, Huestis
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Footnotes:

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Figure legends

Fig. 1. Time-concentration profiles for MDMA (panel A) and MDA (panel B) in groups of rats receiving sc injections of 2.5, 5 and 10 mg/kg MDMA. Rats received MDMA at time 0 and blood specimens were collected via indwelling jugular catheters at 0.5, 1, 2, 4, 6, 8, 16 and 24 h after dosing. Analytes were assayed in plasma by LC-MSMS. Data are mean±SEM for N=6-7 rats/group.

Fig. 2. Expected (EXP) versus observed (OBS) area-under-the-curve (AUC) values for MDMA (panel A) and MDA (panel B) at 5 and 10 mg/kg MDMA doses. Expected values were calculated by multiplying the observed AUC at 2.5 mg/kg by the proportionate increase in dose. Values are the mean±SEM for N=6-7 rats/group. *Significant difference at p<0.05.

Fig. 3. Time-concentration profiles for HHMA (panel A) and HMMA (panel B) in groups of rats receiving sc injections of 2.5, 5 and 10 mg/kg MDMA. Rats received MDMA at time 0 and blood specimens were collected via indwelling jugular catheters at 0.5, 1, 2, 4, 6, 8, 16 and 24 h after dosing. Analytes were assayed in plasma by LC-MSMS. Data are mean±SEM for N=6-7 rats/group.

Fig. 4. Expected (EXP) versus observed (OBS) area-under-the-curve (AUC) values for HHMA (panel A) and HMMA (panel B) at 5 and 10 mg/kg MDMA doses. Expected values were calculated by multiplying the observed AUC at 2.5 mg/kg by proportionate increase in dose. Values are the mean±SEM for N=6-7 rats/group. *Significant difference at p<0.05.
Fig. 5. Time-effect curves for 5-HT syndrome (panel A) and core temperature (panel B) in rats receiving sc injections of 2.5, 5 and 10 mg/kg MDMA. Rats received MDMA at time 0; syndrome score and colonic temperature were monitored at 0.5, 1, 2, 4, 6, 8, 16 and 24 h after dosing. Data are mean±SEM for N=6-7 rats/group.

Fig. 6. Relationships between plasma MDMA concentration and 5-HT syndrome (panel A) or core temperature (panel B). Data from individual rats receiving 10 mg/kg MDMA were used to construct plots. Only data from time points post-injection were used (56 points/plot). Pearson’s correlation coefficient was calculated for each data set.

Fig. 7. Relationships between plasma MDA concentration and 5-HT syndrome (panel A) or core temperature (panel B). Data from individual rats receiving 10 mg/kg MDMA were used to construct plots. Only data from time points post-injection were used (56 points/plot). Pearson’s correlation coefficient was calculated for each data set.
Table 1. Pharmacokinetic constants (mean±SD) for plasma MDMA, MDA, HHMA, HMMA and HMA after subcutaneous administration of 2.5, 5 and 10 mg/kg to rats.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MDMA Dose</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>AUC (h*ng/ml)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDMA</td>
<td>2.5 (n=7)</td>
<td>164.1±47.1</td>
<td>0.6±0.2</td>
<td>272.1±71.6</td>
<td>1.1±0.9</td>
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<td>5 (n=6)</td>
<td>370.8±41</td>
<td>0.9±0.6</td>
<td>879.1±133.2</td>
<td>0.9±0.1</td>
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<td>10 (n=7)</td>
<td>893.9±90.7</td>
<td>1.1±0.4</td>
<td>2879.9±491.5</td>
<td>2±0.6</td>
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<tr>
<td>MDA</td>
<td>2.5 (n=7)</td>
<td>15.4±3.7</td>
<td>1.9±0.4</td>
<td>47.4±23.5</td>
<td>1.2±0.2</td>
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<td>5 (n=6)</td>
<td>57.5±11.9</td>
<td>3±1.1</td>
<td>262.3±83.3</td>
<td>1.7±0.9</td>
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<td>10 (n=7)</td>
<td>193.7±43.6</td>
<td>3.7±0.8</td>
<td>1157.9±244.2</td>
<td>1.8±0.2</td>
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<tr>
<td>HHMA</td>
<td>2.5 (n=7)</td>
<td>61.1±11.2</td>
<td>3.7±1.8</td>
<td>531.6±97.3</td>
<td>4.1±1.4</td>
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<tr>
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<td>5 (n=6)</td>
<td>93.4±29.9</td>
<td>4±1.3</td>
<td>789.6±193.7</td>
<td>3.7±0.9</td>
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<td>10 (n=7)</td>
<td>102.7±35.1</td>
<td>5.1±2.5</td>
<td>969.9±246.7</td>
<td>3.1±0.9</td>
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<tr>
<td>HMMA</td>
<td>2.5 (n=7)</td>
<td>159.1±46.9</td>
<td>5.4±1.0</td>
<td>1032.4±160.5</td>
<td>2.7±1</td>
</tr>
<tr>
<td></td>
<td>5 (n=6)</td>
<td>212±74.6</td>
<td>5±1.1</td>
<td>1442.4±429.9</td>
<td>2.9±0.3</td>
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<tr>
<td></td>
<td>10 (n=7)</td>
<td>276±100.6</td>
<td>7.1±1.1</td>
<td>2132.5±654.3</td>
<td>2.7±0.5</td>
</tr>
</tbody>
</table>
Figure 1

(A) MDMA concentrations (ng/ml) over time (h) for different doses: 
- MDMA 2.5 mg/kg (closed circles)
- MDMA 5.0 mg/kg (open squares)
- MDMA 10 mg/kg (open triangles)

(B) MDA concentrations (ng/ml) over time (h) for different doses:
- MDMA 2.5 mg/kg (closed circles)
- MDMA 5.0 mg/kg (open squares)
- MDMA 10 mg/kg (open triangles)
Figure 4

A

HHMA

AUC (ng/ml*h)

5-EXP  5-OBS  10-EXP  10-OBS

B

HMMA

AUC (ng/ml*h)

5-EXP  5-OBS  10-EXP  10-OBS

* indicates statistically significant difference.
Figure 5

A

Vehicle

MDMA 2.5 mg/kg

MDMA 5.0 mg/kg

MDMA 10 mg/kg

Syndrome score

Time (h)

B

Temperature (°C)

Time (h)
Figure 6

A

Syndrome Score

r = 0.8083
p < 0.0001

MDMA (ng/ml)

B

Temperature (°C)

r = 0.1353
NS

MDMA (ng/ml)
Figure 7

A

Syndrome Score

r = 0.1598
NS

MDA (ng/ml)

B

Temperature (°C)

r = 0.7595
p < 0.0001

MDA (ng/ml)