Enhanced Methamphetamine Metabolism in Rhesus Macaque as Compared with Human: An Analysis Using a Novel Method of Liquid Chromatography with Tandem Mass Spectrometry, Kinetic Study, and Substrate Docking[□]

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ABSTRACT

Methamphetamine (MA), which remains one of the widely used drugs of abuse, is metabolized by the cytochrome P450 (P450) family of enzymes in humans. However, metabolism of methamphetamine in macaques is poorly understood. Therefore, we first developed and validated a very sensitive liquid chromatography with tandem mass spectrometry (LC-MS/MS) method using solid phase extraction of rhesus plasma with a lower limit of quantitation at 1.09 ng/ml for MA and its metabolites, 4-hydroxy methamphetamine (4-OH MA), amphetamine (AM), 4-OH amphetamine (4-OH AM), and norephedrine. We then analyzed plasma samples of MA-treated rhesus, which showed >10-fold higher concentrations of AM (~29 ng/ml) and 4-OH AM (~28 ng/ml) than MA (~2 ng/ml). Because the plasma levels of MA metabolites in rhesus were much higher than in human samples, we examined MA metabolism in human and rhesus

microsomes. Interestingly, the results showed that AM and 4-OH AM were formed more rapidly and that the catalytic efficiency ($V_{\max}/K_m$) for the formation of AM was ~8-fold higher in rhesus than in human microsomes. We further examined the differences in these kinetic characteristics using three selective inhibitors of each human CYP2D6 and CYP3A4 enzymes. The results showed that each of these inhibitors inhibited both D- and L-MA metabolism by 20%–60% in human microsomes but not in rhesus microsomes. The differences between human and rhesus CYP2D6 and CYP3A4 enzymes were further assessed by docking studies for both D and L-MA. In conclusion, our results demonstrated an enhanced MA metabolism in rhesus compared with humans, which is likely to be caused by differences in MA-metabolizing P450 enzymes between these species.

Introduction

Methamphetamine (MA) remains one of the widely used drugs of abuse. MA abuse can cause euphoria, dysphoria, paranoia, cognitive impairments, and neuronal toxicity. It also decreases the dopamine and serotonin levels in the brain (Thompson et al., 2004; Krasnova and Cadet, 2009). Similar findings were also observed in other species, including monkey, rat, and mouse brains (Melega et al., 2008; Krasnova and Cadet, 2009). Although, the altered level of dopamine can result in MA-mediated neurotoxicity and an increase in oxidative

stress, (Fitzmaurice et al., 2006), the underlying mechanisms remain unclear. Metabolism mediated by cytochrome P450 (P450) of various drugs, including MA, can lead to oxidative stress, which may cause neurotoxicity (Lin et al., 1997; Dorman et al., 2008; Cherner et al., 2010; Moszczynska and Yamamoto, 2011; Pendyala et al., 2011; de la Torre et al., 2012; Shah et al., 2013).

Although many pathologic conditions that afflict humans can be accurately recapitulated in mouse models, nonhuman primate (NHP) models still play the preeminent role in research concerning many diseases. This is especially so in the area of infectious disease research concerning pathogens such as human immunodeficiency virus type 1 and tuberculosis (Legrand et al., 2009; Holder et al., 2014; Phillips et al., 2014; Scanga and Flynn, 2014), as the host-pathogen interactions involved in these pathologies are most accurately reproduced in a NHP model. In these diseases as well as others, NHP models are often used to test the efficacy of therapeutic agents that are metabolized by the

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ABBREVIATIONS: AM, amphetamine; CV, coefficient of variation; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IS, internal standard; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LLOQ, lower limit of quantitation; MA, methamphetamine (D and L); MRM, multiple reactions monitoring; m/z , mass-to-charge ratio; NHP, nonhuman primate; 4-OH AM, 4-hydroxyamphetamine; 4-OH MA, 4-hydroxymethamphetamine; P450, cytochrome P450; QC, quality control; SPE, solid phase extraction; ULOQ, upper limit of quantitation.

P450 pathway (Nishimuta et al., 2011; Uno et al., 2014). Even though the NHP models can accurately reproduce the pathogenic effects of the pathogen as well as model drug disposition, the use of drugs of abuse must also be taken into account. Not only do these agents have the potential to alter the pathologic course of disease by affecting the inflammatory process, they also have the ability to modify the effects of therapeutic agents by affecting their metabolism and/or disposition (Nishimuta et al., 2010; Moss et al., 2012; Uno et al., 2014). However, MA metabolism in macaques is poorly understood. Therefore, given the wide use of rhesus macaques in studying infectious diseases that have a high prevalence among users of MA, here we investigated P450-mediated MA metabolism in rhesus macaques.

MA is primarily metabolized to amphetamine (AM) and 4-hydroxy methamphetamine (4-OH MA) (de la Torre et al., 2012) by human CYP2D6 and, to a lesser extent, CYP3A4 (Welter et al., 2013). AM is further metabolized to 4-hydroxymethamphetamine (4-OH AM) and norephedrine by CYP2D6 (Miranda-G et al., 2007; de la Torre et al., 2012). Many species of monkeys, including rhesus macaques, also express various P450-isozymes that are homologous to human CYP2D6 and CYP3A4 with >90% homology (Carr et al., 2006; Uno et al., 2010). These enzymes in monkeys may also be responsible for the metabolism of MA, but that connection is unknown. Therefore, we studied MA metabolism in rhesus macaques and compared the results with MA metabolism in human. However, to study MA metabolism in rhesus, we first needed to develop a sensitive liquid chromatography with tandem mass spectrometry (LC-MS/MS) method to measure MA and its metabolites in rhesus plasma and liver microsomes.

In previous studies, MA and its metabolites have been quantified in brain, urine, serum, plasma, hair, and nails using immunoassay, high-performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS), and liquid chromatography mass spectrometry (LC-MS) (Fitzgerald et al., 1988; Thurman et al., 1992; Armstrong and Noguchi, 2004; Berankova et al., 2005; Hendrickson et al., 2006; Kim et al., 2008; Wongniramaikul et al., 2012; Koster et al., 2014). However, these methods have moderate sensitivity with a lower limit of quantitation (LOQ) at 5–10 ng/ml. In addition, these methods can be laborious and time consuming (Armstrong and Noguchi, 2004; Kim et al., 2008). Recently, additional LC-MS/MS methods have been developed to study MA metabolism in humans, but these methods are less sensitive, time consuming, and tedious (Djozan and Baheri, 2007; Sergi et al., 2009; Lee et al., 2012). To study extensive MA metabolism, a suitable and sensitive analytic method is needed for the concurrent determination of MA and its four metabolites: 4-OH MA, AM, 4-OH AM, and norephedrine. These four metabolites are important because they are the most active sympathomimetic agents compared with other MA metabolites (Kraemer and Maurer, 2002). As such, there is no appropriate validated LC-MS/MS method reported for the determination of MA and its metabolites in rhesus plasma and liver microsomes. Therefore, we developed and validated a novel, sensitive, and rapid electrospray ionization (ESI) LC-MS/MS method using solid phase extraction (SPE) from plasma and liver microsomes of human and rhesus monkey for concurrent analysis of MA and four of the metabolites. We then studied the relative metabolism of MA stereoisomers (d- and l-MA) in rhesus and human liver microsomes using enzyme kinetics and inhibition methods. Further, we performed a docking study to explain the results obtained from the kinetic and inhibition studies.

Materials and Methods

Chemical and Reagents. MA, 4-OH MA, AM, 4-OH AM, norephedrine, MA-d5, and AM-d8, and P450 inhibitors were purchased from Cerilliant Analytical Reference Standards (Sigma-Aldrich, Round Rock, TX). HPLC grade methanol, acetonitrile, ammonia, and formic acid were purchased

from Fisher Scientific (New Brunswick, NJ). All HPLC grade chemicals were used without further purification. An Xbridge HPLC reverse phase C18 column and HLB Oasis solid phase extraction cartridges (Waters Corporation, Milford, MA) were employed for the determination of analytes.

Method Development. MA, 4-OH MA, AM, 4-OH AM, norephedrine, MA-d5, and AM-d8 stocks were made in methanol, and the concentrations were corrected using a formula described elsewhere (Earla et al., 2012). Standard curves (800.00, 640.00, 486.40, 389.12, 214.02, 74.91, 14.98, 4.94, 1.09 ng/ml) for each analyte were generated using drug-free rhesus macaque plasma. Similarly, the quality control (QC) samples at four concentrations (486.40, 214.02, 14.98, and 1.09 ng/ml) were separately prepared in the rhesus plasma as mentioned elsewhere (Earla et al., 2012). The system suitability test for each analyte was performed independently by using six replicate injections of 800 ng/ml of the reference standard with internal standard (IS). The carryover test was performed by injecting an extract blank followed by immediate injection of an extracted upper limit of quantitation (ULOQ) of the standard curve with an IS.

The LC-MS/MS method was developed using rhesus plasma. The mass spectrometer (3200 QTRAP LC-MS/MS system; AB Sciex, Framingham, MA) was optimized for detection of MA and its four metabolites along with IS. The most suitable proton adduct in ESI $[M+H]^+$ precursor ions was determined for MA (150.5), 4-OH MA (166.3), AM (136.4), 4-OH AM (152.3), norephedrine (152.3), MA-d5 (155.5), and AM-d8 (144.5) (Fig. 1 and Supplemental Table 1). These precursor ions were optimized by adjusting the curtain gas, declustering potential, ion spray voltage, and source gas 1. The precursor ions of MA and four of its metabolites along with IS were fragmented by applying collisionally activated dissociation gas and collision energy to obtain their most abundant product ions. The product ions for MA (91.2), 4-OH MA (135.4), AM (91.3), 4-OH AM (135.2), norephedrine (134.4), MA-d5 (92.3), and AM-d8 (97.2) were optimized as described in our earlier report (Earla et al., 2014) (Fig. 1 and Supplemental Table 1). The multiple reactions monitoring (MRM) transitions (m/z) $[M+H]^+$, (Q_1/Q_3) selected for quantitative analyses were 150.5/91.2 for MA, 166.3/135.4 for 4-OH MA, 136.4/91.3 for AM, 152.3/135.2 for 4-OH AM, 152.3/134.4 for norephedrine, 155.5/92.3 for MA-d5, and 144.5/97.2 for AM-d8 (Fig. 2 and Supplemental Table 1). A dwell time of 200 milliseconds and a source temperature of 450°C was employed for all the analyte determinations.

The chromatographic separation was achieved using a reverse phase Xbridge MS C18 column (50 × 4.6 mm, i.d., 5 μ m) in conjunction with a UFLC Shimadzu LC-20AD HPLC (Shimadzu Scientific Instruments, Columbia, MD). An isocratic mobile phase composed of 65% acetonitrile and 35% of water containing 0.05% of formic acid at a flow rate of 0.3 ml/min was used. The samples were reconstituted in 200 μ l of mobile phase, and 10 μ l of each sample was injected into the LC-MS/MS for quantitative analysis over a 4-minute run time. The LC-MS/MS acquired MRM data were processed using Analyst software (version 6.2; AB Sciex, Foster City, CA).

A simple SPE technique was used for extraction of analytes. Macaque plasma was spiked with 20 μ l of 10 μ g/ml IS (final concentration of ~1 μ g/ml). The mixture was vortex-mixed for 30 seconds followed by the addition of 20 μ l of an aqueous 10% formic acid solution, which was again vortex-mixed for 1 minute before SPE. The SPE columns (HLB 30 mg, 1-ml cartridge) were conditioned with 1 ml of methanol followed by 1 ml of water. After loading, washing, and drying of the SPE cartridge as described previously elsewhere (Earla et al., 2014), analytes were eluted with 1 ml of methanol. After elution, samples were evaporated using a speed vacuum at 35°C for 60 minutes.

Method Validation. The LC-MS/MS method validation was performed by testing specificity, selectivity, accuracy, precision, recovery, matrix effect, and stability of each analyte in rhesus plasma and liver microsomes as described previously elsewhere (Earla et al., 2010, 2012, 2014). The specificity and selectivity of the method were tested by analyzing blank plasma samples from six rhesus macaques for the extracted lower limit of quantitation (LLOQ, 1 ng/ml). The blank matrix signal to noise ratio did not show measurable interference at the analyte peak of interest for MA and its metabolites (Fig. 2A). The percentage of interference determined in the blank was calculated by comparing the mean peak area of LLOQ of the analyte with the peak response obtained from the blank samples ($\leq 20\%$).

Within-assay and between-assay precision and accuracy experiments were performed by analyzing eight extracted calibration standards and four levels of QC standards as described elsewhere (Earla et al., 2010). The precision and accuracy were calculated within the acceptable range (20%–25%) according

Fig. 1. Development of LC-MS/MS method to quantitate methamphetamine (MA) and four of its metabolites in rhesus plasma. MS/MS spectra of (A) MA, (B) 4-hydroxymethamphetamine (4-OH MA), (C) amphetamine (AM), (D) 4-hydroxyamphetamine (4-OH AM), (E) norephedrine, (F) methamphetamine-d5 (IS), (G) Amphetamine-d8 (IS) with ESI proton adducts $[M+H]^+$ in positive mode. (H) Simultaneous analysis of multiple reaction monitoring (MRM) chromatogram peaks of a mixture of reference standard containing MA, 4-OH MA, AM, 4-OH AM, norephedrine, MA-d5(IS) and AM-d8 (IS) which were separated based on their mass to charge (m/z). The y-axis shows intensity (CPS, count per second); the x-axis shows the mass to charge ratio (m/z , amu) in A–G and run time (min) in H.

to the guidance for industry standards for bioanalytic method validation in the U.S. Food and Drug Administration guidelines (www.fda.gov) and also as described previously elsewhere (Earla et al., 2010, 2014).

Recovery of MA and its metabolites was estimated by analyzing two sets of six replicates of plasma extracted low and high QC standards and a postspiked (representing 100% recovery) sample along with IS. Similarly, the matrix effect

Fig. 2. Concurrent analysis of LC-MS/MS-MRM chromatogram peaks of methamphetamine (MA), 4-hydroxymethamphetamine (4-OH MA), amphetamine (AM), 4-hydroxyamphetamine (4-OH AM), and norephedrine, MA-d5 (IS) and AM-d8 (IS) in rhesus plasma. (A) Extracted blanks. (B) Extracted lower limit of quantitation (LLOQ, 1.09 ng/ml). (C) Extracted upper limit of quantification (ULOQ, 800 ng/ml). (D) Extracted deuterated internal standards. (E) Extracted low quality control (LQC, 14.94 ng/ml) standard. (F) Extracted middle quality control (MQC, 214.0 ng/ml). (G) Extracted high quality control (HQC, 800 ng/ml). (H) Extracted deuterated internal standards. PA, peak area; PAR, peak area ratio (analyte/IS); IS, internal standard. The y-axis shows intensity (CPS, count per second); x-axis shows run time (min).

of MA and its metabolites was evaluated by analyzing two sets of six replicates each of low and high QC standards from postspiked (extracted blank plasma samples) and spiked standards in aqueous solutions (representing no matrix effect) (Earla et al., 2014). The stability of analytes such as bench top, and freeze-thaw in rhesus plasma was studied at -80°C and 25°C , respectively, for several weeks to estimate the degradation of analytes in the matrix. Six replicates of each stability experiment sample at concentrations of 486.40 and 14.98 ng/ml were prepared for each analyte in pooled rhesus plasma according to our published methods (Earla et al., 2010, 2014).

Analysis of MA Metabolism in Plasma. The rhesus macaques (*Macaca mulatta* of Indian origin) were bred in the Emory University breeding facility and were housed at the Yerkes National Primate Research Center as per the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care. All the studies were carried out in accordance with the recommendations of the Emory University Institutional Animal Care and Use Committee under the National Institutes of Health guidelines. After the initial quarantine phase, the animals were adapted to saline injection twice every day for 4 weeks. The animals were then

treated with injections of increasing doses of MA (0.1 mg/kg once in a day to 0.75 mg/kg twice a day, 5 days a week) over a 4-week period. The animals were then maintained at 0.75 mg/kg of MA twice every day for an additional 16 weeks to mimic conditions of chronic MA use. The blood samples were collected from the femoral vein on Monday morning after the monkeys had been injected with 10 doses of MA every week. The blood samples in heparin were collected after 20 weeks of MA injections. The samples were then centrifuged at 2500 rpm at 4°C for 10 minutes, and the plasma from each sample was collected (Kumar et al., 2000, 2001). All the samples were stored at -80°C until analysis and were processed according to the sample preparation and extraction protocols described herein.

MA Metabolism in Liver Microsomes. The human and rhesus liver microsomes (Invitrogen, Carlsbad, CA) were used to study MA metabolism. The reaction was performed in HEPES buffer (0.1 M, pH 7.4) using $1\ \mu\text{M}$ MA, $1\ \text{mg/ml}$ of microsomes (major source of P450 enzymes), $10\ \text{mM}$ MgCl_2 , and $1\ \text{mM}$ NADPH at 37°C as described elsewhere (Meyer et al., 2008). The reaction was initiated by the addition of NADPH, and it was quenched by freezing the sample at -80°C . The reaction was performed at various time points (0, 0.5, 1, 2, 3, 6, 12, and

24 hours). MA and its metabolites were analyzed using SPE and LC-MS/MS methods by optimizing these methods in liver microsomes (Supplemental Tables 3–5). The apparent kinetic parameters for the degradation of MA and formation of AM and 4-OH AM were determined by fitting the hyperbolic equation using Sigma Plot 11 (Systat Software, San Jose, CA). We also performed concentration-dependent kinetics using both d and l-MA stereoisomers at various concentrations (2.5, 5, 10, 25, 50, 100, 250, and 500. μM) with human and rhesus microsomes. The reaction was performed as described earlier for 20 minutes at 37°C. The analysis of the formation of AM from both the MA stereoisomers by human and monkey liver microsomes were performed with Michaelis-Menten kinetics using nonlinear regression (Sigma Plot software). For inhibition studies, the reaction mixture was preincubated with various inhibitors (0.5 μM of each of quinidine, paroxetine, fluoxetine, ketoconazole, ritonavir, or indinavir) at 37°C for 10 minutes. The reaction then was performed as described above for 60 minutes. The formation of the major metabolite, AM, was measured with/and without P450 inhibitors.

MA Docking to Human and Rhesus P450 Models. The initial models of human CYP2D6 and CYP3A4 for docking were taken from the Protein Data Bank (PDB, www.rcsb.org/pdb/). To date, there are 4 crystal structures available for human CYP2D6 and 17 for human CYP3A4. These structures were resolved with or without a ligand bound to the respective P450s. On the basis of the resolution, completion, and ligand size, 3DTA (2.67 Å) and 3NXU (2.00 Å) were selected for human CYP2D6 and CYP3A4, respectively. Chain A of the two structures was used for docking simulations. The initial model of rhesus CYP2D6, constructed by the comparative protein modeling method by satisfaction of spatial restraints, was used to predict the three-dimensional structural models of CYP2D6 and CYP3A64 (Larkin et al., 2007; Edmund et al., 2013). Rhesus CYP3A64, which is a homolog to human CYP3A4 (93% sequence identity) and shows similar metabolic characteristics to human CYP3A4 (Carr et al., 2006), was used to build the protein model. The 3DTA CYP2D6 and 3NXU CYP3A4 structures were chosen as the templates for the model construction of rhesus CYP2D6 and CYP3A64, respectively. Construction of the homology models was accomplished by using Modeller 9 (<http://salilab.org/modeller/>, Fiser and Sali, 2003). The sequence alignment was performed using the default parameters by ClustalX (<http://www.clustal.org/>; Larkin et al., 2007; Edmund et al., 2013). A blocks substitution matrix (BLOSUM) was used for the sequence alignments. Because of the high sequence identity between the template and the target sequence (~93% for both), there are no gap inserts for both CYP2D6 and CYP3A4 sequence alignments. Automated docking of the substrate MA into the active site of four

enzymes (human CYP2D6 and CYP3A4 as well as rhesus CYP2D6 and CYP3A64) was performed using Glide 5.8 with the standard precision mode (<http://www.schrodinger.com/citations/41/5/1/>). The center of the grid was placed on the heme iron atom, and the sizes of the grid box and the inner box were set to 20 Å and 14 Å, respectively. Each run had 30 outputs of the ligand poses. The first 10 binding poses with the lowest scores were analyzed in detail.

Statistical Analysis. The concentration of all analytes in the plasma from subjects was calculated by Analyst software (AB Sciex). The statistical significance (p-values were) calculated using one-way analysis of variance (ANOVA). The MA kinetic parameters were calculated using Sigma Plot software (Systat Software, San Jose, CA).

Results

Method Developments. The maximum intensity precursor ion with proton adducts $[\text{M}+\text{H}]^+$ for MA, 4-OH MA, AM, 4-OH AM, norephedrine, MA-d5, and AM-d8 was optimized by modifying the compound parameters of quadrupole 1 (Q1), especially declustering potential at 30, 30, 30, 26, 30, 30, and 26 V, respectively. The collision energy of Q2 was applied to optimize the product ions of MA, 4-OH MA, AM, 4-OH AM, norephedrine, MA-d5, and AM-d8 at 15, 15, 15, 26, 12, 20, and 15 V, respectively (Table 1). The parameters for MRM transitions of Q1 and Q3 for quantitative analysis of MA and its metabolites are shown in Fig. 1 and Supplemental Table 1. The system performance test resulted in <3% variation for MA and its metabolites. The percentage coefficient of variation (% CV) for MA and 4-OH MA with respect to MA-d5 as IS was 2.8 and 2.2, respectively (Supplemental Table 2). Similarly, the % CV for AM, 4-OH AM, and norephedrine with respect to AM-d8 was 2.5, 2.1, and 2.7, respectively. The carryover test for MA and its metabolites at the ULOQ of the calibration curve standard did not produce any carryover to the rhesus blank sample. The results are summarized in Supplemental Table 2.

Method Validation. MA and its metabolites as well as the IS were separated from endogenous intervention peaks of the rhesus blank plasma matrix (Fig. 2A). The signal to noise ratio of extracted blank

TABLE 1

Between day precision and accuracy of calibration curve standards ($n = 6$) and four-level quality control ($n = 18$) standards for methamphetamine, 4-hydroxymethamphetamine, amphetamine, 4-hydroxyamphetamine, and norephedrine in monkey plasma

All CC and QC standard concentration values were presented in one decimal place except CC-8 and 9, and LQC and LLOQ which were presented in two decimal places.

Name	Calibration Curve Standard									Quality Control			LLOQ
	1	2	3	4	5	6	7	8	9	High	Middle	Low	
Nominal concentration (ng/ml)	800	640	486.4	389.1	214	74.9	14.9	4.94	1.09	640	214	14.9	1.09
MA													
Actual (ng/ml)	827.8	655.4	447.4	383.5	228.3	84.1	15.6	5.35	1.2	544.6	205.9	15.2	1
% Accuracy	103.5	102.4	92	98.6	106.4	112.2	104.7	108.3	110.1	85.1	96.2	101.5	91.9
% CV	7.8	10.1	11.7	14.8	4.3	18.1	9.6	9	9.6	13.7	11.5	7.6	23.1
4-OH MA													
Actual (ng/ml)	869.7	605.4	497.4	383.3	228.2	83.9	15.5	5.34	1.23	578	214.4	15.3	1.1
% Accuracy	108.7	94.6	102.3	98.7	106.7	112.3	104.5	108.3	112.8	90.3	100.2	102.1	100.9
% CV	10.8	16.3	17.5	14.6	4.3	17.9	9.5	9.1	12.2	14.06	10.91	6.78	21.2
AM													
Actual (ng/ml)	807.8	655.7	445.3	385.8	240.8	75.5	15.2	4.5	1.28	549.2	212.6	14.6	0.97
% Accuracy	101	102.5	91.6	99.2	112.5	100.8	102	91.1	117.4	85.8	99.3	97.5	89.4
% CV	7.1	8.7	10.2	12.8	8.1	10.4	10.6	19	8.6	13.5	10.4	10.4	27.2
4-OH AM													
Actual (ng/ml)	779.4	671.7	465.3	439.8	245	78.7	16	4.7	1.29	560.6	232.7	15.4	1.06
% Accuracy	97.4	105	95.7	113	114.5	105.1	106.7	95.2	118.5	87.6	108.7	102.8	97.6
% CV	9	10.8	13	7.8	18.6	14.6	8.9	20.5	1.4	12.5	15.7	9.6	17
Norephedrine													
Actual (ng/ml)	787.8	635.7	425.3	399.8	244.8	74.7	15.6	4.7	1.29	584.6	210.3	15.6	1.06
% Accuracy	98.5	99.3	87.4	102.7	114.4	99.7	104	95.2	118.5	91.3	98.3	104.1	97.6
% CV	7.6	9.2	11.2	7.2	7.6	9.4	12.7	16.2	6.6	15.4	10.2	6.2	19.5

CV (precision), coefficient of variation; HQC, high QC; LQC, lower QC; MQC, middle QC.

were found to be <5% of MA and its metabolites at the mean peak area ratio LLOQ and ULOQ with IS (Fig. 2A-D). The LLOQ for MA and its metabolites in plasma was obtained at 1.09 ng/ml. The mean LLOQ peak area ratios (analyte/IS) for MA, 4-OH MA, AM, 4-OH AM, and norephedrine were 0.0021, 0.0013, 0.0021, 0.0018, and 0.0015, respectively (Fig. 2B). Similarly the mean ULOQ peak area ratios for MA, 4-OH MA, AM, 4-OH AM, and norephedrine were 1.1956, 1.8917, 1.7240, 0.7168, and 1.0796, respectively (Fig. 2C). An example of extracted validation QC sample of low QC, middle QC, and high QC chromatogram peak area ratios of MA and its metabolites that were proportional to the concentrations of two IS are shown in Fig. 2E-H. The low QC peak area ratios for MA, 4-OH MA, AM, 4-OH AM, and norephedrine were between 0.0226 and 0.0403 (Fig. 2E). Similarly, middle QC and high QC peak area ratios for MA, 4-OH MA, AM, 4-OH AM, and norephedrine ranged between 0.1167 and 0.3676, and 0.4391 and 1.4571, respectively (Fig. 2F and 2G). The extracted IS peak area is shown in Fig. 2H.

Accuracy and precision were calculated using the best linear fit and least-square residuals for the standards. The assay was linear over the range of 1.09–800 ng/ml with R^2 ($n = 6$) ≥ 0.9866 . The regression equation and coefficient of determination were obtained as follows: MA: $y = 0.0014x - 0.0011$, 4-OH MA: $y = 0.0017x + 0.0003$, AM: $y = 0.0018x - 0.0087$, 4-OH AM: $y = 0.0006x - 0.0075$, and norephedrine: $y = 0.001x + 0.0054$. The accuracy and precision results of plasma were shown in Table 1 and for liver microsomes are shown in Supplemental Table 3. The recovery, matrix effects, bench top, and freeze-thaw stabilities of these analytes in plasma and liver microsomes were measured. These results are shown in the Table 2, Table 3, Supplemental Table 2, and Supplemental Table 3.

MA Metabolism in Rhesus Plasma. MA and its metabolites were determined at a linear range of 1.09–800 ng/ml levels in rhesus plasma. MA concentrations in two rhesus plasma samples were ~1–3 ng/ml. Surprisingly, the concentrations of both AM and 4-OH AM were ~30 ng/ml (Table 4). In contrast, the 4-OH MA concentration was <1 ng/ml, and norephedrine was ≤ 3 ng/ml. These results demonstrated that AM and 4-OH AM are the major metabolites of MA. In addition, the relative concentrations of total MA metabolites were >40-fold higher than MA, suggesting that MA metabolism is very rapid in rhesus macaque (Table 4).

TABLE 2

Recovery and matrix effect of two levels of quality control (QC, $n = 6$) standards for MA, 4-OH MA, AM, 4-OH AM, and norephedrine in monkey plasma

All CC and QC standard values were presented in one decimal place except CC-8 and 9, and LQC and LLOQ which were presented in two decimal places.

Name	Nominal concentration. (ng/ml)	Recovery		Matrix Effect	
		HQC 640.0	LQC 14.94	HQC 640.0	LQC 14.94
MA	Actual (ng/ml)	685.8	13.15	625.1	14.5
	% Accuracy	107.2	88.0	97.7	97.1
	% CV	6.7	13.3	9.5	15.8
4-OH MA	Actual (ng/ml)	619.7	12.8	598.9	14.4
	% Accuracy	96.8	85.7	93.6	96.4
	% CV	10.4	15.1	16.4	13.6
AM	Actual (ng/ml)	639.2	14.3	599.6	14.9
	% Accuracy	99.9	95.7	93.7	99.7
	% CV	6.2	8.3	10.6	7.8
4-OH AM	Actual (ng/ml)	699.2	16.3	659.6	16.5
	% Accuracy	109.3	109.1	103.1	110.4
	% CV	9	10.8	3.6	7.5
Norephedrine	Actual (ng/ml)	640	14.94	640	14.94
	% Accuracy	113.9	117.8	114.7	119.8
	% CV	7.6	9.7	11.8	8.2

CV (precision), coefficient of variation, HQC, high QC; LQC, lower QC; MQC, middle QC.

TABLE 3

Bench top (25°C) and freeze thaw (–80°C) for stability four cycle quality control (QC, $n = 6$) standards of methamphetamine; 4-hydroxymethamphetamine; Amphetamine; 4-hydroxyamphetamine and norephedrine in plasma

All CC and QC standard values were presented in one decimal place except CC-8 and 9, and LQC and LLOQ which were presented in two decimal places.

Name	Nominal concentration. (ng/ml)	Bench Top (25°C)		Freeze Thaw (–80°C)	
		HQC 640.0	LQC 14.94	HQC 640.0	LQC 14.94
MA	Actual (ng/ml)	661.8	15.3	592.4	14.7
	% Accuracy	103.5	102.4	92.0	98.6
	% CV	7.8	10.1	11.7	14.8
4-OH MA	Actual (ng/ml)	695.8	14.15	655.1	14.7
	% Accuracy	108.7	94.6	102.3	98.7
	% CV	10.8	16.3	17.5	14.6
AM	Actual (ng/ml)	645.8	15.3	586.1	14.8
	% Accuracy	101.0	102.5	91.6	99.2
	% CV	7.1	8.7	10.2	12.8
4-OH AM	Actual (ng/ml)	623.3	15.7	612.1	16.9
	% Accuracy	97.4	105.0	95.7	113.0
	% CV	9.0	10.8	13.0	7.8
Norephedrine	Actual (ng/ml)	629.7	14.8	558.9	15.4
	% Accuracy	98.5	99.3	87.4	102.7
	% CV	7.6	9.2	11.2	7.2

CV (precision), coefficient of variation, HQC, high QC; LQC, lower QC; MQC, middle QC.

MA Metabolism in Liver Microsomes. The metabolism of MA in rhesus and human liver microsomes was examined by analyzing the levels of MA and its three metabolites, AM, 4OH AM, and 4-OH MA at different time points. Before measuring these analytes in microsomes with the LC-MS/MS method, we optimized the detection of these metabolites in liver microsomes by using SPE extraction. Upon optimization, these analytes also showed a sensitivity of 1.09 ng/ml for all metabolites in liver microsomes. MA metabolism for the N-demethylation and hydroxylation showed a pseudo-first-order hyperbolic reaction (Fig. 3). Therefore, we determined maximum enzyme activity (highest product formation/substrate consumed) and apparent $t_{1/2}$ (time it takes for the formation of half the total products or consumption of half the total substrate) using equation for pseudo-first-order hyperbolic reaction (Fig. 3A-D, Table 5).

The maximum level of MA degradation in human microsomes was significantly lower than for rhesus microsomes (40.1 ± 11.1 vs. 60.0 ± 5.1 ng/ml/mg microsomes) (Fig. 3B, Table 5). Furthermore, the apparent $t_{1/2}$ for MA in humans was much higher than rhesus microsomes (7.18 ± 4.7 vs. 1.46 ± 0.41 hours). Along with MA degradation, the maximum formation of AM in rhesus microsomes was significantly higher than in human microsomes (35.5 ± 4.2 vs. 27.5 ± 3) (Fig. 3C and Table 5), but the formation of 4-OH AM was similar in both rhesus and human microsomes (Fig. 3D and Table 5). Furthermore, the $t_{1/2}$ of AM and 4-OH AM were significantly lower in human than rhesus microsomes (0.29 ± 0.25 and 1.31 ± 0.60 vs. 6.04 ± 2.0 and 11.1 ± 9.8 , respectively) (Table 4). These results clearly suggest that the rate of MA metabolism is much higher in rhesus liver microsomes than in human microsomes.

We further determined the kinetic parameters (V_{max} and K_m) for the formation of AM in human and monkey liver microsomes using both the (l- and d-) MA stereoisomers (Fig. 4). The K_m for d-MA was marginally different (not statistically significant) in human ($15 \pm 4 \mu M$) compared with rhesus ($27 \pm 11 \mu M$) microsomes. Similarly, the K_m for l-MA was also similar in both human ($16 \pm 0.2 \mu M$) and rhesus ($16 \pm 4 \mu M$). However, the V_{max} for both d-MA and l-MA in rhesus microsomes was ~6-fold higher ($19 \text{ pM AM/min/mg microsomes}$ for both stereoisomers) than in human microsomes (3.5 and

TABLE 4

Determination of the methamphetamine and its metabolites 4-hydroxymethamphetamine, amphetamine, 4-hydroxyamphetamine and norephedrine in the rhesus monkey plasma after administration of methamphetamine for 20 weeks

Drug/Metabolites	Monkey-1	Monkey-2
	ng/ml	
Methamphetamine	2.83	1.22
4-Hydroxymethamphetamine	0.28	0.24
Amphetamine	29.7	28.4
4-Hydroxyamphetamine	27.3	30.1
Norephedrine	2.11	2.39

3.1 pM AM/min/mg microsomes for d-MA and l-MA, respectively) (Fig. 4). These results suggest that MA metabolism in rhesus microsomes is catalytically more efficient ($V_{\max}/K_m$) than in human microsomes.

Inhibition of MA Metabolism by Human CYP Inhibitors. We determined the relative contributions of human CYP2D6 and CYP3A4 in MA metabolism for both the MA stereoisomers by using their specific inhibitors: quinidine, paroxetine, and fluoxetine for CYP2D6, and ketoconazole, ritonavir, and indinavir for CYP3A4 (Fig. 5). The results showed that human CYP2D6 inhibitors such as quinidine, paroxetine, and fluoxetine inhibited d-MA metabolism by approximately 35%, 27%, and 60%, respectively, and they inhibited l-MA metabolism by approximately 13%, 33%, and 20%, respectively. Similarly, human CYP3A4 inhibitors such as ketoconazole, ritonavir, and indinavir inhibited d-MA metabolism by approximately 50%, 30%, and 45%, respectively, while they inhibited l-MA metabolism by approximately 27%, 18%, and 22%, respectively. However, no

significant inhibition of either d- or l-MA metabolism was observed in rhesus microsomes by these human CYP2D6 and CYP3A4 inhibitors, except with fluoxetine and indinavir for the metabolism of d-MA or l-MA, respectively (Fig. 5). These results clearly suggest that human CYP2D6 and CYP3A4 are different from their homologous enzymes (CYP2D6 and CYP3A64) in rhesus.

MA Docking to Human and Rhesus CYP Models. The differences observed in the inhibition of MA-metabolism using human versus rhesus microsomes may be due to different binding patterns of human and rhesus CYP3A4/3A64 and CYP2D6 enzymes. Therefore, we investigated the binding patterns of both d- and l-MA with human and rhesus CYP2D6 and CYP3A enzymes using substrate docking. The docking results showed that d-MA binds the active sites of all the four P450 enzymes in three different orientations (Table 6). From the top 10 ranking in docking scores, the binding poses were classified into three cases: 1) the *N*-methyl group approaching to the heme iron, which would undergo *N*-demethylation; 2) the phenyl ring pointing to the heme iron, which would undergo hydroxylation at the C4 site; and 3) other atoms approaching to the heme iron, which would lead to other reactions. The detailed results have been summarized in Table 6.

From the statistical data, human CYP2D6, rhesus CYP3A4, and rhesus CYP3A64 preferred both the cases (cases 1 and 2), suggesting that these enzymes can yield both *N*-demethylation and 4-hydroxylation of MA. On the other hand, human CYP3A4 preferred only case 2, suggesting this enzyme can only yield *N*-demethylation. Further, based on the number of conformers, human CYP2D6 prefers 4-hydroxylation over *N*-demethylation, whereas rhesus CYP2D6 prefers *N*-demethylation over 4-hydroxylation (Table 6, Fig. 6). However, both human and rhesus CYP3A4 mainly prefer *N*-demethylation.

Fig. 3. Kinetics of methamphetamine (MA) degradation and formation of its metabolites, amphetamine (AM) and 4-hydroxyamphetamine (4-OH AM) in rhesus liver microsomes and human liver microsomes. (A) MA remaining amount (ng/mL). (B) MA degradation (ng/mL). (C) Formation of AM. (D) Formation of 4-OH MA. The degradation of MA was plotted by using the remaining amount of MA (A) and by using the amount that was metabolized (B). The enzyme activities were performed using human and rhesus microsomes as described in *Materials and Methods*. The maximum activity and apparent $t_{1/2}$ were determined by fitting the data using hyperbolic equation. LM, liver microsomes.

TABLE 5

Determination of apparent kinetic constants of the microsomal N-demethylation and hydroxylation of methamphetamine into amphetamine and 4-hydroxyamphetamine in rhesus monkey and human

Apparent kinetic constants for the hydroxylation and N-demethylation of methamphetamine by hepatic microsomes (HM) from human and monkey were obtained by measuring the consumption of methamphetamine and formation of metabolites.

Name	Monkey Microsomes		Human Microsomes		Ratio: Monkey/Human Relative activity/(t _{1/2})
	Activity	Apparent (t _{1/2})	Activity	Apparent (t _{1/2})	
	ng/ml/mg LM	h	ng/ml/mg LM	h	
MA	67.9 ± 5.1	1.46 ± 0.41	40.1 ± 11	7.18 ± 4.7	8.34
AM	35.5 ± 4.2	0.29 ± 0.25	27.3 ± 4.5	6.04 ± 2.0	27.1
4-OH AM	4.79 ± 0.61	1.31 ± 0.60	4.57 ± 1.9	11.1 ± 9.8	9.00

The results from docking energy and average distance from the heme to the site of reaction for all the P450 enzymes suggest that N-demethylation is preferred over 4-hydroxylation with both rhesus and human CYP2D6 and CYP3A enzymes. Furthermore, the results of l-MA docked into the active sites of four P450 enzymes were very similar to those of d-MA. The docking results of top 10 binding poses for each P450 are summarized in the supplemental data (Supplemental Fig. 1; Supplemental Table 7). Briefly, the binding poses of l-MA-docking were also classified into three cases, which are similar to those of d-MA.

One point that is worth noting is that there was one binding pose leading to l-MA 4-hydroxylation by human CYP3A4, but no such binding pose was observed for d-MA. Similarly, there was no binding pose yielded for the l-MA bond to rhesus CYP3A64, but one binding pose was observed for d-MA (Supplemental Data; Supplemental Table 7 and Supplemental Fig. 1).

Discussion

In this study, we developed and validated a reliable, rapid, simple, and sensitive ESI-tandem mass spectrometry method for simultaneous determination of MA and its four metabolites in rhesus plasma and liver microsomes. Surprisingly, the plasma levels of MA metabolites AM and 4-OH AM were much higher than MA and other metabolites.

This finding was further confirmed in rhesus liver microsomes. In addition, we found that the metabolism of MA was more efficient in rhesus than human microsomes, suggesting differences in MA-metabolizing P450 enzymes between human and rhesus. The basis of these differences, in part, was supported by inhibition and docking studies. This is the first report on the metabolism of MA in rhesus, and we demonstrate that the metabolism of MA is more rapid in rhesus than in humans.

LC-MS/MS is a widely used technique in clinical and preclinical research. However, LC-MS/MS results are often inconsistent because of ineffective optimization of MRM parameters, sample preparation, and extraction methods. These types of issues can be overcome by optimizing sample extraction and mass spectrometry parameters including MRM transitions with LC chromatogram conditions. Liquid-liquid and solid-phase extractions are usually the most effective approaches, but they are expensive and time consuming (Earla et al., 2012). The elimination of water-soluble inorganic metallic substances such as phosphates, sodium, and sulfates from plasma is important in ESI LC-MS/MS analysis to reduce ion suppression (Bogusz et al., 2007; Clavijo et al., 2009). In addition, the pH of the reconstitution solution and mobile phase is very important to achieve maximum chromatographic peak separation, resolution, reliability, ionization, and reproducibility. By considering these factors, we have validated a novel, rapid, and robust ESI-LC-MS/MS assay that uses a hydrophilic-lipophilic balance

Fig. 4. Substrate kinetic studies using both enantiomers of methamphetamine (d- and l-MA) and determination of kinetic parameters K_m and V_{max} of human and monkey microsomes for formation of AM. The velocity of formation of amphetamine [pM AM/min/mg liver microsomes (LM)] are shown on the y-axis, and the MA concentrations (μ M) are shown on the x-axis. (A) Formation of AM in human and monkey LM with d-MA. (B) Formation of AM in human and monkey LM with l-MA. The kinetic parameters K_m and V_{max} were determined by fitting the curve to nonlinear regression analysis using Michaelis-Menten model, and the data are presented in the inset. Mean $\pm$ S.E.M. were calculated from the fitting of the curve.

Fig. 5. Enzyme inhibition using selective inhibitors of human CYP2D6: quinidine (QND), paroxetine (PXN), and fluoxetine (FXN); and CYP3A4: ketoconazole (KTZ), ritonavir (RTV), and indinavir (INV). The formation of amphetamine (AM) was determined in the absence and presence of these inhibitors in rhesus and human liver microsomes (LM). (A) Formation of AM in human LM at 1 hour. (B) Formation of AM in rhesus LM at 1 hour. The y-axis represents the percentage of AM formation (mean + S.E). The 100% activity in human and rhesus corresponds to ~2 ng/ml/mg LM and ~11 ng/ml/mg LM, respectively.

SPE cartridge to analyze rhesus plasma. In this method, we used 1 ml of methanol followed by water, which is simpler and faster than the previously reported methods (Bogusz et al., 2007; Clavijo et al., 2009).

In previously described methods, a higher amount of solvents was used followed by additional washing steps that also contained ammonium hydroxide, dichloromethane, and isopropanol. All previously reported LC-MS/MS methods have used ammonium acetate or ammonium formate buffers in their mobile phase systems with proton adducts in negative and positive modes (Djozan and Baheri, 2007; Kim et al., 2008; Lee et al., 2012). Such ammoniated buffer mobile phase systems may enhance ionization of analytes; however, ammoniated buffers commonly clog the peak tubes, pump seals, and precipitate in the flow line of the mobile phase, which can lead to an increase in backup pressure causing leakage. Therefore, the optimization of MA and its metabolites in a formic acid mobile phase system, which does not clog the pump and maintains a uniform pressure, is superior to the previous method (Earla et al., 2012).

Second, this mobile phase system is easy to clean because this mobile phase does not precipitate. Further, MA and its metabolites are all eluted and separated from the column within the same retention time (~1 minute) with sharp resolution. Thus, this LC-MS/MS method is robust, fast, and effective compared with other published method

(Mueller et al., 2008); which had a longer run time and asynchronous analyte elution. The recovery efficiency of this method is not only higher than the previous methods, but the peak response is reproducible and consistent. In addition, MA and its metabolites are very stable in freeze-thaw cycles at -80°C as well as at bench top ambient temperature.

Using the newly developed LC-MS/MS technique in rhesus plasma and liver microsomes, we have shown that MA is rapidly metabolized to a major metabolite AM and a minor metabolite 4-OH AM in the liver microsomes. However, the levels of both the metabolites are similar; they >20-fold higher than MA in plasma when the rhesus macaque was treated for 20 weeks with MA. These results suggest that MA is first metabolized to AM through N-demethylation followed by conversion to 4-OH AM through 4-OH AM. In addition, our results suggest that MA is also metabolized to 4-OH MA, which may be converted into 4-OH AM. Further, 4-OH AM is metabolized into norephedrine. Thus, in rhesus that were treated for 20 weeks with MA, production of the major metabolite AM and the terminal metabolite 4-OH MA is expected, rather than the production of AM alone as observed in liver microsomes treated for up to 12 hours by MA.

This is the first *in vitro* as well as *in vivo* report on MA metabolism in rhesus macaques. Our results are consistent with the literature,

TABLE 6
Statistical results of MA docked into the active sites of four CYP enzymes

P450	SOM1 (4-OH)				SOM2 (de-methyl)				Other
	No. of Configurations	Average Score ^a	Lowest Score	Average Distance (Å) ^b	No. of Conf	Average Score	Lowest Score	Average Distance (Å) ^b	No. of Configurations
Human CYP2D6	6	-5.65	-5.87	3.54	2	-5.447	-5.485	3.16	2
Rhesus CYP2D6	3	-5.1	-5.38	3.86	4	-4.95	-5.04	3.31	3
Human CYP3A4	0	0	0	0	8	-5.12	-5.62	3.42	2
Rhesus CYP3A64	1	-5.58	-5.58	3.43	7	-5.39	-5.87	3.27	2

^aThe average docking scores of all poses in this case.

^bThe average distance between C4 in the phenyl ring or the C atom of the N-methyl group.

Fig. 6. Autodocking of MA with human CYP3A4, rhesus CYP3A64, human CYP2D6, and rhesus CYP2D6. Binding of methamphetamine (MA) with human CYP2D6 active site in orientation leading to N-demethylation (A) and 4-hydroxylation (B), with rhesus CYP2D6 in orientation leading to N-demethylation (C) and 4-hydroxylation (D), with human CYP3A4 in orientation leading to N-demethylation (E–F) and 4-hydroxylation (G–H). MA is shown in blue, P450 amino acids are shown in green, and heme of the P450 is shown in red.

which report that MA is primarily metabolized to AM and 4-OH MA by N-demethylation and 4-hydroxylation, respectively, in human plasma and liver microsomes. AM is further metabolized to 4-OH AM and norephedrine by hydroxylation (Miranda-G et al., 2007; Mueller et al., 2008; de la Torre et al., 2012). Similarly, in vitro analysis of MA metabolism in human microsomes also suggests that MA is first metabolized to the major metabolite AM and the minor metabolite 4-OH AM. They also produce 4-OH MA, but its levels are too low to be able to determine its rate of formation (data not shown). As expected, norephedrine was not detectable in short reaction times in in vitro experiments using liver microsomes. However, norephedrine is expected to be formed in the plasma upon chronic treatment with MA, as observed in rhesus plasma in our study and in humans as shown previously elsewhere (Brodie et al., 1969; Lewander, 1971; Lin et al., 1997).

The most interesting observation in our study was that the rate of MA metabolism, especially for the formation of AM, is much faster in rhesus liver microsomes than in human microsomes. Further, our kinetic study clearly suggests that rhesus microsomes are much more efficient (~ 8 -fold when compared for $V_{\max}/K_m$) than human microsomes for the formation of AM due to the metabolism of both d-MA and l-MA. However, there is no observable difference in the K_m between d-MA and l-MA, suggesting that both the stereoisomers bind with similar affinity with either the human or rhesus enzymes. These

differences could be a result of differences in the P450 enzymes between human and rhesus. MA is mainly metabolized by CYP2D6, and to some extent CYP3A4, in the human liver (Ramamoorthy et al., 2001; de la Torre et al., 2012; Welter et al., 2013). However, our results from the in vitro study using human microsomes show that CYP2D6 and CYP3A4 contribute almost equally to the metabolism of MA stereoisomers. Most importantly, human CYP2D6 and CYP3A4 inhibitors did not inhibit MA metabolism in rhesus microsomes.

These results clearly suggest significant differences between human and rhesus MA-metabolizing P450 enzymes, which may also explain differences in catalytic properties of human and rhesus MA-metabolizing enzymes. Furthermore, it is possible that MA-metabolism in rhesus is attributed to entirely different CYP450 enzymes.

Species differences are expected to cause differences in drug metabolism between rhesus and humans (Kumar et al., 2009). Moreover, there is very little known about the rhesus homologs of human CYP2D6 and CYP3A4 (Selvakumar et al., 2014). The literature suggests that there is a rhesus P450 enzyme that is homologous to human CYP2D6, which has $>90\%$ sequence homology (Yasukochi and Satta, 2011). However, the metabolic characteristics of rhesus macaque CYP2D6 have not been studied. Similarly, rhesus CYP3A64 is 93% identical to human CYP3A4 and also shows similar metabolic characteristics to human CYP3A4 for some substrates tested (Carr et al., 2006). In contrast, our results from

MA metabolism, as well as inhibition by human CYP3A4 selective inhibitors, clearly suggest that rhesus CYP3A64 is different from the human CYP3A4. Similarly, the differences in MA metabolism and inhibition by human CYP2D6 selective inhibitors also suggest that rhesus CYP2D6 is different from human CYP2D6. Therefore, we developed rhesus CYP2D6 and CYP3A64 models using the crystal structures of human CYP2D6 and CYP3A64, respectively, and then docked these models with MA in the active site.

The findings from docking studies are consistent with the experimental findings that AM is the major product and 4-OH MA or 4-OH AM are minor products in both human and rhesus microsomes. However, based on docking data, it would be difficult to explain why rhesus P450 enzymes are more active than human P450 enzymes. To some extent, the docking data suggest that N-demethylation, which is the main reaction, is more preferred by rhesus CYP2D6 than the human CYP2D6. Similarly, the formation of 4-hydroxylation product is more preferred by rhesus CYP3A64 than human CYP3A4. Taken together, our data suggest that CYP2D6 may be the major contributor of MA metabolism in rhesus, while both CYP2D6 and CYP3A4 may contribute equally in human.

In conclusion, we developed and validated a highly sensitive LC-MS/MS method for determining MA metabolism using rhesus plasma and liver microsomes, and we used the method for analysis of differential MA metabolism in rhesus and human. Overall, our results suggest that rhesus MA metabolic P450 enzymes are different from those of humans. Further studies are needed to understand structure-function relationships of rhesus CYP2D6 and CYP3A64 that contribute to rapid MA metabolism. It is also important to study whether P450 pathway-mediated MA metabolism contributes to oxidative stress and subsequent neurotoxicity in rhesus as well as in humans.

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Authorship Contributions

Participated in research design: Earla, S. Kumar, Fox, W. Li, A. Kumar.

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Contributed new reagents or analytic tools: Earla, W. Li.

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Wrote or contributed to the writing of the manuscript: Earla, S. Kumar, Fox, W. Li., Silverstein, A. Kumar.

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