Journal of Chromatography B, 876 (2008) 266–276

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A validated gas chromatographic–electron impact ionization mass spectrometric method for methamphetamine, methylenedioxymethamphetamine (MDMA), and metabolites in mouse plasma and brain

Karl B. Scheidweiler, Allan J. Barnes, Marilyn A. Huestis*

Chemistry and Drug Metabolism, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Biomedical Research Center, 251 Bayview Boulevard, Baltimore, MD 21224, USA

ARTICLE INFO

Article history:
Received 16 July 2008
Accepted 2 November 2008
Available online 7 November 2008

Keywords:
Methylenedioxymethamphetamine
Methamphetamine
Gas chromatography
Mass spectrometry
Tissue
Mouse

ABSTRACT

A method was developed and fully validated for simultaneous quantification of methamphetamine (MAMP), amphetamine, hydroxy-methamphetamine, methylenedioxymethamphetamine (MDMA, ecstasy), methylenedioxyamphetamine, 3-hydroxy-4-methoxy-methamphetamine, and 3-hydroxy-4-methoxy-amphetamine in 100 μL mouse plasma and 7.5 mg brain. Solid phase extraction and gas chromatography–electron impact ionization mass spectrometry in selected-ion monitoring mode achieved plasma linear ranges of 10–20 to 20,000 ng/mL and 0.1–0.2 to 200 ng/mg in brain. Recoveries were greater than 91%, bias 92.3–110.4%, and imprecision less than 5.3% coefficient of variation. This method was used for measuring MAMP and MDMA and metabolites in plasma and brain during mouse neurotoxicity studies.

Published by Elsevier B.V.

1. Introduction

The sympathomimetic amines, methamphetamine (MAMP) and its derivative methylenedioxymethamphetamine (MDMA, ecstasy) are psychostimulants widely abused worldwide [1–4]. MAMP and MDMA can cause serious health abnormalities [5–8]. Research in humans has shown that MAMP is highly toxic and can produce hyperthermia, heart failure, aggression, psychotic behavior, memory loss and potential brain damage [5]. MAMP caused prolonged depletion of the neurotransmitter dopamine and dopamine transporter protein in brain [5,7]; additional studies provided evidence of neuronal death in MAMP users' brains [9].

Although MDMA is structurally similar to MAMP, MDMA's effects differ by possessing both stimulant and hallucinogenic properties [6]. In most species, MDMA is selectively neurotoxic to serotonergic terminals, except in mice MDMA depletion of dopaminergic terminals predominates [7,10]. Studies have demonstrated long-term impairments of memory and learning in human subjects reporting heavy MDMA use [8].

Transgenic mice are genetically engineered to lack expression of particular proteins and are useful for delineating sympathomimetic amine mechanisms of action and neurotoxicity [11–24]. Results from transgenic mice can be confounded by compensatory changes in protein expression. Therefore, it is important to identify whether drug metabolism and distribution are altered in the transgenic mice, confounding interpretation of experimental results.

Sympathomimetic amines have been measured in plasma and tissue using gas chromatography-nitrogen-phosphorous detection [25,26] gas chromatography–mass spectrometry (GC–MS) [27–36], high pressure liquid chromatography (HPLC)-diode array detection [37], HPLC-fluorescence detection [38–40] and HPLC–mass spectrometry [41–45]. Most methods have been developed for measuring AMP and MAMP or MDMA and metabolites in human plasma, although Peters et al. detailed a validated GC–MS method for measuring AMP, MAMP, MDMA and MDA in human plasma [34]. Three validated LC–MS methods for measuring MAMP, MDMA and metabolites in plasma have also been reported [41,42,45]. Only a few reports detail measuring MAMP and AMP [16] or MDMA and metabolites [40,46] in mouse brain. Comprehensive validation of these brain analytical methods was not presented [16,40,46]. MAMP- and MDMA-induced depletion of dopaminergic and serotonergic nerve terminals have been identified in multiple regions of the brain, predominantly in striatum and cortex [7,10,47,48]. Therefore, it is important to measure drug concentrations in brain regions most affected by MAMP and MDMA.

* Corresponding author at: National Institute on Drug Abuse, National Institutes of Health, Biomedical Research Center, 251 Bayview Boulevard, Suite 200, Room 05A-721, Baltimore, MD 21224, USA. Tel.: +1 443 740 2524; fax: +1 443 740 2823.
E-mail address: mhuestis@intra.nida.nih.gov (M.A. Huestis).

1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2008.11.001

Measuring drug concentrations in mouse striatum presents a considerable challenge as the brain volume is small, typically weighing 15–20 mg. Collection of blood from mice via cardiac puncture typically yields 100–300 μL of plasma for analysis. Therefore, analytical sensitivity is critical for measuring concentrations of MAMP, MDMA and metabolites in small specimens collected during mice neurotoxicity studies.

This manuscript details the development and validation of a gas chromatographic electron impact ionization mass spectrometric (GC–MS-EI) method for simultaneous quantification of MAMP, its metabolites amphetamine (AMP) and para-hydroxy-methamphetamine (OH-MAMP) and MDMA and its metabolites methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxy-methamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA) in mouse plasma and brain to support our studies investigating the role of drug metabolism and distribution in MAMP- and MDMA-induced neurotoxicity in transgenic mice. This manuscript details the first validated method capable of simultaneously measuring MAMP, MDMA and metabolites in mouse plasma and striatum and will be applied to the investigation of MAMP and MDMA metabolism and distribution in wild-type and transgenic mice during neurotoxicity studies.

2. Experimental

2.1. Reagents

Racemic mixtures of MAMP, AMP, MDMA, MDA (1 mg/mL in methanol) and internal standards MAMP-d_{14}, AMP-d_{11}, MDMA-d_5, and MDA-d_5 (100 μg/mL) were purchased from Cerilliant Corporation (Round Rock, TX, USA). Racemic HMMA and HMA (1 mg/mL in methanol) were obtained from Lipomed Inc. (Cambridge, MA, USA) and *p*-hydroxy-methamphetamine powder (greater than 98% purity) from Sigma–Aldrich (St. Louis, MO, USA). Racemic MDMA hydrochloride and racemic MAMP hydrochloride administered to mice were from Lipomed Inc. and the National Institute on Drug Abuse's drug inventory maintained by Research Triangle Institute, Inc. (Research Triangle Park, NC, USA), respectively.

p-Methoxy-methamphetamine, *p*-methoxyamphetamine, ephedrine, pseudoephedrine, phenylpropanolamine (norephedrine), fenfluramine and phentermine for evaluation of potential interferences were obtained from Cerilliant Corporation; methylenedioxyethylamphetamine was from Alltech Associates, Inc. (Deerfield, IL, USA).

Concentrated hydrochloric acid, acetic acid, ammonium hydroxide, ACS reagent grade potassium phosphate monobasic, potassium phosphate dibasic and sodium hydroxide were acquired from JT Baker (Phillipsburg, NJ, USA). All solvents were HPLC grade purchased from JT Baker (Phillipsburg, NJ, USA) unless indicated otherwise. GC grade n-heptane and β-Glucuronidase from *Helix pomatia* (107,100 units/mL and 7500 units/mL glucuronidase and sulfatase activity, respectively) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Heptafluorobutyric acid anhydride (HFAA), triethylamine (ionate grade) and 10 mL reacti-vials were bought from Pierce Chemical Co. (Rockford, IL, USA). Brain tissue was homogenized using a 750-W ultrasonic processor equipped with a 1/8″ diameter probe operated at 60% amplitude (Cole-Parmer, Vernon Hills, IL, USA). SPEC MP1, 10 mL reservoir/70 mg bed mass, mixed mode monolithic disc solid phase extraction columns were acquired from Varian Inc. (Lake Forest, CA, USA). Mouse blood was collected in heparinized tubes from Braintree Scientific (Braintree, MA, USA). Pooled blank human plasma was obtained from the National Institutes of Health Clinical Center (Bethesda, MD, USA).

2.2. Internal standard, calibrator and quality control solutions

Working internal standard solution containing 1 μg/mL of MAMP-d_{14}, AMP-d_{11} and MDMA-d_5 and 0.5 μg/mL of MDA-d_5 was prepared in methanol. Internal standard working solution was stored at −20 °C.

OH-MAMP was weighed and diluted in methanol to produce a 1 mg/mL solution. Working standard solutions containing all seven analytes (MAMP, AMP, OH-MAMP, MDMA, MDA, HMMA, and HMA) at concentrations of 0.2, 0.5, 1, 2, 5, 10, 25, 50 and 100 μg/mL were prepared in methanol. All calibrator solutions were stored at −20 °C.

Quality control (QC) solutions were prepared in methanol using different ampules of reference standards than were used for calibrator preparation. OH-MAMP was weighed and diluted in methanol to produce a 1 mg/mL solution. QC working solutions of 0.3, 2.25, 9.0, 36 and 90 μg/mL were prepared in methanol and stored at −20 °C.

2.3. Plasma specimen preparation

Calibration and QC samples were prepared in plasma verified as negative prior to use. Addition of 10 μL (20 μL for 20,000 ng/mL calibrator) of each calibrator working solution to 100 μL of blank human plasma in 12 mm × 75 mm polypropylene tubes yielded calibrators from 10 to 20,000 ng/mL. Quality controls (30, 225, 900, and 3600 ng/mL) were prepared by adding 10 μL of each QC working solution to 100 μL of blank human plasma. 20 μL of 90 μg/mL QC working solution was added to 100 μL blank plasma to prepare 18,000 ng/mL QC. Working internal standard solution (10 μL) was added to each calibrator and QC, so that each contained 100 ng/mL of MAMP-d_{14}, AMP-d_{11} and MDMA-d_5 and 50 ng/mL of MDA-d_5.

Samples (100 μL) were vortexed briefly and 800 μL of ice-cold 0.388 M trichloroacetic acid in water added. Tubes were capped, vortexed and protein pelleted via centrifugation at 3000 × *g*, 4 °C for 10 min. Supernatants were decanted into 10 mL reacti-vials. Pellets were re-suspended by vortexing after addition of 775 μL of room temperature 0.2 M trichloroacetic acid in water. Tubes were centrifuged again at 3000 × *g*, 4 °C for 10 min and supernatants were decanted into the same 10 mL reacti-vials. Reacti-vials were capped with Teflon-lined caps and stored overnight at 4 °C. Vials were equilibrated at room temperature for 15 min and 100 μL of concentrated hydrochloric acid (12 M) was added. After re-capping, vials were vortexed and incubated at 100 °C for 45 min to hydrolyze OH-MAMP, HMMA and HMA conjugates. After equilibration at room temperature for 20 min, samples were neutralized by addition of 200 μL 6 M sodium hydroxide in water and diluted with 4 mL of 0.2 M sodium acetate buffer, pH 4.5.

2.4. Brain specimen preparation

7.5 mg of mouse brain was homogenized in 800 μL of homogenization solution (0.05 M trichloroacetic acid and 0.025 M thiourea in water). Blank mouse brains were frozen in dry ice-isopentane bath immediately upon harvest from male CD-1 mice and stored at −80 °C.

For preparation of calibrators and QC samples, an entire mouse brain was weighed into a polypropylene tube and homogenization solution added to yield a 100 mg brain/mL concentrated blank brain stock solution. Tubes were placed in an ice-water bath and homogenized for 1 min. Concentrated blank brain stock solution was diluted with homogenization solution to yield a 9.375 mg/mL brain homogentate solution that was aliquoted for preparation of calibrators and QC samples.

Brain calibrators (0.1–100 ng/mg of brain tissue) were prepared by adding 7.5 μL of each calibrator working solution to

800 μL of 9.375 mg/mL blank brain homogenate aliquoted into a 12 mm × 75 mm polypropylene test tube. 15 μL of 100 μg/mL calibrator working solution was added to 800 μL of 9.375 mg/mL blank brain homogenate to prepare 200 ng/mg calibrator. Quality controls (0.3, 2.25, 9.0 and 36 ng/mg) were prepared by adding 7.5 μL of each QC working solution to 800 μL of 9.375 mg/mL blank brain homogenate aliquoted into a 12 mm × 75 mm polypropylene test tube. 15 μL of 90 μg/mL QC working solution was added to 800 μL of 9.375 mg/mL blank brain homogenate to prepare 180 ng/mg QC. Working internal standard solution (7.5 μL) was added yielding 1.0 ng/mg MAMP-d_{14}, AMP-d_{11} and MDMA-d_5 and 0.5 ng/mg MDA-d_5.

Our research protocol requires measurement of MAMP, MDMA and metabolite concentrations in cortex, striatum and hippocampus of wild-type and knock-out mice. 800 μL of homogenization solution was added to one hemisphere of each frozen brain region (weighed into a 12 mm × 75 mm polypropylene test tube). Specimens were homogenized for 30 s while tubes were in an ice-water bath. An aliquot of specimen homogenate was diluted with homogenization solution such that each contained the equivalent of 7.5 mg of tissue in 800 μL of homogenization solution. Working internal standard solution (7.5 μL) was added producing 1.0 ng/mg MAMP-d_{14}, AMP-d_{11} and MDMA-d_5 and 0.5 ng/mg MDA-d_5.

Samples were precipitated by centrifugation at 3500 × *g*, 4 °C for 10 min. Supernatants were decanted into 13 mm × 100 mm glass, screw-top test tubes. Pellets were re-suspended via vortexing in 775 μL of room temperature 0.05 M trichloroacetic acid in water. Tubes were centrifuged again at 3500 × *g*, 4 °C for 10 min and supernatants combined. Tubes were capped with Teflon-lined caps and stored overnight at 4 °C. Tubes were re-capped after addition of 100 μL of concentrated hydrochloric acid (12 M) and heated at 100 °C for 45 min. Samples were neutralized with 200 μL of aqueous 6 M sodium hydroxide and diluted with 4 mL of 0.2 M sodium acetate buffer, pH 4.5.

2.5. Solid phase extraction and derivatization

Prepared plasma and brain specimens were decanted onto SPEC MP1 columns preconditioned with 1 mL of methanol, 1 mL distilled water and 1 mL 0.2 M sodium acetate buffer, pH 4.5. After gravity flow application, columns were washed with 1 mL of 0.1 M acetic acid, 1 mL of tetrahydrofuran and 2 mL of methanol. Following each wash step, columns were dried under vacuum for 2 min at −17 to −34 kPa. Analytes of interest were eluted into clean 5 mL glass centrifuge tubes using 1.5 mL of ethyl acetate:methanol:ammonium hydroxide (77:20:3; v/v/v).

Eluates were completely dried under nitrogen at 35 °C and reconstituted in 100 μL of 0.05 M triethylamine in heptane. Tubes were capped after adding 10 μL of heptafluorobutyric acid anhydride and incubated at 60 °C for 20 min. After tubes cooled to room temperature, 200 μL of 0.1 M potassium phosphate buffer (pH 7.4) was added. Tubes were vortexed for 2 min with a multi-tube vortexer, centrifuged at 3000 × *g* 20 °C for 10 min, and the upper (organic) layer was transferred to autosampler vials.

2.6. Chromatographic instrumentation and analytical conditions-plasma

Analysis was performed on an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) with mass selective detector (Agilent 5975) operated in electron impact mode. The injection port temperature was 250 °C. To extend the linear dynamic range, calibrators, controls and specimens were injected in pulsed splitless and pulsed split (1:5) modes. For the plasma assay, both pulsed split and pulsed splitless modes employed a 1 μL injection volume and pulse pressure of 34.5 kPa (5 psi). The GC capillary column was an Agilent DB-17 ms (30 m length × 0.32 mm inner diameter, 0.25 μm film thickness). The GC oven temperature program began at 70 °C, was immediately increased at 20 °C/min to 150 °C (no hold time), 15 °C/min to 195 °C (no hold time), 5 °C/min to 205 °C, and finally increased at 35 °C/min to 320 °C and held for 2 min. High-purity helium (99.999%) was the carrier gas with a flow rate of 1.5 mL/min operated in constant flow mode. Quadrupole, ion source and mass selective interface temperatures were 150, 230 and 280 °C, respectively. The MS system was operated in the selected-ion monitoring mode with the electron multiplier voltage set at the daily tune value. Table 1 details the ions monitored; dwell time for all ions was 20 ms. Daily GC–MS maintenance included clipping the GC column and replacing the injector septum, liner and gold seal.

Table 1
Analytes, internal standards, quantification and qualifier ions for analysis of sympathomimetic amines in plasma and brain.

Analyte[a]	Selected ions (*m*/*z*)[b]	
	Plasma	Brain
MAMP	118, 210, (254)	118, 210, (254)
MAMP-d14	213, (261)	213, (261)
AMP	118, 192, (240)	118, 192, (240)
AMP-d11	98, (244)	98, (244)
OH-MAMP	210, (254), 330	(254), 303, 330
MDMA	162, (210), 389	162, (254), 389
MDMA-d5	(213), 394	213, (258)
MDA	135, (162), 375	135, (162), 375
MDA-d5	(167), 380	(167), 380
HMMA	(254), 333, 360	(254), 333, 360
HMA	163, (240), 360	163, (240), 360

[a] *Abbreviations*: methamphetamine (MAMP), amphetamine (AMP), hydroxymethamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).
[b] Quantification ions are noted in parentheses.

The instrumentation and conditions for the brain assay were identical to those for the plasma except 2 μL was injected with a 69 kPa (10 psi) pulse pressure for pulsed splitless and pulsed split (1:5) modes.

2.7. Data analysis

Analytes were identified by comparison of retention times (±0.02 min) and qualifier ion ratios (±20%) to corresponding average calibrator values assayed in the same batch. Peak abundance ratios of analytes to corresponding internal standards were calculated for each concentration. In the absence of commercially available stable isotopes for OH-MAMP, HMA and HMMA, MAMP-d14, MDA-d5 and MDMA-d5 were employed as internal standards for OH-MAMP, HMA and HMMA, respectively. Daily calibration was performed using Agilent MSD Chemstation software (version E.01). Calibrator concentrations calculated against the full calibration curve were required to be within 20% of target. Two calibration curves were established for each analyte (low curve: splitless injections, high curve: split injections) to achieve extended linear dynamic ranges.

2.8. Hydrolysis conditions

Plasma collected from male CD-1 mice 2 h after a single intraperitoneal (i.p.) 20 mg/kg MDMA dose (n_{total} = 8 mice) was pooled and 100 μL aliquots were used for evaluation of conjugated OH-MAMP hydrolysis parameters in triplicate. Plasma collected from male FVB-1 mice 0.33, 1, 2, 3 and 4 h after a single i.p. 20 mg/kg MDMA dose (n_{total} = 50 mice) was pooled and 100 μL aliquots were

used for evaluation of conjugated HMMA and HMA hydrolysis parameters in triplicate. Acidic hydrolysis for 45 min at 100 °C, after addition of 100 μL 12 M hydrochloric acid was compared to enzyme hydrolysis. Enzyme hydrolysis conditions were addition of 47 μL of β-glucuronidase solution (for totals of 5000 units of β-glucuronidase and 350 units of sulfatase activity per sample) to TCA-protein precipitated plasma samples that had been neutralized with 30 μL concentrated ammonium hydroxide and buffered with 2 mL of 0.2 M sodium acetate buffer, pH 5.0. After 16 h, 37 °C enzyme hydrolysis, samples were diluted with 4 mL 0.2 M sodium acetate buffer, pH 4.5 prior to SPE. Non-hydrolyzed pooled mouse plasma was also evaluated in triplicate, for which supernatants collected following TCA-plasma protein precipitation were neutralized with 30 μL concentrated ammonium hydroxide and diluted with 4 mL of 0.2 M sodium acetate buffer, pH 4.5 prior to SPE.

2.9. Validation experiments and acceptance criteria

Specificity, sensitivity, linearity, carry-over, bias, imprecision (inter-assay and intra-assay), recovery, and stability were evaluated during method validation.

2.9.1. Specificity

To evaluate endogenous interferences, internal standard working solution was added to 100 μL blank human and mouse plasma and to 800 μL of 9.375 mg/mL blank mouse brain homogenate. Three separate pools of blank human plasma in addition to plasma and brains collected from 12 drug-naive CD-1 mice were prepared and analyzed for interfering peaks.

Additionally, eight structurally related, potentially interfering drugs were investigated by adding individually to low QC samples. Plasma (100 μL) fortified with all seven analytes of interest at 30 ng/mL had 5000 ng/mL of one of the following drugs: pseudoephedrine, ephedrine, phenylpropanolamine, phentermine, fenfluramine, *p*-methoxy-methamphetamine, *p*-methoxyamphetamine and methylenedioxyethylamphetamine. Similarly, each of the eight potential interferences was added individually at a final concentration of 50 ng/mg to the 0.3 ng/mg brain QC sample. Criteria for lack of interference were acceptable ion ratios (within 80–120% average of calibrator ratios) and quantification of low QC within 80–120% of target concentration.

2.9.2. Sensitivity and linearity

Limit of detection (LOD) was evaluated in quadruplicate and defined as concentration producing a peak eluting within ±0.02 min of the analytes' retention time for the lowest calibration standard, a signal-to-noise ratio of at least 3, Gaussian peak shape, and qualifier ion ratios within ±20% of mean calibrator qualifier ion ratios. Limit of quantification (LOQ) also was evaluated in quadruplicate and defined as the concentration that met LOD criteria, signal-to-noise ratio at least 10 and measured concentration within 20% of target in four replicates.

Preliminary experiments using four sets of calibrators were conducted to determine the most appropriate calibration model comparing goodness-of-fit for calibration curves fit using unweighted linear least squares vs. linear least squares employing $1/x$ weighting. Daily calibration curves for the plasma assay were prepared by analyzing 100 μL blank human plasma fortified to contain 10, 20, 50, 100, 200, 500, 1000, 2500, 5000, 10,000, and 20,000 ng/mL analytes of interest. Daily calibration curves for the brain assay were prepared by analyzing 0.8 mL of 9.375 mg brain/mL of homogenate solution fortified to contain 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 25, 50, 100, and 200 ng/mg for analytes of interest. To achieve extended linear ranges in both plasma and brain, two sets of calibration curves were prepared for each analyte. All calibrators, QC samples and specimens were injected twice, once in splitless mode and immediately thereafter in 1:5 split mode. The low curves were constructed with data from splitless injections, and high curves from 1:5 split injections. Specimens were initially processed with splitless data. If a specimen injected in splitless mode quantified greater than 1000 ng/mL in plasma or 10 ng/mg in brain, quantification occurred by split injection and from the high curve. Calibration curves were fit with linear least squares regression. Each point on the calibration curve was required to have acceptable chromatography, ion ratios within ±20% of calibrators' average and quantify within 20% of target when back-calculated against the full calibration curve.

2.9.3. Carry-over

Potential carry-over in the chromatographic system was evaluated in each batch by analyzing a negative sample (blank plasma or brain containing internal standard) injected in splitless mode after splitless injection of the highest calibrator containing all analytes at 20,000 ng/mL or 200 ng/mg for plasma and brain, respectively. The negative sample injected after the highest calibrator was required to not fulfill LOD criteria for any of the analytes.

2.9.4. Bias and imprecision

Bias and imprecision were evaluated over the methods' linear dynamic ranges. Low, medium and high QC samples were 30, 225 and 900 ng/mL and 0.3, 2.25 and 9.0 ng/mg for the splitless curves for the plasma and brain methods, respectively. Low, medium and high QC concentrations were 900, 3600 and 18,000 ng/mL and 9.0, 36 and 180 ng/mg for the split curves for plasma and brain methods, respectively. Intra- and inter-assay performances were evaluated in separate studies. Intra-assay bias was determined comparing measured concentrations of four samples in one batch, inter-assay bias by duplicates in seven batches ($n_{total} = 14$). Imprecision was expressed as percent of coefficient of variation (% CV). Intra-assay imprecision was evaluated by four determinations in one batch, inter-assay imprecision by duplicates in seven batches ($n_{total} = 14$).

2.9.5. Recovery and stability

Recovery from fortified plasma and brain homogenates was evaluated by analyzing two sets of samples, each consisting of four replicates at 10 and 20,000 ng/mL for plasma and 0.1 and 200 ng/mg for brain. Set A was blank plasma or brain homogenate that was fortified with non-deuterated calibrator and internal standard working solutions prior to protein precipitation. Set B was blank plasma or brain homogenate that was extracted and subsequently the SPE eluates were fortified with non-deuterated calibrator and internal standard working solutions. Comparison of mean non-deuterated analyte peak areas (set A/set B) × 100, yielded overall analyte recoveries expressed as percentages.

Stability of stored plasma samples was investigated with blank plasma fortified at 225 ng/mL. Non-deuterated analyte QC working solution was added to 100 μL aliquots of blank plasma in 12 mm × 75 mm polypropylene test tubes. Tubes were capped and stored at room temperature for 12 h, 4 °C for 48 h or −20 °C with room temperature thawing for three freeze/thaw cycles. Each condition was evaluated with five replicates. Samples were fortified with internal standard immediately prior to specimen preparation.

Stability and recovery of analytes during brain homogenization process was investigated with a set of samples prepared with blank mouse brain fortified at three QC concentrations prior to homogenization, and quantification to another set of QC samples fortified after homogenization. Set A was prepared by weighing 5–12 mg portions of blank mouse brain into 12 mm × 75 mm polypropy-

lene test tubes and fortifying each sample with 7.5 μL of 300 or 9000 ng/mL or 15 μL of 90,000 ng/mL QC working solution. 800 μL homogenization solution was added and each sample homogenized for 0.5 min while in an ice-water bath. Internal standard was added to samples from set A after homogenization (prior to protein precipitation and SPE). Set B was prepared by adding 7.5 μL 300 or 9000 ng/mL or 15 μL 90,000 ng/mL QC working solution to 800 μL aliquots of 9.375 mg/mL of blank brain homogenate in 12 mm × 75 mm polypropylene test tubes. Internal standard also was added to brain homogenates from Set B prior to protein precipitation and SPE. Sets A and B were evaluated in quadruplicate.

2.9.6. Human-mouse plasma cross-validation

Since it was not practical to obtain sufficient mouse plasma to prepare calibrators and QC samples daily, pooled human plasma was employed. A cross-validation was performed to verify validity of human plasma for preparing calibrators to measure MAMP, MDMA and metabolites in mouse plasma specimens. Trunk blood was collected and processed to obtain plasma from 12 drug-naive CD-1 mice as detailed in the animal study protocol section of this manuscript. Plasma specimens from 12 mice were combined to form a single pool for preparing QC samples. Calibrators were prepared with pooled human plasma and pooled blank mouse plasma was used to prepare 30, 900 and 18,000 ng/mL QC samples ($n = 3$).

2.10. Animal study protocol

Male CD-1 mice, 12–16 weeks old and weighing 35–45 g, were purchased from Charles River Laboratories (Raleigh, NC, USA). Male FVB-1 mice, 8–12 weeks old and 25–35 g were purchased from Taconic (Germantown, NY, USA). Animals were housed individually and maintained in a temperature- and light-controlled environment. Food and water were available ad libitum. Mice received a single intra-peritoneal injection of MAMP hydrochloride or MDMA hydrochloride and were euthanized 2 h after dosing. Plasma and brain were harvested immediately upon euthanasia. Trunk blood was collected from mice with a pasteur pipet, transferred to a heparinized tube, centrifuged at 1500 × *g* for 7 min and plasma transferred to 1.5 mL screw top polypropylene tubes for storage at −20 °C. Brains were removed and dissected on an ice-chilled steel plate using forceps. Each brain region was frozen in a 1.5 mL screw top polypropylene tube on dry ice immediately upon dissection and stored at −80 °C. Animal use procedures followed the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local National Institute on Drug Abuse Animal Care and Use Committee.

3. Results

3.1. Hydrolysis of conjugated metabolites

Preliminary studies during method development compared assay characteristics obtained employing conditions similar to those validated for human urine [49] using 100 μL HCl and 45 min 100 °C incubation and overnight 37 °C enzyme hydrolysis with β-glucuronidase from *H. pomatia* as per Pizarro et al. [29] and found that enzyme hydrolysis produced elevated matrix interferences that resulted in 2–5-fold decreases in AMP and MAMP sensitivities compared to sensitivities achieved with acidic hydrolysis. Considering the decreased sensitivity obtained using β-glucuronidase and the cost of enzyme, we elected to use acidic hydrolysis for the method presented in this manuscript. However, it is important to understand hydrolysis efficiency for de-conjugating OH-MAMP, HMMA and HMA conjugates in mouse plasma. Completion of preliminary METH and MDMA neurotoxicity studies using CD-1 and FVB-1 mice during which mice were administered 20 mg/kg METH or MDMA i.p. yielded sufficient plasma to create plasma pools containing METH, MDMA and conjugated metabolites. Addition of 100 μL concentrated HCl and 100 °C incubation for 45 min is only 33% as efficient as overnight 37 °C hydrolysis with 5000 units of β-glucuronidase for hydrolyzing conjugated OH-MAMP (Table 2). Similarly acidic hydrolysis was 60% as efficient at hydrolyzing HMMA and HMA conjugates as was found after overnight enzyme hydrolysis (Table 2). Results obtained via both acidic and enzyme hydrolysis were reproducible with the % CV's not exceeding 4.5% between triplicates for acidic and enzyme hydrolysis studies.

Table 2
Hydrolysis of conjugated hydroxy-methamphetamine (OH-MAMP), 3-hydroxy-4-methoxy-methamphetamine (HMMA), and 3-hydroxy-4-methoxy-amphetamine (HMA) in mouse plasma.

	Mean concentration in ng/mL (% CV), $n = 3$		
	OH-MAMP	HMMA	HMA
Non-hydrolyzed	<LOQ[a]	<LOQ[a]	<LOQ[a]
Acid hydrolyzed	125.0 (2.2)	282.4 (4.3)	49.2 (3.9)
Enzyme hydrolyzed	376.6 (1.4)	483.1 (1.4)	78.4 (2.8)

Mouse plasma collected from 8 mice 2 h after 20 mg/kg METH was pooled and evaluated for efficiency of hydrolyzing conjugated OH-MAMP. Mouse plasma collected from 50 mice 0.33–4 h after 20 mg/kg MDMA was pooled and evaluated for efficiency of hydrolyzing conjugated HMMA and HMA. Acid hydrolysis conditions were: addition of 100 μL concentrated hydrochloric acid and 100 °C incubation for 45 min. Enzyme hydrolysis conditions were: addition of 5000 units β-glucuronidase activity to plasma buffered at pH 5.0 and 37 °C incubation for 16 h.

[a] Limits of quantification are 20 ng/mL for OH-MAMP, HMMA and HMA.

3.2. Specificity

No human plasma pools or plasma from 12 drug-naive CD-1 mice contained interfering compounds with any peaks of interest. Similarly, there were no interfering peaks observed in blank mouse brain collected from 12 drug-naive CD-1 mice. None of eight potential exogenous interferences fortified at 5000 ng/mL into 30 ng/mL plasma QC samples caused ion ratios or quantification criteria to fail. Likewise, eight potential interferences at 50 ng/mg fortified in 0.3 ng/mg brain QC samples did not interfere with QC quantification.

3.3. Sensitivity and linearity

Preliminary experiments were conducted using four sets of calibration curves that were fit using both unweighted linear least squares and with linear least squares employing $1/x$ weighting factor to determine the most appropriate calibration model. Inspection of residuals obtained from the unweighted vs. $1/x$ weighted models clearly revealed linear least squares employing $1/x$ weighting produced better fit for the calibration data (data not shown). Calibration by linear least squares employing $1/x$ weighting was used for all data presented in this manuscript. Low calibration curves for plasma employed seven calibrators ranging from 10 to 1000 ng/mL for MAMP, AMP, MDMA and MDA, and six calibrators ranging from 20 to 1000 ng/mL for OH-MAMP, HMMA and HMA. High calibration curves for all seven analytes in plasma were constructed with six calibrators from 500 to 20,000 ng/mL. All correlation coefficients (R-squared) exceeded 0.996. Slight matrix interference was consistently encountered producing some peak distortion, but LOD criteria were fulfilled at 2.5–5.0 ng/mL of plasma. Table 3 summarizes calibration data for the plasma assay with LODs. Fig. 1 contains extracted ion chromatograms from samples fortified at each analyte's LOQ in plasma.

Table 3
Limits of detection (LOD) and calibration curves for sympathomimetic amines in plasma.

Analyte	LOD[a] (ng/mL)	Calibrator range[b] (ng/mL)	Regression equation $y = m^c x + b^d$	Coefficients of determination[e] (R^2)
MAMP	2.5	10–1,000	y = 1.0e−2 (4.1e−4) x − 1.7e−2 (3.0e−3)	0.999–1.000
		500–20,000	y = 1.0e−2 (4.4e−4) x − 3.2e−2 (2.2e−1)	0.999–1.000
AMP	5.0	10–1,000	y = 9.9e−3 (3.4e−4) x − 1.1e−2 (2.6e−3)	0.999–1.000
		500–20,000	y = 9.9e−3 (3.8e−4) x + 9.8e−2 (2.1e−1)	0.999–1.000
OH-MAMP	2.5	20–1,000	y = 1.7e−2 (9.1e−4) x − 8.3e−2 (1.9e−2)	0.998–1.000
		500–20,000	y = 1.7e−2 (7.8e−4) x − 1.3e+0 (5.8e−1)	0.998–1.000
MDMA	5.0	10–1,000	y = 1.0e−2 (4.8e−4) x − 3.0e−3 (7.3e−3)	0.999–1.000
		500–20,000	y = 1.1e−2 (5.4e−4) x − 4.8e−1 (1.8e−1)	0.999–1.000
MDA	5.0	10–1,000	y = 3.7e−2 (1.6e−3) x − 4.7e−3 (3.4e−2)	0.998–1.000
		500–20,000	y = 3.7e−2 (1.7e−3) x + 6.6e−1 (1.5e+0)	0.996–1.000
HMMA	2.5	20–1,000	y = 2.4e−2 (1.5e−3) x − 2.0e−1 (2.1e−2)	0.997–0.999
		500–20,000	y = 2.8e−2 (1.4e−3) x − 3.9e+0 (8.4e−1)	0.998–1.000
HMA	2.5	20–1,000	y = 9.4e−2 (5.3e−3) x − 3.7e−1 (3.5e−2)	0.998–1.000
		500–20,000	y = 9.1e−2 (4.0e−3) x − 7.4e−1 (3.8e+0)	0.996–1.000

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] (n = 4).

[b] 10 or 20–1000 ng/mL calibration using 1 μL pulsed splitless injection. 500–20,000 ng/mL calibration using 1 μL pulsed split injection (1:5).

[c] Mean slope (S.D.) from calibration curves fit using linear regression (n = 7).

[d] Mean intercept (S.D.) from calibration curves fit using linear regression (n = 7).

[e] Range of coefficients of determination from calibration curves (n = 7).

Low calibration curves for brain analyses were constructed from seven calibrators ranging from 0.1 to 10 ng/mg for MAMP, AMP, MDMA, MDA and HMMA, while low curves for OH-MAMP and HMA utilized six points from 0.2 to 10 ng/mg. High curves included six points (5–200 ng/mg) for all seven analytes. All correlation coefficients (R-squared) exceeded 0.995. Similar to plasma, slight matrix interference was consistently encountered producing some peak distortion, but LOD criteria were fulfilled at 0.05–0.1 ng/mg in brain. Table 4 summarizes brain calibration data. Fig. 2 contains extracted ion chromatograms from samples fortified at each analyte's LOQ in brain.

3.4. Carry-over

None of the negative samples injected in splitless mode after the most concentrated calibrator (20,000 ng/mL or 200 ng/mg for plasma and brain, respectively) met LOD criteria (n = 7).

3.5. Bias and imprecision

Bias and imprecision were evaluated at three concentrations across the linear dynamic range. Bias in plasma ranged from 93.9 to 107.5% and 92.0 to 110.4% of target concentrations for intra-

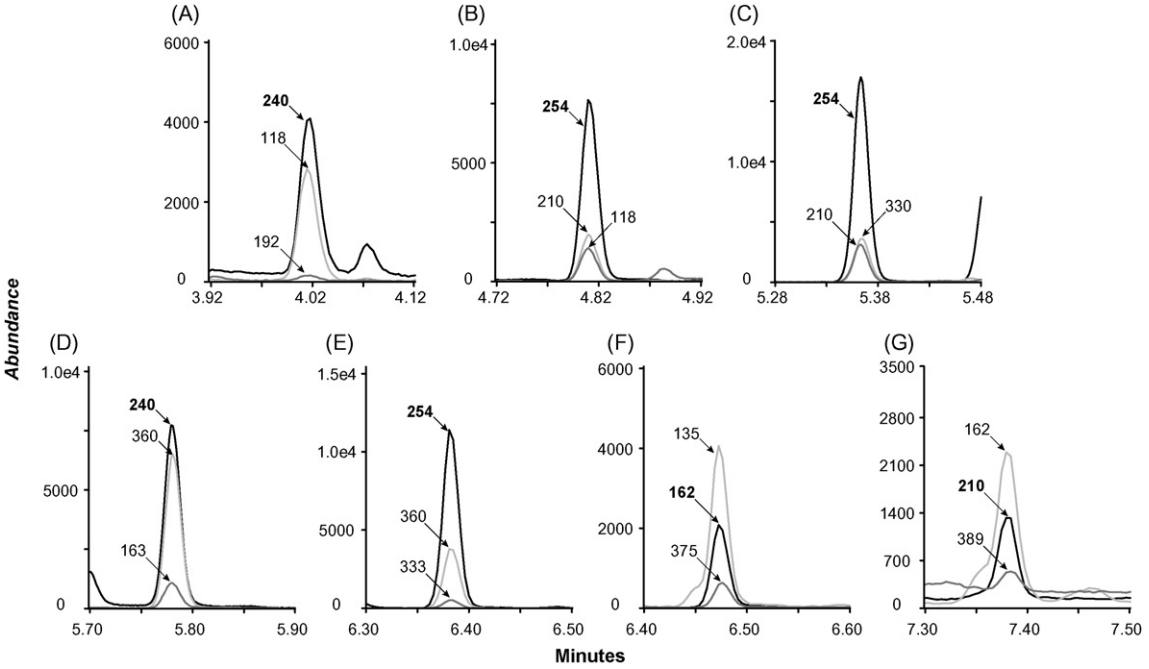

Fig. 1. 100 μL of plasma fortified with drug standards at each analyte's limit of quantification (A: 10 ng/mL amphetamine, B: 10 ng/mL methamphetamine, C: 20 ng/mL hydroxymethamphetamine, D: 20 ng/mL 3-hydroxy-4-methoxy-amphetamine, E: 20 ng/mL 3-hydroxy-4-methoxy-methamphetamine, F: 10 ng/mL methylenedioxyamphetamine and G: 10 ng/mL methylenedioxymethamphetamine).

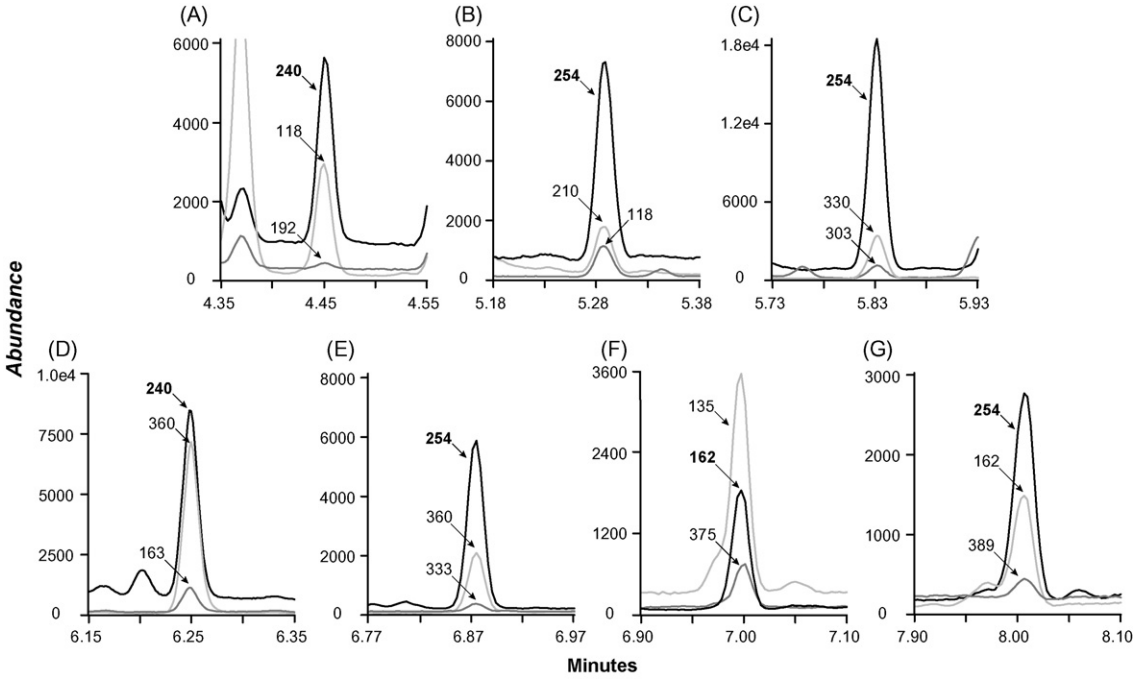

Fig. 2. 7.5 mg blank brain fortified with drug standards at each analyte's limit of quantification (A: 0.1 ng/mg amphetamine, B: 0.1 ng/mg methamphetamine, C: 0.2 ng/mg hydroxy-methamphetamine, D: 0.2 ng/mg 3-hydroxy-4-methoxy-amphetamine, E: 0.1 ng/mg 3-hydroxy-4-methoxy-methamphetamine, F: 0.1 ng/mg methylenedioxyamphetamine and G: 0.1 ng/mg methylenedioxymethamphetamine). *Note*: injection was made after capillary column replacement, therefore retention times do not match Fig. 1.

and inter-assay bias, respectively (Table 5). Intra- and inter-assay imprecision in plasma were 0.2–5.1 and 2.0–5.3% CV, respectively (Table 5). Brain bias and imprecision are presented in Table 5. Bias of sympathomimetic amines in brain was 94.9–108.2 and 95.9–109.0% of target concentrations for intra- and inter-assay bias. Intra- and inter-assay imprecision of sympathomimetic amines in brain ranged from 0.4 to 5.2 and 2.4 to 4.8% CV, respectively.

3.6. Recovery and stability

Recoveries of sympathomimetic amines from plasma and brain homogenate are presented in Table 6. Recoveries of all analytes from plasma and brain were greater than 91 and 90%, respectively.

Concentrations of all analytes were stable in plasma stored at room temperature for 12 h, 4 °C for 48 h and after three

Table 4
Limits of detection (LOD) and calibration curves for sympathomimetic amines in brain.

Analyte	LOD[a] (ng/mL)	Calibrator range[b] (ng/mL)	Regression equation $y = m^c x + b^d$	Coefficients of determination[e] (R^2)
MAMP	0.05	0.1–10	y = 1.0e0 (8.6e−2) x − 1.7e−2 (1.2e−2)	0.999–1.000
		5–200	y = 1.1e0 (6.6e−2) x − 4.3e−1 (3.5e−1)	0.998–1.000
AMP	0.1	0.1–10	y = 9.9e−1 (8.1e−2) x − 5.8e−3 (8.2e−3)	0.999–1.000
		5–200	y = 1.0e0 (6.1e−2) x − 2.5e−1 (3.3e−1)	0.998–1.000
OH-MAMP	0.1	0.2–10	y = 1.5e0 (1.7e−1) x − 6.7e−2 (2.0e−2)	0.999–1.000
		5–200	y = 1.5e0 (1.3e−1) x − 1.0e+0 (4.9e−1)	0.998–1.000
MDMA	0.05	0.1–10	y = 9.8e−1 (8.6e−2) x − 9.9e−3 (1.4e−2)	0.999–1.000
		5–200	y = 1.1e0 (4.5e−2) x − 8.0e−1 (3.9e−1)	0.998–1.000
MDA	0.05	0.1–10	y = 3.4e0 (2.7e−1) x − 3.8e−2 (2.7e−2)	0.999–1.000
		5–200	y = 8.5e0 (7.9e−1) x − 1.7e0 (1.0e+0)	0.995–1.000
HMMA	0.05	0.1–10	y = 2.3e0 (2.4e−1) x − 8.0e−2 (2.3e−2)	0.996–0.999
		5–200	y = 2.6e0 (1.4e−1) x − 2.6e0 (1.0e0)	0.998–1.000
HMA	0.05	0.2–10	y = 7.8e0 (7.9e−1) x − 2.7e−1 (1.5e−1)	0.997–1.000
		5–200	y = 8.5e0 (7.9e−1) x − 6.3e0 (3.1e+0)	0.996–1.000

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] (n = 4).

[b] 0.1 or 0.2–10 ng/mL calibration using 2 μL pulsed splitless injection. 5–200 ng/mL calibration using 2 μL pulsed split injection (1:5).

[c] Mean slope (S.D.) from calibration curves fit using linear regression (n = 7).

[d] Mean intercept (S.D.) from calibration curves fit using linear regression (n = 7).

[e] Range of coefficients of determination from calibration curves (n = 7).

Table 5
Sympathomimetic amines in plasma and brain: bias and imprecision data.

Analyte	Intra-assay, $n = 4$ (ng/mL)						Inter-assay, $n = 14$ (ng/mL)					
	30[a]	225[a]	900[a]	900[b]	3,600[b]	18,000[b]	30[a]	225[a]	900[a]	900[b]	3,600[b]	18,000[b]
Bias in plasma (% of target concentration)												
MAMP	102.0	101.5	101.8	99.2	104.8	102.6	101.2	102.6	102.4	100.4	105.2	100.0
AMP	100.3	100.1	100.8	99.0	104.0	102.2	100.2	101.4	101.5	99.8	104.7	99.3
OH-MAMP	99.3	93.9	98.0	96.6	95.8	96.6	97.6	92.0	95.4	92.3	95.4	94.3
MDMA	103.7	101.5	100.1	96.4	100.3	102.8	100.6	102.0	102.0	99.1	102.8	99.6
MDA	104.3	100.5	100.6	93.9	101.7	100.1	101.2	102.7	101.3	99.4	100.9	98.4
HMMA	107.5	100.1	103.7	101.0	101.7	106.6	110.4	100.7	105.0	101.5	103.5	103.2
HMA	103.1	102.3	104.0	100.1	103.3	101.8	102.7	102.4	102.3	102.0	101.4	99.0
Imprecision in plasma (% coefficient of variation)												
MAMP	2.0	1.1	3.4	3.0	1.2	1.4	2.3	3.5	4.3	4.1	3.3	3.3
AMP	1.4	0.9	2.5	3.0	1.5	1.7	2.4	3.0	3.6	3.7	2.6	3.0
OH-MAMP	3.8	1.4	2.0	1.6	0.8	2.2	5.3	3.9	4.7	4.0	3.3	3.1
MDMA	0.6	0.8	3.8	4.1	1.3	1.1	4.4	3.2	4.1	4.4	3.3	3.6
MDA	0.2	0.9	3.8	5.1	2.9	1.2	3.6	2.7	4.0	4.8	2.6	3.6
HMMA	3.2	1.6	3.0	2.5	0.6	3.1	3.0	3.8	4.4	3.1	3.0	2.8
HMA	3.0	2.0	1.5	2.2	2.6	1.4	4.0	2.0	3.7	4.3	2.6	3.7
	Intra-assay, $n = 4$ (ng/mg)						Inter-assay, $n = 14$ (ng/mg)					
	0.3[c]	2.25[c]	9.0[c]	9.0[d]	36[d]	180[d]	0.3[c]	2.25[c]	9.0[c]	9.0[d]	36[d]	180[d]
Bias in brain (% of target concentration)												
MAMP	99.6	102.4	105.4	101.4	104.1	97.9	102.4	99.4	103.0	101.6	102.2	98.9
AMP	96.7	99.6	102.1	98.9	101.4	95.8	99.1	97.7	100.5	99.8	100.4	96.7
OH-MAMP	108.2	104.2	108.1	101.4	103.6	98.2	109.0	101.0	106.0	102.8	103.8	100.5
MDMA	99.2	101.4	106.1	101.0	100.8	100.3	102.7	98.9	103.5	101.5	101.0	99.4
MDA	99.3	99.2	105.8	99.4	102.1	96.5	102.4	100.3	103.9	100.8	102.7	97.6
HMMA	94.9	96.3	101.6	97.9	101.8	99.5	99.6	95.9	102.9	100.9	101.1	98.8
HMA	107.7	99.2	106.9	102.2	105.4	98.3	106.8	100.7	106.9	104.5	105.6	100.3
Imprecision in brain (% coefficient of variation)												
MAMP	1.7	1.3	1.3	1.1	3.1	0.8	3.1	2.9	3.1	2.4	4.6	4.6
AMP	1.9	1.3	1.5	1.5	3.5	0.7	2.7	2.5	3.2	2.5	4.5	4.8
OH-MAMP	1.3	1.0	1.3	0.9	5.2	1.2	3.5	3.0	3.5	3.0	4.2	4.1
MDMA	1.2	1.4	0.8	1.3	3.2	0.4	3.8	2.9	3.2	2.5	4.5	4.7
MDA	1.7	1.2	2.8	2.1	2.1	1.5	3.8	3.3	3.2	4.1	4.0	4.0
HMMA	2.2	2.3	1.4	1.7	4.1	0.6	3.3	3.5	3.5	3.2	4.5	4.4
HMA	3.5	2.6	4.8	1.9	4.2	1.0	4.4	3.7	3.7	4.6	3.3	3.6

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] Measurements made using 1 μL splitless injection.

[b] Measurements made using 1 μL split 1:5 injection.

[c] Measurements made using 2 μL splitless injection.

[d] Measurements made using 2 μL split 1:5 injection.

freeze/thaw cycles. Concentrations of 225 ng/mL QC samples stored under these conditions were within ±10% of target concentrations (Table 7).

Concentrations of all analytes were stable in brain during homogenization. Concentrations of 0.3, 9.0 and 180 ng/mg QC samples fortified into intact brain tissue prior to homogenization were within ±10% of target and demonstrated 90% recovery of analytes following homogenization (Table 8). It should be noted that during initial method development there were stability/recovery problems for HMMA and HMA during homogenization. Homoge-

Table 6
Recovery of sympathomimetic amines from plasma and brain.

	% Recovery from plasma[a]		% Recovery from brain[b]	
	10 ng/mL	20,000 ng/mL	0.1 ng/mg	200 ng/mg
MAMP	96.3	91.2	102.0	103.5
AMP	94.4	93.1	99.6	103.1
OH-MAMP	96.3	93.4	90.0	101.0
MDMA	93.3	91.2	99.0	103.0
MDA	93.2	93.5	98.8	102.0
HMMA	92.7	93.9	98.8	102.3
HMA	96.8	97.0	96.3	98.1

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] Recovery was determined by comparing peak areas of plasma fortified with calibrator solution prior to trichloroacetic acid precipitation and solid phase extraction (SPE) vs. blank plasma extracts fortified with calibrator solution after SPE, $n = 4$ for each group.

[b] Recovery was determined by comparing peak areas of brain homogenate fortified with calibrator solution prior to trichloroacetic acid precipitation and SPE vs. blank brain extracts fortified with calibrator solution after SPE, $n = 4$ for each group.

Table 7
Stability of sympathomimetic amines in plasma.

	% Target concentration (% CV)		
	RT, 12 h	4 °C, 48 h	Freeze/thaw, ×3
MAMP	100.8 (1.3)	102.3 (1.8)	101.7 (3.3)
AMP	100.3 (1.3)	102.1 (1.9)	100.9 (3.5)
OH-MAMP	91.3 (5.2)	96.9 (2.9)	99.3 (0.7)
MDMA	101.5 (1.2)	101.4 (2.1)	101.4 (3.1)
MDA	99.7 (1.8)	99.8 (2.5)	100.2 (3.3)
HMMA	94.8 (4.2)	96.0 (4.2)	97.4 (1.2)
HMA	95.9 (4.7)	100.6 (3.8)	100.2 (1.3)

Stability studies were evaluated using 225 ng/mL quality control samples that were fortified in blank plasma stored 12 h at room temperature, 48 h at 4 °C or that underwent 3 cycles of freezing at −20 °C and thawing at room temperature prior to analysis. Measured concentrations are expressed as mean percentage of target concentration (% CV), $n = 5$ for each storage condition. *Abbreviations*: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

nization with 0.05 M trichloroacetic acid without thiourea yielded variable HMMA and HMA recoveries. Mean recoveries of 0.3 ng/mg QC samples were 79.8 and 79.5% for HMMA and HMA, respectively ($n = 5$). Additionally, imprecision for QC samples fortified prior to homogenization were 14.9 and 18.1% CV for HMMA and HMA, respectively. Addition of the anti-oxidant thiourea to the homogenization solution prevented HMMA and HMA degradation during homogenization.

3.7. Human-mouse plasma cross-validation

Human-mouse plasma cross-validation was investigated by quantifying 30, 900 and 18,000 ng/mL QC samples ($n = 3$) prepared in pooled mouse plasma against calibrators prepared in pooled human plasma. Bias for all analytes prepared in mouse plasma was 85.6–115.4% of target concentration (Table 8). HMA quantification was consistently lower than target (85.6–89.3% of target

Table 8
Stability and recovery of sympathomimetic amines during brain homogenization and cross-validation for measuring sympathomimetic amines in human vs. mouse plasma.

Analyte	Stability and recovery in brain				Human vs. mouse	
	Target (ng/mg)	Set A[a] mean (%CV), $n = 4$	Set B[b] mean (%CV), $n = 4$	Recovery[c] %	Target (ng/mL)	Mouse QC[d] % target (%CV), $n = 3$
MAMP	0.3[e]	0.31 (4.8)	0.33 (3.7)	95.8	30[e]	107.4 (1.9)
	9.0[e]	9.15 (3.7)	8.78 (1.7)	104.3	900[e]	103.3 (3.8)
	9.0[f]	9.09 (3.7)	8.72 (1.9)	104.3	900[f]	99.7 (3.3)
	180[f]	174.91 (2.2)	173.35 (1.6)	99.7	18,000[f]	95.0 (1.4)
AMP	0.3[e]	0.30 (4.2)	0.31 (1.3)	96.6	30[e]	105.5 (2.4)
	9.0[e]	8.87 (3.5)	8.56 (1.8)	103.6	900[e]	102.8 (3.1)
	9.0[f]	8.84 (3.8)	8.51 (1.7)	103.9	900[f]	99.4 (2.9)
	180[f]	170.95 (2.4)	171.65 (1.9)	99.6	18,000[f]	93.2 (0.9)
OH-MAMP	0.3[e]	0.33 (2.9)	0.33 (4.8)	100.1	30[e]	115.4 (4.3)
	9.0[e]	9.33 (4.2)	8.82 (6.3)	105.7	900[e]	103.5 (10.2)
	9.0[f]	8.99 (4.4)	8.46 (6.5)	106.3	900[f]	98.2 (7.8)
	180[f]	182.41 (1.9)	175.87 (3.2)	103.7	18,000[f]	85.7 (2.5)
MDMA	0.3[e]	0.33 (3.4)	0.33 (2.1)	99.3	30[e]	108.6 (2.8)
	9.0[e]	9.20 (4.0)	8.87 (1.7)	103.6	900[e]	104.9 (3.6)
	9.0[f]	9.06 (3.5)	8.73 (1.7)	103.7	900[f]	102.6 (3.6)
	180[f]	176.89 (2.0)	178.21 (1.9)	99.3	18,000[f]	95.9 (3.3)
MDA	0.3[e]	0.31 (3.9)	0.31 (1.7)	99.3	30[e]	104.6 (2.9)
	9.0[e]	9.00 (3.9)	8.57 (1.8)	105.0	900[e]	107.4 (3.4)
	9.0[f]	9.37 (2.7)	9.10 (0.8)	103.0	900[f]	101.8 (4.9)
	180[f]	170.00 (1.1)	171.62 (2.6)	99.1	18,000[f]	96.2 (1.3)
HMMA	0.3[e]	0.31 (3.6)	0.31 (4.0)	100.0	30[e]	97.9 (3.7)
	9.0[e]	9.23 (4.9)	8.91 (4.0)	103.5	900[e]	96.2 (4.0)
	9.0[f]	9.15 (4.9)	8.88 (4.4)	103.0	900[f]	93.2 (3.3)
	180[f]	180.22 (1.9)	179.82 (1.3)	100.22	18,000[f]	91.2 (2.6)
HMA	0.3[e]	0.33 (3.5)	0.33 (4.7)	99.8	30[e]	88.5 (2.8)
	9.0[e]	9.16 (5.4)	8.65 (8.6)	105.9	900[e]	88.2 (4.2)
	9.0[f]	9.18 (4.4)	8.84 (8.5)	103.9	900[f]	85.6 (4.9)
	180[f]	180.97 (2.5)	174.89 (6.3)	103.5	18,000[f]	89.3 (1.3)

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] Set A: quality control working solution added prior to homogenization.
[b] Set B: quality control working solution added after homogenization, but prior to solid phase extraction.
[c] Recovery was calculated as mean target ion areas of Set A divided by the mean target ion areas of Set B, ×100.
[d] Calibrators were prepared in blank human plasma and quality control (QC) samples were prepared in blank mouse plasma.
[e] Sample injected in splitless mode.
[f] Sample injected in split 1:5 mode.

Table 9
Analyte concentrations in mouse plasma and brain specimens collected 2 h after intra-peritoneal administration of 20 mg/kg methamphetamine (MAMP) or methylenedioxymethamphetamine (MDMA).

(A) Mouse #1: 2 h after 20 mg/kg MAMP administration

	MAMP	AMP	OH-MAMP
Plasma, ng/mL	970.2	225.5	66.3
Cortex, ng/mg	2.2	0.5	<0.2[a]

(B) Mouse #2: 2 h after 20 mg/kg MDMA administration

	MDMA	MDA	HMMA	HMA
Plasma, ng/mL	597.8	252.1	252.5	75.0
Cortex, ng/mg	6.8	1.9	<0.1[a]	<0.2[a]

Abbreviations: methamphetamine (MAMP), amphetamine (AMP), hydroxy-methamphetamine (OH-MAMP), methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), 3-hydroxy-4-methoxy-methamphetamine (HMMA), 3-hydroxy-4-methoxy-amphetamine (HMA).

[a] Brain limits of quantification (LOQ) are 0.2 ng/mg for OH-MAMP and HMA and 0.1 ng/mg for HMMA, respectively.

concentration); however, bias was acceptable, within 80–120% of target for all analytes. Imprecision was within 0.9–10.2% CV (Table 8).

3.8. Proof of method

Plasma and brain cortex from a CD-1 mouse 2 h after a single 20 mg/kg intra-peritoneal (i.p.) MAMP injection and from an FVB mouse 2 h after a single 20 mg/kg i.p. MDMA injection demonstrated method applicability (Table 9). OH-MAMP was less than the LOD in cortex from the mouse administered MAMP. MDMA, MDA, HMMA and HMA were less than the LOD in plasma and cortex after MAMP. In cortex collected 2 h after MDMA, HMMA and HMA concentrations were less than the LOQ. MAMP, AMP and OH-MAMP were less than the LOD in plasma and cortex after MDMA administration.

4. Discussion

It has been shown that OH-MAMP, HMMA and HMA are highly conjugated being excreted in urine conjugated to sulfate and glucuronide with only small amounts of parent drug present in urine [50,51]. Shima et al. directly measured HMMA, HMMA-glucuronide and HMMA-sulfate in human urine and found that HMMA-sulfate predominates 3-fold compared to HMMA-glucuronide [51]. Interspecies differences between amounts of OH-MAMP and HMMA glucuronidation and sulfation were noted between humans and rats [50,51]. Therefore, it is important to evaluate hydrolysis efficiency for cleaving conjugated OH-MAMP, HMMA and HMA in mouse plasma to be able to predict total OH-MAMP, HMMA and HMA in mouse plasma. We found that HMMA and HMA were highly conjugated in mouse plasma and that both enzyme hydrolysis using β-Glucuronidase type HP-2 from *H. pomatia* and acidic hydrolysis using HCl were effective for de-conjugation of OH-MAMP, HMMA and HMA. Acid hydrolysis using 100 μL of HCl was selected for de-conjugating OH-MAMP, HMMA and HMA for validation of this method based upon results of our previous studies [49] using human urine collected after MDMA administration that found acid was equally efficient as enzyme for hydrolysis of conjugated HMA and acid was more efficient than enzyme for hydrolysis of conjugated HMMA. Furthermore acid was preferred for hydrolysis due to its speed, low cost and since cleaner chromatographic background was obtained in extracts after acid hydrolysis compared to enzyme hydrolysis. Acidic hydrolysis using 100 μL HCl is not as optimal as enzyme hydrolysis for de-conjugating OH-MAMP, HMMA and HMA conjugates in mouse plasma specimens but use of enzyme hydrolysis would have resulted in reduced AMP and MAMP sensitivity. It is important to note that use of 100 μL HCl relative to enzyme hydrolysis consistently produces 33 and 60% hydrolysis efficiency for OH-MAMP and HMMA, HMA, respectively. These results highlight the importance of evaluating hydrolysis conditions within any species.

Mouse studies examining the role of drug metabolism and distribution in MAMP- and MDMA-induced neurotoxicity require a sensitive assay with limited specimen size to accurately measure MAMP, MDMA and metabolites across a large dynamic range. This method requires a plasma specimen volume of 100 μL and achieved LOQs of 10 ng/mL for MAMP, AMP, MDMA, and MDA and 20 ng/mL for OH-MAMP, HMMA and HMA. The method detailed in this manuscript achieves similar sensitivity to another fully validated GC–MS-EI method which achieved LOQs of 5 ng/mL for MAMP, AMP, MDMA and MDA, but employed a larger specimen volume of 1 mL [34]. Sensitivity obtained using the method presented here compares favorably with another method employing more expensive GC–MS negative ion chemical ionization instrumentation along with a custom-made derivatization reagent to measure enantiomers of MAMP, MDMA and metabolites in plasma [33], which achieved LOQs of 5 ng/mL for AMP, MAMP, MDMA and MDEA enantiomers along with 1 ng/mL for MDA using a 200 μL plasma volume. Use of LC–MS/MS instrumentation enables less tedious sample preparation and obtained LOQs of 0.5–1.0 ng/mL when AMP, MAMP, MDMA and MDA were monitored in 50 μL plasma [41]. This LC–MS/MS assay did not measure OH-MAMP, HMA or HMMA. Other LC–MS assays for MAMP, MDMA and metabolites achieved analyte sensitivities similar to those obtained in the method presented in this manuscript [42,45]. Construction of two calibration curves employing splitless and 1:5 split injections enabled upper limits of quantification of 20,000 ng/mL. The brain method requires a sample size of 7.5 mg and achieves LOQs of 0.1 ng/mg for MAMP, AMP, MDMA, MDA and HMMA and 0.2 ng/mg for OH-MAMP and HMA. The same splitless and split injection approach as employed for plasma, enabled broad linear ranges with upper limits of quantification of 200 ng/mg brain. Inter-assay bias for plasma and brain was between 92 and 110% of target concentration. Inter-assay imprecision, expressed as coefficient of variation, was less than 5.3 and 4.8% for plasma and brain, respectively. It is difficult to obtain sufficient plasma from mice under acute effects of neurotoxic doses of sympathomimetic amines, making analytical sensitivity a priority for mouse plasma analysis.

MAMP- and MDMA-induced neurotoxicity is pronounced in discrete brain regions such as striatum, making it critical to measure tissue drug concentrations in these small brain regions. Adult mouse striatum typically weighs 15–20 mg, so it was critical to develop a method with adequate sensitivity for a sample size less than 15 mg. Importantly, the 7.5 mg sample size employed for brain analysis affords sufficient leftover sample for repeat analysis in case of experimental error during sample processing. A previous method for measuring MAMP in mouse brain achieved MAMP and AMP LOQ of 33 pg/mg using a larger sample than used for the method presented in this manuscript [16]. Previous methods for measuring MDMA and metabolites in mouse brain did not include detailed validation [46] or used the entire mouse brain [40]. This fully validated method enables characterization of MAMP, MDMA and metabolites in plasma and brain tissue collected from wild-type and transgenic mice during studies investigating the role of drug metabolism and distribution in MAMP- and MDMA-induced neurotoxicity.

Acknowledgements

The authors thank the co-investigators of the MAMP and MDMA neurotoxicity study conducted at the Intramural Research Program, National Institute on Drug Abuse, Jean Lud Cadet and Bruce Ladenheim for generously providing mouse plasma and brain specimens for proof of method. This research was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health.

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