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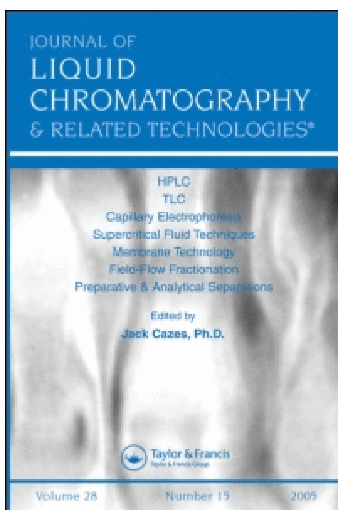

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Simultaneous Quantitation of Amphetamine and 4'-Hydroxyamphetamine by High Performance Liquid Chromatography

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SIMULTANEOUS QUANTITATION OF AMPHETAMINE AND 4'-HYDROXYAMPHETAMINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A relatively simple HPLC procedure used to detect and quantify amphetamine and 4'-hydroxyamphetamine in rat urine has been developed. Following Bond Elut® solid phase extraction, the two analytes – amphetamine and 4'-hydroxyamphetamine, and the two internal standards – methamphetamine and 4'-hydroxymethamphetamine were separated by HPLC using a phenyl column and detected by UV at 215 nm. The limit of quantitation for amphetamine and 4'-hydroxyamphetamine was 0.92 and 0.81 μg/ml, respectively. Based on replicated analysis of controls at 1.6, 6.5, and 16.0 μg/ml , the method is accurate (94 – 103% of target), and precise (% CVs of 1.3 – 5.2). The linear range of the assay is suitable for the quantitation of both analytes at urine concentrations that commonly result from administration of a single dose of amphetamine to the rat.

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INTRODUCTION

A quantitative method to simultaneously determine the concentrations of amphetamine and 4'-hydroxyamphetamine would be beneficial for *in vivo* studies on the oxidative metabolism of amphetamine. Simultaneous determination of amphetamine and 4'-hydroxyamphetamine has been achieved using gas chromatography/mass spectrometry (1, 2). High performance liquid chromatography (HPLC) offers an alternative approach to analysis where analyte derivatization is not desirable. Although a number of HPLC methods have been developed to detect amphetamines (3), the only methods described for simultaneous measurement of the parent amphetamine and 4'-hydroxyamphetamine have required either radioactive drug (4) which is not commercially available, or specialized chiral columns with derivatization (5). The ability to perform relatively simple HPLC analysis, without derivatization, in a single step would be highly desirable.

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The rat, when compared to other species, is an excellent model for studying *in vivo* 4-hydroxylation of amphetamine since rats predominantly utilize the 4-hydroxylation pathway as observed by Axelrod (6). Conjugation of amphetamine metabolites was characterized by Dring and coworkers (7) who found that 4'-hydroxyamphetamine was conjugated by glucuronidation, and benzoic acid was conjugated by sulfation. It is, therefore, necessary to hydrolyze rat urine in order to quantify the amount of 4'-hydroxyamphetamine formed in any *in vivo* studies.

Reverse-phase HPLC is a suitable approach to achieve the separation of nonderivatized 4'-hydroxyamphetamine and amphetamine because 4'-hydroxyamphetamine is water soluble and amphetamine is readily soluble in acids. HPLC with ultraviolet (UV) detection, however, was not widely considered by most investigators because of amphetamine's poor UV absorption characteristics. The derivatization requirement of GC/MS detection for the quantification of 4'-hydroxyamphetamine and/or amphetamine prompted Farrell and Jefferies (8) to suggest that an HPLC method maybe more attractive. Subsequent success in the

use of silica-based reverse-phase HPLC-UV to separate drugs of forensic interest including amphetamine and 4'-hydroxyamphetamine (3, 9-12) suggest this alternative approach to detect amphetamine and 4'-hydroxyamphetamine.

Preliminary studies in our laboratory demonstrated chromatographic conditions that could be used for UV detection of amphetamine and 4'-hydroxyamphetamine standards (13), but did not address matrix differences which may arise from hydrolysis of urine samples. The combination of solid phase extraction (SPE) by Bond Elut® C18 columns and reverse phase HPLC with a phenyl column resulted in a satisfactory analytical method.

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MATERIALS

4'-Hydroxyamphetamine bromide and 4'-hydroxymethamphetamine were generously provided by Dr. Anthony S. Murabito of SmithKline Beecham (Philadelphia, PA). ß-Glucuronidase (Type H-1), *d*-amphetamine sulfate, and *d*-methamphetamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Bond-Elut® C18 SPE columns were purchased from Varian (Harbor City, CA). All solvents were of HPLC grade from Burdick and Jackson (Muskegon, MI). All other reagents were of reagent grade or better.

Rat urine was collected from male Sprague Dawley (SD) rats purchased from Sasco, Inc. (Omaha, NE). Following acclimatization to environmentally controlled animal housing facilities, these animals were kept in individual Nalgene (Rochester, NY) metabolic cages for urine collection. Food and water were available *ad libitum.*

The spectra of the analytes in the appropriate solvent were obtained using a Varian (Walnut Creek, CA) Cary 2200 UV-VIS scanning spectrophotometer.

The separation and detection of amphetamines was achieved by a Varian (Walnut Creek, CA) HPLC system, which consisted of a VISTA 5500 pump, UV200 scanning detector, and VISTA 402 Data Integrator. It was also equipped with a Rheodyne (Cotati, CA) injector with a 10 µl loop, a Microsorb® (Rainin, Emeryville, CA) phenyl reverse phase column, and a C18 column from Alltech

(Deerfield, IL), both columns had the respective Alltech (Deerfield, IL) pre-column cartridge system attached.

METHODS

Urine Hydrolysis and Solid Phase Extraction

Urine hydrolysis was performed as described by Yamamoto and coworkers (14) to account for the total amount of amphetamine metabolites excreted. Briefly, 0.5 ml of rat urine and 0.5 ml (1000 units) of ß-glucuronidase with sulfatase activity in 0.1 M acetate (pH 5.0) were added to screw cap test tubes and incubated at a 37° C overnight. The tubes were stored at 4° C until extraction.

The SPE procedure described by Shimosato (15) was modified to elute both amphetamine and 4'-hydroxyamphetamine. The Bond Elut® C18 columns were conditioned by successively washing with 1 ml each of methanol and 50 mM potassium phosphate (pH 11). While the columns were being conditioned, the following was added to the hydrolyzed urines: 0.5 g sodium chloride, 0.5 ml 50 mM potassium phosphate (pH 11), and internal standards (4'-hydroxymeth-amphetamine, 1.3 μg, and methamphetamine, 3.1 μg). The mixtures' pHs were adjusted to 11 using ammonium hydroxide. The solutions were thoroughly mixed and loaded onto the conditioned columns. After the urinary solutions had passed through, the columns were washed in succession with 1 ml each of 50 mM potassium phosphate (pH 11), freshly prepared 30% methanol, and acetonitrile. Successive elution with 1 ml each of freshly prepared 2% glacial acetic acid in acetonitrile for the amphetamines, and 2% hydrochloric acid in acetonitrile for the hydroxy-metabolites was performed. The eluates were pooled and dried under a stream of air at room temperature and stored dry at 4° C until ready for analysis.

Analytes Extraction at various Loading and Washing pHs.

To examine the effect of pH on the loading and washing steps, 0.5 ml aliquots of 50 mM potassium phosphate solutions (pH 8.5 to 13.0) were added to

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preparations of 0.5 ml spiked (4'-hydroxyamphetamine and amphetamine) and hydrolyzed urines prior to adding sodium chloride and internal standards. Potassium phosphate (50 mM) solutions at respective pHs were used to condition and wash the SPE columns. The pH of all other steps was not changed.

HPLC

Analysis was performed using the HPLC system described above. An injection volume of 25 µl was delivered to the 10 µl injector loop to achieve complete loop loading. Detection was at 215 nm, with a sensitivity of 0.005 AU/mV. The mobile phase was 50 mM potassium phosphate (pH 3), methanol, and acetonitrile (85:10:5), at a flow rate of 1 ml/min. The run time was 18.5 minutes.

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Analyte concentration estimation

Standards were drug-free rat urine spiked with 4'-hydroxyamphetamine (0.8, 1.6, 3.3, 4.9, 6.5, 8.1, 16.3, and 32.6 µg/ml) and amphetamine (0.9, 1.8, 3.7, 5.5, 7.3, 9.2, 18.3, and 36.7 µg/ml). These concentrations are for free drugs and do not reflect the actual weighed amount of the respective salt. Standards were processed through the hydrolysis and extraction procedures described above, including addition of the internal standards. The standard curve was constructed based on the peak area ratios of analyte to internal standard (4'-hydroxy-amphetamine/4'-hydroxymethamphetamine; amphetamine/methamphetamine), with the curves equation calculated by the least-squares method. Peak area ratios for urine sample data were determined, and the respective analyte concentrations were calculated from the standard curve equation.

Validation of Method

The precision and accuracy, limit of quantitation (LoQ) and recovery of this analytical method were determined in accordance with established procedures (16). The linearity of the standard curves was determined by calculating the coefficient of linear regression (Pearson product-moment correlation coefficient, r). Within-

and between-run precision and accuracy of control reference solutions (QC) at three concentrations and low standards for determination of LoQ were performed in three runs: all samples were in replicates of five in one run, and replicates of three in the other two runs. The former was used for within-run determinations, and the means of all three runs were used for between-run determinations. Precision was defined by the relative standard deviation (RSD), where RSD = (standard deviation ÷ mean) x 100%. Accuracy was calculated by dividing the mean analyte concentrations determined from standard curve by the weighed-in analyte concentrations, and multiplying the result by 100%.

Analyte recovery was determined at three 4'-hydroxyamphetamine (1.6, 6.5, and 16.3 μg/ml) and amphetamine (1.8, 7.3, and 18.3 μg/ml) concentrations. Ten urine samples per concentration were aliquoted; five tubes were prepared and extracted normally (internal calibrators). The remaining five tubes (external calibrators) went through the hydrolysis and extraction steps with amphetamine and 4'-hydroxyamphetamine added just before the dry down step. The internal standards were added before extraction as described above. Percentage recovery was calculated by dividing the mean of internal calibrator ratios by the mean of the external calibrator ratios and multiplying by 100%.

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RESULTS AND DISCUSSION

Solid Phase Extraction

Liquid to liquid (L/L) extraction has been the method of choice for extracting amphetamine and 4'-hydroxyamphetamine from biological samples over the years. Preliminary data, however, revealed L/L extraction of hydrolyzed rat urine was insufficient for obtaining usable HPLC chromatograms due to high background; up to 50 peaks were seen in a chromatogram. The alternative approach of using back extraction which would partition the analytes into the appropriate phase under acidic or basic conditions did not resolve the problem of numerous background

peaks. SPE technology offered another approach. When Bond Elut® C18 columns were used, the resulting chromatograms had only five major peaks, very few minor peaks, and the highest recovery of analytes.

The pKa of amphetamine is 9.9, and that of 4'-hydroxyamphetamine is 10.7 (17). The SPE column loading and washing pHs can affect the recovery of these analytes. SPE column recovery was determined as the peak area achieved from the eluate relative to the peak area of unextracted material. The concentrations of 4'-hydroxyamphetamine and amphetamine used were 6.5 and 7.3 μg/ml, respectively. At pH 8.5 – 11, the recoveries of 4'-hydroxyamphetamine and 4'-hydroxymethamphetamine were between 20 and 30% for both compounds, whereas those of amphetamine and methamphetamine were greater than 90%, at pH 8.5 to 11.5. At higher pH, 12 and 13, the recovery of all four compounds suffers, decreasing to between 3 and 12% for the hydroxylated amphetamines and between 70 to 85% for amphetamine and methamphetamine (Figure 1). This study demonstrated that maximum recovery from the SPE columns can be achieved when loading and washing pHs of 10-11 were used.

Determination of the percentage recovery of 4'-hydroxyamphetamine over the entire extraction procedure (i.e., using internal and external calibrators) resulted in the recoveries of 31, 24, and 24% for the three concentrations (1.6, 5.3, and 16.3 μg/ml) of QC samples used. For the three concentrations of amphetamine (1.8, 7.3, and 18.3 μg/ml), the current procedure yields 93, 93, and 96% recovery, respectively. There was excellent agreement between recoveries determined during pH optimization experiments (see above) and this subsequent recovery determination experiment.

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UV Spectra

The spectra (200 to 300 nm) of amphetamine, methamphetamine, 4'-hydroxy-amphetamine and 4'-hydroxymethamphetamine in the HPLC mobile phase are shown in Figure 2. The UV spectra for amphetamine, methamphetamine, and 4'-hydroxyamphetamine in HPLC mobile phase are essentially the same as those

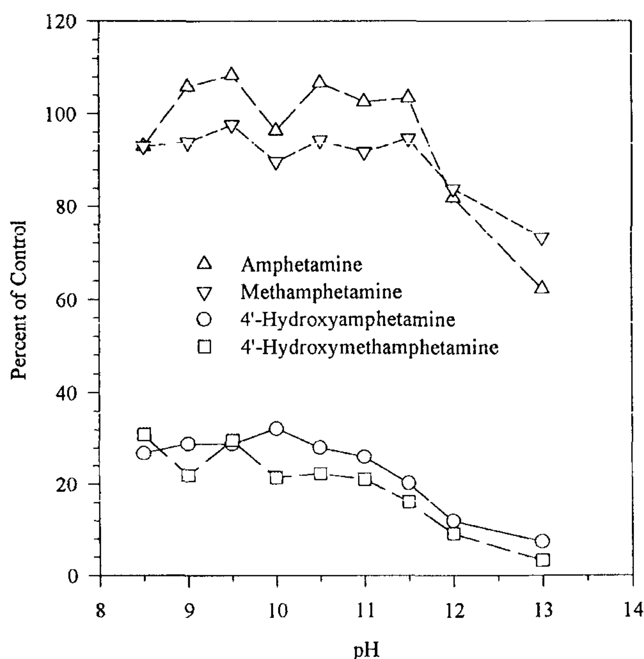

FIGURE 1. Recovery of analytes from SPE columns when the loading and washing pHs vary (see method section).

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determined previously in 0.1 N HCl (18). The listed λ_{max} values for 4'-hydroxy-amphetamine in 0.1 N HCl are 220 and 274. In HPLC mobile phase they were 230 and 274 for both 4'-hydroxyamphetamine and 4'-hydroxymethamphetamine. Those for both amphetamine and methamphetamine are 252, 257, and 263 nm in 0.1 N HCl and in HPLC mobile phase. Using detection wavelengths at any of these λ_{max} would only allow detection of a single set of analytes, i.e., either the hydroxylated amphetamines, or the set of amphetamine and methamphetamine. At 215 nm significant UV absorption is exhibited by all four analytes (Figure 2). Although it is not the ideal wavelength for selective detection of the individual analyte, it is optimal when detection of all four analytes simultaneously is important.

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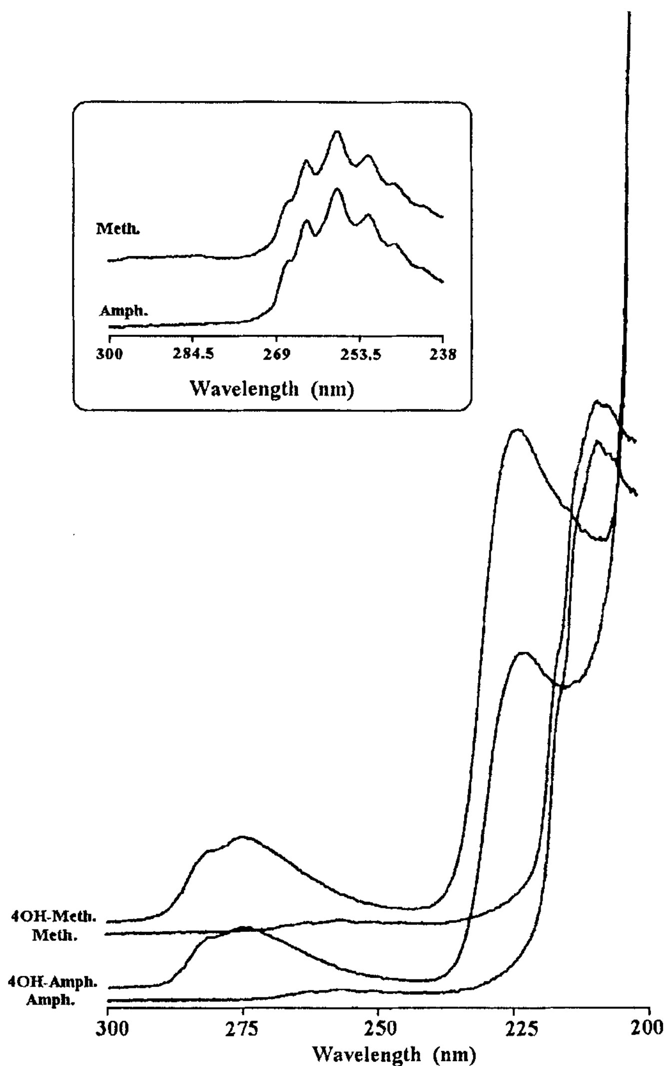

FIGURE 2. UV absorption spectra of 4'-hydroxyamphetamine (4OH-Amph), 4'-hydroxymethamphetamine (4OH-Meth), amphetamine (Amph), and methamphetamine (Meth). HPLC mobile phase (50 mM potassium phosphate:methanol:acetonitrile; 85:10:5) was used as solvent. Analyte concentrations were 7.5 µg/ml, and the wavelengths scanned were between 200 and 300 nm. The inset spectra were 100 µg/ml of amphetamine and methamphetamine in the solvent as above, and the wavelengths scanned were between 238 and 300 nm.

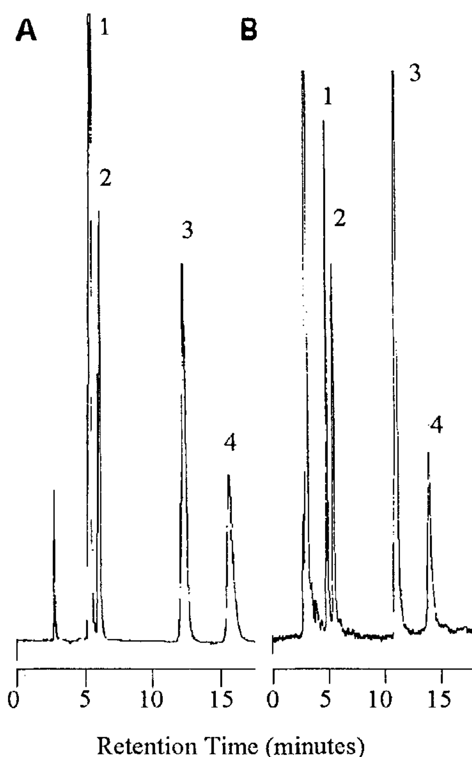

FIGURE 3. High performance liquid chromatograms of unextracted amphetamine standards (Figure 3A), and urine extract from female SD rat (Figure 3B). Peaks labeled 1, 2, 3, and 4 are 4'-hydroxyamphetamine, 4'-hydroxymeth-amphetamine (internal standard), amphetamine, and methamphetamine (internal standard), respectively. The standards in 3A contained 16.3, 2.6, 18.3, and 6.2 μg/ml, respectively.

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Chromatography

When HPLC was used to separate the four analytes from hydrolyzed urine using C8 or C18 columns, background material interfered with the detection of the hydroxylated compounds, and this could not be overcome by changes in flow rate or mobile phase composition. Using a phenyl column, chromatography with fairly low background interference was obtained. By increasing the phosphate

concentration of mobile phase from 25 mM to 50 mM, the 4-hydroxylated analytes were adequately separated from both the solvent front and background interference as shown for unextracted amphetamine standards and an urine extract from a SD rat (Figure 3). Changing the flow rate to higher or lower than 1 ml/min did not markedly improve the shape or separation of peaks. Retention times for the amphetamines were 5.3, 6.0, 12.2, and 15.6 minutes for 4'-hydroxyamphetamine, 4'-hydroxymethamphetamine, amphetamine, and methamphetamine, respectively.

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Method Validation

The coefficients of linear regression of the standard curves for both 4'-hydroxyamphetamine and amphetamine were consistently greater than 0.98. Tables 1 and 2 summarize the precision and accuracy of analysis for 4'-hydroxyamphetamine and amphetamine. During the process of determining the LoQ, 4'-hydroxyamphetamine at 0.4 and 0.6 μg/ml and amphetamine at 0.5 and 0.7 μg/ml were also evaluated; however, their peaks signals were too weak for integration. The precision and accuracy values calculated for LoQs of 4'-hydroxyamphetamine at 0.8 μg/ml and amphetamine at 0.9 μg/ml were within the recommended 20% limits (16). Using the rejection limit of 15% (16), the precision and accuracy values of the QCs for between- and within-run were acceptable. The between- and within-run precisions of QCs for 4'-hydroxyamphetamine remained below 5% RSD, and the RSDs for amphetamine were within 7%.

This HPLC separation and detection method demonstrated adequate linearity, precision, and accuracy to detect amphetamine and 4'-hydroxyamphetamine in *in vivo* experimental samples (Tables 1 and 2). The LoQ of the method is approximately 1 μg/ml, which is sufficient for *in vivo* studies where animals were dosed with mg quantities of amphetamine. In the rat, a dose of amphetamine excreted in the urine over 12 to 24 hours was approximately 20% parent

TABLE 1

Summary Table of 4'-Hydroxyamphetamine Precision and Accuracy.

Samples	Weighed-in concentrations (μg/ml)	Determined concentrations (Mean ± SD)	Relative Standard Deviation (RSD)	Accuracy (%)
	Between-runs			
LoQ	0.81	0.82 ± 0.03	3.50	101.0
QC1	1.63	1.68 ± 0.07	4.42	102.8
QC2	6.51	6.53 ± 0.08	1.27	100.4
QC3	16.29	16.03 ± 0.59	3.70	98.4
	Within-run			
LoQ	0.81	0.79 ± 0.10	12.26	98.0
QC1	1.63	1.60 ± 0.05	3.31	98.3
QC2	6.51	6.44 ± 0.34	5.23	99.0
QC3	16.29	15.38 ± 0.38	2.46	94.4

Note: The means ± SD are presented in the table. A total of three separate analytical runs were done, with n=3 for the first two and n=5 for the third run. Between-run values were calculated using the mean for each run. The within-run values were calculated from the last analytical run (n=5). See method section for equations.

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TABLE 2

Summary Table of Amphetamine Precision and Accuracy.

Samples	Weighed-in concentrations (μg/ml)	Determined concentrations (Mean ± SD)	Relative Standard Deviation (RSD)	Accuracy (%)
	Between-runs			
LoQ	0.92	0.90 ± 0.18	19.44	97.9
QC1	1.83	1.86 ± 0.08	4.55	101.7
QC2	7.34	7.49 ± 0.06	0.75	102.1
QC3	18.34	18.70 ± 0.84	4.51	101.7
	Within-run			
LoQ	0.92	0.77 ± 0.03	4.34	84.1
QC1	1.83	1.77 ± 0.03	1.85	96.6
QC2	7.34	7.55 ± 0.28	3.74	102.9
QC3	18.34	18.97 ± 1.30	6.87	103.4

Note: The means ± SD are presented in the table. A total of three separate analytical runs were done, with n=3 for the first two and n=5 for the third run. Between-run values were calculated using the mean for each run. The within-run values were calculated from the last analytical run (n=5). See method section for equations.

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compound and 40% 4'-hydroxyamphetamine (7). If a 200 g rat was given 1 mg of amphetamine (5 mg/kg) and excreted 10 ml of urine over this time, that should translate to 20 μg/ml amphetamine and 40 μg/ml 4'-hydroxyamphetamine. These concentrations are well within the LoQ of this method.

ACKNOWLEDGMENTS

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