Rapid and Accurate Simultaneous Quantitation of Low Concentration Amphetamines and Metabolites from Urine using Strata™-X*-C and LC/MS/MS on Gemini® C18

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Use of Strata-X-C SPE cartridges allows for an aggressive organic wash of biological samples while maintaining high recoveries of amphetamines and their metabolites. In combination with LC/MS/MS, limits of quantitation (LOQ) of 0.54 ng/mL are achieved for MAMPH, HMMA, and MDMA and LOQ of 1.0 ng/mL are achieved for AMPH and MDA on Gemini C18 HPLC columns. Accuracy and reproducibility are not compromised with this simple and effective approach.

Introduction

Amphetamines are synthetic amines similar to the body's own catecholamines (i.e. dopamine and norepinephrine), and to the hormone adrenaline, which is produced by the adrenal glands during a sympathetic response.[1] These substances stimulate the reticular activating system as stimulants of the central nervous system (CNS), and have been used medically since 1930.[2] Prior to 1970, amphetamines were prescribed for a large number of conditions, including depression, fatigue, and long-term weight reduction. Amphetamines have also been used by athletes for their strong stimulant effect in an attempt to boost performance and to overcome fatigue. This led to their ban by many sports organizations, both amateur and professional. As a result of the psychological effects and the dependence that users can develop, amphetamines have become, along with cocaine and marijuana, some of the most commonly abused illicit drugs in the United States, and consequently constitute a substantial risk to public health. The analysis of amphetamines and their metabolic derivatives has received a great deal of attention, thus promoting the development of adequate methods for their screening, especially for their simultaneous monitoring in toxicological, clinical or forensic laboratories.

In drug testing, the analysis for amphetamines is commonly performed by immunochemical assay techniques (e.g. EMI, RIA, and FPIA). Because the antibodies used in many immunoassays may cross react with chemically related drugs, the immunoassay techniques used for drug screens usually require an additional confirmatory test (like GC/MS and HPLC) to eliminate potential false positive results.

GC/MS is a highly sensitive and specific analytical technique. Its ability to couple extensive mass spectral data (when used in "full scan" mode) with chromatographic data, allows for a definitive identification of target analytes. However, the use of GC is limited to compounds which are volatile or semi-volatile, otherwise labor intensive chemical derivatization is required prior to analysis by GC/MS. The use of HPLC for determining amphetamines [3,4] has not received as much attention because of the poor response of UV spectrometric detectors to these compounds when present at low concentration levels. However, HPLC coupled with mass spectrometry can provide the sensitivity required for the analysis of analytes at low concentrations in biological samples. In comparison to GC/MS and LC/UV analyses, LC/MS/MS offers superior sensitivity, selectivity and robustness for a wide range of analytes without any need for derivatization. LC/MS/MS technology also makes possible the development of highly selective, sensitive and precise methods for the simultaneous analysis of a class of compounds like amphetamines and their metabolic derivatives.[5]

Experimental

Chemicals and Reagents

3,4-Methylenedioxyamphetamine-d_5 (MDA-d_5) and 3,4-Methylenedioxymethamphetamine-d_5 (MDMA-d_5) were obtained from Medical Isotopes (Pelham, NH). (±)-Amphetamine (AMPH), (±)-Methamphetamine (MAMPH), 3,4-Methylenedioxyamphetamine (MDA), 3,4-Methylenedioxymethamphetamine (MDMA), and 4-Hydroxy-3-methoxymethamphetamine (HMMA) were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used were obtained from Sigma-Aldrich and used without further purification. HPLC grade water (Milli-Q, Millipore, Billerica, MA) was used to prepare HPLC mobile phase and for sample preparation. HPLC grade acetonitrile (ACN) was obtained from Honeywell Burdick & Jackson (Muskegon, MI).

Sample Preparation

Amphetamines and metabolites standard solutions were made up in water at six different concentrations. Samples were prepared by the addition of 200 µL amphetamines and metabolites standard solution and 100 µL of internal standard solution to 200 µL of acid hydrolyzed urine (adjusted to neutral pH); 100 µL of 1 M acetic acid and 400 µL water was then added to create the calibration curve samples. Final spiked sample concentrations (Level 1-6) are outlined in Table 1. Internal standard concentrations were 50 and 25 ng/mL for MDA-d_5 and MDMA-d_5, respectively.

Table 1.
Amphetamines and Metabolites: Calibration Standard and Control Samples

Compound	AMPH	MAMPH	MDA	MDMA	HMMA
Level 1	1.0 ng/mL	0.5 ng/mL	1.0 ng/mL	0.5 ng/mL	0.5 ng/mL
Level 2	10.0	5.0	10.0	5.0	5.0
Level 3	50.0	25.0	50.0	25.0	25.0
Level 4	100.0	50.0	100.0	50.0	50.0
Level 5	500.0	250.0	500.0	250.0	250.0
Level 6	1000.0	500.0	1000.0	500.0	500.0
Control 1	10.0	5.0	10.0	5.0	5.0
Control 2	500.0	250.0	500.0	250.0	250.0

* Strata-X is patented by Phenomenex, Inc.

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SPE Procedure	
Sorbent	Strata™-X-C 30 mg/1 mL (Part Number 8B-S029-TAK) or 30 mg/well 96-Well Plate (Part Number 8E-S029-TGB)
Condition 1:	1 mL methanol
Condition 2:	1 mL water
Load:	1 mL of spiked urine
Wash 1:	1 mL water
Wash 2:	1 mL 0.1 M acetic acid
Wash 3:	1 mL methanol
Elution:	1 mL 2 % ammonium hydroxide (28-30 %) in methanol (2 aliquots of 500 µL).
Evaporate to dryness under gentle nitrogen flow.	
NOTE: To prevent the loss of amphetamine and methamphetamine during solvent evaporation, add two drops of conc. HCl prior to the evaporation step. Reconstitute in 200 µL mobile phase.	

Chromatographic Conditions

The chromatographic system consisted of an Agilent 1100 series binary pump equipped with on-line solvent degasser, autosampler, and column temperature module (Palo Alto, California); interfaced with an Applied Biosystems API3000 tandem mass spectrometer with TurboIonSpray® electrospray ionization interface (ESI). The system was controlled using Analyst 1.41 software.

HPLC Conditions	
Column:	Gemini 5 µm C18
Dimensions:	150 x 3.0 mm
Part No.:	00F-4435-Y0
Injection:	5 µL of the reconstituted extract
Flow Rate:	0.6 mL/min
Mobile Phase:	90:10 (A:B), Isocratic
	A. 10 mM Ammonium formate with 0.1 % formic acid
	B. Acetonitrile
MS Detection:	TurbolonSpray - heater gas flow 8000 cc/min - heater temperature 450 °C - ESI, Positive Ion Mode - MRM

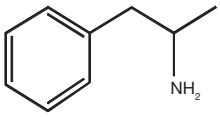
Analyte	MRM Pair (Q1/Q3)
HMMA	196.2 / 165.2
MAMPH	150.1 / 91.1
MDMA	194.2 / 163.3
MDMA-d_5	199.2 / 165.2
AMPH	136.2 / 91.1
MDA	180.2 / 105.1
MDA-d_5	185.2 / 110.3

Results and Discussion

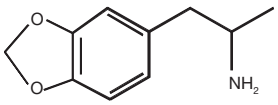
The structures of the amphetamines are very closely related **(Figure 1**) and their basic nature makes them ideally suited for concentration and cleanup from biological sample matrices using solid phase extraction (SPE) media with a strong cation exchange functionality. Sample preparation of the urine samples prior to LC/MS/MS analysis was performed via SPE using Strata-X-C in order to eliminate biological matrix components that could potentially interfere via ion suppression of the target analytes. Strata-X-C is a polymer-based sorbent possessing a binary (hydrophobic – hydrophilic) nature, as well as cation exchange capability. The strong cation exchange mechanism is ideal for retention of the basic amphetamines, which form strong ionic bonds with the sulfonic acid moieties on the SPE sorbent under acidic and neutral pH conditions. A judicious choice of wash solvents can result in extremely clean extracts. In this method an initial wash with water and acidic water removes polar matrix components and salts. A second stronger wash with methanol removes more hydrophobic matrix components. The addition of 2 % ammonium hydroxide to the methanol elution solvent neutralizes the amine groups on the amphetamines allowing for complete elution.

Figure 1.
Molecular Structures of Amphetamines

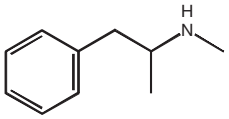
(±)-Amphetamine (AMPH) MW=135.21, pK_a=9.9



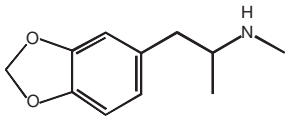
3,4-Methylenedioxyamphetamine (MDA) MW=179.22



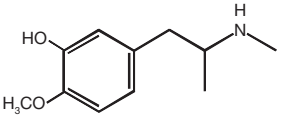
(±)-Methamphetamine (M-AMPH) MW=149.24, pK_a=10.1



3,4-Methylendioxymethamphetamine (MDMA) MW=193.25



4-Hydroxy-3-Methoxymethamphetamine (HMMA) MW=195.26



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The resulting chromatographic separation is shown in **Figures 2** and **3**, for amphetamines standards and amphetamines spiked in urine, respectively, at Level 5 (**Table 1**) and **Figure 4** shows the chromatogram obtained for low level (Level 1) amphetamines spiked in urine. Good peak shapes for all compounds of interest were obtained on the Gemini C18 column under isocratic conditions in less than eight minutes. The chromatogram obtained for a urine blank sample is shown in **Figure 5**.

Figure 2.
Amphetamine Standards (Level 5) - LC/MS/MS

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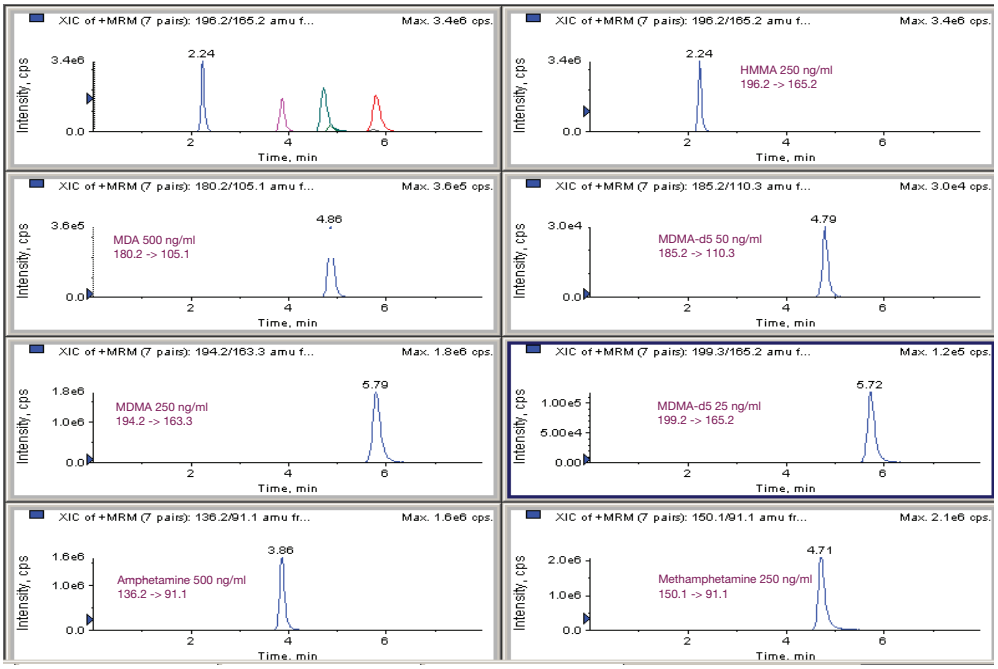

Figure 3.
Amphetamines in Urine (Level 5) - LC/MS/MS

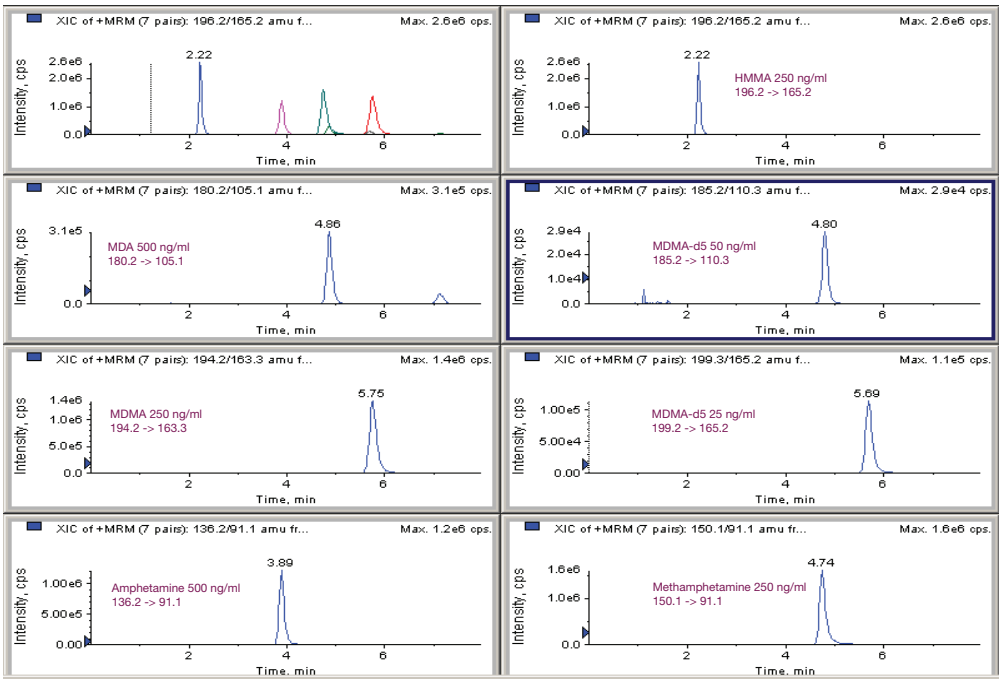

Figure 4.
Amphetamines in Urine (Level 1) - LC/MS/MS

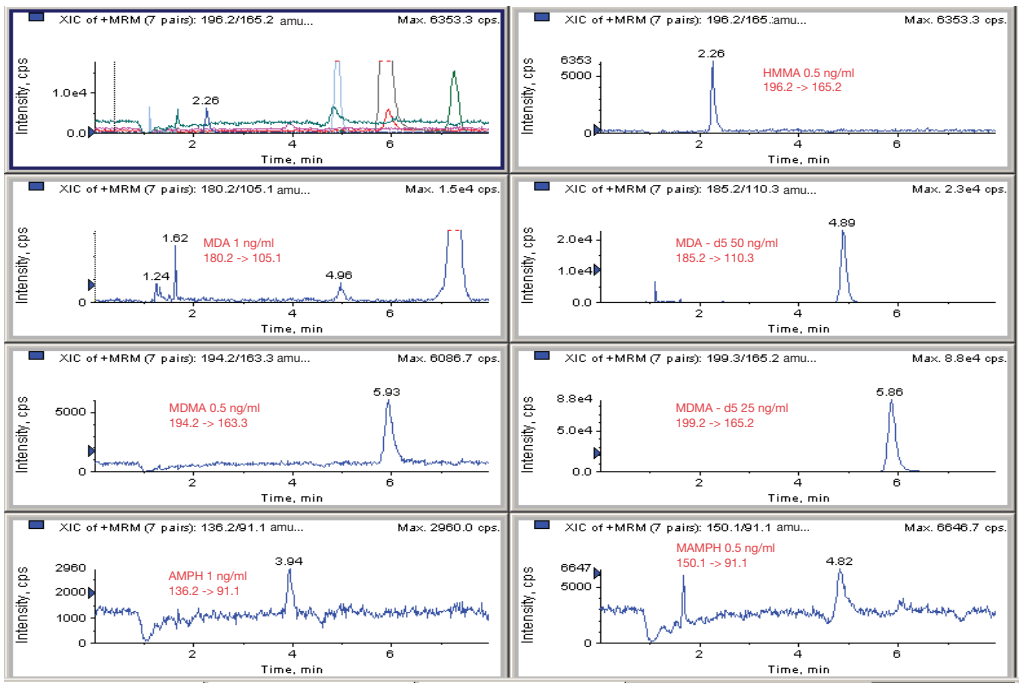

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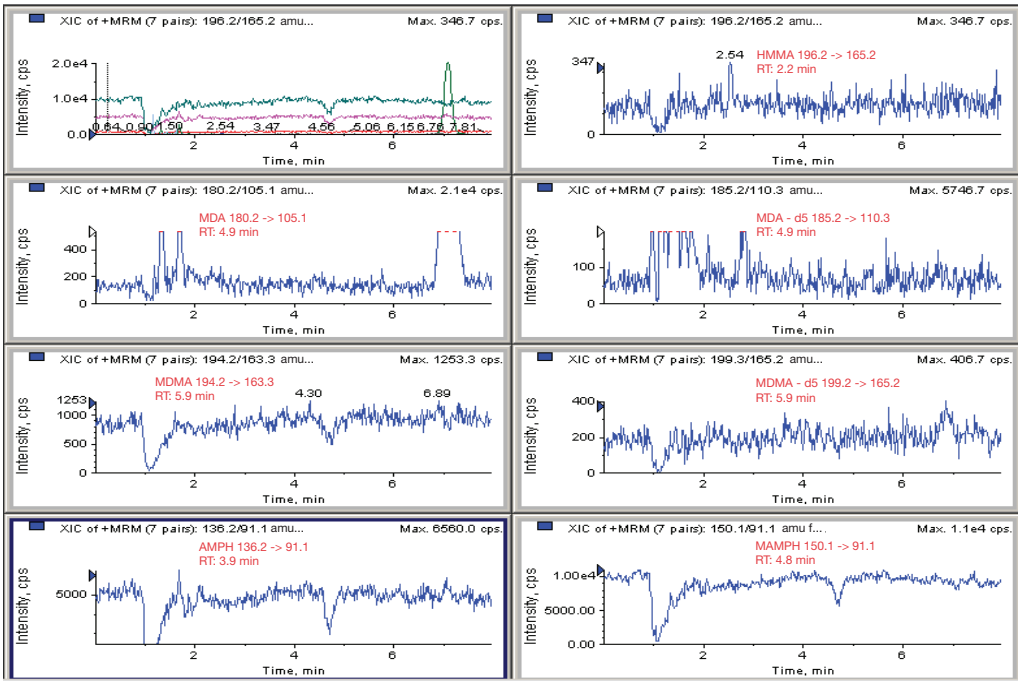
Figure 5.
Urine Blank – LC/MS/MS



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Calibration curves were generated utilizing a 6-point extracted standard curve over a concentration range of 0.5 to 500 ng/mL for HMMA, MDMA, MAMPH using MDMA-d_5 as internal standard (IS) at 25 ng/mL, and 1.0-1000 ng/mL for MDA and AMPH with MDA-d_5 as IS at 50 ng/mL. The calibration curves were constructed for each analyte by plotting MRM peak area ratios (analyte/IS) versus concentration and are shown in **Figures 6** and **7**. For each of the analytes the calibration curves demonstrate good linearity with values of $R^2 > 0.997$.

Figure 6.
Amphetamines - Standard Calibration Curves (1)

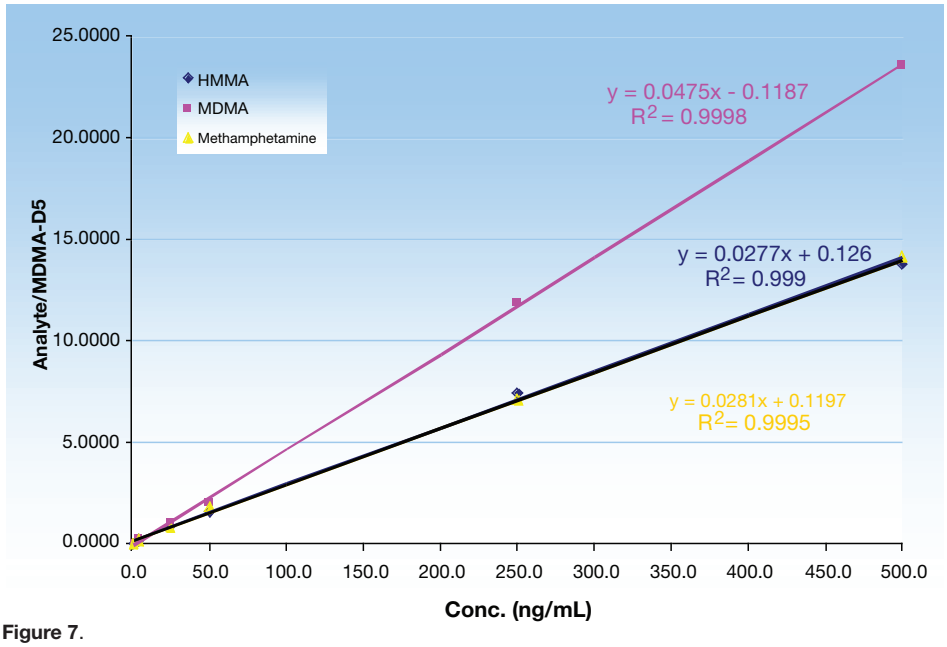

Figure 7.
Amphetamines - Standard Calibration Curves (2)

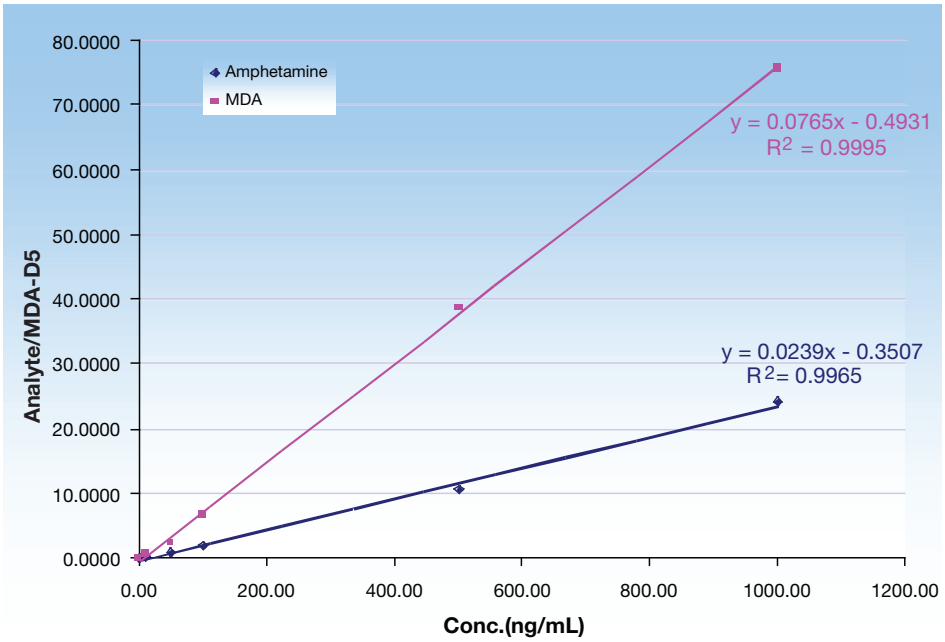

Table 2.
Quantification of Amphetamines in Urine

Compound		AMPH	M-AMPH	MDA	MDMA	HMMA	MDA-d_5 (IS)	MDMA-d_5(IS)
MRM transition		136.2☯91.1	150.1☯91.1	180.2☯105.1	194.2☯163.3	196.2☯165.2	185.2☯110.3	199.3☯165.2
Internal Standard (ISTD) Reference		MDA–d_5	MDA–d_5	MDA–d_5	MDMA–d_5	MDMA–d_5	–	–
R^2 * (3 sets)		≥ 0.997	>0.998	>0.998	>0.999	≥ 0.997		
% RSD* (n=6)	Level 3	13.21	5.52	2.77	5.09	5.50		
	Level 5	6.44	6.33	7.56	10.83	3.58	9.74	9.54
% Recovery * (n=6, absolute)	Level 3	71.9	108.65	96.59	97.00	71.4		
	Level 5	70.4	64.7	75.8	66.2	67.5	86.3	82.6
Accuracy (%) (n=8)	Control 1	103.1	117.7	92.9	106.7	102.61		
	% RSD	3.56	13.31	9.83	4.82	11.80		
	Control 2	92.1	107.1	81.0	112.7	120.57		
	% RSD	6.40	5.02	7.74	3.12	7.82		

* See Table 1.

Accuracy and precision were evaluated with 8 replicate assays of amphetamines and metabolites spiked into acid hydrolyzed urine at both low (5-10 ng/mL) and high (250-500 ng/mL) concentrations, denoted as Control 1 and Control 2, respectively in **Table 2**. Accuracy was determined by comparing actual results for spiked urine samples with expected levels. Accuracy was found to be 92-118 % at low concentration levels and 81-120 % at high concentration levels by inter-assay. Method precision was <14 % RSD at low concentration and <8 % RSD at high concentration. Recovery (absolute) was determined by spiking acid hydrolyzed urine samples at two concentrations, one low (25 – 50 ng/mL) and one high (250 - 500 ng/mL). The results are summarized in **Table 1** and indicate acceptable average sample recoveries at both low and high concentrations.

The limit of quantification (LOQ) was defined as the lowest concentration of analyte that could be reproducibly and accurately measured (accuracy >80 % and % RSD <20 %).[5] The LOQ for this method was determined to be 1 ng/mL for AMPH and MDA, and 0.5 ng/mL for MAMPH, HMMA and MDMA, respectively.

Additional Method Applicability

This method should also be applicable to the analysis of amphetamines in plasma and hair samples. Although the concentration of amphetamines is much lower in hair than in urine, it is noteworthy that the decomposition of residual stimulants deposited in hair is extremely slow. This can provide a trace record of the history of drug usage.

For hair samples, the sample preparation proposed is as follows: extract 0.25 g hair sample with 2 mL of 1.5 N sodium hydroxide solution overnight at room temperature, neutralize the solution with 2 mL of 1.5 N hydrochloric acid solution; sample 400 µL of the extract and add 100 µL of 1 N acetic acid and 500 µL of water, and then follow the SPE steps detailed on page 2.

Conclusion

A rapid and sensitive LC/MS/MS method utilizing Strata-X-C, 30 mg/1 mL SPE cartridges for sample preparation and a Gemini 5 µm C18 100 Å column for chromatographic separation for the simultaneous quantitative determination of amphetamines and their metabolites in human body fluids has been developed. Sample preparation from the biological sample matrix using Strata-X-C concentrates the amphetamines while eliminating biological matrix components that could potentially interfere with detection following rapid separation on Gemini C18. The LOQ for this method was determined to be 0.54 ng/mL for MAMPH, HMMA and MDMA with good linearity (R^2 > 0.997) over the concentration range 0.5 to 500 ng/mL. For AMPH and MDA, the LOQ was 1.0 ng/mL with good linearity (R^2 > 0.997) from 1.0 to 1000 ng/mL.

This extraction and chromatography method is well suited for the analysis of amphetamines and metabolites from other biological matrices, such as hair and should provide an attractive alternative for analyzing drugs of abuse in forensic, toxicological, and clinical laboratories.

References

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2. Edward M. Brecher & Editors of Consumer Reports “Licit & Illicit Drugs” Litle, Brown and Company, 1972.
3. M. E. Soares, M. Carvalho, H. Carmo et. al. “Simultaneous determination of amphetamine derivatives in human urine after SPE extraction and HPLUV analysis”; Biomed. Chromatogr. 18: 125-131 (2004).
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5. M. Wood, G. De Boeck, N. Samyn et. al. “Development of a Rapid and Sensitive Method for the Quantitation of Amphetamine in Human Plasma and Oral Fluid by LC-MS-MS” J. Anal. Tox., Vol. 27, March 2003..

Ordering Information

Sample Preparation

Part No.	Description	Unit
8B-S029-TAK	Strata-X-C Tubes (30 mg/1 mL)	100/box
8B-S029-UBJ	Strata-X-C Tubes (60 mg/3 mL)	50/box
8B-S029-FBJ	Strata-X-C Tubes (200 mg/3 mL)	50/box
8B-S029-HBJ	Strata-X-C Tubes (500 mg/3 mL)	50/box
8B-S029-ECH	Strata-X-C Tubes (100 mg/6 mL)	30/box
8B-S029-FCH	Strata-X-C Tubes (200 mg/6 mL)	30/box
8B-S029-HCH	Strata-X-C Tubes (500 mg/6 mL)	30/box
8B-S029-HDG	Strata-X-C Giga™ Tubes (500 mg/12 mL)	20/box
8B-S029-JDG	Strata-X-C Giga™ Tubes (1 g/12 mL)	20/box
8E-S029-AGB	Strata-X-C 96-Well Plate (10 mg)	2 Plates/Box
8E-S029-TGB	Strata-X-C 96-Well Plate (30 mg)	2 Plates/Box
8E-S029-UGB	Strata-X-C 96-Well Plate (60 mg)	2 Plates/Box

HPLC Columns			SecurityGuard™ Cartridges
Part No.	Description	Unit	4 x 2.0* (10pk)
00F-4435-Y0	Gemini 5 µm C18 (150 x 3.0 mm)	ea	AJ0-7596
00B-4439-B0	Gemini 3 µm C18 (50 x 2.0 mm)	ea	AJ0-7596
00F-4439-B0	Gemini 3 µm C18 (150 x 2.0 mm)	ea	AJ0-7596
00F-4439-Y0	Gemini 3 µm C18 (150 x 3.0 mm)	ea	AJ0-7596

*SecurityGuard™ Analytical Cartridges require holder, Part No.: KJ0-4282

guarantee

If Strata-X SPE products and Gemini analytical HPLC columns do not provide at least equivalent results and separation as compared to products of similar dimension, phase, and particle size, return the column with comparative data within 45 days for a FULL REFUND.

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