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Dilute and shoot approach for the screening of stimulants by LC-dynamic-MS/MS

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Abstract

There is a growing need to improve the sensitivity of determination for multiple chemical constitutes in human urine because the Minimum Required Performance Levels (MRPL) for the detection of prohibited substances is continuously updated by the World Anti-Doping Agency (WADA). The MRPL for stimulants, for instance, was dropped from 500 to 100 ng/mL in early 2013. The conventional multiple reaction monitoring (cMRM) mode, however, is not well suited for multi-component identification due to its low sensitivity. Here we apply a dynamic MRM (dMRM) technique for the screening of 78 stimulants and metabolites in human urine using an Agilent triple-quadrupole 6410B mass spectrometer. By allowing extended dwell times, dMRM provides much higher sensitivity and reproducibility than cMRM. After precipitation of protein, the urine sample was injected into LC-MS/MS system directly without sample pre-concentration. For comparison of the sensitivity, both cMRM and dMRM were performed under same chromatographic conditions in this study. The result showed that both of the sensitivity and peak symmetry of extracted chromatogram for each stimulant improved significantly using dMRM. The LODs for the stimulants under investigation met the requirement set by WADA. The method also provided satisfactory results in terms of intra- and inter-day precisions, accuracy, matrix effect and specificity. This approach has been employed for routine analysis in our laboratory and External Quality Assessment Scheme (EQAS), which is designed by WADA to continuously monitor the capabilities of the laboratories, to evaluate laboratory proficiency, and to improve test result uniformity between laboratories.

Introduction

Detection of stimulants in human urine has been performed by gas chromatography with nitrogen phosphorous detector (NPD) [1], gas chromatography-mass spectrometry (GC-MS) [2] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [3,4]. Conventional liquid chromatography-tandem mass spectrometry using a triple-quadrupole mass spectrometer has been applied successfully in doping control analysis in sports. Data acquisitions are usually done by multiple reaction monitoring mode allowing multiple targets to be covered in a single run. A major drawback of cMRM is the limited number of target transitions that can be included in a single time segment.
This study aims at developing a dynamic MRM approach to screen 78 stimulants and metabolites using low resolution instruments (Agilent triple-quadrupole 6410B mass spectrometer). Unlike cMRM, dMRM automatically associates MRM transitions with retention time and it monitors each MRM transition only around its expected RT instead of monitoring all transitions throughout the entire operation as is the case with cMRM. Thus, dMRM allows more MRM transitions to be monitored in a single acquisition while maintaining high quality, sensitivity, selectivity, and reproducibility of the chromatographic results than with cMRM.

Experimental

Reference materials of stimulants were purchased from Sigma, Anpu, Alltech, NMI of Australia. Some analytical standards were kind gifts from Canadian and other WADA accredited Laboratories. Lead acetate was of analytical gradeand obtained from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China). Acetonitrile of HPLC grade was obtained from Sigma-Aldrich (St. Louis, USA). Ammonium formate and formic acid of HPLC grade were purchased from Fluka (Pittsburgh, USA) and

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DikmaPure (Lake Forest, USA) respectively. Deionized water was purified with a Milli-Q Academic ultra-pure water system (Millipore, Milford, USA).
Chromatography was performed on an Agilent 1200 series HPLC system. Separation was achieved on an Eclipse XDB-C18 column (2.1×100 mm, 3.5 µm, Agilent Technologies). The mobile phase was composed of 10 mM aqueous ammonium formate buffer (which was adjusted to pH 3.5 with formic acid) (eluent A) together with acetonitrile (eluent B). Gradient elution was as follows: 90% eluent A for 5 min, then decreased linearly to 50% eluent A in 5 min, followed by an decrease linearly to 10% eluent A in 5 min, and held at 10% eluent A for 1 min. Then the system was equilibrated for 4 min before the next injection (total run time 20 min). A constant flow rate of 0.4 mL/min was maintained.
Mass spectrometric detection was carried out using an Agilent triple-quadrupole 6410B mass spectrometer equipped with an ESI source. For comparison, both dMRM and cMRM mode were employed to detect the analytes in positive ionization mode, monitoring two ion transitions per compound. The spray voltage was set at 4000 V and the ion source was operated at 330°C. Nitrogen was used as the nebulizing and the drying gas, and the pressure was set at 40 psi.
After precipitation of protein using an equivalent volume of 5% $PbAc_2$ aqueous solution, the urine sample was injected into the LC-MS/MS system directly without sample pre-concentration or cleanup.

Results and Discussion

The “dilute and shoot” method was developed to analyze 78 stimulants and metabolites from WADA's prohibited list. The conventional MRM and dynamic MRM modes were performed for comparison. The result demonstrated that the dMRM had superior advantages over cMRM in terms of sensitivity and quality of the chromatographic peaks (Figure 1).

Method validation

Limit of detection (LOD)
Aliquots of six different blank urine samples with no detectable concentration of stimulants were spiked with the IS (mefruside) and an additional six aliquots were spiked with stimulants and the IS. The samples were prepared and analyzed according to the established protocol. The LOD was defined as the lowest concentration that can be detected with a signal-to-noise ratio > 3. For 80% of the stimulants the LODs were below 1 ng/ml, and for 18% the LODs were 1-5 ng/mL. Because of low proton affinity, the LODs for caffeine, dobutamine and amphetaminil were 25 ng/mL, which still complies with the MRPL criteria.

Precision and accuracy
Table 1 shows that the intra-and inter-day precision of stimulants was less than 20% at three concentration levels (low, medium and high). The accuracy at the three concentrations was within the range of 85-120%. The results demonstrated that the values were all within the acceptable range and the method was shown to be accurate and precise.

Specificity
The specificity of the method was evaluated by analyzing 20 individual blank urine samples prepared according to the established protocol. The results indicated that no other compounds co-eluted or interfered with the analyte or the IS at the same retention time or exhibited the same fragmentation pattern.

Matrix effect
Matrix effect was performed in ten different blank urines at three different concentrations (10, 100, 500 ng/mL) respectively (Table 1). The matrix effect was determined by comparing the peak areas of stimulants and IS from the spiked urine samples with those of the standard solutions in the mobile phase. The observed variation at low concentration (10 ng/mL) did not exceed the range of 75-125%. The matrix effects at higher concentration levels (100, 500 ng/mL) were between 80-120%. It can be concluded that the matrix effect for the analyte was not significant in the present LC-dynamic-MS/MS method.

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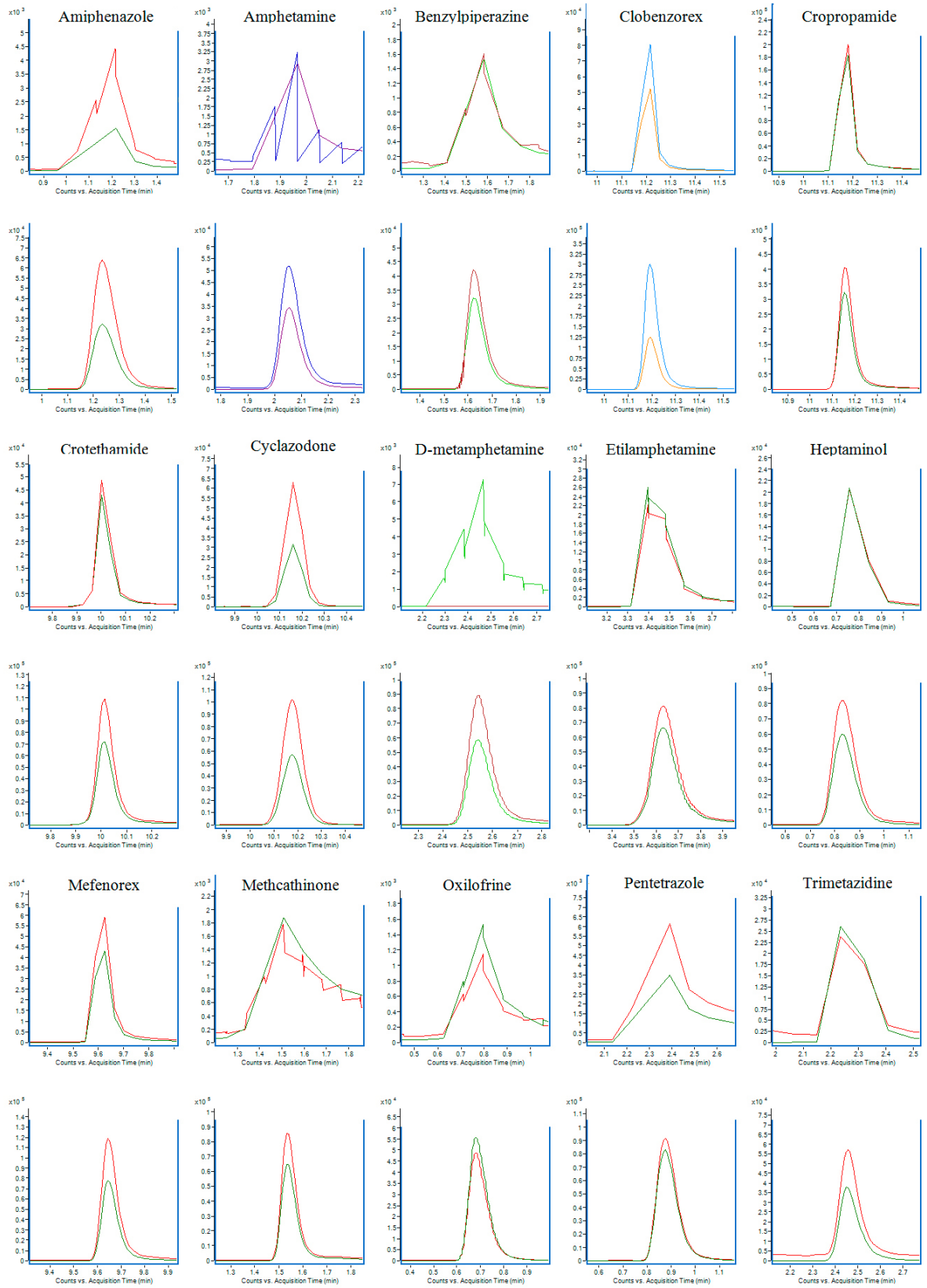

Figure 1. Comparison of selected chromatograms between cMRM (upper, without smoothening) and dMRM (lower, without smoothening) for the same stimulant at the same concentration (50 ng/mL) and under the same LC-MS conditions. The quality of the chromatograms by dMRM was greatly improved than by cMRM, and the peaks were well-defined since there were sufficient data points (more than 30) across the chromatographic peaks in each of the stimulants.

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No. Compound	Intra-day precision CV (%) (n = 6/6/6)			Inter-day precision CV (%) (n = 18/18/18)			Accuracy (%) (n = 6/6/6)			Matrix effect (%) (n = 6/6/6)		
	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}
1 2-amino-N-ethyl-1-phenyl-butane	5,2	4,8	3,5	8,6	6,1	4	86	92	105	83	85	110
2 adrafinil	—	6,1	4,5	—	7,8	6,1	—	95	90	—	112	90
3 amfepramone	—	5,8	4	—	6,7	4,6	—	97	90	—	109	86
4 amiphenazole	6,8	5,6	4,1	7,3	5,8	5	108	105	93	85	107	89
5 amphetamine	5,6	5,4	4,6	6,8	6,1	3,9	105	111	90	93	105	88
6 amphetaminil	—	16,9	4,5	—	19	4,8	—	107	112	—	96	110
7 benfluorex	5,2	5	3,9	6,3	6,4	4,7	118	98	105	112	92	116
8 benzoylecgonine	4,5	4,6	2,9	5,4	5	3,2	109	105	112	89	108	119
9 benzphetamine	4,8	5	3,8	5,9	5,6	4,5	115	106	102	121	96	89
10 benzylpiperazine	5,4	4,9	4,5	6,4	4,8	4,9	92	110	86	89	107	113
11 bupropion	6,6	6,2	4	7,6	6,4	4,5	87	103	90	80	94	85
12 caffeine	—	17,8	5,8	—	20	6,4	—	115	110	—	120	117
13 carphedone	—	10,8	3	—	12,4	3,5	—	108	90	—	112	85
14 cathine	5,3	5,4	3,8	6	5,6	4,5	87	105	110	85	109	120
15 chlorphentermine	5,8	5,2	4	6,5	5,4	5,4	92	103	90	86	93	85
16 clobenzorex	4,9	4	2,9	6,7	4,9	3,9	90	108	112	86	119	109
17 cocaine	6	5,5	3,5	7,9	5	4,2	89	98	105	84	89	111
18 cotinine	6,1	4,7	4	8,1	5,1	4,8	105	95	110	113	91	118
19 cropropamide	5,5	5	4,3	7,9	6	4,8	108	105	110	120	93	91
20 crotetamide	5	4,5	3,8	6,9	5,1	4,1	87	102	106	80	93	115
21 cyclazodone	4,9	4	2,7	5,8	4,8	3,5	88	97	88	79	91	85
22 dimethylamphetamine	5,7	3,9	3,7	7,8	4,5	4,2	85	102	93	90	92	83
23 dobutamine	—	12,8	3,9	—	15,9	4,8	—	96	105	—	91	114
24 ephedrine	6,9	2,5	4,2	8	4,2	3,8	90	103	85	83	89	104
25 etamivan	6,4	6	3,9	8,3	6,9	6,7	89	106	90	77	110	83
26 etilamphetamine	5,8	3,9	2,9	6,3	4,5	3,4	113	102	96	109	95	91
27 etilefrine	6,6	6	4,6	8,3	6,2	5	107	95	105	93	105	117
28 famprofazone	5	3,9	3,1	6,1	4,9	3,9	88	99	90	78	93	89
29 fenbutrazate	4,9	3,7	3	6,2	4,6	3,5	115	95	109	125	92	117
30 fencamfamin	5,1	4,6	3,9	7	5,2	4,4	89	94	110	116	85	116
31 fencamine	4,8	4	3,4	6,4	5	4,1	85	93	108	75	83	109
32 fenetylline	5,3	1,8	2,8	8,7	5,4	3,9	94	113	96	123	95	115
33 fenfluramine	6	3,8	4,3	7,8	4,9	5,4	112	106	115	124	92	120
34 fenproporex	5,5	3,7	2,7	4,8	4,6	2,9	89	102	88	84	89	104
35 furfenorex	5,6	2,8	4,2	7,3	4,8	4,3	90	97	93	80	107	112
36 heptaminol	6,2	5,2	4	8,2	5,7	4,9	86	95	88	109	85	115
37 p-hydroxyamphetamine	6,1	4,8	4,1	7,9	5,2	5	88	114	90	79	109	89
38 isometheptene	6,3	5	3,8	8,1	5,4	4,7	91	112	110	123	89	119
39 MDA	5,7	4,3	2,9	6,8	4,8	3,7	86	109	89	115	91	117
40 MDMA	5,1	3,7	4,1	7	4,5	5	88	107	90	82	111	110

Table 1. Summary of method validation results (QC_{low} = 10 ng/mL; QC_{medium} = 100 ng/mL; QC_{high} = 500 ng/mL).

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No.	Compound	Intra-day precision CV (%) (n = 6/6/6)			Inter-day precision CV (%) (n = 18/18/18)			Accuracy (%) (n = 6/6/6)			Matrix effect (%) (n = 6/6/6)		
		QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}	QC_{low}	QC_{middle}	QC_{high}
41	mefenorex	4,9	4,8	3	6,7	5,2	5,3	85	110	88	92	93	109
42	mephedrone	6,2	5,1	4	8,4	5,5	4,9	115	94	110	89	109	119
43	mephentermine	6	3,9	4,1	7,4	6	5,4	85	108	106	113	91	90
44	D-methamphetamine	5,8	6	3,8	6,7	6,2	4	87	109	110	80	88	118
45	L- methamphetamine	6,1	5,2	3,5	8	7	4,1	85	109	105	85	91	114
46	methcathinone	6	4,2	2,9	8,2	5,1	3,5	86	105	110	116	95	113
47	methoxyphenamine	6,7	5	4,1	7,4	5,2	5	91	109	88	76	97	109
48	p-methylamphetamine	6,3	4	4,5	8,1	4,7	4,7	90	110	91	82	113	89
49	methylephedrine	5,6	3,9	4,1	6,1	4,2	4,5	87	94	88	85	108	80
50	methylhexaneamine	4,3	3,7	3,6	5,4	4,8	4,2	85	103	87	97	90	95
51	methylphenidate	4,4	3,2	3,5	8,3	6,5	5,6	96	113	99	85	110	109
52	modafinil	5,2	5	4,2	6,7	5,9	5	85	109	88	82	105	109
53	modafinil acid	5,6	4,2	4,6	6,9	5	4,8	111	105	109	120	97	119
54	nikethamide	6,3	3,8	4,6	5,6	3,1	3,9	97	113	87	91	95	110
55	norephedrine	—	10,5	6,2	—	12,4	7,8	—	112	90	—	115	105
56	norfenfluramine	4	3,8	3,1	5,5	4,3	3,8	85	107	88	80	112	84
57	ortetamine	6,3	5,1	4,6	7,8	5,9	5,1	120	109	115	124	109	118
58	oxilofrine	7,1	5	3,9	8,4	5,7	4,5	87	115	89	84	119	81
59	pemoline	—	6,3	3	—	7,8	3,7	—	110	86	—	116	82
60	pentetrazol	4,2	3,7	4	5	4,7	4,9	87	109	85	91	118	83
61	phendimetrazine	—	5,2	3,9	—	6,2	4,2	—	109	92	—	89	95
62	phenmetrazine	5,6	5	3,1	6,7	5,5	3,8	115	110	90	120	105	116
63	phenpromethamine	5,3	4,6	4	6,4	5,4	4,8	120	96	112	113	109	91
64	phentermine	6,4	5	4,5	7	5,8	5,1	85	94	88	109	83	85
65	pholedrine	7	5,3	4,1	7,6	6	5	108	91	110	105	112	91
66	pipradrol	6,8	5	4	8	5,6	4,7	85	92	85	81	107	95
67	prenylamine	6,5	3,8	3	7,9	4,6	3,4	86	95	90	84	112	80
68	prolintane	5	5,8	4,6	8,4	6	4,9	104	109	93	82	90	91
69	propylhexedrine	6,3	5,1	4,5	7,4	5,6	5,2	109	95	113	92	107	110
70	pseudoephedrine	7	3,9	3,1	6,8	3,6	3	86	92	109	79	89	117
71	pyrovalerone	6,5	4,5	3	6,9	5,3	3,8	89	109	113	85	116	107
72	ritalinic acid	5,9	4,3	2,9	6,7	4,8	3,5	116	94	110	121	90	114
73	selegiline	6,3	5	3,2	7,6	5,7	4,4	90	105	108	88	110	107
74	sibutramine	6	3,1	2,7	7,8	3,9	3,4	112	91	110	110	89	118
75	strychnine	—	4,5	4	—	5	3,9	—	113	85	—	110	91
76	trans-3'-OH-cotinine	—	11,4	4,2	—	10,3	8	—	108	87	—	120	89
77	trimetazidine	—	10,5	2,9	—	12,5	4,5	—	95	85	—	90	84
78	tuaminopheptane	7	4,9	3,8	8	6,1	4,6	89	94	85	109	89	83

Table 1 (continued). Summary of method validation results (QC_{low} = 10 ng/mL; QC_{medium} = 100 ng/mL; QC_{high} = 500 ng/mL).

Conclusions

A precise and highly sensitive analytical LC-dynamic-MS/MS method with minimum sample preparation was developed and validated for the quanlitative determination of 78 stimulants and metabolites in human urine in this study. The dMRM acquisition mode displayed superior efficiency and sensitivity to cMRM. The advantages of this approach include easy workup, improved sensitivity and peak symmetry of extracted ion chromatogram for each stimulant under investigation. All assays performed within the acceptable parameters in terms of LOD, intra- and inter-day precision, accuracy, matrix effects and specificity. This study could provide a valuable means employing low resolution instruments for doping-control purposes.

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