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Hydrolysis of 3,4-methylenedioxymethamphetamine (MDMA) metabolite conjugates in human, squirrel monkey, and rat plasma

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Abstract

Characterizing the formation of metabolites of 3,4-methylenedioxymethamphetamine (MDMA, “Ecstasy”) in different species (rat, squirrel monkey, and human) may provide insight into mechanisms of MDMA neurotoxicity. Two prominent MDMA metabolites, 3,4-dihydroxymethamphetamine (HHMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA), are conjugated with glucuronic or sulfuric acid, but reference standards are not available; therefore, quantification is only possible after conjugate cleavage. Different concentrations of HHMA and HMMA were obtained in human, squirrel monkey, and rat plasma specimens when acid or enzymatic cleavage was performed. Our data document that these differences are due to species-specific influences on conjugate cleavage. Acidic hydrolysis should be used for analyzing free HHMA and HMMA in human or squirrel monkey plasma, while enzymatic hydrolysis with glucuronidase or sulfatase maximizes recovery of free HHMA and HMMA in rat plasma. Optimization of cleavage conditions showed that sulfate conjugates were more readily cleaved by acid hydrolysis and glucuronides by glucuronidase.

Keywords

MDMA; Metabolites; LC-ESI-MS; Hydrolysis; Human; Squirrel monkey; Rat; Plasma

Introduction

3,4-Methylenedioxymethamphetamine (MDMA; “Ecstasy”) is a psychotropic drug that has gained popularity as a recreational drug [1, 2]. Abuse of MDMA is sometimes associated with severe reactions such as malignant hyperthermia [3–6]. In addition, there is considerable evidence to indicate that MDMA has neurotoxic potential [1, 2, 7–10]. In rats, squirrel monkeys, rhesus monkeys, and baboons, MDMA damages primarily serotonergic nerve endings, while in mice, it selectively destroys dopaminergic nerve endings [11].

MDMA metabolism may have an important role in MDMA neurotoxicity, as direct injection of MDMA into the brain fails to reproduce neurotoxic effects seen after systemic administration [12]. A report that alteration of cytochrome P450 (CYP450) mediated MDMA metabolism influences neurotoxicity is also often cited [13]. However, in that study, the tested CYP450 modulators affected acute (3 h) but not long-term (1 wk) MDMA-induced serotonin depletions.

MDMA metabolism proceeds via two pathways. The first involves *O*-demethylenation to 3,4-dihydroxymethamphetamine (HHMA) followed by *O*-methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA) and subsequent *O*-conjugation with sulfate or glucuronic acid [13–15]. HHMA and 3,4-dihydroxyamphetamine can also be oxidized to quinones which, in turn, can form adducts with glutathione and other thiols [16–18]. Recently, such adducts have been implicated in MDMA neurotoxicity [19]. The second metabolic pathway entails initial *N*-demethylation to 3,4-methylenedioxyamphetamine (MDA), followed by deamination and oxidation to the corresponding benzoic acid derivatives that are subsequently conjugated with glycine [13]. Both pathways are operant in all species [11]. However, the first pathway seems to be predominant in primates (humans, squirrel monkeys, rhesus monkeys) [11, 18, 19], whereas the second pathway is more active in rodents (rats, mice).

The above-mentioned species differences in neurotoxicity also suggest that species differences in drug metabolism may be an important factor. Indeed, comparing metabolic patterns in different species may improve understanding of differences in neurotoxicity and may provide insight into mechanisms underlying MDMA neurotoxicity.

Methods for simultaneous quantification of MDMA and its metabolites (including glucuronide and sulfate conjugates of MDMA metabolites) have been described [20, 21]. However, the conjugates are measured indirectly (by determination of the respective precursor metabolites after conjugate cleavage) because glucuronide and sulfate conjugates are not amenable to gas chromatography (GC)-based analysis and, more importantly, because reference standards of such conjugates are not commercially available. The two most common methods of cleaving these conjugates are enzymatic hydrolysis with β -glucuronidase/arylsulfatase and acidic hydrolysis with hydrochloric acid [22]. Recently, we compared a limited number of human, squirrel monkey, and rat plasma specimens employing either enzymatic hydrolysis as described by Mueller et al. [23] or acidic hydrolysis described by Kolbrich et al. [21]. After liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis (for details, see Mueller et al. [23]), both cleavage methods yielded comparable results with respect to MDMA and MDA but considerably different results with respect to HHMA and HMMA. For the latter two

analytes, higher concentrations were found in human and squirrel monkey plasma after acidic hydrolysis, while in rat plasma, higher concentrations were found after enzymatic cleavage.

The purpose of the present study was 2-fold: (1) to carry out a systematic evaluation of the above-described discrepancies and determine species-specific influences on conjugate cleavage; and (2) to optimize cleavage conditions for HHMA and HMMA conjugates in plasma of human, squirrel monkey, and rat.

Experimental

Chemicals, reagents, and solutions

Methanolic solutions (1,000 mg/l) of racemic HMMA and methanolic solutions (100 mg/l) of racemic MDMA-*d*₅ and MDA-*d*₅ were obtained from Cerilliant (Round Rock, TX, USA); HHMA hydrochloride and methanolic solutions of racemic MDMA hydrochloride and MDA hydrochloride were from Lipomed (Cambridge, MA, USA). 4-Hydroxymethamphetamine (pholedrine), 4-methylcatechol, ethyl-enediamine tetraacetic acid disodium salt dihydrate (EDTA), glucuronidase type HP-2 from *Helix pomatia* (glucuronidase activity, 115,800 units/ml and sulfatase activity, 880 units/ml; enzyme code (EC), 232-606-8), and sulfatase type H-2 from *H. pomatia* (sulfatase activity, 2,490 units/ml and glucuronidase activity, 276,600 units/ml; EC, 232-772-1) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Unit definitions for both enzyme solutions are as follows: at pH 5.0 and 37 °C, one unit of β -D-glucuronidase liberates 1.0 μ g of phenolphthalein from phenolphthalein glucuronide per hour, while one unit of sulfatase hydrolyzes 1.0 μ mol of 4-nitrocatechol sulfate (PNCS) per hour. Racemic MDMA hydrochloride was received from the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA), and its identity was confirmed by means of gas chromatography-mass spectrometry. Ammonium formate was obtained from Fluka (Steinheim, Germany), sodium metabisulfite (SMBS) from E. Merck (Darmstadt, Germany), formic acid and acetonitrile from Fischer Scientific (Fair Lawn, NJ, USA), and perchloric acid (PCA) from J.T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid was purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade or highest purity available.

Human plasma specimens

Two different batches of pooled human plasma specimens positive for MDMA were provided by Chemistry and Drug Metabolism, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA. One batch contained low, the other high, analyte concentrations following controlled MDMA administration. Participants provided informed consent for this Institutional Review Board-approved protocol that adhered to Federal guidelines for the protection of human subjects.

Squirrel monkey plasma specimens

Two different batches of pooled plasma specimens from squirrel monkeys (*Saimiri sciureus*) treated with racemic MDMA were evaluated. One batch contained low, the other high analyte concentrations.

Rat plasma specimens

Two different batches of pooled plasma specimens from Sprague–Dawley rats treated with racemic MDMA were tested. One batch contained low, the other one high analyte concentrations. All animal experiments were carried out according to *The Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Methods

Comparison of hydrolysis conditions for each species and determination of interspecies differences were achieved through the experiments described below. Statistical comparisons were performed using Graphpad Prism 3.02 software (San Diego, CA, USA). *P* values <0.05 were considered significant.

Specimen preparation was performed as previously described by Mueller et al. [23] with minor changes. Briefly, specimen volume was 100 μ l plasma preserved with SMBS and EDTA. Calibrators were prepared by adding 100 μ l of the corresponding aqueous analytical standard solution. Accordingly, 100 μ l of aqueous vehicle were added to each noncalibrator sample to adjust the volume. Internal standard (IS) solution (100 μ l) was added to each sample. To perform acidic hydrolysis, samples were mixed with 300 μ l 0.5 M HCl and subsequently incubated for 80 min at 100 °C. To perform enzymatic hydrolysis, 10 μ l of either enzyme solution were added to samples; samples were mixed and incubated for 180 min at 60 °C. After cooling to room temperature, 20 μ l of 4-methylcatechol was added to reduce adsorption of HHMA onto the precipitated proteins [24] and samples briefly mixed. Protein precipitation was performed by adding 10 μ l PCA, centrifugation for 5 min at 16,000 $\times$ g, and 5 μ l supernatant injected into the LC-ESI-MS system. MDMA and its main metabolites MDA, HMMA, and HHMA were analyzed in two different batches of pooled plasma for each species, one pool containing low and one containing high concentrations. Five replicates of each concentration level for each species were tested. For each species, mean quantitative values of each analyte in each hydrolysis condition were compared with one-way analysis of variance with grouping variable “cleavage procedure,” followed by Bonferroni’s multiple comparison test.

LC-ESI-MS analysis

All samples were analyzed with an Agilent Technologies (AT, Waldbronn, Germany) AT Series 1100 LC-MSD, VL version, equipped with an ESI interface (ESI inlet conditions: drying gas, nitrogen (12 l/min, 350 °C) and nebulizer gas, nitrogen (172.5 kPa); capillary voltage, 4,000 V) coupled with an AT 1100 Series HPLC system consisting of a degasser, a quaternary pump, a column thermostat, and an autosampler.

Glucuronide conjugates of HHMA and HMMA as well as the respective free analytes were separated by LC using a Zorbax 300-SCX column (Narrow-Bore 2.1 $\times$ 150 mm, 5 μ m) with a Zorbax SCX guard column (4.6 $\times$ 12.5 mm, 5 μ m) and an isocratic mobile phase consisting of 70% (v/v) 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid and 30% acetonitrile. Flow rate and run time were 18 min and 0.8 ml/min, respectively, for analysis of HHMA glucuronide (HHMA-gluc) and HMMA glucuronide (HMMA-gluc). MS settings for the analysis of HHMA, HMMA, HHMA-gluc, and HMMA-gluc were as follows: polarity, positive; selected ion monitoring (SIM) of the ions *m/z* 182 (t) and *m/z* 151 for HHMA, *m/z* 196 (t) and *m/z* 165 for HMMA, *m/z* 358 (t) and *m/z* 182 for HHMA-gluc; *m/z* 372 (t) and *m/z* 196 for HMMA-gluc; fragmentor voltage, 100 V.

For analysis of HHMA sulfate (HHMA-sulf) and HMMA sulfate (HMMA-sulf), isocratic elution was performed on a Spherisorb C18 column (4.6 $\times$ 150 mm, 3 μ m). The mobile phase consisted of 70% (v/v) 5 mM aqueous ammonium formate adjusted to pH 9 with sodium hydroxide and 30% (v/v) acetonitrile. The flow rate was 0.8 ml/min and the run time 4 min. For detection of HHMA-sulf and HMMA-sulf, MS settings were as follows: polarity, negative; SIM of the ions *m/z* 260 (t) and *m/z* 180 for HHMA-gluc, *m/z* 274 (t) and *m/z* 194 for HMMA-gluc; fragmentor voltage, 100 V.

Simultaneous LC-MS analysis and quantification of MDMA and its metabolites HHMA, HMMA, and MDA were performed as previously described [23].

Results and discussion

Incubation conditions in the experiments were derived from preliminary data on the extent of hydrolysis with respect to temperature and time using 4-nitrophenyl β -D-glucuronide, 4-nitrophenyl sulfate, PNCS, and morphine 3- β -D-glucuronide, well-established test compounds for studying cleavage of glucuronide and sulfate conjugates [25–34]. Both aryl sulfates were easily and completely cleaved by acidic hydrolysis, while cleavage of glucuronides required higher temperatures and/or longer incubation times (data not shown). Additional experiments with the same test compounds in the presence and absence of matrix indicated that plasma matrix reduces the rate of enzymatic and acidic conjugate cleavage. The effect was more pronounced with enzymatic hydrolysis, and for acidic hydrolysis, it was limited to the glucuronic acid conjugates. Matrix effects were independent of species type (data not shown) [23].

MDMA, HMMA, HHMA, and MDA were determined using the validated LC-MS methods previously described [23, 35]. Comparison of plasma concentrations of MDMA, HMMA, and HHMA in human plasma specimens obtained after different methods of conjugate cleavage showed significant differences following acid and enzyme hydrolysis for HMMA and HHMA. HMMA and HHMA plasma concentrations in both batches were significantly higher ($p < 0.001$) after acidic cleavage than after either enzymatic cleavage. Plasma concentrations (mean $\pm$ SD) for each analyte in both batches are shown in Table 1. Acidic hydrolysis of HMMA resulted in about 2.5-fold higher plasma levels in the first batch and in about 4-fold higher plasma levels in the second batch. The differences between HHMA concentrations after acidic and enzymatic hydrolysis in both batches were about 10-fold. There was no significant difference between plasma levels after hydrolysis with either enzyme. MDA levels were below the limit of quantification (LOQ) of the method (LOQ = 10 ng/ml) and, therefore, not presented.

Taking into consideration that acidic hydrolysis was less efficient in cleaving glucuronides than sulfate conjugates, a possible explanation for the present findings could be more extensive formation of sulfate conjugates as compared to glucuronic acid conjugates of MDMA metabolites in humans. Indeed, only HMMA-gluc, HHMA-sulf, and HMMA-sulf could be detected in authentic human plasma specimens analyzed without previous conjugate cleavage (Figs. 1a and 2a). As shown in Fig. 1b, after acidic hydrolysis of the same human plasma specimen, HMMA-gluc was still detectable, but its absolute peak area had decreased by 45%. Additionally, free HHMA and HMMA could be found. HHMA-sulf and HMMA-sulf could not be detected after performing acidic hydrolysis (Fig. 2b). As shown in Fig. 1c, after enzymatic hydrolysis of the same human plasma specimen with glucuronidase, HMMA-gluc was no longer detectable. From the free analytes, only HMMA could be found but to a lesser extent (about three times) than after cleavage with acid. Accordingly, HHMA-sulf and HMMA-sulf were still present, as shown in Fig. 2c. However, one would expect higher recovery of HMMA and HHMA by using the enzyme solution with high sulfatase activity.

Comparison of MDMA, MDA, HMMA, and HHMA plasma concentrations in squirrel monkey plasma specimens obtained after different methods of conjugate cleavage showed significant differences between the acidic, glucuronidase, and sulfatase hydrolysis for HMMA and HHMA (Table 2). HMMA and HHMA plasma concentrations in both batches were significantly higher ($p < 0.001$) after acidic cleavage than after either enzyme solution. HMMA levels found in the first batch were six to ten times higher and two to three times higher in the second batch. HHMA levels after acidic hydrolysis in the first batch were 10- to 12-fold higher than after enzymatic hydrolysis; differences in the second batch were 6- to 7-fold. HMMA concentrations after hydrolysis with sulfatase solution were about 1.5 times

lower than after cleavage with glucuronidase solution. Plasma concentrations (mean $\pm$ SD) for each analyte in both batches are shown in Table 2. Again, an explanation for the significant differences between acidic and enzymatic hydrolysis could be the more extensive formation of sulfate conjugates as compared to glucuronic acid conjugates in squirrel monkeys. Matrix-based enzyme inhibition could serve as an explanation for the differences found between the enzyme solutions. As already shown in humans, analysis of authentic squirrel monkey plasma samples without conjugate cleavage and after acid and enzymatic hydrolysis with glucuronidase solution supported this hypothesis. After simple protein precipitation, HMMA-gluc, HHMA-sulf, HMMA-sulf, and free HMMA could be detected in squirrel monkey plasma samples (Figs. 3a and 4a). As shown in Fig. 3b, after acidic hydrolysis of the same squirrel monkey plasma sample, the absolute peak area of the HMMA-gluc was about 30% decreased. HHMA could be detected, and HMMA was about 50 times higher than without hydrolysis. HHMA-sulf and HMMA-sulf could no longer be detected after acidic hydrolysis (Fig. 4b). As shown in Fig. 3c, after enzymatic hydrolysis with glucuronidase solution of the same plasma sample, the HMMA-gluc was no longer detectable. HMMA was about 16 times higher after cleaving the conjugates. However, compared to the sample that was prepared using acidic hydrolysis, amounts of HHMA and HMMA were decreased, HHMA about seven times less and HMMA about three times less. Accordingly, HHMA-sulf and HMMA-sulf could still be detected after performing enzymatic cleavage (Fig. 4c).

Comparison of plasma concentrations of MDMA, MDA, HMMA, and HHMA in rat plasma specimens after different methods of conjugate hydrolysis showed significant differences between acid and enzyme hydrolysis for HMMA and HHMA (Table 3). In rat plasma, notably different from results obtained with human and monkey plasma specimens, HMMA and HHMA concentrations were significantly higher ($p<0.001$) in both batches after enzymatic hydrolysis than after acidic cleavage. HMMA levels in the first batch were about three times higher and about 2.5 times higher in the second batch. HHMA concentrations after enzymatic hydrolysis in the first batch were five times higher and 3-fold higher in the second pool. There was no advantage of one enzyme solution over the other. Plasma concentrations (mean $\pm$ SD) for each analyte in both batches are presented in Table 3. Given the fact that both enzymes have high glucuronidase activity and that acidic hydrolysis was less effective in cleaving glucuronides, formation of glucuronides rather than sulfate conjugates of MDMA metabolites in rat plasma could explain the presented findings. Again, this hypothesis was supported by results after analysis of authentic rat plasma specimens without conjugate cleavage and after acid and enzymatic hydrolysis with glucuronidase solution. As shown in Figs. 5a and 6a, without conjugate cleavage, HHMA-gluc, HMMA-gluc, free HHMA, free HMMA, HHMA-sulf, and HMMA-sulf were detected in rat plasma. After acidic hydrolysis of the same rat plasma sample, HHMA-gluc and HMMA-gluc were still present. However, the absolute peak areas of glucuronic acid conjugates were decreased by 17% for HHMA-gluc and 30% for HMMA-gluc. Absolute peak areas of free analytes increased 2-fold for HHMA and 9-fold for HMMA (Fig. 5b). Peaks for HHMA-sulf and HMMA-sulf could no longer be detected (Fig. 6b). As shown in Fig. 5c, after analyzing the same rat plasma following enzymatic hydrolysis using glucuronidase solution, neither glucuronide could be detected. Again, absolute peak areas of free analytes were increased. Despite the fact that peak areas for HHMA and HMMA were 2.5-fold higher than after acidic hydrolysis, HHMA-sulf and HMMA-sulf could still be found in samples treated with enzyme solution (Fig. 6c).

These results are in accordance with those of Shima et al. [36]. After comparing the amount of HMMA glucuronide and HMMA sulfate excreted in urine of human MDMA users and rats treated with MDMA, Shima et al. found that sulfation of HMMA was predominant in humans, whereas in rats, more than 99% of conjugated HMMA was excreted as

glucuronide. These data strongly indicate considerable species differences in formation rates of glucuronic acid and sulfate conjugates. Indeed, such differences between species also have been reported for other substances. Boettcher et al. [37], when comparing rats and humans, found that in rats, the sulfate conjugation of antipyrine was the predominant pathway, whereas in humans, antipyrine glucuronides were most prominent, with the sulfate formation being dose-dependent. Kokkinakis et al. [38], for example, found that *N*-nitroso(2-hydroxy-propyl)(2-oxopropyl) amine in hamsters is conjugated with sulfuric acid ten times faster than in rats, where the glucuronic acid conjugates are predominant. Wong [39] found that sulfate conjugation of 4-hydroxy-3-methoxyphenylethanol is predominant in most animal species such as mouse, cat, rat, rabbit, and guinea-pig, whereas in humans, only the glucuronide was detected.

By employing analytical procedures which are suitable of simultaneous determination of conjugated and free analytes, discrepancies in quantification due to such species differences could be prevented. LC-MS methods seem to be superior over GC-MS procedures because glucuronide and sulfate conjugates are not amenable to GC-based analysis. However, the lack of commercially available standard solutions for glucuronide and sulfate conjugates of HHMA and HMMA presents a problem for developing such analytical methods. Recently, Shima et al. [40] published an LC-ESI-MS procedure for the simultaneous detection of MDMA and its metabolites including free HMMA, as well as the intact phase II metabolites HMMA-sulf and HMMA-gluc in urine. For their studies, the authors used in-lab-synthesized HMMA conjugates.

Conclusions

The results of this study indicate that differences in the hydrolysis of MDMA metabolites exist between species and that these differences are important to consider in specimen preparation. Acidic hydrolysis should be utilized if MDMA metabolites are to be analyzed in human or squirrel monkey plasma, while in rat, enzymatic hydrolysis with either glucuronidase or sulfatase should be employed to maximize recovery of MDMA metabolites. Whenever standard solutions for the intact conjugate are available, analytical procedures which are suitable of simultaneous determination of conjugated and free analytes should be preferred to avoid discrepancies in quantification due to species differences. LC-MS seems to be the analytical method of choice.

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Fig. 1. Merged mass chromatograms of the given ions (for HHMA-gluc, HMMA-gluc, HHMA, and HMMA) of an authentic human plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase and protein precipitation (c)

Fig. 2. Merged mass chromatograms of the given ions (for HHMA-sulf and HMMA-sulf) of an authentic human plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase solution and protein precipitation (c)

Fig. 3.

Merged mass chromatograms of the given ions (for HHMA-gluc, HMMA-gluc, HHMA, and HMMA) of an authentic squirrel monkey plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase solution and protein precipitation (c)

Fig. 4. Merged mass chromatograms of the given ions (for HHMA-sulf and HMMA-sulf) of an authentic squirrel monkey plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase solution and protein precipitation (c)

Fig. 5. Merged mass chromatograms of the given ions (for HHMA-gluc, HMMA-gluc, HHMA, and HMMA) of an authentic rat plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase solution and protein precipitation (c)

Fig. 6. Merged mass chromatograms of the given ions (for HHMA-sulf and HMMA-sulf) of an authentic rat plasma specimen without conjugate cleavage and after protein precipitation (a), after acidic conjugate cleavage and protein precipitation (b), and after enzymatic conjugate cleavage using glucuronidase solution and protein precipitation (c)

Table 1

Plasma concentrations (ng/ml, mean±SD) for MDMA, HMMA, and HHMA in two different pools of authentic human plasma specimens after hydrolysis with acid, glucuronidase, or sulfatase solution

Samples		1st batch		2nd batch	
Method of hydrolysis					
Analyte	IS	Acidic		Enzymatic (glucuronidase)	
		(Mean±SD)	(n=5)	(Mean±SD)	(n=5)
MDMA	MDMA-d5	138.0±7.9		137.9±2.3	
HMMA	Pholedrine	97.0±1.9		39.7±1.0	
HHMA	Pholedrine	90.8±0.8		9.7±1.1	
		Enzymatic (sulfatase)		Acidic	
		(Mean±SD)	(n=5)	(Mean±SD)	(n=5)
				Enzymatic (glucuronidase)	Enzymatic (sulfatase)
				(Mean±SD)	(Mean±SD)
				(n=5)	(n=5)
				146.8±2.1	141.5±2.3
				64.8±1.1	55.8±2.5
				11.4±0.5	10.0±1.4

Table 2

Plasma concentrations (ng/ml, mean±SD) for MDMA, MDA, HMMA, and HHMA in two different plasma pools of squirrel monkeys treated with MDMA after hydrolysis with acid, glucuronidase, or sulfatase solution

Samples		1st batch				2nd batch			
		Method of hydrolysis							
Analyte	IS	Acidic (Mean±SD) (n=5)	Enzymatic (glucuronidase) (Mean±SD) (n=5)	Enzymatic (sulfatase) (Mean±SD) (n=5)	acidic (Mean±SD) (n=5)	Enzymatic (glucuronidase) (Mean±SD) (n=5)	Enzymatic (sulfatase) (Mean±SD) (n=5)		
MDMA	MDMA-d5	272.8±8.2	245.3±2.8	225.7±3.9	777.5±25.5	786.5±6.0	771.7±9.6		
HMMA	Pholedrine	597.7±17.9	107.2±2.0	63.6±3.1	823.5±33.1	353.9±6.8	277.5±5.9		
HHMA	Pholedrine	268.7±7.2	20.6±1.5	26.8±3.1	330.0±8.8	46.4±1.9	55.3±1.6		
MDA	MDA-d5	9.5±1.2	10.6±0.5	9.8±0.7	18.6±0.5	20.5±1.1	22.1±0.6		

Table 3

Plasma concentrations (ng/ml, mean±SD) for MDMA, MDA, HMMA, and HHMA in two different plasma pools of Sprague-Dawley rats treated with MDMA after hydrolysis with acid, glucuronidase, or sulfatase solution

Samples		1st batch				2nd batch			
		Method of hydrolysis							
Analyte	IS	Acidic (Mean±SD) (n=5)	Enzymatic (glucuronidase) (Mean±SD) (n=5)	Enzymatic (sulfatase) (Mean±SD) (n=5)	Acidic (Mean±SD) (n=5)	Enzymatic (glucuronidase) (Mean±SD) (n=5)	Enzymatic (sulfatase) (Mean±SD) (n=5)		
MDMA	MDMA-d5	85.0±4.7	97.3±3.0	90.1±3.4	608.2±5.0	614.3±5.1	576.2±22.8		
HMMA	Pholedrine	93.9±4.6	272.4±4.8	271.0±6.1	78.8±3.2	196.4±3.2	195.5±9.3		
HHMA	Pholedrine	85.2±4.4	448.8±13.9	443.6±12.8	112.9±2.5	381.9±6.5	357.4±9.0		
MDA	MDA-d5	196.0±7.3	177.4±5.0	198.3±13.2	643.4±11.0	614.1±21.6	619.4±29.9		