Determination of Cathinone Derivatives and Other Designer Drugs in Serum by Comprehensive LC-Triple Quadrupole MS/MS Analysis

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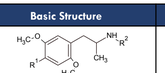
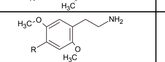
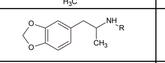
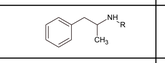
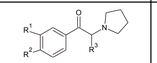
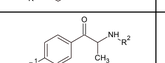
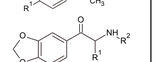
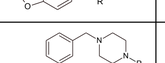
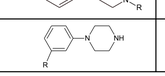
INTRODUCTION

In recent decades, clandestine drug lab operators have attempted to bypass controlled substance laws and legal regulations with "designer" compounds similar to current drugs of abuse, including methamphetamine, Ecstasy, and khat. Presently, "bath salts" have erupted onto the drug scene as "legal highs" containing cathinone analogs that have produced severe side effects in users across the globe[1]. These products have sparked concern among law enforcement agencies, and emergency bans have been placed on the sale of such items. Designer drugs often carry unknown safety profiles, a high potential for abuse, unknown potency, and serious health consequences, especially when ingested unknowingly. While such compounds only account for about 3% of all drug seizures worldwide, severe intoxications and fatalities are not uncommon[2]. These drugs are difficult to identify from a forensic standpoint due to the large numbers of compounds classified as designer drugs, the frequent introduction of new structures, and inadequate accessibility to standards.

Despite the increasing number of designer drugs on the market, there are few comprehensive screening techniques available for their detection in biological specimens. Extensive confirmatory techniques are required for the detection and quantification of multiple classes of designer drugs in human specimens, particularly serum. The LC-MS/MS method presented here encompasses over twenty compounds amongst the most prominent classes of designer drugs, including cathinone derivatives.

DRUGS SELECTED FOR STUDY

The drugs chosen were based on prevalence in literature reports, DEA schedule, and availability as standards.

Drug Class	Basic Structure	Compounds	
Phenethylamines		R^1 = Br, R^2 = H R^1 = C_2H_5, R^2 = H R^1 = CH_3, R^2 = H	DOB DOET DOM
		R = Br R = C_2H_5 R = I	2C-B 2C-E 2C-I
		R = H R = CH_3 R = C_2H_5	MDA MDMA MDEA
		R = H R = CH_3 R = C_2H_5	Amphetamine Methamphetamine Ethylamphetamine
		R^1 = R^2 = O-CH_2-O, R^3 = C_3H_7	MDPV
		R^1 = R^2 = CH_3 R^1 = R^2 = H R^1 = H, R^2 = CH_3 R^1 = O-CH_3, R^2 = CH_3 R^1 = CH_3, R^2 = C_2H_5 R^1 = F, R^2 = CH_3	Mephedrone Cathinone Methcathinone Methedrone 4-MEC Flephedrone
		R^1 = R^2 = CH_3 R^1 = C_2H_5, R^2 = CH_3	Methylone Butylone
Piperazines		R = H	BZP
		R = Cl R = CF_3	mCPP TFMPP

MATERIALS AND METHODS

- Analyses were performed on an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6490 triple quadrupole MS/MS with Jet Streaming technology and electrospray ionization (ESI).
- Separation occurred on an Agilent Zorbax Rapid Resolution HD Eclipse Plus C_{18} threaded column (50 x 2.1 mm, 1.8 µm particle size).
- Data acquisition was performed in Dynamic MRM mode with positive ESI using one principal MRM transition for quantitation and one additional transition to serve as a qualifier for each analyte.
- After the chromatographic method was optimized for all compounds, the drug mixtures were spiked into blank human serum with deuterated internal standards, and then extracted using mixed-mode solid-phase extraction cartridges with hydrophobic C_{18} and cation exchange sites (Resprep Drug Prep I cartridges, 200 mg, 10 mL).
- The solid phase extraction method, adapted from published methods[3,4], was performed manually with a Supelco Visiprep-DL Disposable Liner SPE vacuum manifold using analytical grade solvents.
- Validation parameters were evaluated, including selectivity, matrix effects, recovery, process efficiency, stability, linearity, precision, and accuracy as recommended by Peters, et al.[5]

RESULTS

Agilent Optimizer software was used to optimize the data acquisition parameters for MRM mode by automatically selecting the best precursor ions and associated fragmentor voltages in addition to selecting the best fragment ions and collision energies for each transition.

Prior to matrix samples, various concentrations of neat standards were analyzed to determine the instrument detection limits for each analyte. LOQs, with a SNR of at least ten, were calculated in the range of 1-100 pg/mL.

The assay was selective for all of the tested analytes in a run-time of less than 6 minutes under gradient conditions. Figure 1 depicts the quantifier MRM transitions for all of the targeted analytes and internal standards extracted from spiked blank human serum at a nominal concentration of 10 ng/mL. Enhanced sensitivity was achieved with the Dynamic MRM acquisition capabilities of the Agilent system, which utilizes analyte retention times, detection windows (Δt_R), and a constant scan cycle time for precise detection of multiple analytes in a small window.

Agilent MassHunter Quantitative Analysis was used for analysis of calibration and QC samples during the method validation stages. The experiments performed to evaluate the validation parameters are summarized below, including selectivity, matrix effects, recovery, process efficiency, processed sample stability, linearity, LOQ, precision, and accuracy.

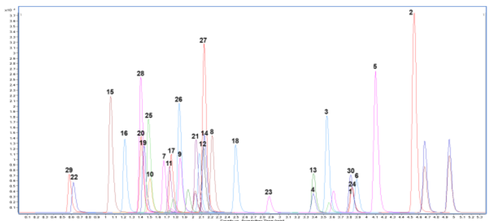


Figure 1: Dynamic MRM Transitions of Analytes after SPE

Selectivity

First, all drugs were run individually with the Dynamic MRM method. No interfering signals were observed. Compounds with similar transitions, such as DOM and 2C-E, were still able to be differentiated due to the difference in retention times.

In order to determine selectivity for processed matrix samples, samples of blank pooled serum were analyzed for interferences. Interfering peaks were negligible and did not elute at the same time as any analytes or internal standards.

Only deuterated compounds were chosen as internal standards to avoid over-estimation of the internal standard signal that can occur when using therapeutic drugs as IS.

The method proved to be selective for all targeted analytes.

ME/RE/PE

Matrix effects (ME), recovery (RE), and process efficiency (PE) were evaluated by preparing three sets of samples (5 each, for a total of 15). The first set, Set A, consisted of neat samples that were dried down and reconstituted in 50 µL of mobile phase. The second set, Set B, consisted of blank serum samples that were extracted. The elutions were spiked with the same amount of analytes and IS before drying down and reconstituting in 50 µL of mobile phase. The third set, Set C, consisted of blank serum samples that were spiked with analytes (nominal concentration of 50 ng/mL) and IS before SPE. Absolute peak areas (drug/IS) were used for the following calculations:

ME = (B/A)*100
RE = (C/B)*100
PE = (C/A)*100

Analyte	Matrix Effects (mean ± SD, %)	Recovery (mean ± SD, %)	Process Efficiency (mean ± SD, %)
BZP	227 ± 6.8	108 ± 3.9	242 ± 5.8
Cathinone	85 ± 9.1	133 ± 14.8	112 ± 2.7
Methcathinone	55 ± 4.8	122 ± 12.2	67 ± 2.3
Methylone	60 ± 5.5	123 ± 12.1	73 ± 2.7
Flephedrone	67 ± 4.9	125 ± 10.9	83 ± 2.5
Amphetamine	84 ± 6.0	110 ± 6.2	92 ± 1.5
MDA	119 ± 7.9	102 ± 5.9	122 ± 1.5
Methedrone	87 ± 4.2	113 ± 7.0	97 ± 2.5
Methamphetamine	106 ± 8.4	102 ± 6.1	108 ± 2.9
MDMA	84 ± 7.4	106 ± 6.8	89 ± 3.4
Butylone	81 ± 10.4	113 ± 11.4	90 ± 2.6
Ethylamphetamine	99 ± 5.8	100 ± 3.0	98 ± 4.8
Mephedrone	86 ± 5.3	107 ± 7.3	91 ± 2.1
MDEA	77 ± 6.2	101 ± 7.3	78 ± 4.7
4-MEC	58 ± 7.8	106 ± 11.8	61 ± 3.5
mCPP	83 ± 7.9	130 ± 14.0	108 ± 5.6
MDPV	57 ± 11.3	106 ± 17.9	60 ± 5.3
2C-B	147 ± 12.0	95 ± 6.9	141 ± 16.0
DOM	139 ± 16.6	86 ± 10.6	118 ± 1.8
DOB	127 ± 19.8	79 ± 12.4	98 ± 4.6
TFMPP	115 ± 5.2	108 ± 2.1	124 ± 3.7
2C-I	126 ± 17.4	89 ± 9.0	112 ± 12.2
2C-E	99 ± 7.9	98 ± 2.8	97 ± 7.1
DOET	117 ± 18.0	81 ± 11.4	93 ± 3.4

Processed Sample Stability

Ten blank serum samples were spiked with analytes (at a nominal concentration of 50 ng/mL) and IS. The extracts were pooled, mixed, and aliquotted out into vials with liners. The aliquots were left in the auto-sampler and injected every four hours for 20 hours. Absolute peak areas were plotted versus time. Stability was determined by comparing the final peak area to the initial peak area based on the regression lines. Changes within ±10% were considered stable.

Drug	Change (%)	Drug	Change (%)
BZP	3.46	Mephedrone	2.11
Cathinone	3.01	MDEA	7.27
Methcathinone	2.64	4-MEC	7.96
Methylone	2.83	mCPP	3.27
Flephedrone	2.71	MDPV	9.65
Amphetamine	1.19	2C-B	8.08
MDA	2.32	DOM	1.28
Methedrone	-0.53	DOB	0.14
Methamphetamine	0.09	TFMPP	-3.16
MDMA	3.62	2C-I	4.89
Butylone	0.83	2C-E	4.29
Ethylamphetamine	4.77	DOET	2.30

Precision and Accuracy

Quality control (QC) samples were analyzed at LLOQ (10 ppb), LOW (100 ppb) and HIGH (750 ppb) concentrations relative to the calibration curve in triplicate on each of four days. QC samples were made up on the first day and then aliquots were frozen for use in subsequent days. Calibrators were made up fresh daily using blank serum for a curve using six levels in triplicate. The concentrations in the QC samples were calculated based on the daily calibration curves using MassHunter Quantitative Analysis. Accuracy (% bias) and repeatability (inter-day precision) were evaluated for each analyte. The Agilent Software was used to calculate the percent accuracy for the daily QC samples while Analyse-it Software was used in Excel for the calculation of repeatability. Acceptance criteria requires ±15% bias (±20% around the LLOQ) and <15% R.S.D. for precision (<20% R.S.D. around the LLOQ). These were met for a majority of the analytes but could require further optimization, including the evaluation of freeze-thaw stability.

Analyte	Repeatability, RSD (%)			Accuracy, bias (%)		
	LLOQ	LOW	HIGH	LLOW	LOW	HIGH
BZP	84.3	12.2		24.3	-3.3	7.1
Cathinone	23.6	5.1	17.3	-1.2	-18.0	-10.7
Methcathinone	14.1	4.9	9.0	10.1	3.4	1.6
Methylone	23.1	3.7	3.4	-11.0	3.4	-4.2
Flephedrone	18.6	6.9	31.9	7.0	-6.3	-10.8
Amphetamine	17.7	7.4	10.3	13.1	9.9	8.4
MDA	24.1	9.9	7.9	14.9	37.1	5.3
Methedrone	21.8	9.2	8.7	-0.2	10.2	7.7
Methamphetamine	39.8	7.5	4.2	-0.5	-1.6	-2.5
MDMA	24.7	3.0	3.5	8.8	3.3	4.4
Butylone	18.2	3.8	14.0	-12.3	1.5	-4.5
Ethylamphetamine	26.1	5.9	4.2	11.1	4.7	-3.6
Mephedrone	19.7	2.4	2.5	4.1	6.1	-4.1
MDEA	21.6	4.8	27.4	14.1	21.9	12.1
4-MEC	12.0	7.8	14.1	5.9	12.1	8.5
mCPP	12.9	3.7	4.7	2.9	-3.7	-8.2
MDPV	10.0	12.5	19.7	-14.6	10.9	10.9
2C-B	41.1	16.1	11.6	57.3	-12.7	-11.7
DOM	18.3	20.6	37.2	-14.6	29.5	8.9
DOB	25.6	16.4	19.1	6.3	70.5	12.1
TFMPP	28.0	6.2	6.0	-12.3	7.1	-4.5
2C-I	50.2	5.5	35.4	79.6	24.3	27.9
2C-E	26.4	3.7	56.5	8.4	13.6	2.7
DOET	20.2	18.6	12.7	-9.1	57.9	15.1

Linearity and LLOQ

Triplicates of matrix calibrators at eight concentrations from 1 pg/mL to 1000 ng/mL were analyzed in order to determine the LLOQ and linear range. Agilent Mass Hunter Quantitative Analysis software was used to create calibration curves and also to examine the precision and accuracy for each analyte. Bias within ±15% (±20% around LLOQ) and precision within ±15% R.S.D. (±20% around LLOQ) are required for acceptance. Linear regression models were used, except in the instance of slight curvature where quadratic models were utilized. All models were weighted by a factor of 1/x to account for heteroscedasticity. All R^2 values were a minimum of 0.990 in this experiment. LLOQs were in the range of 1 ng/mL to 10 ng/mL, though still detectable in the 10 pg/mL to 100 pg/mL range.

For all future experiments, daily calibration curves were prepared with each batch of validation samples. The calculated concentrations were compared with their respective nominal values. Values that did not meet acceptance criteria were excluded from calculations.

METHOD PARAMETERS

No.	Drug	Transitions	CE (V)	Frag. (V)	t_R (min)	IS
1	DOB	**274.01 → 256.9** 274.01 → 228.9	14 10	100	3.846	d6-Amphetamine
2	DOET	**224.3 → 207** 224.3 → 91	5 49	85	4.547	d6-Amphetamine
3	DOM	**210.3 → 193.1** 210.3 → 165	5 13	75	3.538	d6-Amphetamine
4	2C-B	**260.01 → 242.9** 260.01 → 227.9	4 6	90	3.403	d5-MDMA
5	2C-E	**210.3 → 193** 210.3 → 163	5 25	80	4.119	d5-MDMA
6	2C-I	**308.1 → 290.9** 308.1 → 91	9 49	90	3.906	d5-MDMA
7	MDA	**180.1 → 163** 180.1 → 105	4 20	70	1.658	d6-Amphetamine
8	MDEA	**208.14 → 163** 208.14 → 105	8 24	90	2.220	d5-MDMA
9	MDMA	**194.1 → 163** 194.1 → 105	8 24	85	1.849	d5-MDMA
10	Amphetamine	**136.11 → 91** 136.11 → 119	16 4	75	1.490	d6-Amphetamine
11	Methamphetamine	**150.13 → 91** 150.13 → 119	16 4	80	1.715	d5-MDMA
12	Ethylamphetamine	**164.11 → 91** 164.11 → 119	20 8	85	2.093	d5-MDMA
13	MDPV	**276.3 → 126** 276.3 → 135	25 25	130	3.383	d3-Methylone
14	Mephedrone	**178.25 → 160** 178.25 → 144	10 30	85	2.123	d3-Mephedrone
15	Cathinone	**150.2 → 132** 150.2 → 117	10 22	80	1.031	d3-Mephedrone
16	Methcathinone	**164.23 → 146** 164.23 → 130	10 34	85	1.196	d3-Mephedrone
17	Methedrone	**194.25 → 176** 194.25 → 161	10 18	80	1.745	d3-Mephedrone
18	4-MEC	**192.28 → 174.1** 192.28 → 145	10 18	95	2.482	d3-Mephedrone
19	Flephedrone	**182.21 → 164** 182.21 → 148	10 34	85	1.422	d3-Mephedrone
20	Methylone	**208.24 → 160** 208.24 → 132	14 26	80	1.397	d3-Methylone
21	Butylone	**222.26 → 174** 222.26 → 204	14 10	95	2.035	d3-Methylone
22	BZP	**177.11 → 91** 177.11 → 65	20 50	100	0.589	d7-BZP
23	mCPP	**197.11 → 153.9** 197.11 → 118	20 36	120	2.878	d4-TFMPP
24	TFMPP	**231.11 → 188** 231.11 → 118	20 44	125	3.826	d4-TFMPP
25	d6-Amphetamine (IS)	**142.25 → 93** 142.25 → 125.1	13 5	75	1.470	-
26	d5-MDMA (IS)	**199.29 → 165** 199.29 → 107	9 25	90	1.839	-
27	d3-Mephedrone (IS)	**181.27 → 163** 181.27 → 148	9 21	90	2.115	-
28	d3-Methylone (IS)	**211.21 → 163** 211.21 → 135	13 29	85	1.390	-
29	d7-BZP (IS)	**184.11 → 98.1** 184.11 → 70.1	21 57	105	0.562	-
30	d4-TFMPP (IS)	**235.11 → 190** 235.11 → 46.1	21 21	125	3.815	-

CONCLUSIONS

- The developed LC-QQQ-MS/MS method met many of the acceptance criteria for analysis of more than twenty designer drug entities, including the most recent cathinone derivatives, in human serum. Further validation parameters will be evaluated to account for low precision & accuracy for certain compounds.
- The selective method allowed for the separation and quantitation of 24 designer drugs after extraction from human serum, with LLOQ in the range of 1 to 10 ng/mL.
- Future work will incorporate additional compounds (*e.g.* tryptamines, metabolites, and unknowns) while also adapting the methods to other matrices, such as urine.
- The fully validated method will be applied to case samples obtained during DUI, DUID, drug overdose, and/or post-mortem investigations in order to assess the utility of the confirmatory method for real-life analysis of forensic specimens.

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