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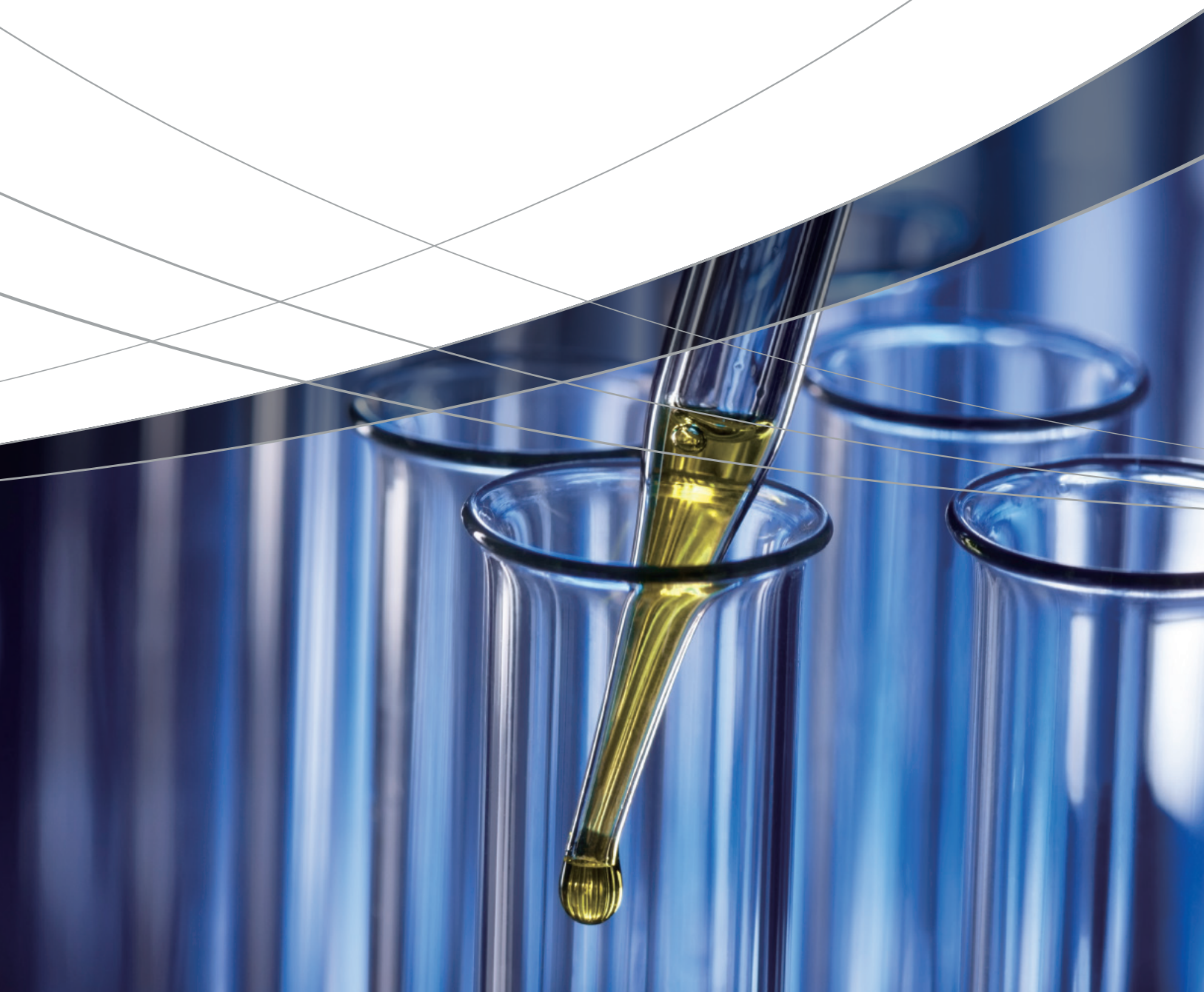

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High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

ASMS 2017 WP358

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High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

Introduction

LC-MS/MS has become a preferred method for the routine analysis for forensic toxicology. LC-MS/MS allows for the simultaneous analysis of multiple compounds in a single run, thus enabling a fast and high throughput analysis. In recent years that it seems the number of incident and accident is increasing caused by dosed with psychotropic drugs and the number of drug testing with LC-MS/MS is also increasing to investigate the cause of death. However, manual sample preparation often involves several complicated manual steps which can introduce error into the results. In this study, we investigated the processing capability to analyze serum, whole blood and urine spiked sixty psychotropic drugs by LC-MS/MS with automated sample preparation unit.

Group 1. Eight Barbiturate drug and Bromovalerylurea

Allobarbital Amobarbital Barbital Pentobarbital Phenobarbital
Secobarbital Thiamylal Thiopental Bromovalerylurea

Group 2. twelve Tri-/Tetra-cyclic antidepressant

Amitriptyline Amoxapine Clomipramine Desipramine Dosulepin Imipramine
Maprotiline Mianserin Nortriptyline Promethazine Setiptiline

Group 3. Thirty-nine Benzodiazepines and their metabolites

Alprazolam Bromazepam Brotizolam Chlordiazepoxide Clorazepic acid Clotiazepam Cloxazolam Diazepam Estazolam Ethyl loflazepate Etizolam Fludiazepam Flunitrazepam Flurazepam Flutazolam Flutoprazepam Haloxazolam Lorazepam Lormetazepam Medazepam Mexazolam Midazolam Nimetazepam Nitrazepam Oxazolam Prazepam
Quazepam Rilmazafone Tofisopam Triazolam Zolpidem

7-Aminoflunitrazepam 7-Aminonimetazepam 7-Aminonitrazepam
α-Hydroxyetizolam (M-VI) α-Hydroxyalprazolam α-Hydroxybrotizolam
α-Hydroxytriazolam Zolpidem M-1

Figure 1 Target drugs

High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

Methods and Materials

The analysis of 60 psychoactive drugs (eight Barbiturate drug, thirty-nine Benzodiazepines and their metabolites, twelve Tri-/Tetra- cyclic antidepressant and bromovalerylurea) were performed using a fully automatic LCMS preparation unit (CLAM-2000, Shimadzu) online with HPLC-LCMS (NexeraX2-LCMS-8060, Shimadzu).

Samples were trapped on Imtakt Unison UK-C18 (10x2mm, 3.0µm), then separated by Imtakt Unison UK-C18 (75x2mm, 3.0µm) with a binary gradient system. Water with ammonium formate and methanol were used for mobile phases.

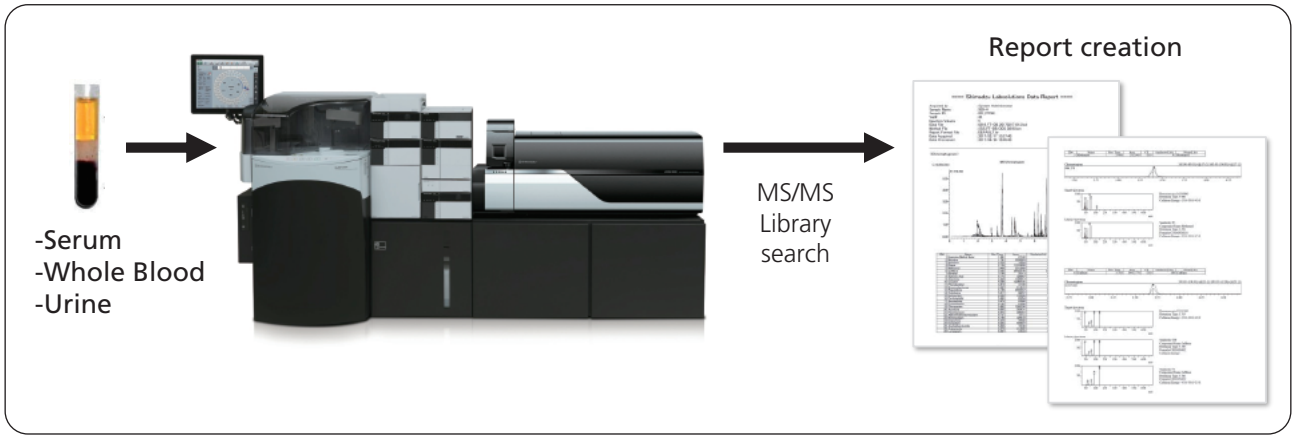

Figure 2 CLAM-2000 and LCMS-8060 system

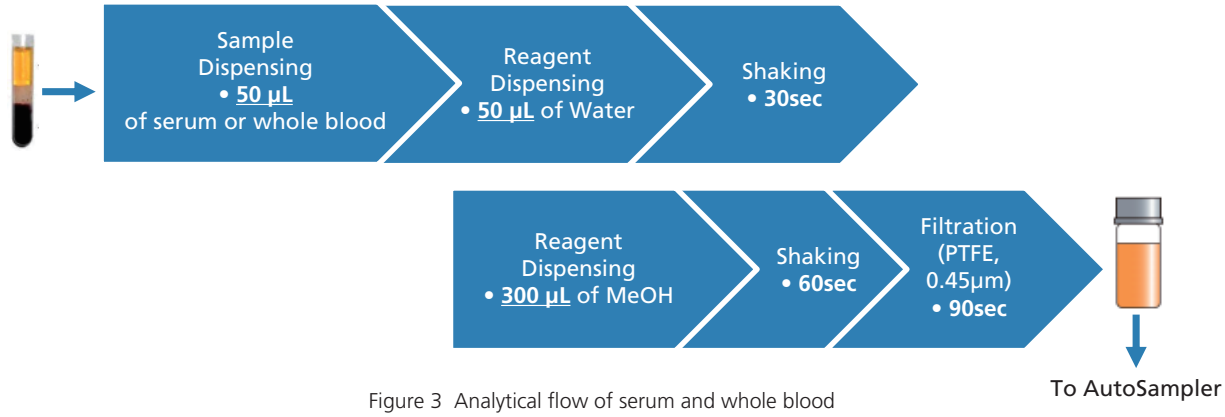

Figure 3 Analytical flow of serum and whole blood

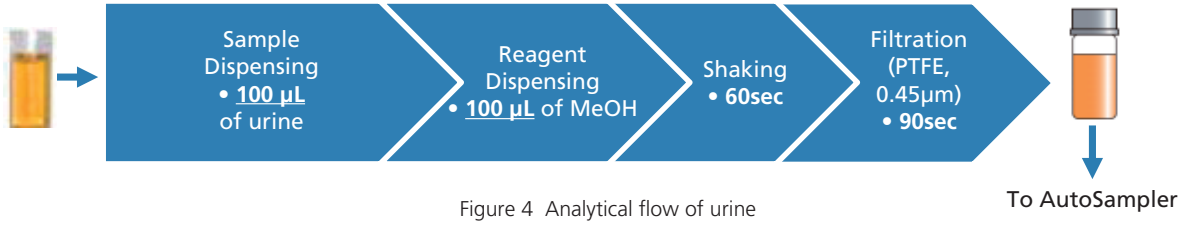

Figure 4 Analytical flow of urine

High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

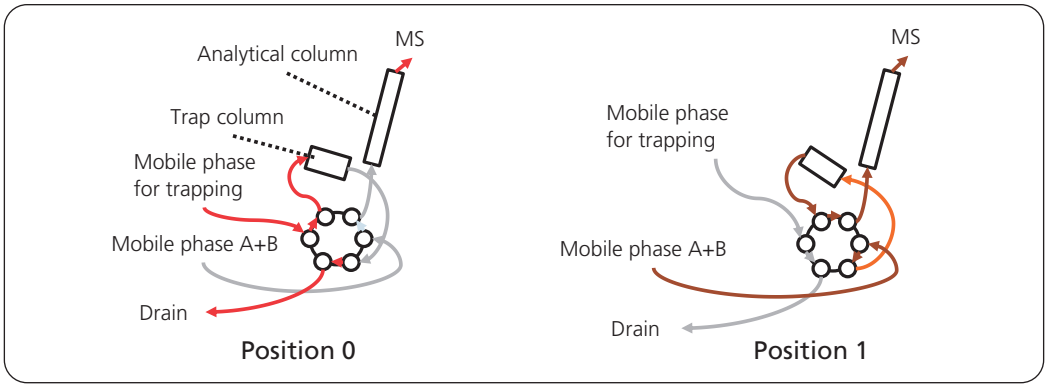

Figure 5 Flow diagram of trapping system

LC/MS/MS conditions (Nexera system and LCMS-8060)	
Ionization	: ESI, Positive/Negative MRM mode
Trap column	: Unison UK-C18 (10×2 mm, 3 µm, Imtakt)
Analytical column	: Unison UK-C18 (75×2 mm, 3 µm, Imtakt)
Mobile phase for traping	: 5% MeOH / 0.1% Formic acid
Mobile phase A	: 10mM Ammonium formate, 5% Methanol
B	: 10mM Ammonium formate, 95% Methanol
Time program	: B conc. 0 % - (1 min) - 5 % - (7 min) - 95 % (3 min)

Result

Recovery rate

Usually LC-MS/MS analysis of biological samples require some manual preparation steps such as protein precipitation, solid phase extraction or liquid/liquid extraction before the injection. With the aim to reduce the operator involvement, to increase the throughput and the data quality, we completely eliminated the manual sample preparation procedure by the use of a novel automatic preparation unit including precipitation, filtration, incubation, shaking and pipetting. Serum and whole blood spiked with sixty psychoactive drugs were pretreated with organic solvent and filtration by the unit. On the other hands, urine spiked with their drugs were only filtration. The treated samples were trapped for cleaning and concentration, then separated by Unison UK-C18 in HPLC Unit.

The recovery of whole blood spiked with sixty psychoactive drugs were more than 70% and the recovery of serum and urine spiked with them were more than 80%. We completed analysis of their psychoactive drugs in several biological matrices using the automated sample preparation system coupled to LC-MS/MS

High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

Concentration in sample (μg/mL)	Serum			Whole blood			Urine		
	0.1	1	10	0.1	1	10	0.1	1	10
Allobarbital (neg)	81%	84%	80%	67%	77%	76%	90%	103%	89%
Amobarbital (neg)	81%	86%	80%	78%	79%	78%	88%	103%	92%
Barbital (neg)	84%	82%	91%	73%	84%	79%	87%	102%	89%
Pentobarbital (neg)	77%	78%	80%	72%	80%	76%	95%	109%	92%
Phenobarbital (neg)	77%	83%	92%	70%	74%	81%	92%	101%	86%
Secobarbital (neg)	69%	92%	80%	67%	73%	77%	78%	107%	88%
Thiamylal (neg)	76%	83%	84%	72%	79%	77%	76%	97%	84%
Thiopental (neg)	75%	84%	81%	68%	75%	77%	81%	110%	95%
Bromovalerylurea	71%	84%	84%	75%	79%	79%	91%	104%	92%

Concentration in sample (μg/mL)	Serum			Whole blood			Urine		
	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Amitriptyline	74%	69%	80%	64%	66%	70%	87%	84%	102%
Amoxapine	72%	73%	80%	66%	66%	73%	90%	86%	101%
Clomipramine	67%	70%	74%	63%	65%	83%	82%	78%	99%
Desipramine	74%	72%	77%	57%	60%	89%	90%	83%	103%
Dosulepin	70%	68%	80%	63%	64%	77%	88%	86%	100%
Imipramine	76%	70%	81%	64%	67%	87%	86%	84%	100%
Maprotiline	68%	68%	76%	56%	58%	67%	91%	83%	101%
Mianserin	79%	84%	76%	78%	77%	81%	90%	85%	111%
Nortriptyline	71%	73%	80%	55%	64%	70%	91%	83%	100%
Promethazine	71%	69%	80%	89%	104%	116%	89%	84%	103%
Setiptiline	83%	75%	83%	68%	70%	83%	87%	87%	106%

High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

Concentration in sample (μg/mL)	Serum			Whole blood			Urine		
	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Alprazolam	69%	70%	82%	78%	75%	86%	98%	93%	106%
Bromazepam	73%	74%	78%	82%	76%	77%	103%	96%	112%
Brotizolam	72%	72%	86%	79%	73%	85%	98%	93%	108%
Chlordiazepoxide	76%	73%	81%	77%	73%	82%	99%	93%	111%
Clorazepic acid	84%	70%	80%	78%	77%	90%	97%	91%	126%
Clotiazepam	73%	73%	74%	81%	76%	85%	96%	93%	116%
Cloxazolam	90%	79%	82%	81%	80%	89%	N.A	77%	83%
Diazepam	82%	77%	77%	80%	74%	83%	96%	94%	109%
Estazolam	72%	73%	79%	80%	74%	82%	99%	95%	110%
Ethyl loflazepate	82%	72%	81%	76%	73%	80%	90%	92%	112%
Etizolam	77%	73%	80%	81%	75%	95%	105%	95%	103%
Fludiazepam	79%	76%	79%	74%	74%	87%	95%	93%	115%
Flunitrazepam	79%	74%	80%	76%	73%	82%	100%	95%	111%
Flurazepam	75%	73%	93%	73%	73%	97%	92%	93%	99%
Flutazolam	75%	71%	79%	70%	70%	81%	92%	97%	111%
Flutoprazepam	82%	79%	73%	79%	77%	92%	96%	89%	106%
Haloxazolam	56%	64%	87%	55%	68%	75%	86%	89%	122%
Lorazepam	76%	73%	76%	86%	70%	76%	115%	85%	103%
Lormetazepam	75%	73%	78%	75%	73%	79%	95%	89%	106%
Medazepam	80%	70%	79%	77%	76%	91%	96%	90%	123%
Mexazolam	81%	77%	73%	83%	75%	103%	79%	93%	123%
Midazolam	74%	70%	80%	79%	72%	84%	94%	91%	104%
Nimetazepam	75%	73%	81%	76%	75%	85%	105%	98%	115%
Nitrazepam	80%	73%	77%	75%	74%	77%	97%	93%	107%
Oxazolam	N.A.	73%	89%	N.A.	73%	88%	77%	70%	81%
Prazepam	78%	82%	73%	81%	78%	86%	94%	90%	107%
Quazepam	73%	78%	70%	81%	77%	81%	86%	88%	217%
Rilmazafone	78%	76%	82%	71%	73%	70%	98%	97%	126%
Tofisopam	73%	71%	82%	78%	75%	85%	101%	93%	109%
Triazolam	72%	70%	83%	80%	76%	85%	93%	93%	110%
Zolpidem	71%	72%	87%	76%	74%	95%	99%	97%	102%
α-Hydroxyalprazolam	84%	70%	76%	81%	72%	77%	96%	95%	110%
α-Hydroxybrotizolam	70%	74%	79%	74%	70%	76%	94%	89%	107%
α-Hydroxyetizolam (M-VI)	79%	70%	79%	71%	70%	80%	86%	90%	104%
7-Aminoflunitrazepam	75%	72%	78%	79%	75%	79%	94%	91%	108%
7-Aminonimetazepam	75%	74%	78%	81%	75%	80%	93%	90%	104%
7-Aminonitrazepam	74%	71%	78%	77%	73%	88%	94%	90%	103%
α-Hydroxytriazolam	74%	72%	78%	79%	75%	79%	109%	92%	106%
Zolpidem M-1	74%	71%	86%	76%	74%	95%	99%	98%	103%

High-sensitivity and simultaneous analysis of Psychoactive drugs using LC-MS/MS with full-automated pretreatment system

Conclusions

These results shows the capability of the system for large sample set analyses with improved accuracy and precision by eliminating human error associated with manual sample handling.

* Disclaimer: LCMS-8060 and CLAM-2000 are not registered as a Class I device, and it is available for Research Use Only (RUO). Not for use in diagnostic procedures.

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Application News

No. AD-0135

Clinical Research / LCMS-8060

A Fast LC/MS/MS Method for Quantitative Analysis of Five β-Lactam Antibiotics in Human Plasma

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❑ Introduction

The β-lactam type antibiotics are used in the treatment of various bacterial infections in human over decades. One of the consequences of continuous usage of antibiotics is the progressive development of drug resistance of bacteria in human [1]. Therapeutic Drug Monitoring (TDM) aims at obtaining pharmacokinetic pattern of an antibiotic in patient to develop personalized medicine treatment. Conventional TDM methods such as immunoassays are well-established. However, one of the drawbacks of immunoassays is lack of specificity due to cross-reactivity with metabolites, which may give false positives result [2,3]. Recently, LC/MS/MS has been used for fast and direct measurement of β-lactam antibiotics such as amoxicillin [4] and piperacillin, etc. [5,6] in human plasma. In this application news, a fast LC/MS/MS method with a simple sample pre-treatment procedure for quantitative analysis of five β-lactam antibiotics meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT) is described. A small injection volume of sample of this MRM-based method is required only, which minimizes the contamination of sample matrix, as such, reducing the cleaning and maintenance time of the interface of LC/MS/MS in clinical research work.

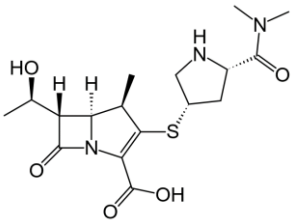

Figure 1: Structure of meropenem (MER) with a β-lactam ring.

❑ Experimental

Sample preparation and analytical conditions

Five antibiotics used in this study are meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT). The compounds and four stable isotope-labelled meropenem-d6, piperacillin-d5, cefepime-cd3 and ceftazidime-d6 as internal standards were purchased from certified suppliers. Pool human plasma was obtained from i-DNA Biotechnology Pte Ltd and used as matrix. The sample pre-treatment and spiked sample preparation procedure are illustrated in Figure 1. A simple protein crash method was applied by adding ACN:MeOH (1:1) to plasma in a ratio of 3:1, followed by vortex and centrifuge. A calibration series of spiked standard samples were prepared: 20, 40, 80, 200, 400, 2000 and 4000 ng/mL in plasma. The concentrations of internal standards were 200 ng/mL or 800 ng/mL in these calibrants. A LCMS-8060, a triple quadrupole LC/MS/MS system with heated ESI was employed in this work. The analytical conditions and instrumental parameters are compiled into Table 1.

Table 1: Analytical conditions and parameters on LCMS-8060

Column	Kinetex 1.7µ C18 100A (100 mmL x 2.10mm I.D.)
Mobile Phase	A: Water with 0.1% FA B: Acetonitrile with 0.1% FA
Elution Program	Gradient elution (5.5 minutes) B: 5% (0 to 0.2 min) ➔ 90% (3.5 to 4.0 min) ➔ 5% (4.1 to 5.5 min)
Flow Rate	0.5 mL/min
Oven Temp.	40ºC
Injection	2 µL
Interface	ESI (heated)
MS Mode	MRM, Positive
Block Temp.	400ºC
DL Temp.	250ºC
Interface Temp.	300ºC
CID Gas	Ar, 270 kPa
Nebulizing Gas	N_2, 3.0 L/min
Drying Gas	N_2, 5.0 L/min
Heating Gas	Zero Air, 15L/min

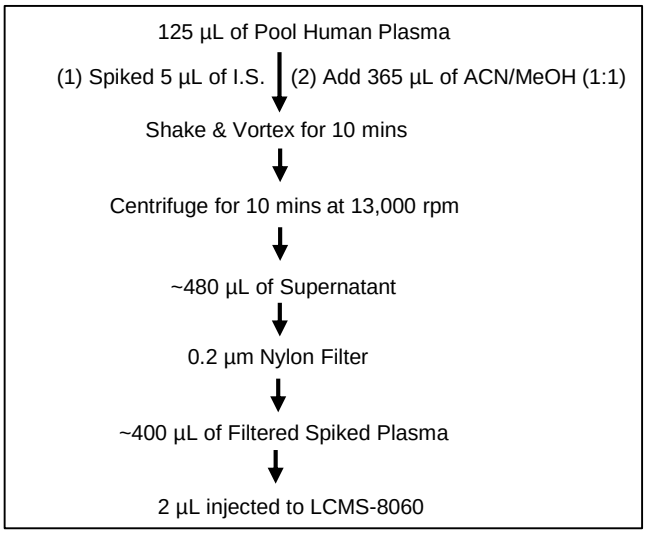

Figure 2: Procedure of protein crash and spiked-sample preparation

❑ Results and Discussion

Fast MRM-based method for five β-lactam antibiotics

Table 2 shows the summarized results of optimized MRM transitions and parameters of the five β-lactam antibiotics studied and four stable isotope-labelled internal standards. Two MRM transitions were selected for each compound, with one as the quantitation ion and the other for confirmation.

Furthermore, a fast gradient elution MRM method was established with a total run time of 5 minutes. The MRM chromatograms of a mixed standard sample in plasma are shown in Figure 3. Due to lack of stable isotope-labelled tazobactam, MER-d6 was also used as the internal standard for tazobactam (TAZ) in this work.

Table 2: MRM transitions and parameters of five β-lactam antibiotics and internal standards on LCMS-8060

Compd.	Formula	E. Mass	MRM (m/z)	CE (V)	Int (%)
Tazobactam (TAZ)	$C_{10}H_{12}N_4O_5S$	300.05	301.1>168.2	-15	100
			301.1>122.1	-22	92
Cefepime (CEF)	$C_{19}H_{24}N_6O_5S_2$	480.13	481.1>86.2	-15	100
			481.1>396.0	-13	63
Meropenem (MER)	$C_{17}H_{25}N_3O_5S$	383.15	384.1>68.1	-41	100
			384.1>141.1	-16	64
Ceftazidime (CFT)	$C_{22}H_{22}N_6O_7S_2$	546.10	547.1>468.0	-13	100
			547.1>396.1	-19	42
Piperacillin (PIP)	$C_{23}H_{27}N_5O_7S$	517.16	518.2>143.1	-21	100
			518.2>160.1	-15	25
CEF-cd3	$C_{18}{}^{13}CH_{21}D_3N_6O_5S_2$	484.13	485.2>86.1	-16	100
			485.2>400.1	-13	66
MER-d6	$C_{17}H_{19}D_6N_3O_5S$	389.15	390.2>147.2	-18	100
			390.2>114.1	-27	74
CFT-d6	$C_{22}H_{16}D_6N_3O_7S_2$	552.10	553.1>474.0	-16	100
			553.1>319.1	-20	59
PIP-d5	$C_{23}H_{22}D_5N_5O_7S$	522.16	523.1>148.1	-21	100
			523.1>160.1	-14	23

Table 3: MRM-based quantitation method of five β-lactam antibiotics with internal standards on LCMS-8060

Compd.	RT (min)	qMRM (m/z)	IS	IS (ng/mL)	Range (ng/mL)	R^2
TAZ	1.18	301.1 > 168.1	MER-d6	200	20~4000	0.9993
CEF	1.21	481.1 > 86.1	CEF-cd3	800	20~4000	0.9991
MER	1.47	384.1 > 68.1	MER-d6	200	20~4000	0.9989
CFT	1.47	547.1 > 468.0	CFT-d6	800	20~4000	0.9971
PIP	2.33	518.2 > 143.1	PIP-d5	200	20~4000	0.9999

Calibration curves with IS

As shown in Figure 4, linear calibration curves with IS method were constructed using the standard samples prepared by pre-spiked in plasma matrix. The method parameters are summarized in Table 3. It can be seen that good *linearity* with

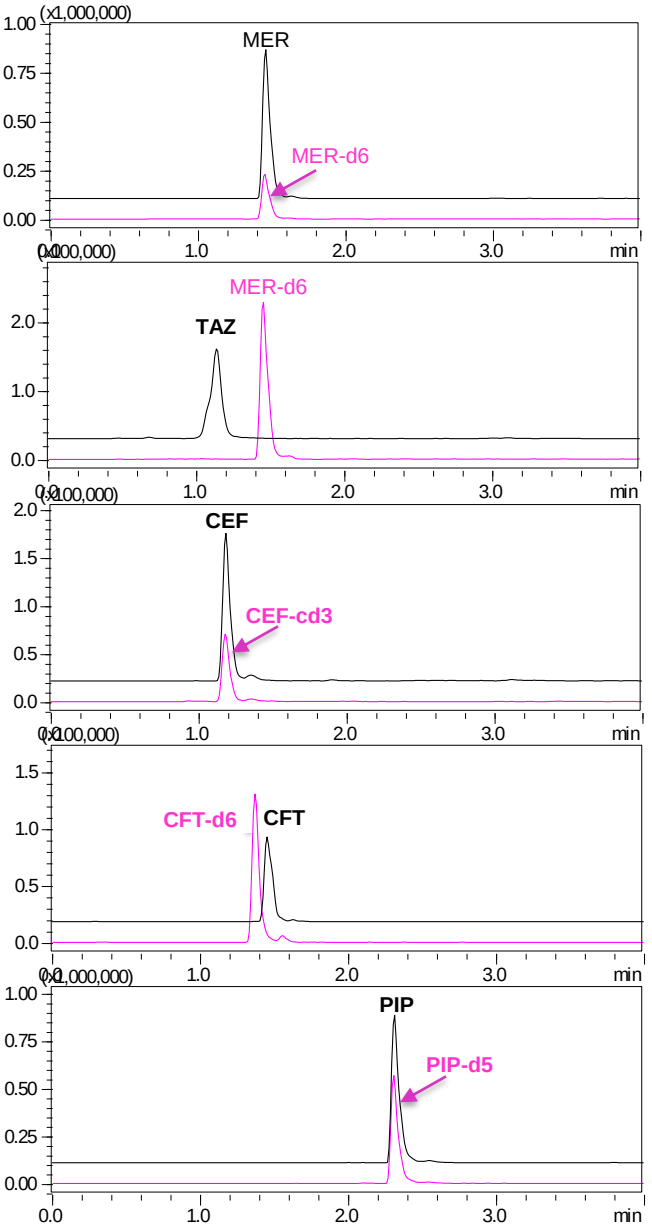

Figure 3: MRM chromatograms of five β-lactam antibiotics each (400 ng/mL) with internal standards in plasma on LCMS-8060

R2 greater than 0.997 was obtained for the five compounds in the range from 20 ng/mL to 4000 ng/mL in plasma.

Evaluation of method performance

Accuracy of the quantitation method was evaluated with pre-spiked standard samples at all concentration levels with duplicate injections. The results are shown in Table 4, which indicate that reliable quantitation accuracy was obtained, except CFT at 20 ng/mL, due to employing IS method.

Table 4: Results of RSD (%, n=5) of five β-lactam antibiotics with IS in plasma samples on LCMS-8060

Compd.	Accuracy (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	91	97	100	106	107	101	99
CEF	87	97	102	105	109	101	98
MER	97	102	102	99	102	95	102
CFT	126	103	93	87	91	96	104
PIP	99	100	97	103	102	100	100

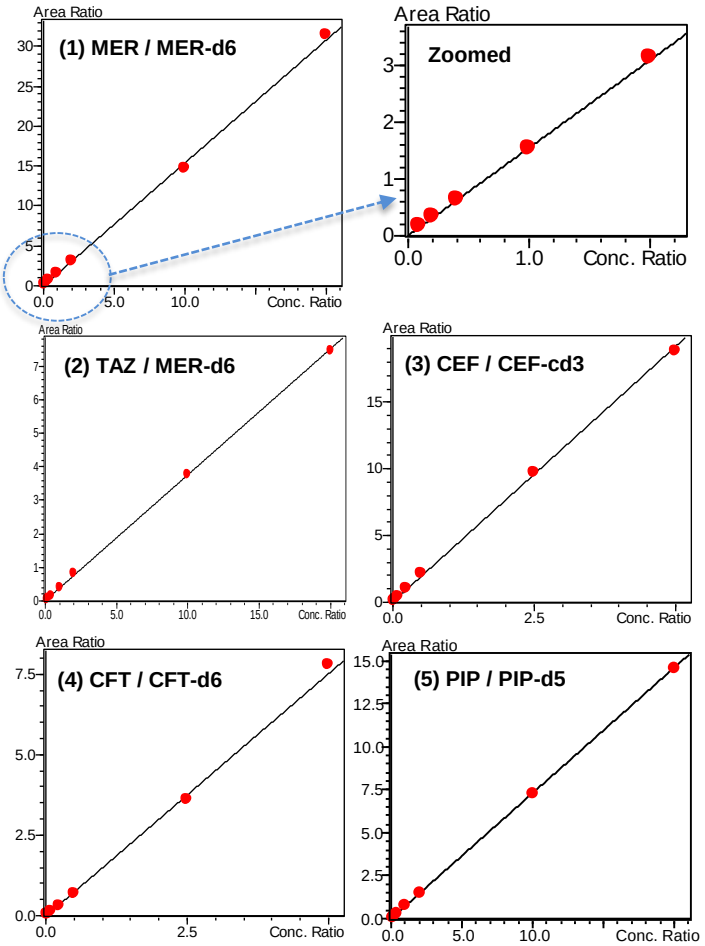

Figure 4: Calibration curves of five β-lactam antibiotics with stable isotope labelled internal standards in human plasma on LCMS-8060. Details of the calibration information are shown in Table 3.

Repeatability of the method on LCMS-8060 was evaluated with pre-spiked samples, post-spiked samples and mixed standards in solvent at low, middle and high concentration levels. The %RSD results of pre- and post-spiked sample are shown in Table 5. The results indicate excellent repeatability achieved, which is believed to be due to employing IS method and the excellent operation stability of the LCMS-8060 system.

Table 5: Results of RSD (%, n=5) of five β-lactam antibiotics with internal standards in plasma samples on LCMS-8060

Compd.	At 40 ug/mL		At 200 ng/mL		At 2000 ng/mL	
	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked
TAZ	4.1	1.8	4.0	4.2	4.9	3.9
CEF	5.1	5.0	4.2	2.0	1.9	3.8
MER	4.2	4.5	1.2	1.1	1.0	2.0
CFT	6.4	5.4	7.2	7.2	4.9	5.1
PIP	5.1	3.8	5.3	3.6	3.6	1.9

Recovery of the sample pre-treatment method was evaluated based on the peak area ratios of pre-spiked samples and post-spiked samples at all concentration levels. The results shown in Table 6 indicate excellent recovery were obtained.

Table 6: Recovery (%) of five β-lactam antibiotics in plasma samples by protein crash pre-treatment and determined on LCMS-8060

Compd.	Recovery (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	86	89	90	94	97	92	97
CEF	94	87	87	88	90	88	89
MER	103	104	98	102	102	97	103
CFT	113	94	93	97	103	100	101
PIP	104	106	98	105	103	98	102

Matrix effect of the method was determined by the peak area ratios of spiked samples and mixed standards in pure solvent at all concentration levels. The results are shown in Table 7. It can be seen that strong matrix effect occurred for CFT (33%~40%) and TAZ (128%~149%). This could be due to interference from plasma, which causes ion suppression and ion amplification. By further dilution of 2.5 times of the plasma samples with pure water before injection into LCMSMS, the matrix effects of CFT and TAZ were improved significantly to 62%~85% and 95%~109%, respectively.

Table 7: Results of matrix effect (%) of five β-lactam antibiotics in plasma samples on LCMS-8060

Compd.	Matrix effect (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ*	149	140	145	139	137	134	128
CEF	118	115	115	120	117	123	117
MER	93	93	101	98	99	99	103
CFT*	33	38	37	39	40	36	34
PIP	96	94	102	99	99	101	97

**Note: the matrix effect was improved significantly by diluting the plasma sample with pure water before injection to LCMS-8060.*

Specificity of the method for detection and confirmation of the five β-lactam antibiotics is demonstrated in Figure 5. In addition, the confirmation criteria include the MRM transitions, the ratios with reference MRM transitions (variation < 30%) as well as retention time (shift < 5%).

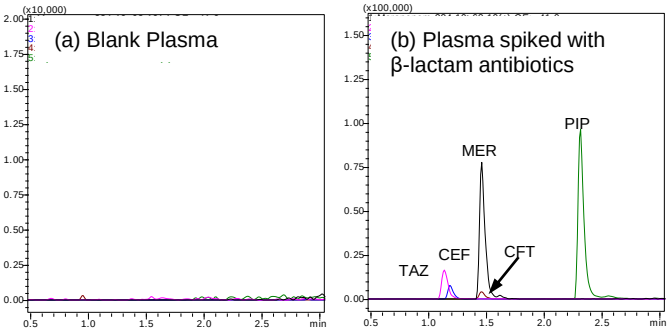

Figure 5: MRM chromatograms of blank plasma and plasma spiked with five β-lactam antibiotics (40 ng/mL) on LCMS-8060

Limit of quantitation (LOQ) of the method was estimated from the chromatograms of the lowest level spiked sample (40 ng/mL). Based on S/N = 10, the estimated LOQ of the method are 5.8, 6.0, 1.9, 2.9 and 0.7 ng/mL for TAZ, CEF, MER, CFT and PIP, respectively.

❑ Conclusions

A fast MRM-based method for quantitation of five β-lactam antibiotics tazobactam, cefepime, meropenem, ceftazidime and piperacillin in human plasma was developed on LCMS-8060. A simple sample pre-treatment with protein crash by organic solvent was applied and a small injection volume of 2 µL was required due to the high sensitivity of the LCMS-8060 employed. The method performance was evaluated on the linearity, accuracy, repeatability, recovery, matrix effect, specificity and limit of quantitation (LOQ). The estimated LOQs of the method for the five antibiotics are in the range from 0.7 ng/mL to 6.0 ng/mL with an injection volume of 2 µL.

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Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

ASMS 2017 MP-474

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Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

Introduction

Recently, metabolomics has been developed and applied to a variety of research fields, such as the food science, agriculture, engineering, and medical fields. In the medical research field, metabolomics is used to search for novel metabolite biomarkers of a variety of diseases and elucidate pathogenic mechanisms, etc., and there have been a considerable number of metabolite biomarker studies. As a step toward the practical use of metabolite biomarkers, a simple and quick automated sample preparation method involving metabolite extraction and metabolite measurement should be developed. In this study, we assessed whether the plasma levels of metabolites could be quantitatively measured using a fully automatic pre-treatment system for LC/MS that can be connected online to an LC/MS device.

Methods and Materials

Reagents: Acetonitrile (LC/MS grade), formic acid (LC/MS grade) and methanol (MeOH; LC/MS grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Morpholinoethanesulfonic acid (MES), which was employed as an internal standard of primary metabolites, was purchased from Sigma Aldrich. L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine were acquired from Sigma Aldrich (MO, USA). Isotopically labeled L-valine (D_8), L-leucine ($^{13}C_6$), L-isoleucine (D_{10}), L-tyrosine ($^{13}C_9$, ^{15}N), and L-phenylalanine (D8) were purchased from Cambridge Isotope Laboratories (MA, USA). Commercially available pooled plasma (Kohjin-Bio Co., Saitama, Japan), which was collected using EDTA-Na as an anticoagulant, was utilized as human plasma, and pooled plasma with the same lot number was used for all experiments.

Manual method: 20 µL of plasma (N=5) were mixed with 230 µL of MeOH containing 10 µM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine and MES as internal standards. Next, the mixture was shaken at 1,200 rpm for 30 min at room temperature, before being passed through an ultrafiltration filter (Amicon Ultra 0.5-mL centrifugal filters, Ultracel-3K). The mixture was then centrifuged at 14,000 g for 60 min at 4°C, and the collected solution was subjected to the LC/QqQMS-based analysis of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine.

Automatic method: The automatic method used to analyze L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine levels was performed using an CLAM-2000 (Shimadzu Corporation, Kyoto, Japan). In the CLAM-2000, MeOH containing 10 µM isotopically labeled L-valine, L-leucine, L-isoleucine, L-tyrosine, L-phenylalanine and MES (as internal standards) was added into the solvent container, and 20 µL of plasma (N=5) were also applied into another tubes. By running the CLAM-2000, 20 µL of plasma were automatically mixed with 230 µL of MeOH and the internal standards, before the resultant mixture was shaken at 1,900 rpm for 30 min at room temperature. Then, the mixture was automatically subjected to suction filtration for 90 sec, and the filtered solution was transferred to an SIL-30AC autosampler online, before being subjected to LC/QqQMS analysis.

Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

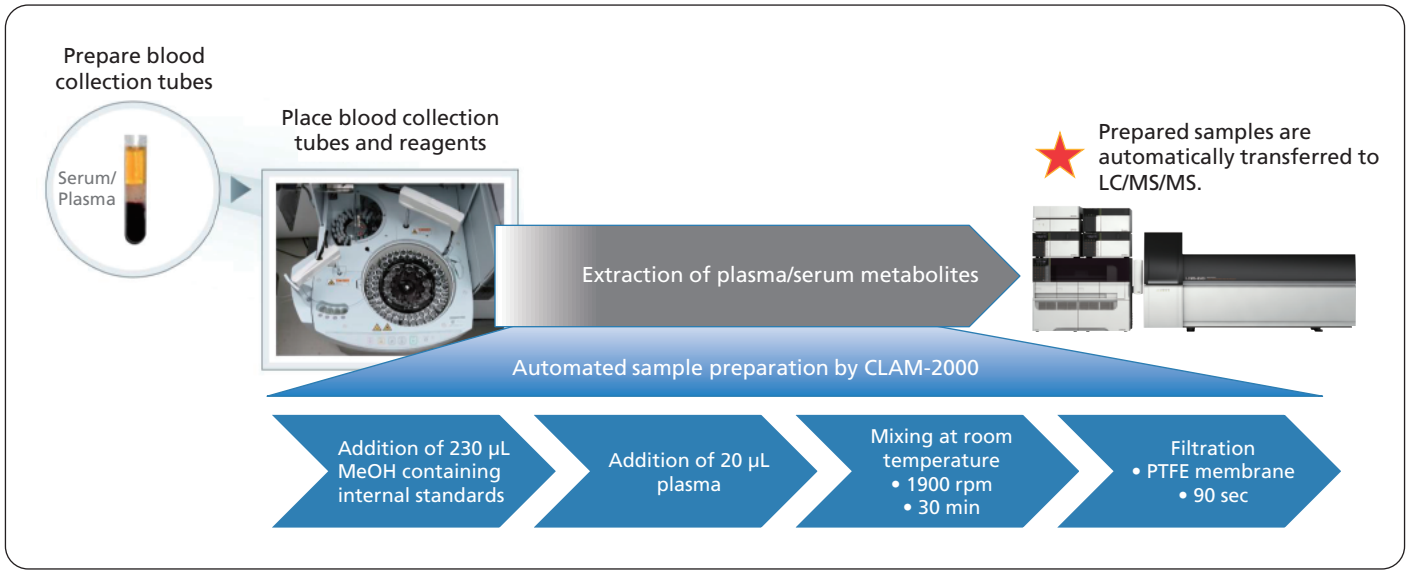

Figure 1. Workflow for analysis of metabolites using fully automated sample preparation LC/MS/MS system

HPLC conditions	
Column	: Discovery HS F5 2.1 mm × 150 mm, 3.0 µm
Mobile phase A	: 0.1% Formic acid/Water
Mobile phase B	: 0.1% Formic acid/Acetonitrile
Time program	: B conc. 25%(5 min) - 35%(11 min) - 95%(15 min) - 95%(20 min) - 0%(20.01-25 min)
Injection vol.	: 1 µL
Flow rate	: 0.25 mL/min.
Column temperature	: 40°C
MS conditions (LCMS-8040)	
Ionization	: ESI (Positive/Negative)
Nebulizing Gas Flow	: 2.0 L/min.
Drying Gas Flow	: 15.0 L/min.
DL temperature	: 250°C
Block Heater Temperature	: 400°C

For the analysis of primary metabolites except L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, LC/MS/MS Method Package for Primary Metabolites (Shimadzu Corporation, Kyoto, Japan) was used. The multiple reaction monitoring (MRM) transitions of the native and stable isotopes of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine are shown in Table 1.

An amino acid analysis of plasma samples with the same lot number was also performed by SRL (Tokyo Japan), and the plasma concentrations of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine (µM) were measured. The Fischer ratio was calculated based on the quantitative results. The resultant data are shown in the 'Reference concentration (µM)' (Table 2).

Table 1. The MRM transitions of native and stable isotope molecules of L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine

Product name	Precursor ion (*m/z*)	Product ion (*m/z*)
L-valine	118.1	72.15
L-tyrosine	182.1	136.1
L-isoleucine	132.1	86.2
L-leucine	132.1	86.05
L-phenylalanine	166.1	120.1
L-valine (D_8)	126.2	80.15
L-tyrosine ($^{13}C_9$, ^{15}N)	192.2	145.2
L-isoleucine (D_{10})	142.25	96.15
L-leucine ($^{13}C_6$)	138.15	91.15
L-phenylalanine (D_8)	174.2	128.2

Results and Discussion

The utility of the CLAM-2000 as an automatic pre-treatment system for analyzing hydrophilic blood metabolites was evaluated in the present study (Table 2). In this experiment, stable isotopes corresponding to the 5 targeted native metabolites; i.e., L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, were used for the quantitative analysis because the quantitative performance of MS is affected by various factors, such as ion suppression, and stable isotopes are required to obtain detailed quantitative information about the targeted molecules. The targeted metabolites included branched-chain and aromatic amino acids, and the Fischer ratio was calculated based on the quantitative results. In a comparison between the automatic method involving the CLAM-2000 and the manual method, the quantitative results, including the data regarding the Fischer ratio, obtained using the two methods were almost the same. In addition, these quantitative results were almost the same as those acquired by SRL. The measurement stability of each method was also high, and the metabolites' RSD% values were very low (<6%). Regarding the Fisher ratio data obtained using the two methods, the associated RSD% values were <1.5%. Regarding the metabolites except L-valine, L-leucine, L-isoleucine, L-tyrosine, and L-phenylalanine, the measurement stability of the automatic method is higher than that of the manual method (Table 3). These results suggest that the CLAM-2000 could be used for automatic pre-treatment during the analysis of hydrophilic blood metabolites.

Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

Table 2. Comparison between the manual and automatic methods for analyzing branched-chain and aromatic amino acids

Product name	Pre-treatment	Concentration (μM)	SD	RSD (%)	Reference concentration (μM)
L-valine	Manual method	210.6	8.19	3.89	215
	Automatic method	200.2	6.65	3.32	
L-leucine	Manual method	132.8	7.20	5.42	127
	Automatic method	126.4	4.30	3.40	
L-isoleucine	Manual method	65.6	3.38	5.15	69
	Automatic method	66.8	2.81	4.22	
L-tyrosine	Manual method	71.8	2.37	3.30	67
	Automatic method	67.8	2.58	3.80	
L-phenylalanine	Manual method	54.6	1.81	3.32	55
	Automatic method	54.5	1.97	3.61	
Fischer ratio	Manual method	3.24	0.048	1.47	3.4
	Automatic method	3.22	0.033	1.03	

Table 3. Comparison between the measurement stability of manual and automatic methods

	The number of detected metabolites	
Method	Manual method	Automatic method
Total	45	46
0%<RSD%£20%	33 (73.3%)	40 (87.0%)
20%<RSD%£50%	11 (24.4%)	6 (13.0%)
50%<RSD%	1 (2.2%)	0 (0%)

To the best of our knowledge, this is the first study in which the CLAM-2000 was utilized for metabolomics. The CLAM-2000 is a fully automatic pre-treatment device for LC/MS, and it can be connected online to an LC/MS system. Therefore, metabolome analysis using the CLAM-2000 might be suitable for measuring larger numbers of serum/plasma samples, because CLAM-2000 has no manual step leading to the decreased accident error by hand working, and moreover CLAM-2000 automatically can do the metabolite extraction and the following measurement of 60 serum/plasma samples in one batch. However, there are some issues related to our CLAM-2000-based procedure that remain to be evaluated. For example, the extracted solutions are directly transferred into an autosampler, but it might be better to dilute the extracted solutions with H_2O to reduce the percentage of organic solvent in the solution because a higher percentage might lead to column flooding and poor chromatography. In addition, removal of lipids from the extracted solutions may be also needed for the stable measurement. If these problems could be resolved, metabolome analysis using the CLAM-2000 could become more practical.

Evaluation of an automated LC-MS/MS system for analyzing hydrophilic blood metabolites

Table 4. Metabolites detected by using automated sample preparation

Amino acids				
Asymmetric dimethylarginine	Alanine	Arginine	Asparagine	Aspartic acid
Citrulline	Cysteine	Cystathionine	Cystine	Dimethylglycine
Glutamine	Glutamic acid	Glycine	Histidine	Hydroxyproline
Isoleucine	Leucine	Lysine	Methionine	Methionine-sulfoxide
Ornitine	Phenylalanine	Proline	Symmetric dimethylarginine	Serine
Threonine/Homoserine	Tryptophan	Tyrosine	Valine	
Organic acids				
cis-Aconitate	Citrate	Creatine	Isocitrate	Lactate
Malate	Pantothenate	Pyruvate	Uric acid	
Nucleosides and Nucleotides				
Adenosine	Guanosine	Inosine	Thymidine	Uracil
Uridine	AMP			
Others				
Carnitine	Kynurenine	Adenine	Choline	Acetylcarnitine
Creatinine				

Conclusions

- The use of the CLAM-2000 as a fully automatic pre-treatment system for LC/MS-based metabolomics facilitates the identification of metabolite biomarker candidates, the validation of metabolite biomarker candidates, and the practical use of metabolite biomarkers.
- Further optimization of our method for CLAM-2000-based metabolome analysis is necessary.

Acknowledgements

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Application News

No.C139

Liquid Chromatography Mass Spectrometry

Measurement of Enzymatic Activities in Dried Blood Spots with On-Line Solid Phase Extraction-LC/MS/MS System

Lysosomes are a type of intracellular organelle that uses a variety of hydrolytic enzymes to digest waste matter. To measure the enzymatic activity of lysosomes, methods using artificial fluorescent dyes and tandem mass spectrometry are used. Of these methods, tandem mass spectrometry offers the advantage of being able to measure multiple enzymatic activities at the same time.
In this example, a protocol developed at the Meyer Children's Hospital, Mass Spectrometry, Clinical Chemistry and Pharmacology Laboratory (Florence, Italy) was used to measure the enzymatic activity in dried blood spots (DBS) using an online solid phase extraction (SPE) - liquid chromatograph - tandem mass spectrometer (LCMS-8050) system. Because using this system results in samples being cleaned up during SPE, samples can be inserted directly for measurement after enzymatic reaction, without any pretreatment processes.

Sample Preparation and Analytical Conditions

Three enzymes were targeted, alpha-iduronidase (IDUA), acid alpha-glucosidase (GAA), and alpha-galactosidase A (GLA). DBS was used as sample. 3.2 mm diameter disks were punched from the DBSs and placed in a 96-well plate. Then a reaction solution containing respective enzyme substrates and internal standard substance (Genzyme) was added to each well and incubated for 22 hours at 37 ˚C. A flowchart of the preparation process is shown in Fig. 1.
Samples were analyzed using online SPE-LC and LCMS-8050 system. Respective reaction products were measured as the target compounds based on multiple reaction monitoring (MRM) using an internal standard substance. The structures of the target enzymatic reaction products and the internal standards are shown in Fig. 2. MRM transitions are listed in Table 1 and the LC and MS conditions in Table 2.

Online Solid Phase Extraction-Tandem Mass Spectrometer System

The online SPE-LC/MS/MS system configuration is shown in Fig. 3. When the enzymatic reaction was complete, the sample was injected directly and measured. The trapping and cleanup procedure was centered on a Perfusion column POROS® R1 and separation chromatography was performed through a Shim-pack XR-ODS. The two operations are articulated through the following steps. Upon the injection, the sample is cleaned through the Perfusion column with an aqueous solution (solvent A) and delivered by pump A at 1.2 mL/min for 1 min. With the activation of the valve, the Perfusion column is connected in line with the ODS column and both are flowed by 300 µL/min of organic solvent (solvent B). With the switching-back of the valve, occurring at 3 min, the Perfusion column is re-equilibrated with a solvent A and delivered by pump A at 1.2 mL/min for 2.2 min. Using this system eliminates the need for desalting and purification processes.

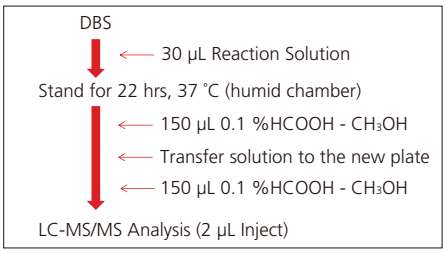

Fig. 1 Preparation Process Flowchart

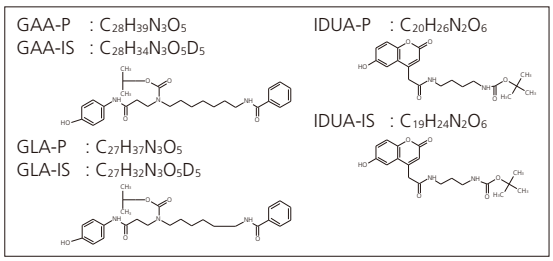

Fig. 2 Structures of Enzymatic Reaction Products and Internal Standards

Table 1 MRM Transitions

Compounds	Polarity	Precursor ion *m/z*	Product ion *m/z*
IDUA-P	+	391.2	291.2
IDUA-IS	+	377.3	277.2
GAA-P	+	498.4	398.3
GAA-IS	+	503.4	403.3
GLA-P	+	484.3	384.3
GLA-IS	+	489.3	389.3

•Note: P: Product, IS: Internal Standard

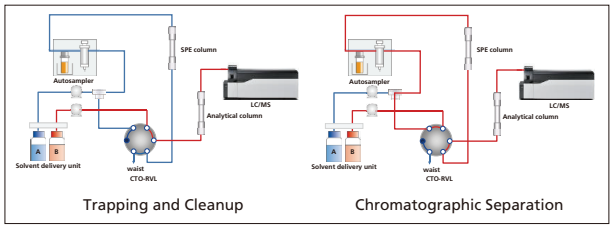

Fig. 3 MRM Chromatograms of Each Target Compound

Table 2 Analytical Conditions

<LC>

Analytical Column	: Shim-pack XR-ODS (75 mm L. × 2.0 mm I.D., 2.2 µm)
Trapping Column	: POROS® R1 (30 mm L. × 2.1 mm I.D., 20 µm)
Solvent A	: 0.05 % HCOOH-5 mM $HCOONH_4$-H_2O
Solvent B	: 0.1 % HCOOH-CH_3OH
Ratio	: 50 %B
Flowrate	: 0.6 mL/min
Oven Temperature	: 30 ˚C
Injection Volume	: 2 µL
Analysis Time	: 5.5 min

<MS>

Instrument	: LCMS-8050
Ionization Mode	: ESI (+)
Interface Temperature	: 100 ˚C
DL Temperature	: 100 ˚C
Heat Block Temperature	: 100 ˚C
Nebulizing Gas Flow	: 3 L/min.
Heating gas Flow	: 5 L/min.
Drying Gas Flow	: 15 L/min.

Measurement Results

The enzymatic activities of IDUA, GLA, and GAA were measured. Examples of enzymatic activity measurement results are shown in Table 3 and MRM chromatograms of each target compound in Fig. 4. Filter paper without any blood was used as blanks. Sample A is a sample that contains enzymes with each activity, Sample B is missing IDUA, Sample C is missing GLA, and Sample D is missing GAA. Whereas peaks were clearly detected for enzyme decomposition products in Sample A, there was a decrease in target peaks in Samples B to D.

Table 3 Example of Enzymatic Activity Measurement Results

	IDUA (μmol/h/L)	GLA (μmol/h/L)	GAA (μmol/h/L)
Blank	0.3	0.1	0.1
Control	15.2	10.1	6.0
Sample A	13.2	6.6	7.3
Sample B	1.8	6.9	5.8
Sample C	17.6	0.5	9.8
Sample D	8.4	2.0	0.8

Activity (μmol/h/L) = [(P/IS) × [IS] × 30/3.4] / 22
· (P/IS): Area ratio between product (P) and internal standard (IS)
· [IS]: Concentration (μmol/L) of internal standard (IS)
· 30: Volume of solution added (μL)
· 3.4: Volume of blood in DBS (μL)
· 22: Reaction time (hr)

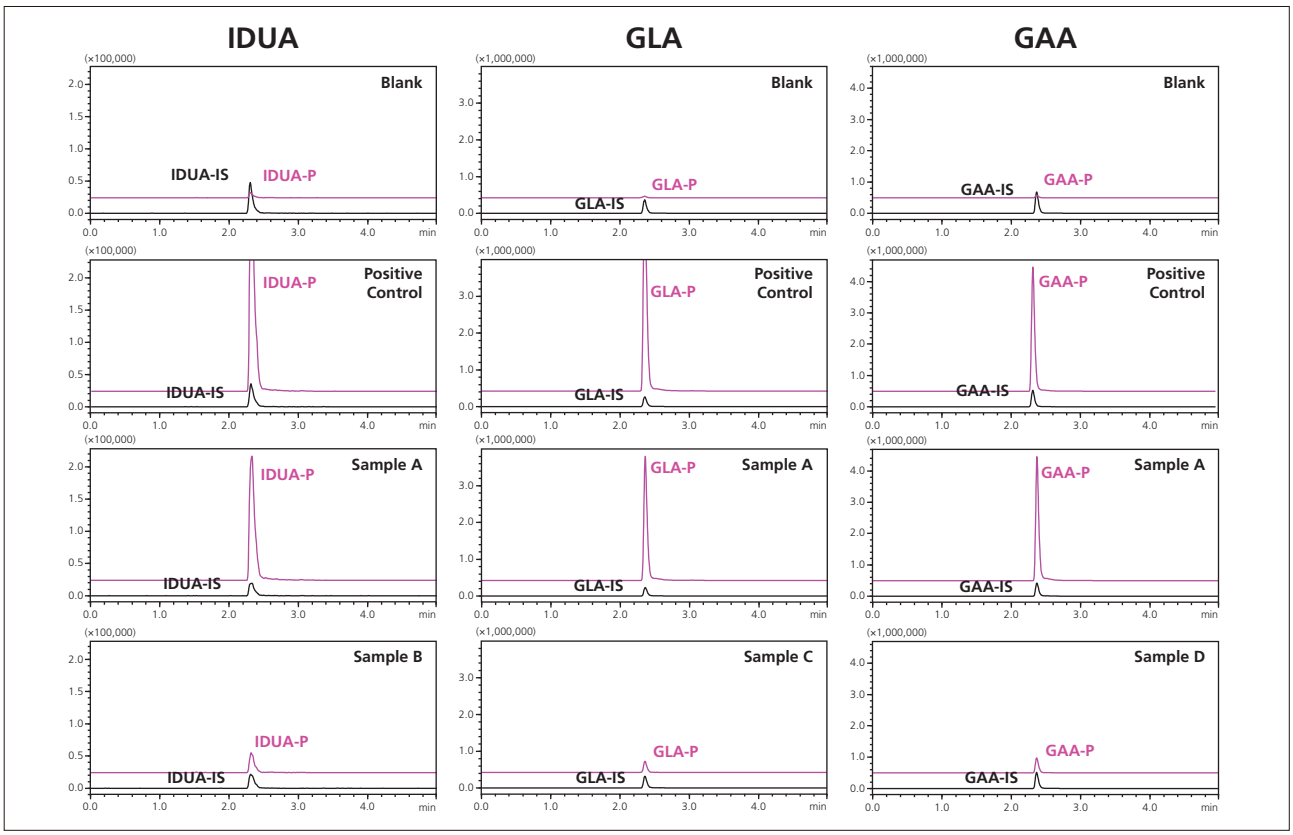

Fig. 4 MRM Chromatograms of Each Target Compound
Blank: Sample filter paper containing no blood; Sample A: Sample containing enzymes with all activities; Sample B: Sample missing IDUA; Sample C: Sample missing GLA; and Sample D: Sample missing GAA

[References]
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[Acknowledgments]
This Application News bulletin was prepared based on guidance and samples provided by Dr. G. la Marca (Meyer Children's Hospital, Mass Spectrometry, Clinical Chemistry and Pharmacology Laboratory, Florence, Italy). We are sincerely grateful for his help.

Notes: The equipment mentioned in this article has not been approved/certified as medical equipment under Japan's Pharmaceutical and Medical Device Act.
The analytical methods described in this article cannot be used for diagnostic purposes.

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PO-CON1531E

Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

ASMS 2015 ThP-251

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Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

Introduction

Analysis of amino acids (AA) and acylcarnitines (AC) in dried blood spot (DBS) sample collection method by flow injection analysis (FIA) is now widely used. On the other hand, traditionally, analysis of steroid such as 17-hydroxyprogesterone is done by immunoassays but LC/MS/MS will be an attractive analytical alternative because it can also screen for other related steroids.

The use of LC/MS/MS results in a reduction of false positives and a more accurate quantitative performance. The requirements against steroid analysis by LC/MS/MS are getting more stringent issues. In this study, we present a strategy for performing both AA/AC and steroids analysis within a single LC/MS/MS platform.

Methods and Materials

The experimental setup designed to combine a FIA measurement covers 8 AAs and 17 ACs and a LCMS measurement for 5 steroids includes cortisol, 21-deoxycortisol (21-DOF), 11-deoxycortisol (11-DOF), androstenedione (4-AD) and 17-hydroxyprogesterone (17-OHP) with 2 position 6 port high pressure valve.

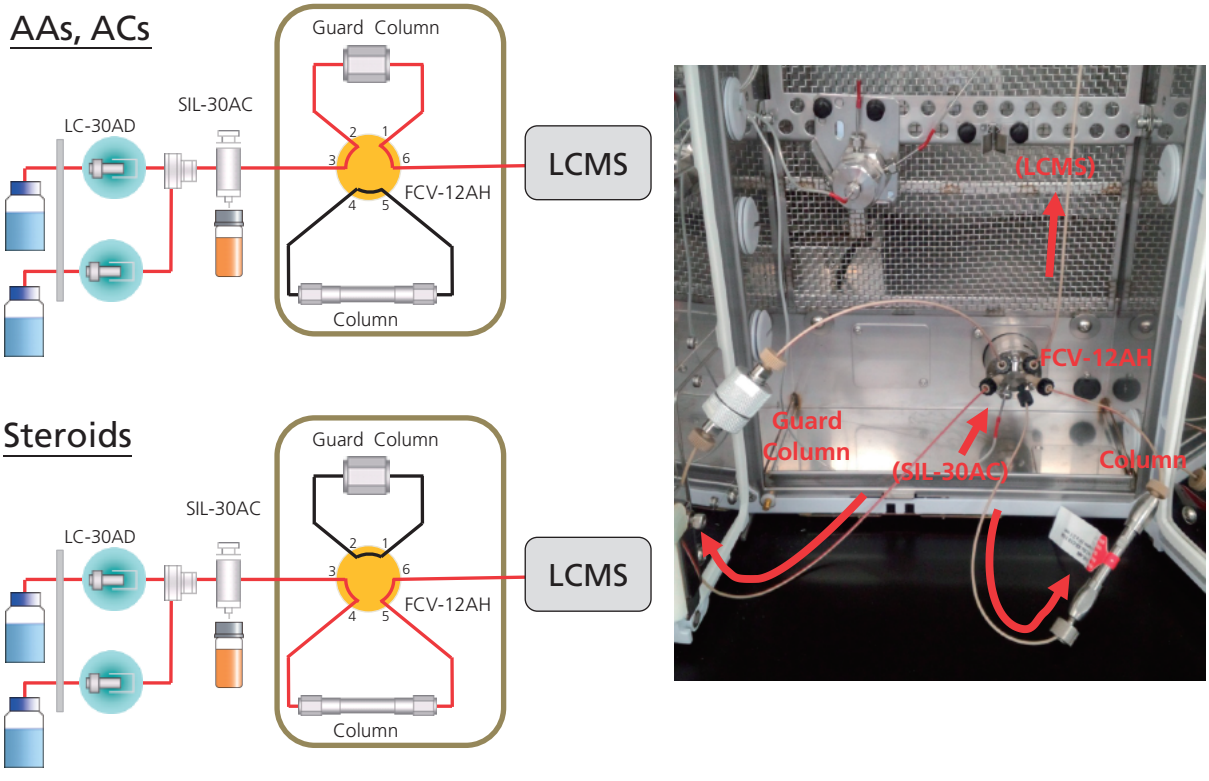

Figure 1 Flow Diagram of FIA/LC-MS/MS system

Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

The isotopically labeled internal standards for amino acids, acylcarnitines, and steroids were purchased from Cambridge Isotope Laboratories, Inc. Quality control materials were obtained from the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC).

Analytical Conditions

HPLC

Mobile Phase A : 0.1% Formic acid - water
Mobile Phase B : Methanol
Column temperature : 40 °C

[for Amino Acids and Acylcarnitines]

Guard Column : GL Sciences Cartridge Guard Column E (10 mmL x 1.5 mm I.D.)
Gradient Program :

Time	B conc. (%)	Flow rate (mL min)
0	90	0.13
0.65	90	0.13
0.66	90	0.7
1.00	90	0.7

Injection Volume : 1 µL

[for Steroids]

Column : Phenomenex Kinetex 2.6u XB-C18 (50 mmL x 2.1 mm I.D., 2.6µm)
Flow Rate : 0.3 mL/min
Gradient Program :

Time	B conc. (%)
0	50
0.5	55
1.5	55
3.0	90
5.0	90

Injection Volume : 10 µL

Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

Mass (LCMS-8050 triple quadrupole mass spectrometry)

Ionization	: heated ESI		
Nebulizing Gas Flow	: 3 L / min	Drying Gas Pressure	: 10 L / min
Heating gas flow	: 10 L/min	DL Temperature	: 250 °C
BH Temperature	: 500 °C	Interface Temperature	: 400 °C
MRM parameter	:		

Target	Q1>Q3		IS	Q1>Q3
Phe	166.10>120.10	-	Phe IS	172.10>126.10
Leu	132.10>86.10	-	Leu IS	135.10>89.10
Met	150.10>104.10	-	Met IS	153.10>107.10
Tyr	182.10>136.10	-	Tyr IS	188.10>142.10
Val	118.10>72.10	-	Val IS	126.10>80.10
Cit	176.10>113.10	-	Cit IS	178.10>115.10
Arg	175.10>70.10	-	Arg IS	180.10>75.10
Ala	90.00>44.00	-	Ala IS	94.00>48.00
C0	162.10>103.00	-	C0 IS	171.10>103.00
C2	204.10>85.00	-	C2 IS	207.10>85.00
C3	218.10>85.00	-	C3 IS	221.10>85.00
C4	232.20>85.00	-	C4 IS	235.20>85.00
C4OH	248.20>85.00			

Target	Q1>Q3		IS	Q1>Q3
C5	246.20>85.00	-	C5 IS	255.20>85.00
C5DC	276.10>85.00	-	C5DC IS	279.10>85.00
C5OH	262.20>85.00	-	C5OH IS	265.20>85.00
C6	260.20>85.00	-	C8 IS	291.20>85.00
C8	288.20>85.00			
C10	316.20>85.00	-	C12 IS	347.30>85.00
C12	344.30>85.00			
C14	372.30>85.00	-	C14 IS	381.30>85.00
C16	400.30>85.00	-	C16 IS	403.30>85.00
C16OH	416.30>85.00			
C18	428.40>85.00	-	C18 IS	431.40>85.00
C18OH	444.40>85.00			

	R.T(min)	Q1>Q3	CE (V)
Cortisol	1.50	363.15>121.10	-31
		363.15>327.10	-17
21-DOF	1.85	347.15>311.15	-15
		347.15>121.10	-28
11-DOF	2.20	347.15>109.10	-31
		347.15>97.05	-27
4-AD	2.65	287.15>97.05	-24
		287.15>109.10	-24
17-OHP	3.02	331.15>109.05	-29
		331.15>97.10	-30

	R.T(min)	Q1>Q3	CE (V)
Cortisol-D2	1.50	365.15>122.10	-27
		365.15>329.10	-18
21-DOF-D8	1.82	355.20>319.25	-18
		355.20>46.10	-45
11-DOF-D2	2.23	349.20>109.05	-33
		349.20>97.10	-29
4-AD-13C3	2.65	290.15>100.00	-22
		290.15>112.00	-28
17-OHP-D8	3.00	339.20>100.10	-27
		339.20>113.10	-31

DBS samples (d = 5mm) were placed in 96-well plates, and AAs, ACs and steroids were extracted with 180 µL of 80% acetonitrile-water solution consists of the known concentrations of stable isotope labeled standards of each compounds. The extraction were performed in an ultrasonic bath for 30 min. Samples were measured using a Nexera UHPLC system coupled to LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan).

Result

DBS provides a number of advantages, for examples, a less invasive and much simpler sample collection method rather than venipuncture technique. Furthermore, it provides you simpler storage and transportation as well as it can lower the infection risk of various pathogens, and requires a smaller blood volume. To date, DBS-LC-MS/MS has emerged as an important method for quantitative analysis of small molecules.

Previously we developed an innovative AAs and ACs screening method makes it possible to inject just 1 µl of sample and successfully reduce analytical run time as fast as 74 seconds (conventional method >120 sec.) using the combination of Nexera MP and LCMS-8040 (Shimadzu Corporation, Japan). In addition to that, we independently developed a method for steroids in DBS. Steroids were separated on a Phenomenex kinetex XB-C18 (50x2mm, 2.6µm) at a column temperature of 40 °C for 5 min.

In this study, we present a strategy for performing AAs, ACs and steroids analysis within a single LC/MS/MS platform. AAs, ACs and steroids were extracted from only one dried blood spot. This system enables to automatically analyze 7 min in all target analytes in 2 injections.

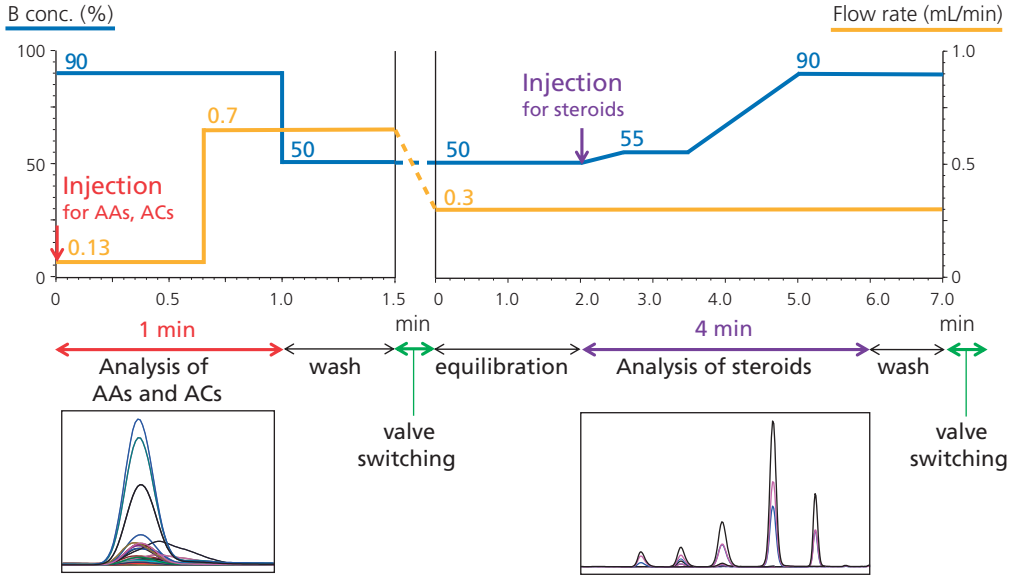

Figure 2 Time Program of amino acids, acylcarnitines and steroids analysis

Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

Table 1 Data Summary of 8 amino acids, 17 acylcarnitines and 5 steroids

	Phe	Leu	Met	Tyr	Val	Cit	Arg	Ala		C0	C2	C3	C4
No.1321	65.3	172.91	17.57	54.12	132.99	32.19	14.33	192.19	No.1361	22.25	14.65	1.43	0.1
CV	1%	5%	9%	1%	4%	4%	9%	3%	CV	4%	9%	2%	11%
Target	66.1	131.6	15.9	49.1	127.2	26.2	15	179.1	Target	17	12.4	1.2	0.1
No.1322	166.28	291.55	58.11	236.31	315.48	55.37	100.26	295.09	No.1362	32.23	25.4	4.75	0.97
CV	1%	4%	8%	2%	1%	3%	2%	3%	CV	1%	9%	4%	10%
Target	157	221.2	52.2	211	262.2	52.5	97.4	261	Target	29	21.5	4	0.9
No.1323	253.12	418.99	141.41	415.87	438.18	127.41	192.38	391.76	No.1363	47.27	32.72	9.78	2.28
CV	1%	1%	9%	5%	1%	4%	1%	5%	CV	4%	4%	6%	8%
Target	245.1	345.3	123.8	375.5	360.2	118.7	184.2	348.6	Target	40.1	30.5	8	2.2
No.1324	319.28	598.09	221.83	554.31	500.46	254.09	269.5	441.5	No.1364	62.3	48.82	16.36	5.05
CV	0%	3%	5%	2%	1%	1%	0%	5%	CV	4%	5%	5%	7%
Target	330.5	552.8	192.5	516.9	464.4	237.2	260.5	422.2	target	55.6	40.6	13.2	4.4

	C4OH	C5	C5DC	C5OH	C6	C8	C10	C12	C14	C16	C16OH	C18	C18OH
No.1361	0.05	0.1	0.12	0.72	0	0.02	0.02	0.01	0.05	0.78	0.01	0.57	0
CV	17%	19%	14%	2%	-	31%	35%	24%	15%	2%	-	1%	-
Target	0.1	0.1	0	0.6	0	0	0	0	0.1	0.8	0	0.6	0
No.1362	0.28	0.48	0.51	1.09	0.45	0.49	0.49	0.42	0.46	3.14	0.08	1.23	0.07
CV	11%	4%	7%	5%	7%	2%	4%	4%	1%	2%	9%	2%	3%
Target	0.4	0.5	0.5	1	0.4	0.5	0.5	0.4	0.5	3.5	0.1	1.5	0.1
No.1363	0.53	1.51	0.93	2.11	0.93	1.04	1	0.87	1.44	7.23	0.36	2.08	0.33
CV	10%	4%	8%	4%	6%	2%	1%	1%	2%	2%	6%	1%	2%
Target	0.7	1.3	1	1.8	0.8	1	1	0.9	1.4	7.2	0.4	2.2	0.3
No.1364	1.42	2.79	2.45	3.07	2.37	2.54	2.5	2.1	2.83	10.41	0.72	4.49	0.68
CV	8%	4%	3%	6%	2%	1%	2%	0%	2%	1%	1%	0%	4%
Target	1.6	2.7	2.4	2.7	1.9	2.4	2.4	2	2.6	10.5	0.7	4.8	0.7

Integration of amino acid, acylcarnitine and steroids analysis in single FIA/LC-MS/MS platform

	Conc. (ng/mL)	Area	%RSD	S/N	LOD (ng/mL)	LOQ (ng/mL)
Cortisol	5	15,919	6.05	66.1	0.23	0.76
	25	54,064	2.11	246.3		
	75	186,970	0.85	681.0		
	125	322,782	1.68	971.9		
	150	345,172	0.26	994.9		
	500	1,307,317	0.16	1606.4		

	Conc. (ng/mL)	Area	%RSD	S/N	LOD (ng/mL)	LOQ (ng/mL)
4-AD	5	97,910	0.62	184.1	0.08	0.27
	25	387,155	0.92	946.5		
	75	1,247,236	0.08	2338.6		
	125	2,187,611	0.26	5318.0		
	150	2,406,566	0.36	4459.4		
	500	9,143,414	0.69	36564.1		

	Conc. (ng/mL)	Area	%RSD	S/N	LOD (ng/mL)	LOQ (ng/mL)
21-DOF	5	23,687	1.87	27.8	0.54	1.80
	25	98,627	3.64	103.0		
	75	388,221	2.04	473.1		
	125	701,690	1.16	181.9		
	150	758,860	0.43	537.1		
	500	3,000,289	0.70	214.5		

	Conc. (ng/mL)	Area	%RSD	S/N	LOD (ng/mL)	LOQ (ng/mL)
17-OHP	5	40,107	6.12	79.3	0.19	0.63
	25	157,773	2.85	366.5		
	75	504,027	1.03	830.9		
	125	874,209	1.56	3824.8		
	150	951,763	0.62	1635.5		
	500	3,694,638	0.20	1932.9		

	Conc. (ng/mL)	Area	%RSD	S/N	LOD (ng/mL)	LOQ (ng/mL)
11-DOF	5	39,161	1.53	60.5	0.25	0.83
	25	167,525	2.43	218.1		
	75	534,404	0.39	436.3		
	125	964,525	0.26	94.2		
	150	1,029,424	0.53	695.0		
	500	3,872,397	0.60	97.9		

Conclusions

This platform is an effective tool for an initial screening and able to minimize sample consumption down to 1 uL. However, the use of a high performance LC/MS/MS system is highly recommended in order to achieve the appropriate level of sensitivity for the steroids especially. Using high performance LC/MS/MS (LCMS-8050, Shimadzu Corporation, Japan), the observed limit of detection (LOD) for the analysis of 17-hydroxyprogesterone was 0.19 ng/mL.

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

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).

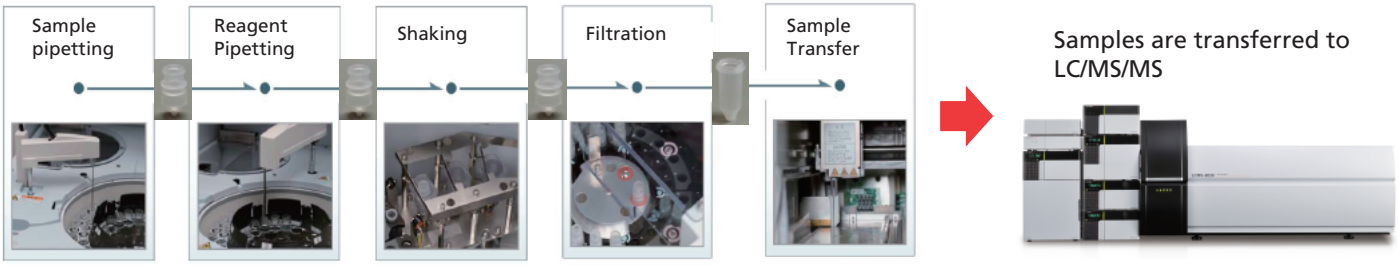

Figure 1: Procedure of protein crash and spiked-sample preparation

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

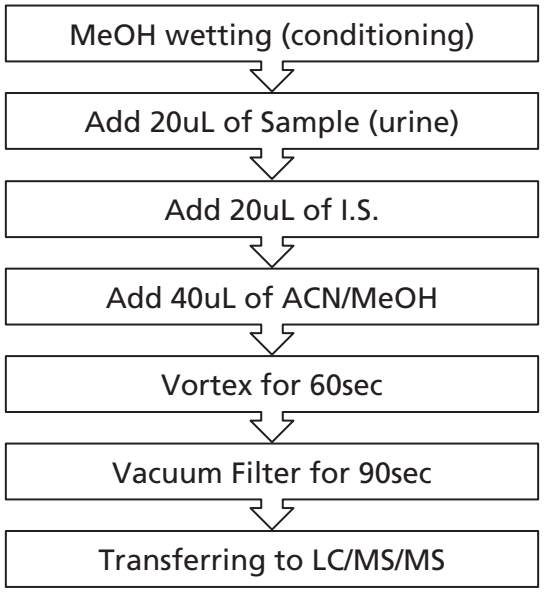

Figure 2: Typical auto-workflow of urine sample via protein-crash and adding IS for LC/MS/MS by CLAM-2000

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

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.

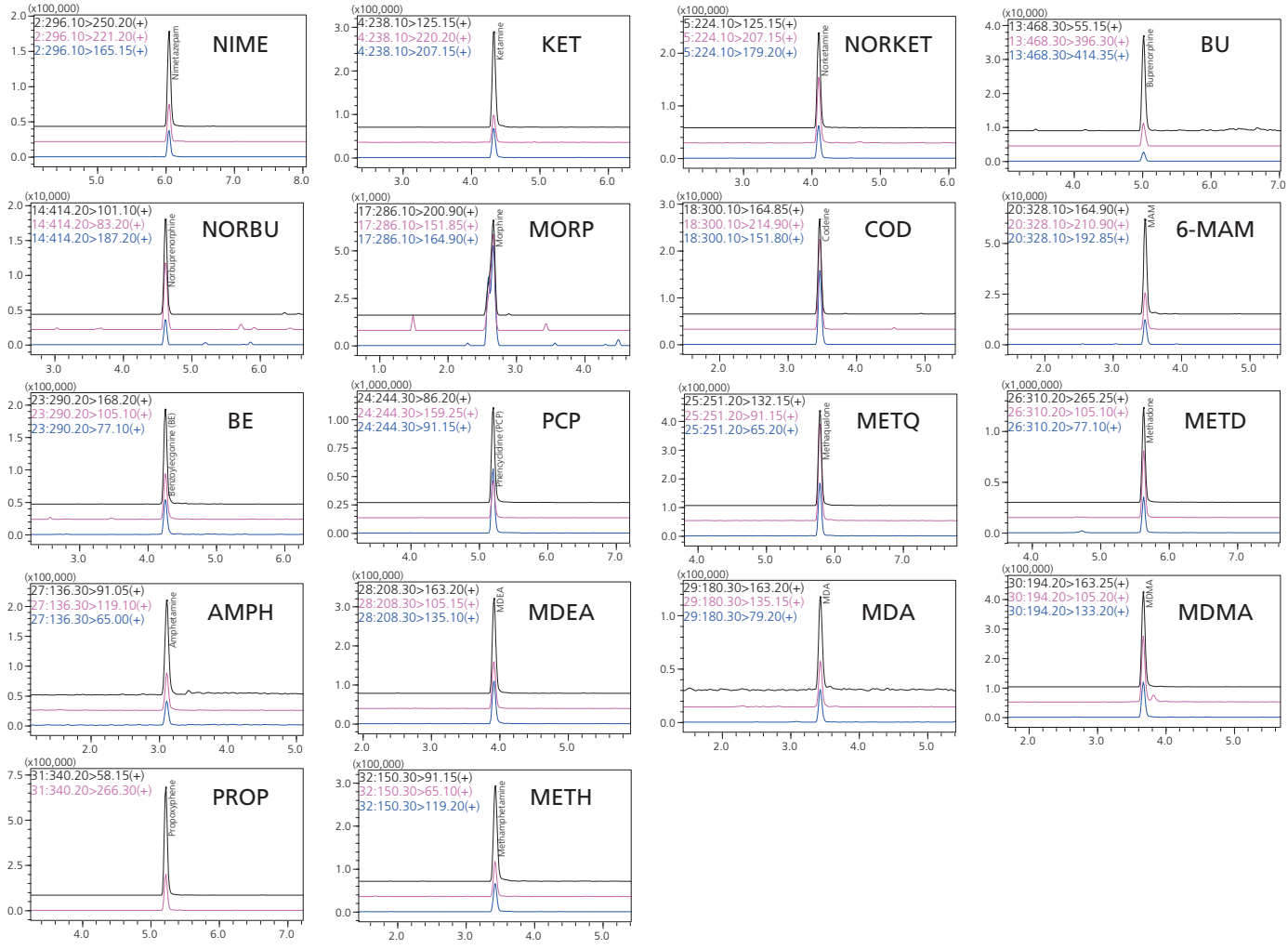

Figure 3: Individual MRM chromatograms of eighteen illicit drugs each (200 ng/mL) and fourteen ISs (100 ng/mL) spiked in urine obtained on CLAM-LC/MS/MS platform.

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

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

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

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.

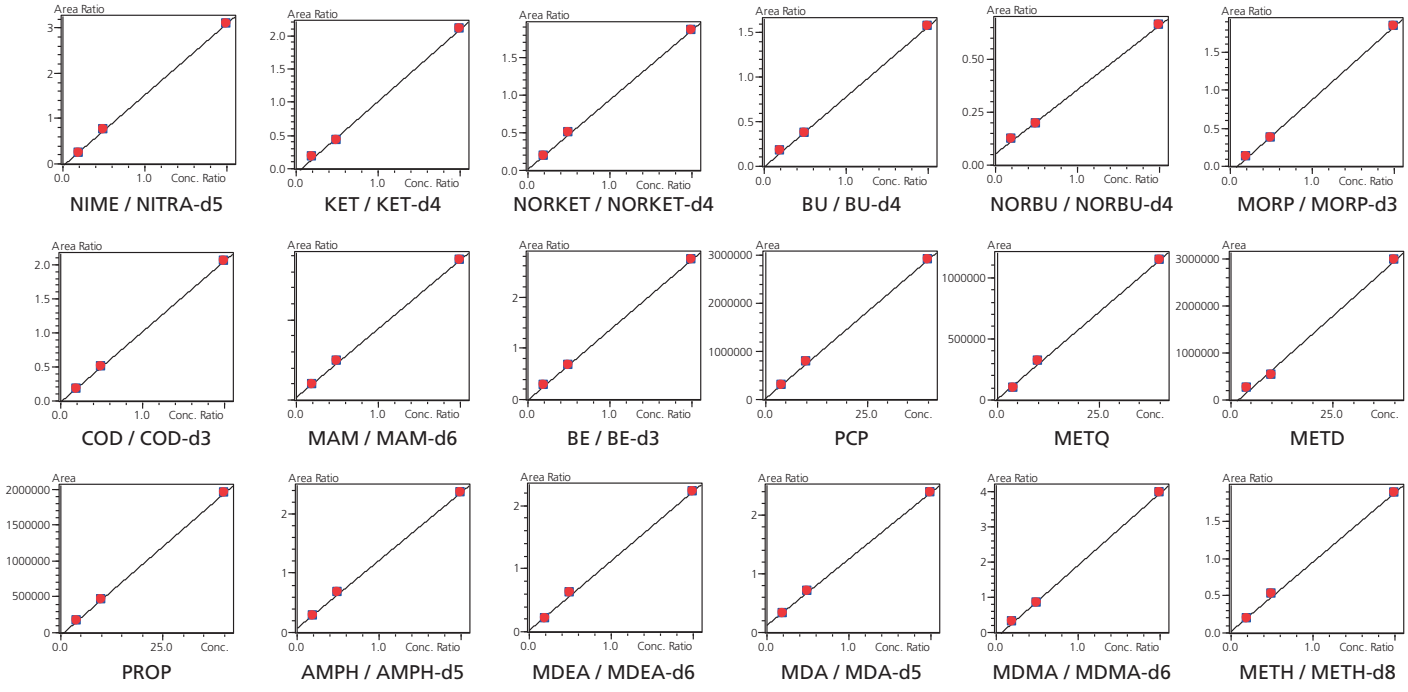

Figure 4: Calibration curves of 14 illicit drugs with isotope-labelled internal standards and 4 illicit drugs with external standard in human urine on LCMS-8040. Details are shown in Table 3.

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

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

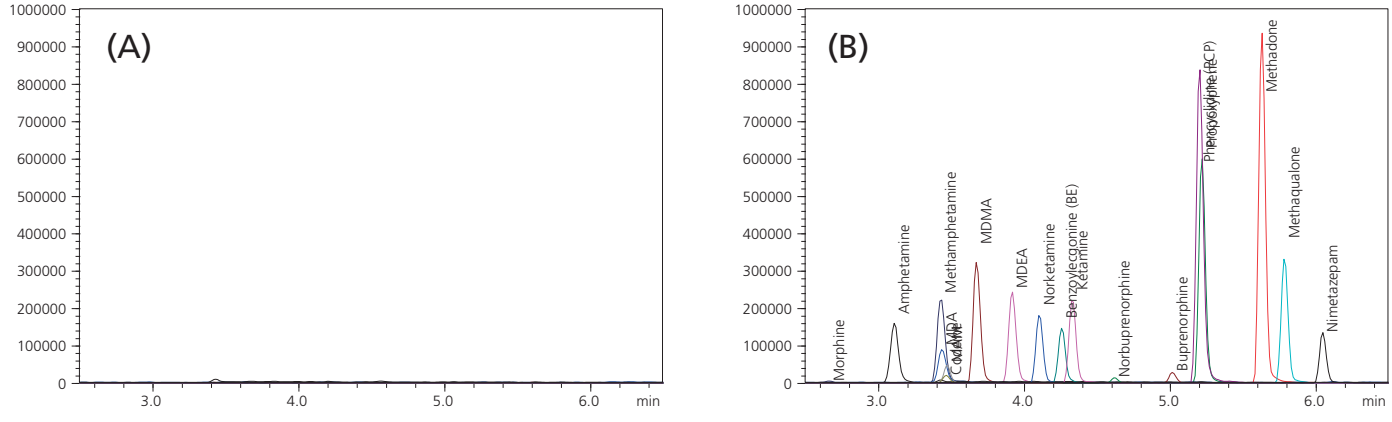

Figure 5: Total MRM chromatograms of (A) blank urine and (B) spiked urine with eighteen illicit drugs (200 ng/mL).

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

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.

Disclaimer: The data and instruments presented in this Application News are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

First Edition: June, 2017

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Application News

No.C142A

Liquid Chromatography Mass Spectrometry

Screening Analysis of Highly Polar Doping Agents in Urine Using 2DLC/MS/MS

The use of performance-enhancing drugs, or "doping," has been recognised for decades and since 1999 the World Anti-Doping Agency (WADA) has governed and harmonized the worldwide sports drug testing efforts. However, these needs are changing and the continuing. discovery of new doping strategies with naturally occurring substances, such as androgenic steroids, pro-hormones and related metabolites, peptide hormones, as well as the emergence of designer drugs and the manipulation of blood and blood components results in sports drug testing methods which are capable of a range of tests. In this application news, we report the simultaneous analysis of highly polar doping agents including meldonium and adrenergic agents such as synephrine, norfenefrine, etilefrine, oxilofrine and octopamine using 2D LC/MS/MS.

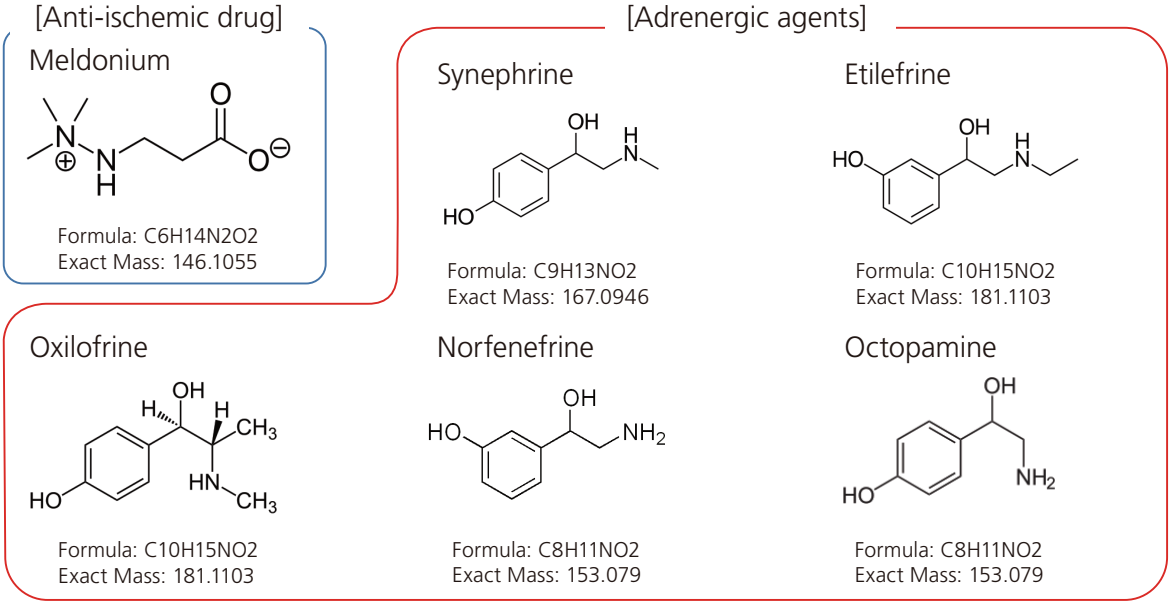

Fig. 1 Structures of 6 Compounds

Table 1 Analytical Conditions

[LC] NexeraX2 System		[MS] LCMS-8060	
Analytical Column	: Nucleodur HILIC (100 mm L. × 2 mm I.D., 1.8 µm)	Ionization	: ESI (+/-)
Trapping Column	: Nucleodur HILIC (20 mm L. × 2 mm I.D., 3 µm)	Nebulizing Gas Flow	: 3.0 L/min.
Mobile Phase	: A: H_2O + 5 % buffer,	Drying Gas Flow	: 15.0 L/min.
	B: Acetonitrile + 5 % buffer,	Heating Gas Flow	: 15.0 L/min.
	C: Acetonitrile + 5 % buffer	HB Temp.	: 500 ˚C
	(buffer: 200 mM Ammonium Acetate + 0.15 % glacial acetic acid)	DL Temp.	: 300 ˚C
		Interface Temp.	: 400 ˚C
Column Oven Temp.	: 40 ˚C		
Injection Volume	: 30 µL		

MRM parameter:

#	Name	Polarity	Q1	Q3 Qualifier 1	Q3 Qualifier 2	Ret. Time (min)	CE Qualifier 1	CE Qualifier 1
1	Meldonium	+	147.20	58.25	59.25	8.18	-27	-18
2	Etilefrine	+	182.30	135.25	91.25	5.34	-20	-27
3	Norfenefrine	+	154.20	91.25	65.25	6.01	-21	-35
4	Octopamine	+	154.20	91.25	119.20	6.00	-21	-15
5	Oxilofrine	+	182.30	149.25	105.25	5.69	-20	-22
6	Synefrine	+	168.20	135.20	107.25	5.87	-20	-31
7	Meldonium-d3	+	150.20	62.25	60.25	8.18	-18	-30
8	Etilefrine sulphate	+	262.20	164.15		5.19	-19	
9	Synefrine sulphate	+	248.20	150.25	135.20	5.68	-15	-30
10	Norfenefrine sulphate	+	234.20	136.20	91.20	5.62	-18	-35
11	Etilefrine sulphate_neg	-	260.20	180.20	121.10	5.19	18	39
12	Oxilofrine sulphate_neg	-	260.20	77.10	178.20	5.49	26	12
13	Synefrine sulphate_neg	-	246.20	148.20	106.10	5.70	20	30
14	Norfenefrine sulphate_neg	-	232.20	152.20	121.15	5.69	17	36
15	Octopamine sulphate_neg	-	232.20	134.15	107.10	5.81	22	30

#7 : Internal Standard
#8 ～ 15 : Confirmation of Sulpho-conjugate

Compound list including MRM transitions for unchanged parent drug molecules and corresponding sulfonated metabolites. Rapid polarity switching was used during the analysis to confirm peak identification.

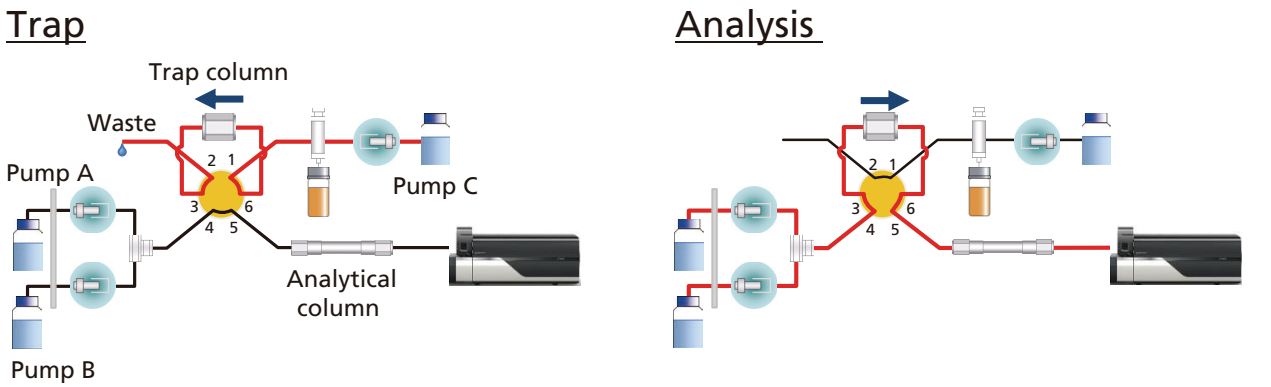

Fig. 2 Flow Diagram of 2D-HILIC System

Diluted urine samples were injected directly onto the 2D HILIC system using a HILIC trapping column for clean-up and pre-concentration followed by an effective HILIC analytical separation.

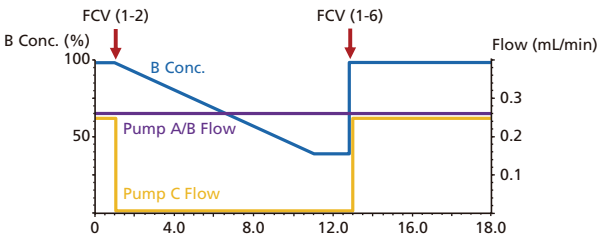

Fig. 3 Flow Rate and Gradient Program

Sample Preparation of Urine Sample

1. Centrifuge urine samples at 3,000 rpm for 10 min at room temperature.
2. Transfer 60 µL supernatant to new tube and add 10 µL IS solution (*) and 140 µL acetonitrile, mix the solution by vortex mixing.
3. Centrifuge at 13,000 rpm for 5 min.
4. Transfer 180 µL supernatant to vial.
   (*) Meldonium-d3 in 200 mM Ammonium Acetate

Calibration Curves

Fig. 4 shows calibration curves of 6 compounds spiked into urine. Meldonium was included in the World Anti-Doping Agency (WADA) Prohibited List on 1 January 2016, the guidance for meldonium in urine samples collected after 30 September 2016 applies normal results management to samples above a concentration of 100 ng/mL. In this method, the urine calibration range between 1 to 200 ng/mL resulted in a linear response for all compounds with regression coefficients r2 > 0.997.

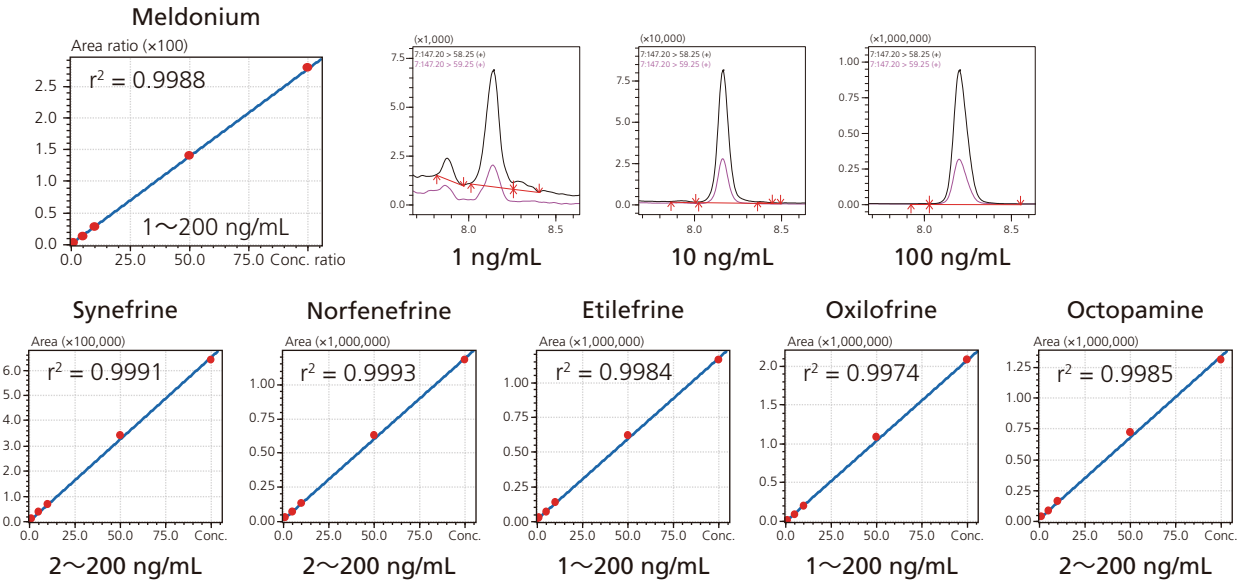

Fig. 4 Calibration Curves and MRM Chromatograms of 6 Compounds

Analysis of Synephrine, Etilefrine and Oxilofrineine in Urine

Each urine samples were collected from volunteers being separately administered with synephrine, etilefrine and oxilofrine were analyzed using 2D-HILIC System. In all samples, both the unchanged form and sulphated metabolites were detected.

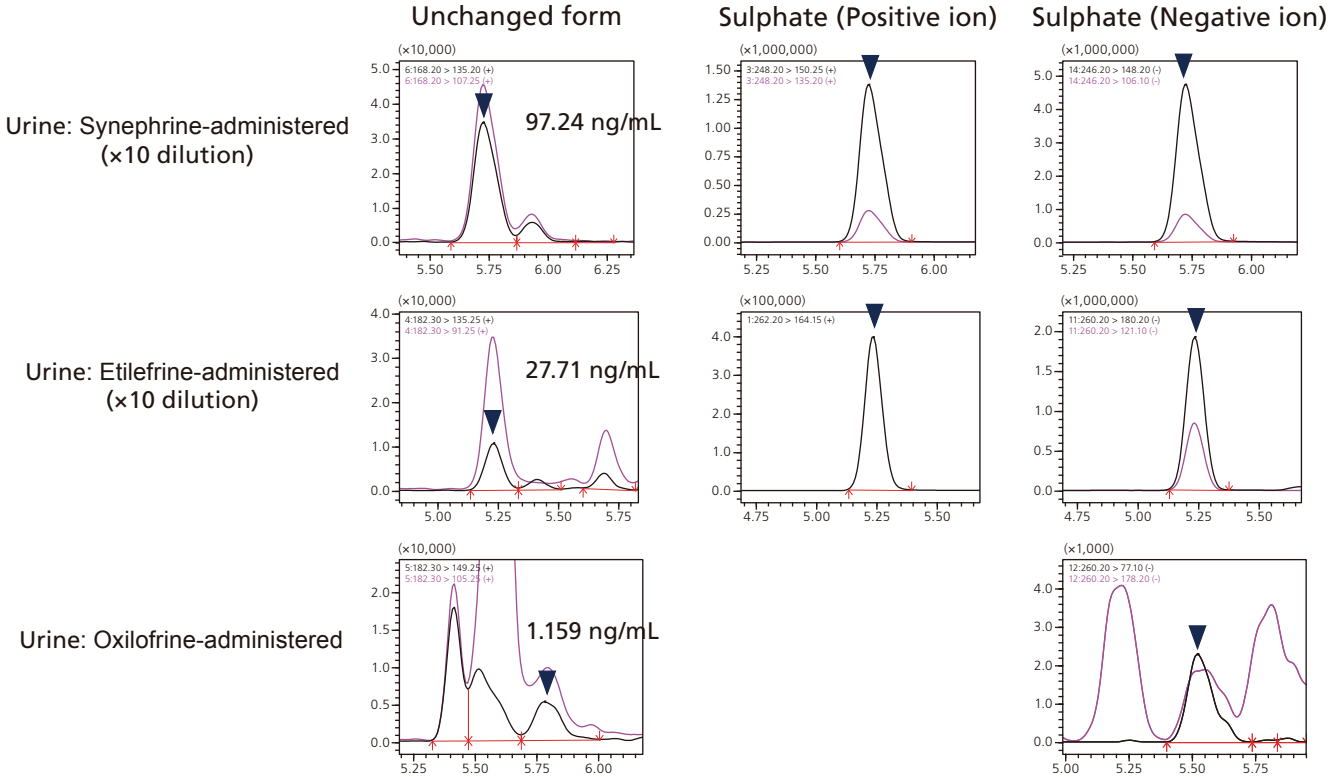

Fig. 5 Results of Urine: Synephrine, Etilefrine and Oxilofrine were separately administered

Distinguishing Norfenefrine and Octopamine in Urine

Norfenefrine is a positional isomer of octopamine resulting in the same retention time and MRM transitions for the unchanged parent drug molecule. However, by detecting the corresponding sulphate metabolite using rapid polarity switching enabled a positive identification.

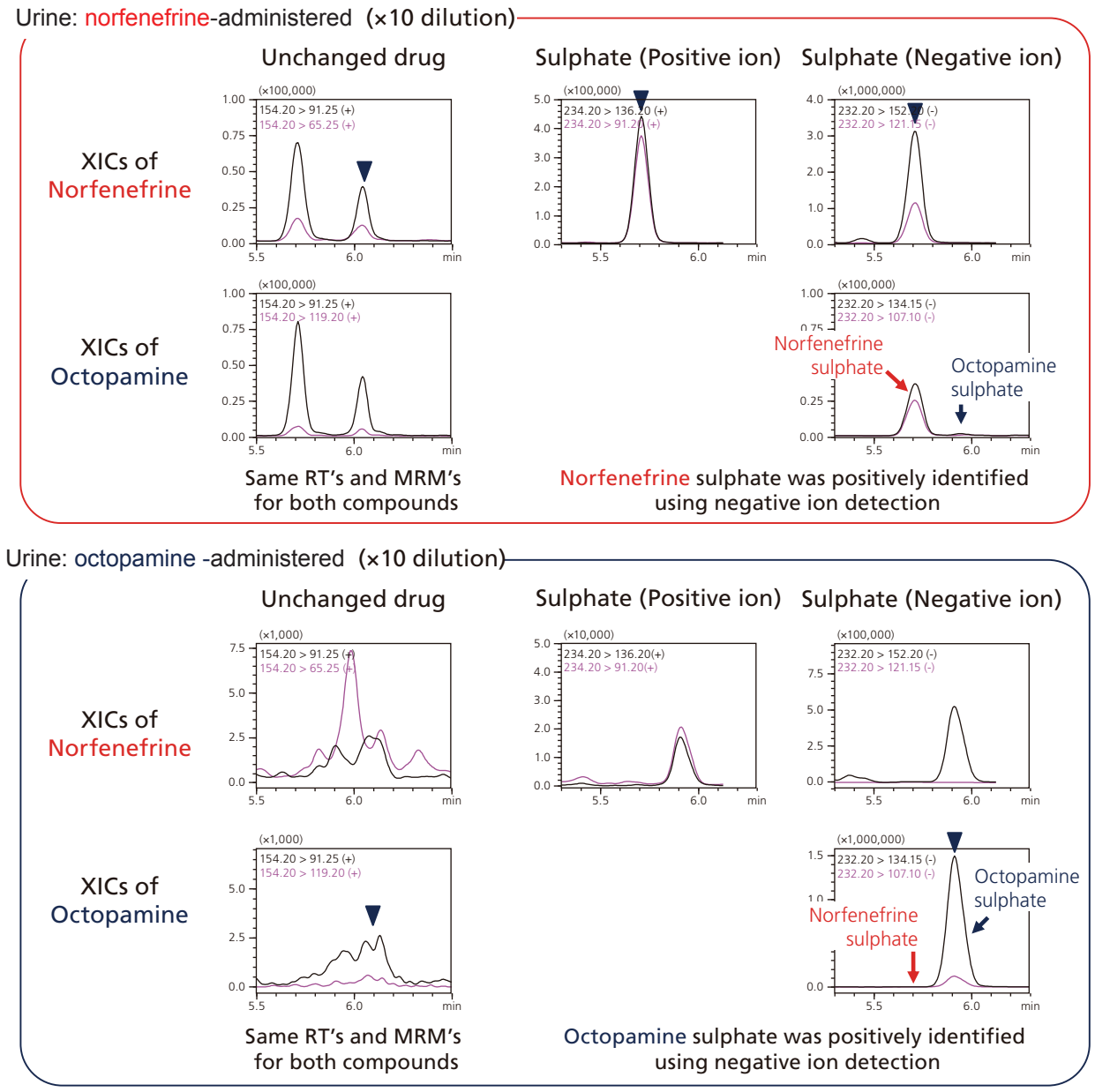

Fig. 6 Results of Urine: Norfenefrine and Octopamine were separately administered

The sample used for this analysis was provided by Anti-Doping Laboratory, LSI Medience Corporation, Tokyo, Japan

References: Anal Bioanal. Chem. (2015), 407, 5354-5379
Drug Test. Analysis (2015), 7, 973–979

Notes: • The products mentioned in this article have not received approval for use as medical devices based on the Pharmaceutical and Medica Device Act.
• The analytical methods mentioned in this article cannot be used for diagnostic purposes, for Research Use Only (RUO).

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Application News

No. C158

Direct Probe Ionization Mass Spectrometer

15 Second Screening Analysis of Cyanide in Blood Serum Without Pretreatment

Recent years have witnessed an increasing trend in incidents of crime and poisoning involving various legal drugs and toxic substances. The diversity in the types of used substances has lead to such incidents becoming a social problem. In the fields of forensic medicine, forensic toxicology, and critical care, finding and identifying causative agents is a problem that requires establishment of an analysis method that is both quick and highly sensitive. There is also growing demand in these workplaces to further simplify the complex pretreatment processes and instrument operations as well as to increase analysis speed. While various analysis instruments have been utilized until now for analyzing specific components in blood, most instruments require complex pretreatment, such as extracting the target component from blood. What is needed is a screening method that best reduces the time and labor required to perform analysis.

Probe electrospray ionization (PESI) is a direct ionization technique that ionizes sampled target components by sampling samples using an ultrafine and minimally invasive probe and applying high voltage to the probe tip. This technique enables sample analysis without the need for a chromatograph (Fig. 1).

The DPiMS-2020, which combines PESI with a mass spectrometer, is suitable for simple screening analysis because it enables quick analysis of target components in samples without pretreatment regardless of whether samples are in liquid or solid form.

This article introduces a rapid screening method for detecting cyanide in blood serum that does not require pretreatment by utilizing the DPiMS-2020 and In-Source CID.

T. Murata

Analysis Sequence Without Pretreatment

Potassium cyanide was added to blood serum to obtain a final concentration of 10 µg/mL and then taurine and naphthalene dialdehyde were added to perform derivatization.[*1] The obtained cyanide derivative (Fig. 2) was added to blood serum and used as the sample.

While complex pretreatment, such as that shown in Fig. 3, is required in conventional blood serum analysis, analysis that utilizes PESI can be performed using blood serum that contains cyanide derivatives either as-is or diluted with water by injecting it onto a small (10 µL) sample plate and setting the sample plate in the instrument.

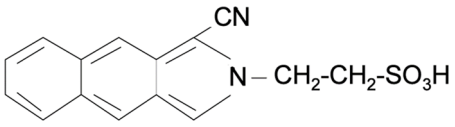

Fig. 2 Cyanide Derivative (MW 300)

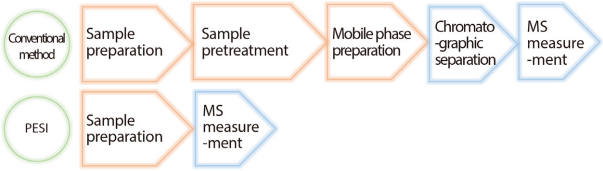

Fig. 3 Analysis Sequence Without Pretreatment

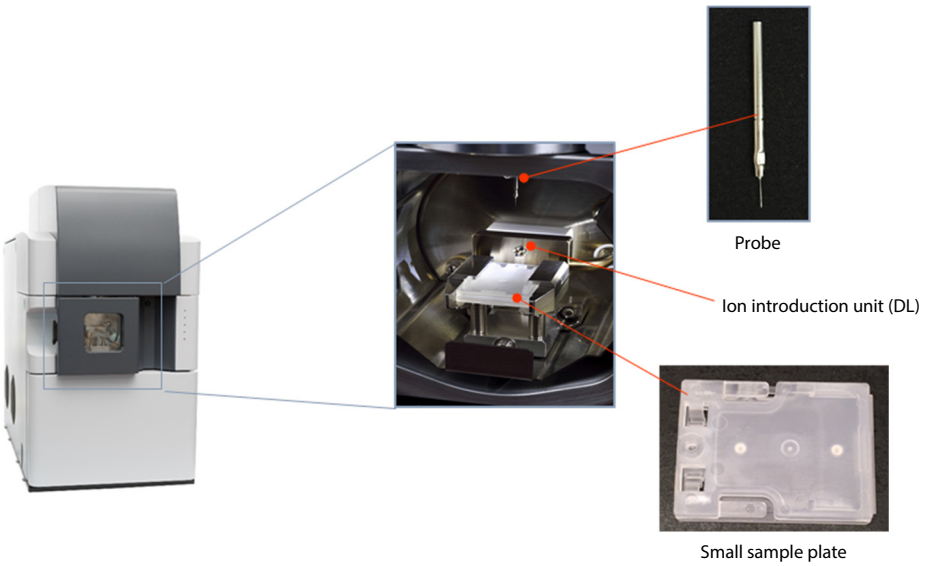

Fig. 1 DPiMS-2020

Structural Analysis Using In-Source CID Analysis

While a triple quadrupole mass spectrometer is usually used for structural analysis, a single quadrupole mass spectrometer can also obtain molecular structure information as well as molecular weight information by setting the lens system to a high voltage.
Analysis that utilizes PESI results in a unique mass chromatogram (Fig. 4) because the probe is driven at a constant frequency to repeat a process of sampling followed by ionization by applying a high voltage.
Fig. 5 shows the mass chromatogram obtained in our example. Applying a voltage of –80 V to DL bias and Q-array bias allows molecular structure information to be obtained and enables quick and simple screening analysis for cyanide in blood serum.
For reference, Fig. 6 shows the product ion (MS/MS) mass spectrum of the cyanide derivative obtained using the LCMS-8040 triple quadrupole mass spectrometer.

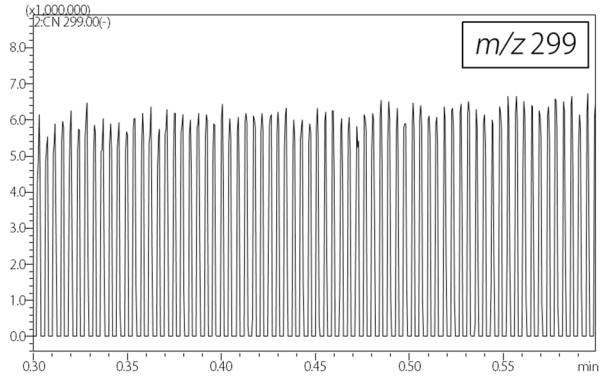

Fig. 4 Mass Chromatogram from DPiMS-2020

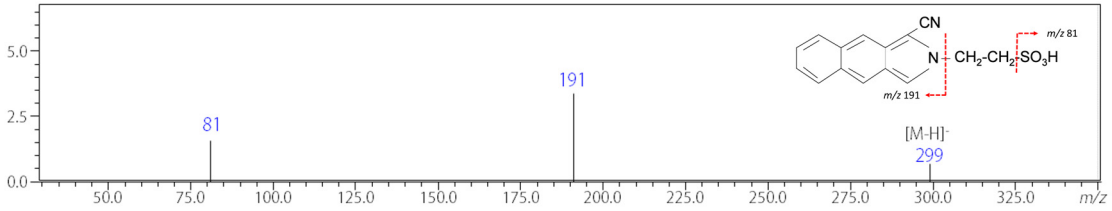

Fig. 5 Mass Spectrum from DPiMS-2020

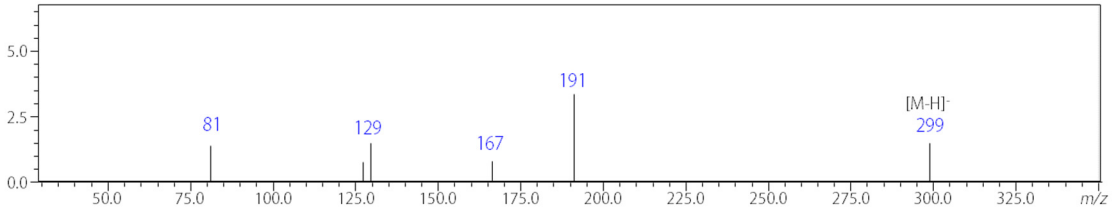

Fig. 6 Product Ion Mass Spectrum from LCMS-8040 (Reference)

DPiMS-2020 Analysis Conditions

When performing analysis using the DPiMS-2020, the drive conditions of the PESI probe and the analysis conditions of the mass spectrometer must be set.

Table 1 and 2 list the drive and analysis conditions respectively.

Table 1 PESI Drive Conditions

Ionization position	: –37 mm
Ionization stop time	: 100 msec
Sampling position	: –46 mm
Sampling stop time	: 50 msec
Probe speed	: 250 mm/s
Probe acceleration	: 0.63 G

Table 2 Mass Spectrometer Analysis Conditions

DL temperature	: 250 °C
Heater block temperature	: 35 °C
Interface voltage	: –2.45 kV (ESI – Negative mode)
DL bias voltage	: –80 V (*m/z* 299)
Q-array bias voltage	: –80 V (*m/z* 299)

References
*1 S. Chinaka, N. Takayama, Y. Michigami, and K. Ueda. *J. Chromatogr. B.* 713: 353–359 (1998)

Acknowledgments
We would like to thank associate professor Kei Zaitsu and assistant professor Yumi Hayashi at the Nagoya University Graduate School of Medicine for their guidance regarding data acquisition and sample preparation.

The product described in this document has not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan. It cannot be used for the purpose of medical examination and treatment or related procedures.

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Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

ASMS 2016 WP 271

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Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

Introduction

Multi Target Screening (MTS) has been applied to systemic toxicological analysis to reduce false positive and negative reporting using MS/MS spectral library based identification. MTS methods uses threshold triggered multiple reaction monitoring (MRM) and MS/MS product ion scans at three collision energies to confirm the compound identification based on mass spectral library searching. The MS/MS library was created using certified reference materials and included electrospray spectral data from over 1200 compounds relevant to clinical and forensic toxicology in both positive and negative ion modes. The MTS approach was applied to screening whole blood samples at three concentration levels to evaluate screening at therapeutic, overdose and toxic concentrations.

Methods and Materials

MTS methods were developed to screen whole blood spiked with a range of commonly observed compounds including antidepressant compounds, anxiolytic drugs, analgesics and antipsychotic agents. Samples were prepared by QuEChERS method with inclusion of ten internal standard compounds to normalise sample matrix effects. Data acquisition parameters were set to a single MRM per compound with threshold triggered MS/MS at 3 collision energies (10, 35, 55V) enabling confirmation of parent ion (low) and fragment ions at medium and high CE voltages. Library searching was performed on all CE spectral data in addition to a merged-CE spectrum.

Table 1. LC-MS/MS data acquisition conditions. The method included full scan and MRM data acquisition in both positive and negative ion mode. 10 internal standard compounds were also included in the method.

Liquid chromatography

UHPLC	: Nexera LC system
Analytical column	: Restek Raptor Biphenyl 2.7 µm 100 x 2.1 mm
Column temp.	: 50°C
Injection cycle	: 5 µL injection volume
Flow rate	: 0.3 mL/min
Solvent A	: Water + 2mM ammonium formate + 0.002% formic acid
Solvent B	: Methanol + 2mM ammonium formate + 0.002% formic acid
Binary Gradient	:

Time (mins)	%B
1.00	5
2.00	40
10.50	100
13.00	100
13.01	5
17.00	Stop
11-14.2	0.5 mL/min

Mass spectrometry

LC-MS/MS	: LCMS-8060
Ionisation mode	: Heated ESI
Scan speed	: 30,000 u/sec
Polarity switching time	: 5 msec
MRM Dwell time	: 5 msec
Pause time	: 3 msec
Interface temp.	: 300°C
Heating block	: 400°C
Desolvation line	: 250°C
Heating gas	: 10 L/min
Drying gas	: 10 L/min
Nebulising gas	: 3 L/min
CID gas pressure	: 250kPa
Interface voltage	: 4 kV

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

LC-MS/MS method set up for simultaneous full scan and MRM data acquisition with polarity switching

Type	Event	Polarity	Name | m/z	Time (0-13mins)
MRM	5	+	Target | 7-aminonitrazepam 252.10>121.10	
Product Ion Scan	6	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	7	+	> CE: -35, 30.00-1000.00	
Product Ion Scan	8	+	> CE: -50, 30.00-1000.00	
MRM	9	+	Target | 7-aminoclonazepam 286.05>121.10	
Product Ion Scan	10	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	11	+	> CE: -35, 30.00-1000.00	
Product Ion Scan	12	+	> CE: -50, 30.00-1000.00	
MRM	13	+	Target | 3-Hydroxybromazepam 322.00>287.00	
Product Ion Scan	14	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	15	+	> CE: -35, 30.00-1000.00	

Spectral Library >1200 compounds

Each library spectrum was acquired by authentic standard flow injection at collision energies 10-60V. Compounds that ionised efficiently with more than one adduct state were saved resulting in 1476 Library entries from 1207 compounds (1278 positive mode, 229 negative mode). Spectral Library information was registered for CE 10, 35 and 55V. Optimised MRM transitions were determined for all compounds with chromatographic retention time and peak area measured to enable reference ion-ratio calculation. RT analysis included internal standard compounds for relative RT calculation. Compound information was populated including: CAS number, formula, synonyms, compound class/properties, ChemSpider URL and ID number, mol file, InChI and InChIKey.

Toxicological Screening

Compounds were spiked into whole blood, prepared in triplicate at a concentration range 1-1000 µg/L (calibration curves typically ranged 5-500 µg/L). Quality control samples were prepared (5x) at three concentrations (20, 100, 500 µg/L). Two MTS methods were prepared, the first measuring benzodiazepines (36 compounds), the second measuring antiepileptics, antipsychotics, barbiturates and cannabinoids (35 compounds).

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

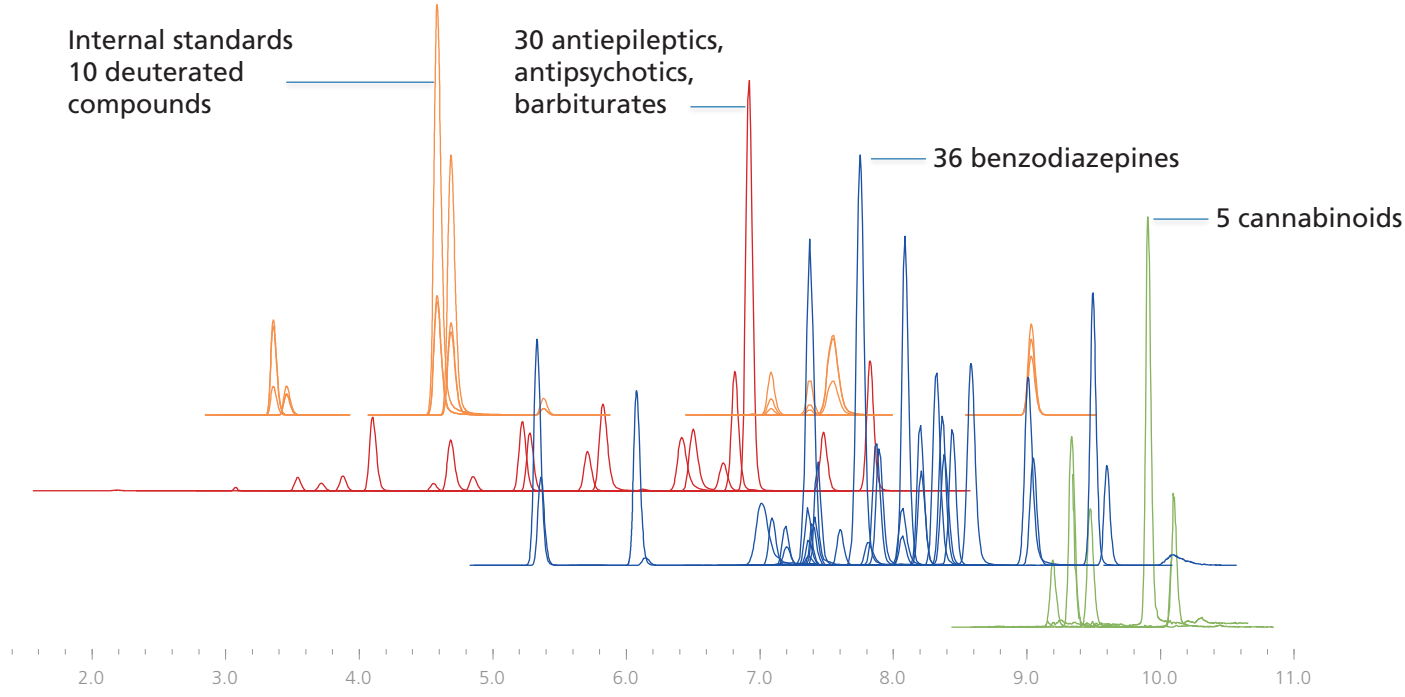


Figure 1. MRM chromatograms for a panel of drugs extracted from whole blood using a QuEChERS method corresponding to a concentration of 100 µg/L

Results

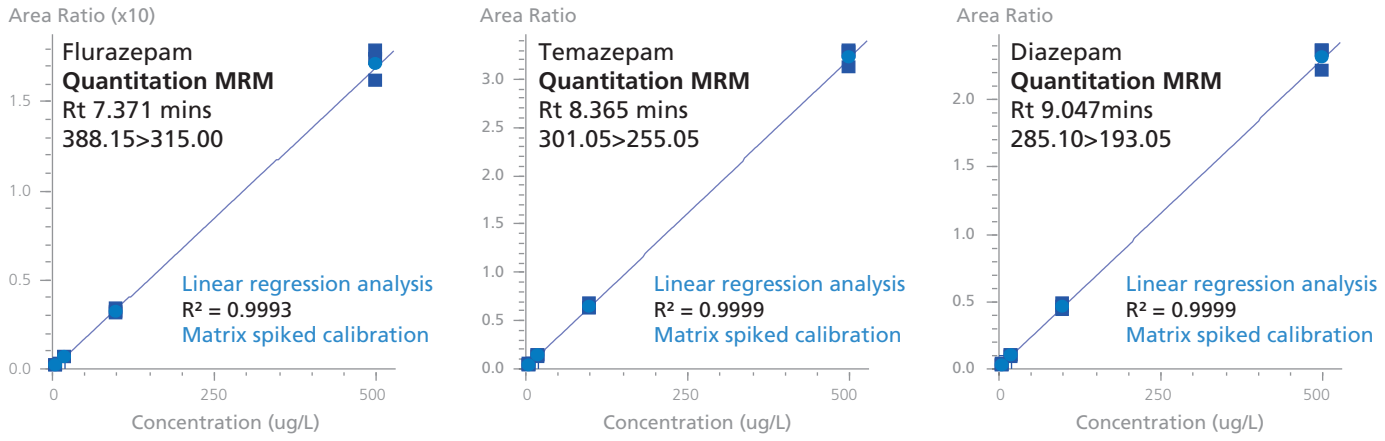
Quantitative analysis

The scope of the method was to ensure robust quantitation and a high level of confidence in the reported result. Using a MRM method followed by three product ion scans at different collision energies resulted in linear calibration curves over the concentration range of 5-500 µg/L (r2 >0.996 for all compounds). With regard to accuracy and precision; accuracy was between 80-120% and precision <20% throughout the calibration range.

Using a pause time of 3msecs and a dwell time of 5msec, the scan time was set to 50msecs (scanning from 30-1000u). As a result of fast data scanning, the peak sampling rate resulted in more than 20 data points across a peak.

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

MRM quantitation



	Rt (mins)	MRM	Mean Accuracy				Repeatability			
			Calibration standards (n=3 for each calibration level)				20ug/L replicate (n=5)		100ug/L replicate (n=5)	
			5ug/L	20ug/L	100ug/L	500ug/L	Mean Conc (ug/L)	%RSD	Mean Conc (ug/L)	%RSD
Flurazepam	7.371	388.15>315.00	109.5	95.5	95.3	101.1	18.1	3.0	100.2	7.7
Temazepam	8.365	301.05>255.05	103.4	97.8	99.1	100.2	18.8	1.9	101.5	6.0
Diazepam	9.047	285.10>193.05	102.0	99.5	98.6	100.3	18.6	2.5	100.2	5.6

Figure 2. Calibration curve data for flurazepam, temazepam and diazepam spiked into whole blood and extracted using QuEChERS together with results for accuracy and reproducibility at two different concentrations (20 µg/L and 100 µg/L; n=5; %RSD less than 8%).

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

MRM triggered product ion spectrum

MRM data was used to generate robust quantitation and also to help trigger product ion scans at three different collision energies.

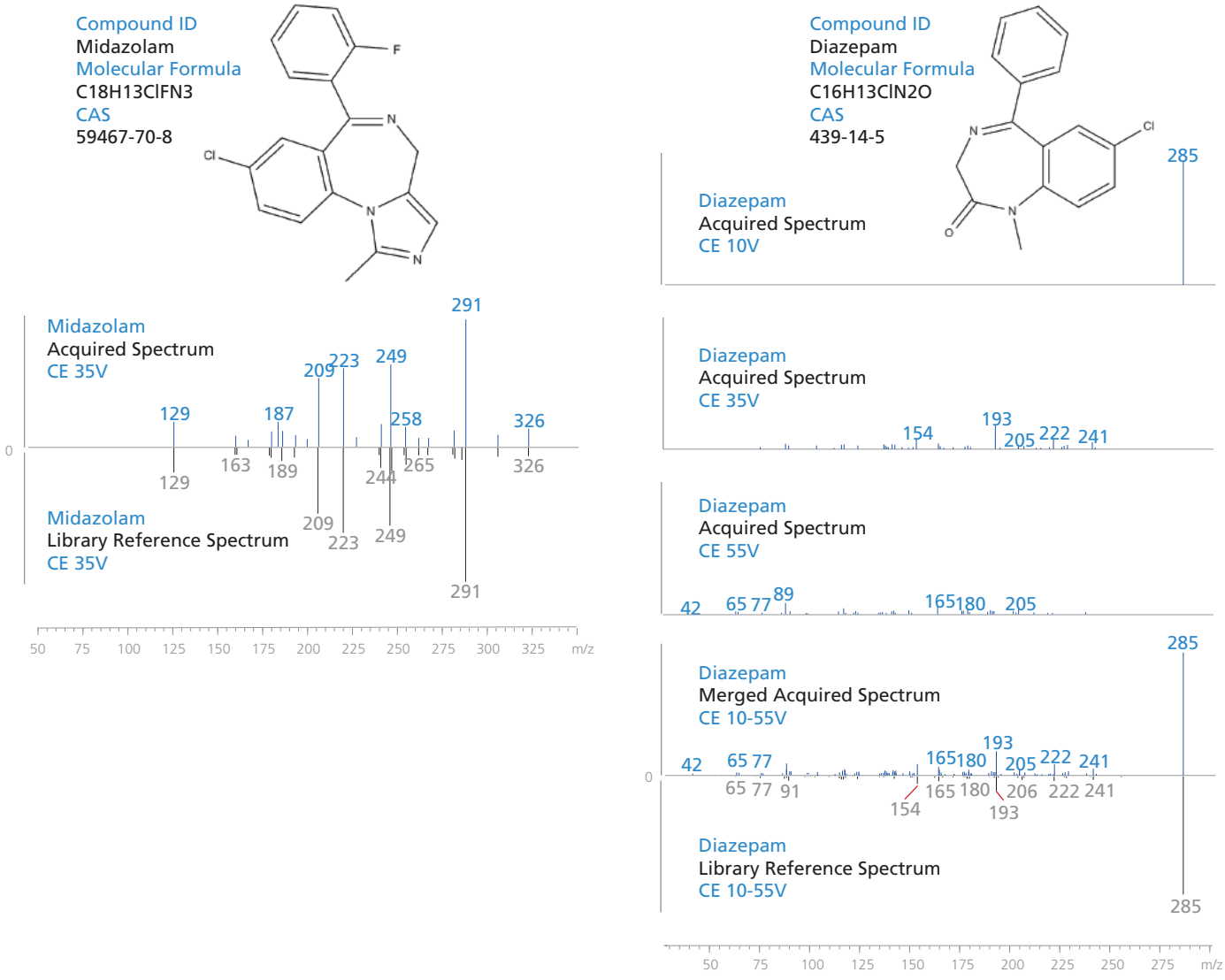


Figure 3. MRM triggered product ion spectrum data for midazolam and diazepam. The library included spectra for each collision energy and a separate library for merged spectra enabling match criteria to be set for a specific fragmentation voltage (as shown for midazolam) or to use a broad band fragmentation and merged spectra (as in the case for diazepam).

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

Library hits

A MTS procedure for clinical and forensic toxicology screening was developed for a single LC/MS/MS method following a QuEChERS extraction of whole blood. This approach results in robust quantitation using MRM data and enables a higher degree of confidence in compound identification as shown in Table 2.

Table 2. Library search results for a panel of drugs spiked into whole blood and extracted by QuEChERS from three QC levels (low, medium and high QC's correspond to 20, 100, 500 ug/L). Most compounds can be identified as the first hit in a spectral based library match (5 compounds are identified as the second candidate in the library; for 4 compounds the hit was not identified as either the first or second candidate).

Compound	RT (min)	Quality control level			Compound	RT (min)	Quality control level		
		Low	Medium	High			Low	Medium	High
		Library Hit					Library Hit		
		Merged CE spectrum					Merged CE spectrum		
Paracetamol	3.02	2	1	1	2-(2-amino-5-bromobenzoyl)pyridine	7.43	1	1	1
Levetiracetam	3.49	1	1	1	Dextropropoxyphene	7.44	1	1	1
Theophylline	3.67	1	1	1	Desalkylflurazepam	7.60	-	1	1
Scopolamine	4.05	1	1	1	Zolpidem	7.75	1	1	1
Felbamate	4.51	1	2	1	Hydroxyzine	7.80	1	1	1
Lamotrigine	4.81	2	2	1	Hydroxyalprazolam	7.82	1	1	1
Tramadol	5.18	1	1	1	4-hydroxymidazolam	7.87	1	1	1
10-hydroxycarbamazepine	5.24	1	1	1	Chlordiazepoxide	7.89	1	1	1
7-aminonitrazepam	5.33	1	1	1	1-hydroxymidazolam	8.07	1	1	1
7-aminoclonazepam	5.36	1	1	1	Nordiazepam	8.07	-	1	1
Ketamine	5.67	1	1	1	Clobazam	8.09	1	1	1
Niaprazine	5.77	1	1	1	Flunitrazepam	8.20	1	1	1
Norbuprenorphine	6.08	1	1	1	Lormetazepam	8.21	1	1	1
3-Hydroxybromazepam	6.15	1	1	1	Estazolam	8.32	1	1	1
Doxylamine	6.36	1	1	1	Temazepam	8.37	1	1	1
LSD	6.45	1	1	1	Triazolam	8.38	1	1	1
Diphenhydramine	6.78	1	-	1	Ethyl loflazepate	8.44	1	1	1
Carbamazepine	6.90	1	1	1	Alprazolam	8.58	1	1	1
Zopiclone	6.99	1	1	1	Midazolam	9.01	2	1	1
Desmethylflunitrazepam	7.09	1	1	1	Diazepam	9.05	1	1	1
N-desmethylclobazam	7.19	1	1	1	11-OH-THC	9.23	1	1	1
Lorazepam	7.20	1	1	1	THC	9.37	1	1	1
3-hydroxy-flunitrazepam	7.36	1	1	1	Clotiazepam	9.49	1	1	1
Oxazepam	7.37	-	1	1	THC-COOH	9.51	1	1	1
Flurazepam	7.37	1	1	1	Tetrazepam	9.60	1	1	1
Clonazepam	7.40	1	1	1	Cannabinol	9.94	1	1	1
Nitrazepam	7.41	1	1	1	Loprazolam	10.09	1	1	1

Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

Conclusions

A spectral based library of more than 1200 compounds has been created using certified reference materials acquired at three collision energies on a triple quadrupole mass spectrometry platform.
A MRM triggered product ion spectra method to quantify and identify a panel of compounds commonly found in clinical and forensic toxicology was successfully applied to whole blood samples spiked with a panel of compounds. All compounds were detected at highest concentration and positively identified using product ion scan MS/MS library based searching generating higher data quality for compound identification.

Disclaimer: The Shimadzu LCMS-8060 is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

First Edition: June, 2016

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Application News

No.C98

Liquid Chromatography Mass Spectrometry

Analysis of Steroids and NSAIDs Using the Shimadzu LCMS-8050 Triple Quadrupole Mass Spectrometer

With performance enhancing drug use considered contrary to fair play, along with the adverse effects they may have on the health and social welfare of athletes, sports doping testing is increasing and has been conducted according to the provisions of WADA (World Anti-Doping Agency).
Drugs that are registered as prohibited substances mainly fall into the categories of anabolic steroids (AAS) used primarily for building muscle strength, steroidal anti-inflammatory drugs for their anti-inflammatory and immunosuppressive effects, and narcotic and designer drugs. Also, non-steroidal anti-inflammatory drugs (NSAIDs) are drugs used to treat pain and inflammation as well as fever, and although they are not specified as prohibited drugs, their abuse by athletes is being viewed as a problem due to their side effects.
Since doping tests provide information for making critical decisions that actually affect athletes' lives, accuracy at the time of testing, as well fairness, are necessary. In this Application News, we introduce an accurate identification method for typical steroidal and non-steroidal anti-inflammatory drugs using multiple reference ion ratios, in addition to an example of high-sensitivity measurement.

MRM Analysis of Standards and Matrix-Matched Calibration Curves

We conducted MRM measurement of a mixed standard solution consisting of 14 typical steroids and non-steroidal anti-inflammatory drugs. Fig. 1 shows the MRM chromatograms obtained using the mixed standard solution (each component at 50 ng/mL), and Fig. 2 shows MRM chromatograms obtained from analysis of typical compounds at concentrations near their respective LOQs. Table 1 shows minimum and maximum concentrations used for generating the respective calibration curves. The lower limits of quantitation ranged from 10 to 100 pg/mL (20 – 200 fg on column), and excellent linearity was obtained over a wide range of more than 3 orders of magnitude for each substance.

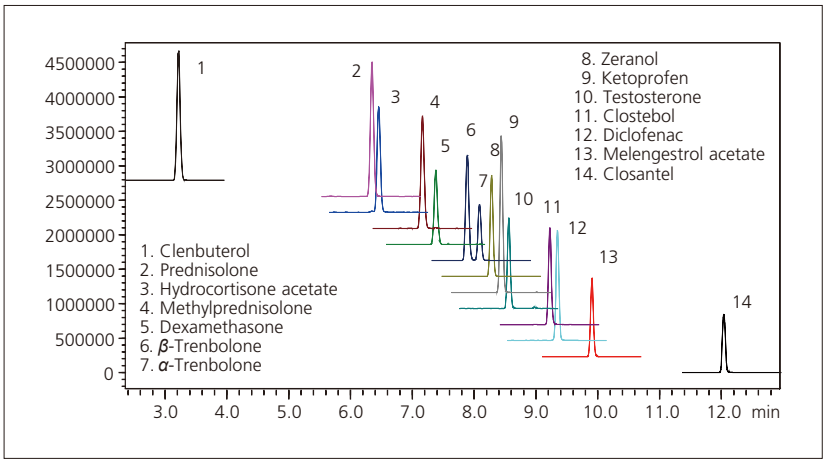


Fig. 1 Chromatograms of Steroids and NSAIDs

Table 1 Calibration Curve Min. /Max. Concentrations

Compounds	Min. Conc.	Max. Conc.
Clenbuterol	0.01	10
Prednisolone	0.05	20
Hydrocortisone acetate	0.1	50
Methylprednisolone	0.5	50
Dexamethasone	0.5	50
α-, β-Trenbolone	0.1	50
Zeranol	0.1	50
Ketoprofen	0.05	50
Testosterone	0.05	10
Clostebol	0.05	50
Diclofenac	0.01	50
Melengestrol acetate	0.05	50
Closantel	0.01	10

(Unit: ng/mL)

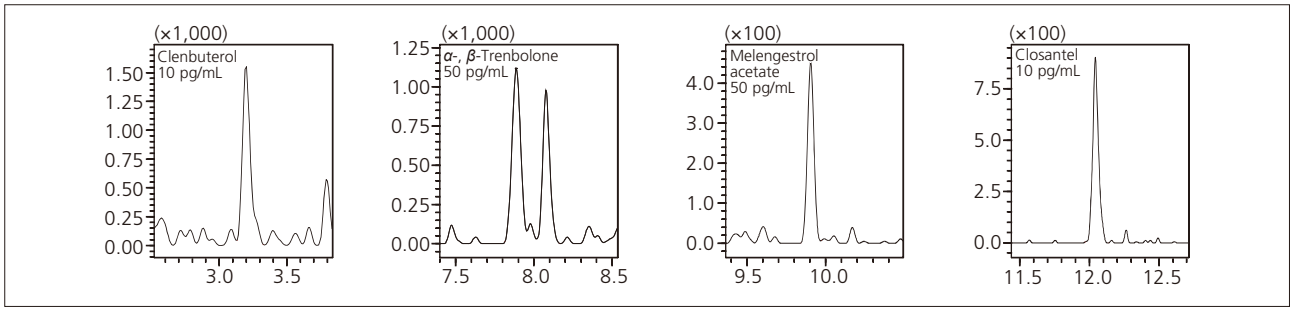


Fig. 2 MRM Chromatograms Near the LOQ of Typical Compounds

Peak Determination Using Multiple Reference Ions

When using multiple reference ions to conduct high-accuracy identification, the process of selecting and making the associated entries becomes complicated.
As of Labsolutions Ver. 5.65, however, this selection and entry process for qualifier MRM transitions now provides for automatic selection and entry as reference ions.

< Examples of New Features >

(1) Multiple reference ions are automatically entered (desired transitions can be selected and changed using drop-down menu).
(2) The ion ratio of the STD is automatically set as the reference value.
(3) A different allowable width of relative ion ratio can be set for each reference ion.
(4) The identification range (%) is automatically calculated from the ion ratio, allowable width and reference ion mode.

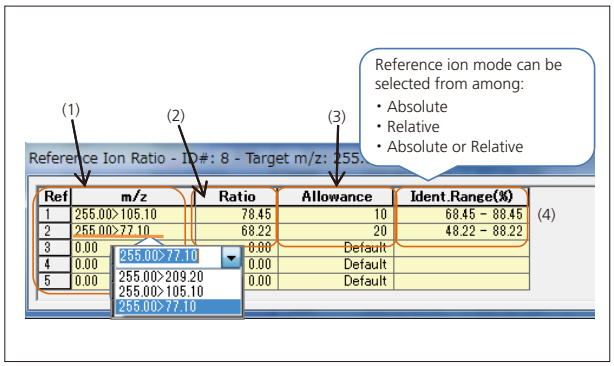
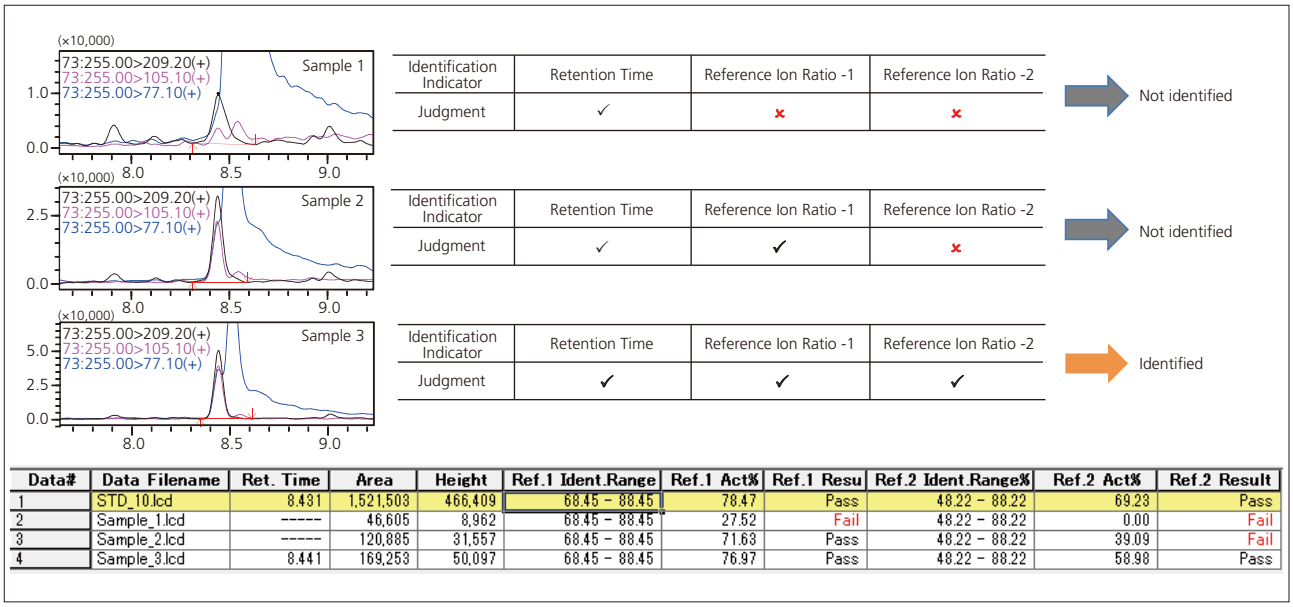


Fig. 3 Reference Ion Setting Window



Data#	Data Filename	Ret. Time	Area	Height	Ref.1 Ident.Range	Ref.1 Act%	Ref.1 Resu	Ref.2 Ident.Range%	Ref.2 Act%	Ref.2 Result
1	STD_10.lcd	8.431	1,521,503	466,409	68.45 - 88.45	78.47	Pass	48.22 - 88.22	69.23	Pass
2	Sample_1.lcd	-----	46,605	8,962	68.45 - 88.45	27.52	Fail	48.22 - 88.22	0.00	Fail
3	Sample_2.lcd	-----	120,885	31,557	68.45 - 88.45	71.63	Pass	48.22 - 88.22	39.09	Fail
4	Sample_3.lcd	8.441	169,253	50,097	68.45 - 88.45	76.97	Pass	48.22 - 88.22	58.98	Pass

Fig. 4 Example of Peak Determination Using Multiple Reference Ions

Table 2 Analytical Conditions

Column	: Shim-pack XR-ODS Ⅱ (2.0 × 75 mm, 2.2 µm)
Mobile Phase A	: 0.1 % Formic acid – Water
Mobile Phase B	: Acetonitrile
Time Program	: 1 %B (0 min) → 15 %B (1 min) → 40 %B (6 min) → 100 %B (10 - 13 min) → 1 %B (13.01 - 16 min) (12.01 - 15 min)
Flowrate	: 0.2 mL/min.
Injection Volume	: 2 µL
Oven Temperature	: 40 °C
Ionization Mode	: ESI (Positive / Negative)
Probe Voltage	: +4.5 kV / -3.5 kV
Neburizing Gas Flow	: 3.0 L/min.
Drying Gas Flow	: 10.0 L/min.
Heating Gas Flow	: 10.0 L/min.
Interface Temperature	: 400 °C
DL Temperature	: 200 °C
Block Heater Temperature	: 400 °C

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Application News

No. G286

Gas Chromatography

Analysis of Carbon Monoxide in Blood

Carbon monoxide (CO) is known as a toxic gas produced from the incomplete combustion of organic compounds. Since CO is responsible for many cases of poisoning, the carboxyhemoglobin saturation level is measured to be used as an index to determine whether poisoning by carbon monoxide has occurred. Gas chromatography thermal conductivity detectors (GC-TCD) employ an indirect measurement method that isolates carbon monoxide in blood for analysis, but sensitivity is not very high. On the other hand, barrier discharge ionization detectors (BID) are able to detect most compounds, with the exception of helium and neon, at high sensitivity compared to TCD. BID analysis is useful because measuring at higher sensitivities allows the volume of a blood sample used in testing to be reduced, enabling any remaining blood in the sample to be used in other tests. This article introduces an example of measuring carbon monoxide in blood using GC-BID.

S. Uchiyama

Analysis Method

The pretreatment method was performed as follows by referencing "Quantitative Testing 1-2 (2)" under "II-1 Toxic Gas Testing Methods" in "Testing Methods and Annotation for Toxic Pharmaceuticals 2006".

1. Preparation of potassium ferricyanide aqueous solution (oxidizing agent)
   20 g of potassium ferricyanide and 5 g of saponin were dissolved in distilled water to precisely obtain a volume of 100 mL.
2. Preparation of sample solution
   0.25 mL of blood sample, 0.5 mL of distilled water, and 0.25 mL of oxidizing agent were added to a 9-mL vial and the vial was sealed immediately.
3. Measurement
   The blood sample was kept warm at 30 °C for 90 minutes and then measurement was performed by injecting 0.1 mL of headspace gas into the GC using a gas-tight syringe. The Rt-Msieve 5A column was used.

Table 1 Analysis Conditions

Model	: Tracera (GC-2010 Plus + BID-2010 Plus)
Column	: RESTEK Rt-Msieve 5A (30 m × 0.53 mm I.D., df = 50 µm) with Particle Trap 2.5 m
Column Temp.	: 100 °C
Inj. Mode	: Split 1:7
Inj.Temp	: 250 °C
Carrier Gas	: He 45 cm/sec (constant linear velocity mode)
Det. Temp.	: 280 °C
Discharge Gas	: 50 mL/min (He)
Inj. Volume	: 0.1 mL

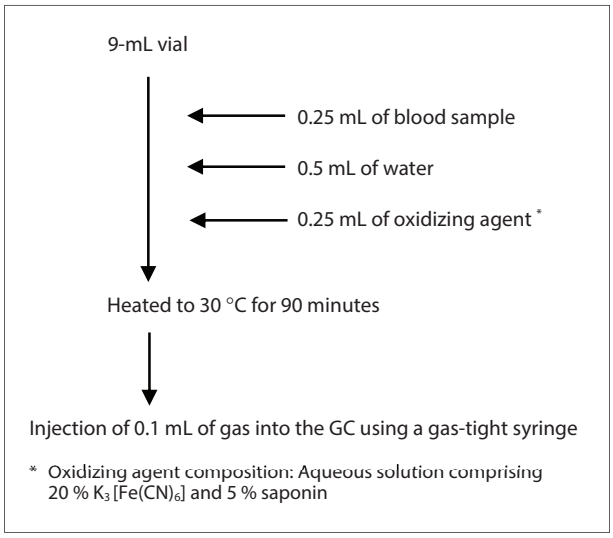

Fig. 1 Example of Sample Pretreatment

Measurement of Blood Sample Saturated with Carbon Monoxide

A blood sample saturated with carbon monoxide was created by bubbling 10 mL of CO through a 25 mL blood sample and mixing, and this process was repeated nine times. An untreated blood sample and the blood sample saturated with carbon monoxide were analyzed according to steps 2 and 3 of the analysis method and the resulting chromatograms are shown in Fig. 2.

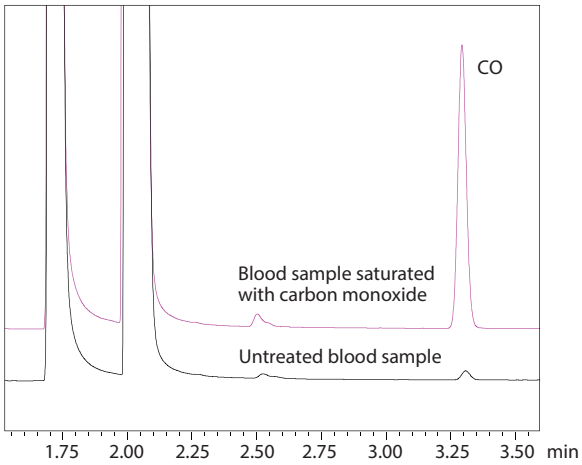

Fig. 2 Comparison of Untreated Blood Sample and Blood Sample Saturated with Carbon Monoxide

Linearity of Calibration Curve

A calibration curve from 2 to 3900 ppm was created by diluting carbon monoxide standard gas with air. Fig. 3 shows the calibration curve. There is sufficient sensitivity even with an extremely low concentration of 2 ppm, indicating that detection is possible at low concentrations which cannot be detected using a TCD.

The calibration curve shows good linearity with a correlation coefficient (R^2) of 0.999 or greater in the 2 to 3900 ppm concentration range.

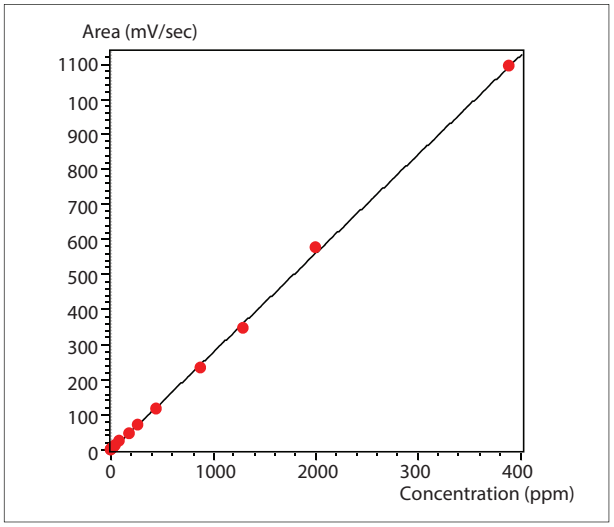

Fig. 3 Calibration Curve

Analysis of Carbon Monoxide in Blood

Fig. 4 shows the results of analyzing carbon monoxide in the blood of a smoker and non-smoker. We can observe a significant difference in CO concentration between the smoker and non-smoker.

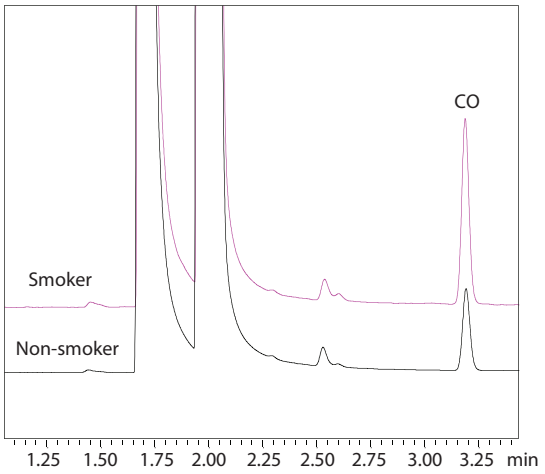

Fig. 4 Comparison of a Smoker and Non-Smoker

Calculating Carboxyhemoglobin Saturation Levels

The percentage of carboxyhemoglobin saturation (hereafter CO-Hb (%)) must be determined because CO-Hb (%) relates to the degree of CO poisoning. The concentration of carbon monoxide in the blood of six smokers and six non-smokers was determined and the CO-Hb (%) calculation results are listed in Table 2.

Table 2 Calculating Carboxyhemoglobin Saturation Levels

		1	2	3	4	5	6
Smoker	Analysis quantitative value (ppm)	414	452	285	240	339	318
	CO-Hb binding amount (µmol)	0.133	0.146	0.092	0.077	0.109	0.102
	CO-Hb max. binding amount (µmol)	2.191	2.412	2.558	2.601	2.586	2.657
	CO-Hb (%)	6.084	6.034	3.587	2.971	4.211	3.854
Non-smoker	Analysis quantitative value (ppm)	146	158	218	188	207	255
	CO-Hb binding amount (µmol)	0.047	0.051	0.07	0.061	0.067	0.082
	CO-Hb max. binding amount (µmol)	2.617	2.357	2.613	2.530	2.395	2.766
	CO-Hb (%)	1.794	2.156	2.689	2.393	2.777	2.964

* The CO-Hb maximum binding amount (µmol) was determined using a spectrophotometer.

Equations

CO-Hb binding amount (µmol) = total CO amount in headspace
= A * B / 0.082 / 303 / 1000
CO-Hb max. binding amount (µmol) = total hemoglobin in blood sample
= C * D * 4 * 369.2 * 1000 / 64500
CO-Hb (%) = CO-Hb binding amount / CO-Hb max. binding amount * 100

A : CO quantitative value (ppm)
B : Headspace volume (mL)
C : Absorbance at 540 nm, according to "Quantitative Testing 1-2 (2)" under "II-1 Toxic Gas Testing Methods" in "Testing Methods and Annotation for Toxic Pharmaceuticals 2006"
D : Used blood sample volume (mL)

We would like to thank Takeshi Omori and Yasuo Seto at the National Research Institute of Police Science for providing and creating the data that was used to produce this issue of Application News.

References: The Pharmaceutical Society of Japan: Testing Methods and Annotation for Toxic Pharmaceuticals 2006 - Analysis, Toxicity, and Coping Methods

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