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Development and Validation of Direct Analysis Method for Screening and Quantitation of Amphetamines in Urine by LC/MS/MS

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Introduction

Amphetamines are among the most commonly abused drugs type worldwide. The conventional analytical procedure of amphetamines in human urine in forensic laboratory involves initial immunological screening followed by GCMS confirmation and quantitation [1]. The new guidelines of SAMHSA under U.S. Department of Health and Human Services effective in Oct 2010 [2] allowed use of LC/MS/MS for screening, confirmation and quantitation of illicit drugs including amphetamines. One of the advantages by using LC/MS/MS is that derivatization of amphetamines before analysis is not needed, which was a standard procedure of GCMS method. Since analysis speed and throughput could be enhanced significantly, development and use of LC/MS/MS methods are in demand and many such efforts have been reported recently [3]. The objective of this study is to develop a fast LC/MS/MS method for direct analysis of amphetamines in urine without sample pre-treatment (except dilution with water) on LCMS-8040, a triple quadrupole system featured as ultra fast mass spectrometry (UFMS). The compounds studied include amphetamines (AMPH), methamphetamine (MAMP) and three newly added MDMA, MDA and MDEA by the new SAMHSA guidelines, four potential interferences as well as PMPA as a control reference (Table 1). Very small injection volumes of 0.1uL to 1uL was adopted in this study, which enabled the method suitable for direct injection of untreated urine samples without causing significant contamination to the ESI interface.

Experimental

The stock standard solutions of amphetamines and related compounds as listed in Table 1 were prepared in the Toxicology Laboratory in the Department of Scientific Services (MOH, Brunei). Five urine specimens were collected from healthy adult volunteers. The urine samples used as blank and spiked samples were not pre-treated by any means except dilution of 10 times with Milli-Q water. An LCMS-8040 triple quadrupole coupled with a Nexera UHPLC system (Shimadzu Corporation) was used. The analytical column used was a Shim-pack XR-ODS III UHPLC column (1.6 µm) 50mm x 2mm. The mobile phases used were water (A) and MeOH (B), both with 0.1% formic acid. A fast gradient elution program was developed for analysis of the ten compounds: 0-1.6min, B=2%->14%; 1.8-2.3min, B=70%; 2.4min, B=2%; end at 4min. The total flow rate was 0.6 mL/min. Positive ESI ionization mode was applied with drying gas flow of 15 L/min, nebulizing gas flow of 3 L/min, heating block temperature of 400 °C and DL temperature of 250 °C. Various injection volumes from 0.1 uL to 5 uL were tested to develop a method with a lower injection volume to reduce contamination of untreated urine samples to the interface.

Results and Discussion

Method development of direct injection of amphetamines in urine

MRM optimization of the ten compounds (Table 1) was performed using an automated MRM optimization program with LabSolutions workstation. Two MRM transitions were selected for each compound, one for quantitation and second one for confirmation (Table 1). The ten compounds were separated and eluted in 0.75~2.2 minutes as sharp peaks as shown in Figure 1. In addition to analysis speed and detection sensitivity, this method development was also focused on evaluation of small to ultra-small injection volumes to develop a method suitable for direct injection of urine samples without any pre-treatment while it should not cause significant contamination to the interface. The Nexera SIL-30A auto-sampler enables to inject as low as 0.10 uL of sample with excellent precision.

Figure 1 shows a few selected results of direct injection of urine blank (a) and mixed standards spiked in urine with 1 uL (c and d) and 0.1 uL (b) injection. It can be seen that all compounds (12.5 ppb each in urine) could be detected with 0.1uL injection except MDA and Norpseudo-E. With 1uL injection, all of them were detected.

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Table 1: MRMs of amphetamines and related compounds

Cat.	Compound	Abbr.	RT (min)	MRM	CE (V)
B1	Nor pseudo ephedrine	Nor pseudo-E	0.75	152>134	-13
				152>115	-23
B2	Ephedrine	Ephe	0.94	166>148	-14
				166>91	-31
B3	Pseudo ephedrine	Pseudo-E	1.01	166>148	-14
				166>91	-30
A1	Amphetamine	AMPH	1.20	136>91	-20
				136>119	-14
A2	Methampheta-mine	MAMP	1.42	150>91	-20
				150>119	-14
A3	3,4-methylenedi oxyamphetamine	MDA	1.49	180>163	-12
				180>163	-38
A4	3,4-methylene dioxymeth amphetamine	MDMA	1.59	194>163	-13
				194>105	-22
A5	3,4-methylene dioxy-N-ethyl amphetamine	MDEA	1.94	208>163	-12
				208>105	-24
B4	Phentermine	Phent	1.93	150>91	-20
				150>119	-40
R	Propyl amphetamine	PAMP	2.20	178>91	-22
				178>65	-47

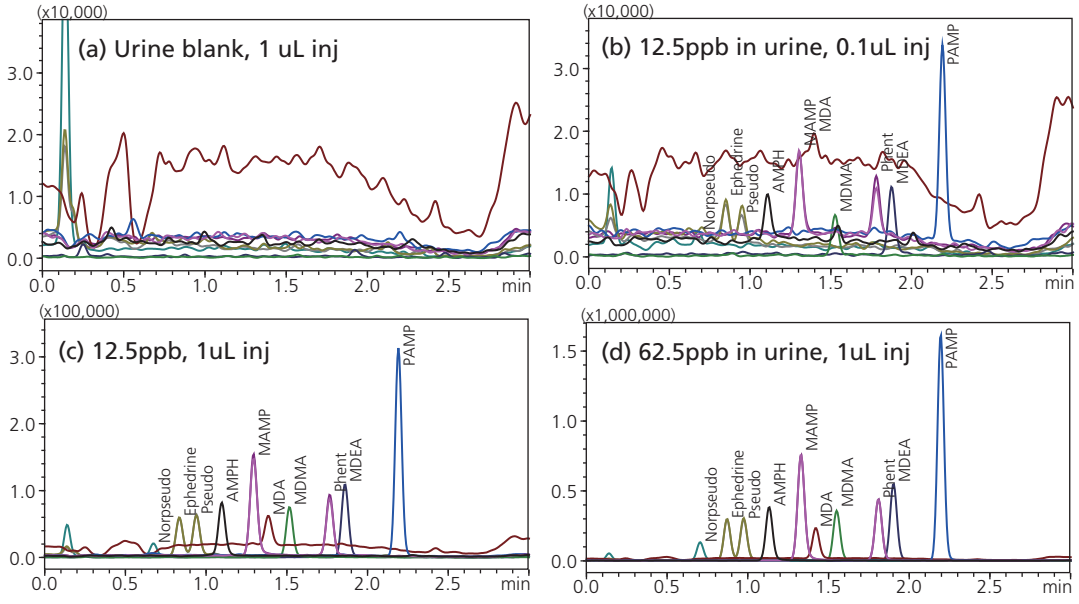

Figure 1: MRM chromatograms of urine blank (a) and spiked samples of amphetamines and related compounds in urine by LC/MS/MS method with 1uL and 0.1uL injection volumes.

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Calibration curves with small and ultra-small injection volumes

Linear calibration curves were established for the ten compounds spiked in urine with different injection volumes: 0.1, 0.2, 0.5, 1, 2 and 5 uL. Good linearity of calibration curves (R2>0.999) were obtained for all injection volumes including 0.1uL, an ultra-small injection volume. The calibration curves with 0.1 uL injection volume are shown in Figure 2. The linearity (r2) of all compounds with 0.1 uL and 1 uL injection volumes are equivalently good as shown in Table 2.

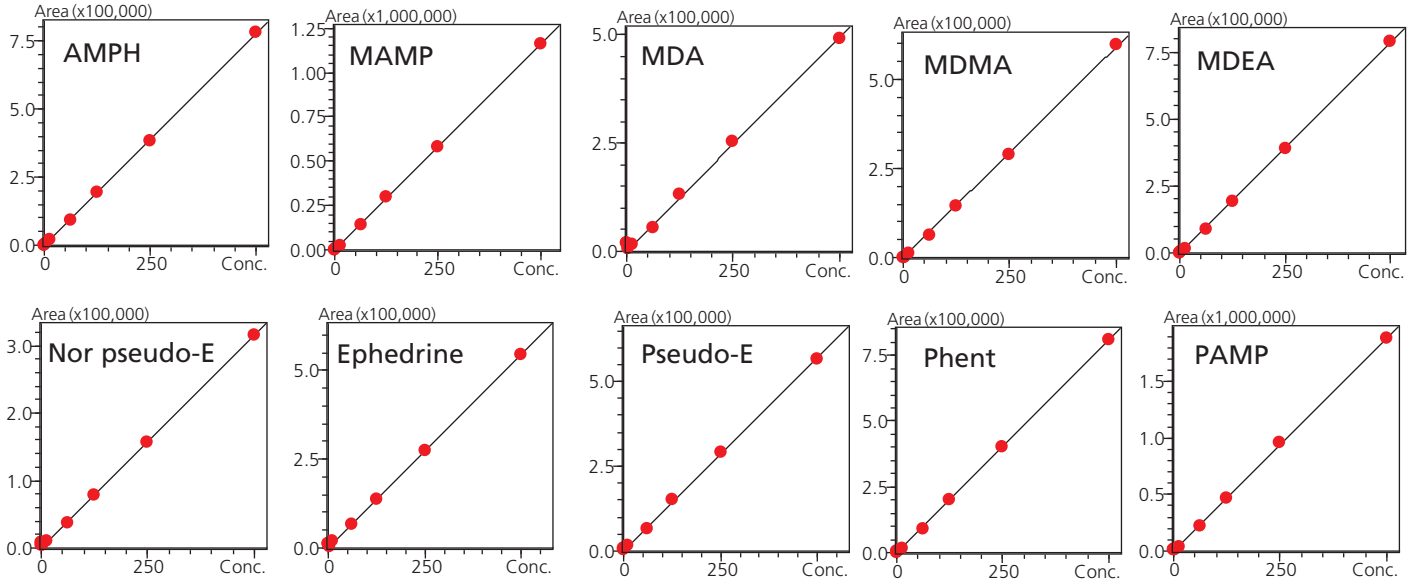

Figure 2: Calibration Curves of amphetamines spiked in urine with 0.1uL injection

Performance validation

Repeatability of peak area was evaluated with a same loading amount (6.25 pg) but with different injection volumes. The RSD shown in Table 2 were 1.6% ~ 7.9% and 1.6 ~ 7.8% for 0.1uL and 1uL injection, respectively. It is worth to note that the repeatability of every compounds with of 0.1uL injection is closed to that of 1uL injection as well as 5uL injection (data not shown).

Matrix effect of the method was determined by comparison of peak areas of mixed standards in pure water and in urine matrix. The results of 62.5ppb with 1uL injection were at 102-115% except norpseudoephedrine (79%) as shown in Table 2.

Accuracy and sensitivity of the method were evaluated with spiked samples of low concentrations. The results of LOD and LOQ of the ten compounds in urine are shown in Table 3. Since the working samples (blank and spiked) were diluted for 10 times with water before injection, the concentrations and LOD/LOQ of the method described above for source urine samples have to multiply a factor of 10. Therefore, the LOQs of the method for urine specimens are at 2.1-17.1 ng/mL for AMPH, PAMP, MDMA and MDEA and 53 ng/mL for MDA. The LOQs for the potential interferences (Phentermine, Ephedrine, Pseudo-Ephedrine and Norpseudo-Ephedrine) are at 17-91 ng/mL, 2.4 ng/mL for the internal reference MAMP. The sensitivity of the direct injection LC/MS/MS method are significantly higher than the confirmation cutoff (250 ng/mL) required by the SAMHSA guidelines.

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Table 2: Method Performance with different inj. volumes

Name	Calibration curve, R2			RSD% area (n=6)		M.E. %[1]
	(ppb)[2]	(0.1uL)	(1uL)	(0.1uL)	(1uL)	(1uL)
Norpseudo-E	1-500	0.9992	0.9996	4.5	5.7	79
Ephe	2.5-500	0.9995	0.9998	3.2	2.9	115
Pseudo-E	1-500	0.9994	0.9986	3.7	3.3	113
AMPH	1-500	0.9997	0.9998	3.5	2.4	102
MAMP	1-500	0.9998	0.9999	1.6	2.3	110
MDA	2.5-500	0.9978	0.9995	7.9	7.8	103
MDMA	1-500	0.9993	0.9998	1.8	4.5	115
MDEA	1-500	0.9996	0.9998	3.5	2.9	115
Phent	2.5-500	0.9998	0.9998	4.1	1.6	106
PAMP	1-500	0.9998	0.9932	2.9	2.0	102

1: Measured with mixed stds of 62.5 ppb in clear solution and spiked in urine
2: For 0.1uL injection, the lowest conc. is 2.5 or 12.5 ppb

Table 3: Method performance: sensitivity & accuracy (1uL)

Name	Conc. (ppb)		Accuracy	Sensitivity (ppb)		
	Prep.	Meas.	(%)	S/N	LOD	LOQ
Norpseudo-E	1.0	1.2	118.7	2.3	1.53	5.09
Ephe	2.5	2.2	88.2	2.7	2.41	8.04
Pseudo-E	1.0	1.0	99.5	5.9	0.50	1.67
AMPH	1.0	1.1	114.1	6.7	0.51	1.71
MAMP	1.0	1.0	103.6	21.8	0.14	0.47
MDA	2.5	2.4	96.3	4.5	1.60	5.34
MDMA	1.0	1.1	106.4	51.9	0.06	0.21
MDEA	1.0	1.1	111.8	28.5	0.12	0.39
Phent	2.5	2.6	105.3	2.9	2.73	9.10
PAMP	1.0	1.0	101.7	42.2	0.07	0.24

Method operational stability

The method operational stability with 1uL injection was tested with spiked samples of 25 ppb in five urine specimens, corresponding to 250 ng/mL in the source urine samples. Continuous injections of accumulated 120 times was carried out in about 10 hours. The purpose of the experiment was to evaluate the operational stability against the ESI source contamination by urine samples without pre-treatment. Figure 3 shows the first injection and the 120th injection of the same spiked sample (S1) as well as other spiked samples (S2, S3, S4 and S5) in between. Decrease in peak areas of the compounds occurred, but the degree of the decrease in average was about 17% from the first injection to the last injection. This result indicates that it is possible to carry out direct analysis of urine samples (10 times dilution with water) by the high sensitivity LC/MS/MS method with a very small injection volume.

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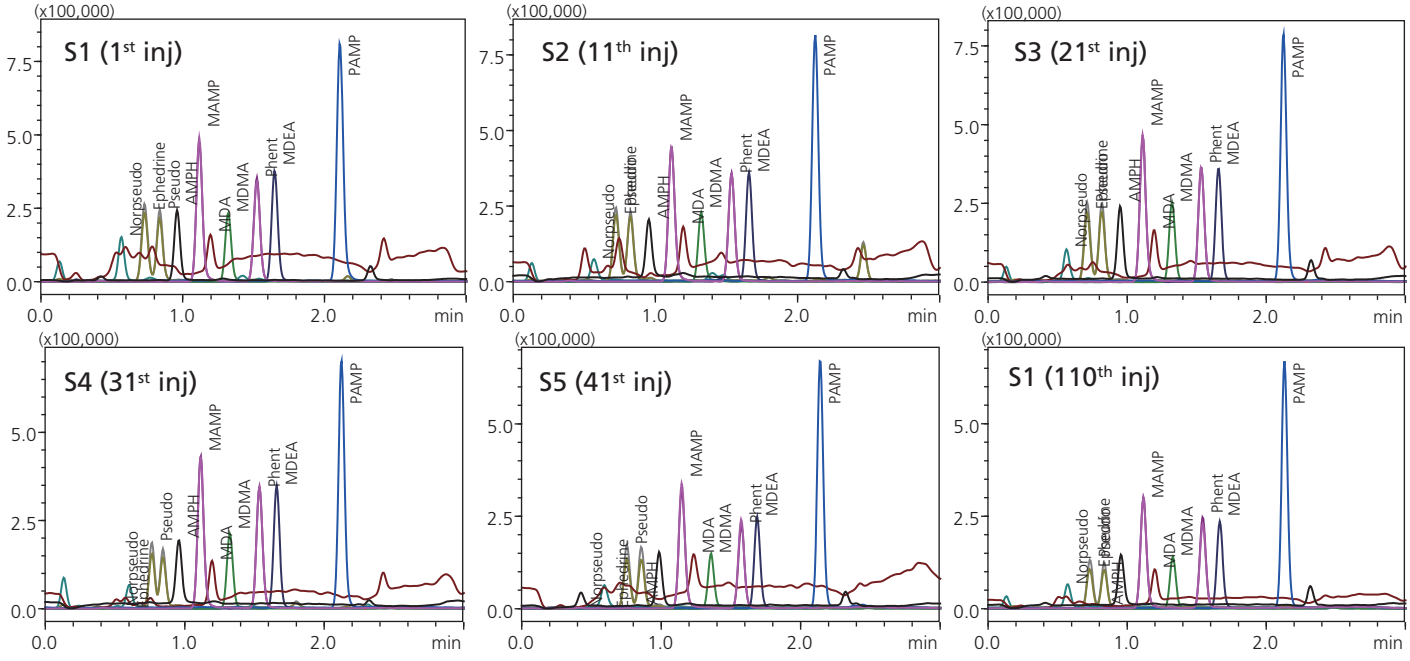

Figure 3: Selected chromatograms of continuous injections of spiked samples (25 ppb) with 1 µL injection. Five urine specimens S1, S2, S3, S4 and S5 were used to prepare these spiked samples.

Conclusions

In this study, we developed a fast LC/MS/MS method for direct analysis of five amphetamines and related compounds in human urine for screening and quantitative confirmation. Very small injection volumes of 0.1~1.0 uL were adopted to minimize ESI contamination and enhance operational stability. The good performance results observed reveals that screening and confirmation of amphetamines in human urine by direct injection to LC/MS/MS is possible and the method could be an alternative choice in forensic and toxicology analysis.

References

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