Amphetamines, Phentermine, and Designer Stimulant Quantitation Using an Agilent 6430 LC/MS/MS

Application Note

Forensics

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Abstract

A method was developed for the quantitation of amphetamines, phentermine, and designer stimulants in biological samples using an Agilent 6430 Triple Quadrupole Mass Spectrometer. Thirteen compounds were evaluated using linear weighted and quadratic weighted calibration models to establish method feasibility. Sufficient resolution and peak shape was achieved within a cycle time of 9 minutes. Additional validation studies confirmed that this method met all criteria required for routine analysis of amphetamines, phentermine, and designer stimulants in whole blood.

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Introduction

Amphetamines and phentermine are analyzed in biological matrices in many forensic toxicology laboratories. Quantitative assessment of amphetamines is important in the investigation of Driving Under the Influence of Drugs (DUID) cases due to the *Per Se* limits set forth by many state governments. Standard GC/MS analysis requires time consuming sample preparation, including liquid-liquid extraction and derivatization prior to analysis. Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is becoming an increasingly common technique in forensics and clinical toxicology due to instrumental accuracy and sensitivity.

This application note addresses the development of a LC/MS/MS method for the quantitative analysis of amphetamines, phentermine, and designer stimulants. Validation studies were conducted in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) method validation guidelines in conjunction with Virginia Department of Forensic Science validation guidelines [1,2]. This method was determined to meet all predetermined acceptance criteria for the qualitative and quantitative analysis of amphetamines, phentermine, and designer stimulants.

Experimental

Equipment and instrumentation

- Agilent 6430 Triple Quadrupole Mass Spectrometer System
- Agilent 1260 Infinity Series LC System
- Agilent Poroshell 120 EC-C18, 2.1 × 75 mm, 2.7 µm column
- Agilent 1290 Automatic Liquid Sampler
- Autosampler vials with inserts
- Agilent MassHunter Optimizer Software
- Zymark TurboVap Evaporator
- Screw capped extraction tubes with 12 mL or greater capacity
- Kimble/Chase tapered glass tubes for evaporation and reconstitution (p/n 73785-5)
- Glass Pasteur pipets
- Pipets for accurate dispensing of volumes 10 µL to 250 µL, and 1 mL to 10 mL
- Test tube rocker or rotator
- Centrifuge capable of 2,000–3,000 rpm

Materials

- Sodium phosphate tribasic, ACS powder
- 1-Chlorobutane, HPLC grade
- Hydrochloric acid, Optima grade
- 2-Propanol, HPLC grade
- Formic acid, eluent additive ~98 %
- Water, Type I or HPLC grade
- Acetonitrile, Optima grade or higher
- Methanol, HPLC grade or higher

Mobile phase solutions

- 0.1 % formic acid in water (mobile phase A)
- 0.1 % formic acid in acetonitrile (mobile phase B)

The calibrators, controls, and internal standards for this method were purchased from Cerilliant and Grace-Alltech. The targets and associated internal standards are described in Table 1.

Table 1. Targets and Corresponding Internal Standards

Target	Internal standard
Amphetamine	Amphetamine-D_{11}
Methamphetamine	Methamphetamine-D_{11}
Phentermine	
3,4-Methylenedioxyamphetamine (MDA)	MDA-D_5
3,4-Methylenedioxymethamphetamine (MDMA)	MDMA-D_5
Mephedrone	Mephedrone-D_3
Methedrone HCl	
α-Pyrrolidinopentiophenone (α-PVP)	
3,4-Methylenedioxypyrovalerone HCl (MDPV)	
Bupropion HCl	
Methcathinone	
Pseudoephedrine	Pseudoephedrine-D_3
Methylone HCl	Methylone-D_3

Sample preparation

Samples were prepared according to procedures defined by the Virginia Department of Forensic Science [2]. Amphetamines, phentermine, and designer stimulants were extracted from biological matrixes by adding trisodium phosphate buffer and extracting with 1-chlorobutane as delineated in Figure 1. The extract was quantitatively assessed using an Agilent 6430 LC/MS/MS system with an Agilent 1260 Infinity LC.

Preparation of solutions, calibrators, and internal standard

0.2 % Hydrochloric acid in 2-propanol: Add 1 mL of concentrated HCl (12 N) into 500 mL of 2-propanol.

Saturated trisodium phosphate buffer: Add trisodium phosphate to Type I or HPLC grade water until no more dissolves after vigorous shaking.

Working standard solution (10 µg/mL): Pipette 100 µL of 1.0 mg/mL standard into a 10-mL volumetric flask and bring to final volume with methanol.

Working standard solution (1 µg/mL): Pipette 1.0 mL of 10 µg/mL working standard solution into a 10-mL volumetric flask and bring to final volume with methanol.

Working internal standard solution (1 µg/mL): Pipette 10 µL of 1.0 mg/mL (or 100 µL of 0.1 mg/mL) internal standard into a 10-mL volumetric flask and bring to final volume with methanol.

To prepare the calibration curve, pipette the following volumes of the 10 µg/mL or 1 µg/mL working standard solutions into appropriately labeled 16 × 125 mm screw cap test tubes. Add 1 mL of blank blood to each tube to obtain the final concentration listed in Table 2.

Table 2. Preparation of Calibrators

Volume of 10 µg/mL standard solution (µL)	Volume of 1 µg/mL standard solution (µL)	Final concentration of target compounds (mg/L)
200		2.0
100		1.0
50		0.50
25		0.25
	100	0.10
	50	0.05
	20	0.02
	10	0.01

Procedure

1. Label clean 16 × 125 mL screw cap tubes appropriately for calibrators, controls, and case samples.
2. Prepare calibrators and controls.
3. Add 1 mL case specimens to the appropriately labeled tubes.
4. Add 100 µL of internal standard into each tube and vortex briefly.
5. Add 2 mL of saturated trisodium phosphate buffer to each tube and vortex.
6. Add 6 mL of 1-chlorobutane to each tube.
7. Cap and rotate tubes for 15 minutes.
8. Centrifuge at approximately 2,500 rpm for 15 minutes to achieve separation. If emulsion or suspension forms, knock it down with wooden stick and centrifuge again.
9. Transfer organic (upper layer) to appropriately labeled tubes.
10. Add 100 µL of 0.2 % HCl in 2-propanol to each tube and evaporate samples to dryness at approximately 40 °C.
11. Reconstitute in 200 µL of 0.1 % formic acid in water. Transfer to autosampler vials with inserts for LC/MS/MS analysis.
12. Run the samples in a Worklist, using LC/MS/MS method delineated in Tables 3 and 4.

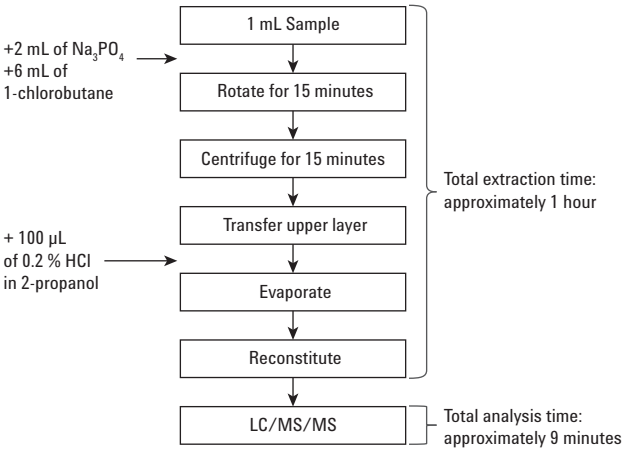

Figure 1. Procedure flowchart.

Table 3. Instrument Conditions

MS parameters		
Ionization	ESI	
Polarity	Positive	
Drying gas	10.0 L/min	
Gas temperature	350 °C	
Nebulizer pressure	45 psi	
Capillary	3,000 V	
LC parameters		
Column	Agilent Poroshell 120 EC-18, 201 × 75 mm, 2.7 µm	
Injection volume	2 µL	
Column thermostat	50 °C	
Needle wash	5.0 seconds	
Mobile phase A	0.1 % formic acid in water	
Mobile phase B	0.1 % formic acid in acetonitrile	
Flow rate	0.5 mL/min	
Gradient	Time (min)	% B
	Initial	2
	2	5
	4	10
	6	30
	7	90
	8.5	90
	9	2
Post time	1 minute	

Sample acquisition method

Instrument	Agilent LC/MS/MS with Agilent MassHunter Software
	Positive Dynamic MRM Mode
Run time	9 minutes
Dynamic range	0.01—2.0 mg/L

The method contains 13 target compounds and seven deuterium-labeled internal standards in positive dynamic MRM mode.

Table 4. Instrumental Method Ion Selection

Compound	Precursor ion	Product ions	Frag (V)	CE (V)	Cell Acc (V)	Retention time (min)
Methamphetamine-D_{11} (IS)	161.2	127.1, 97.1	85	8, 20	7	3.78
Amphetamine-D_{11} (IS)	147.2	130.1, 98.1	75	4, 16	7	3.23
MDA-D_5 (IS)	185.1	168.1, 110.1	80	8, 24	7	3.84
Methylone-D_3 (IS)	211.2	163, 135	85	13, 29	7	3.25
Pseudoephedrine-D_3 (IS)	169.1	151.1, 115	80	8, 28	7	2.77
Mephedrone-D_3 (IS)	181.3	163, 148	90	9, 21	7	4.84
MDMA-D_5 (IS)	199.1	165.1, 107.1	90	8, 24	7	4.26
α-PVP	232.2	126.1, 91	115	28, 24	7	5.97
Amphetamine	136.1	119.1, 91.1	75	4, 16	7	3.3
Bupropion	240	184,166	80	5, 10	7	6.39
MDA	180.1	163.1, 105.1	75	4, 24	7	3.89
MDMA	194.1	163.1, 105.1	90	8, 24	7	4.29
MDPV	276.3	135, 126	130	25, 25	7	6.12
Mephedrone	178.3	160, 144	85	10, 30	7	4.85
Methamphetamine	150.1	119.1, 91.1	90	8, 20	7	3.86
Methcathinone	164.2	146, 130	85	10, 34	7	2.65
Methedrone	194.2	176, 161	90	8, 20	7	4.11
Methylone	208.2	190, 132	80	14, 26	7	3.26
Phentermine	150.1	91.1, 65.1	70	21, 45	7	4.61
Pseudoephedrine	166.1	148.1, 133.1	81, 80	5, 21	7	2.79

Results and Discussion

The method achieved separation of 13 amphetamines and designer stimulant compounds using dynamic MRM analysis in positive mode. The method achieved acceptable resolution of target compounds in an overall cycle time of 9 minutes. The total ion chromatogram for the target analytes and internal standards is shown in Figure 2A. Figures 2B and 2C illustrate examples of MRM transitions for MDPV and amphetamine. The transitions were developed using MassHunter Optimizer Software. The chromatography establishes good peak shape with no significant tailing or other chromatographic abnormalities.

A total of nine calibration curves were analyzed to establish the calibration model for each target. The calibration model was established based on predetermined acceptance criteria of R^2 value ≥ 0.985, the back calculated concentration of ± 20 % for calibration, residual plots, and statistical analysis. The dynamic range was established to be 0.01–2.0 mg/L for all target compounds.

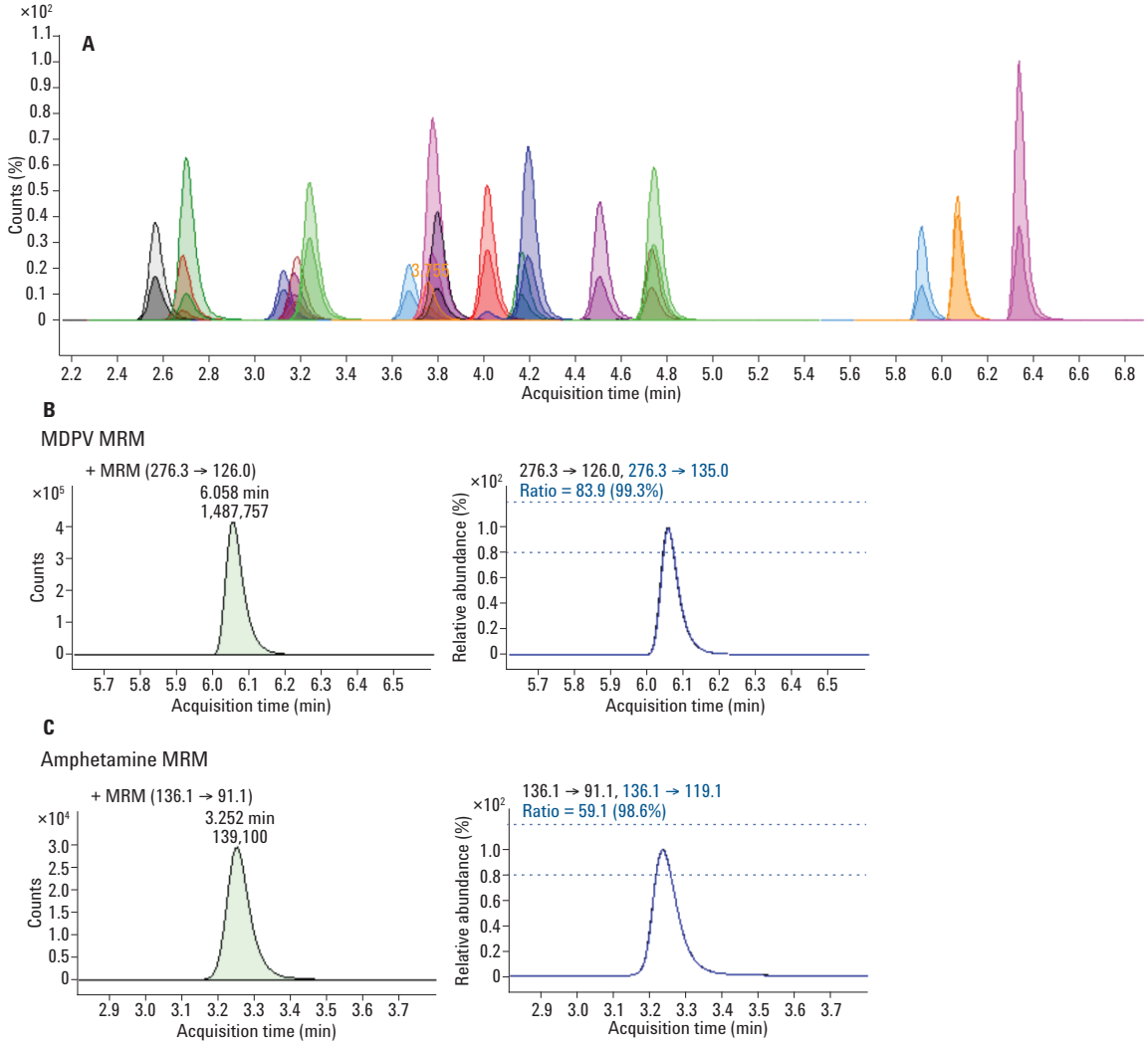

Figure 2. A) Total ion chromatogram, B) MRM transitions for MDPV, and C) MRM transitions for amphetamine.

Example calibration curves are shown in Figures 3A-3B. The best fit calibration model for methcathinone, pseudoephedrine, methylone, and mephedrone was linear weighted (1/x). The remainder of the targets were determined to have a quadratic weighted (1/x) best fit calibration model. Table 5 depicts the best fit calibration models for each target compound. To further validate the calibration model, controls were assessed at various concentrations across the calibration range, including a control between the two highest calibrator concentrations.

Validation studies were conducted using the SWGTOX Standard Practices for Method Validation in Forensic Toxicology guidelines in conjunction with the Virginia Department of Forensic Science validation guidelines [1,2]. This method was determined to meet all acceptance criteria for the quantitative analysis of amphetamines and designer stimulants. Items assessed during the validation study included linearity and calibration model fit, precision and accuracy, sensitivity (limits of detection (LOD) and limits of quantitation (LOQ)), interference, robustness, carryover, dilution integrity, stability, ion suppression/enhancement, and recovery. A comprehensive explanation of the validation study can be found in "Validation of a Quantitative Method for Amphetamines, Phentermine, and Designer Stimulants Using an Agilent 6430 LC/MS/MS" [3].

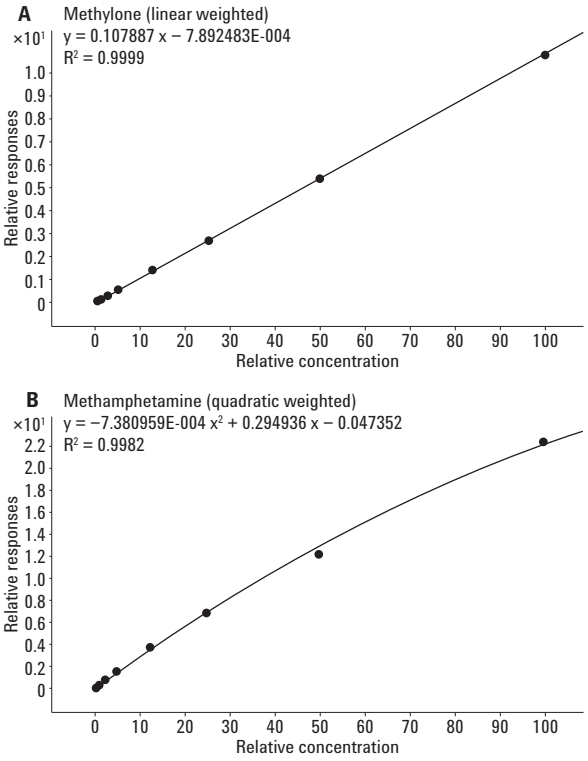

Figure 3. Calibration curves for methylone and methamphetamine.

Conclusion

This method provides a rapid and sensitive technique for the detection and quantitation of amphetamines, phentermine, and designer stimulants by LC/MS/MS. Sample preparation for LC/MS/MS analysis is less time consuming and does not require derivatization when compared to traditional GC/MS analysis. Validation studies as performed in [3] indicate that the method meets all criteria required for the routine analysis of amphetamines, phentermine, and designer stimulants in whole blood. This method is part of a long term initiative to develop and validate methods to include multiple drugs and drug classes.

Table 5. Regression Analysis for Calibration Model Determination

Target	Linear/Quadratic	Weighting
Methcathinone	Linear	Weighted (1/x)
Pseudoephedrine	Linear	Weighted (1/x)
Methylone	Linear	Weighted (1/x)
Amphetamine	Quadratic	Weighted (1/x)
Methamphetamine	Quadratic	Weighted (1/x)
MDA	Quadratic	Weighted (1/x)
Methedrone	Quadratic	Weighted (1/x)
MDMA	Quadratic	Weighted (1/x)
Phentermine	Quadratic	Weighted (1/x)
Mephedrone	Linear	Weighted (1/x)
α-PVP	Quadratic	Weighted (1/x)
MDPV	Quadratic	Weighted (1/x)
Bupropion	Quadratic	Weighted (1/x)

For More Information

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References

1. "Standard Practices for Method Validation in Forensic Toxicology", SWGTOX, Doc 003, Revision 1, May 20, **2013**.
2. http://www.dfs.virginia.gov/wp-content/uploads/2015/01/220-D100-Toxicology-Procedures-Manual.pdf
3. J. Hudson, J. Hutchings, R. Wagner, P. Friel "Validation of a Quantitative Method for Amphetamines, Phentermine, and Designer Stimulants Using an Agilent 6430 LC/MS/MS" *Agilent Technologies Application Note*, publication number 5991-5129EN, **2015**.

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