Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

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Overview

Purpose: To develop an HPLC-MS method for the forensic toxicological analysis of the three Schedule I cathinones: MDPV, methylone and mephedrone, as well as other substituted cathinones: methedrone, ethylone, butylone and naphyrone in urine with limits of quantitation (LOQs) of 0.5 ng/mL.

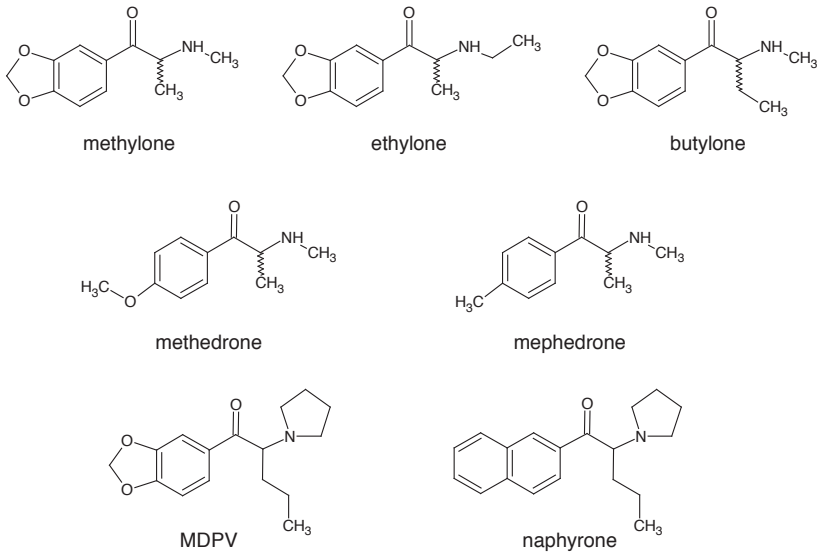
Methods: Liquid/liquid extraction followed by HPLC/MS/MS analysis on a Thermo Scientific™ Q Exactive™ benchtop Orbitrap mass spectrometer.

Results: We achieved LOQs of 0.5 ng/mL with good reproducibility and accuracy for MDPV, mephedrone, methylone, methylone, ethylone and butylone. Naphyrone showed more variability and is considered qualitative using this method.

Introduction

Substituted cathinones, or "Bath Salts," have become the latest abused designer drugs. Based on cathinone, a substance found in the African *Catha edulis* (khat) plant, substituted cathinones are stimulants with amphetamine- and cocaine-like effects. As with many designer drugs classes, variations on base structure abound (Figure 1). On October 21, 2011 the United States Drug Enforcement Agency (US DEA) listed three of the most common chemicals – methylenedioxy pyrovalerone (MDPV), methylone and mephedrone – as Schedule I drugs, thereby making them illegal. As these drugs are not detected by current ELISA drug screening tests, new methods are needed to detect and quantitate these compounds.

FIGURE 1. Structures of designer cathinones

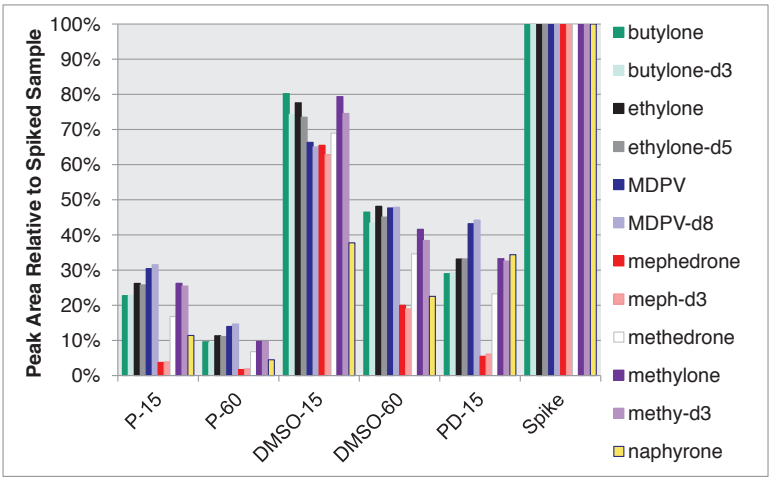


Methods

Method Development

This assay was originally developed as a urine dilution method. When a lower limit of quantitation (LOQ) was desired, liquid/liquid extraction was developed to concentrate the samples. Initial experiments showed good linearity and detection limits, but also low recovery and highly variable internal standard responses. To investigate and mediate the possible loss of analytes during the evaporation step, the following experiments were performed: 1 & 2) Evaporate samples for either 15 minutes or 60 minutes; 3 & 4) Add 20 µL of DMSO to the tubes before evaporation to prevent samples from evaporating to dryness, again for 15 or 60 minutes; 5) Add 20 µL DMSO to tubes after evaporation to determine if solubility is an issue; and 6) Spike a blank processed sample with analytes after evaporation as 100% recovery. Results shown in Figure 2 indicate that evaporation time is critical, especially for mephedrone, the smallest molecule tested, and solubility might be an issue for naphyrone.

FIGURE 2. Results for method development experiments to determine effects of evaporation step in sample processing. P = plain tubes after -15 and -60 min evaporation; DMSO = tubes with DMSO added prior to -15 and -60 min evaporation; PD = evaporated without DMSO, add DMSO after 15 min evaporation; Spike = compounds spiked after 15 min evaporation



Sample Preparation

Deuterated internal standards were available for all compounds except methedrone and naphyrone. Butylone-d3 was used as internal standard for methedrone and MDPV-d8 was used for naphyrone. Samples preparation is a liquid-liquid extraction (LLE). 200 µL of urine and 10 µL of internal standard mix solution (2 µg/mL of each deuterated IS) were basified with 100 µL of 1 N NaOH. Extraction was performed by adding 1 mL of ethylacetate:hexane (1:1), mixing and centrifuging. 800 µL of the resulting supernatant was transferred to a clean test tube containing 20 µL of DMSO to prevent complete evaporation of solvent. Analytes are small and slightly volatile, and will evaporate if left too long in the evaporator. Solvent was evaporated at 37 °C under nitrogen for 15 minutes. 200 µL of 5% methanol was added, mixed and transferred to an HPLC vial with limited-volume insert. 20 µL was then injected onto HPLC-MS.

Liquid Chromatography

Chromatographic analysis was performed using the Thermo Scientific™ Accela™ 600 HPLC pump and a Thermo Scientific™ Hypersil™ GOLD C18 column (50 x 2.1 mm, 3 µm particle size) under gradient conditions (Figure 3). Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase C was acetonitrile:1-propanol:acetone (45:45:10). The total run time was 5 minutes.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific Q Exactive bench-top Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (Figure 5). The Q Exactive was operated in t-MS^2 mode at a resolution of 17,500 (@*m/z*=200). Exact masses, collision energies and fragment ions are listed in Figure 6.

FIGURE 3. HPLC gradient for cathinone analysis.

Time (min)	%A	%B	%C	Flow (µL/min)
0	90	10	0	500
0.15	90	10	0	500
2.15	5	95	0	500
2.45	5	95	0	500
2.46	0	0	100	500
3.30	0	0	100	500
3.31	90	10	0	500
5.00	90	10	0	500

FIGURE 4. Mass spectrometer source conditions.

Parameter	Value
Sheath Gas	35
Aux gas	15
Sweep gas	1
Discharge current	4
Capillary temp	320
S-Lens RF Level	60
Vaporizer Temp	350

Data Analysis

Data acquisition and processing were performed using Thermo Scientific™ TraceFinder™ software.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at low (LQC), middle (MQC) and high (HQC) concentrations. Intra-run variability and robustness were determined by processing six replicates of each QC level along with a calibration curve as outlined in the Sample Preparation section on three different days. Matrix effects were investigated by comparing peak areas of analyte at 10 ng/mL, and internal standard prepared in 12 different lots of urine to those of a sample prepared in water.

FIGURE 5. Diagram of Q Exactive Mass Spectrometer

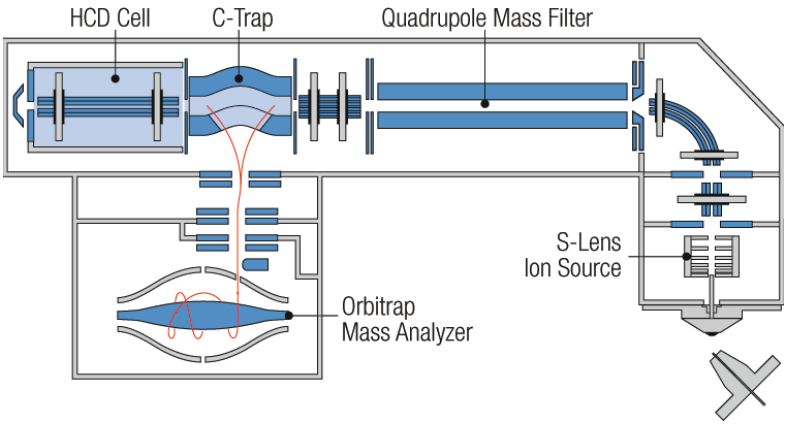


FIGURE 6. Exact masses and normalized collision energies (NCE) for cathinones

Analyte	m/z	NCE	Quantifier m/z	Qualifier m/z
Mephedrone	178.1226	35%	160.1119	145.0885
Mephedrone-d3	181.1415	35%	163.1306	148.1072
Methylone	208.0968	35%	160.0756	190.0861
Methylone-d3	211.1156	35%	163.0943	193.1049
MDPV	276.1594	60%	126.1278	135.0440
MDPV-d8	284.2096	60%	134.1779	135.0440
Naphyrone	282.1852	45%	141.0697	211.1115
Ethylone	222.1125	45%	174.0911	204.1016
Ethylone-d5	227.1438	45%	179.1224	209.1329
Butylone	222.1125	45%	174.0912	204.1017
Butylone-d3	225.1313	45%	177.1099	207.1205
Methedrone	194.1176	35%	176.1069	161.0834

Results

MDPV, methylone, mephedrone, methedrone, ethylone and butylone were all linear from 0.5 to 1000 ng/mL. Figure 7 shows representative calibration curves for all compounds. Figure 8 shows representative chromatogram at 0.5 ng/mL for all compounds tested. Inter-assay quality control statistics shown in Figure 9 demonstrate the method to be reproducible across the calibration range for the above compounds. Limited matrix effects were seen for the above compounds, and those were largely mediated by deuterated internal standards. The absolute recoveries of all cathinones tested in various lots of urine compared to a sample prepared in water ranged from 89% to 163%. Relative recoveries ranged from 105% to 136%. Precision across all lots also improved when deuterated internal standards were used.

Although naphyrone was detected at 0.5 ng/mL, it showed more variability than the other compounds and a greater matrix effect from lot to lot. Absolute recoveries for naphyrone ranged from 146% to 754% while relative recoveries using MDPV-d8 as internal standard ranged from 150% to 596%. All available internal standards were tried, and MDPV-d8 showed the best results. A lack of a deuterated analog for naphyrone does not allow for matrix effect corrections and negatively effects method precision. In this assay, naphyrone should be considered qualitative.

FIGURE 7. Representative calibration curves of cathinones in urine.

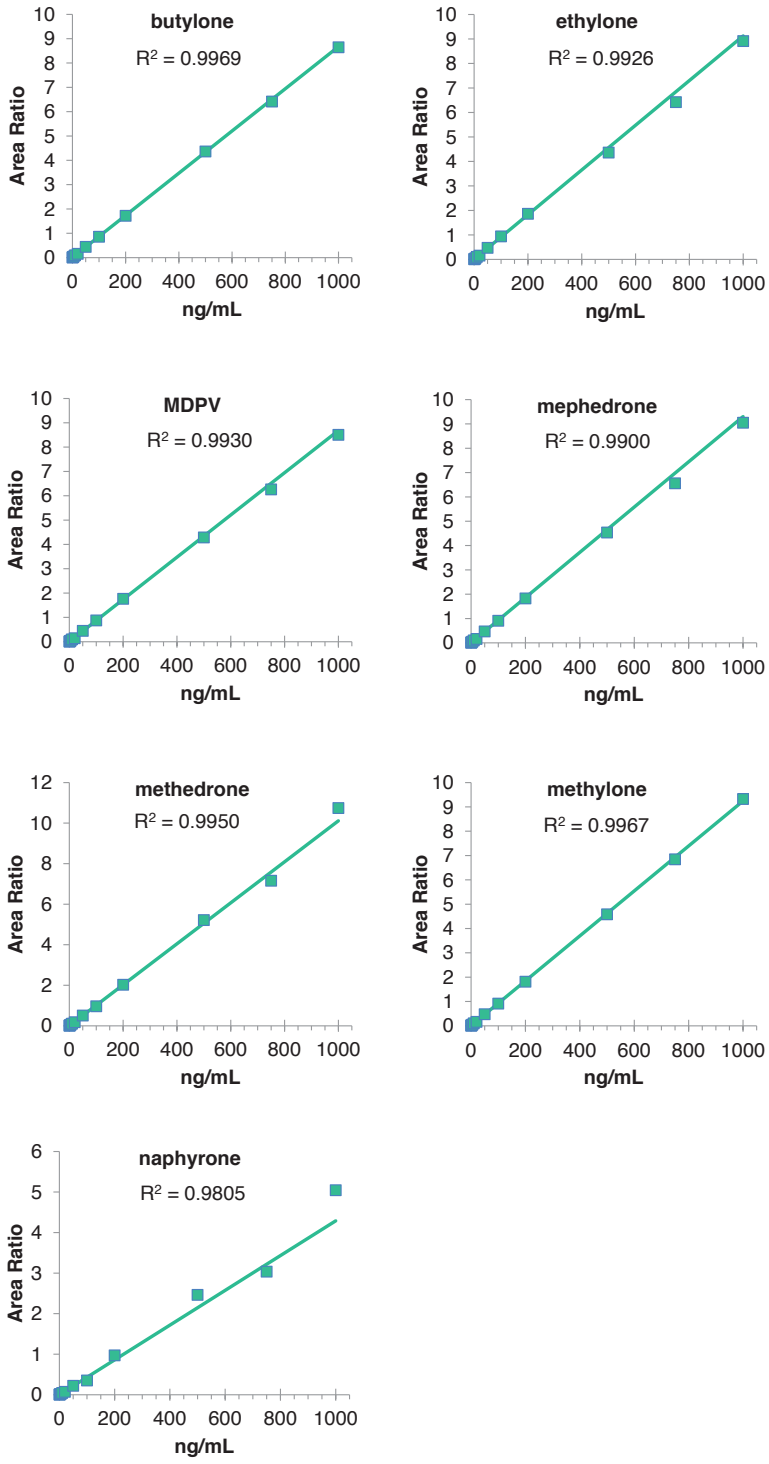


FIGURE 8. Representative chromatogram of cathinones at 0.5 ng/mL in urine reconstructed at 5 ppm mass accuracy.

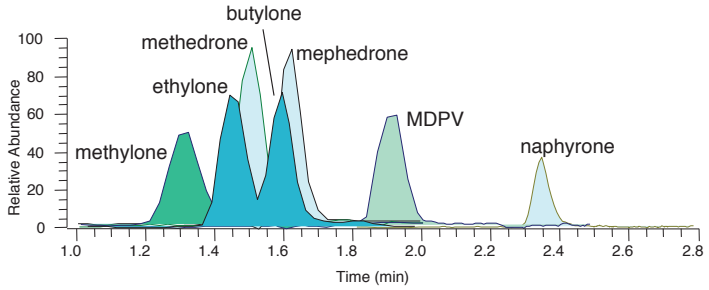


FIGURE 9. Inter-assay QC results

	butylone			ethylone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.28	26.0	99.0	2.31	25.9	99.6
%Bias	-8.7	4.1	-1.0	-7.4	3.4	-0.4
%CV	3.7	3.8	4.4	3.4	3.0	1.7

	MDPV			mephedrone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.30	25.7	98.4	2.38	26.7	97.8
%Bias	-8.1	2.9	-1.5	-4.7	6.8	-2.2
%CV	5.8	6.3	5.1	7.1	1.9	2.7

	methedrone			methylone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.49	28.1	105	2.34	26.1	99.6
%Bias	-0.3	12.5	5.0	-6.5	4.6	-0.4
%CV	4.4	6.4	3.1	5.1	1.8	3.1

	naphyrone		
	LQC 2.5 ng/mL	MQC 25 ng/mL	HQC 100 ng/mL
Mean	2.55	22.9	103
%Bias	2.1	-8.3	3.0
%CV	22	8.5	5.2

Conclusion

We achieved our goal of a 0.5-ng/mL LOQ for the three newly-regulated cathinones, MDPV, mephedrone and methylone, as well as methylone, ethylone and butylone in urine. Naphyrone, which shows greater variability, can be detected down to 0.5 ng/mL in a qualitative manner. Deuterated internal standards are essential for rigorous quantitation of these compounds.

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