SHIMADZU

EU Workplace Drug Testing by Dilute and Shoot LC-MS/MS with Enhanced MRM Data Quality

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1. Introduction

In this study we present a single dilute and shoot methodology using a high sensitivity triple quadruple mass spectrometer in order to meet the required European Work Place Drug testing cut-off concentrations (and other international regulatory guidelines, e.g. SAMSHA). A comprehensive suite of drugs were studied including amphetamines, benzodiazepines, opiates, cannabis, barbiturates, cocaine, methadone, and some of their metabolites. This list included amobarbital, pentobarbital, phenobarbital, secobabital and butalbital which are typically difficult to incorporate into multi-residue methods due to low sensitivity and the required chromatographic separation of structural isomers amo/pentobarbital. To enhance data quality the method was optimized to acquire 6 MRM transitions for each compound, rather than the conventional 2 MRMs.

Opiates	Benzodiazepines	Stimulants	Hallucinogens
6-Monoacetylmorphine	7-Aminoflunitrazepam	Amphetamine	Ketamine
Codeine	Alprazolam	AEME	LSD
Dihydrocodeine	Bromazepam	Benzoylecgonine	Mescaline
EDDP	Chlordiazepoxide	Cathinone	
Fentanyl	Clonazepam	Cocaethylene	**Cannabinoids**
Methadone	Diazepam	Cocaine	11-OH-THC
Morphine	Flunitrazepam	MBDB	THC
Oxycodone	Flurazepam	MDA	THC-COOH
Propoxyphene	Lorazepam	MDEA	
	Midazolam	MDMA	**Other**
Barbituates	Nitrazepam	Mephedrone	Clozapine
Amobarbital	Nordiazepam	Methamphetamine	Fluoxetine
Butalbital	Oxazepam	Methcathinone	Haloperidol
Pentobarbital	Temazepam	Norcocaine	Meperidine
Phenobarbital		Zolpiclone	Thebaine
Secobarbital			Trazodone
			Zolpidem

2. Materials and Methods

Liquid chromatography	
UHPLC	Nexera UHPLC system
Analytical column	Restek Raptor biphenyl (100 x 2.1 mm, 2.7 µm)
Column temperature	30°C
Flow rate	0.5mL/minute
Solvent A	0.15mM ammonium fluoride
Solvent B	Methanol
Binary Gradient	20% B (0min) → 48% B (1.5min) → 53% B (4min) → 100%B (6min) → 100% B (7.5min) → 20% B (7.51min) → 20% B (9.5min)
Mass Spectromtery	
LC-MS/MS	LCMS-8050
Dwell times	3 - 20ms
Polarity switching time	5ms
Interface/block/DL temp.	400°C / 400°C / 200°C
Heating/Drying/Nebulising gas	10 / 10 / 3 L/min
Sample Preparation	
	Enzymatically hydrolyzed human urine was spiked with target compounds between 10 - 1000% of the required cut off concentration. Samples were subsequently diluted 5x with 0.1% formic acid.

3. Results and discussion

3-1 Enhanced MRM data quality

Conventional multi-residue LC-MS/MS methods typically use two MRM transitions (quantifier and qualifier ion.) However, in complex matrices there is the potential for matrix interferences to lead to false positives, as has been reported (Schurmann et al, Rapid Comm. Mass Spectr. 23 (2009) 1196; Kaufmann et al, Anal. Chim. Acta. 1 (2010) 673).

In this study we compared the data quality between two methods. Method 1 acquired two MRMs per compound, whilst 'Method 2' acquired 6 MRMs per compound. Results for several example compounds are displayed below. These results show that response and precision were not effected by acquiring these extra transitions.

Method 1	**Method 2**
2 MRMs per compound: 220 transitions in total	6 MRMs per compound: 436 transitions in total

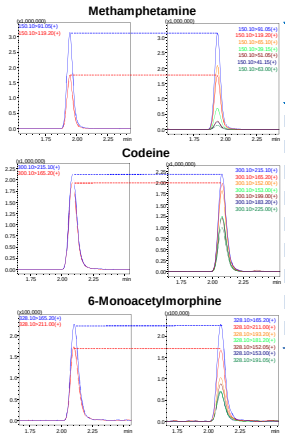

Figure 1. Example chromatograms in urine comparing 'Method 1' and 'Method 2'

	%CV peak area (n=6)	
	2 MRMs per compound: 220 transitions in total	6 MRMs per compound: 436 transitions in total
6-MAM	1.6	2.7
7-AFNZ	1.6	1.5
Amobarbital	2.4	3.5
Amphetamine	2.3	2.7
Benzoylecgonine	1.9	1.3
Butalbital	2.4	2.0
Cocaethylene	0.8	1.5
Codeine	1.5	2.5
Dihydrocodeine	1.3	1.6
Ketamine	0.6	0.6
LSD	2.0	2.7
Mephedrone	4.5	4.3
Methamphet.	1.2	2.6
Norcocaine	1.5	1.3
PCP	1.0	1.2
Pentobarbital	3.0	2.7
Propoxyphene	1.3	2.7
Tramadol	1.2	0.8
Zolpidem	2.2	3.0

Table 1. Peak area %CV (n=6) in urine at the cut off concentration. Comparison of 'Method 1' (2 MRMs per compound) and 'Method 2' (6 MRMs per compound)

An important chromatographic consideration was the separation of isobaric analytes; MBDB/MDEA, benzoylecgonine/norcocaine, amobarbital/pentobarbital. All compounds were eluted from the column within 6.7 minutes. The separation of amobarbital/pentobarbital is shown in Figure 2.

3-2 Final method performance

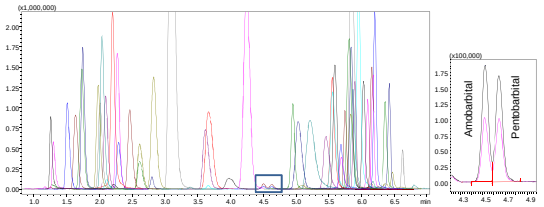

Figure 2. Chromatogram of 56 compounds in human urine spiked at the cut off concentration

To test the performance of the developed method, linearity, repeatability (low and high) accuracy (low and high), carryover and matrix effects were assessed. This validation was performed using a method with 6 MRMs per compound.

Linearity was assessed from 10% - 1000% of the required cut off concentration. The concentration range for each compound is listed in table 2. Calibration standards were analysed in duplicate at the following percentage of the cut-off; 10%, 20%, 50%, 100%, 500%, 1000%. All 56 target compounds achieved excellent correlation coefficients R^2>0.993 (Linear, 1/C, zero not forced). Calibration curves for methamphetamine, ketamine, bromazepam and pentobarbital are shown in Figure 3. Corresponding internal standards are listed in Table 2.

Accuracy and %CV were determined at 50% of the cut off concentration (n=6). Accuracy was within the range 90.5 – 112.1 % for all target compounds, and %CV was less than 7.9%. Carryover, following injection of the highest calibration point, was less than 0.05% compared to the lowest calibration point.

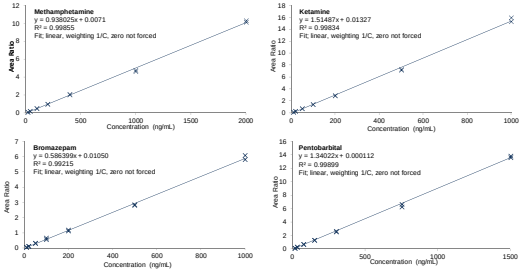

Figure 3. Calibration curves for methamphetamine (20-2000ng/mL), ketamine (10-1000ng/mL), bromazepam (10-1000ng/mL) and pentobarbital (15-1500ng/mL) spiked into enzymatically hydrolyzed human urine

	Ret. Time	MRM transitions	Q1/Q3 Resolution	ESI Polarity	Linear range (ng/mL)	R^2	%CV at 50% cut off conc.	%Accuracy at 50% cut off conc.	Corresponding ISTD
6-Monoacetylmorphine	2.1	328 > 165, 211	Unit/Unit	ESI+	1 - 100	0.9968	5.3	97.5	6-Acetylmorphine-D3
7-Aminoflunitrazepam	5.0	284 > 135, 227	High/High	ESI+	10 - 1000	0.9989	2.1	103.4	7-Aminoflunitraze.-D7
AEME	1.3	182 > 91, 118	Unit/Unit	ESI+	15 - 1500	0.9973	2.7	97.3	AEME-D3
Alprazolam	6.3	309 > 281, 205	High/High	ESI+	10 - 1000	0.9990	4.9	99.5	Alprazolam-D5
Amobarbital	4.5	225 > 182, 42	Unit/Unit	ESI-	15 - 1500	0.9985	4.7	105.9	Amobarbital-D5
Amphetamine	1.7	136 > 91, 119	High/High	ESI+	20 - 2000	0.9963	3.5	105.8	Amphetamine-D5
Benzoylecgonine	2.8	290 > 168, 77	High/High	ESI+	15 - 1500	0.9987	4.2	101.2	Benzoylecgonine-D3
Bromazepam	5.7	316 > 182, 209	Unit/Unit	ESI+	10 - 1000	0.9992	3.9	102.6	Bromazepam-D4
Chlordiazepoxide	6.0	300 > 227, 283	High/High	ESI+	10 - 1000	0.9968	6.4	103.3	Chlordiazepoxide-D5
Clozapine	5.6	327 > 270, 192	High/High	ESI+	20 - 2000	0.9982	3.3	106.8	Clozapine-D4
Cocaethylene	5.0	318 > 196, 82	High/High	ESI+	15 - 1500	0.9980	2.3	106.5	Cocaethylene-D3
Thebaine	5.2	312 > 58, 251	High/Unit	ESI+	10 - 1000	0.9955	2.0	109.1	Cocaethylene-D3
Cocaine	4.0	304 > 182, 82	High/Unit	ESI+	15 - 1500	0.9988	5.0	106.8	Cocaine-D3
Codeine	2.1	300 > 215, 165	High/High	ESI+	30 - 3000	0.9934	3.9	94.4	Codeine-D6
Clonazepam	5.9	316 > 270, 214	Unit/Unit	ESI+	10 - 1000	0.9963	4.8	90.5	Diazepam-D5
Diazepam	6.4	285 > 193, 154	High/High	ESI+	10 - 1000	0.9979	3.3	101.1	Diazepam-D5
Flurazepam	5.8	388 > 315, 317	High/Unit	ESI+	10 - 1000	0.9993	3.4	100.4	Diazepam-D5
Midazolam	6.4	326 > 291, 249	High/High	ESI+	10 - 1000	0.9995	3.1	106.0	Diazepam-D5
Dihydrocodeine	2.0	302 > 199, 128	High/High	ESI+	30 - 3000	0.9973	1.9	104.1	Dihydrocodeine-D6
EDDP	6.1	278 > 157, 218	High/High	ESI+	25 - 2500	0.9976	1.2	100.3	EDDP-D3
Fentanyl	5.7	337 > 188, 105	High/High	ESI+	30 - 3000	0.9974	2.3	107.7	Fentanyl-D5
Flunitrazepam	6.2	314 > 268, 239	High/High	ESI+	10 - 1000	0.9993	3.9	104.5	Flunitrazepam-D7
Fluoxetine	5.6	310 > 44,	High/High	ESI+	10 - 1000	0.9979	1.4	96.4	Fluoxetine-D6
Haloperidol	5.5	376 > 165, 123	High/High	ESI+	20 - 2000	0.9991	5.1	107.3	Haloperidol-D4
Ketamine	3.7	238 > 125, 220	High/High	ESI+	10 - 1000	0.9983	1.0	98.2	Ketamine-D4
Lorazepam	5.8	321 > 275, 303	High/Unit	ESI+	10 - 1000	0.9957	3.9	99.2	Lorazepam-D4
LSD	5.1	324 > 223, 207	Unit/Unit	ESI+	0.1 - 10	0.9976	7.9	102.4	LSD-D3
MBDB	2.8	208 > 135, 77	High/High	ESI+	20 - 2000	0.9986	1.0	100.7	MBDB-D5
MDA	2.0	180 > 135, 105	High/High	ESI+	20 - 2000	0.9978	1.4	96.5	MDA-D5
MDEA	2.6	208 > 163, 105	High/High	ESI+	20 - 2000	0.9984	1.7	106.9	MDEA-D5
MDMA	2.3	194 > 163, 105	High/High	ESI+	20 - 2000	0.9985	0.8	103.2	MDMA-D5
Meperidine	3.6	248 > 220, 174	High/Unit	ESI+	10 - 1000	0.9994	1.4	96.4	Meperidine-d4
Mephedrone	2.4	178 > 160, 145	High/High	ESI+	20 - 2000	0.9978	3.3	102.4	Mephedrone-D3
Mescaline	1.7	212 > 195, 180	High/High	ESI+	10 - 1000	0.9993	2.4	106.7	Mescaline-D9 HCl
Methadone	6.4	310 > 223, 105	High/High	ESI+	25 - 2500	0.9987	1.8	100.9	Methadone-D3
Cathinone	1.5	150 > 117, 132	High/High	ESI+	20 - 2000	0.9995	4.3	101.9	Methamphet.-D11
Methamphetamine	2.0	150 > 91, 119	High/High	ESI+	20 - 2000	0.9986	2.1	103.3	Methamphet.-D11
Methcathinone	1.7	164 > 146, 131	High/High	ESI+	20 - 2000	0.9996	1.9	107.1	Methamphet.-D11
Morphine	1.3	286 > 152, 201	High/Unit	ESI+	30 - 3000	0.9981	3.8	100.1	Morphine-D3
Nitrazepam	5.9	282 > 236, 180	High/High	ESI+	10 - 1000	0.9960	5.4	97.7	Nitrazepam-D5
Norcocaine	4.2	290 > 168, 136	High/High	ESI+	15 - 1500	0.9986	1.5	97.4	Norcocaine-D3 HCl
Nordiazepam	6.1	271 > 140, 208	High/Unit	ESI+	10 - 1000	0.9928	3.5	97.4	Nordiazepam-D5
Oxazepam	5.8	287 > 269, 104	Unit/Unit	ESI+	10 - 1000	0.9989	3.7	112.1	Oxazepam-D5
Oxycodone	2.2	316 > 241, 256	High/High	ESI+	30 - 3000	0.9987	2.3	105.8	Oxycodone-D3
PCP	5.8	244 > 86, 91	Unit/Unit	ESI+	2.5 - 250	0.9973	2.2	96.4	PCP-D5
Butalbital	3.7	223 > 180, 42	Unit/Unit	ESI-	15 - 1500	0.9984	4.3	103.4	Pentobarbital-D5
Pentobarbital	4.6	225 > 182, 42	Unit/Unit	ESI-	15 - 1500	0.9990	4.5	97.5	Pentobarbital-D5
Phenobarbital	3.4	231 > 42, 188	Unit/Unit	ESI-	15 - 1500	0.9994	5.7	103.8	Phenobarbital-D5
Propoxyphene	5.9	340 > 58, 266	High/High	ESI+	30 - 3000	0.9997	1.3	96.8	Propoxyphene-D5
Secobarbital	5.1	237 > 194, 42	Unit/Unit	ESI-	15 - 1500	0.9977	1.6	108.6	Secobarbital-D5
Temazepam	6.2	301 > 255, 283	High/High	ESI+	10 - 1000	0.9987	4.4	95.7	Temazepam-D5
THC-COOH	6.5	345 > 299, 299	Unit/Unit	ESI+	3 - 150	0.9961	3.1	97.3	THC-COOH-D9
THC	6.6	315 > 193, 123	Unit/Unit	ESI+	10 - 1000	0.9992	4.5	96.3	THC-D3
Trazodone	6.2	372 > 148, 148	High/High	ESI+	10 - 1000	0.9975	2.4	98.3	Trazodone-D6-HCL
Zopiclone	5.5	389 > 245, 217	Unit/Unit	ESI+	10 - 1000	0.9992	3.0	104.1	Zopiclone-D4
Zolpidem	6.0	308 > 235, 236	High/High	ESI+	10 - 1000	0.9973	3.1	104.3	Zolpidem-D7

Table 2 – Method performance data in enzymatically hydrolysed human urine

3. Conclusion

- A simple 'dilute and shoot' method was developed for the quantitation of 56 dugs of abuse and metabolites in human urine. The use of a high speed mass spectrometer allowed the acquisition of 6 MRMs per compound to enhance data quality.