The AAPS Journal 2006; 8 (4) Article 80 (http://www.aapsj.org).

Themed Issue: NIDA/AAPS Symposium on Drugs of Abuse: Mechanisms of Toxicity, Toxicokinetics and Medical Consequences, November 4-5, 2005
Guest Editors - Rao S. Rapaka and Jagitsing H. Khalsa

Quantitative Determination of Total Methamphetamine and Active Metabolites in Rat Tissue by Liquid Chromatography With Tandem Mass Spectrometric Detection

Submitted: June 19, 2006; Accepted: September 20, 2006; Published: November 22, 2006

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ABSTRACT

High-throughput liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) methodology for the determination of methamphetamine (METH), amphetamine (AMP), 4-hydroxymethamphetamine (4-OH-METH), and 4-hydroxyamphetamine (4-OH-AMP) was developed and validated using simple trichloroacetic acid sample treatment. The method was validated in rat serum, brain, and testis. Lower limits-of-quantitation (LOQ) for METH and AMP were 1 ng•mL^{-1} using positive ion electrospray tandem mass spectrometry (MS/MS). The accuracy of the method was within 25% of the actual values over a wide range of analyte concentrations. The within-assay precision was better than 12% (coefficient of variation). The method was linear over a wide dynamic range (0.3-1000 ng•mL^{-1}). Quantitation was possible in all 3 matrices using only serum standards because of minimal matrix-associated ion effects or the use of an internal standard. Finally, the LC-MS/MS method was used to determine serum, brain, and testis METH and AMP concentrations during a subcutaneous infusion (5.6 mg kg^{-1} day^{-1}) of METH in rats. Concentrations of 4-OH-AMP and 4-OH-METH were below the LOQ in experimental samples. The bias introduced by using serum calibrators for the determination of METH and AMP concentrations in testis and brain was less than 8% and insignificant relative to the interanimal variability.

KEYWORDS: methamphetamine, rats, LC-MS/MS, matrix ion effects

INTRODUCTION

(+)-Methamphetamine (METH) abuse has become a significant health concern in the United States and internationally with cognitive behavioral intervention being the only effective treatment option currently available.[1] While there are no effective pharmacotherapeutic agents available for the treatment of this disease, 2 primary approaches toward the development of medications have shown promise in animal models. One approach is to inhibit the reinforcing effects of the drug with small molecule receptor antagonists.[2-6] A second approach, and one pursued by our laboratory, is to block distribution of METH into the brain, its primary site of action, by administration of an anti-METH monoclonal antibody (mAb).[7-10] These high molecular weight (150 kDa) proteins interact strongly with METH (K_d < 100 nM), decreasing the volume of distribution of METH and blocking METH penetration into the brain.[7] Monoclonal antibodies for cocaine, nicotine, methamphetamine, and phencyclidine are currently in either preclinical or clinical development by our group or other investigators. Several reviews recently have been published on this promising treatment strategy.[11-13] Monoclonal antibodies, when used in this manner, are pharmacokinetic antagonists to a specific drug (eg, methamphetamine or nicotine). Therefore, the evaluation of mAb effects on the tissue disposition of a drug is critical during the development of mAbs as potential drug-abuse treatment medications.

An evaluation of brain distribution in the presence and absence of mAb as a function of time is most important for psychoactive drugs such as methamphetamine and other stimulants because its rate of distribution into the brain is thought to affect the abuse liability of these drugs.[14] Brain imaging techniques (eg, positron emission topography [PET] and magnetic resonance imaging [MRI]) have improved our ability to follow changes in brain concentration of drugs that are abused.[15] These noninvasive techniques have been used to determine the brain concentration-time course for cocaine and nicotine in humans and methamphetamine in dogs.[16-18] The expense and lack of radiolabeled compounds are likely the reasons for the limited number of publications in the area of drug/brain pharmacokinetics using these imaging techniques. There are also limitations with temporal resolution (<2 minutes) that

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may limit the utility of these techniques when applied to rapidly distributed drugs like phencyclidine and methamphetamine.[19,20] Therefore, despite the advantages of brain imaging techniques, more traditional bioanalytical techniques are still necessary. In our own efforts to develop mAbs to specific drugs-of-abuse, we must test these mAbs first in rodents since the mAbs are of murine origin when first produced.

Because of the high costs of producing sufficient quantities of these mAbs, efficient in vivo screening methods that are indicative of the mAb function are desirable for the successful development of these medications. These studies are best conducted in the rat or mouse because the amount of mAb used per animal can be minimized in these small animals and because the physiology of these animals is well defined. Our approach toward development of a predictive in vivo screening method is to determine the effect of anti-METH mAb on METH and its primary active metabolites, (+)-amphetamine (AMP), (+)-4-hydroxymethamphetamine (4-OH-METH), and (+)-4-hydroxyamphetamine (4-OH-AMP), in rats. The magnitude of the $[METH]_{serum}/[METH]_{brain}$ ratio should reflect the efficacy of a given mAb. Following the distribution of METH (and metabolites) is a direct measure mAb function, and it is significantly more cost-effective analytically, since selective determination of mAb concentrations in biological samples is not a trivial matter relative to small molecule determinations.

Liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) has become the preferred analytical technique for the determination of small molecules (ie, molecular weight [MW] <1 kDa) in complex biological samples. Papac and Shahrokh have recently reviewed the effect mass spectrometry has had in drug discovery and development and stressed the need for high-throughput techniques because of the fast-paced nature of drug discovery and development.[21] Certainly the compound selectivity of tandem mass spectrometry (MS/MS) implies that other aspects of the bioanalytical method (eg, sample preparation and analytical chromatography) can be minimized or eliminated. But mass spectrometric detection and particularly electrospray ionization (ESI) mass spectrometry is limited by a phenomenon referred to as matrix-associated ion suppression or ion enhancement.[22-25]

While there are working theories describing the mechanism of ion suppression/enhancement,[26,27] this phenomenon is complex and must be evaluated for each analyte and matrix. Several approaches have been taken to minimize or eliminate matrix ion effects. Perhaps the simplest approach is to choose atmospheric chemical ionization (APCI) over ESI. On modern instruments, APCI and ESI probes can be switched easily without breaking vacuum. King et al, have shown that APCI is less susceptible to matrix ion effects than ESI for 3 model compounds, but they also point out that APCI will not always reduce matrix ion effects because of the complexity of the process.[28] Another limitation of APCI is that its sensitivity is not optimized for polar molecules with basic or acidic functional groups like those present in drugs of abuse.[29] A second approach is to eliminate the interfering material using extraction techniques combined with analytical separation. A review of the recent literature indicates that on-line or off-line solid-phase extraction (SPE) before analytical separation is by far the most popular means of sample preparation prior to MS/MS.[30-40] Liquid-liquid extraction (LLE) has been touted as advantageous over SPE because the purity of the solvents used is easier to control when compared with potential impurities in SPE materials. Despite this advantage, LLE is not a popular means of biological sample preparation as shown by a search of recent literature.[41-44] This dissatisfaction with LLE is likely because of the labor-intensive nature of the technique. While there are automated LLE systems commercially available, it is necessary to purchase components from several manufacturers,[45,46] and automated control of these components with a single data system is not trivial.

In most cases, the endogenous components causing the greatest interference to the quantitation of small molecules in biological samples are high concentrations of biopolymers (eg, proteins, lipids, polysaccharides). The solubility of these molecules under reversed-phase chromatographic conditions is the greatest obstacle to the direct analysis of small molecules in biological tissues. Still, the initial promise of MS/MS detection held that the high selectivity of MS/MS detection would all but eliminate the need for elaborate extraction methods and analytical separation (LC) prior to detection and would, thereby, greatly increase the throughput of the analytical method. The premise was that these biopolymers could be precipitated by changing the ionic strength (salting-out) or by changing the hydrophobicity of the solution with an organic solvent. But as clearly demonstrated recently by Mallet et al, when this simple precipitation method is used for bioanalytical methods, interferences from matrix ions can adversely affect the robustness of a quantitative method.[22]

Recently, investigators have explored means of precipitating the large molecules with a variety of solvent systems and, in some cases, have found that incubating the combined solutions at lower temperatures results in a cleaner sample for LC-MS/MS analysis. Polson et al. have recently described a general approach for high throughput sample clean-up of dog, rat, mouse, and human plasma prior to LC-MS/MS using 4 classes of precipitants (acids, metal ions, organic solvent, and salts).[47] They concluded that pure organic compounds (eg, methanol, ethanol, acetonitrile) produce the greatest matrix ion effects. Trichloroacetic acid (TCA, 10% wt/vol) was the most efficient at protein removal across species. These researchers also observed the least

amount of matrix ion effects if 0.1% formic acid was added to the mobile phase for analytical separation. Despite all the efforts to create a general sample preparation step compatible with quantitative LC-MS/MS of small molecules in biological matrices, this step must be evaluated for each analyte.

In this article we describe the development and validation of an LC-MS/MS method for the determination of METH and 3 active metabolites (ie, AMP, 4-OH-METH, and 4-OH-AMP) in serum, brain, and testis. We previously published an LC-MS/MS method for the determination of METH and AMP in rat serum.[48] The application of this method for the quantitation of *p*-hydroxylated metabolites (4-OH-METH and 4-OH-AMP) was not demonstrated in our previous work. In brief, sample clean-up of serum samples was accomplished with guanidine and zinc sulfate treatment. These reagents were used to break the strong interactions between METH and the anti-METH mAb. In this manner, the total drug concentration could be determined. The resulting supernatant was subjected to SPE prior to LC-MS/MS analysis. While the previous method was robust and sensitive, we show in this article an improved method with a simple one-reagent sample (20% TCA) clean-up step followed by quantitation of METH and 3 active metabolites in serum, brain, and testis by LC-MS/MS.

Materials and Methods

Instrumentation

The LC system was a Waters Alliance 2695 (Waters Corp, Milford, MA), coupled to a Waters/Micromass (Beverly, MA) Quattro LC triple quadrupole mass spectrometer with an electrospray interface and equipped with a Mark II source. The analytical column was a BDS Hypersil C8 column, 100 × 2.1 mm (3 μm), and the guard-column was a BDS Hypersil C8, 10 × 2.1 mm (3 μm), both from Keystone/Thermo-Electron (Bellefonte, PA). A binary linear gradient was used for analytical separation. Solvent A was 5 mM ammonium acetate buffer (pH 3.7) with 5% (vol/vol) acetonitrile. Solvent B was 5 mM ammonium acetate buffer (pH 3.7) with 95% (vol/vol) acetonitrile. The flow rate was 0.3 mL min^{-1}. The following gradient profile was used. Solvent B was held at 0% for the first 2.5 minutes and was increased over 5 minutes to 65% and held at 65% for 2.5 minutes. Solvent B was decreased to 0% over the next 2 minutes. The total run time was 14 minutes. The eluent from the column was directed to waste for the first 2.5 minutes of each chromatographic run. The flow was then switched back to the mass spectrometer using an automated 6-port switching valve attached to the Quattro LC. Each sample (20 μL) was injected into a 100-μL sample loop using the Alliance 2695 autosampler. The desolvation gas (nitrogen) was operated at 325°C and 680 L hr^{-1}. The cone gas was operated at 115° C and 120 L hr^{-1}. Collision-induced fragments were produced in the collision cell with argon at a pressure of 2×10^{-3} torr. Positive parent/daughter ions were monitored in the multiple reaction-monitoring mode with mass-to-charge ratios of 136 and 91 (AMP), 147 and 98 (amphetamine-d_{11} as internal standard), 150 and 91 (METH), 155 and 92 (methamphetamine-d_5 as internal standard), 152 and 107 (4-OH-AMP), and 166 and 107 (4-OH-METH). The cone voltage and collision energy were optimized separately for each parent ion and daughter ion by infusing a 10 μg mL^{-1} solution of pure analyte with mobile phase (0.3 mL min^{-1}) into the mass spectrometer. The dwell time and interchannel delay were 0.1 seconds and 0.1 seconds, respectively. The LC-MS system was washed with 6 mL of water containing 10% methanol followed by 6 mL of methanol:isopropanol:acetonitrile after each batch run. Instrument control, data acquisition, and sample quantitation were performed using Masslynx 3.4 (Waters Corp).

Materials

Methamphetamine-d_5 ((±)-1-Phenyl-2-(methyl-$^2H_3$-amino) propane-1,2-$^2H_2$) and amphetamine-d_{11} ((±)-1-Phenyl-$^2H_5$-2-aminopropane-1,1,2,3,3,3-$^2H_6$) were purchased from Sigma/Isotech (St Louis, MO). Methamphetamine hydrochloride ((+)-(N-α-dimethylphenethylamine chloride), amphetamine sulfate ((+)-α-methylphenethylamine sulfate), 4-hydroxymethamphetamine ((±)-4-hydroxy-N-α-dimethylphenethylamine bromide), and 4-hydroxyamphetamine ((±)-4-hydroxy-α-methylphenethylamine bromide) were obtained from the National Institute on Drug Abuse (NIDA, National Institutes of Health, Bethesda, MD). The identity of each compound was confirmed by mass spectrometry and used as received without further purification. Structures for METH and the 4 metabolites studied here are shown in Figure 1.

Sodium hydroxide and zinc sulfate heptahydrate were purchased from Sigma/Aldrich. Methanol, acetonitrile, and isopropanol were Optima grade and purchased from Fisher Scientific (Pittsburgh, PA). Trichloroacetic acid solutions (10% and 20% wt/vol), acetic acid (glacial), ammonium hydroxide, and all other reagents were purchased from Fisher. Water was purified using a Millipore Synthesis A10 water purification system (Millipore Corp, Billerica, MA).

Preparation of Standards

Stock solutions of analytical standards (1 mg mL^{-1}) were prepared in methanol. All concentrations are expressed as the free base. Serial dilutions containing all 4 analytes (METH, AMP, 4-OH-METH, and 4-OH-AMP) were prepared in drug-free rat serum. Serum standards contained 0.3, 1, 3, 10, 30, 100, 300, 1000 ng•mL^{-1} of each analyte.

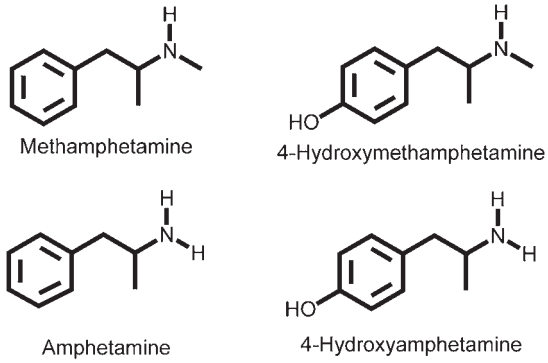

Figure 1. Structures for methamphetamine (METH); 4-hydroxymethamphetamine (4-OH-METH); amphetamine (AMP); and 4-hydroxyamphetamine (4-OH-AMP).

Separate calibration curves using standards at 5, 50, 500, and 1000 ng•mL^{-1} (or ng•g^{-1}) were prepared by serial dilutions from separate stock solutions. These calibration standards were prepared in serum, brain, testis, and mobile phase. Serial dilutions were made using the appropriate tissue. Brain and testis tissue were homogenized in 4 volumes of water prior to dilution or analysis.

Sample Clean-up and Calibration

Optimization of Precipitation

Three reagents ($ZnSO_4$, acetonitrile, and TCA) were investigated for their ability to efficiently precipitate samples and recover METH from rat tissues. These tissues (serum, brain, and testis) were obtained from a previous study in our laboratory in which rats were dosed with METH and a tracer dose of [^{3}H]-METH ((+)-[2',6'-$^3H_2$]-methamphetamine).[8] The scintillations were counted from an aliquot of tissue (10 µL in 20 mL of scintillation fluid) prior to precipitation to determine total counts. Separate aliquots of tissues were treated with either a 1:1 or 2:1 ratio (agent:tissue) of precipitating agent. This combination was mixed on a rotary mixer at 4°C (15 minutes). Scintillation counts were determined from an aliquot (10 µL) of the resulting supernatant. The reagent providing the highest recovery of radioactivity and best precision from all 3 tissues was evaluated further as a sample clean-up reagent prior to LC-MS/MS.

Sample Clean-up Prior to LC-MS/MS

A mixture containing 100 ng•mL^{-1} of AMP-d_{11} and METH-d_5 (10 µL) was added to each experimental sample (10-100 µL) and standard (100 µL). Tissue samples were stored in Fisher brand siliconized polypropylene tubes (Fisher Scientific, Fair Lawn, NJ). The volume of all samples was brought to 100 µL with normal rat serum (Pel-Freez Biologicals, Rogers, AR) such that the concentration of internal standard was the same in the samples and standards. Cold TCA (100 µL of 20% wt/vol) was added to each sample, vortex-mixed for 5 to 10 seconds, and mixed on a rotary mixer for 15 minutes at 4°C. Samples were centrifuged for 5 minutes (20 000 relative centrifugal force [rcf]) and the supernatant filtered using 0.2-µm nylon centrifugal filters (Millipore). The filtered supernatant was injected (20 µL) onto the LC-MS/MS system.

Calibration curves for METH, AMP, and 4-OH-AMP were generated by plotting the peak height ratio of analyte to the internal standard against the standard concentration in serum. The internal standard was METH-d_5 for METH and AMP-d_{11} for AMP and 4-OH-AMP. A suitable internal standard was not identified for 4-OH-METH, so the external standard approach was used. Because we used a simple precipitation step for sample clean-up, the external standard approach is applicable here. Concentration-response (peak height ratio or peak area) data were fit to a linear least squares regression with 1/concentration weighting. Analyte concentrations from tissue standards and experimental samples were determined from the slope and y-intercept of these calibration curves. The inter-day variability (CV) in the slope of the calibrations curve for each analyte was assessed over time (n = 6; 8 weeks). Statistical comparison of calibration curve slopes obtained from each matrix was performed using GraphPad Prism 4.0c for Macintosh (GraphPad Inc, San Diego, CA). Each slope was compared with the serum slope using a 2-tailed Student *t* test. A *P* value greater than .05 suggested the slopes were the same. Absolute recovery from serum, brain, and testis was calculated using external standard calibration curves generated in solvent to predict analyte concentrations in each tissue. The accuracy of the method in predicting METH, AMP, and 4-OH-AMP concentration in each tissue was determined using internal standard calibration curves generated in serum to predict analyte concentrations in serum, brain, and testis.

Methamphetamine and Metabolite Distribution Studies

Experimental samples from male Sprague-Dawley rats (n = 7; Charles River Laboratories, Wilmington, MA) were obtained in order to assess the interanimal variability and bias introduced by quantitating brain and testis drug concentrations with serum calibrators. The relative bias introduced by quantitation of all samples using serum calibration standards was determined according to the following equation:

$$\text{Bias} = \frac{C_S - C_T}{C_T} \times 100\% \qquad (1)$$

where C_S was the value predicted from serum calibrators and C_T was the value predicted from brain or testis calibrators in the corresponding tissue.

Each rat was shipped from the supplier with an indwelling jugular vein catheter (average weight ± standard deviation

[SD] was 255 ± 10 g). The animals were conditioned to handling for 3 days prior to surgical implantation of osmotic minipumps as described by Wessinger and Owens.[20] In brief, under halothane anesthesia, Alzet osmotic minipumps (model 1003D, DURECT Corp, Cupertino, CA) were surgically implanted subcutaneously (sc) after equilibrating the pumps in saline for 2 hours. Pumps were filled to deliver 5.6 mg kg^{-1} day^{-1} METH. Time was allowed for drug concentrations to reach steady-state at which time animals received either an intravenous (IV) bolus of mAb preparation buffer (n = 3) or an IV bolus dose of anti-METH mAb (mAb4G9, n = 4). Blood, brain, and testis from each animal were collected 13 days following administration of mAb4G9. Blood samples were allowed to stand at room temperature for ~1 hour and then centrifuged, and the serum was collected as the supernatant. Brain and testis samples were homogenized in 4 volumes of water. All samples were stored at −80°C until analyzed. All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Results

Sample Clean-up

The efficiency of analyte recovery from the supernatant for 10% zinc sulfate, acetonitrile, and TCA was compared for each reagent in serum, brain, and testis (Table 1). These 3 reagents were chosen because they were shown to be more efficient at protein precipitation and produce fewer matrix ion effects than other reagents (ie, methanol, ethanol) when tested with serum samples from various species.[47] We found a clear advantage in extraction efficiency using an equal volume of 20% TCA. Because this radiochemical method lacked specificity, the extraction efficiency of 20% TCA was further evaluated with LC-MS/MS.

LC-MS/MS Matrix Effects, Calibration, and Quantitation

The retention times for METH, AMP, 4-OH-METH, and 4-OH-AMP were 8.22 (±0.04), 7.95 (±0.04), 6.10 (±0.07), 5.39 (±0.06) minutes, respectively. Postcolumn infusion studies were conducted for each analyte and internal standard in each of the matrices (data not shown). These infusion studies showed significant matrix ion enhancement for each analyte and internal standard. An injection (20 µL) of 20% TCA mixed with an equal volume of 0.05 M ammonium acetate (pH 3.7) onto the column produced a similar postcolumn infusion trace for the compounds studied, suggesting that TCA is responsible for much of the ion enhancement effects observed. Despite the significant contribution of TCA to the matrix ion enhancement, there were matrix-dependent differences in the ion intensity for the analytes. To determine whether these differences would affect the analytical recovery, calibration, and quantitation of the assay, we evaluated the matrix ion enhancement using a more quantitative approach.

Another important aim for this study was to determine if rat serum could be used as a universal matrix for quantitation of these compounds in serum, brain, and testis from rats. Calibration curves were generated in each matrix. The internal standard approach was used for METH, AMP, and 4-OH-AMP, while the external standard approach was used for 4-OH-METH. Slopes from the calibration curve for each analyte, in all 4 matrices are shown in Table 2. The data in Table 2 show that the matrix did not alter the calibration curve slope for METH, AMP, and 4-OH-METH ($P > .05$), suggesting that the relative matrix ion effects will not adversely affect quantitation of these analytes. The data in Table 2 also show that the slope obtained for 4-OH-AMP in testis was significantly different from the slope generated in serum, suggesting that quantitation of this analyte may be adversely affected if serum calibrators are used to quantitate 4-OH-AMP in experimental testis samples. The coefficient of variation (CV) in the slope between all 3 matrices is also shown in Table 2 as an evaluation of the contribution of

Table 1. ^{3}H-METH method: Effects of Precipitating Reagent on Total Analyte Recovery (%) With Experimental Rat Tissues*

	Total Recovery % (CV) of Radioactivity From Tissue Supernatant				
Tissue	**$ZnSO_4$**	**ACN**	**0.5 Volume TCA (10%)**	**1 Volume TCA (10%)**	**1 Volume TCA (20%)**
Brain	56 (6.2)	95 (1.1)	82 (2.8)	83 (1.3)	92 (5.8)
Testis	20 (3.3)	49 (5.7)	94 (1.2)	94 (2.4)	97 (1.8)
Serum	17 (4.8)	17 (17)	89 (4.2)	50 (20)	86 (0.2)

*CV indicates coefficient of variation; ACN, acetonitrile; and TCA, trichloroacetic acid. Samples from a single rat were obtained following 1 mg kg^{-1} intravenous METH (with ^{3}H-METH tracer) dose and an equimolar dose of anti-(+)-METH monoclonal antibody 30 minutes later.[10] Aliquots of homogenized tissue or serum (10 µL) were mixed with 20 mL of scintillation fluid and the total scintillations counted. A separate aliquot (10 µL) was taken of the supernatant following precipitation and the scintillations counted. The recovery was determined from the following equation: (Supernatant Counts/Tissue Counts) × 100%.

Table 2. Effects of Matrix on the Calibration Curve Slope and Precision*

Compound	Slope of Calibration Curve in Matrix (CV)			Between Tissue CV
	Serum	Brain	Testis	
4-OH-AMP	5.18×10^{-3} (1.7%)	5.7×10^{-3} (7.2%)	4.64×10^{-3} (1.7%)†	9.8%
4-OH-METH	254 (1.2%)	277 (6.3%)	268 (0.5%)	4.4%
AMP	2.33×10^{-2} (1.5%)	2.5×10^{-2} (4.8%)	2.32×10^{-2} (1.8%)	3.9%
METH	2.46×10^{-1} (1.1%)	2.4×10^{-1} (4.1%)	2.33×10^{-1} (3.8%)	2.8%

*CV indicates coefficient of variation; 4-OH-AMP, 4-hydroxyamphetamine; 4-OH-METH, 4-hydroxymethamphetamine; AMP, amphetamine; and METH, methamphetamine. Values are the mean (CV), n = 6.
†Indicates a slope is significantly different from the calibration curve slope obtained in serum.

relative matrix effects. Matuszewski has recently suggested that a CV in calibration slopes between separate lots of tissue of >5% is a good indicator that relative matrix effects will adversely affect the assay.[49] We tested this principle in our study. Calibration curve slopes for a given analyte were pooled and the CV in the slope was calculated from this pooled data (Table 2). The poorest precision was observed for 4-OH-AMP (9.8%), suggesting that relative matrix ion effects may adversely affect its quantitation. CVs for the other 3 analytes were <5%.

Calibration curves generated using solvent standards were used to quantitatively evaluate any matrix ion effects. Quantitative assessment of matrix ion effects is shown in Table 3. The linear least squares equations obtained from external standard curves for solvent calibration standards were $y = 28x + 80$, $y = 245x + 2352$, $y = 88x + 295$, $y = 376x + 1776$ for 4-OH-AMP, 4-OH-METH, AMP, and METH, respectively. Tissue standards samples, containing a mixture of each analyte (5, 50, 500, and 1000 ng•mL^{-1}) were quantitated using solvent calibration curves. Absolute recovery values in each tissue were determined for each analyte. An absolute recovery of greater than 100% would indicate matrix ion enhancement, while a value less than 100% would suggest matrix ion suppression. Percentage matrix ion effect values are shown in Table 3 and were calculated by subtracting 100% from the absolute recovery. With the exception of AMP matrix ion enhancement in testis (37%), the magnitude of ion effects observed for the other 3 analytes was small in comparison with that observed for 4-OH-AMP. Ion suppression was observed with METH in serum (−12%). The smallest amount of matrix ion effects was recorded for 4-OH-METH and justifies the external standard approach used to quantitate this compound. Because matrix ion effects were significant for 4-OH-AMP, METH, and AMP (Table 3), the internal standard approach was employed for the quantitation of these analytes. While the matrix ion effects for the internal standards were not quantitatively assessed here, the precision and accuracy of the method was satisfactory for 4-OH-AMP, METH, and AMP when the internal standard approach was used for these analytes. These results are shown in Table 4. The relative effects of matrix on both the calibration curve slope and the magnitude of ion enhancement can produce bias when determining analyte concentrations in experimental samples. On the one hand, serum and brain calibration curves were not different for any of the compounds studied (Table 2, $P > .12$). On the other hand, the calibration curve generated in testis for 4-OH-AMP was different from the serum calibration curves ($P = .02$). Similarly, a high level of matrix ion enhancement in testis was observed for AMP (Table 3), 37%.

Table 3. Matrix-associated Ion Effects of Serum, Brain, and Testis on MS/MS Response*

Analyte	Matrix Ion Effect (%)†			
	4-OH-AMP	4-OH-METH	AMP	METH
Serum	51	10†	23†	-12
Brain	40	30†	11†	22
Testis	37	4†	37	17

*MS/MS indicates tandem mass spectrometry; 4-OH-AMP, 4-hydroxyamphetamine; 4-OH-METH, 4-hydroxymethamphetamine; AMP, amphetamine; and METH, methamphetamine.
†Indicates that the suppression or enhancement was not significant. The matrix ion effect was calculated according to the following formula: Absolute Recovery% − 100%. Absolute Recovery% was determined by predicting the tissue standard concentrations in each tissue matrix using a calibration curve generated in solvent. The peak area for the analyte in serum, brain, or testis was then used to determine predicted concentration from the least squares equation generated from solvent calibration data.

Quantitation of Samples Using Only Serum Calibration Standards

We have used several of approaches to evaluate the matrix ion effects from rat serum, brain, and testis. The infusion method, precision of calibration slope values between tissues, and absolute recovery all suggested that quantitation of 4-OH-AMP and AMP from testis might be biased if serum standards are used to quantitate these compounds.

Table 4. Within-assay Accuracy and Precision for Each Analyte*

	Accuracy of Analytes From Each Tissue							
Analyte	**4-OH-AMP**		**4-OH-METH**		**AMP**		**METH**	
Added (ng•g^{-1})	5	1000	5	1000	5	1000	5	1000
Serum	114 ± 11	97 ± 6	90 ± 12	100 ± 7	81 ± 7	100 ± 7	79 ± 5	102 ± 4
Brain	112 ± 7	98 ± 8	85 ± 12	101 ± 8	74 ± 10	102 ± 8	86 ± 5	97 ± 7
Testis	87 ± 3	72 ± 1	85 ± 12	87 ± 5	104 ± 5	103 ± 7	99 ± 20	118 ± 7

*4-OH-AMP indicates 4-hydroxyamphetamine; 4-OH-METH, 4-hydroxymethamphetamine; AMP, amphetamine; and METH, methamphetamine. Values are the mean ± standard deviation (SD) (n = 6).

Despite the quantitative approach taken to evaluate the matrix ion effects, there are shortcomings to this approach for method validation. The *accuracy* is a more reliable measure of the reliability of the method, and these values are shown in Table 4 for each analyte. In each tissue, concentrations were determined using serum calibration standards. The equations from these serum calibration curves were

$$y = 0.25(\pm 0.003)x + 1.2(0.3),$$

$$y = 254(\pm 3)x + 2610(\pm 258),$$

$$y = 5.2x10^{-3}(\pm 9 * 10^{-5})x + 8 * 10^{-2}(\pm 8 * 10^{-2}),$$

and

$$y = 254(\pm 3)x + 2610(\pm 258)$$

for METH, AMP, 4-OH-AMP, and 4-OH-METH, respectively. These data show that the method accuracy was 74% to 118% for all compounds, and the precision was 1% to 11% as determined by the within-assay CV (Table 4). The limits-of-quantitation (LOQ) were 1 ng•mL^{-1} in serum (100 µL) and 5 ng g^{-1} in brain and testis (100 µL homogenized tissue) for METH and AMP. The LOQ were 5 ng•mL^{-1} in serum and 25 ng g^{-1} in brain for 4-OH-METH and 4-OH-AMP. The LOQ was determined using the criteria suggested by the Food and Drug Administration in the Guidance for Industry Bioanalytical Method Validation, (ie, <20% deviation from the added concentration and within the linear dynamic range of the calibration standards). Eight serum calibration standards were run with every set of samples. These were used for quantitation of all analytes in each of the 3 matrices.

Distribution of METH and AMP Following Subcutaneous Infusion of METH

The method was further validated by determining the interanimal variability of METH and AMP concentrations in serum, brain, and testis (Table 5). The mAb-treated animals received a dose of mAb4G9 (180 mg kg^{-1}) equal to the molar body burden of METH and administered via the jugular vein catheter. Tissue samples were collected 13 days after mAb administration. The data in Table 5 show significantly higher serum concentrations of METH in the mAb-treated animals compared with the control animals ($P < .05$). AMP serum concentrations were noticeably higher in the mAb-treated animals, but the differences were not significant. Brain and testis concentrations of METH and AMP were not significantly different between the 2 groups. METH and AMP brain concentrations were then quantitated using brain calibrators to evaluate the bias introduced by using serum calibrators. The bias was also evaluated for testis samples. The bias introduced by using serum calibrators was <8% and is insignificant relative to the large interanimal variability observed. 4-OH-METH concentrations were below the LOQ in all 3 tissues and 4-OH-AMP was not detected. Glucuronidation of 4-OH-METH is a major route of metabolism in the rat and is a likely explanation for why 4-OH-METH was not detected in these samples.[50]

SUMMARY

We have validated simple and rugged LC-MS/MS methodology for the determination of methamphetamine and 3 active metabolites in serum, brain, and testis of the rat. Sample clean-up was a rapid precipitation of tissue with TCA and injection of the filtered supernatant onto the LC-MS/MS system. The TCA served to remove biopolymers from the sample and effectively break up strong protein-analyte interactions between METH and anti-METH mAb. Determination of analyte concentrations in all 3 tissues was possible using calibration standards prepared in a single tissue (serum). We observed matrix ion effects for all 4 analytes and the internal standards in each of 3 tissues using the postcolumn infusion technique. The majority of observed matrix ion effects were attributable to the precipitating reagent (20% TCA). The relative matrix ion effects were eliminated by using appropriate internal standards for METH, AMP, and 4-OH-AMP. Stable isotopes were used as internal standards for METH and AMP, while an analog of 4-OH-AMP served as an internal standard for this analyte. The external standard approach provided better quantitation of 4-OH-METH and was justified by the excellent accuracy and precision obtained (Table 4).

Table 5. Effects of Anti-METH mAb on the Distribution of METH and AMP 14 Days Following IV Administration of mAb4G9*

	METH Concentration		AMP Concentration	
Tissue	**Control**	**mAb-treated**	**Control**	**mAb-treated**
Serum†	65 ± 15	553 ± 117	12 ± 5	31 ± 9
Brain†	388 ± 78	466 ± 72	121 ± 33	137 ± 42
Testis†	299 ± 61	353 ± 84	88 ± 26	92 ± 39

*METH indicates methamphetamine; mAb, monoclonal antibody; and AMP, amphetamine.

†Serum concentrations are expressed in ng•mL^{-1}

‡Brain and testis concentrations are expressed in ng g^{-1}. Values are the mean ± standard deviation (SD). Control (n = 3); mAb-treated (n = 4). METH was infused subcutaneously at a dose of 5.6 mg kg^{-1} d^{-1} through an osmotic mini-pump.

Fielding and Ryall observed that anions and large cationic species were adsorbed to TCA-precipitated serum proteins, while small cations remained in the supernatant.[51] These observations also may explain why TCA was a more efficient solvent in our assay, since under these acidic conditions METH, AMP, 4-OH-METH, and 4-OH-AMP are small cationic species and will likely remain in the supernatant, while anions and large cations remain in the pellet and are removed from the sample. This finding may explain why analyte recovery following TCA precipitation was higher than with other precipitating reagents (acetonitrile and zinc sulfate) that were studied here.

ACKNOWLEDGMENTS

This study was supported by grants P01DA14361, R01DA11560, and K2514601 from the National Institute on Drug Abuse, National Institutes of Health, Bethesda, MD, and grant EPS-9977816 from the National Science Foundation, Arlington, VA.

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