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Application Notebook
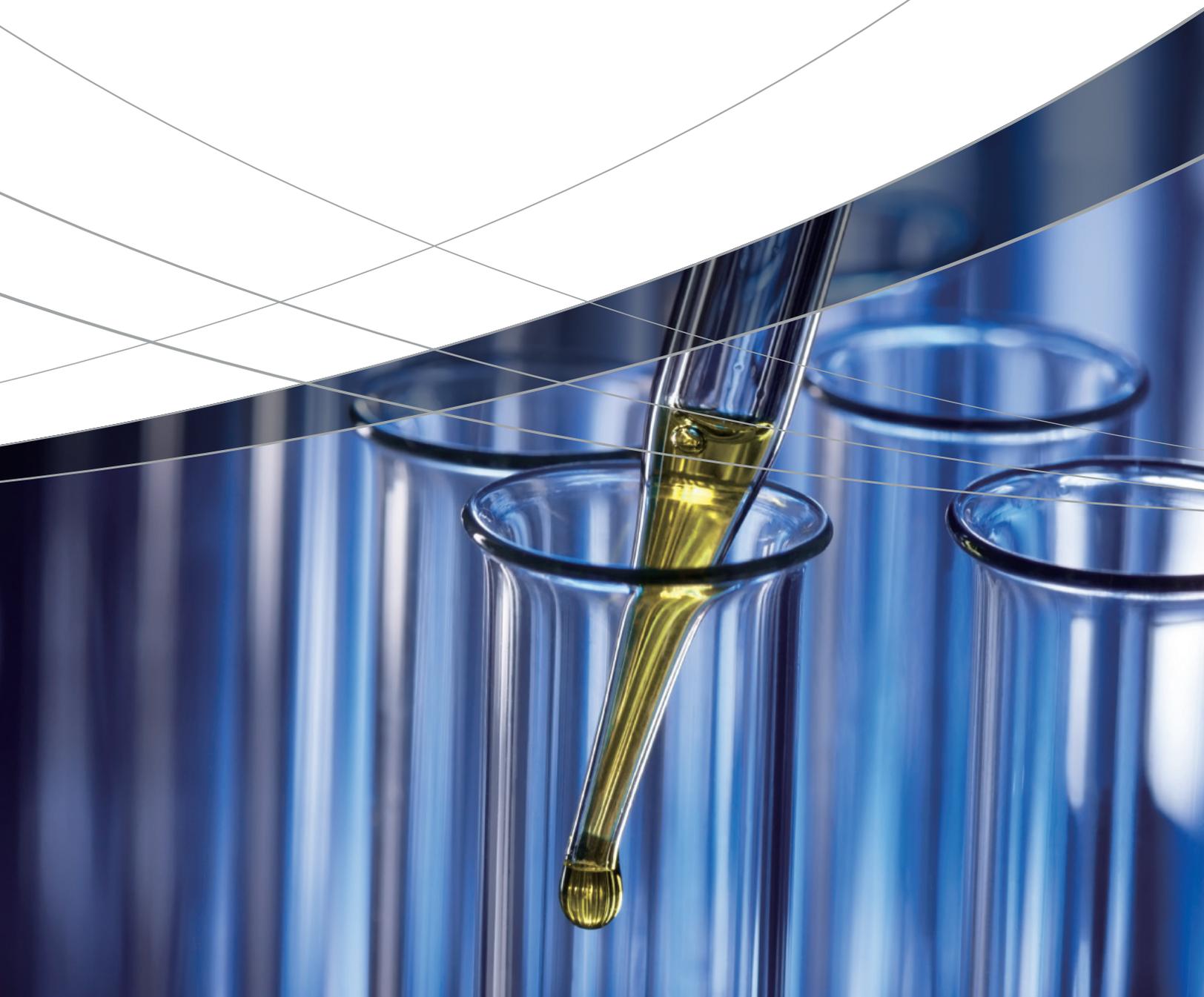
Therapeutic Drug Monitoring

High-Throughput Optimization of Therapeutic Drug Monitoring Using Fully Automated Sample Preparation LC-MS/MS System (CLAM-2000 + LCMS-8040)

This article introduces the results of TDM using a fully automated sample preparation LC-MS/MS system comprised of the CLAM-2000 fully automated LCMS sample preparation unit and the LCMS-8040 high performance liquid chromatograph-mass spectrometer. This system resolves the problems associated with TDM, and achieves TDM research results on a fast and high-precision analytical workflow.

Simultaneous Analysis of Antiarrhythmic Drugs in Human Blood Plasma Using the Fully Automated Sample Preparation LC-MS/MS System

This article introduces a study which achieves a fast and simultaneous analysis workflow of six antiarrhythmic drugs with the fully automated sample preparation LC-MS/MS system. The analysis method used by this system is a fast and low-burden analysis technique that achieves quantitative results equivalent to conventional manual pretreatment methods and we anticipate its utilization into the future.

Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS

Absorption value was measured by UV-Visible spectrophotometer to evaluate Lysis efficiency for automated sample treatment LC-MS/MS analysis.

High-sensitivity and Simultaneous Analysis of Psychoactive Drugs Using LC-MS/MS with Full-Automated Pretreatment System

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

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

A fast MRM-based method for quantitation of five β-lactam antibiotics tazobactam, cefepime, meropenem, ceftazidime and piperacillin in human plasma was developed. A simple sample pretreatment 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.

Endocrinology

Evaluation of an Automated LC-MS/MS System for Analyzing Hydrophilic Blood Metabolites

In this study, we assessed whether the plasma levels of metabolites could be quantitatively measured using a fully automatic pretreatment system for LC/MS that can be connected online to an LC/MS device.

Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pretreatment

The aim of this study is to develop a reliable LC-MS/MS method for quantitation of 22 free amino acids and creatinine in urine samples. A derivatization-free LC-MS/MS amino acid method with stable isotope labelled IS was employed.

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

In this application, 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) – LC-MS/MS system.

Integration of Amino Acid, Acylcarnitine and Steroids Analysis in Single FIA/LC-MS/MS Platform

In this study, we present a strategy for performing both amino acids (AA)/ acylcarnitines (AC) and steroids analysis within a single LC-MS/MS platform. All compounds were extracted from only one dried blood spot. This system enables to automatically analyze 7 min in all target analytes in 2 injections.

Clinical Toxicology

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

This platform, CLAM-2000 module coupled with Shimadzu LCMS-8040 was applied and evaluated for quantitation of 18 illicit drugs with 14 isotope-labelled internal standards. The method performance was evaluated on the linearity, accuracy, specificity and process efficiency.

Screening Analysis of Highly Polar Doping Agents in Urine Using 2D LC-MS/MS

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.

15 Second Screening Analysis of Cyanide in Blood Serum Without Pretreatment

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

Quantitative Multi Target Screening (MTS) Using Liquid Chromatography-tandem Mass Spectrometry with MS/MS Library Based Identification for Forensic Toxicology

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.

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

In this article, 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.

Analysis of Carbon Monoxide in Blood

This article introduces an example of measuring carbon monoxide (CO) in blood, which is known as a toxic gas produced from the incomplete combustion of organic compounds. The barrier discharge ionization detector (BID) was applied to detect CO because of higher sensitivity compare to TCD.

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Application News

Liquid Chromatography Mass Spectrometry

No.C123

High-Throughput Optimization of Therapeutic Drug Monitoring Using Fully Automated Sample Preparation LC/MS/MS System (CLAM-2000 + LCMS-8040)

Therapeutic drug monitoring (TDM) is a series of processes where the blood concentration of drugs in a patient is measured to determine the optimal dose and method of administration for an individual based on pharmacokinetic and pharmacodynamic analysis. TDM is used during drug treatment with drugs that pose administration management difficulties, such as drugs with a narrow therapeutic range or with an effective range and toxic range that are close to each other. High performance liquid chromatography (HPLC) has been the main analytical method used with TDM, but recently liquid chromatography-mass spectrometry (LC/MS/MS) is being used to improve analytical accuracy and precision based on its superior selectivity.

LC/MS/MS normally requires sample preparation steps such as deproteinization and dilution to analyze a blood serum or blood plasma sample. These steps introduce the risk of error or variability occurring based on operator skill. The volume of work performed by an operator also increases in accordance with the number of samples. Therefore, the sample preparation process can become the bottleneck of an analytical workflow when analyzing a large number of samples.

This article introduces the results of TDM using a fully automated sample preparation LC/MS/MS system comprised of the CLAM-2000 fully automated LCMS sample preparation unit and the LCMS-8040 high performance liquid chromatograph-mass spectrometer. This system resolves the above-mentioned problems associated with TDM, and achieves TDM research results on a fast and high-precision analytical workflow.

Fully Automated Sample Preparation LC/MS/MS System

■ High-Throughput Analytical Workflow for Antiepileptic Drug Analysis

We introduce an example simultaneous analysis of seven antiepileptic drugs and drug active metabolites in blood serum using a fully automated sample preparation LC/MS/MS system.

Preparation of blood serum samples for analysis normally requires deproteinization by the addition of organic solvent, and then centrifugal separation of solid components followed by supernatant recovery. The fully automated sample preparation LC/MS/MS system only

requires placing of the blood collection tube in the system, as the system performs all these preparation steps automatically, followed by LC/MS/MS analysis (Fig. 1).

Preparation of the next sample can also be performed in parallel with LC/MS/MS analysis, which can greatly reduce the time required for each sample analysis. In our example, a per-sample cycle time including analysis of 9 minutes is achieved.
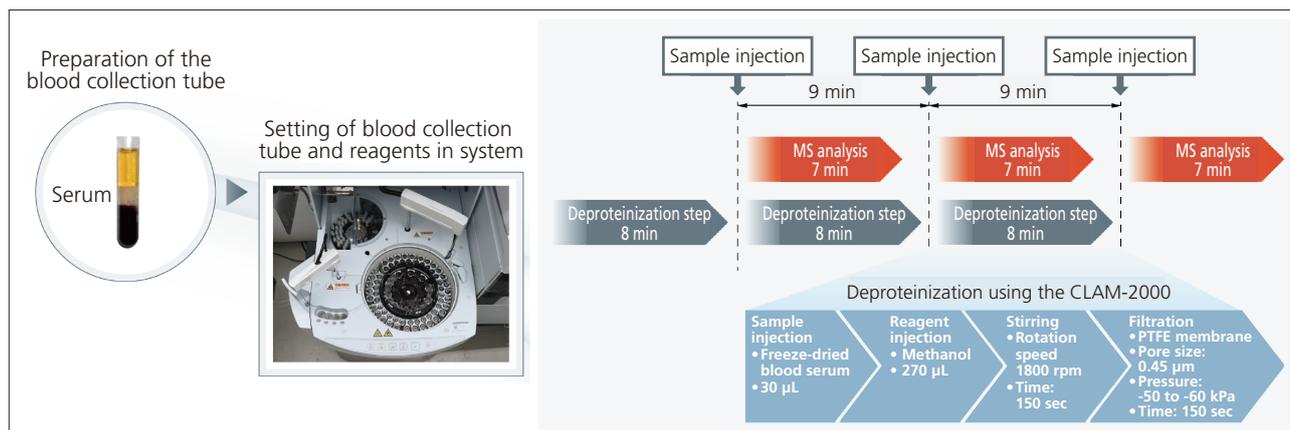

Fig. 1 Workflow for Simultaneous Analysis of Antiepileptic Drugs in Blood Serum Using Fully Automated Sample Preparation LC/MS/MS System

Fig. 2 shows the mass chromatogram for a control sample consisting of seven antiepileptic drugs and drug metabolites added to human blood serum. Because LC/MS/MS can detect target drugs selectively based on the mass and structure of those drugs, the results show no apparent interference from other constituents in the blood serum.
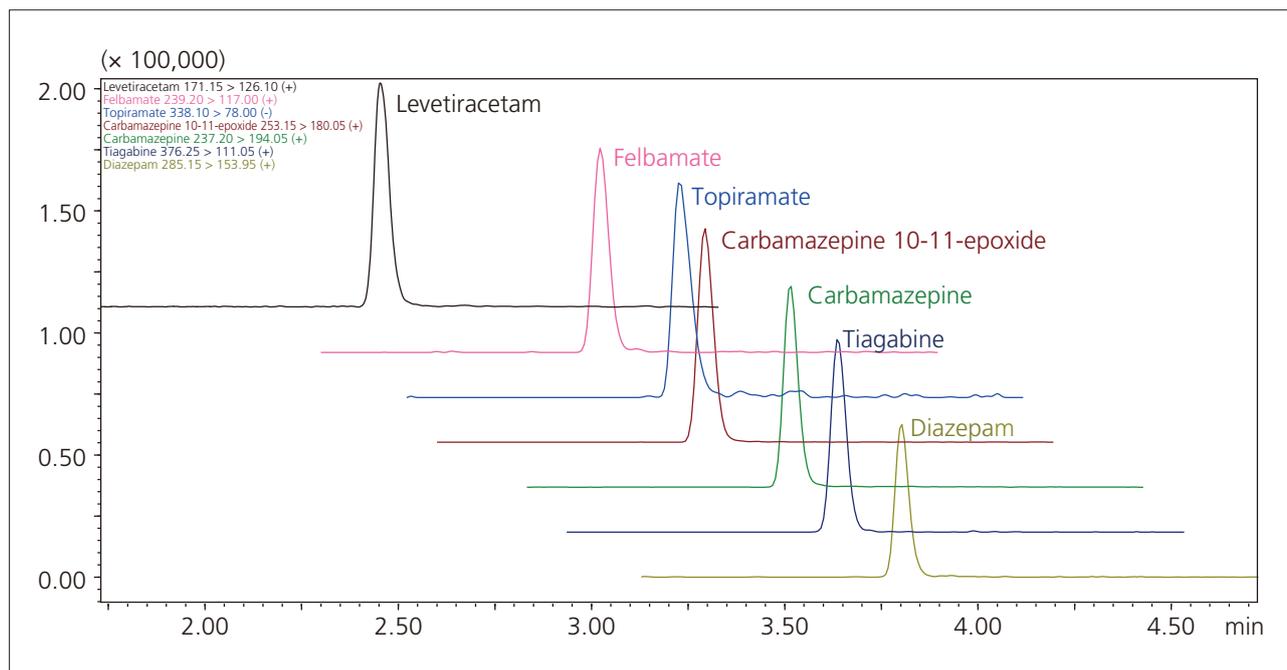

Fig. 2 Mass Chromatogram of Seven Antiepileptic Drugs and Drug Metabolites in a Control Serum Sample

Calibration curves were prepared by continuous analysis with fully automated sample preparation and analysis, and used to assess accuracy and precision (repeatability). Good linearity was obtained across the set calibration curve range for each antiepileptic drug (Fig. 3), with accuracy within 100 % ±15 % over the entire measurement range including the minimum limit of quantification. Similarly, precision was measured at a %RSD of within 15 %, showing that good repeatability was achieved (Table 1).
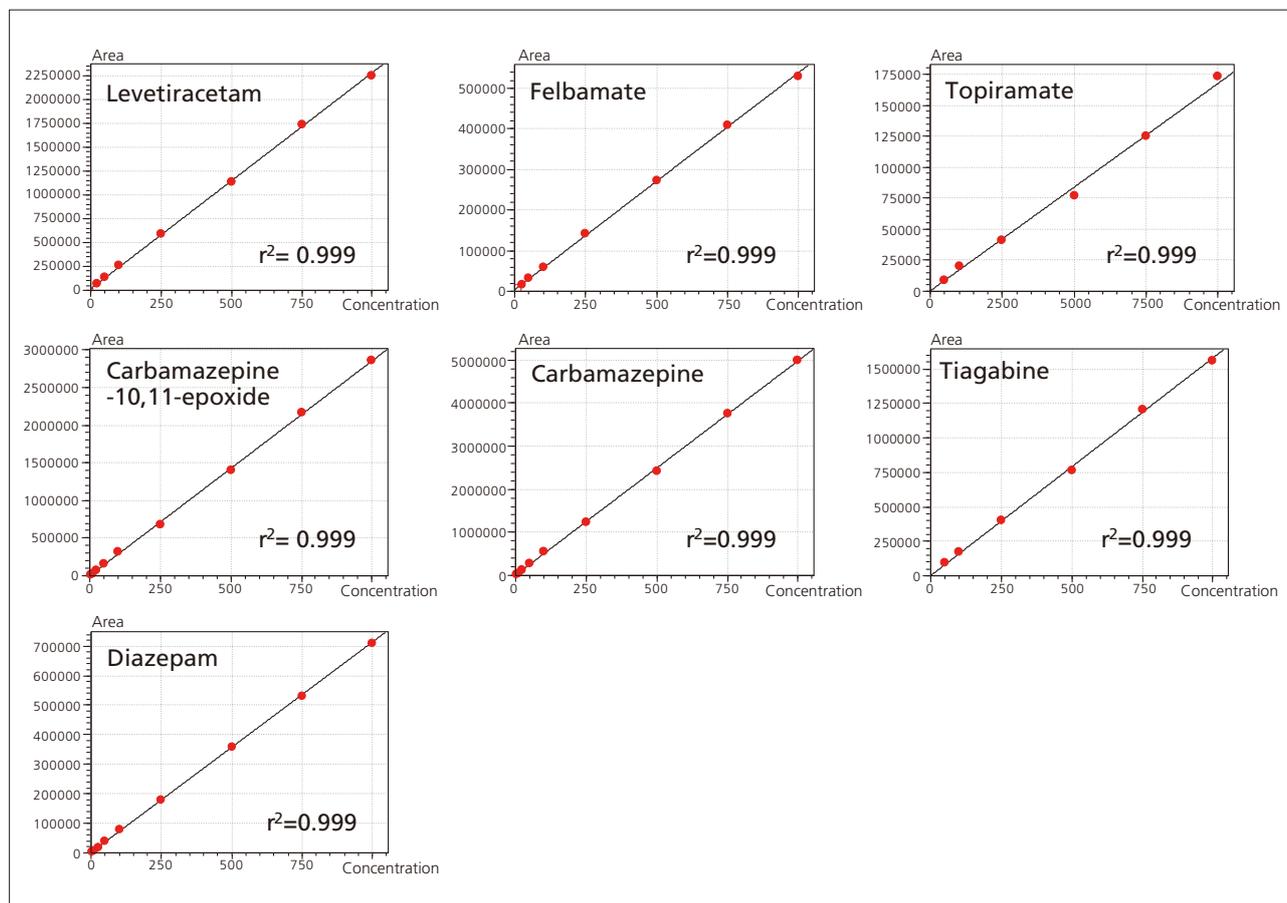

Fig. 3 Calibration Curves of Seven Antiepileptic Drugs and Drug Metabolites

Table 1 Results of Validation Test for Simultaneous Analysis of Antiepileptic Drugs

Compounds	Range (ng/mL)	QC samples concentration (ng/mL)			Accuracy (%)			% RSD (n=6)		
		LLOQ	Medium	ULOQ	LLOQ	Medium	ULOQ	LLOQ	Medium	ULOQ
Levetiracetam	10 - 750	10	100	750	94.6	106.1	99.2	3.42	1.23	1.98
Felbamate	25 - 1000	25	250	1000	98.6	101.8	99.6	6.28	1.88	1.50
Topiramate	500 - 10000	500	2500	10000	102.3	97.1	100.6	6.71	3.58	2.96
Carbamazepine-10, 11-epoxide	5 - 1000	5	100	1000	92.9	107.8	99.3	7.48	3.32	1.41
Carbamazepine	10 - 1000	10	100	1000	90.6	110.3	99.1	3.79	3.42	1.19
Tiagabine	50 - 1000	50	250	1000	98.5	101.9	99.6	1.95	2.00	1.26
Diazepam	5 - 1000	5	250	1000	98.1	102.4	99.5	4.61	1.50	1.53

Table 2 Analytical Conditions for Antiepileptic Drugs

Column	: Inertsil ODS-4 (50 mm L. × 2.1 mm I.D., 2 µm)
Mobile Phase	: A 10 mmol/L Ammonium acetate - Water
	: B Methanol
Flowrate	: 0.4 mL/min
Time Program	: B. Conc. 3 % (0 - 0.5 min) - 90 % (3.0 - 5.0 min) - 3 % (5.01 - 7.0 min)
Column Temperature	: 40 °C
Injection Volume	: 1 µL
Probe Voltage	: 4.5 kV / - 3.5 kV (ESI-positive / negative mode)
DL Temperature	: 150 °C
Block Heater Temperature	: 400 °C
Nebulizing Gas Flow	: 3 L/min
Drying Gas Flow	: 10 L/min
MRM Transition	: Levetiracetam (+) m/z 171.15 > 126.10, Felbamate (+) m/z 239.20 > 117.00, Carbamazepine-10,11-epoxide (+) m/z 253.15 > 180.05, Carbamazepine (+) m/z 237.20 > 194.05, Tiagabine (+) m/z 376.25 > 111.05, Diazepam (+) m/z 285.15 > 153.95, Topiramate (-) m/z 338.10 > 78.00

■ **System Validation for Antiarrhythmic Drugs Analysis**

TMD is used with a wide variety of drugs, and the physicochemical properties of these drugs differ individually. Therefore, confirming whether a given series of standard operations, which includes the process steps, tools, instruments and equipment used in an analytical workflow, are appropriate for the target drug is important for ensuring the analytical results obtained are valid. We introduce an example validation of sample preparation and analysis operations using antiarrhythmic drugs with very different physicochemical properties, and in particular very different hydrophilic properties.

We chose the highly hydrophilic drug sotalol (partition coefficient: log P=2.6342) and the highly hydrophobic drug amiodarone (log P=6.9326) and its active metabolite N-desethylamiodarone were chosen, and performed simultaneous analysis using the fully automated sample preparation LC/MS/MS system (Fig. 4).
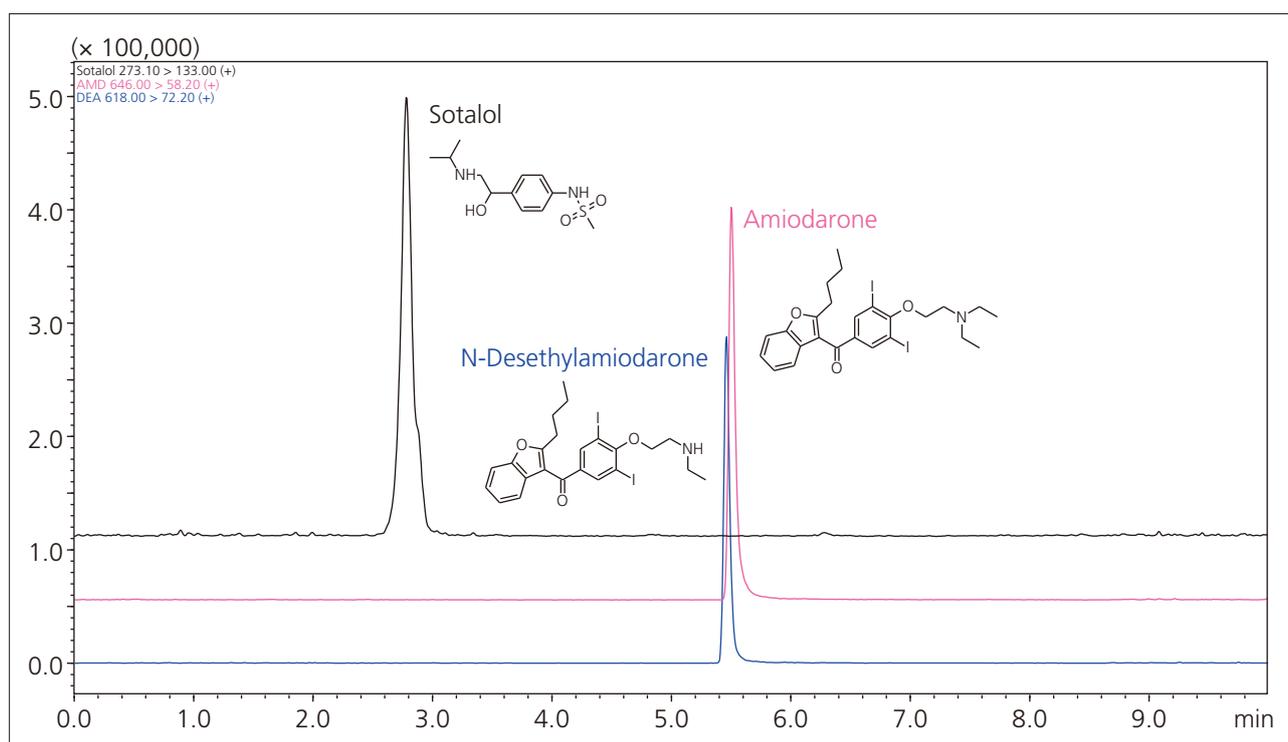

Fig. 4 Mass Chromatogram of Three Antiarrhythmic Drugs and Drug Metabolite in a Control Serum Sample

Calibration curves were prepared by continuous analysis, then used to validate accuracy and precision (repeatability). Good linearity was obtained across the set calibration curve range for each of the highly hydrophilic drug sotalol and the highly hydrophobic drug amiodarone and its active metabolite N-desethylamiodarone, with accuracy within 100 % ±15 % over the entire measurement range including the minimum limit of quantification. Similarly, precision was measured at a %RSD of within 15 %, showing that good repeatability was achieved (Table 3). These results indicate that sample preparation and analysis performed using the fully automated sample preparation system is suitable for a wide range of hydrophilic and hydrophobic drugs.

Table 3 Results of Validation Test for Simultaneous Analysis of Antiarrhythmic Drugs

Compounds	Range (ng/mL)	QC samples concentration (ng/mL)			Accuracy (%)			% RSD (n=6)		
		LLOQ	Medium	High	LLOQ	Medium	High	LLOQ	Medium	High
Sotalol	100-5000	100	1000	2000	107.0	101.2	101.1	3.20	1.83	1.80
Amiodarone	100-5000	100	1000	2000	99.2	102.6	100.6	3.78	1.66	1.99
N-Desethylamiodarone	100-5000	100	1000	2000	101.2	103.3	100.1	4.22	1.48	3.01

Table 4 Preparation Conditions for Antiarrhythmic Drugs

Sample Volume : 50 µL
Reagent   : Acetonitrile 200 µL
Shaking   : 90 sec, 1900 rpm
Filtration   : 150 sec

Table 5 Analytical Conditions for Antiarrhythmic Drugs

Column   : Mastro C18 (100 mm L. × 2.1 mm I.D., 3 µm)
Mobile Phase   : A 0.1 % Formic acid - Water
                                     : B 0.1 % Formic acid - Methanol
Flowrate   : 0.4 mL/min
Time Program   : B. Conc. 5 % (0 - 1.5 min) - 100 % (5.5 - 7.5 min) - 5 % (7.51 - 10 min)
Column Temperature   : 40 °C
Injection Volume   : 0.3 µL
Probe Voltage   : 4.5 kV (ESI-positive mode)
DL Temperature   : 250 °C
Block Heater Temperature : 400 °C
Nebulizing Gas Flow   : 3 L/min
Drying Gas Flow   : 15 L/min
MRM Transition   : Sotalol (+) m/z 273.1 > 133.0,
                                     Amiodarone (+) m/z 646.0 > 58.2,
                                     N-Desethylamiodarone (+) m/z 618.0 > 72.2

■ **Conclusion**

Results indicate that the fully automated sample preparation LC/MS/MS system can eliminate the risk of error or variability introduced by manual sample preparation that has been a problem for TDM, and also indicate this system can implement a quick and high-precision analytical workflow that is compatible with drugs with a wide variety of physicochemical properties. We anticipate the fully automated sample preparation LC/MS/MS system will contribute to improved analytical reliability and throughput in TDM.

[References]
1) Guidance for Industry: Bioanalytical Method Validation (2001, US FDA)
2) Guideline on Bioanalytical Method Validation in Pharmaceutical Development (Japan's MHLW, 2013)

Notes
• The products mentioned in this article have not been approved/certified as medical devices according to the Pharmaceutical and Medical Device Act in Japan.
• The analytical methods mentioned in this article cannot be used for diagnostic purposes.

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Application News

No. C153

Liquid Chromatograph Mass Spectrometry

Simultaneous Analysis of Antiarrhythmic Drugs in Human Blood Plasma Using the Fully Automated Sample Preparation LC/MS/MS System

During drug treatment with drugs that pose administration management difficulties, such as drugs with a narrow therapeutic range or drugs with a fine line between toxicity and effectiveness, the blood concentration of drugs in patients is measured to determine the optimal dose and method of administration for individuals based on pharmacokinetic and pharmacodynamic analysis. Application News No. C123 introduced an investigation into optimizing the analysis workflow including pretreatment by using the fully automated sample preparation LC/MS/MS system that comprises the CLAM-2000 fully automated LCMS sample preparation unit and a high performance liquid chromatograph mass spectrometer.

This article introduces a study which achieves a fast and simultaneous analysis workflow of six antiarrhythmic drugs with the fully automated sample preparation LC/MS/MS system.

T. Tsukamoto, D. Kawakami

■ Analysis of Antiarrhythmic Drugs in Blood Plasma with Fully Automated Pretreatment

Pretreatment of blood plasma samples for analysis normally requires a process that involves deproteinization by adding an organic solvent, followed by centrifugal separation of solid components and supernatant isolation. With the fully automated sample preparation LC/MS/MS system, these preparatory steps are done automatically just by setting a blood collection tube after blood plasma separation, and LC/MS/MS analysis is continuously performed (Fig. 1). Pretreatment of the next sample can also be performed in parallel with LC/MS/MS analysis, which can greatly reduce the time required for each sample analysis.

In this analysis example, a per-sample cycle time of 7 minutes was achieved from blood plasma pretreatment to the simultaneous analysis of six antiarrhythmic drugs and metabolites using LC/MS/MS (Table 1 and Fig. 2).
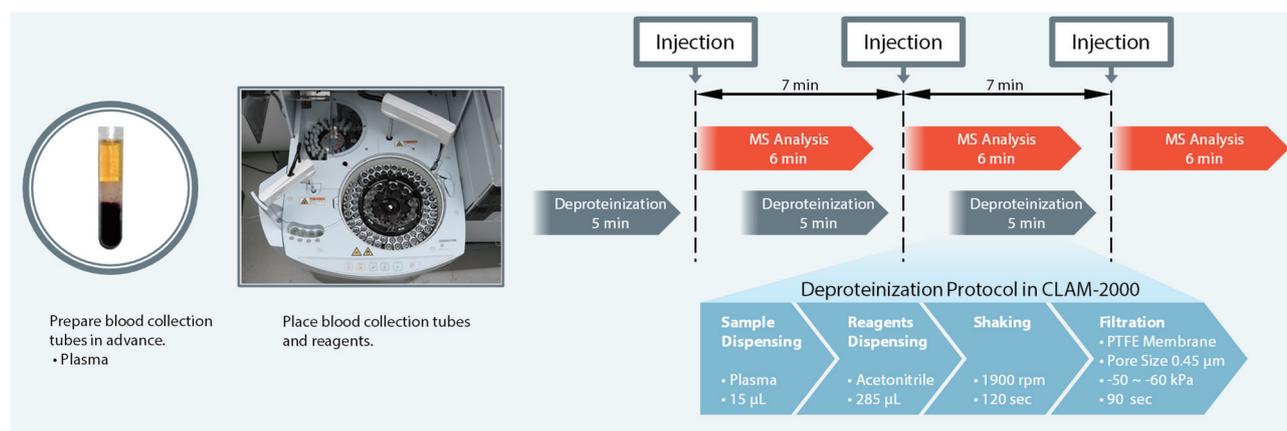

Fig. 1 Pretreatment Workflow of Blood Plasma Samples

Table 1 Antiarrhythmic Drugs and Metabolites

Compound	Molecular Formula	MRM Transition m/z
Amiodarone	$C_{25}H_{29}I_2NO_3$	646.0 > 58.1
Desethylamiodarone*	$C_{23}H_{25}I_2NO_3$	618.0 > 72.1
Bepridil	$C_{24}H_{34}N_2O$	367.1 > 84.1
Flecainide	$C_{17}H_{20}F_6N_2O_3$	415.0 > 301.0
Pilsicainide	$C_{17}H_{24}N_2O$	272.9 > 110.1
Cibenzoline	$C_{18}H_{18}N_2$	262.9 > 115.0
Mexiletine	$C_{11}H_{17}NO$	180.1 > 58.0
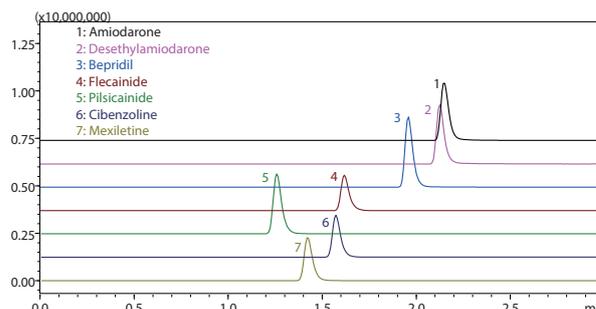
Fig. 2 Mass Chromatograms of Human Blood Plasma with Standard Additives

■ Validation Test of the Fully Automated Pretreatment Analysis Method

Calibration curves were created from the control blood plasma with standards added and the integrity of accuracy and precision were evaluated based on the analysis results of the QC samples (at concentrations of n = 5) (Table 2). Good linearity was obtained in the set concentration range for all antiarrhythmics. The accuracy of the QC samples in the entire range, including the quantitative lower limit, was within 100 ± 15 %. Similarly, precision (%RSD) was within 15 % and good repeatability was obtained.

Immediately after analysis of the highest calibration standard sample, blank blood plasma was measured to check for carryover in the fully automated sample preparation LC/MS/MS system. No significant carryover was detected for any of the drugs upon comparison with the peak intensity of the lowest calibration standard sample (Fig. 3).

The above results show that the fully automated sample preparation LC/MS/MS system used in this article is capable of sufficiently reliable quantitative analysis when performing consecutive analyses of samples of wide-ranging concentrations.

Table 2 Validation Test Results for Simultaneous Analysis of Antiarrhythmic Drugs and Metabolites

Compounds	Cal. Range [ng/mL]	Correlation Coefficient R	Accuracy %				Precision %RSD, n=5			
			LLOQ	Low	Medium	High	LLOQ	Low	Medium	High
Amiodarone[*1]	100-3000	0.9983	98.3	100.6	99.4	103.9	4.1	2.9	3.0	2.7
Desethylamiodarone[*1]	100-3000	0.9987	99.2	98.9	101.1	100.3	5.3	4.2	3.6	4.2
Bepridil[*2]	50-1500	0.9992	100.9	100.5	96.6	103.4	4.1	3.7	2.3	1.8
Flecainide[*2]	50-1500	0.9987	98.1	98.7	96.7	101.4	4.7	3.3	2.4	2.4
Pilsicainide[*1]	100-3000	0.9987	100.4	99.6	97.3	104.8	4.0	3.0	1.8	2.0
Cibenzoline[*2]	50-1500	0.9987	102.4	101.4	99.1	102.9	4.2	3.4	3.0	2.4
Mexiletine[*1]	100-3000	0.9984	104.5	107.4	106.3	107.8	3.8	3.9	2.6	2.6

*1: 100 ng/mL for LLOQ, 250 ng/mL for Low, 1000 ng/mL for Medium, 3000 ng/mL for High
*2: 50 ng/mL for LLOQ, 125 ng/mL for Low, 500 ng/mL for Medium, 1500 ng/mL for High
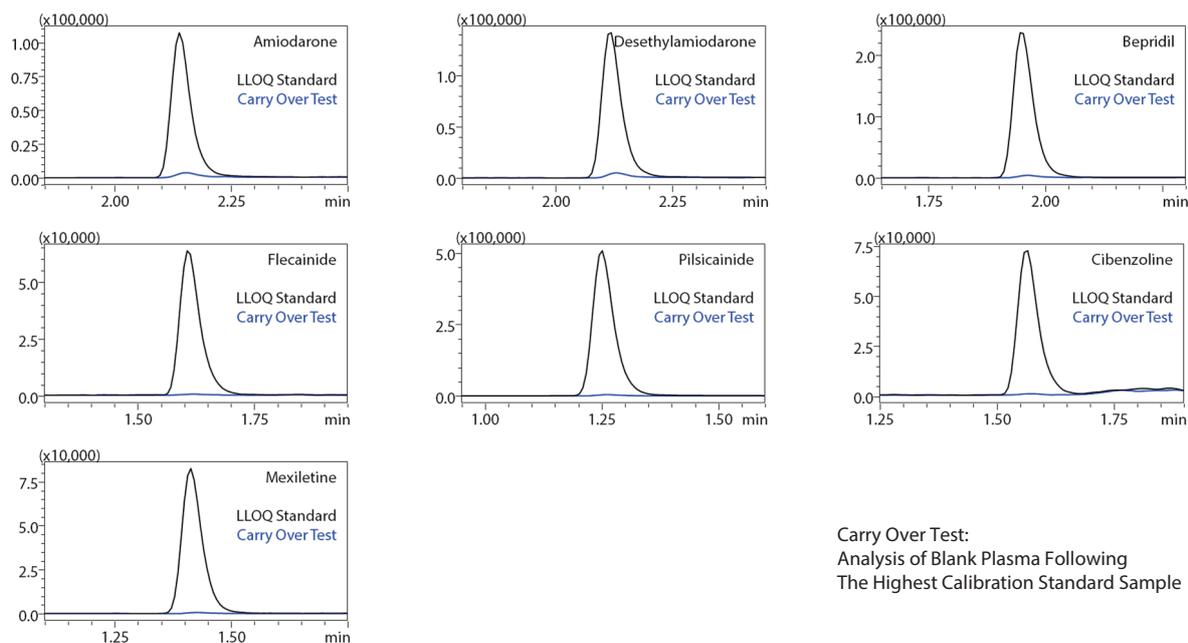
Carry Over Test:
Analysis of Blank Plasma Following
The Highest Calibration Standard Sample

Fig. 3 Carryover Test Results

Table 3 Analysis Conditions (Validation Test)

|	
System	: CLAM-2000 + Nexera + LCMS-8060
Protocol	: Plasma disp. 15 µL - acetonitrile disp. 285 µL - shaking at 1900 rpm, 120 sec - filtration for 90 sec
Column	: Shimadzu GLC Mastro C18 (50 mmL. × 2.1 mmI.D., 3 µm)
Mobile Phase	: A) 0.1% Formic acid - Water, B) 0.1% Formic acid - Methanol
Flow Rate	: 0.4 mL/min
Time program	: B Conc. 10% (0 min) – 100% (2 – 3.5 min) – 10% (3.51 – 6 min)
Column Temp.	50 °C Injection Volume : 0.2 µL
Probe Voltage	: 2.0 kV (ESI-positive mode)
Interface Temp.	: 300 °C DL Temp. : 250 °C
Block Heater Temp.	: 400 °C Nebulizing Gas Flow : 3 L/min
Heating Gas Flow	: 10 L/min Drying Gas Flow : 10 L/min

■ Comparative Test with Manual Pretreatment

A comparative test was performed between a manual pretreatment method and the fully automated pretreatment analysis method that employs the fully automated sample preparation LC/MS/MS system. Human blood plasma for measuring the concentration of amiodarone was used.

The manual pretreatment method involved manually isolating the blood plasma, adding acetonitrile, and mixing to perform deproteinization. After centrifugal separation of this sample, the supernatant was then transferred to a vial for LC/MS/MS analysis. On the other hand, the fully automated pretreatment analysis method enabled the entire analysis process, from blood plasma isolation to LC/MS/MS analysis, to be performed completely automatically using the system described in this article (Fig. 4).

A comparison of quantitative values between the methods was performed for amiodarone and the metabolite desethylamiodarone (Fig. 5 and 6, Table 4 and 5). In the wide range of concentrations detected from the samples, there was favorable agreement between the quantitative results of the manual pretreatment method and the fully automated pretreatment analysis method. The coefficient of determination (R^2) of both methods calculated from these results was 0.95 or higher (Fig. 7 and 8).

The fully automated pretreatment analysis method used by this system is a fast and low-burden analysis technique that achieves quantitative results equivalent to conventional manual pretreatment methods and we anticipate its utilization into the future.

Manual Pretreatment

Plasma 100 µL
↓ Acetonitrile 450 µL
Shaking (60 sec.)
↓
Centrifuge (15000rpm, 5 min.)
↓
Supernatant 50 µL
↓
LC/MS/MS Analysis

Automated Pretreatment with CLAM-2000

Plasma 50 µL
↓ Acetonitrile 225 µL
Shaking (120 sec.)
↓
Filtration (90 sec.)
↓
LC/MS/MS Analysis
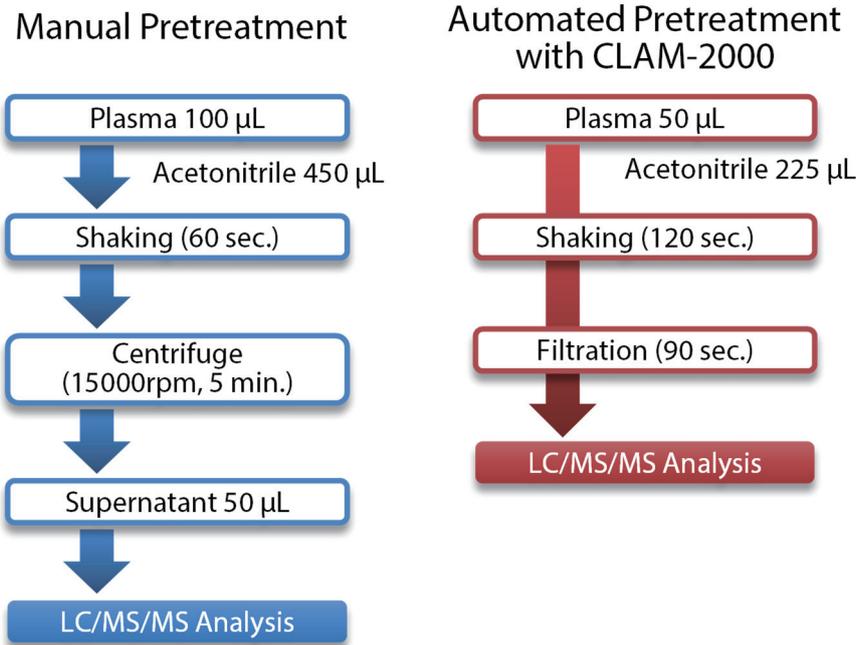
Fig. 4 Pretreatment Workflow of the Manual Pretreatment Method and Fully Automated Pretreatment Analysis Method

(x100,000)
1:AMD 646.00>58.10(+) CE: -52.0
2:DEA 618.00>72.10(+) CE: -32.0
Manual Pretreatment
Amiodarone 546 ng/mL
Desethylamiodarone 416 ng/mL
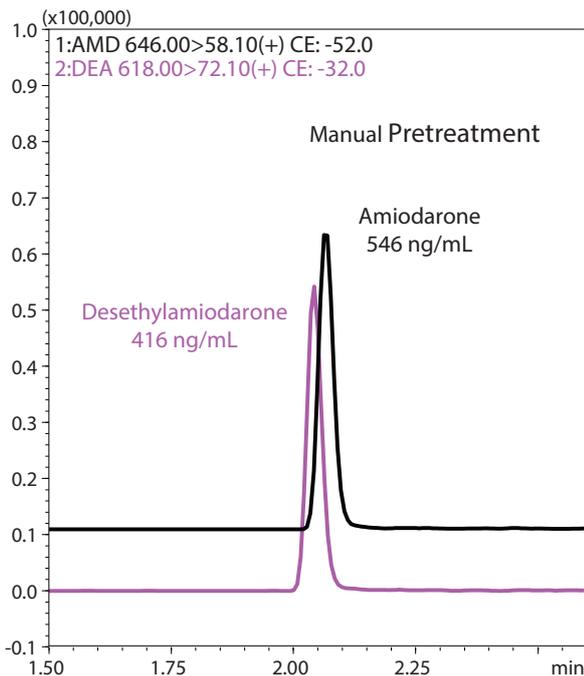
Fig. 5 Human Blood Plasma (Sample 3) Analysis Results Using the Manual Pretreatment Method

(x100,000)
1:AMD 646.00>58.10(+) CE: -52.0
2:DEA 618.00>72.10(+) CE: -32.0
Automated Pretreatment with CLAM-2000
Amiodarone 557 ng/mL
Desethylamiodarone 423 ng/mL
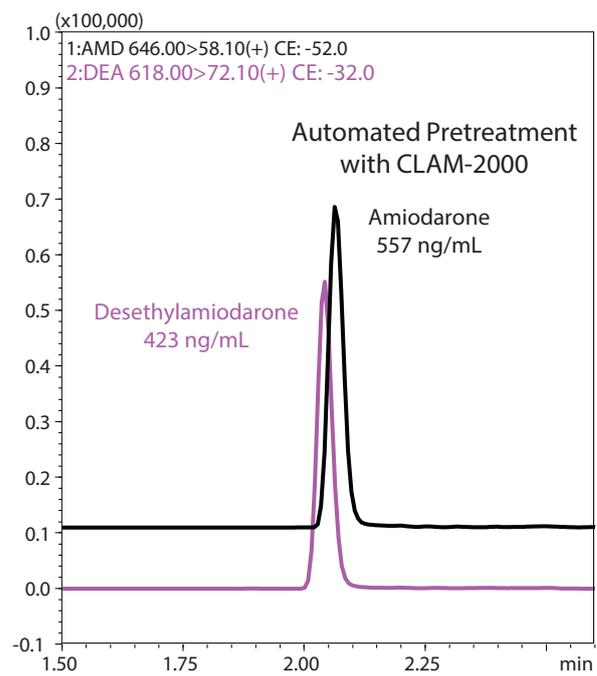
Fig. 6 Human Blood Plasma (Sample 3) Analysis Results Using the Fully Automated Pretreatment Analysis Method

Table 4 Quantitative Results of the Manual Pretreatment Method and Fully Automated Pretreatment Analysis Method (Amiodarone)

	Amiodarone		
	Manual [ng/mL]	Automated [ng/mL]	Ratio %*
Sample 1	373	411	110.2
Sample 2	399	404	101.3
Sample 3	546	557	102.0
Sample 4	205	211	102.9
Sample 5	963	895	92.9
Sample 6	1,318	1,213	92.0
Sample 7	1,271	1,229	96.7
Sample 8	1,233	1,282	104.0
Sample 9	2,259	2,208	97.7
Average			100.0
RSD %			5.8

* Automated Pretreatment / Manual Pretreatment

**Table 5 Quantitative Results of the Manual Pretreatment Method and Fully Automated Pretreatment Analysis Method (Desethylamiodarone)**

	Desethylamiodarone		
	Manual [ng/mL]	Automated [ng/mL]	Ratio %*
Sample 1	304	271	89.1
Sample 2	412	366	88.8
Sample 3	416	423	101.7
Sample 4	271	240	88.6
Sample 5	717	654	91.2
Sample 6	151	150	99.3
Sample 7	431	408	94.7
Sample 8	664	628	94.6
Sample 9	940	1,080	114.9
Average			95.9
RSD %			8.9

* Automated Pretreatment / Manual Pretreatment

AMD

$y = 0.975x$
$R^2 = 0.9946$

Fig. 7 Comparison of Quantitative Results for Amiodarone

DEA

$y = 1.007x$
$R^2 = 0.9541$
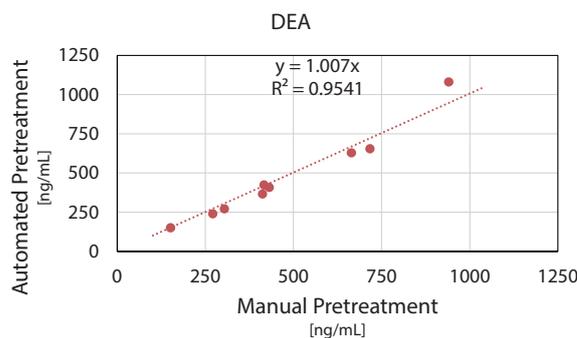
Fig. 8 Comparison of Quantitative Results for Desethylamiodarone

Table 6 Analysis Conditions (Comparative Test of Pretreatment Methods)

System	:	CLAM-2000 + Nexera + LCMS-8040
Protocol	:	Plasma disp. 50 µL - acetonitrile disp. 225 µL - mixing at 1900 rpm, 120 sec - filtration for 90 sec
Column	:	Shimadzu GLC Mastro C18 (50 mmL. × 2.1 mmI.D., 3 µm)
Mobile Phase	:	A) 0.1% Formic acid - Water, B) 0.1% Formic acid - Methanol
Flow Rate	:	0.4 mL/min
Time program	:	B Conc. 10 % (0 min) – 100 % (2 – 3.5 min) – 10 % (3.51 – 6 min)
Column Temp.		50 °C Injection Volume : 0.1 µL
Probe Voltage	:	4.5 kV (ESI-positive mode)
DL Temp.	:	250 °C Block Heater Temp. : 400 °C
Neb. Gas Flow	:	3 L/min Drying Gas Flow : 15 L/min

References • Guidance for Industry : Bioanalytical Method Validation (2001, US FDA)
• Guideline on Bioanalytical Method Validation in Pharmaceutical Development (2013, Japan MHLW)

Notes • 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.
• The samples described in this document were all sampled and measured at the National Cerebral and Cardiovascular Center Hospital in Japan. Permission was obtained regarding the publication of measurement data.

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Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS

ASMS 2017 ThP084

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Introduction

CLAM-2000 (Shimadzu Corp., Japan) fully automates blood or other samples pre-treatment prior to LC-MS analysis (Fig.1). The whole blood has cell debris, fibrin clots and so on. It is desirable to remove such particulates for accurate sample dispensing by centrifugation. However, this poses a challenge to the measurement of immunosuppressants (ISP) in whole blood samples since large proportion of ISP are bound to cytoplasmic proteins in erythrocytes[1]. Thus, it is mandatory to release erythrocytes prior to any centrifugation of the blood. Current practice is to freeze and thaw samples, which is not convenient for emergency analysis. To address this, several protocols of hemolysis were tested. Evaluation of the protocol was based on lysis efficiency, immunosuppressant recovery and time consumption.

Figure 1. Fully automated sample preparation module CLAM-2000 and
triple quadrupole mass spectrometer LCMS-8060.

Methods and Pretreatment

Individual blood sample from healthy volunteer was spiked with tacrolimus (MW: 804.0), sirolimus (MW: 914.2), everolimus (MW: 958.2), and cyclosporine A (MW: 1202.6) and incubated for 30 minutes at room temperature. Then aliquots of each sample were subjected to all lysis protocols. Lysis efficiency was estimated by measuring hemoglobin absorbance in supernatant using a UV-Visible spectrophotometer (UV-1280. Fig.2).

Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS

Lysis Protocols

Lysis reagents were prepared as following. Ammonium chloride mixture was prepared with ammonium chloride, NaHCO3 and EDTA in water. HCl solution was diluted HCl at 2 mol/L in water. Each lysis protocols were performed prior to centrifugation

- Control (1 mL)
- Ammonium Chloride mixture (500 µL) + blood (250 µL)
- HCl solution (100 µL) + blood (1 mL)
- Blood sample (1 mL) with the ultrasound (5 min)
- Blood sample (1 mL) with the ultrasound (10 min)
- Blood sample (1 mL) with the ultrasound (20 min)
- Blood sample (1 mL) with freeze/thaw -20°C (30 min)
- Blood sample (1 mL) with freeze/thaw -80°C(30 min)
- Blood sample (0.2 mL) with freeze/thaw -20°C (30 min)
- Blood sample (0.2 mL) with freeze/thaw -80°C (30 min)

Figure 2. UV-Visible Spectrophotometer UV-1280.

Pretreatment for UV-visible spectrophotometer

Each lysis blood samples (8 µL) are mixed with Drabkin's reagent (2 mL) which has a role of quantitative, colorimetric determination of hemoglobin concentration in whole blood with 540 nm band.

Pretreatment for LC/MS

13C, D2-tacrolimus, D3-sirolimus and D4-everolimus were used for ISTD. Zinc sulfate mixture was prepared with ACN, MeOH, zinc sulfate and ammonium formate. A blood sample (50 µL) was mixed with ISTD (25 µL) and zinc sulfate mixture (350 µL). After centrifugation, ISP recovery was measured using an online SPE-LC-MS/MS method. Supernatant obtained in each tested condition was automatically prepared using CLAM-2000. This included addition of internal standards, protein precipitation, filtration and transfer to the LC autosampler. Acquisition was performed in MRM mode using a triple quad mass spectrometer (LCMS-8060).

MP A : 90%Buffer + 10%MeOH
MP B : 10%Buffer + 90%MeOH
* Buffer: Ammonium formate (3 mM, pH 3.6) in water

Valve Position [0] : Red (2-3), [1] : Blue (1-2)
Valve Position 1 : START METHOD (LOAD)
Valve Position 0 : ELUTION
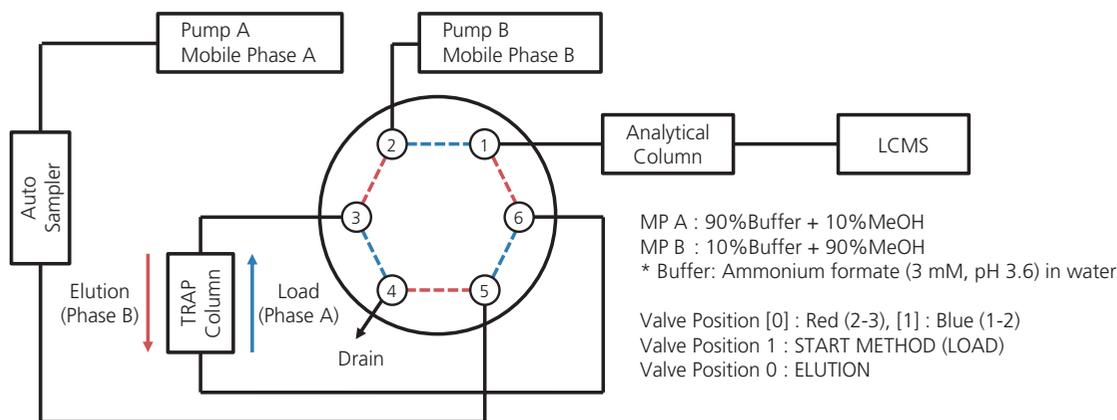
Figure 3. Flow diagram for online SPE

Table 1. LC and MS conditions

[LC] NexeraX2 System	
Column Temp.	: 65 °C
Analytical Column	: Inertsil ODS-3 (5 um 2.1*50 mm)
Trap Column	: MAYI C8 10x4.6 mm
Time Program	: Isocratic
Injection Volume	: 5.0 µL
[MS] LCMS-8060	
Ionization	: ESI Positive
Nebulizer Gas	: 3 L/min
Interface temperature	: 200 °C
Desolvation Line	: 150 °C
Heat Block temperature	: 200 °C
Heating Gas	: 10 L/min
Drying Gas	: 10 L/min

Table 2. MRM transitions for ISP

ISP	transition	CE	Internal Standard
Tacrolimus	821.30 > 768.50	22	13C,D2-Tacrolimus
Sirolimus	931.30 > 864.50	18	D3-Sirolimus
Everolimus	975.30 > 908.40	20	D4-Everolimus
Cyclosporine A	1219.70 > 1202.80	21	-

Results

Lysis efficiency evaluated by UV-Visible spectrophotometer

Absorption value was measured with each lysis protocols (Fig. 4). Absorption value with each lysis protocols was divided by reference which includes RBC for absorption rates. High absorption rates means that there are a lot of hemoglobin which is generated by release of erythrocytes. Freeze/thaw with -80ºC at both volume were efficient for lysis. Freeze/thaw with -20ºC was also efficient. However freezing time is not sufficient for 1.0 mL. In the case of using 0.2 mL, sample pipetting influence the reproducibility (The standard deviation was very high). Ammonium chloride took the place of -80ºC method, because the blood was diluted 3 times with ammonium chloride mixture.

Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS
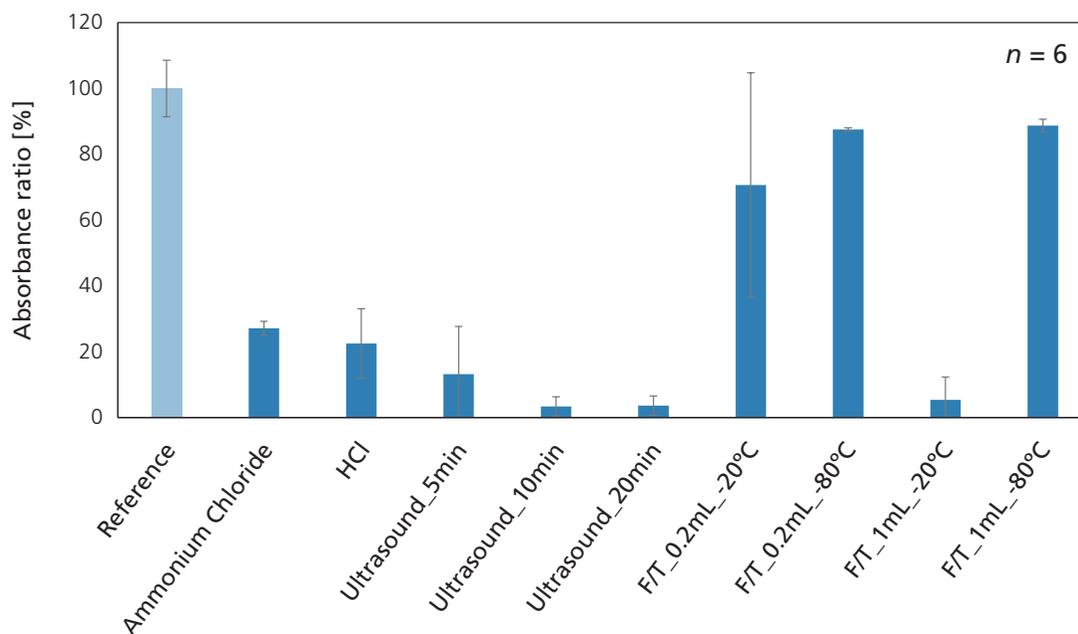
Figure 4. Absorption ratio with each lysis protocols. Freeze/thaw with -80°C was effective lysis procedure. -20°C was also efficient at the volume of 0.2 mL. However, the pipetting technique influenced the number of hemoglobin in such a small volume. Ammonium chloride was also effective method. The reason why is that it was diluted 3 times.

Incorporation of ISP into erythrocytes by LC/MS/MS

ISP are spiked to the whole blood and separated only spiked and centrifuged sample and reference sample which includes particulates. Where the ISP are located was tested by LC/MS/MS (LCMS-8060) for the two samples (plasma and whole blood). Fig.5 shows the MRM chromatogram of the ISP and internal standard.

Compared with these results, about 90% of tacrolimus, sirolimus, everolimus and about 60% of CSA were located to the erythrocytes.
After the experiment, ISP recovery rate with each lysis protocols were evaluated by LC/MS/MS. The whole blood sample were performed for reference.

Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS
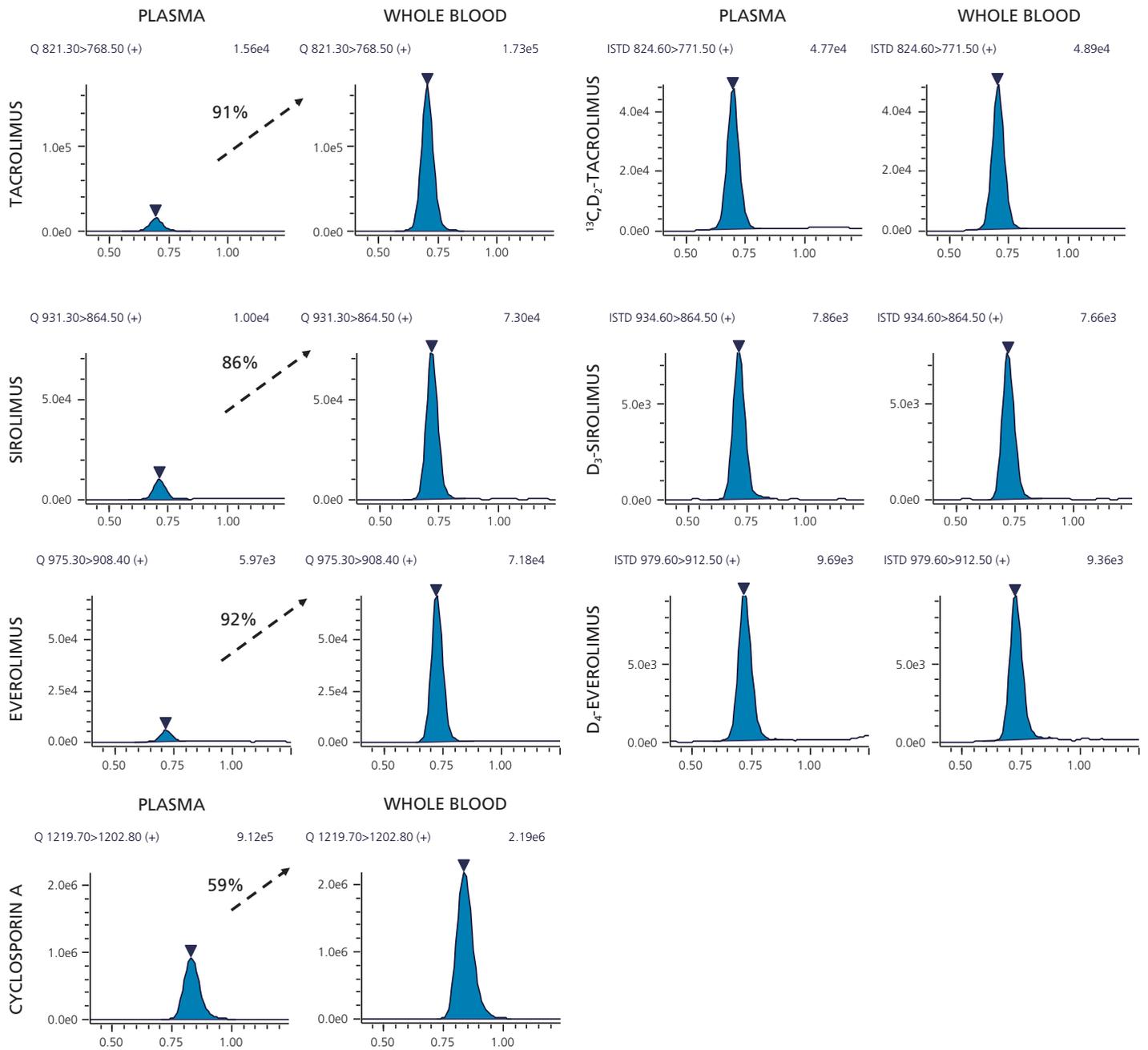
Figure 5. Peak area of ISP and ISTD in a plasma or whole blood. About 90% of tacrolimus, sirolimus and everolimus and about 60% of cyclosporine A were entrapped by erythrocytes.

Evaluation of Blood Lysis Procedures prior to Automated Sample Preparation for Immunosuppressant Assay by LC-MS/MS

ISP recovery rate with each lysis protocols

Area ratio of ISP which was devided by peak area of reference was shown in Fig. 6. There are a correlation between the results of UV spectrophotometer and LC/MS. Freeze/thaw with -80°C was the closest to the reference sample. -20°C was also effective. However, the reproducibility was very low. This is because freezing time was not sufficient. F/T with -80°C was the most efficient pre-treatment method for CLAM-2000. Ammonium chloride was an alternative method. However, it requires sample preparation.
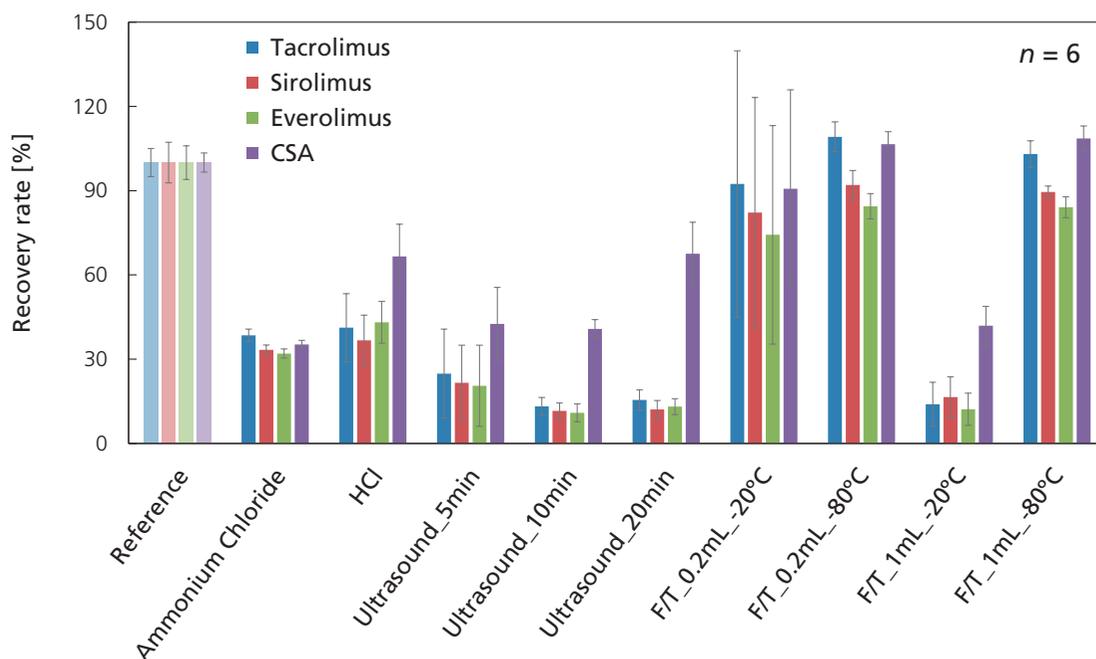
Figure 6. Area ratio of tacrolimus, sirolimus, everolimus, and CSA.
There are a correlation between lysis efficiency and recovery rates of ISP.
(Freeze/thaw with -80°C was the closest to the reference sample)

Conclusion

- Freeze/thaw with -80°C was efficient for blood lysis and recovery of ISP whatever the volume.
- Freezing time was not sufficient for -20°C in a volume of 1.0 mL or 0.2 mL.
- Ammonium chloride can be used as alternative. However, it induce sample dilution.
  Thus, this should be taken into account.
- ISP recovery is correlated with lysis efficiency.

Reference

1) Lugia Rossi, et al., *Advanced Drug Delivery Reviews, 2016*, **106**, 73-87

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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

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.

Report creation

MS/MS
Library
search

-Serum
-Whole Blood
-Urine
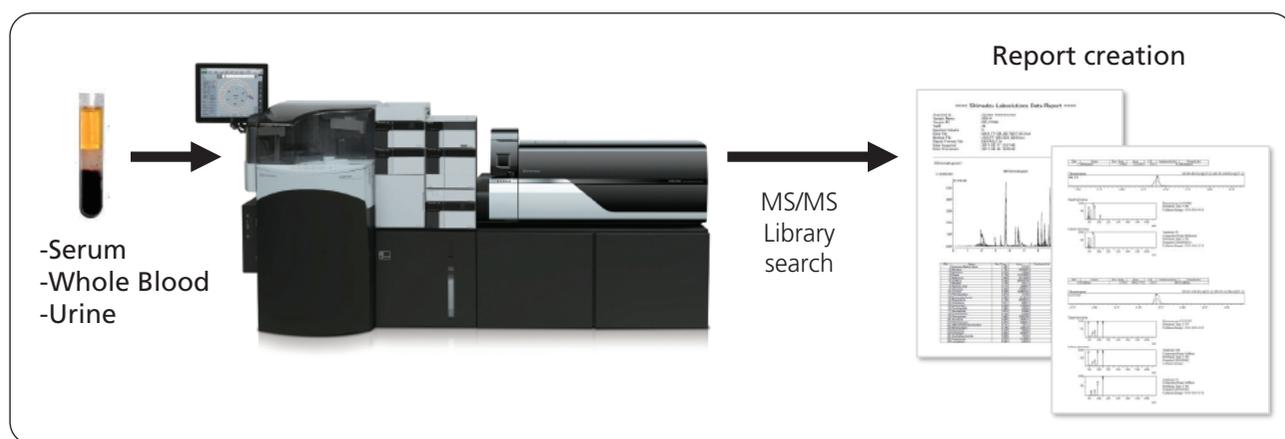
Figure 2 CLAM-2000 and LCMS-8060 system

Sample Dispensing
• **50 μL** of serum or whole blood

Reagent Dispensing
• **50 μL** of Water

Shaking
• **30sec**

Reagent Dispensing
• **300 μL** of MeOH

Shaking
• **60sec**

Filtration (PTFE, 0.45μm)
• **90sec**

To AutoSampler
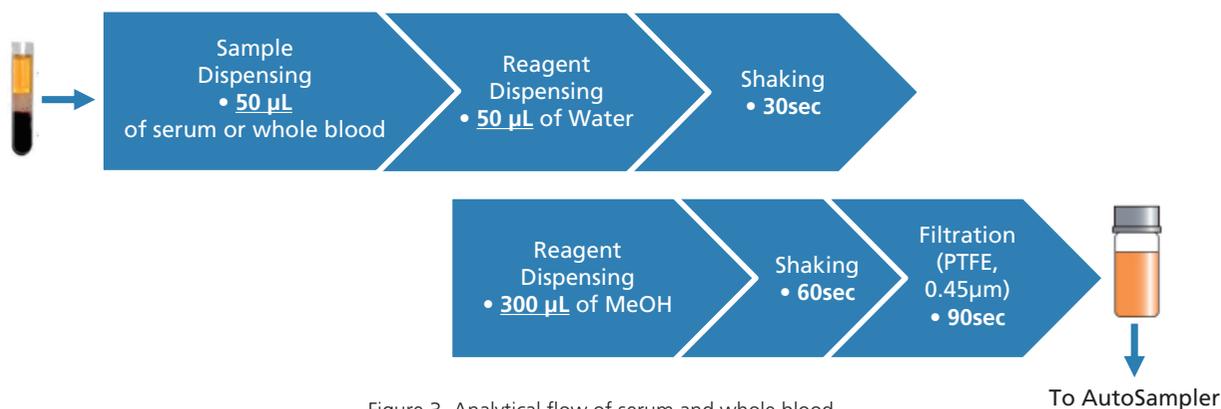
Figure 3 Analytical flow of serum and whole blood

Sample Dispensing
• **100 μL** of urine

Reagent Dispensing
• **100 μL** of MeOH

Shaking
• **60sec**

Filtration (PTFE, 0.45μm)
• **90sec**

To AutoSampler
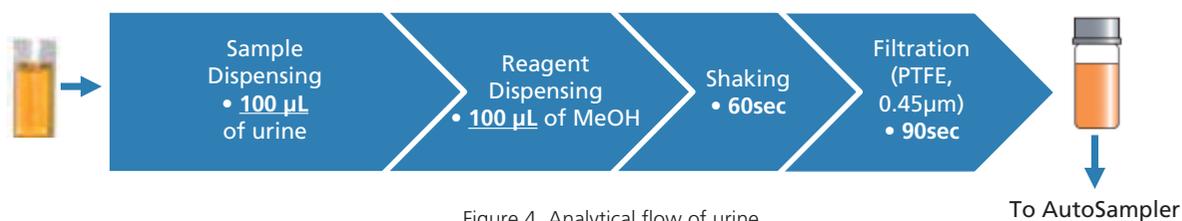
Figure 4 Analytical flow of urine
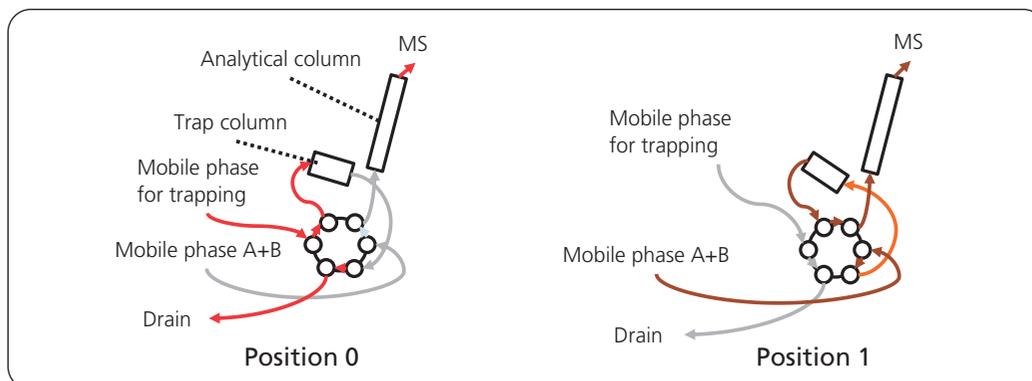
Figure 5 Flow diagram of trapping system

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%

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

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.
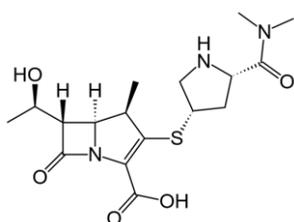

Figure 1: Structure of meropenem (MER) with a β-lactam ring.

❑ 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

125 μL of Pool Human Plasma

(1) Spiked 5 μL of I.S.   (2) Add 365 μL of ACN/MeOH (1:1)

Shake & Vortex for 10 mins

Centrifuge for 10 mins at 13,000 rpm

~480 μL of Supernatant

0.2 μm Nylon Filter

~400 μL of Filtered Spiked Plasma

2 μL injected to LCMS-8060
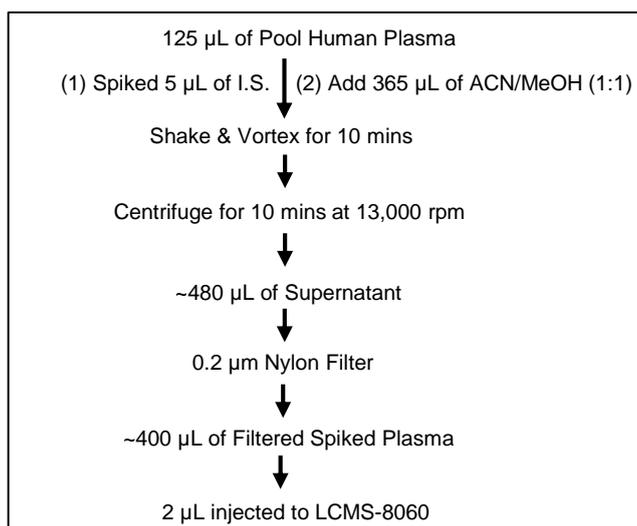
**Figure 2:** Procedure of protein crash and spiked-sample preparation

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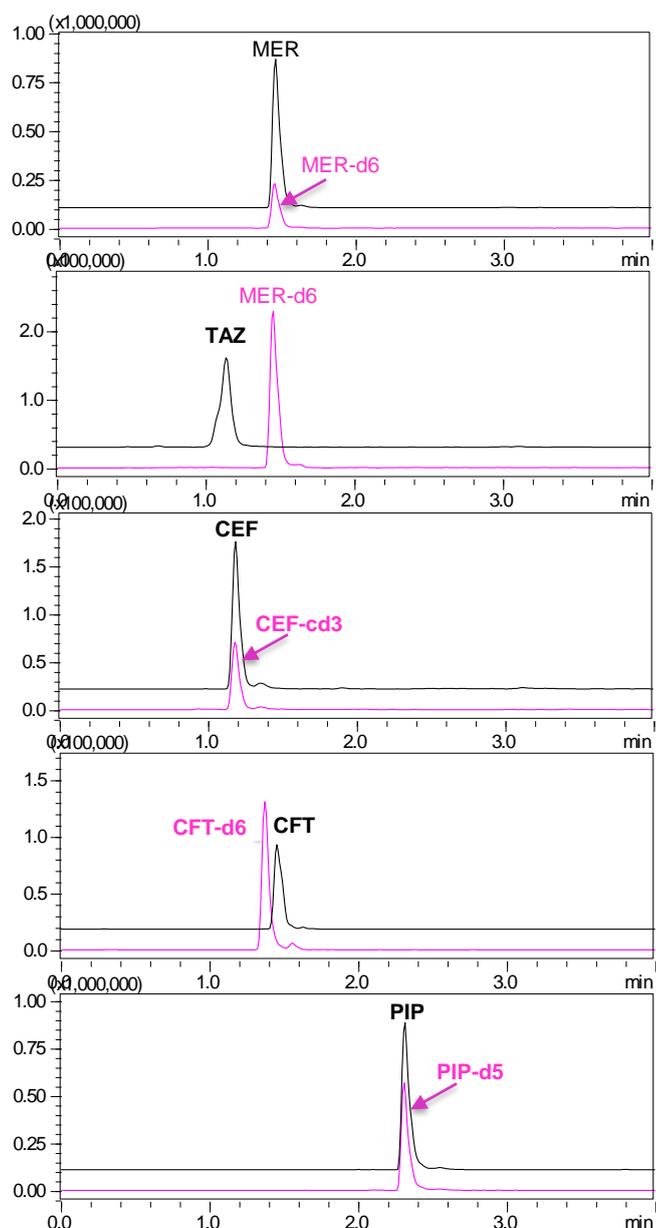
Figure 3: MRM chromatograms of five β-lactam antibiotics each (400 ng/mL) with internal standards in plasma on LCMS-8060

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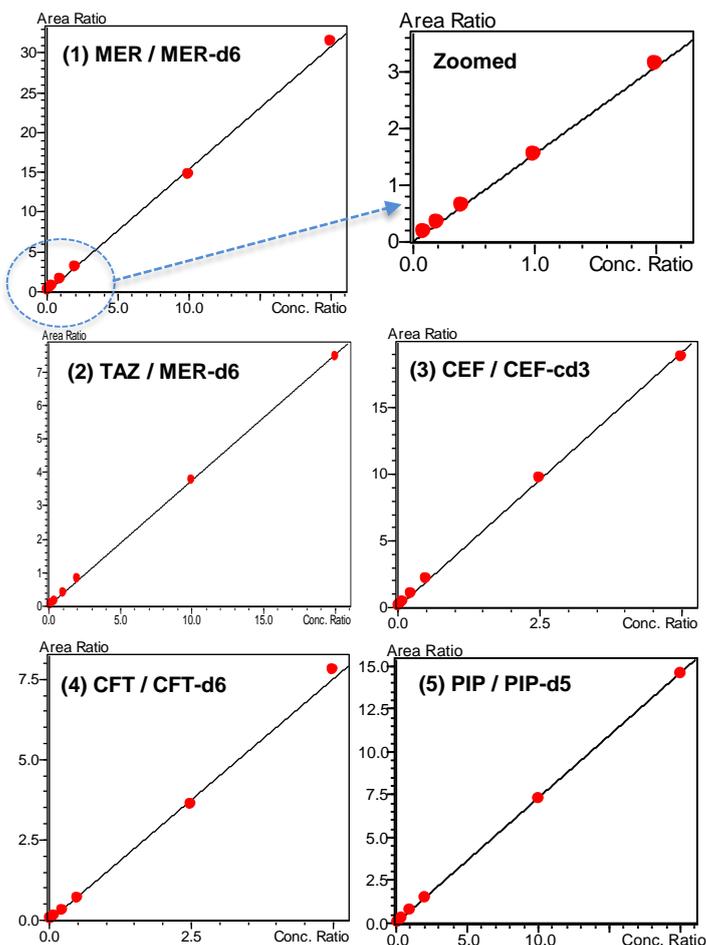
Figure 4: Calibration curves of five β-lactam antibiotics with stable isotope labelled internal standards in human plasma on LCMS-8060. Details of the calibration information are shown in Table 3.

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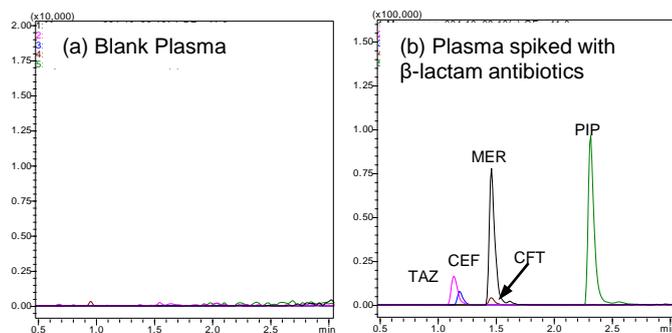
**Figure 5:** MRM chromatograms of blank plasma and plasma spiked with five β-lactam antibiotics (40 ng/mL) on LCMS-8060

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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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 4ºC, 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
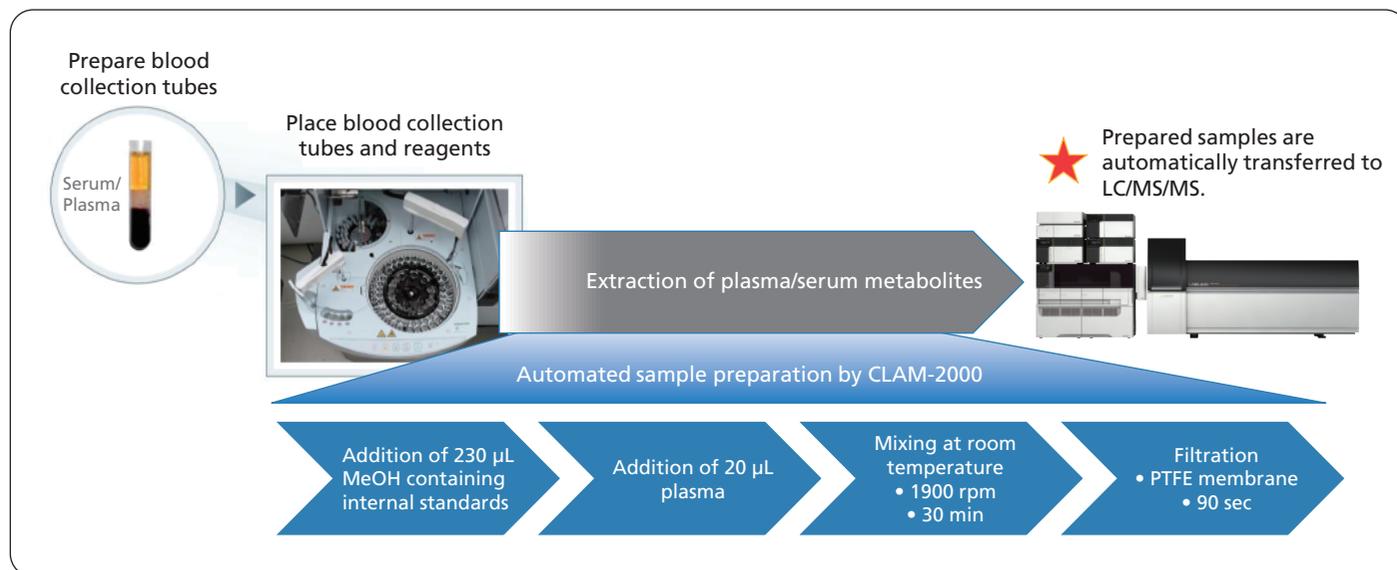
Figure 1. Workflow for analysis of metabolites
using fully automated sample preparation LC/MS/MS system

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	: 40ºC

MS conditions (LCMS-8040)	
Ionization	: ESI (Positive/Negative)
Nebulizing Gas Flow	: 2.0 L/min.
Drying Gas Flow	: 15.0 L/min.
DL temperature	: 250ºC
Block Heater Temperature	: 400ºC

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.

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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Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment

ASMS 2017   WP 348

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Determination of Unbound Urinary Amino Acids Incorporated
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Introduction

Free or unbound amino acids are important metabolites in human blood and urine [1]. The profile of unbound amino acids in urine are the reference indication of metabolic imbalances and amino acid transport disorders as well as dietary protein adequacy and assimilation. Creatinine produced by muscle metabolism is excreted in the urine, which can be used to normalize the metabolite levels to compensate the large variation due to different intakes of water and fluid food [2-4]. The aim of this study is to develop a reliable LC-MS/MS method for quantitation of

22 free amino acids and creatinine in urine samples. A derivatization-free LC-MS/MS amino acid method [5] with stable isotope labelled IS was employed. An on-line sample pre-treatment module CLAM-2000 coupled with LC-MS/MS makes the analysis fully-automated, which enables from adding internal standards, sample and solvent mixing, shaking for protein-crash and filtration to transferring the final sample solution to LC-MS/MS for analysis.

Experimental

A stock solution of 22 amino acids (AA) and creatinine (CRE) were prepared from powder standards. Two isotope-labelled amino acid standards, phenylalanine (ring-d5) and serine (2,3,3-d3) were added to the samples as internal standard by CLAM-2000 prior to LC-MS/MS analysis. A total of 28 urine samples were collected from health individuals of different ages and genders and used for amino acid analysis in this study. The CLAM-2000 is an automated on-line sample pre-treatment module (Shimadzu Corporation), which was employed for urine

sample preparation automatically for a connected LC-MS/MS system. The automated operation includes adding IS, sample-solvent mixing, shaking and filtration etc. The prepared sample was subsequently transferred to LC-MS/MS triple quadrupole system (LCMS-8040) for analysis (Figure 1). An Amino Acid Column (100x3mm, 3µm) was adopted for separation of the 22 compounds with an optimized gradient elution program. The detailed LC and MS/MS conditions are compiled in Table 1.

Automated process takes 3 to 5 minutes per sample

Sample Pipetting | Reagent Pipetting | Shaking | Filtration | Heating | Sample Transfer | Samples are transferred to LC-MS/MS automatically.
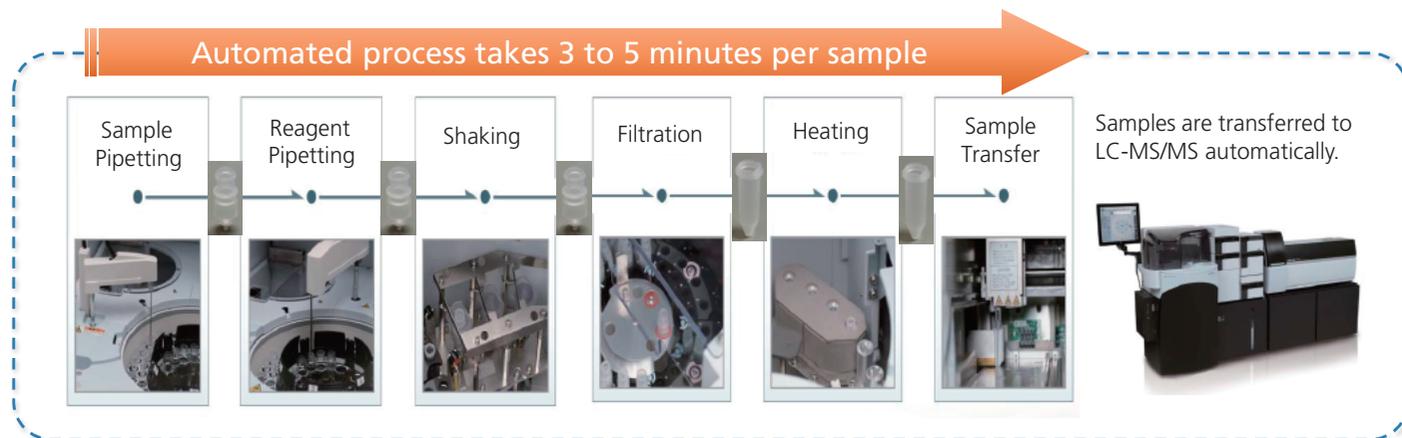
Figure 1: Workflow of CLAM-2000: a fully automated sample preparation module coupled with LCMS-8040

Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment

Table 1: Analytical conditions of twenty two amino acids without derivatization on LCMS-8040 and CLAM-2000

Column	: Intrada Amino Acid (100 mmL x 3 mmID, 3μm)
Flow rate	: 0.6 mL/min
Mobile phase	: A: ACN / THF / 25mM ammonium formate / FA = 9 / 75 /16 / 0.3 (v)
	B: ACN / 100mM ammonium formate = 20 / 80
Elution mode	: Gradient elution, 0-3min (0% B) → 9min (17% B) →
	16-18.5min (100% B) → 19min (0% B)
Oven temp.	: 35°C
Injection vol.	: 5.0 μL
Interface	: ESI
MS mode	: Posi, MRM
Block temp.	: 400°C
DL temp.	: 250°C
CID gas	: Ar (230kPa)
Nebulizing gas flow	: N_2, 2 L/min
Drying gas flow	: N_2, 15 L/min

Results and Discussion

Quantitative method for 22 AA and CRE on CLAM-LC-MS/MS platform

The 20 proteinogenic amino acids (AA), citrulline (Cit) and ornithine (Orn) as well as creatinine (CRE) are the targeted analytes in urine in this study. This is because citrulline and ornithine are the dietary amino acids in the urea cycle along with arginine. A MRM method for quantitative analysis of the 22 AA and CRE (creatinine) was established with IS (internal standard) method as summarized in Table 2. In a previous study [5], it was observed that glycine exhibited low peak intensity and sensitivity in MRM mode (76.1>30.1). Thus, SIM mode (m/z 76.1) was selected for glycine. Figure 2 shows the MRM chromatograms of the 22 AA and CRE mixed standards obtained on CLAM-LC-MS/MS platform. For calibration curve construction, a calibrant series of eight levels (0.1, 0.5, 1, 2, 5, 10, 50 and 100 μM) were prepared and analysed. A few selected calibration curves by IS method are shown in Figure 3. The accuracy and repeatability (based on area) of the method with 10 uM mixed standards are shown in Table 2 and the results obtained with urine samples (not shown in the table) are also satisfied.

Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment
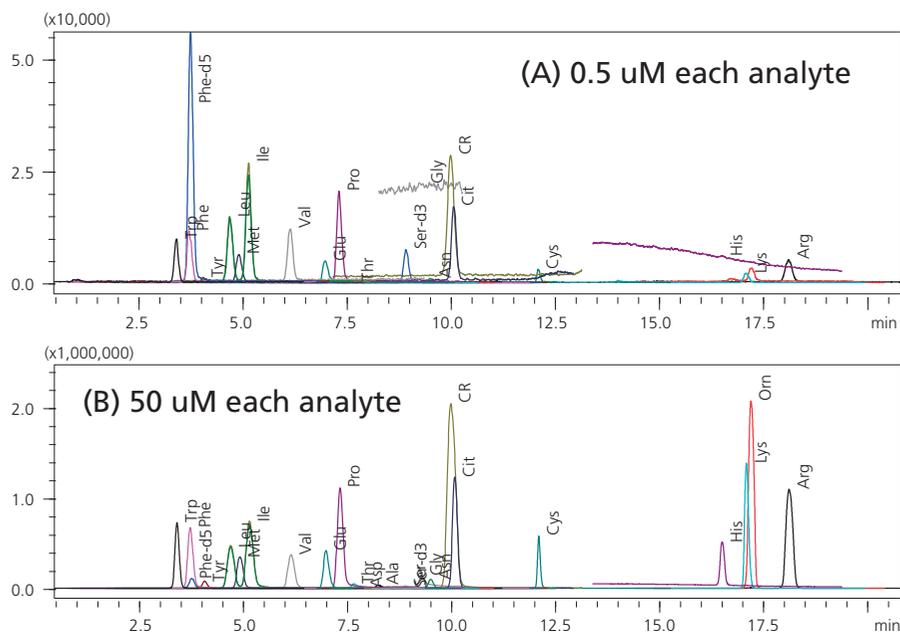

Figure 2: MRM (SIM for Gly) chromatograms of 22 AA and CRE in mixed standards with (5μL inj. volume).
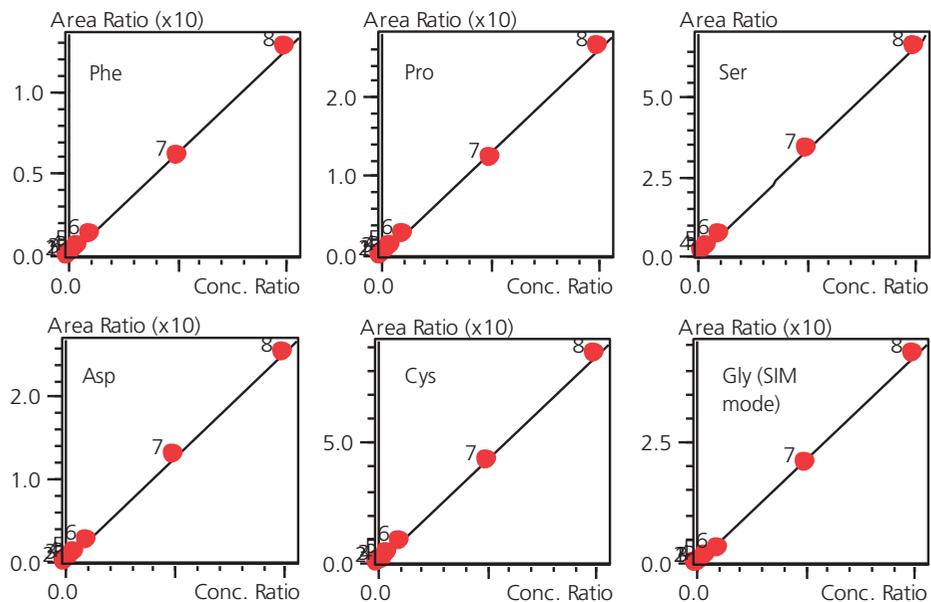
Figure 3: Representative calibration curves of eight levels at from 0.1 μM to 100 μM on CLAM-LC-MS/MS.

4

Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment

Table 2: Summary of MRM quantification method and performance for analysis of 22 AA and CRE on CLAM-2000 coupled with LC-MS/MS: calibration range, linearity, LOQ, accuracy and repeatability (%RSD)

No.	Name	RT (min)	MRM transition (m/z)	IS Calibration Curves and Quantitation Performance				
				Range (µM)	R^2	LOQ (µM)	Accuracy (%)	%RSD (n=6)
1	Trp	3.39	205.10>188.20	0.1-100	0.998	0.14	93.5	2.1
2	Phe	3.67	166.10>120.10	0.1-100	0.999	0.12	99.4	1.6
3	Tyr	3.99	182.10>136.20	0.5-100	0.996	0.78	89.3	0.7
4	Leu	4.62	132.10>86.30	0.1-100	0.998	0.14	98.2	1.5
5	Met	4.81	150.10>56.10	0.1-100	0.999	0.11	104.5	1.6
6	Ile	5.11	132.10>86.30	0.1-100	0.998	0.10	97.7	1.0
7	Val	6.06	118.20>72.05	0.5-100	0.997	0.26	98.8	2.8
8	Glu	7.01	148.10>84.10	0.1-100	0.996	0.12	101.2	2.3
9	Pro	7.26	116.10>70.10	0.1-100	0.999	0.07	104.6	2.2
10	Thr	7.62	120.10>74.00	1-100	0.997	0.9	103.0	4.3
11	Asp	7.81	134.10>73.90	5-100	0.994	5.0	102.5	4.6
12	Ala	8.16	90.10>44.10	1-100	0.995	1.5	103.4	3.3
13	Ser	8.91	106.10>60.20	1-100	0.999	1.6	97.2	3.3
14	Gln	9.10	147.10>84.10	0.1-100	0.999	0.1	106.6	11.8
15	Gly	9.30	76.1 (SIM)	2-100	0.998	4.6	100.3	6.7
16	Asn	9.38	133.10>74.10	0.5-100	0.999	0.66	99.3	3.4
17	Cit	9.98	176.00>70.10	0.1-100	0.997	0.10	101.2	4.6
18	CRE	10.08	114.00>44.00	0.1-100	0.996	0.12	99.3	2.6
19	Cys	12.13	241.00>151.95	0.1-100	0.999	0.12	99.1	8.4
20	His	16.52	156.10>110.10	2-50	0.991	2.2	103.2	7.4
21	Lys	16.95	147.00>84.10	0.5-50	0.996	0.5	109.0	12.9
22	Orn	17.21	133.10>70.05	0.5-50	0.995	0.7	113.3	16.2
23	Arg	17.97	175.10>70.10	0.5-50	0.993	0.3	105.5	8.6

Notes: (1) LOQ values refer to the AA concentrations in neat solution; (2) Accuracy and repeatability values refer to 10 µM level

Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment

Automated batch-run for analysis of 22 AA in 28 urine specimens

A total of 28 urine specimens were collected from 14 male and 14 female health volunteers age between 20 and 30 years old. The urine samples were stored in sealed plastic tubes at -20°C. The samples were analysed in a sequenced batch-run on the CLAM-2000 coupled with LCMS-8040. The automated pre-treatment of each sample on CLAM-2000 involves: (a) pipetting 40 μL of urine, 120 μL of organic solvent (MeOH/ACN) and 40 μL of IS, (b) votexing the mixture, (c) vacuum filtering to obtain clear sample solution and (d) transferring to the autosampler and injecting to LCMS-8040.

A batch-run includes (A) Blank, (B) Calibration series, (C) QCs and (D) samples. The sequence starts from a blank (milli-Q water), followed by calibration series → bank → QC low and QC high → blank → 8-10 samples → blank → QCs → 8-10 samples. The blank was used to ensure no sample carry-over, while QC low and QC high were placed between different group of samples to monitor the loss during sample preparation and ensure the reliability of the results. There was not significant matrix effect of the method as observed in a separate study.

Total AA and Total Creatinine-Normalized AA (TCNA)

Figure 3(a) shows the results of total 22 amino acid (AA) and creatinine (CRE) of 28 urine specimens. The variation of both total AA and CRE are significant, e.g., the total AA scattering in 543~16,640 uM and CRE in 438~2,261 uM. Figure 3(b) displays their molar of Total

AA / CRE, named as Total Creatinine-Normalized AA (TCNA). It can be seen that the 28 urine specimens can be classified into three groups according to the TCNA ratios:
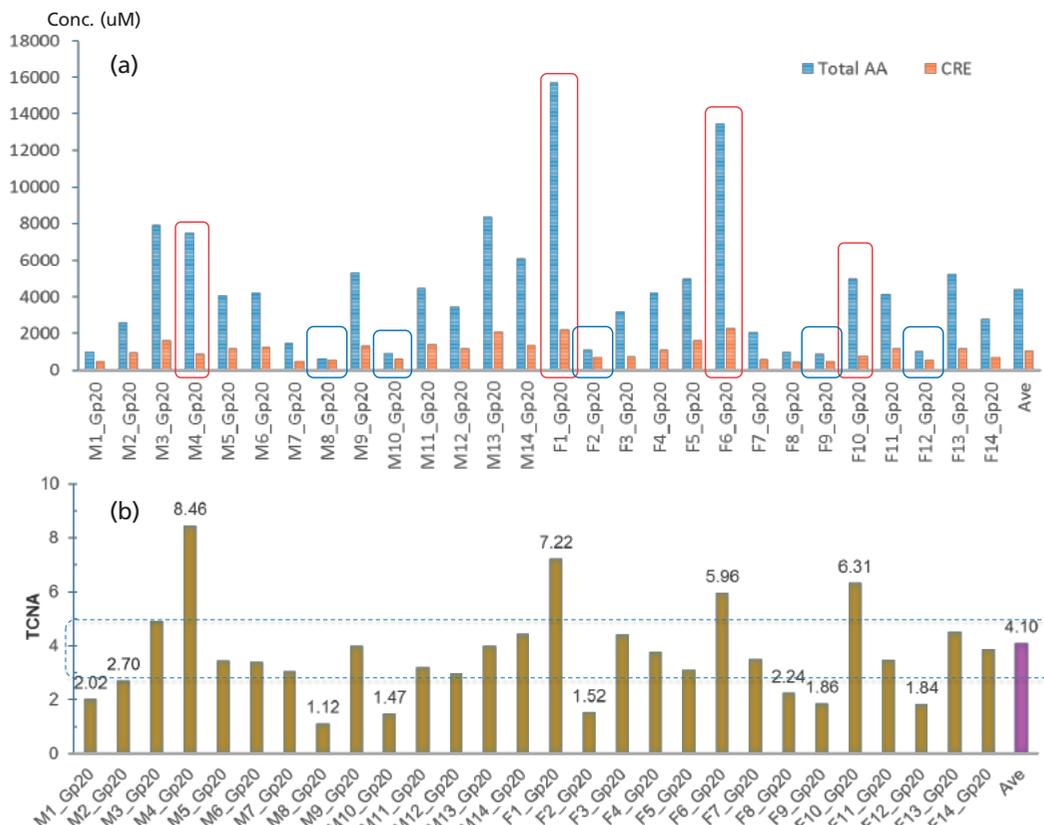

Figure 3: (a) Total AA and creatinine (uM) and (b) Total creatinine-normalized AA (TCNA) of 28 urine specimens from 14 male and 14 female health volunteers age at 20~30 years old

Table 3: P values of the 21 AA variables between two groups identified according to different TCNA

Amino Acid	TCNA (R) = 1.12~3.25			TCNA (R) = 3.45~8.46			P-value
	Ave	Max	Min	Ave	Max	Min	
Trp	3.06	5.96	1.20	4.62	7.82	1.95	0.0056
Phe	2.64	4.00	0.96	4.05	6.32	2.24	0.0005
Tyr	4.73	9.36	1.34	8.04	22.30	3.40	0.0135
Leu	3.35	6.06	0.88	4.07	9.60	0.68	0.1273
Met	0.54	1.48	0.11	1.05	2.20	0.26	0.0022
Ile	1.63	3.38	0.36	2.00	4.11	0.13	0.1477
Val	2.88	4.98	1.48	3.23	5.40	1.26	0.1478
Glu	2.17	4.67	0.52	3.04	10.49	1.16	0.2625
Pro	0.83	1.72	0.14	0.58	1.22	0.13	0.0339
Thr	4.95	10.19	2.08	8.44	19.91	2.95	0.0067
Asp	1.27	4.11	0.11	1.11	6.14	0.06	0.3003
Ala	25.22	51.57	5.90	52.95	114.75	25.96	0.0013
Ser	23.23	42.77	6.12	40.03	90.00	5.20	0.0115
Gln	32.78	51.31	18.40	43.17	67.25	31.47	0.0254
Gly	61.36	125.16	25.27	128.67	249.43	36.18	0.0032
Asn	4.36	7.87	1.00	10.11	24.02	1.07	0.0032
Cys	3.62	6.84	1.19	7.07	22.97	1.87	0.0133
His	49.94	128.20	16.69	140.26	290.32	65.77	0.0000
Lys	3.15	13.77	0.51	24.17	101.00	2.45	0.0075
Orn	0.30	1.18	0.07	1.60	6.60	0.14	0.0088
Arg	1.18	3.74	0.18	2.88	9.47	0.19	0.0092

very low (1.12 ~ 2.24), very high (5.96 ~ 8.46) and the rests (2.70 ~ 4.92) closer to the average of 4.10.

AA profile and statistics analysis

Statistics analysis of 21 AA (Cit is excluded) in urines based on the TCNA model are shown in Table 3 and Figure 4. A best grouping according to TCNA (R) is found at R=1.12~3.25 and 3.45~8.46 based on p-value evaluation. With this assumption, most AA exhibit statistically significant differences *(P<0.05)* except five AA which *p-values* are greater than 0.05. Further multivariate analysis (PCA) reveals that the two groups identified from *p-value* evaluation are distinct, and the few very high values of TCNA (5.96~8.46) are the outliers. This result indicates that the TCNA value can be regarded as a key factor in analysis of AA profile, which may provide a new approach in characterization of AA profiles linking with diagnosis of disease and health conditions [4].
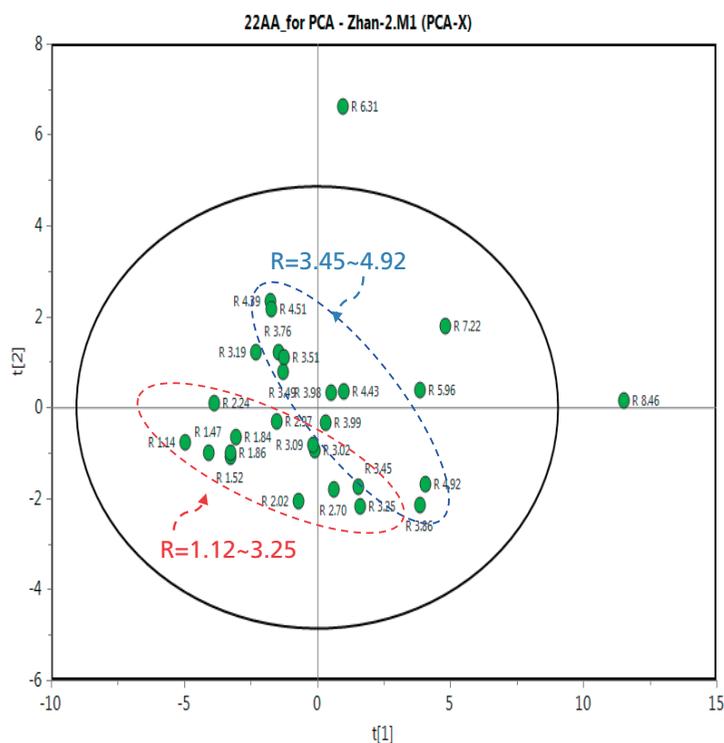
Figure 4: PCA score plot of TCNA (R) for 21 amino acids of 28 urine specimens which are classified in two groups according to TCNA using SIMCA P+ program.

Conclusions

A fully-automated method for quantitative analysis of 22 amino acids (AA) and creatinine (CRE) in urines was established on a novel platform of CLAM-LC-MS/MS. An Intrada Amino Acid column adopted allows the analysis of AA without derivatization by LC/MS/MS. Twenty-eight urine specimens from health volunteers were analysed and the results were used to calculate total creatinine-normalized AA (TCNA), from which two distinct groups of urines are identified. Multivariate analysis of the AA profiles of the 28 specimens confirms the significant importance of the classification based on TCNA, which may provide a new approach in characterization of AA profiles linking with diagnosis of disease and health conditions.

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Disclaimer: The LCMS-8040, CLAM-2000 and the contents are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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Application News

Liquid Chromatography Mass Spectrometry

No.**C139**

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.
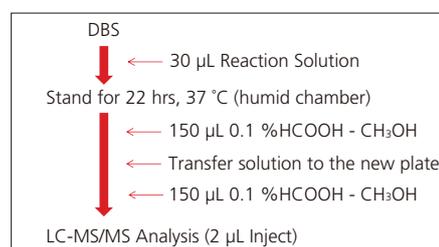

Fig. 1 Preparation Process Flowchart
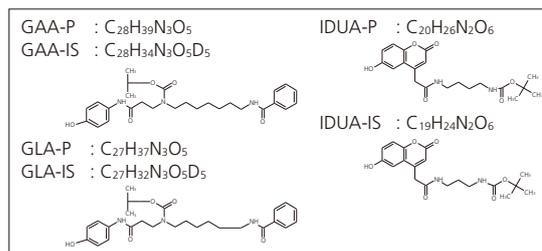
Fig. 2 Structures of Enzymatic Reaction Products and Internal Standards

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

Trapping and Cleanup   Chromatographic Separation
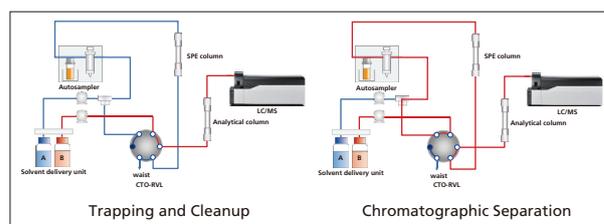
Fig. 3 MRM Chromatograms of Each Target Compound

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$_2$O
Solvent B	: 0.1 % HCOOH-CH$_3$OH
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)
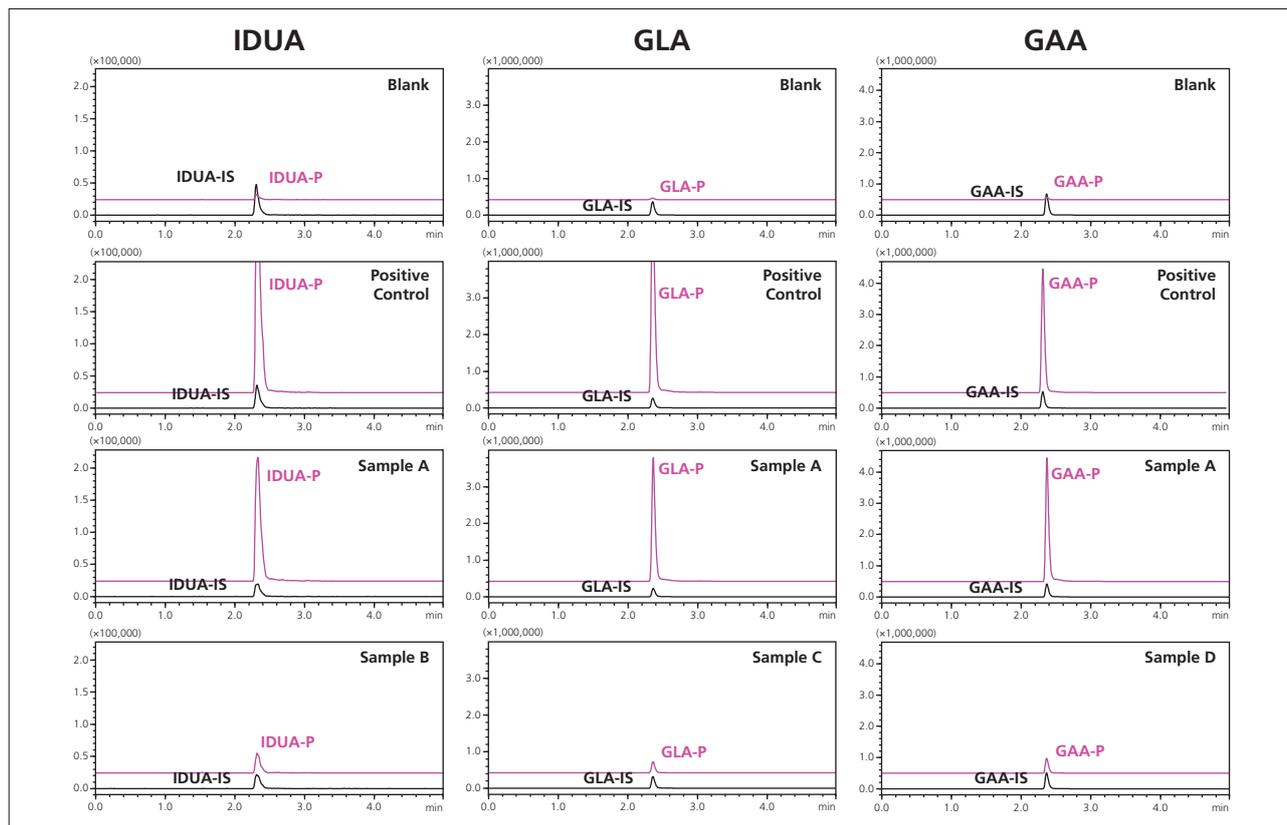
Fig. 4 MRM Chromatograms of Each Target Compound
Blank: Sample filter paper containing no blood; Sample A: Sample containing enzymes with all activities; Sample B: Sample missing IDUA; Sample C: Sample missing GLA; and Sample D: Sample missing GAA

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

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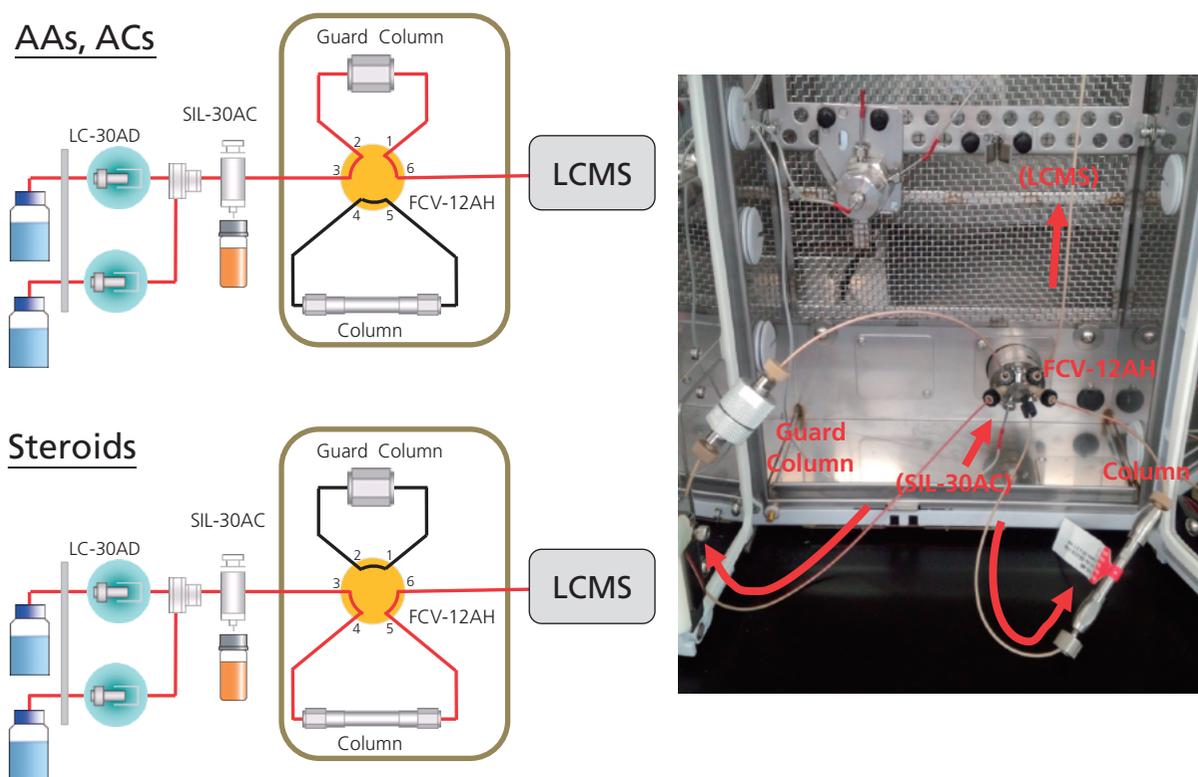
Figure 1 Flow Diagram of FIA/LC-MS/MS system

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 ℃
BH Temperature   : 500 ℃   Interface Temperature : 400 ℃
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.
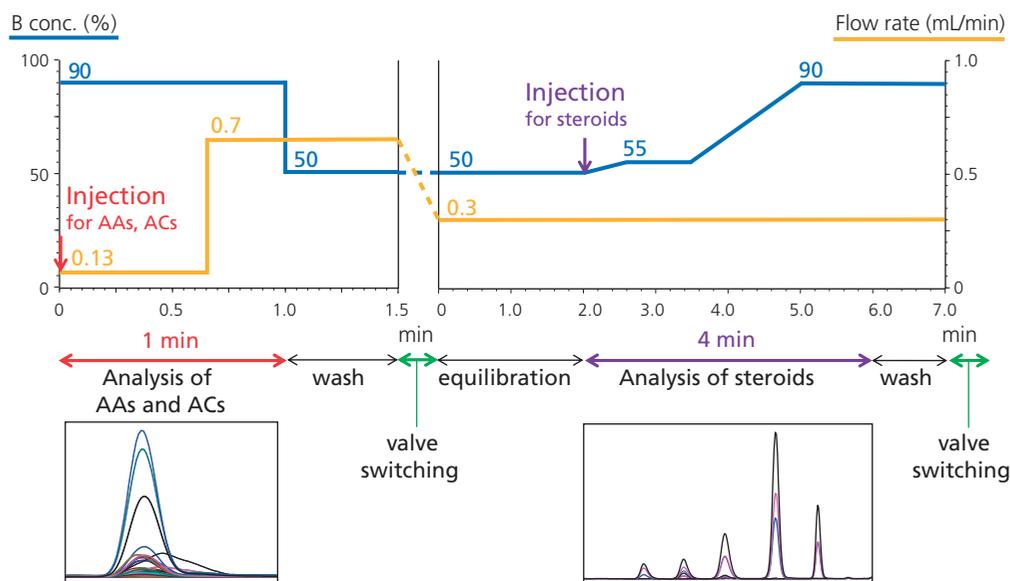

Figure 2 Time Program of amino acids, acylcarnitines and steroids analysis

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

	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)
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)
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		

	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)
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		

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

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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).


| Sample pipetting | Reagent Pipetting | Shaking | Filtration | Sample Transfer |

Samples are transferred to LC/MS/MS
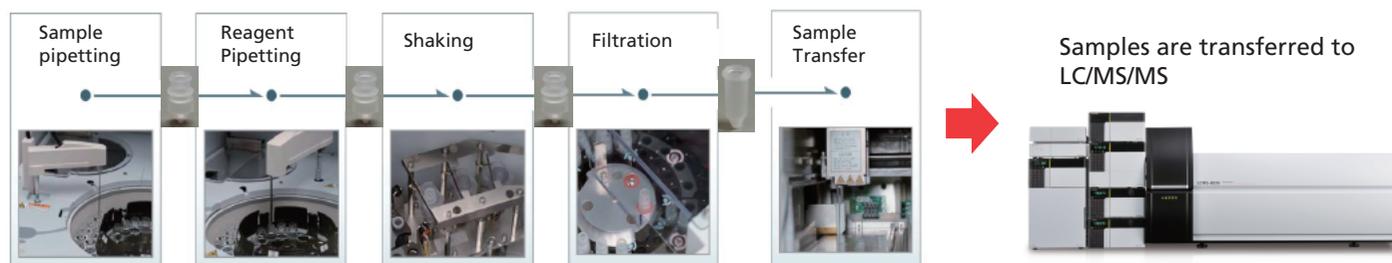
Figure 1: Procedure of protein crash and spiked-sample preparation

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.

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.	: 40ºC
Injection	: 5 μL
Interface	: ESI
MS Mode	: MRM, Positive
Block Temp.	: 400ºC
DL Temp.	: 250ºC
CID Gas	: Ar, 270 kPa
Nebulizing Gas	: N_2, 2.0 L/min
Drying Gas	: N_2, 15.0 L/min

MeOH wetting (conditioning)

↓

Add 20uL of Sample (urine)

↓

Add 20uL of I.S.

↓

Add 40uL of ACN/MeOH

↓

Vortex for 60sec

↓

Vacuum Filter for 90sec

↓

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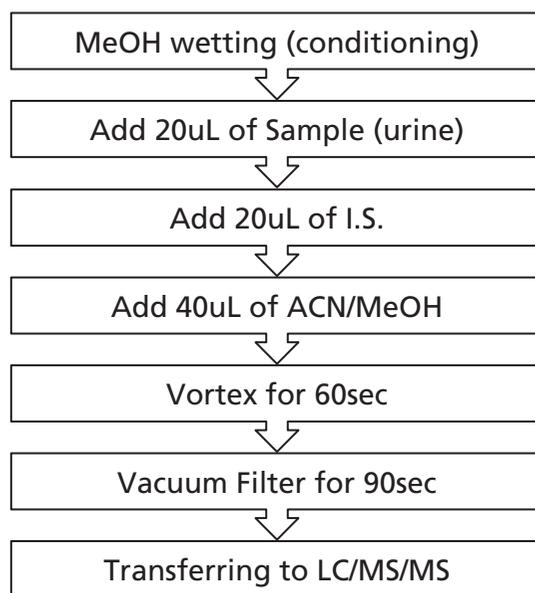
Figure 2: Typical auto-workflow of urine sample via protein-crash
and adding IS for LC/MS/MS by CLAM-2000

![SHIMADZU Excellence in Science]

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.
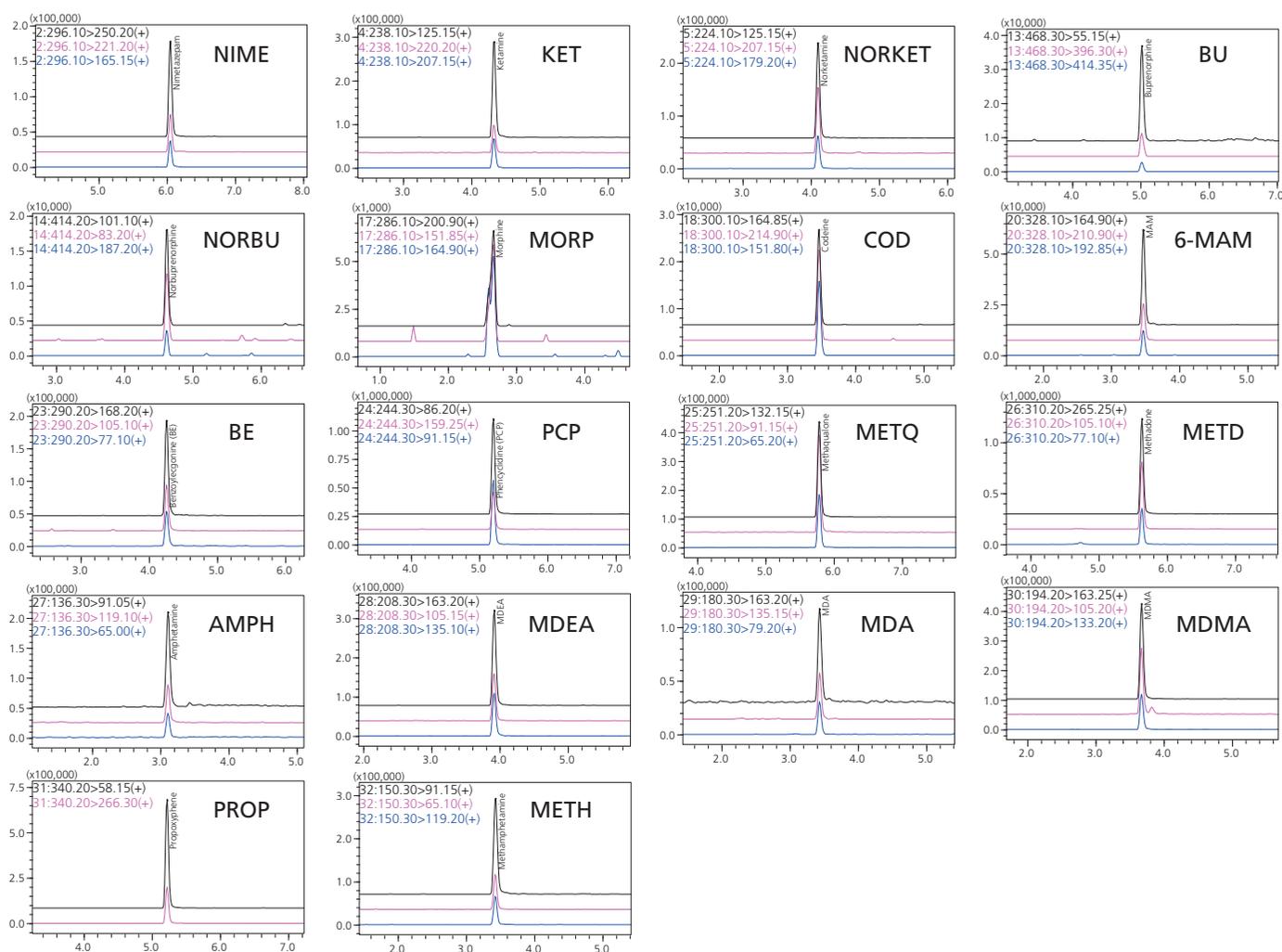

Figure 3: Individual MRM chromatograms of eighteen illicit drugs each (200 ng/mL) and
fourteen ISs (100 ng/mL) spiked in urine obtained on CLAM-LC/MS/MS platform.

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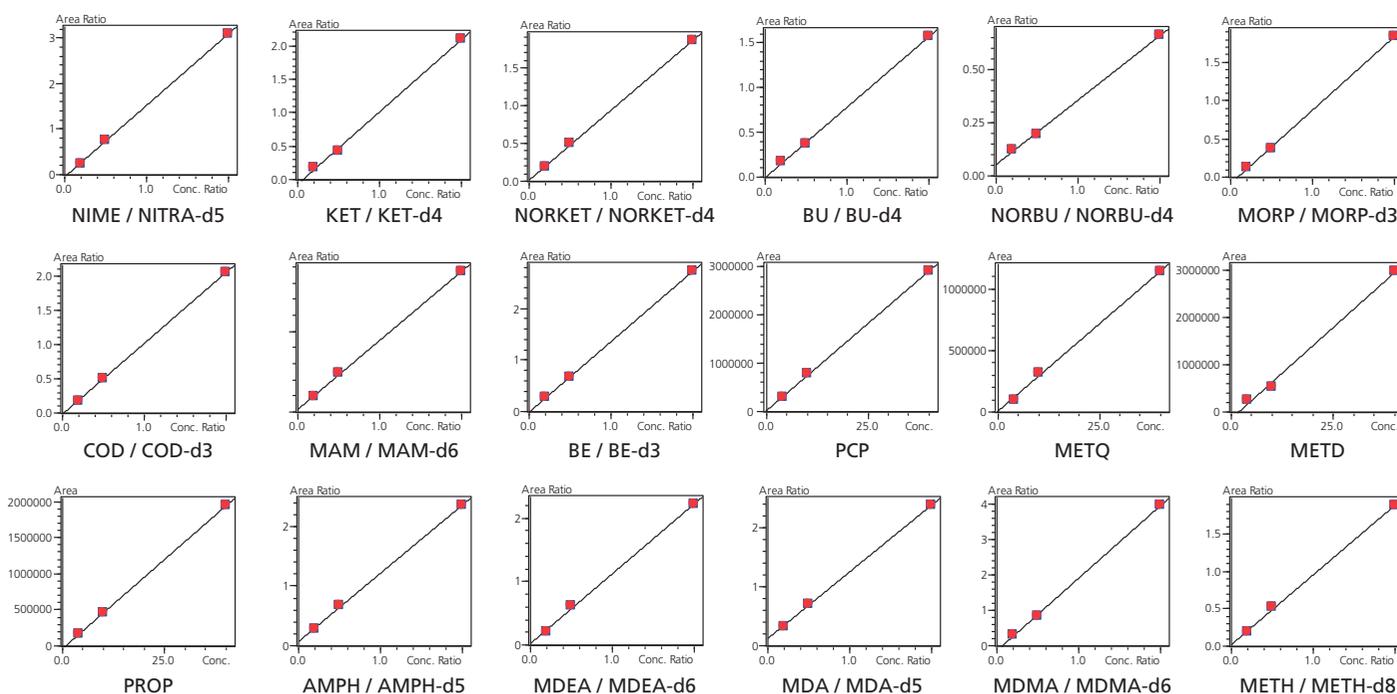
Figure 4: Calibration curves of 14 illicit drugs with isotope-labelled internal standards and 4 illicit drugs with external standard in human urine on LCMS-8040. Details are shown in Table 3.

6

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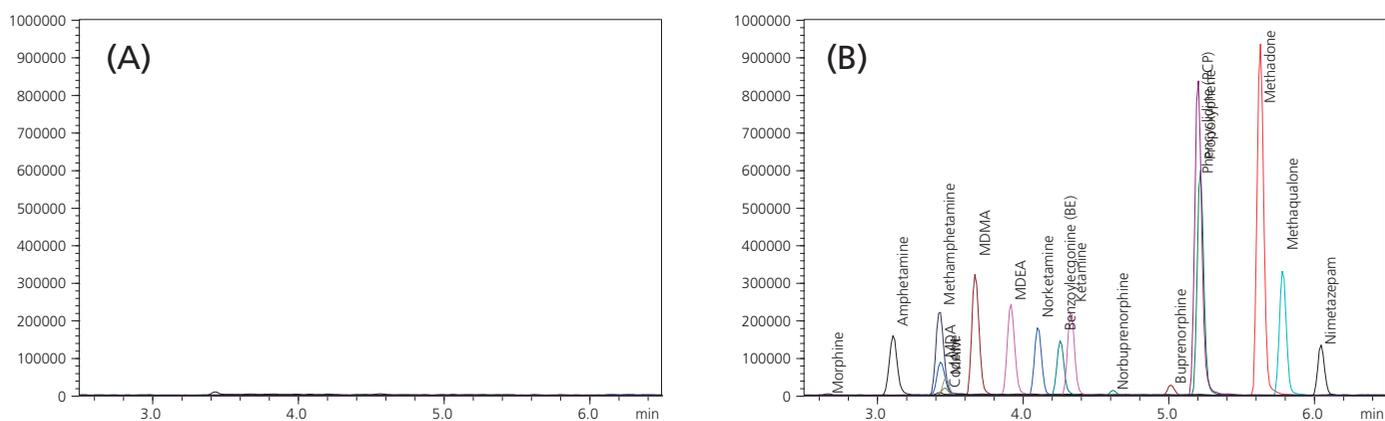
Figure 5: Total MRM chromatograms of (A) blank urine and (B) spiked urine with eighteen illicit drugs (200 ng/mL).

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

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

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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.

[Anti-ischemic drug]

Meldonium

Formula: C6H14N2O2
Exact Mass: 146.1055

[Adrenergic agents]

Synephrine

Formula: C9H13NO2
Exact Mass: 167.0946

Etilefrine

Formula: C10H15NO2
Exact Mass: 181.1103

Oxilofrine

Formula: C10H15NO2
Exact Mass: 181.1103

Norfenefrine

Formula: C8H11NO2
Exact Mass: 153.079

Octopamine

Formula: C8H11NO2
Exact Mass: 153.079
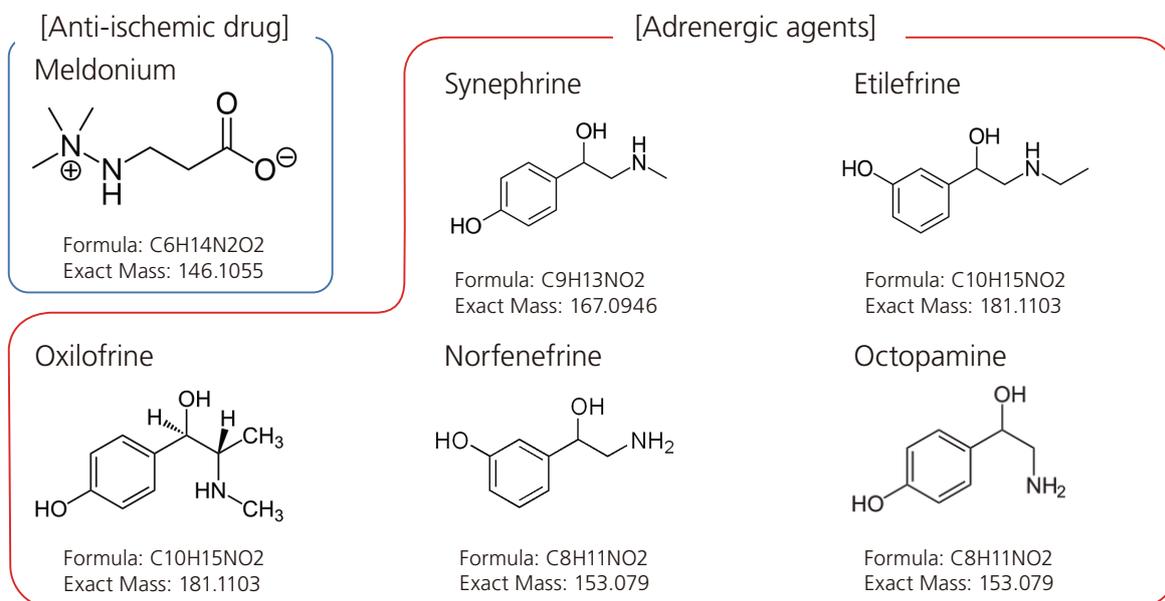
Fig. 1 Structures of 6 Compounds

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 %	DL Temp.	: 300 ˚C
	glacial acetic acid)	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.

Trap
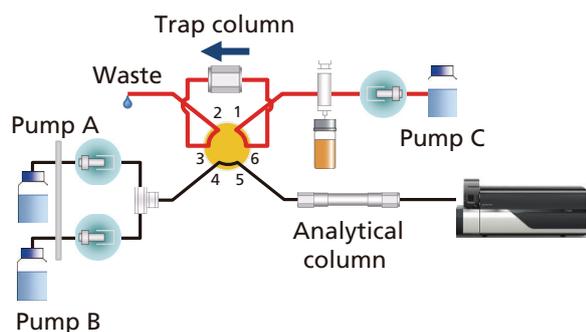
Analysis
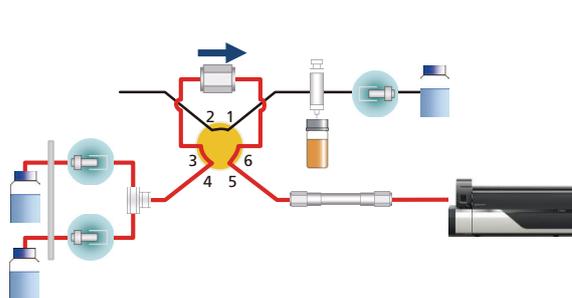
Fig. 2 Flow Diagram of 2D-HILIC System

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.
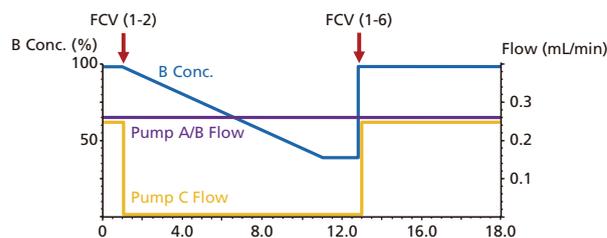
Fig. 3 Flow Rate and Gradient Program

■ **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.
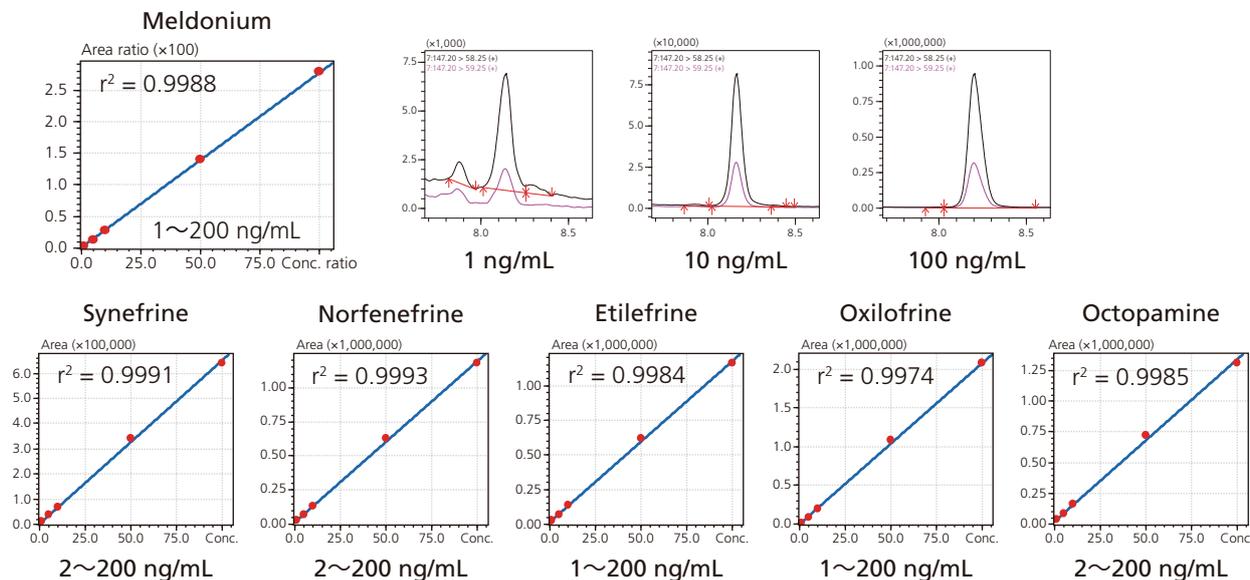

Fig. 4 Calibration Curves and MRM Chromatograms of 6 Compounds

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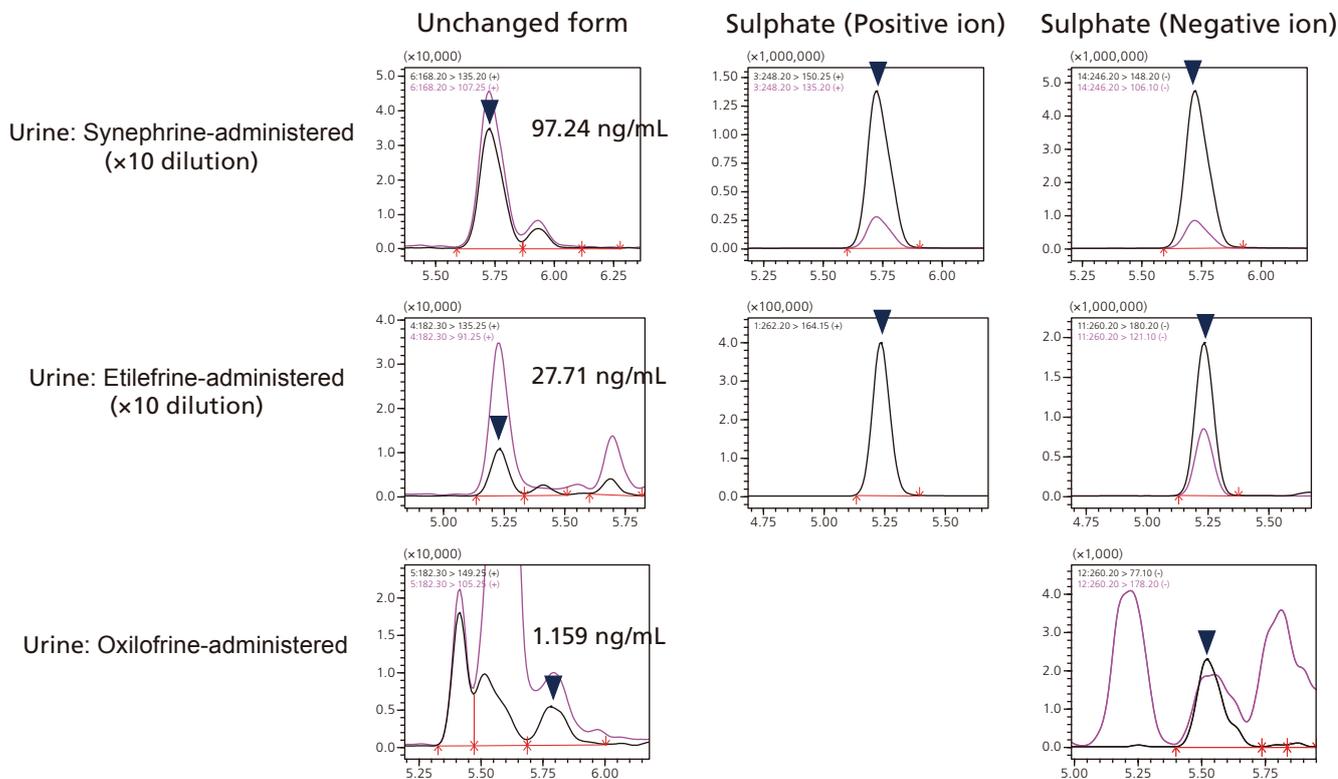
Fig. 5 Results of Urine: Synephrine, Etilefrine and Oxilofrine were separately administered

■ 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.

Urine: norfenefrine-administered (×10 dilution)

	Unchanged drug	Sulphate (Positive ion)	Sulphate (Negative ion)
XICs of Norfenefrine	(×100,000) 154.20 > 91.25 (+) 154.20 > 65.25 (+)	(×100,000) 234.20 > 136.20 (+) 234.20 > 91.20 (+)	(×1,000,000) 232.20 > 152.20 (-) 232.20 > 121.15 (-)
XICs of Octopamine	(×100,000) 154.20 > 91.25 (+) 154.20 > 119.20 (+)		(×100,000) 232.20 > 134.15 (-) 232.20 > 107.10 (-) Norfenefrine sulphate · Octopamine sulphate

Same RT's and MRM's for both compounds

Norfenefrine sulphate was positively identified using negative ion detection

Urine: octopamine -administered (×10 dilution)

	Unchanged drug	Sulphate (Positive ion)	Sulphate (Negative ion)
XICs of Norfenefrine	(×1,000) 154.20 > 91.25 (+) 154.20 > 65.25 (+)	(×10,000) 234.20 > 136.20(+) 234.20 > 91.20(+)	(×100,000) 232.20 > 152.20 (-) 232.20 > 121.15 (-)
XICs of Octopamine	(×1,000) 154.20 > 91.25 (+) 154.20 > 119.20 (+)		(×1,000,000) 232.20 > 134.15 (-) 232.20 > 107.10 (-) Norfenefrine sulphate · Octopamine sulphate

Same RT's and MRM's for both compounds

Octopamine sulphate was positively identified using negative ion detection

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

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

Direct Probe Ionization Mass Spectrometer

15 Second Screening Analysis of Cyanide in Blood Serum Without Pretreatment

No. C158

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.
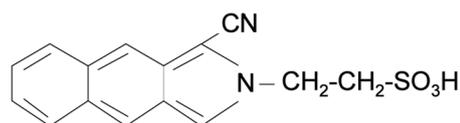
Fig. 2 Cyanide Derivative (MW 300)

Fig. 3 Analysis Sequence Without Pretreatment

Probe

Ion introduction unit (DL)

Small sample plate
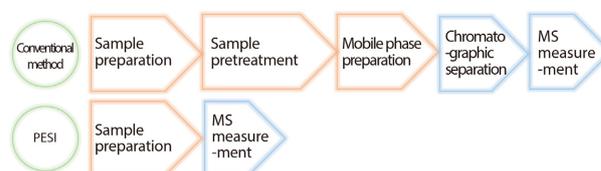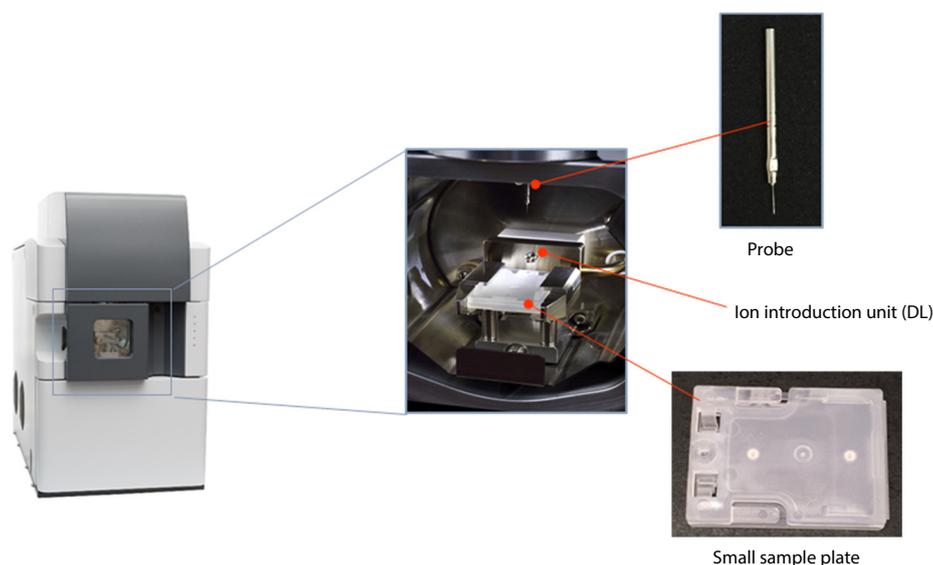
Fig. 1 DPiMS-2020

■ 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.
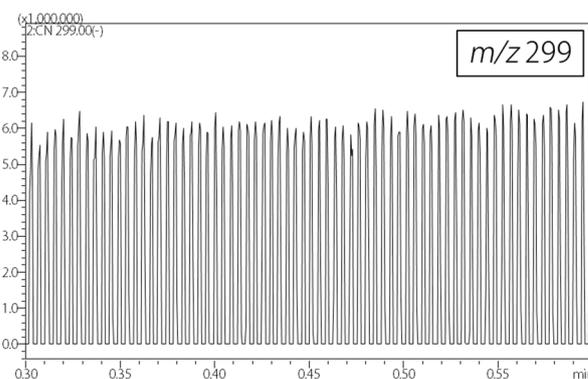
Fig. 4 Mass Chromatogram from DPiMS-2020
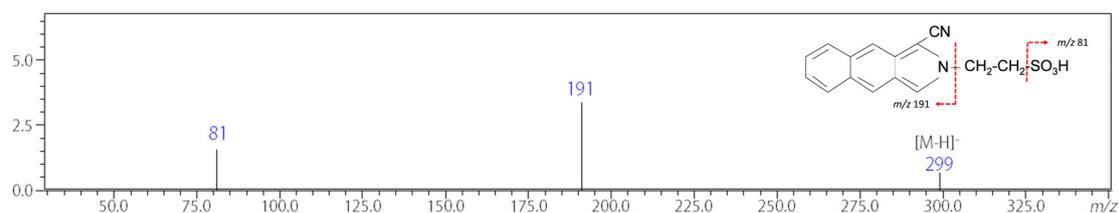
Fig. 5 Mass Spectrum from DPiMS-2020
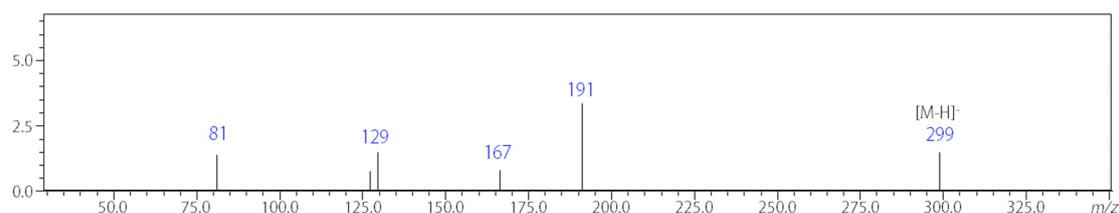
Fig. 6 Product Ion Mass Spectrum from LCMS-8040 (Reference)

■ 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)

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).

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Quantitative Multi Target Screening (MTS) using liquid
chromatography-tandem mass spectrometry with MS/MS library
based identification for forensic toxicology
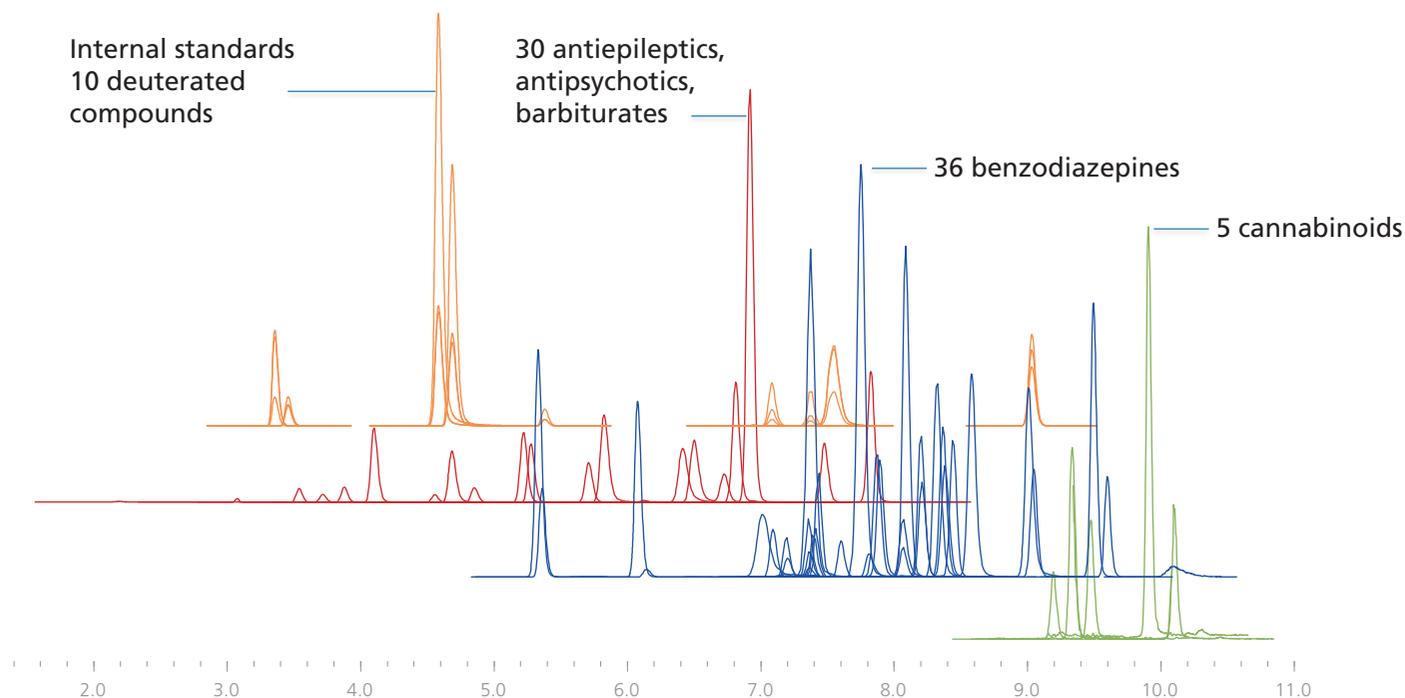
Figure 1. MRM chromatograms for a panel of drugs extracted from whole blood using a QuEChERS
method corresponding to a concentration of 100 µg/L

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.

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

MRM quantitation
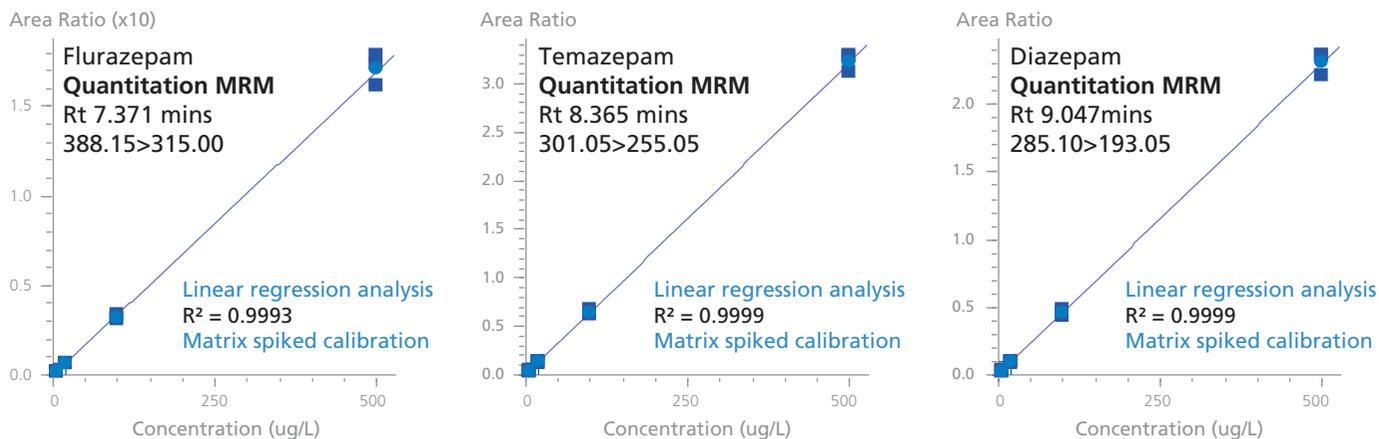
	Rt (mins)	MRM	Mean Accuracy				Repeatability			
			Calibration standards (n=3 for each calibration level)				20ug/L replicate (n=5)		100ug/L replicate (n=5)	
			5ug/L	20ug/L	100ug/L	500ug/L	Mean Conc (ug/L)	%RSD	Mean Conc (ug/L)	%RSD
Flurazepam	7.371	388.15>315.00	109.5	95.5	95.3	101.1	18.1	3.0	100.2	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%).

![Shimadzu – Excellence in Science]

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.
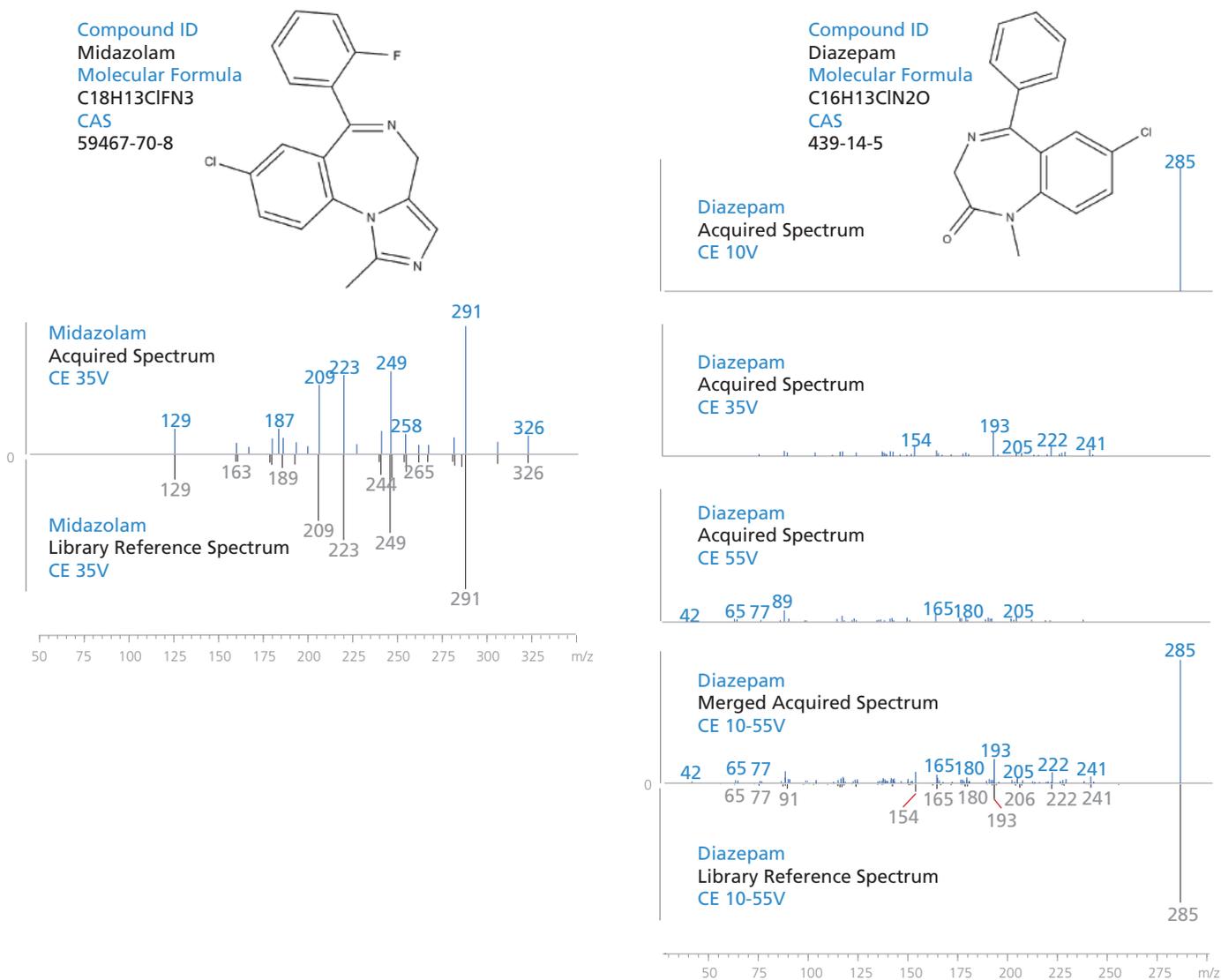
Figure 3. MRM triggered product ion spectrum data for midazolam and diazepam. The library included spectra for each collision energy and a separate library for merged spectra enabling match criteria to be set for a specific fragmentation voltage (as shown for midazolam) or to use a broad band fragmentation 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)	Low	Medium	High	Compound	RT (min)	Low	Medium	High
		Library Hit Merged CE spectrum					Library Hit 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

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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.

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Application News

Liquid Chromatography Mass Spectrometry

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

No.C98

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.
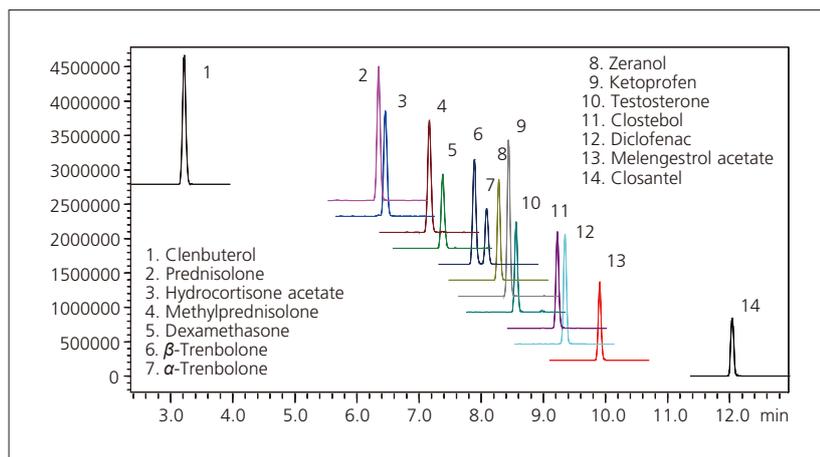
Fig. 1 Chromatograms of Steroids and NSAIDs

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)
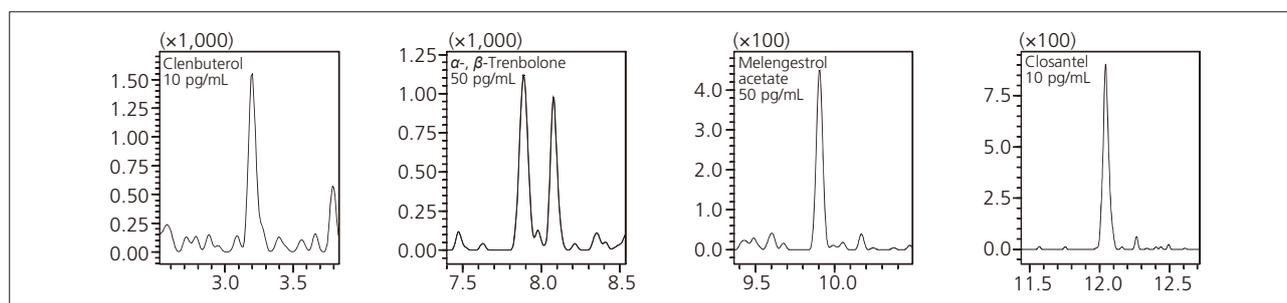
**Fig. 2 MRM Chromatograms Near the LOQ of Typical Compounds**

■ 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.

Reference ion mode can be selected from among:
• Absolute
• Relative
• Absolute or Relative
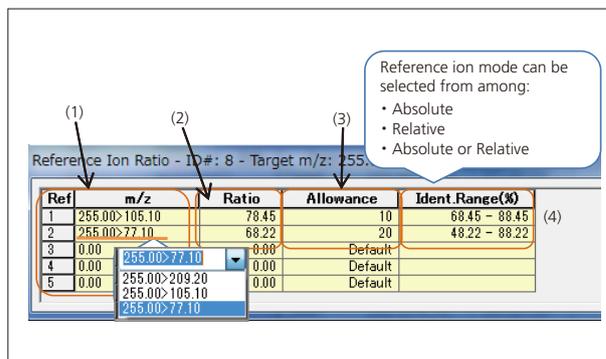
Fig. 3 Reference Ion Setting Window
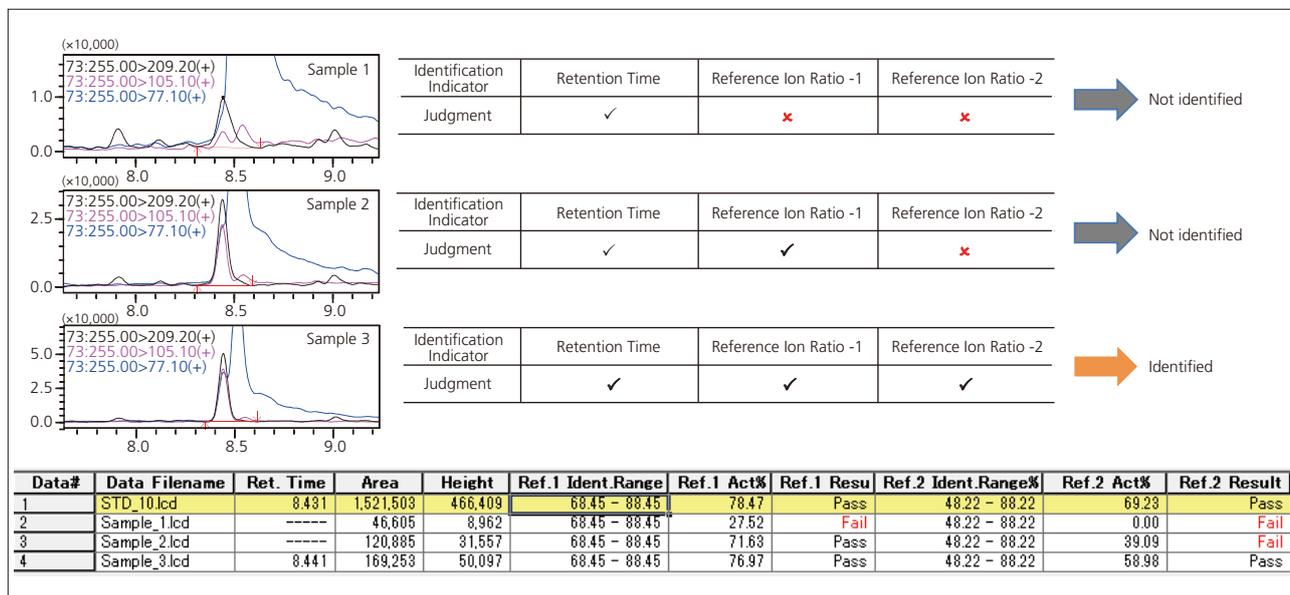
Fig. 4 Example of Peak Determination Using Multiple Reference Ions

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

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

Gas Chromatography

No. G286

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

9-mL vial

0.25 mL of blood sample

0.5 mL of water

0.25 mL of oxidizing agent *

Heated to 30 °C for 90 minutes

Injection of 0.1 mL of gas into the GC using a gas-tight syringe

* Oxidizing agent composition: Aqueous solution comprising 20 % $K_3[Fe(CN)_6]$ and 5 % saponin
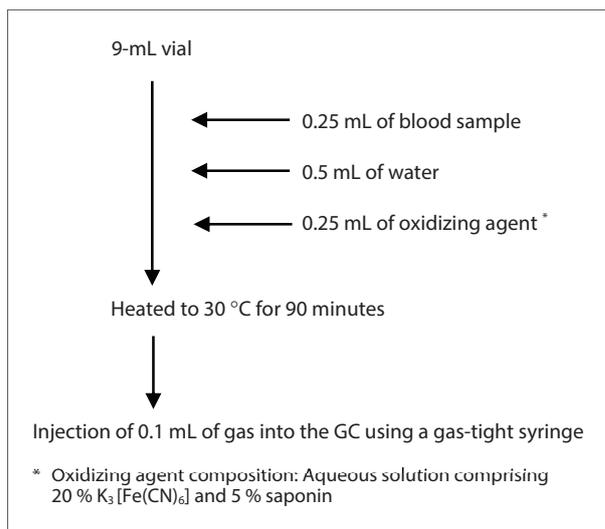
Fig. 1 Example of Sample Pretreatment

■ 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.


CO

Blood sample saturated with carbon monoxide

Untreated blood sample

1.75   2.00   2.25   2.50   2.75   3.00   3.25   3.50 min
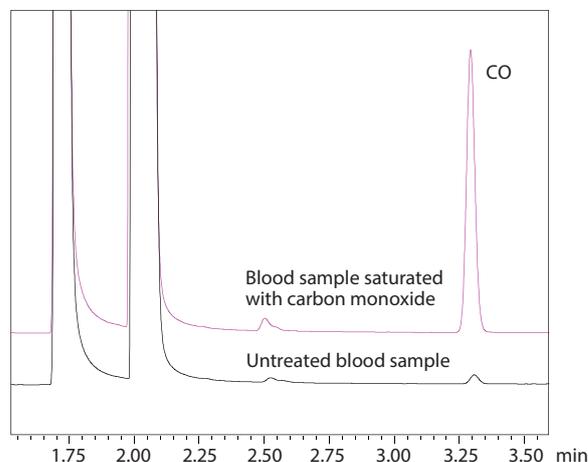
Fig. 2 Comparison of Untreated Blood Sample and Blood Sample Saturated with Carbon Monoxide

◼ 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.
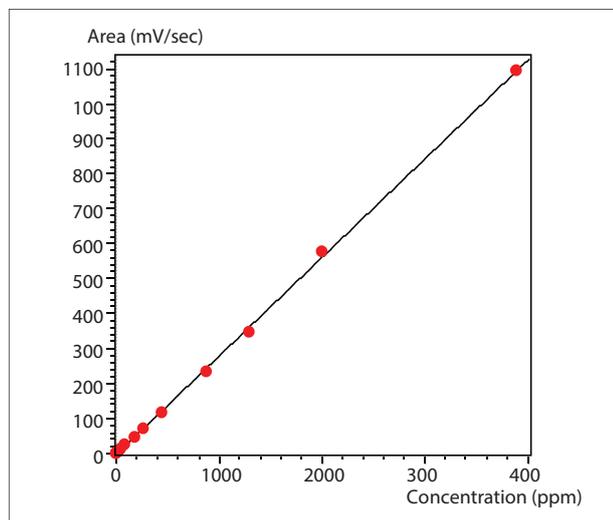

Fig. 3 **Calibration Curve**

◼ 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.
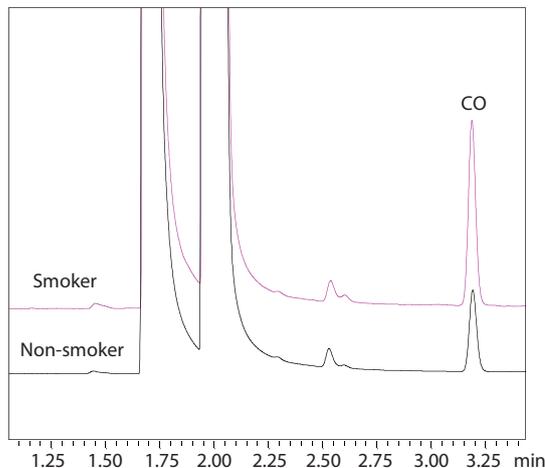

Fig. 4 **Comparison of a Smoker and Non-Smoker**

◼ 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)

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