Quantitative Analysis of Amphetamine-Type Drugs by Extractive Benzoylation and LC/MS/MS

Application

Forensics

Authors

Neil Campbell, B. Sc.
Forensic Science Laboratory
Chemistry Centre (WA)
125 Hay Street
East Perth, Western Australia
Australia

Michael Zumwalt
Agilent Technologies
9780 S. Meridian Blvd.
Englewood, CO 80112
USA

Abstract

A fast, sensitive, and validated technique for confirming the presence of amphetamine drugs in whole blood using the Agilent G6410A Triple Quadrupole Mass Spectrometer (QQQ) is presented. Excellent linearity is demonstrated over the range of approximately 15 to 1,000 ng/mL. The amphetamine drugs analyzed in this work include amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), and methylenedioxymethamphetamine (MDMA) in blood. The drugs have been prepared using an extractive alkylation technique. The sample preparation is then followed by reverse-phase LC/MS/MS using a 1.8-µm particle size C18 column for high chromatographic resolution with a high-speed separation. As a result, elution times for both analytes and internal standards are all less than or equal to 3.6 minutes.

Introduction

Amphetamines are a group of sympathomimetic drugs that exhibit strong central nervous system stimulant effects. Amphetamine (phenylisopropylamine) is the parent drug in this class to which all others are structurally related. Other drugs in the class include: ephedrine, pseudoephedrine, methylamphetamine, phentermine, fenfluramine, chlorphentermine, MDA, and MDMA ("Ecstasy"). The LC/MS/MS method used in this work has applicability to the quantitative analysis of amphetamine-like drugs in both ante- and post-mortem blood and urine samples and post-mortem liver and viscera samples. Primary and secondary aliphatic amines react with pentafluorobenzoyl chloride in alkaline conditions to form the respective amides. The method utilizes this reaction and the principle of extractive alkylation to isolate the products formed by these drugs from blood or urine.

The drugs are quantified by electrospray liquid chromatography/tandem mass spectrometry with multiple reaction monitoring (LC/MS/MS-MRM). For purposes of quantitation, each drug analyte has a quantitative product ion monitored. For confirmation, each analyte has an additional product ion, known as the qualifier ion, monitored. The overall ion ratio of the qualifier to the quantifier ions is fixed to a method-determined value and applied to all samples for confirming the presence of compounds. The tolerance for acceptance of this ratio is ± 20%.

For the associated D5 internal standards, only a quantifier ion is monitored because confirmation is not required.

The compounds' structures are shown in Figure 1.

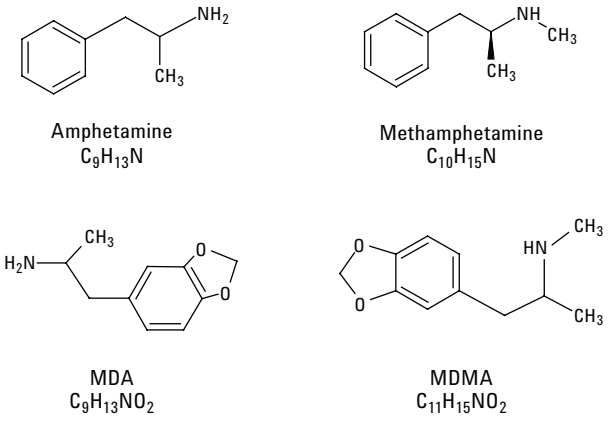

Figure 1. Structures of the compounds analyzed in this work.

Experimental

Reagents (Sigma-Aldrich, Castle Hill, NSW, Australia)

1. 5% Pentafluorobenzoyl chloride (PFBCl) (Prepare fresh by pipetting 0.25 mL PFBCl into 5 mL butyl chloride.)
2. Triethanolamine/cyclohexane (TEA/CH) (Pipette 0.5 mL of TEA/CH into a 500-mL measuring cylinder. Make final volume of 500 mL with cyclohexane. Mix and allow phases to separate.)
3. Ammonia buffer (Place 100 mL of water in a beaker. Dissolve ammonium chloride until a saturated solution is obtained. Adjust to pH 9.4 with concentrated ammonia solution.)
4. Anhydrous sodium sulphate

Standards (Cerilliant, Round Rock, TX, USA)

1. Standard reference solutions of target drugs in methanol made from solid material. The actual amounts vary slightly from one analyte compound to another and are reflected in the concentration ranges reported later. The standards are diluted in methanol and added to the blood to achieve the concentration range of approximately 15 to 1,000 ng/mL.
2. Internal Standards – 10-µg/mL mixture of D5-amphetamine, D5-methylamphetamine, D5-MDMA, and D5-MDA. The standards are purchased from commercial suppliers and are obtained as sealed ampoules, each containing approximately 100 µg of drug in 1 mL of methanol.
3. The response factor(s) is determined by addition of the standards to blood at concentrations that bracket the expected range of significant analytical results. For blood this should be equivalent to concentrations of 0.05, 0.1, 0.25, 0.5, and 1 µg/mL. A blank must be included in each analytical batch.

Sample Preparation

1. Transfer 0.2 mL blood into a 15-mL disposable test tube and dilute to 1 mL with water. Add 5 mL of the TEA/CH solution spiked with the D5-amphetamine standards mixture to a concentration of 50 nanograms per 5 mL, 0.2 mL of ammonia solution, and 0.01 mL of freshly prepared 5% PFBCl solution. Alternatively, the blood can be more conveniently sampled and diluted with the aid of an autodiluter (Hamilton Microlab Series 500) using a 0.2 to 1 mL dilution program.
2. The standard reference solutions are treated as above, with 0.2 mL of blank blood added to the diluted standards.
3. Vortex for 3 minutes, heat at 60 °C for 10 minutes, then centrifuge (see Note 4).
4. Remove the organic phase, dry by passage through a Pasteur pipette packed with anhydrous sodium sulfate, and evaporate to dryness.
5. Reconstitute the residue in 100 µL of methanol, transfer to a low-volume autosampler vial, seal, and then analyze by LC/MS/MS-MRM.

Notes:

1. The IStd (internal standard) quantity described above is equivalent to 250 ng/mL and is appropriate for concentrations in the range 10 to 1,000 ng/mL.
2. The internal standard chosen for analytes where no deuterated analogue is available must match the chemical nature of the analyte, that is, a primary amine is used for a primary amine and a secondary amine for a secondary amine.

3. An emulsion may occur during vortex mixing. It may be broken by stirring with a Pasteur pipette and recentrifuging.
4. For amphetamine, methylamphetamine, MDMA, and MDA, the reaction will proceed without the requirement for heating. If ephedrine is also to be quantitated, the heating step must be included.

LC/MS/MS Instrumentation

The LC/MS/MS system used in this work consisted of an Agilent 1200 Series vacuum degasser, binary pump, autosampler, thermostatted column compartment, the Agilent G6410A Triple Quadrupole Mass Spectrometer (QQQ), and the G1948B electrospray ionization source (ESI). System control and data analysis were provided by the Agilent MassHunter B.01.01 software. Detailed LC and MS conditions are shown below.

LC/MS Method Details

LC Conditions

Column:	Agilent ZORBAX XDB-C18, 4.6 × 50 mm, 1.8 µm (p/n 922975-902)		
Column temp:	60 °C		
Mobile phase:	A = Ammonia buffer (pH = 9), see Reagents B = Methanol		
Flow rate:	0.7 mL/min		
Gradient:	Time (min)	%B	
	0 – 0.2	50	
	3.0 – 4.0	100	Post run time = 1 min.
	4.1 – 6.0	50	
Injection vol:	2 µL		

MS Conditions

Mode:	Positive ESI using the Agilent G1948B ionization source
Nebulizer:	50 psig
Drying gas flow:	6 L/min
Drying gas temp:	350 °C
V_{cap}:	4000 V
Q1 Resolution:	Unit, 0.7 amu (FWHM)
Q2 Resolution:	Unit, 0.7 amu (FWHM)

MRM settings are shown in Table 1. Note that the fragmentor voltage and dwell time for each MRM is fixed for all transitions at 140 V and 40 msec, respectively.

Table 1. MRM Settings for the Compounds Analyzed in This Work (For confirmation, the qualifier ions are also shown in parentheses.)

Compound	Precursor ion	Product ion (qualifier)	Collision Energy
Amphetamine	330	119 (91)	15
D5-Amphetamine	335	124	15
Methylamphetamine	344	119 (91)	15
D5-Methylamphetamine	349	121	15
MDMA	388	163 (135)	20
D5-MDMA	393	165	20
MDA	374	163 (135)	20
D5-MDA	379	168	20

Results and Discussion

The linearity for each compound over the range of approximately 15 to 1,000 ng/mL is shown in Figures 2a through 2d. Note that a quadratic curve fit is applied. There is no weighting and the origin is ignored. The coefficient of determination (R^2) for all four curve fits is excellent at greater than 0.999. As the second-order coefficients are all less than 0.007, see Figures 2a through 2d, making extremely low contributions to the curve fits, the resulting curves can be considered linear for all intents and purposes.

For confirming the presence of the compounds the peak area ratio of the qualifier to quantifier ions must fall within a ± 20% tolerance of an expected value derived during method development. All samples within the batch, including calibrators and quality controls (QCs), must meet this criterion or they are considered negative.

An example of the ion ratio confirmation for each compound is shown in Figures 3a through 3d.

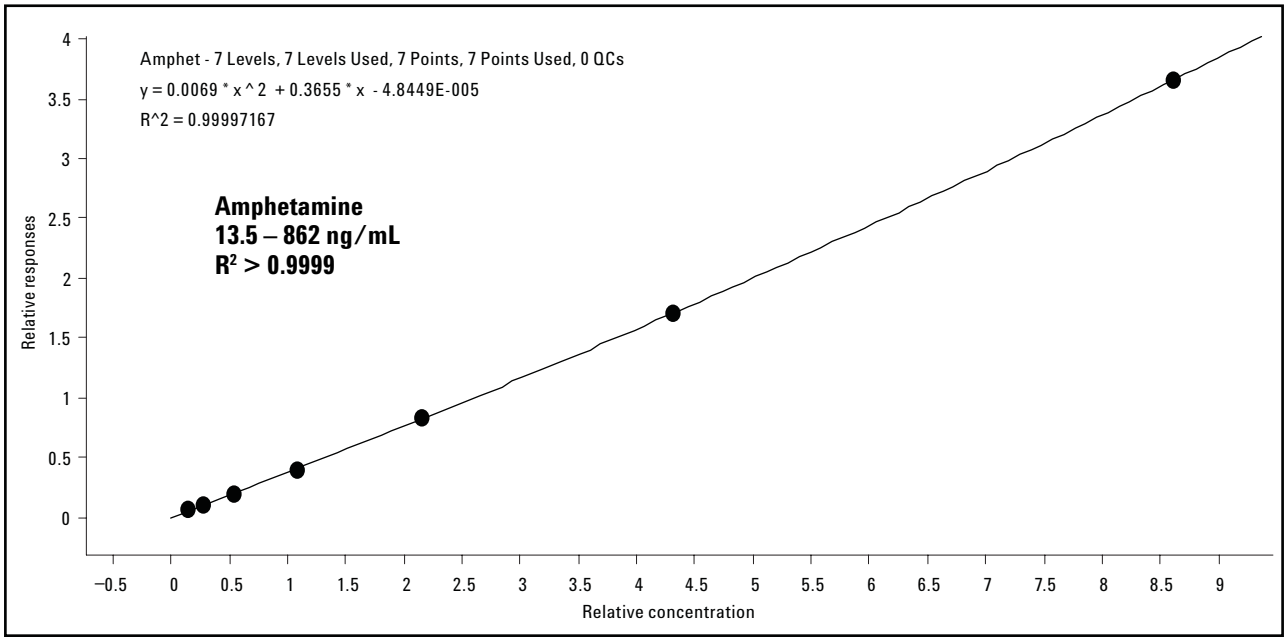

Figure 2a. Linearity of amphetamine in blood.

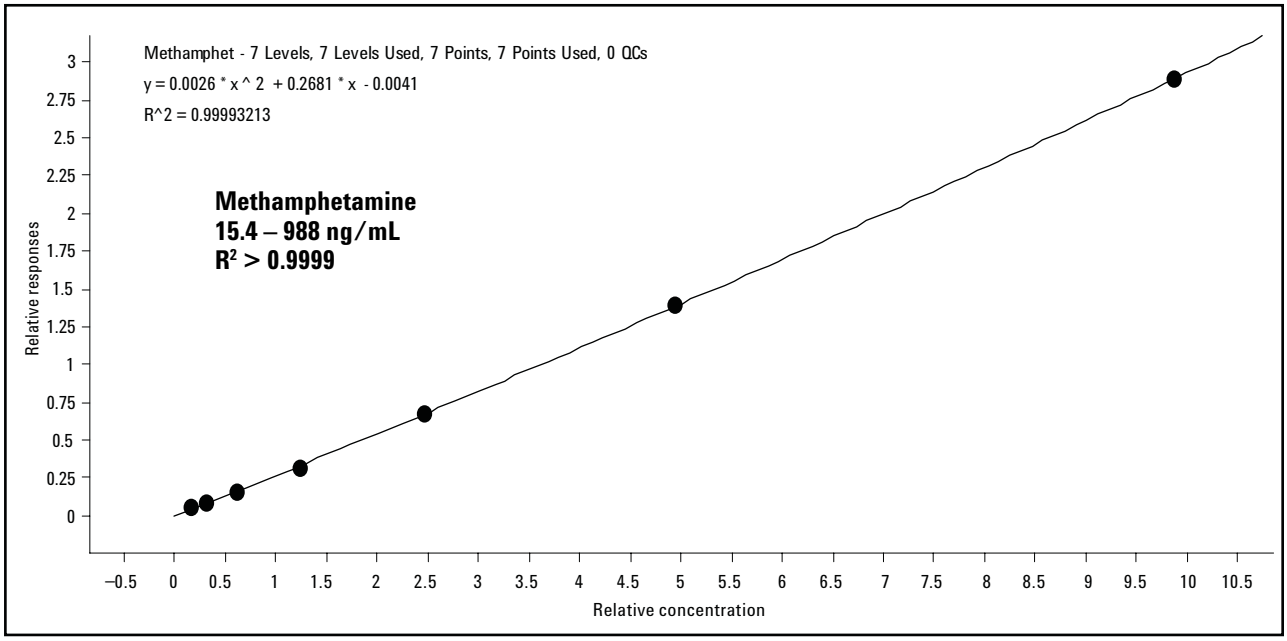

Figure 2b. Linearity of methamphetamine in blood.

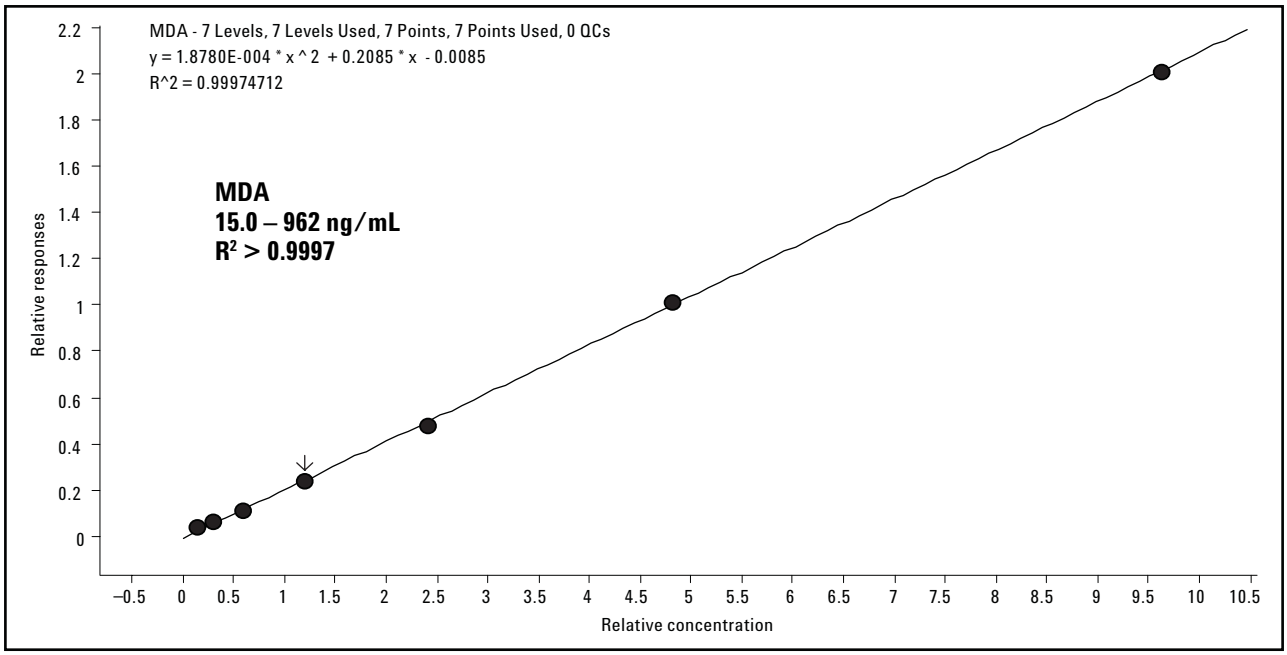

Figure 2c. Linearity of MDA in blood.

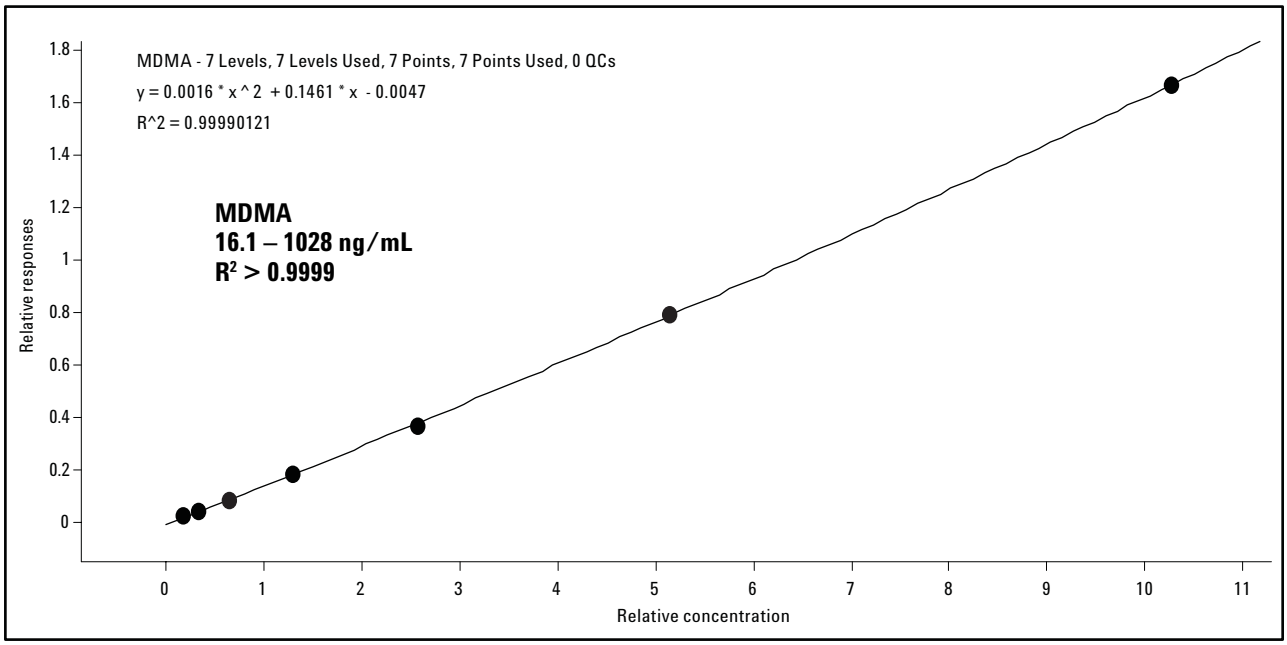

Figure 2d. Linearity of MDMA in blood.

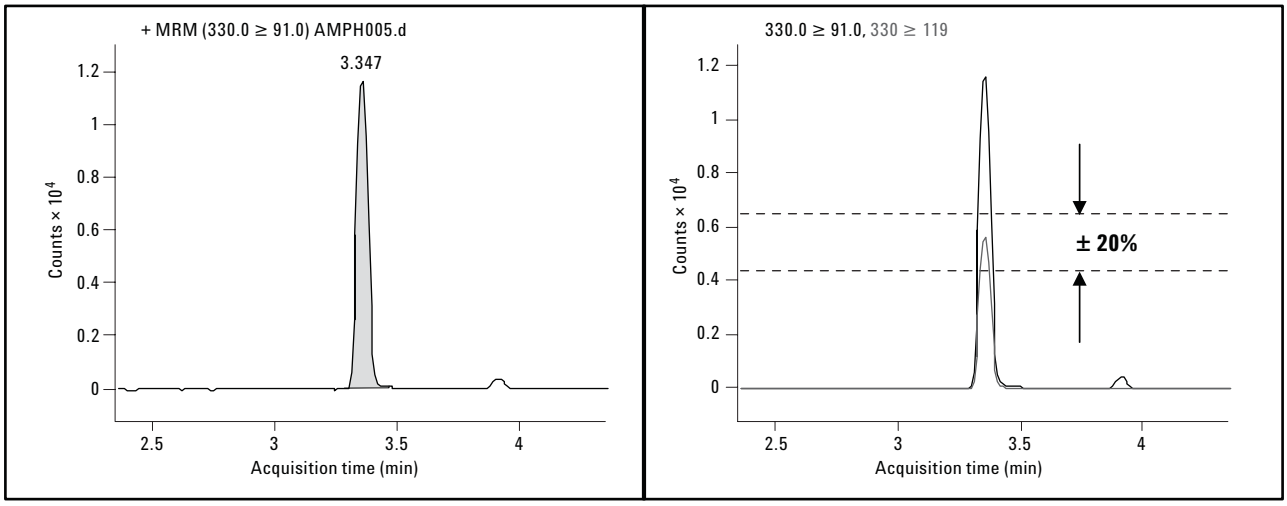

Figure 3a. Ion ratio confirmation for amphetamine in blood. Note retention time of 3.35 min.

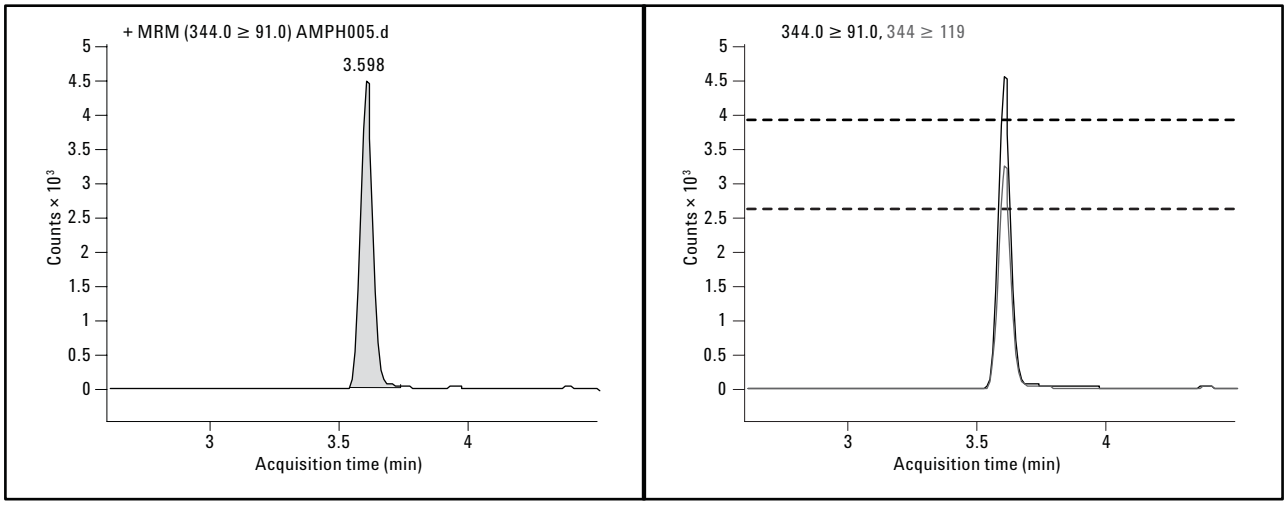

Figure 3b. Ion ratio confirmation for methamphetamine in blood. Note retention time of 3.60 min.

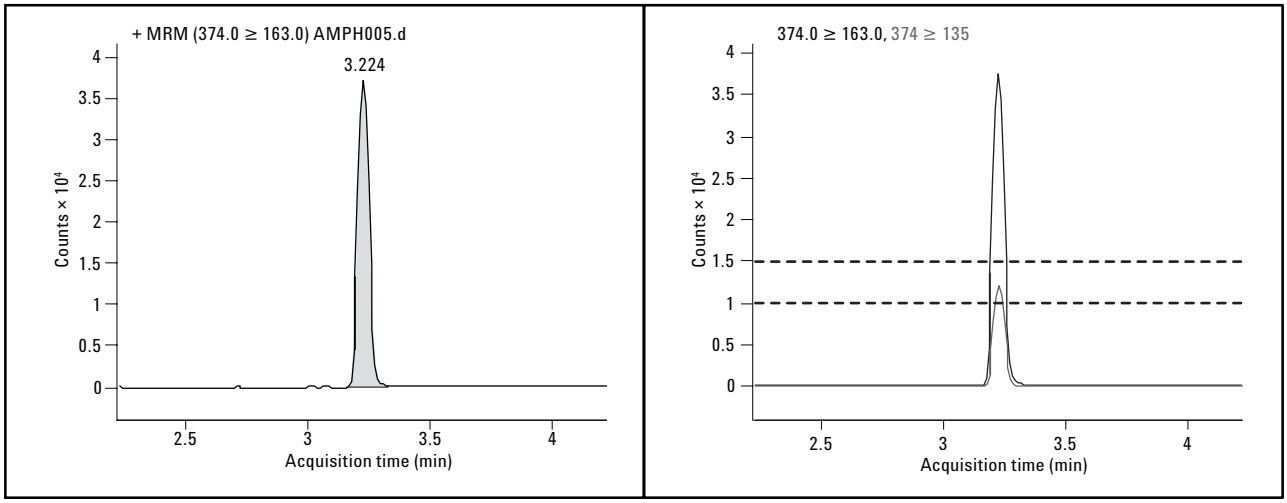

Figure 3c. Ion ratio confirmation for MDA in blood. Note retention time of 3.22 min.

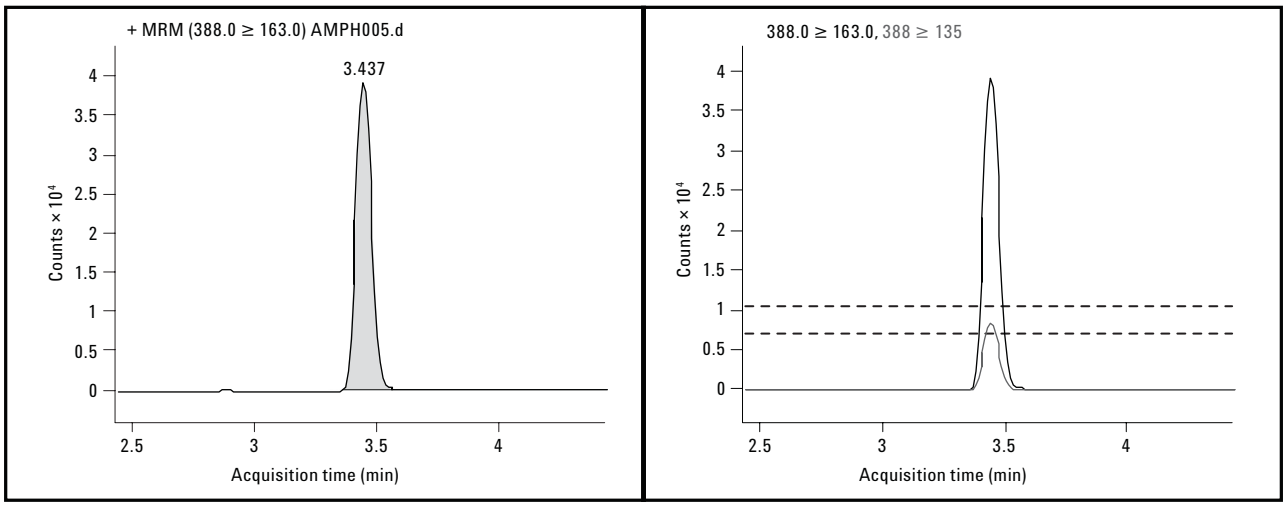

Figure 3d. Ion ratio confirmation for MDMA in blood. Note retention time of 3.44 min.

Also carried out was a study of the reproducibility of amphetamine (amp) and methamphetamine (meth) at two different concentration levels in blood. The results are tabulated below in Tables 2a and 2b in which 10 replicate injections at the 0.5 and 0.25 µg/mL in blood concentration levels are each made. The resulting peak area percent relative standard deviation (%RSD) relative response values of amphetamine and methamphetamine, with respect to the D5 IStd, at the 0.5 µg/mL level are 0.48 and 0.89, respectively. At the 0.25 µg/mL level the corresponding values are 1.12 and 2.27, respectively.

Validation

1. The method is an adaptation of a published validated method and an "in-house" GC-MS method that has been subject to extensive validation. The use of LC/MS/MS-MRM detection is an established technique that does not require further validation.
2. Within-run precision has been established by statistical analysis of replicate samples.
3. Known concentrations of amphetamine and methylamphetamine from commercially available control samples and interlaboratory proficiency trials have been successfully analyzed by the method.

Table 2a. Reproducibility of Amphetamine and Methamphetamine in Blood at the 0.5 µg/mL Level

Injection number	Amp (area cts * 1000)	D5-Amp (area cts * 1000)	Relative response	Meth (area cts * 1000)	D5-Meth (area cts * 1000)	Relative response
1	918	1844	0.498	1060	1600	0.663
2	933	1887	0.494	1077	1599	0.674
3	938	1875	0.500	1087	1620	0.671
4	949	1904	0.498	1076	1627	0.661
5	948	1909	0.497	1082	1648	0.657
6	949	1911	0.497	1081	1641	0.659
7	967	1924	0.503	1109	1650	0.672
8	980	1963	0.499	1132	1689	0.670
9	986	1969	0.501	1128	1678	0.672
10	1006	2011	0.500	1145	1720	0.666
		Std dev	0.002		Std dev	0.006
		%RSD	0.484		%RSD	0.889

Table 2b. Reproducibility of Amphetamine and Methamphetamine in Blood at the 0.25 µg/mL Level

Injection number	Amp (area cts * 1000)	D5-Amp (area cts * 1000)	Relative response	Meth (area cts * 1000)	D5-Meth (area cts * 1000)	Relative response
1	236	966	0.244	167	515	0.324
2	243	957	0.254	173	506	0.336
3	247	972	0.254	173	513	0.342
4	246	972	0.253	166	518	0.324
5	246	973	0.253	175	516	0.338
6	245	978	0.251	173	514	0.335
7	250	994	0.252	176	512	0.342
8	248	989	0.251	178	526	0.348
9	254	1004	0.253	175	536	0.333
10	253	1005	0.252	179	536	0.334
		Std dev	0.003		Std dev	0.008
		%RSD	1.126		%RSD	2.270

4. A calibration curve is established on an analytical batch basis by addition of a range of concentrations of standard amphetamines to blank blood or urine. The method has been shown to be linear in the concentration range of 15 to 1,000 ng/mL. For results greater or less than this range, the result should be reported as "greater than" or "less than." Alternatively, report the result as approximate or the sample may be reanalyzed with the standard range extended to include the concentration encountered.
5. The uncertainty of the method determined from control data and precision studies is 10% at the 95% confidence level.

Conclusions

The LC/MS/MS method described here provides a procedure for the quantitation and confirmation of multiple drugs of abuse in whole blood with very fast analysis times. The multiple reaction monitoring of several fragmentation transitions is carried out not only for quantitation using designated quantifying ions, but also for confirmation using designated qualifier ions. Using the Agilent C18 column with 1.8-µm particle size allows for excellent resolution and peak shape at a relatively high flow rate of 700 µL/min for a 4.6-mm id column and an ESI interface. Less than 1% RSD relative response is shown for both amphetamine and methamphetamine at the 0.5 µg/mL level in blood.

Acknowledgments

A special thanks to Agilent colleague John M. Hughes for very valuable review and comments.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

For more details concerning this application, please contact Michael Zumwalt at Agilent Technologies, Inc.

Printed in the USA
October 25, 2007
5989-7527EN