PO-CON1737E

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A Novel Platform of On-line Sample Pre-treatment and LC/MS/MS Analysis for Screening and Quantitation of Illicit Drugs in Urine

ASMS 2017 WP 353

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Introduction

In recent years, LC/MS/MS methods are adopted in analyses of illicit and prescription drugs in toxicological samples such as urine and serum. Sample pre-treatment is always a critical step in the whole analysis procedure and on-line sample pre-treatment is desired not only for improving analysis throughput, but also minimizing human errors. The CLAM-2000 module is designed for on-line sample pre-treatment in high throughput LC/MS/MS analysis of drugs and metabolites in biological samples such as plasma/serum and urine. Many sample preparation process can be performed automatically such as dispensing solvents, sample-reagent mixing by vortexing, sample filtering by vacuum filtration, and sample derivatisation with heating. Internal standard and reagent for derivatization or other purposes can be added to a sample before or after protein crash. We describe development of an automated sample pre-treatment using a Shimadzu CLAM-2000 module coupled with Shimadzu LCMS-8040 TQ system. It involves IS addition, protein precipitation, filtration and transferring the final solution to LC/MS/MS for analysis. This new platform was applied and evaluated for quantitation of 18 illicit drugs with 14 isotope-labelled internal standards (IS).

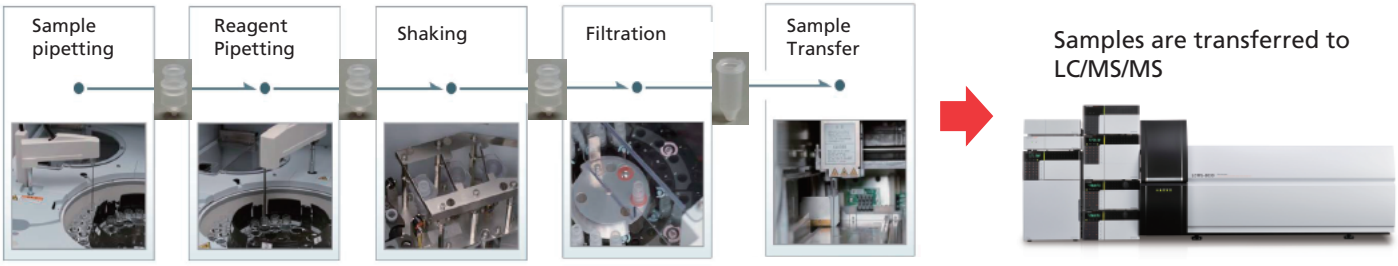

Figure 1: Procedure of protein crash and spiked-sample preparation

Experimental

Sample preparation and analytical conditions

A total of 18 illicit drugs and 14 isotope-labeled internal standards (except for phencyclidine, methaqualone, methadone and propoxyphene) were used for setting up the MRM quantitation method. The urine samples, internal standards mixed solution and organic solvents were pre-loaded onto the CLAM-2000. An automated batch-run program allows sample pre-treatment and analysis to perform concurrently on the CLAM-LC-MS/MS platform. Table 1 shows the analytical conditions on LCMS-8040. Figure 2 illustrates the automated workflow on the CLAM-2000 module. An aliquot of 20 uL of urine sample was dispensed into a filtration vial. Then, 20 µL of mixed internal standard (IS) stock solution was added to the sample, followed by addition of 40 µL of organic solvent (MeOH : ACN = 1 : 1 in volume). The sample mixture was vortexed and filtered into a collection vial before injecting to LCMS-8040. A Phenomenex Biphenyl column (100 x 2.1 mm I.D., 2.6µm) was used for the analysis of 18 analytes and 14 IS with a gradient elution program of 11 minutes. A calibration series of spiked standard samples in urines were prepared in concentrations of 20, 50 and 200 ng/mL. The concentration of each IS was 100 ng/mL. A LCMS-8040 with ESI was employed in this work.

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Table 1: Analytical conditions on LCMS-8040

Column	: Biphenyl 2.6µ, 100A (100 mmL x 2.10mm I.D.)
Mobile Phase	: A: Water with 0.1% FA
	B: Methanol with 0.1% FA
Elution Program	: Gradient elution (11.0 minutes)
	B: 3% (0 to 0.5 min) → 90% (5.5 to 7.0 min) → 3% (7.5 to 11.0 min)
Flow Rate	: 0.4 mL/min
Oven Temp.	: 40°C
Injection	: 5 µL
Interface	: ESI
MS Mode	: MRM, Positive
Block Temp.	: 400°C
DL Temp.	: 250°C
CID Gas	: Ar, 270 kPa
Nebulizing Gas	: N_2, 2.0 L/min
Drying Gas	: N_2, 15.0 L/min

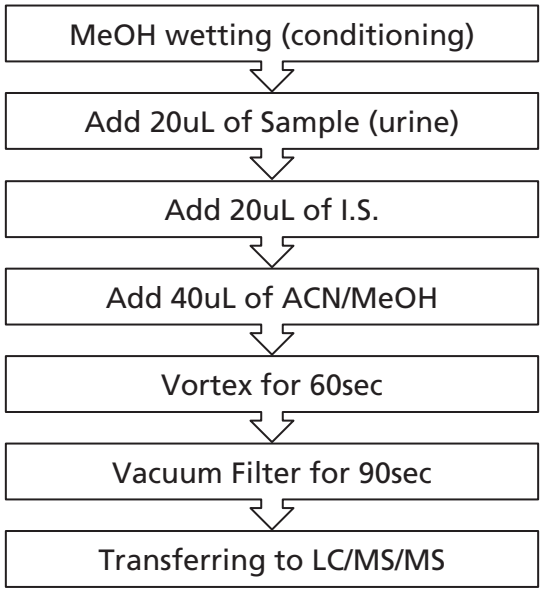

Figure 2: Typical auto-workflow of urine sample via protein-crash and adding IS for LC/MS/MS by CLAM-2000

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Results and Discussion

MRM-based method for eighteen illicit drugs

Table 2 shows the summarized results of optimized MRM transitions and parameters of the eighteen analytes and fourteen isotope-labelled internal standards (IS). However, four isotope-labelled ISs were not available. Three MRM transitions were selected for each compound except PROP with one as the quantitation ion and the other two for confirmation. A gradient elution program was optimized with a total runtime of eleven minutes. The MRM chromatograms of a mixed standard sample in urine are shown in Figure 3.

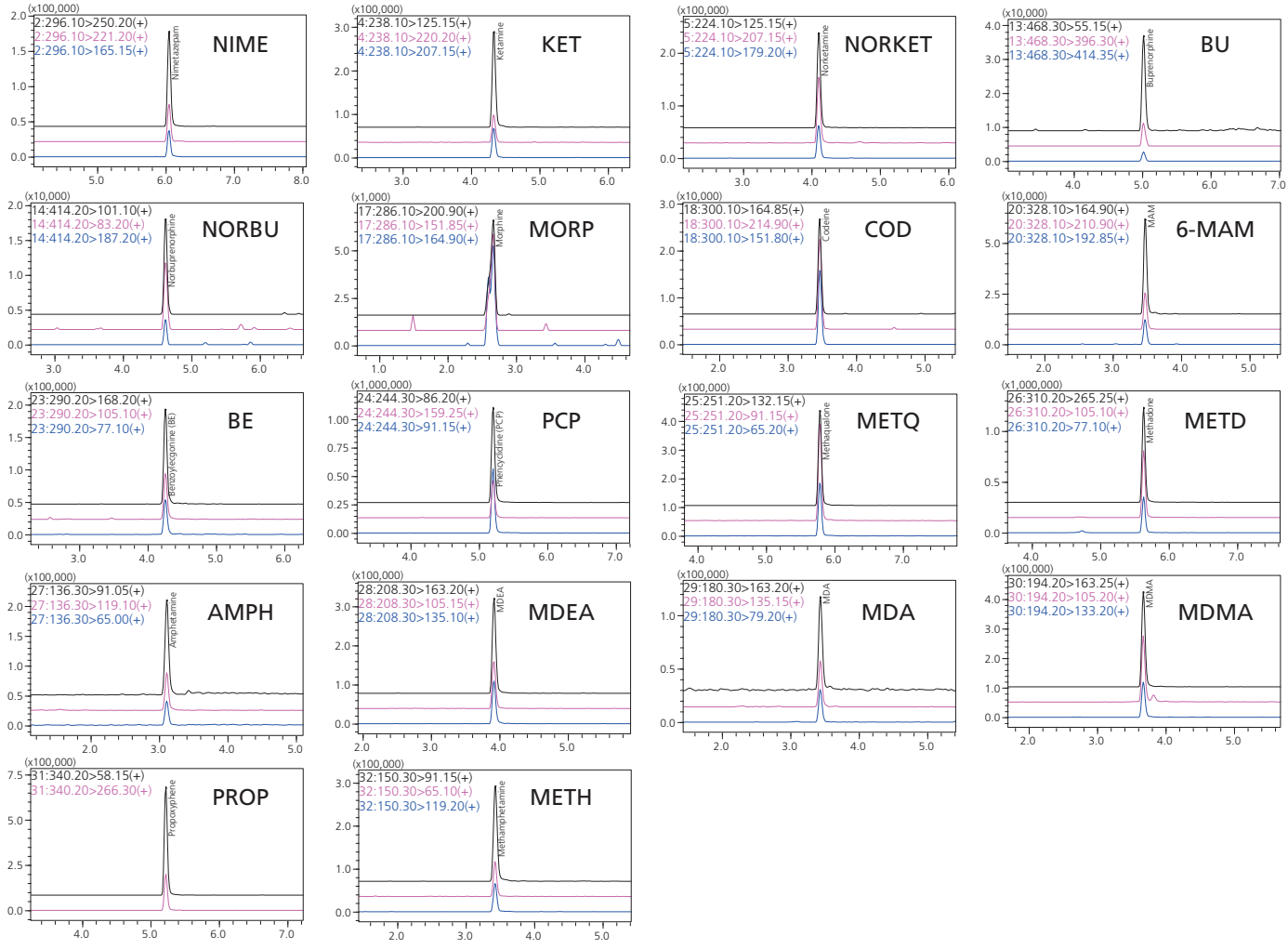

Figure 3: Individual MRM chromatograms of eighteen illicit drugs each (200 ng/mL) and fourteen ISs (100 ng/mL) spiked in urine obtained on CLAM-LC/MS/MS platform.

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Table 2: MRM transitions and parameters of the illicit drugs on LCMS-8060

Standard				Internal Standard			
Compd.	R.T (min)	MRM (m/z)	CE (V)	Compd.	R.T (min)	MRM (m/z)	CE (V)
Nimetazepam (NIME)	6.053	296.1>250.2	-26	D5-Nitrazepam (D5-NITRA)	5.63	287.2>241.2	-26
		296.1>221.2	-34			287.2>185.2	-36
		296.1>165.2	-57			287.2>212.2	-34
Ketamine (KET)	4.334	238.1>125.2	-28	D4-KET	4.32	242.2>129.1	-27
		238.1>220.2	-16			242.2>224.2	-15
		238.1>207.2	-14			242.2>211.2	-15
Norketamine (NORKET)	4.105	224.1>125.2	-24	D4-NORKET	4.09	228.1>129.1	-25
		224.1>207.2	-13			228.1>211.2	-12
		224.1>179.2	-15			228.1>183.2	-16
Buprenorphine (BU)	5.019	468.3>55.2	-60	D4-BU	5.01	472.3>59.2	-54
		468.3>414.4	-36			472.3>400.3	-40
		468.3>396.3	-41			472.3>101.1	-43
Norbuprenorphine (NORBU)	4.622	414.2>83.2	-52	D3-NORBU	4.61	417.3>83.2	-50
		414.2>101.1	-44			417.3>101.2	-41
		414.2>187.2	-38			417.3>187.2	-41
Morphine (MORP)	2.663	286.1>200.9	-26	D3-MORP	2.66	289.1>157.1	-43
		286.1>151.9	-61			289.1>165.1	-41
		286.1>164.9	-41			289.1>153.1	-41
Codeine (COD)	3.474	300.1>164.9	-45	D3-COD	3.46	303.1>151.8	-67
		300.1>214.9	-27			303.1>164.9	-45
		300.1>151.8	-65			303.1>214.9	-27
6-MAM	3.475	328.1>164.9	-39	D6-MAM	3.46	334.1>164.9	-40
		328.1>210.9	-26			334.1>210.9	-27
		328.1>192.9	-29			334.1>192.9	-30
Benzoylecgonine (BE)	4.260	290.2>168.2	-20	D3-BE	4.25	293.2>171.3	-20
		290.2>105.1	-31			293.2>105.1	-29
		290.2>77.1	-53			293.2>77.1	-55
Phencyclidine (PCP)	5.204	244.3>86.2	-12	N.A.			
		244.3>159.3	-14				
		244.3>91.2	-30				
Methaqualone (METQ)	5.786	251.2>132.2	-27	N.A.			
		251.2>91.2	-45				
		251.2>65.2	-61				
Methadone (METD)	5.632	310.2>265.3	-17	N.A.			
		310.2>105.1	-27				
		310.2>77.1	-53				
Propoxyphene (PROP)	5.220	340.2>58.2	-23	N.A.			
		340.2>266.3	-10				
Amphetamine (AMPH)	3.111	136.3>91.5	-21	D5-AMPH	3.066	141.3>124.3	-19
		136.3>119.1	-14			141.3>92.2	-14
		136.3>65.0	-37			141.3>93.2	-17
MDEA	3.919	208.3>163.2	-14	D6-MDEA	3.906	214.3>166.2	-13
		208.3>105.2	-27			214.3>136.2	-20
		208.3>135.1	-22			214.3>108.2	-26
MDA	3.440	180.3>163.2	-12	D5-MDA	3.424	185.3>168.3	-12
		180.3>135.2	-19			185.3>110.3	-23
		180.3>79.2	-32			185.3>138.3	-20
MDMA	3.678	194.2>163.3	-14	D5-MDMA	3.664	199.3>165.3	-13
		194.2>105.2	-24			199.3>107.2	-26
		194.2>133.2	-21			199.3>135.2	-20
Methamphetamine (METH)	3.430	150.3>91.2	-21	D8-METH	3.403	158.3>93.2	-21
		150.3>119.2	-15			158.3>124.3	-15
		150.3>65.1	-43			158.3>92.2	-19

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Performance of MRM-based Quantitative Method

Linearity of the calibration curves with both IS method (14 analytes) and external standard method (4 analytes) were constructed using the standard samples prepared by pre-spiked in urine matrix are shown in Figure 4. The method parameters are summarized in Table 3. It can be seen that good linearity with R^2 greater than 0.995 was obtained for the eighteen illicit drugs in the range from 20 ng/mL to 200 ng/mL in urine.

Accuracy of the quantitation method was evaluated with pre-spiked standard samples at all concentrations. The results are shown in Table 3, which indicate that reliable quantitation accuracy was obtained, except Methadone at 20 ng/mL with an accuracy of 130%.

Process Efficiency (P.E) was evaluated based on the peak area (external standard) or peak ratios (IS method) of pre-spiked samples and neat-spiked sample at all concentrations. The results shown in Table 3 indicate the P.E obtained for the 18 analytes are between 62~122% except four analytes with higher values, Norbuprenorphine, Morphine, MAM, and Methadone. This could be due to interference from urine, which causes ion enhancement.

Specificity of the method for detection and confirmation of the eighteen illicit drugs was evaluated (Figure 5). The confirmation criteria for each target include quantifier MRM peak, its ratios with reference MRM transitions as well as retention time.

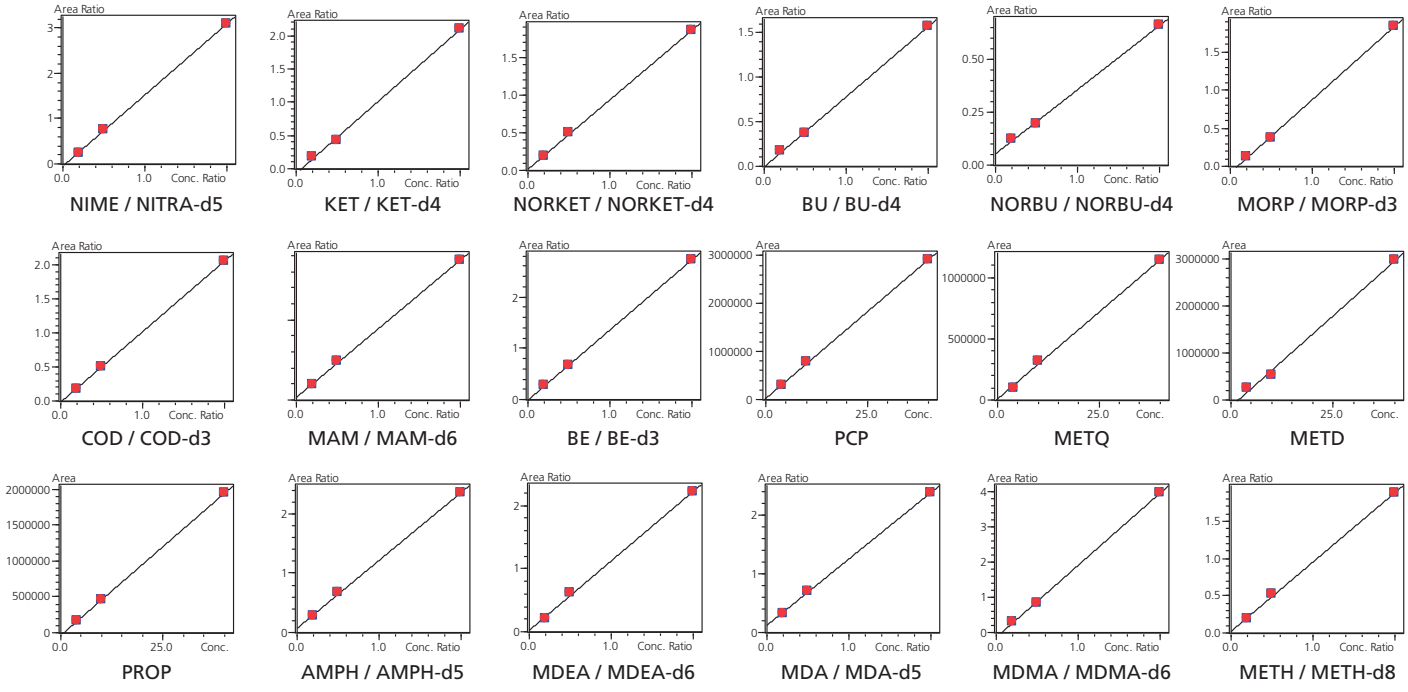

Figure 4: Calibration curves of 14 illicit drugs with isotope-labelled internal standards and 4 illicit drugs with external standard in human urine on LCMS-8040. Details are shown in Table 3.

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Table 3: MRM quantitation method of eighteen illicit drugs

Compd.	Accuracy (%)			R2	*Cut Off ng/mL	Avg. P.E (%)
	20 ng/mL	50 ng/mL	200 ng/mL			
NIME	93.7	103.0	99.9	0.9997	5	99.1
KET	117.5	91.6	100.3	0.9983	100	107.9
NORKET	91.6	104.0	99.8	0.9996	100	106.5
BU	94.5	102.7	99.9	0.9998	2	118.1
NORBU	112.8	93.9	100.3	0.9991	2	183.0
MORP	108.7	95.8	100.2	0.9995	300	62.2
COD	96.0	101.9	99.9	0.9999	300	88.8
MAM	89.6	105.0	99..8	0.9994	10	139.5
BE	102.5	98.8	100.1	0.9999	150	111.3
PCP	92.0	103.8	99.8	0.9996	25	88.3
METQ	80.8	109.2	99.6	0.9980	250	101.9
METD	130.2	85.5	100.6	0.9951	250	161.6
AMPH	91.7	104.0	99.8	0.9996	200	92.3
MDEA	84.0	107.7	99.7	0.9986	200	84.5
MDA	92.1	103.8	99.8	0.9996	200	80.8
MDMA	110.4	95.0	100.2	0.9994	200	98.5
PROP	102.2	98.9	100.0	0.9999	300	122.1
METH	83.7	107.8	99.7	0.9985	200	84.0

*The Cut Off is based on European Guidelines for Workplace Drug Testing in Urine

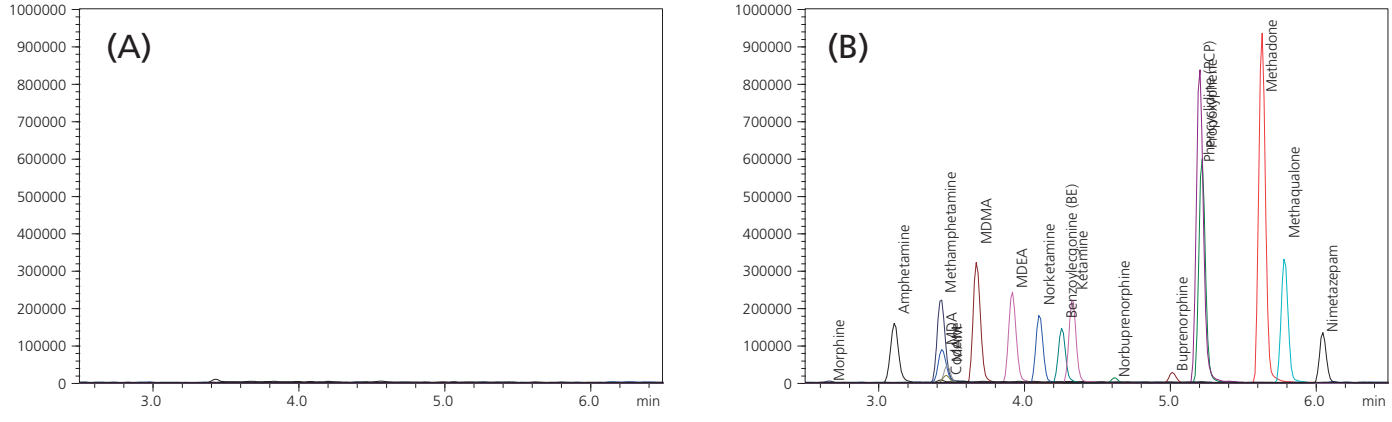

Figure 5: Total MRM chromatograms of (A) blank urine and (B) spiked urine with eighteen illicit drugs (200 ng/mL).

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Conclusions

A fully automated method of sample pretreatment and quantitation for eighteen illicit drugs in human urine was developed on a novel platform of CLAM-LC/MS/MS. The method performance was evaluated on the linearity, accuracy, specificity and process efficiency.

Acknowledgement

The authors thanks Dr Yao Yi Ju and Moy Hooi Yan for their valuable comments and discussion. The authors also thanks Health Science Authority (HSA), Analytical Toxicology Division for providing the analyte standards for this work.

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First Edition: June, 2017

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