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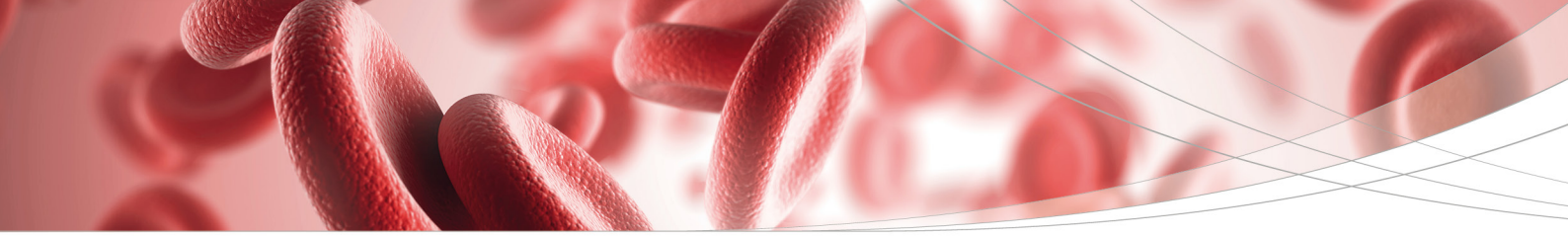
Excellence in Science

Application Handbook

Clinical



Clinical

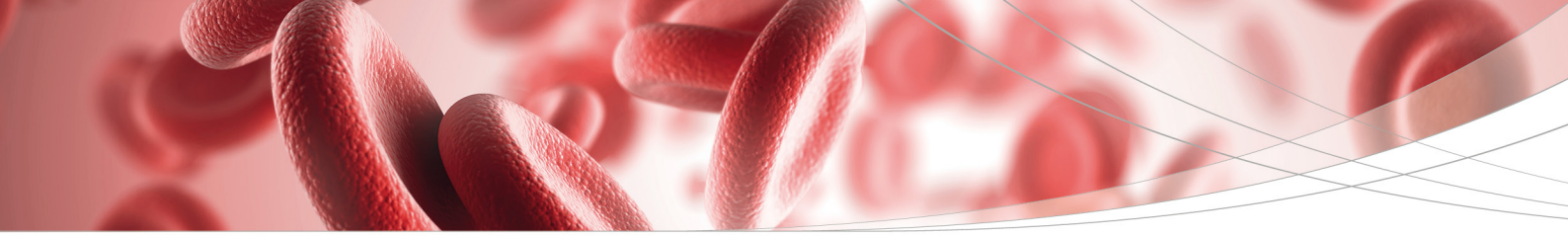
The analysis of whole blood, plasma, serum and urine is a most insightful method in clinical research. Since the sensitivity of analytical instrumentations systems has improved steadily in recent years, research results and reliability have increased as well.

In clinical applications, analytical instruments unfold a multitude of benefits:

- They support the quality of human life. The concentration of medications in Therapeutic Drug Monitoring (TDM), is assured, even though this may change according to age and health conditions and is dependent on gender, genetic constitution or interferences with other drugs.
- They help to save lives, particularly when it comes to time-critical situations, e.g. through acute intoxication, medical or drug abuse.
- They analyze over- and undersupply of vitamins, minerals and trace elements.
- They are applied in genomics, proteomics and metabolomics.
- They uncover fraud in sports, particularly in animal or human doping. At the same time, analytical systems support health protection of animals and humans, even in the long-term.

Clinical applications benefit from Shimadzu's complete portfolio covering chromatography and mass spectrometry (GC, GC-MS, GC-MS/MS, HPLC, UHPLC, LC-MS, LC-MS/MS), spectroscopy (UV-Vis, FTIR, AAS, EDX, ICP-OES), life sciences (MALDI-(TOF)-MS), microchip-electrophoresis, biopharmaceutical (aggregate sizer) and the observation of medical microbubbles in targeted drug delivery using the HPV-X2 ultra high-speed camera.

Shimadzu breaks new grounds by rethinking the use of mature technologies to develop new unique systems such as the iMScope TRIO. It combines an optical microscope with a mass spectrometer for insights on the molecular level. For next-generation brain science, Shimadzu provides LABNIRS, an imaging technology for visualization of brain functions by functional near-infrared spectroscopy (fNIRS).



Contents

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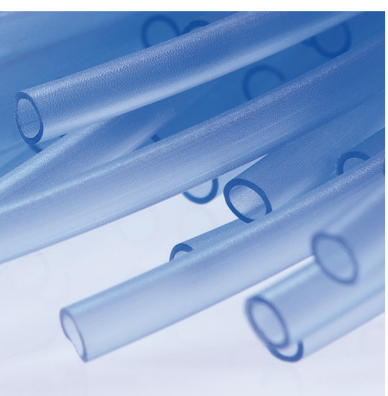
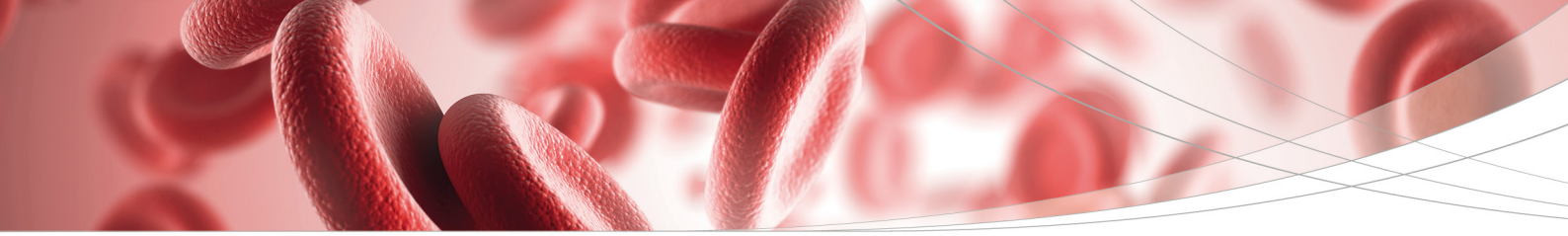
1. Chromatography



1. Chromatography

1.1 Gas Chromatography

Chromatographic separation in gas phase for analysis of volatile and semi volatile components is used in clinical field since many years. Gas chromatography is a key technique for quantitative analysis of alcohol in blood.

LAAN-J-GC-E012	Analysis of alcohol compounds in blood (1)
LAAN-J-GC-E013	Analysis of alcohol compounds in blood (2)

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Application Data Sheet

No.12

GC
Gas Chromatography

Analysis of Alcohol Compounds in Blood (1)

Measurements of oxygenated compounds and alcohols, primarily ethanol, in blood, are frequently performed in forensic medicine, emergency medicine, and other fields. In forensic medicine, such measurements are utilized to determine levels of intoxication from alcohol consumption and to evaluate criminality. In emergency medicine, they are utilized to distinguish between alcohol consumption and other medical cases. For such analyses, systems equipped with a headspace sampler and GC-FID detector are often used.
These measurements must be convenient and quick, and provide high-accuracy results. The Shimadzu HS-20 headspace sampler is capable of meeting these demands. When the HS-20, GC-2010 Plus, and a workstation (LabSolutions LC/GC) are used in combination, everything including the headspace conditions and GC conditions can be controlled from the workstation, which makes it easy to configure settings and operate the system.
This report examines the repeatability and linearity of a standard ethanol solution, and confirms the separation of other oxygenated compound standard solutions. The analysis presented here was performed using the HS-20 and the Rtx-BACPlus series of high-separation columns specifically designed for the analysis of alcohol in blood.

Analysis Conditions

HS-20
Shared Conditions

Oven Temp.:	85 °C	Vial Agitation:	Off
Vial Warming Time:	15 min.	Vial Pressurization:	100 kPa
Vial Pressurization Time:	1 min.	Load Time:	0.5 min.
Injection Time:	0.5 min.	Needle Flash Time:	0.5 min.
Sample Line Temp.:	150 °C	Transfer Line Temp.:	150 °C
Vial Volume:	20 mL		

GC-2010 Plus AF + LabSolutions LC/GC

Columns:	(1) Rtx-BAC Plus 2, 0.32 mm × 30 m, d.f. 0.6 μm (for screening) (2) Rtx-BAC Plus 1, 0.32 mm × 30 m, d.f. 1.8 μm (for separation confirmation)		
Column Temp.:	40 °C		
Carrier Gas Pressure:	100 kPa (helium pressure mode)	Split Ratio:	1:20
FID Temp.:	250 °C	Hydrogen:	40 mL/min.
Makeup Gas:	30 mL/min. (helium)	Air:	400 mL/min.

Results

Table 1 shows area value repeatability for standard solutions (0.1 mg/mL) of ethanol and t-butanol, and area ratio repeatability for ethanol and t-butanol, both obtained using the Rtx-BAC Plus 2 column. Fig. 1 shows overlapping chromatograms for ethanol in the standard solution. As indicated, favorable repeatability was obtained, with an ethanol area value repeatability of 1.42 %, and area ratio repeatability of 0.62 %.
Fig. 2 shows the linearity of ethanol in the 0.1 mg/mL to 1.6 mg/mL range. It is evident that favorable linearity was obtained, with R = 0.9999 or higher.
Fig. 3 shows an example of the separation of a standard solution with seven oxygenated components, including alcohol, using the Rtx-BAC Plus 2. All peaks were successfully separated within 3 minutes. Fig. 4 shows the separation of a standard solution with seven oxygenated components using the Rtx-BAC Plus 1. The separation pattern shown is different from that with the Rtx-BAC Plus 2, so qualitative ability is increased by performing analysis with both columns.
Fig. 5 shows the separation of a standard solution with 10 oxygenated components using the Rtx-BAC Plus 1. The 10 oxygenated components were successfully separated in 5 minutes.

Table 1 Repeatability (n = 7) of Peak Area Values for 0.1 mg/mL of Ethanol and t-Butanol

	1	2	3	4	5	6	7	mean	RSD%
Ethanol	57886	58682	57373	57753	58193	56139	57141	57595	1.422
t-Butanol	969772	980873	964274	958311	969829	949515	953278	963693	1.128
Ethanol/ t-Butanol	0.05969	0.059826	0.059499	0.060265	0.060003	0.059124	0.059942	0.059764	0.622

Table 2 Repeatability (n = 7) of Retention Times for 0.1 mg/mL of Ethanol and t-Butanol

	1	2	3	4	5	6	7	mean	RSD%
Ethanol	1.691	1.691	1.691	1.692	1.691	1.692	1.691	1.691	0.0230
t-Butanol	2.117	2.117	2.117	2.118	2.117	2.118	2.117	2.117	0.0180

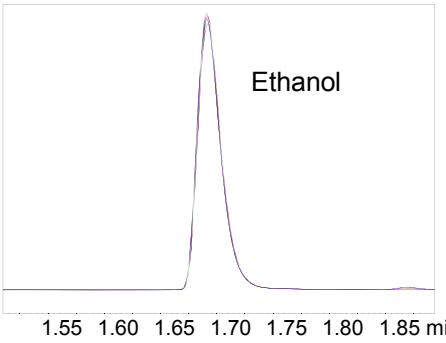
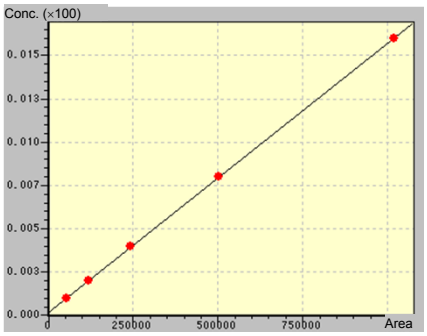


Fig. 1 Repeatability (n = 7) for a 0.1 mg/mL Standard Solution of Ethanol

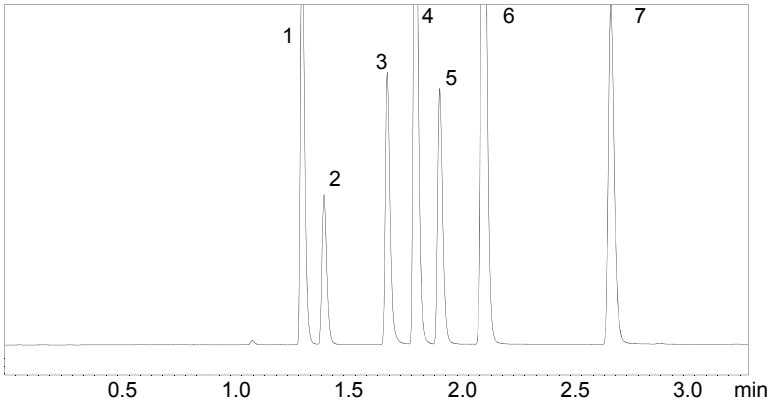


Y = aX + b
a = 1.553005e-006
b = 1.974794e-002
R^2 = 0.9999422
R = 0.9999711

External standard method
Calibration curve: Straight line
Through the origin: No
Weighting: None

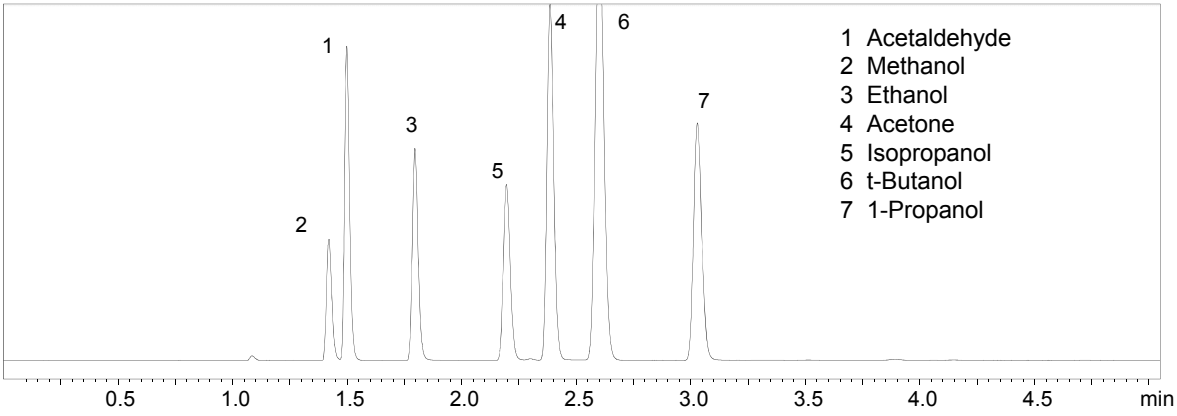
Mean RF: 1.674826e-006
RF standard deviation: 1.019928e-007
RF relative standard deviation: 6.089755

Fig. 2 Linearity of 0.1 mg/mL to 1.6 mg/mL Standard Solutions of Ethanol



1 Acetaldehyde
2 Methanol
3 Ethanol
4 Acetone
5 Isopropanol
6 t-Butanol
7 1-Propanol

Fig. 3 Chromatograms for Seven Components (0.1 mg/mL each) Obtained Using the Rtx-BAC Plus 2



1 Acetaldehyde
2 Methanol
3 Ethanol
4 Acetone
5 Isopropanol
6 t-Butanol
7 1-Propanol

Fig. 4 Chromatograms for Seven Components (0.1 mg/mL each) Obtained Using the Rtx-BAC Plus 1

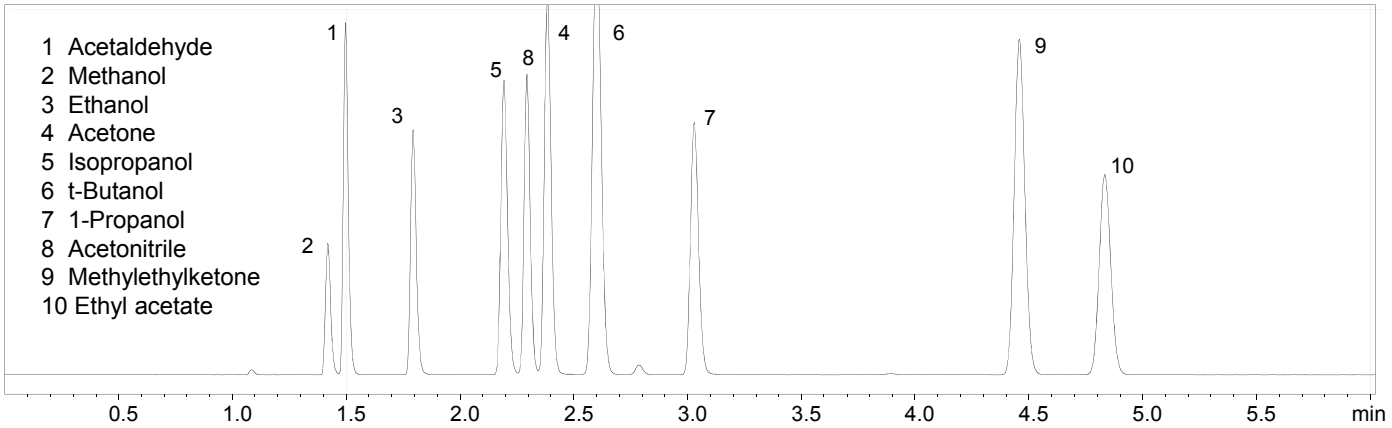


Fig. 5 Chromatograms for 10 Components (0.1 mg/mL each) Obtained Using the Rtx-BAC Plus 1

First Edition: October 2013

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Application Data Sheet

No.13

GC

Gas Chromatography

Analysis of Alcohol Compounds in Blood (2)

Measurements of oxygenated compounds and alcohols, primarily ethanol, in blood, are frequently performed in forensic medicine, emergency medicine, and other fields. In forensic medicine, such measurements are utilized to determine levels of intoxication from alcohol consumption and to evaluate criminality. In emergency medicine, they are utilized to distinguish between alcohol consumption and other medical cases. Application Data Sheet No. 12 introduced results for the repeatability of ethanol and the separation of standard solutions of oxygenated compounds using the HS-20 and the Rtx-BAC Plus series of columns specifically designed for alcohol analysis.

This report introduces the results of an investigation of linearity and repeatability for blood spiked with ethanol.

Analysis Conditions

HS-20
Shared Conditions

Oven Temp.:	85 °C	Vial Agitation:	Off
Vial Warming Time:	15 min.	Vial Pressurization:	100 kPa
Vial Pressurization Time:	1 min.	Load Time:	0.5 min.
Injection Time:	0.5 min.	Needle Flash Time:	0.5 min.
Sample Line Temp.:	150 °C	Transfer Line Temp.:	150 °C
Vial Volume:	20 mL		

GC-2010 Plus AF + LabSolutions LC/GC

Column:	Rtx-BAC Plus 2, 0.32 mm × 30 m, d.f. 0.6 μm		
Column Temp.:	40 °C		
Carrier Gas Pressure:	100 kPa (helium pressure mode)	Split Ratio:	1:20
FID Temp.:	250 °C	Hydrogen:	40 mL/min.
Makeup Gas:	30 mL/min. (helium)	Air:	400 mL/min.
Sample:	Ethanol (EtOH) added to sterilized sheep blood		
Internal Standard Solution (IS):	200 mg/100 mL aqueous t-butanol solution		

Measurement Sequence

Fig. 1 shows the measurement sequence for the blood samples. The pretreatment method was as per "GA/T 842-2009 Analysis Method for Ethanol Concentration in Blood." Sterilized sheep blood was used for the blood samples.

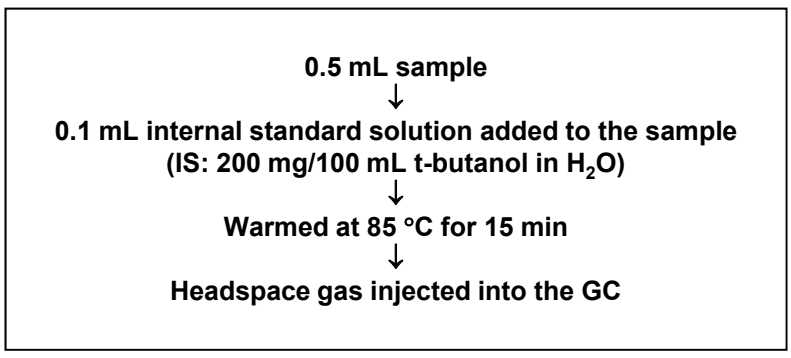

Fig. 1 Measurement Sequence

Results

Fig. 2 shows overlapping chromatograms for a blank blood sample and blood spiked with the equivalent of 8 mg/100 mL to 160 mg/100 mL of EtOH. Fig. 3 shows the linearity obtained when blood was spiked with the equivalent of 8 mg/100 mL to 160 mg/100 mL of EtOH, and Table 1 shows the concentration ratios and area ratios. As indicated, a favorable linearity of R = 0.9999 was obtained.
Tables 2 and 3 show the repeatability of retention times, area values, and area ratios for blood spiked with the equivalent of 40 mg/100 mL of EtOH. Favorable repeatability was obtained, as the retention time RSD% was 0.096 % for EtOH and 0.088 % for the IS; the area value RSD% was 0.83 % for EtOH and 1.18 % for the IS; and the area ratio RSD% was 0.68 %.

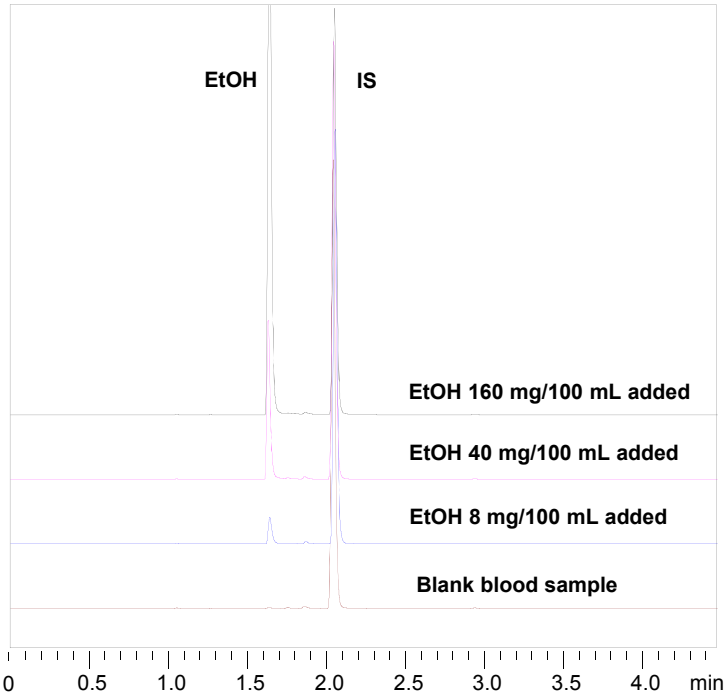

Fig. 2 Chromatograms for a Blank Blood Sample and Blood Spiked with the Equivalent of 8 mg/100 mL to 160 mg/100 mL of EtOH

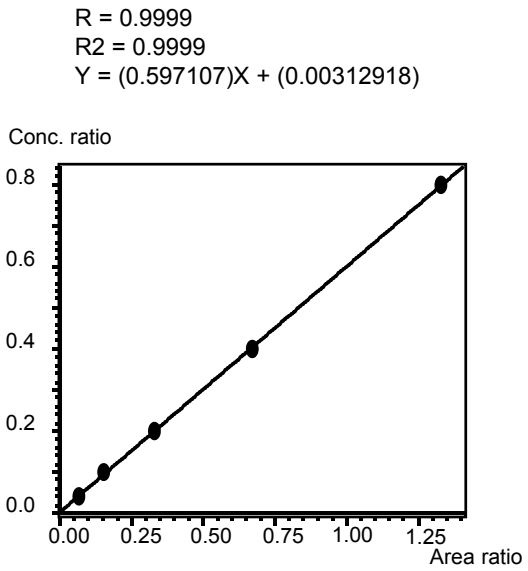

Fig. 3 Linearity of Blood Spiked with the Equivalent of 8 mg/100 mL to 160 mg/100 mL of EtOH

Table 1 Concentration Ratios and Area Ratios

Concentration ratio (EtOH/IS)	0.04	0.10	0.20	0.40	0.80
Area ratio (EtOH/IS)	0.06632	0.15329	0.33028	0.67099	1.3320

Table 2 Retention Time (min) Repeatability (40 mg/100 mL)

	1	2	3	4	5	6	mean	SD	RSD%
EtOH	1.640	1.641	1.642	1.643	1.643	1.644	1.642	0.0016	0.096
IS	2.054	2.055	2.055	2.057	2.057	2.058	2.056	0.0018	0.088

Table 3 Area Value (μV*s) and Area Ratio Repeatability (40 mg/100 mL)

	1	2	3	4	5	6	mean	SD	RSD%
EtOH	384101	374675	376905	378761	377604	378506	378425	3142.8	0.830
IS	1158476	1126253	1135762	1125928	1121669	1130570	1133110	13314.8	1.175
EtOH/IS	0.3316	0.3327	0.3319	0.3364	0.3366	0.3348	0.3340	0.0023	0.679

Reference: Chinese National Standards: GA/T 842-2009 Analysis Method for Ethanol Concentration in Blood

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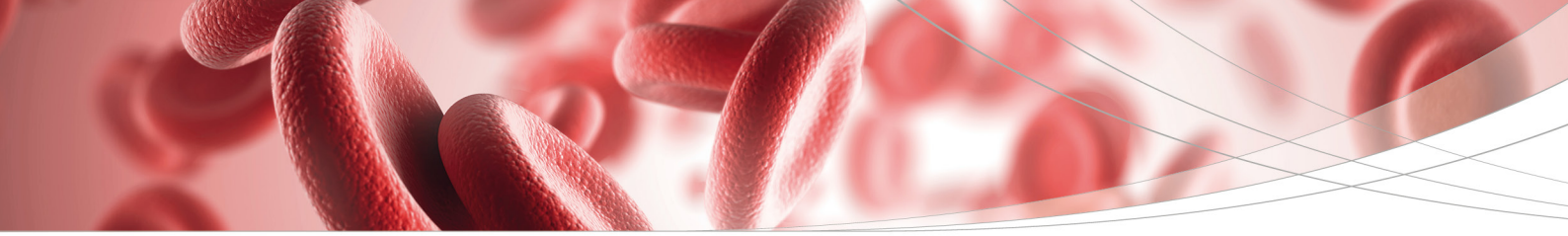

1. Chromatography

1.2 Liquid Chromatography

HPLC and UHPLC systems are able to quantitatively analyze substances in blood, serum, plasma and urines containing multiple compounds by separating and detecting target substances. Shimadzu offers a wide variety of application-specific systems, such as automated sample pretreatment systems for amino acid analysis or on-line sample trapping for quantification of drugs or metabolites.

SCA_190_014	High speed analysis of delta-amino-levulinic acid
LAAN-J-LC-E095	High-speed, sensitive analysis of serotonin
LAAN-J-LC-E097	Ultra-fast, high-sensitivity analysis of degradation products in valsartan
LAAN-J-LC-E100	High-speed and high-sensitivity analysis of cefazolin using SPD-M30A
LAAN-J-LC-E101	Ultra high-sensitivity analysis of degradation products in valsartan using the SPD-M30A photodiode array detector with high-sensitivity cell
SCA_190_012	High speed with high resolution analysis (part 37) analysis of clobazam and cibenzoline in serum by the Nexera UHPLC system
SCA_190_013	High speed with high resolution analysis (part 40) analysis of nucleobases, nucleosides, and nucleotides by the Nexera UHPLC system
LAAN-A-LC-E209	High speed, high resolution analysis (part 41) carryover evaluation of glibenclamide in human plasma by Nexera HPLC
LAAN-A-LC-E227	High speed, high resolution analysis (part 46) analysis of pre-column derivatized biogenic amines by the Nexera SIL-30AC autosampler
LAAN-A-LC-E230A	High sensitivity profiling of glycans in antibody drugs using RF-20Axs

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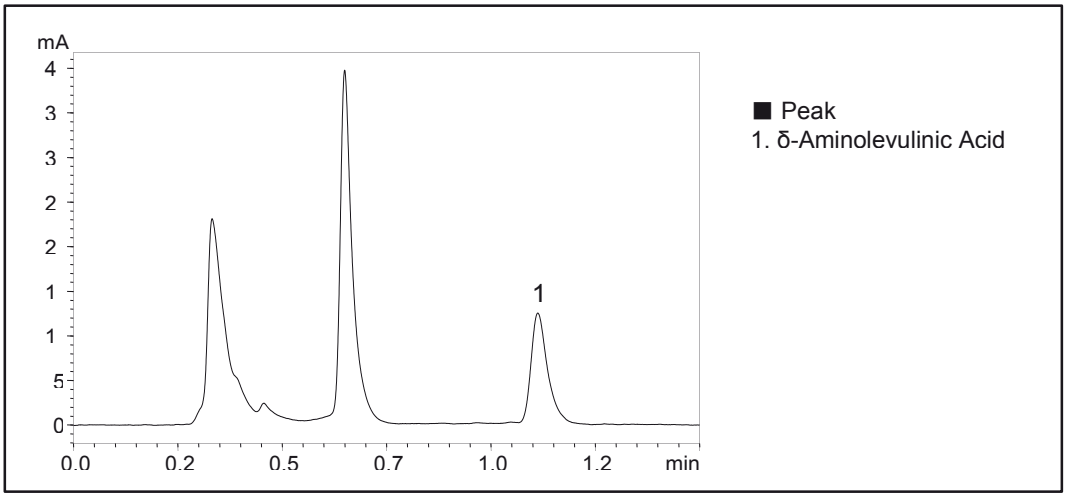
Application News

HPLC

High Speed Analysis of Delta-Aminolevulinic Acid

No. SCA-190-014

Delta-aminolevulinic acid in urine is known as an effective marker of lead (Pb) exposure. This chromatogram shows results of a high speed separation of delta-aminolevulinic acid in urine by Prominence UFLC.



Analysis of Delta-Aminolevulinic Acid in Urine [Note]

[Sample Preparation]

1) 0.45 mL of 8.5% formaldehyde was added to a 20 µL urine sample.
2) 3.5mL of a mixed solution of acetylacetone, ethanol, and distilled water was added to the solution from step 1.
3) After heating the solution for 15 minutes in boiling water then cooling it in a water bath, the solution was injected into the HPLC system.

Note: This data was provided by BML Inc.

Instrument	:	Prominence UFLC system
Column	:	Shim-pack XR-ODS (50 mm × 3.0 mm *i.d.*)
Mobile Phase	:	A) Water, B) Methanol, C) Acetic acidA / B / C = 53 / 45 / 2 (v / v / v)
Flow Rate	:	0.8mL/min
Column Temperature	:	40 °C
Detection	:	Fluorecence (Ex:363nm , Em:473nm) with Semi-micro flow cell
Sample Volume	:	5 µL

Note: The indicated data was not acquired using a system registered by Japanese Pharmaceutical Affairs Act.

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Application Data Sheet

No.21

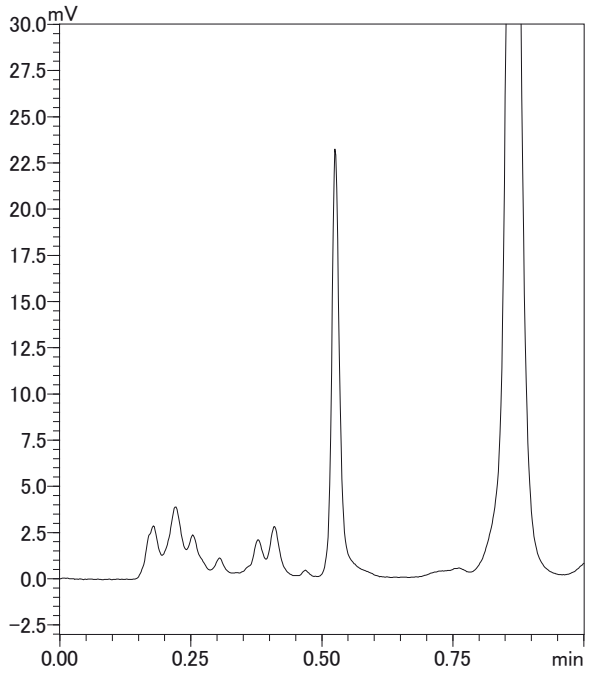
LC
Liquid Chromatograph

High-speed, Sensitive Analysis of Serotonin

Serotonin (5-hydroxytryptamine) is a biologically active substance that plays an important role in the body, functioning in the blood to constrict the vascular smooth muscle, and to promote platelet aggregation. This article introduces an example of ultrafast analysis of serotonin in the blood utilizing the Nexera UHPLC system and the RF-20Axs high-sensitivity fluorescence detector.

Analysis of Serotonin in the Blood

The blood sample was subjected to deproteinization via an aqueous trichloroacetic acid solution. A Shim-pack XR-ODS III (2 mm internal diameter, 50 mm length) with a 1.6 µm particle size was used, and detection was performed via the RF-20Axs fluorescence detector. The maximum system load pressure in this analysis was approximately 79 MPa.



Column	: Shim-pack XR-ODSⅢ (50 mmL. x 2.0 mmI.D., 1.6 µm)
Pressure	: 79 MPa
Mobile Phase	: 0.15 mmol/L Acetate buffer (pH 4.7) / Methanol = 9 / 1 (v / v)
Flow Rate	: 0.7 mL/min
Column Temp.	: 37 ℃
Injection Volume	: 1 µL
Detection	: Fluorescence (RF-20Axs) Ex. 300 nm, Em. 350 nm
Cell Temp.	: 25℃
Flow Cell	: Semi-micro cell

Peak :
1. Serotonin

* This data was provided by BML, Inc.
* The data in this document was not acquired by instruments approved by the Japanese Pharmaceutical Affairs Law.

First Edition: June, 2012

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Application Data Sheet

No.24

LC
Liquid Chromatograph

Ultra-Fast, High-Sensitivity Analysis of Degradation Products in Valsartan

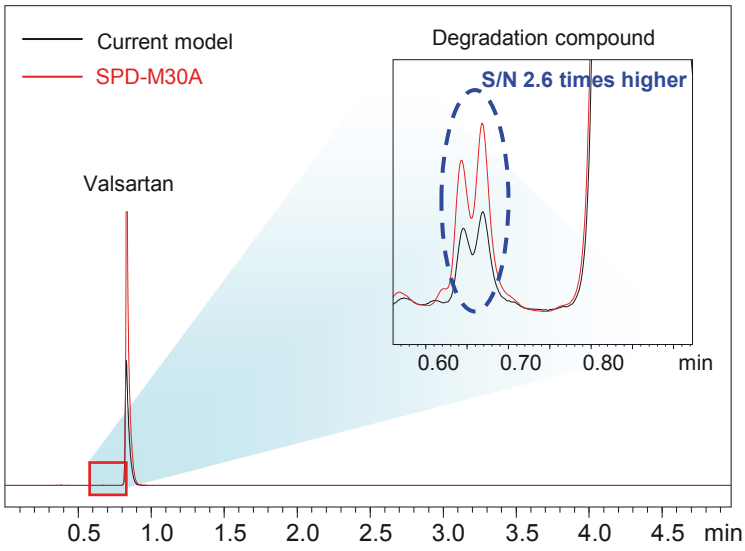
The need for reliable separation and quantification of minute quantities of pharmaceutical impurities suspected of genetic toxicity continues to grow. Using the SPD-M30A high-sensitivity photodiode array detector permits high-sensitivity detection of extremely trace amounts of components.

Introduced here is an example of ultra-fast, high-sensitivity simultaneous analysis of valsartan degradation products.

Example of Analysis of Valsartan

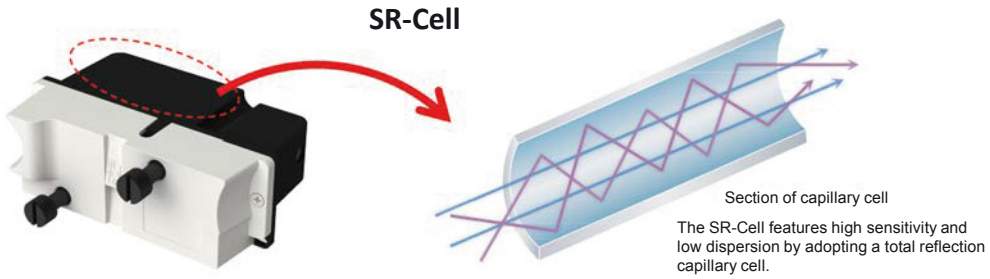
Analysis of the degradation products of valsartan was conducted using the Nexera SR ultra high performance liquid chromatograph (UHPLC). This system, which incorporates the SPD-M30A high-sensitivity photodiode array detector, utilizes the newly designed capillary SR-Cell (Sensitivity and Resolution Cell), successfully achieving both low noise and high sensitivity. Using the SPD-M30A permits high-sensitivity detection of extremely trace amounts of impurities.

Column	: Kinetex 2.6um XB-C18 100A (100 mmL. x 3.0 mmI.D., 2.6µm)
Mobile Phase	: Acetic acid/Acetonitrile/Water =1/500/500
Flow Rate	: 1.5 mL/min
Column Temp.	: 30 ℃
Injection Volume	: 10 µL
Detection	: 245nm



Newly-Designed SR-Cell

If a conventional cell's optical path length is shortened, although scattering will be minimal, sensitivity will be reduced. Conversely, if the optical path length is extended, the peak width will increase, as will noise and drift. The new capillary SR-Cell (Stability and Resolution Cell) overcomes these limitations. By optimizing the cell's optical path length and width, both low noise and high sensitivity have been achieved. It can be applied to analyses from UHPLC to HPLC without replacing the flow cell.



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Application Data Sheet

No.27

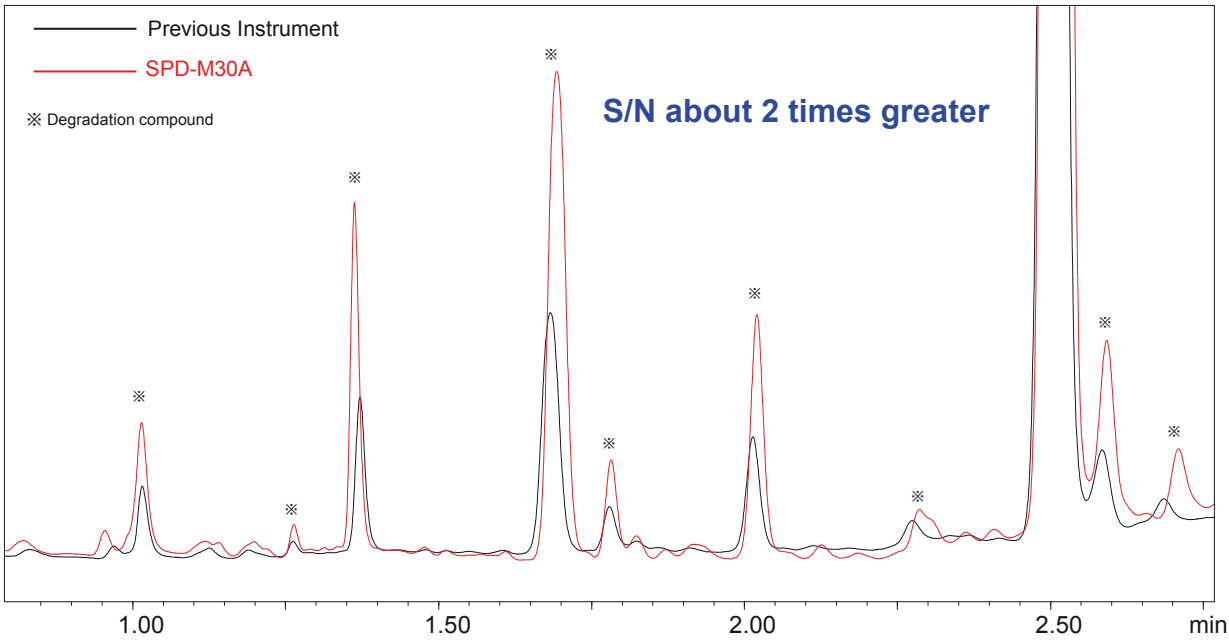
LC
Liquid Chromatograph

High-Speed and High-Sensitivity Analysis of Cefazolin Using SPD-M30A

Impurities present in drugs that are suspected of genotoxicity must be subjected to separation and quantitation at trace levels lower than typically required. With the SPD-M30A high-sensitivity photodiode array detector, even extremely trace level components can be detected and quantitated. Here we introduce an example of ultra-high-speed, high-sensitivity analysis of the degradation products of cefazolin.

Example of Analysis of Cefazolin

We conducted analysis of the degradation products of cefazolin using the Nexera SR ultra high performance liquid chromatograph. This system, which features the SPD-M30A high-sensitivity photodiode array detector, utilizes the newly designed capillary SR-Cell (Sensitivity and Resolution Cell), successfully achieving both low noise and high sensitivity. In the example below, we obtained a signal intensity of the impurity peak that is about twice that seen with the conventional cell of the previous detector model. The SPD-M30A high-sensitivity photodiode array detector permits high sensitivity detection of extremely trace amounts of impurities.



Column	: Shim-pack XR-ODSII (150 mmL. x 3.0 mmI.D., 2.2 µm)
Mobile phase	: A) 20 mmol/L (Sodium) Phosphate buffer (pH2.5) B) Acetonirtile
Gradient	: 15 % (0.0 min.) → 30 % (4.0 min.) → 50 % (9.0 min.)
Flow rate	: 0.9 mL/min
Column temp.	: 40 ℃
Injection volume	: 2 µL
Detection	: 245 nm

First Edition: January, 2013

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Application Data Sheet

No.28

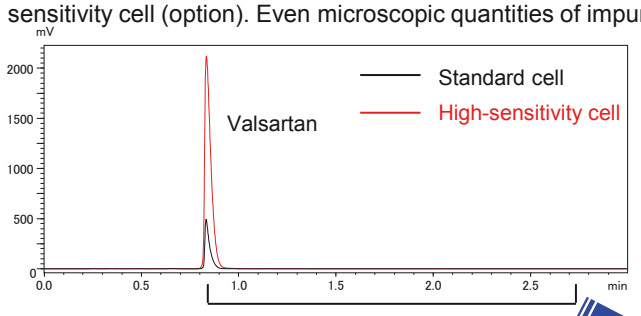
LC
Liquid Chromatograph

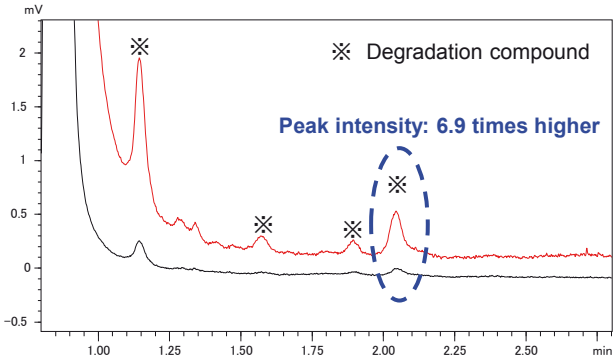
Ultra High-Sensitivity Analysis of Degradation products in Valsartan Using the SPD-M30A Photodiode Array Detector with High-Sensitivity Cell

The need for reliable separation and quantification of minute quantities of pharmaceutical impurities suspected of genetic toxicity continues to grow. Using the SPD-M30A high-sensitivity photodiode array detector permits high-sensitivity detection of extremely trace amounts of components.
Introduced here is an example of ultra-fast, high-sensitivity simultaneous analysis of valsartan degradation products.

Example of Analysis of Degradation products in Valsartan

Using the Nexera SR system, valsartan and its degradation products were analyzed with a standard cell and a high-sensitivity cell (option). Even microscopic quantities of impurities can be detected using the high-sensitivity cell.

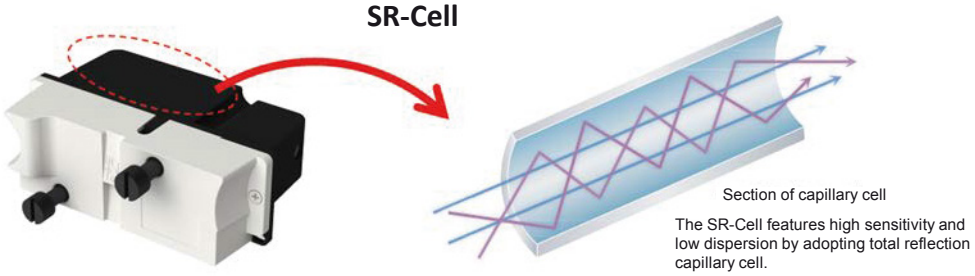




Column	: Kinetex 2.6um XB-C18 100A (100 mmL. x 3.0 mmI.D., 2.6µm)
Mobile Phase	: Acetic acid/Acetonitrile/Water =1/500/500
Flow Rate	: 1.5 mL/min
Column Temp.	: 30 ℃
Injection Volume	: 10 µL
Detection	: SPD-M30A at 290nm

Newly-Designed SR-Cell

If a conventional cell's optical path length is shortened, although scattering will be minimal, sensitivity will be reduced. Conversely, if the optical path length is extended, the peak width will increase, as will noise and drift. The new capilllary SR-Cell (Stability and Resolution Cell) overcomes these limitations. By optimizing the cell's optical path length and width, both low noise levels and high sensitivity have been achieved. It can be applied to analyses from UHPLC to HPLC replacing the flow cell.



First Edition: January, 2013

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Application News

HPLC

High Speed with High Resolution Analysis (Part 37) Analysis of Clobazam and Cibenzoline in Serum by the Nexera UHPLC System

No. SCA-190-012

HPLC is an important technique used for the analysis of drugs in the blood. However, faster analysis is often required to improve sample throughput and productivity at sites handling many specimens.
Here, we introduce an example of ultra-high-speed analysis of clobazam and cibenzoline in serum using the Nexera UHPLC (Ultra High Performance Liquid Chromatography) system and the Shim-pack XR-ODS III high-speed separation column (particle size: 1.6 µm).

Analysis of Clobazam

Clobazam is a benzodiazepine type of antiepilepsy drug. N-desmethylclobazam, one of the substances formed when clobazam is metabolized in the body, displays activity similar to that of clobazam. Fig. 1 shows the structures of these substances.
A serum sample was analyzed after cleanup by liquid-liquid extraction. Analysis was conducted using 2 different columns, the Shim-pack VP-ODS (particle size: 4.6 µm) for conventional analysis, and the Shim-pack XR-ODS III (particle size: 1.6 µm) for ultra-high-speed analysis. Fig. 2 shows the chromatograms, and Table 1 shows the analytical conditions used.
Conducting ultra-high-speed analysis with the Shim-pack XR-ODS III allowed the analysis time to be shortened to about 1/12 that by conventional analysis. The system back pressure in this analysis was about 85 MPa (12,300 psi).

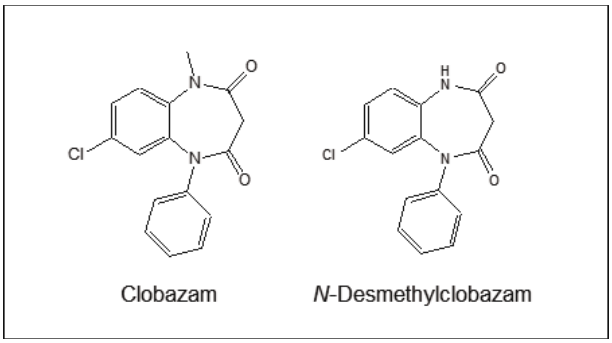

Fig. 1 Structures of Clobazam and N- Desmethyl-clobazam

Table 1: Analytical Conditions

Column	:	Shim-pack VP-ODS (150 mm L. × 4.6 mm I.D., 4.6 µm)
		Shim-pack XR-ODSⅢ (50 mm L. × 2.0 mm I.D., 1.6 µm)
Mobile Phase	:	10 mmol/L NaH_2PO_4 aq./ Acetonitrile = 2 / 1 (v / v)
Flow Rate	:	1.0 mL/min (VP-ODS)
		0.9 mL/min (XR-ODSⅢ)
Column Temp.	:	40 ℃
Injection Volume	:	50 µL (VP-ODS)
		10 µL (XR-ODSⅢ)
Detection	:	SPD-20AV at 230 nm
Flow Cell	:	Conventional Cell (VP-ODS)
		Semi-micro Cell (XR-ODSⅢ)

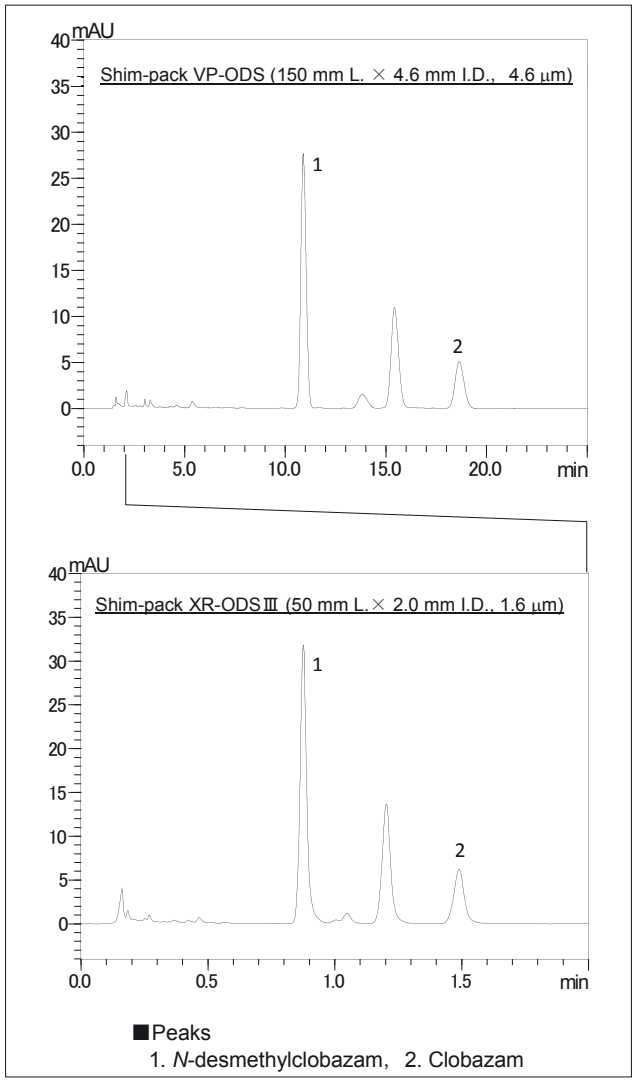

Fig. 2 Chromatograms of Clobazam and N-Desmethylclobazam in Serum Sample
(Upper: Shim-pack VP-ODS, Lower: Shim-pack XR-ODS III)

Analysis of Cibenzoline

Cibenzoline (Fig. 3) is a type of antiarrhythmic drug. A serum sample was analyzed using the Shim-pack XR-ODS III (particle size: 1.6 μm) after cleanup by liquid-liquid extraction. Fig. 4 shows the chromatogram, and Table 2 shows the analytical conditions used. Conducting analysis using these conditions allowed the analysis time to be shortened to about 1/10 that by conventional analysis, which took about 15 minutes. The system back pressure during the high-speed analysis was about 77 MPa (11,100 psi).

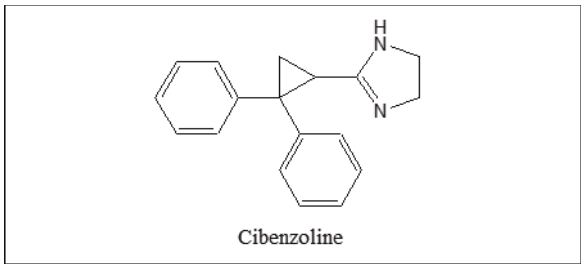

Fig. 3 Structures of Cibenzoline

Tab. 2 Analytical Conditions

Column	:	Shim-pack XR-ODSⅢ (50 mm L. × 2.0 mm I.D., 1.6 μm)
Mobile Phase	:	Phosphate buffer / Acetonitrile / Methanol = 20 / 5 / 4 (v / v / v
Flow Rate	:	0.7 mL/min
Column Temp.	:	40 ℃
Injection Volume	:	10 μL
Detection	:	SPD-20AV at 225 nm
Flow Cell	:	Semi-micro Cell

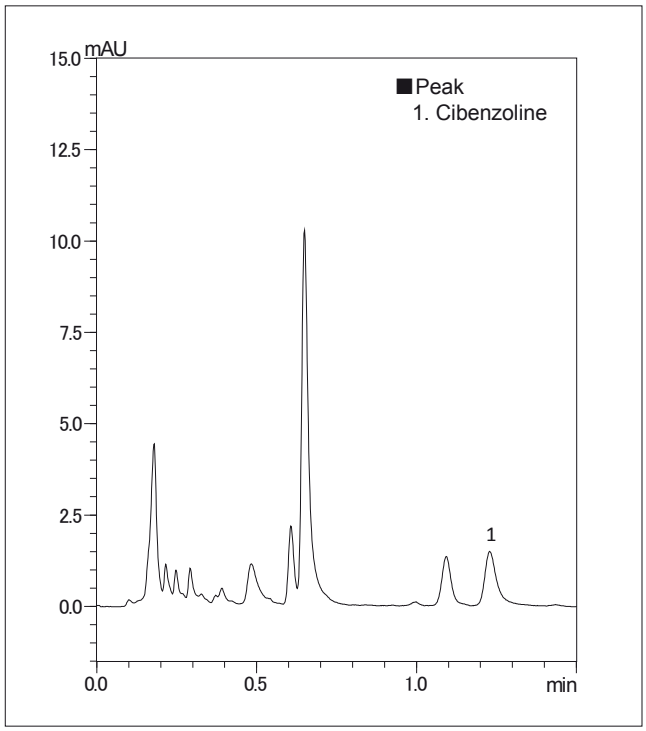

Fig. 4 Chromatogram of Cibenzoline in Serum Sample

Reducing the Total Analysis Time with Overlapping Injection

HPLC productivity is improved when the overall analysis time is shortened. This includes decreasing the run time through method condition changes like temperature and gradient profile, and also reducing the autosampler injection cycle time between samples. The Nexera SIL-30AC autosampler is equipped with an overlapping injection feature that, when enabled, loads the next sample while the current analysis is in progress.

This feature, combined with the world's fastest and cleanest injection performance, greatly shortens the overall analysis time.
Fig. 5 shows the results of overlapping 10 injections of the Cibenzoline sample from Fig. 4. Ten analyses were completed in 15 minutes.

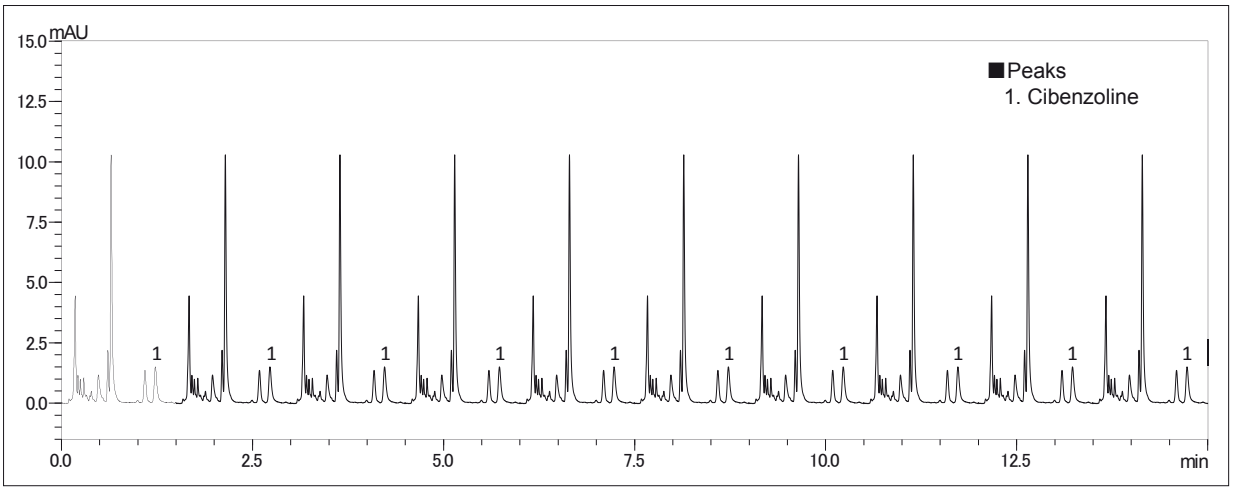

Fig. 5 Chromatograms of Cibenzoline in Serum Sample Using Overlapping Injection (10 Repetitions)

* The published data were acquired with a non-pharmaceutical compliant instrument.

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Application News

HPLC

High Speed with High Resolution Analysis (Part 40) Analysis of Nucleobases, Nucleosides, and Nucleotides by the Nexera UHPLC System

No. SCA-190-013

Nucleic acids are biological macromolecules consisting of linear chains of nucleotides, each of which is made up of a base, a sugar, and a phosphate group, and are important components that bear an organism's genetic code. In addition, nucleic acid-related compounds, including nucleobases, nucleosides, and nucleotides have a variety of functions.

Here, using the Nexera UHPLC (Ultra High Performance Liquid Chromatography) System, and the Shim-pack XR-ODSIII and Phenomenex Kinetex C18 high-speed, high-resolution columns, we introduce examples of ultra-high-speed analysis and ultra-high-resolution analysis of nucleic acid-related compounds.

Analysis of Nucleobases and Nucleosides

We prepared a sample solution consisting of a standard mixture of 10 nucleic acid-related substances, including 5 nucleobases (adenine, guanine, uracil, thymine, cytosine) and 5 nucleosides (adenosine, guanosine, uridine, thymidine, cytidine), each at a concentration of 10 mg/L, and conducted analysis using the Phenomenex Kinetex C18 column (particle size 1.7 µm, 100 mm L. x 2.1 mm I.D.). The Phenomenex Kinetex C18 is a Core-shell column consisting of a 1.25-µm solid core coated with a bonded 0.23 µm multilayer of porous film.

Fig. 1 shows the chromatogram obtained using a 1 µL injection of the prepared standard mixture, and Table 1 shows the analytical conditions used.

This analysis, which took 30 minutes to complete using conventional conditions, took about 1/10 as long (3 minutes) using these analytical conditions. The system back pressure during this analysis was about 75 MPa.

Table 1 Analytical Conditions

Column:	Kinetex 1.7 µm C18 100 A (100 mm L. x 2.1 mm I.D., 1.7 µm)
Mobile Phase:	200 mmol/L Sodium perchlorate, 100 mmol/L (Sodium) phosphate buffer (pH=2.1)aq.
Flow Rate:	0.7 mL/min
Column Temp.:	40 °C
Injection Volume:	1 µL
Detection:	SPD-20AV at 260 nm
Flow Cell:	Semi-micro Cell

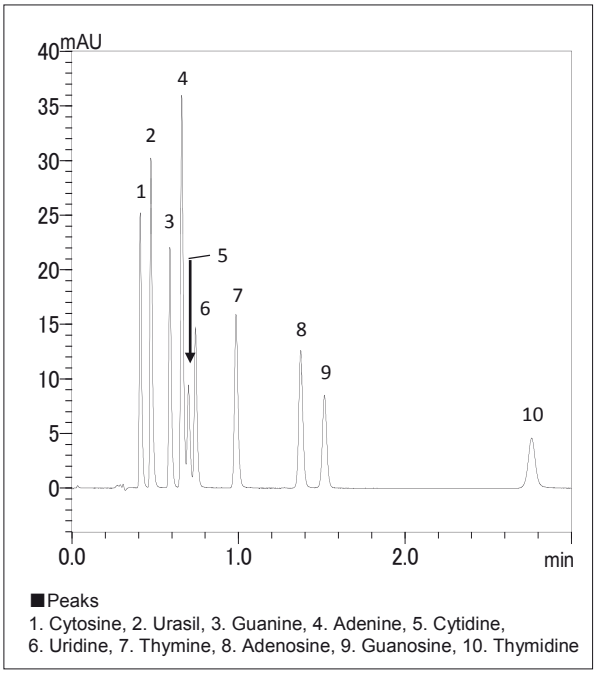

Fig.1 Chromatogram of a Standard Mixture of Nucleobases and Nucleosides（10 mg/L each）

Analysis of ATP-related Compounds

We prepared a sample solution consisting of a standard mixture of 6 ATP-related substances (hypoxanthine, inosine, IMP, AMP, ADP, ATP)*, each at a concentration of 10 mg/L, and conducted analysis using the Shimpack XR-ODS III column (1.6 µm particle size, 50 mm L. × 2.0 mm I.D.).

Note: When using a 100% aqueous mobile phase or a composition close to that, as indicated in the data in this document, the retention times may become smaller by temporarily stopping solvent delivery, and then restarting. To prevent the occurrence of this phenomenon, after completion of the analysis, it is recommended to replace the mobile phase with one containing an organic solvent (example: water/acetonitrile = 1/1) before stopping solvent delivery. In addition, if the retention times gradually become faster, perform a rinse using the same mobile phase.

Fig. 2 shows the chromatogram obtained using a 1-µL injection of the prepared standard mixture, and Table 2 shows the analytical conditions used.
This analysis, which took 25 minutes to complete using conventional conditions, took about 1/10 as long (2.5 minutes) using these analytical conditions. The system back pressure during this analysis was about 83 MPa

Table 2 Analytical Conditions

Column:	Shim-pack XR-ODSⅢ (50 mm L. × 2.0 mm I.D., 1.6 µm)
Mobile Phase:	100 mmol/L Phosphoric acid, 150 mmol/L Triethylamine aq. / Acetonitrile =100/1 (v/v)
Flow Rate:	0.9 mL/min
Column Temp.:	40 °C
Injection Volume:	1 µL
Detection:	SPD-20AV at 260 nm
Flow Cell:	Semi-micro Cell

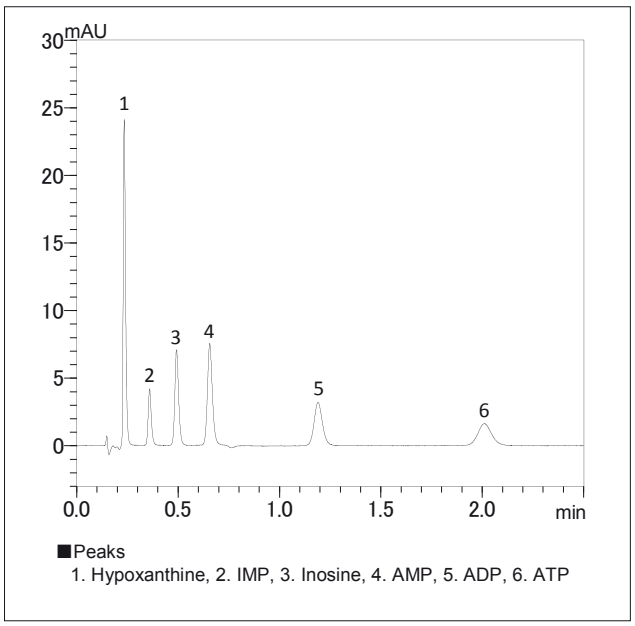

Fig.2 Chromatogram of a Standard Mixture of ATP-related Compounds

Analysis of Nucleotides

We prepared a sample solution consisting of a standard mixture of 18 nucleotides (AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, TMP, TDP, TTP, CMP, CDP, CTP, IMP, IDP, ITP)*, each at a concentration of 50 mg/L, and conducted analysis using the high-resolution Shimpack XR-ODS III column (2.2 µm particle size, 200 mm L. × 2.0 mm I.D.).
Fig. 3 shows the chromatogram obtained using a 1 µL injection of the prepared standard mixture, and Table 3 shows the analytical conditions used.
Analysis of these 18 substances was achieved at high speed and with high resolution using these conditions, and the system back pressure during the analysis was about 78 MPa.

Table 3 Analytical Conditions

Column:	Shim-pack XR-ODSⅢ (200 mm L. × 2.0 mm I.D., 2.2 µm)
Mobile Phase:	A: 100 mmol/L Phosphoric acid, 150 mmol/L Triethylamine aq. B: Mobile Phase A / Acetonitrile = 90/10 (v/v)
Flow Rate:	0.6 mL/min
Column Temp.:	50 °C
Injection Volume:	1 µL
Detection:	SPD-20AV at 260 nm
Flow Cell:	Semi-micro Cell

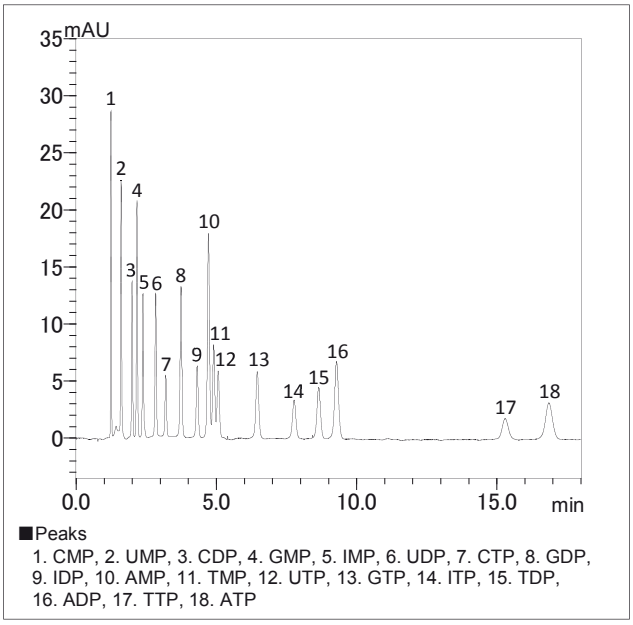

Fig.3 Chromatogram of a Standard Mixture of Nucleotides

* AMP: Adenosine 5′-monophosphate, ADP: Adenosine 5′-diphosphate, ATP: Adenosine 5′-triphosphate, GMP: Guanosine 5′-monophosphate, GDP: Guanosine 5′-diphosphate, GTP: Guanosine 5′-triphosphate, UMP: Uridine 5′-monophosphate, UDP: Uridine 5′-diphosphate, UTP: Uridine 5′-triphosphate, TMP: Thymidine 5′-monophosphate, TDP: Thymidine 5′-diphosphate, TTP: Thymidine 5′-triphosphate, CMP: Cytidine 5′-monophosphate, CDP : Cytidine 5'-diphosphate, CTP : Cytidine 5'-triphosphate, IMP : Inosine 5'-monophosphate, IDP : Inosine 5'-diphosphate, ITP : Inosine 5'-triphosphate

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Application News

No.L431

High Performance Liquid Chromatography

High Speed, High Resolution Analysis (Part 41) Carryover Evaluation of Glibenclamide in Human Plasma by Nexera HPLC

LC/MS/MS high-speed, high-resolution analysis methods with columns packed with particles of 2 µm or less are often used for analysis of drugs in human blood plasma. The small particle columns produce dispersion that is even less than that obtained with conventional columns (5 µm particle size), so peaks are taller, thereby enabling quantitation at even higher sensitivity. While this high-sensitivity analysis has become possible, the effect of autosampler carryover on high-sensitivity LC/MS/MS analysis has become a problem.

In Application News No. L425, we evaluated the performance of the Nexera SIL-30AC autosampler in the analysis of reserpine in human plasma, and reported its low carryover performance. In addition to the high potential of the SIL-30AC, we believe that rinsing with its powerful and sophisticated multi-solvent configuration was very effective.

Here, we report on the effectiveness of the rinse method reported in Application News No. L425, and demonstrate its effectiveness as a rinse method applicable to measurement of a wide range of drugs in plasma.

Preparation of Glibenclamide in Human Plasma

We selected glibenclamide as the target substance for the evaluation of carryover in the SIL-30AC. Glibenclamide is a second-generation oral hypoglycemic medication that was developed in 1966 by Boehringer Mannheim GmbH and Farbwerke Hoechst AG. It acts to lower the sugar level in the blood by stimulating the β cells in the pancreas, causing the discharge of insulin. It is typically used to treat patients with non-insulin dependent diabetes, also referred to as type 2 diabetes.

The structural formula of glibenclamide is shown in Fig. 1. It has an S-phenyl sulfonylurea structure consisting of a para-phenyl group, a sulfonyl group, and a urea bond. Because of the hydrophobic rings, it is a compound that would be expected to be particularly prone to carryover. For the evaluation, LC/MS/MS measurement of glibenclamide in human plasma was conducted using a high-sensitivity MS/MS instrument.

Fig. 2 shows the pretreatment process used for spiking the human plasma with glibenclamide. Commercially available human plasma was used for the experiment. Three µL of the prepared glibenclamide-spiked sample was injected into the Nexera-MS/MS system using the SIL-30AC.

MRM (Multiple Reaction Monitoring) quantitation was conducted using the glibenclamide proton-adduct molecular ion ($[M+H]^+$: *m/z*=494) as the precursor ion, and *m/z*=369 as the product ion.

Quantitation of the glibenclamide in human plasma used the analytical conditions shown in Table 1. With this method, high-speed analysis was conducted with an analysis time of just 4 minutes, and even at a low concentration of 0.005 ng/mL in the plasma, high-sensitivity analysis was achieved with an S/N ratio of 2.

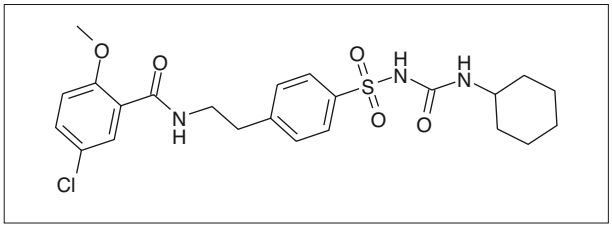

Fig. 1 Structure of Glibenclamide

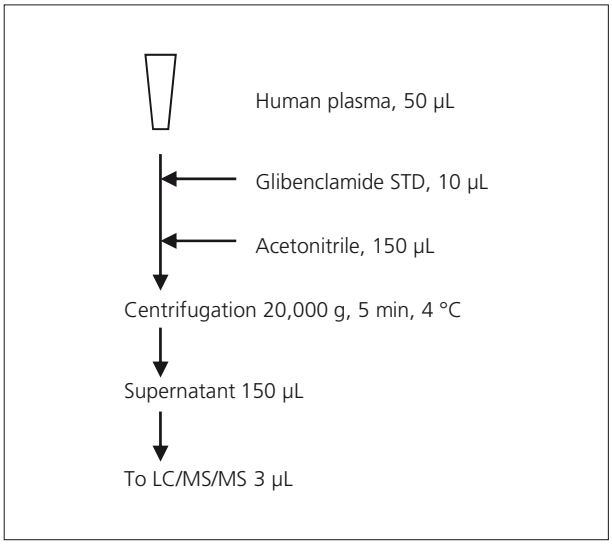

Fig. 2 Pretreatment Protocol

Carryover Evaluation of Glibenclamide in Human Plasma

Table 1 shows the analytical conditions, including the autosampler rinse liquids and rinsing sequence. The SIL-30AC can use 4 different wash solutions to wash the inner and outer needle surfaces.

One possible source of carryover of a drug substance in plasma is the adhesion of phospholipids in the plasma to the sampling device, and the secondary adhesion of the drug substance on top of that. Therefore, considering that phospholipids are easily dissolved in 2-propanol, we programmed internal needle washes with high-concentration 2-propanol as "R1" and a combination of 2-propanol with other solvents as "R2", conducting 2 cycles of alternating washes with R1 and R2, using 300 µL of wash solution for each wash. Additionally, the external surface of the needle was washed for 1 second with "R3" (same solution as "R1"), using the active rinse pump.

Fig. 3 shows the results of the carryover evaluation for glibenclamide in human plasma.

First, we injected a human plasma blank (sample prepared according to Fig. 2, but using methanol instead of glibenclamide). That MRM chromatogram is denoted as "Pre Blank". Next, after injecting 3 µL of sample containing 1000 ng/mL of glibenclamide in human plasma (denoted as "1000 ng/mL" in Fig. 3), the plasma blank was run again (denoted "Post Blank"), and then carryover was evaluated based on the glibenclamide elution position (in the vicinity of 1.7 minutes). A 10-times magnification of that area of the chromatogram is shown in Fig. 3-1, and it is clear that glibenclamide is not detected. These results indicate that carryover was less than 0.005 ng/mL (less than 0.0005 %). Thus, in LC/MS/MS drug analysis using human plasma samples, the Nexera SIL-30AC autosampler can deliver excellent low-carryover performance using the rinse/wash methods described here.

Table 1 Analytical Conditions

<HPLC Conditions>		[Auto Sampler Option]	
Column	: ODS column (50 mm L. × 2.0 mm I.D., 2.3 µm)	Wash Solution	
Mobile Phase	: A: 0.1 % Formic acid in water B: 0.1 % Formic acid in acetonitrile Isocratic elution, A/B = 7/3	R0 : Mobile phase A R1 : 50 % 2-Propanol R2 : Acetonitrile / 2-Propanol / Water / Formic acid (700/200/100/2) R3 : 50 % 2-Propanol Internal Rinse R1 → R2 → R1 → R2 → R0 Wash volume 300 µL each	
Flow Rate	: 0.5 mL/min		
Column Temperature	: 40 °C		
Injection Volume	: 3 µL		
<MS Conditions>		External Rinse	
Ionization Mode	: ESI positive Glibenclamide (494 > 369)	R3 1 s	
		Rinse Mode	: Before and after aspiration
		Rinse Method	: Rinse pump then port

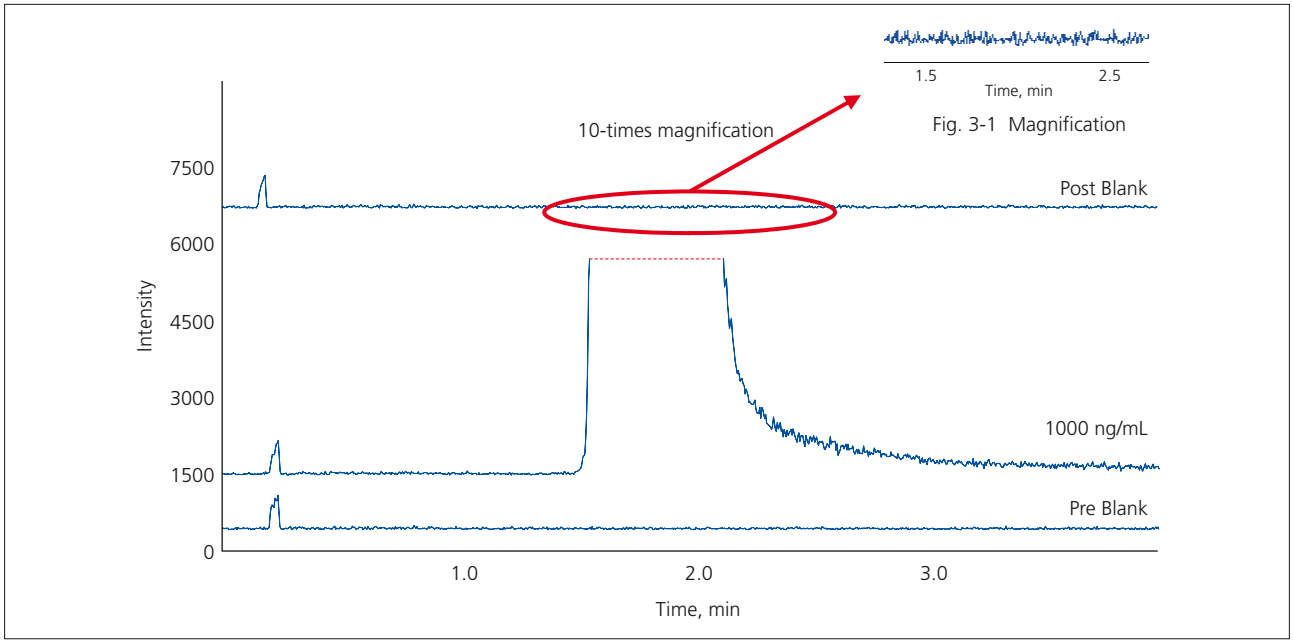

Fig. 3 Carryover Evaluation of Glibenclamide in Human Plasma

Note) The published data was not acquired using an instrument registered by Japanese pharmaceutical affairs law.

First Edition: January, 2012

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Application News

No.L449

High Performance Liquid Chromatography

High Speed, High Resolution Analysis (Part 46) Analysis of Pre-Column Derivatized Biogenic Amines by the Nexera SIL-30AC Autosampler

Biogenic amines are produced naturally by the enzymatic decarboxylation of amino acids in beverages and food. Biogenic amines such as these are also used as an indicator of food spoilage. Histamine, a substance that can cause allergy-like food poisoning, must not exceed 50 ppm in food in general according to FDA standards, 100 ppm in marine products in the EU, and 400 ppm in fish sauce according to the Codex International Food Standards. In addition, biogenic amines such as cadaverine and tyramine appear to intensify the allergy-like food toxicity of histamine.
In Application News articles L432 and L437, we introduced examples of the pretreatment functions of the SIL-30AC autosampler in the analysis of fluorescence-derivatized amino acids using *o*-phthalaldehyde (OPA). Here, we introduce an example of the analysis of fluorescent amines that were derivatized with OPA.

Simultaneous Determination of 7 Biogenic Amines

With this method, the pretreatment functions of the Nexera SIL-30AC autosampler were utilized to conduct automated derivatization of the amines using OPA. Table 1 shows the derivatization reagents used with this method, and Fig. 1 shows the reagent addition and mixing settings that were used for automated derivatization using the Nexera SIL-30AC autosampler. The analytical conditions that were used are shown in Table 2, and the chromatogram obtained from analysis of a standard solution is shown in Fig. 2. In addition to automating the derivatization step, the overall analysis time can be further shortened by using the overlapping injection feature that was introduced in Application News L437. This allows the next sample in the sequence to be derivatized and loaded into the needle for injection immediately after the analysis of the current sample is complete.

Table 1 Derivatization Reagents (10 mg/L each)

- Mercaptopropionic Acid Solution (MPA solution)
  3-Mercaptopropionic Acid 10 µL in 0.1 mol/L Borate Buffer (pH 9.2) 10 mL
- *o* - Phthalaldehyde Solution (OPA solution)
  o - Phthalaldehyde 10 mg in 0.1 mol/L Borate Buffer (pH 9.2) 10 mL

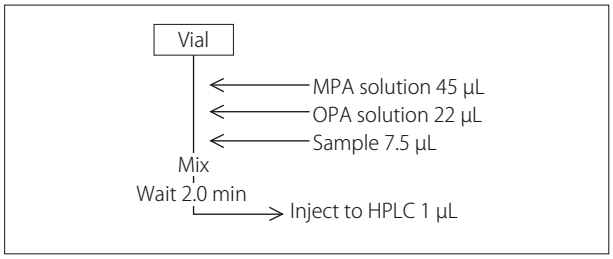

Fig. 1 Flowchart of Derivatization with SIL-30AC

Table 2 Analytical Conditions

Column	: Shim-pack XR-ODSⅢ (75 mm L. × 2.0 mm I.D., 1.6 µm)
Mobile Phase	: A : 100 mmol/L Acetate (Sodium) Buffer (pH 4.7) B : Acetonitrile
Time Program	: B.Conc. 15 % (0 min) →30 % (3 min) →40 % (8 min) →15 % (8.01-11 min)
Flowrate	: 0.5 mL/min
Column Temp.	: 40 °C
Injection Vol.	: 1 µL
Detection	: RF-20Axs Ex. at 330 nm, Em. at 440 nm
Cell Temp.	: 30 °C
Flow Cell	: Semi-micro cell

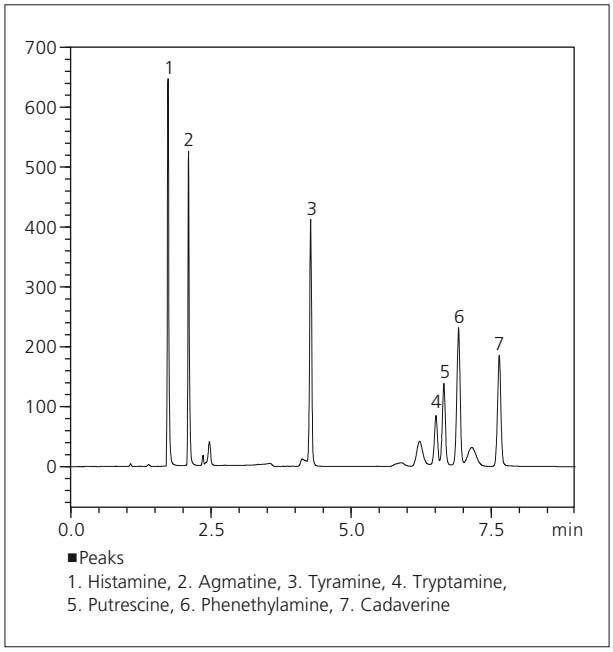

Fig. 2 Chromatogram of Standard Solution of 7 Biogenic Amines (10 mg/L each)

Linearity and Repeatability

The calibration curves generated using amine concentrations from 0.1 to 100 mg/L showed excellent linearity with a coefficient of determination (R^2) greater than 0.999 for all components. Table 3 shows the repeatability of retention times and area values obtained from repeated injections of 7 amines (n=6).

Table 3 Repeatability

	R.T. %RSD	Area %RSD
Histamine	0.067	0.70
Agmatine	0.055	0.72
Tyramine	0.037	0.60
Tryptamine	0.035	0.88
Putrescine	0.037	0.61
Phenethylamine	0.036	0.37
Cadaverine	0.030	0.84

Analysis of Foods and Beverages

Figs. 3 to 6 show the results of analysis of commercially available beer, wine, pork, and tuna samples. The beer and wine were passed through a 0.22 µm membrane filter, and then used as sample solutions. The pork and tuna were first stored at 37 °C for 24 hours to accelerate the production of amines. Then, 0.5 mol/L aqueous trichloroacetic acid solution was added to the homogenized samples, and following centrifugation, the supernatants were neutralized with 0.3 mol/L aqueous sodium hydroxide solution, and then passed through a 0.22 µm membrane filter to serve as sample solutions.

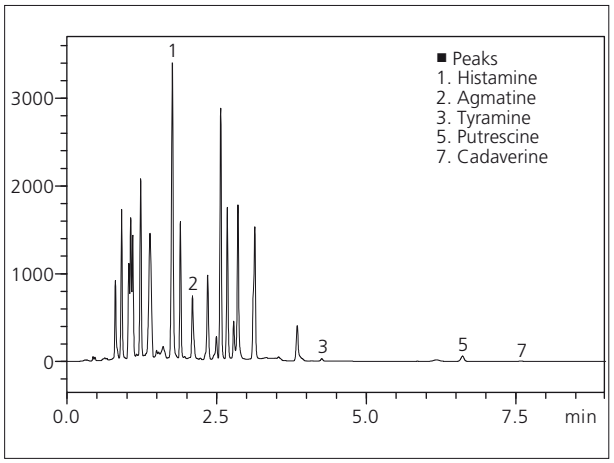

Fig. 3 Chromatogram of Beer

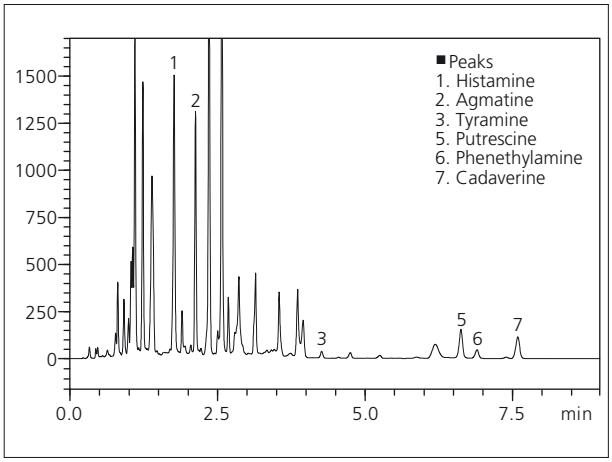

Fig. 4 Chromatogram of Wine

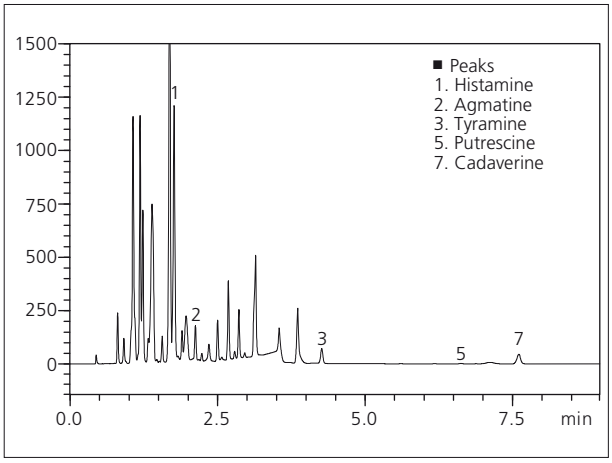

Fig. 5 Chromatogram of Pork

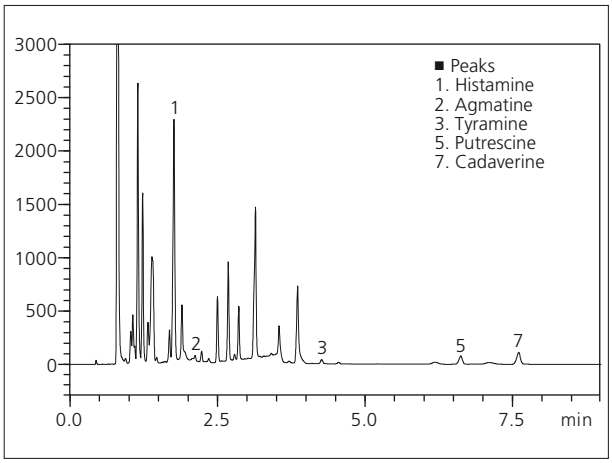

Fig. 6 Chromatogram of Tuna

First Edition: Jun. 2013

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Application News

No.L452A

High Performance Liquid Chromatography

High Sensitivity Profiling of Glycans in Antibody Drugs Using RF-20Axs

Glycans, or sugar chains, in antibody drugs play roles in the antigenicity, pharmacokinetics, and stability of higher-order structure, which could adversely affect their safety and effectiveness. Further, there is also concern about the non-uniformity of these glycans due to instability of antibody drug culture conditions, which has heightened the necessity to manage their production process. However, while there is currently no glycan test method specified in the Japanese Pharmacopoeia, there is wide demand for an assessment method.

Here, we introduce an example of analysis of glycans in antibody drugs using the Nexera X2 ultra high performance liquid chromatograph with the RF-20Axs high-sensitivity fluorescence detector. For the analysis, the Phenomenex core-shell, high-speed analytical Aeris™ PEPTIDE XB-C18 column was used. Since the permeability of the packing material is optimized for analysis of high-molecular compounds such as peptides, the column is useful for separation of glycans and impurities in antibody drugs.

Sensitivity and Linearity of Detectors in PA-Glycan Analysis

The sensitivity and linearity of the RF-20Axs fluorescence detector was evaluated using a pyridylamino (PA)-glycan (PA-Sugar Chain 009, Takara Bio Inc.). Table 1 shows the analytical conditions.

Fig. 1 shows a comparison of the sensitivity obtained in analysis of a PA-glycan at 10 fmol (5 nmol/L, 2 µL injected) using the fluorescence detectors RF-20Axs and the previous model RF-10Axl connected in series. Excellent results were obtained with the RF-20Axs, with a good S/N ratio and low noise. Fig. 2 shows the calibration curve results obtained with the RF-20Axs fluorescence detector over a concentration range of 1 – 100 fmol (0.5 – 50 nmol/L, 2 µL injected).

There is significant improvement in performance compared to the previous model, and these results demonstrate that the RF-20Axs fluorescence detector is suitable for verification not only of the main peak, but of the trace level impurities as well.

Table 1 Analytical Conditions

Instrument	: Nexera X2
Column	: Shim-pack XR-ODS Ⅲ (50 mm L. × 2.0 mm I.D., 1.6 µm)
Mobile Phase*	: A) 20 mmol/L Ammonium Formate 0.0095% (v/v) Formic Acid-Water (pH 4.5)
	B) 20 mmol/L Ammonium Formate 0.0095% (v/v) Formic Acid-Methanol
	A/B=95/5 (v/v)
Flowrate	: 0.5 mL/min
Column Temp.	: 40 ˚C
Detection	: RF-20Axs (Ex = 320 nm, Em = 400 nm)
Injection Vol.	: 2 µL

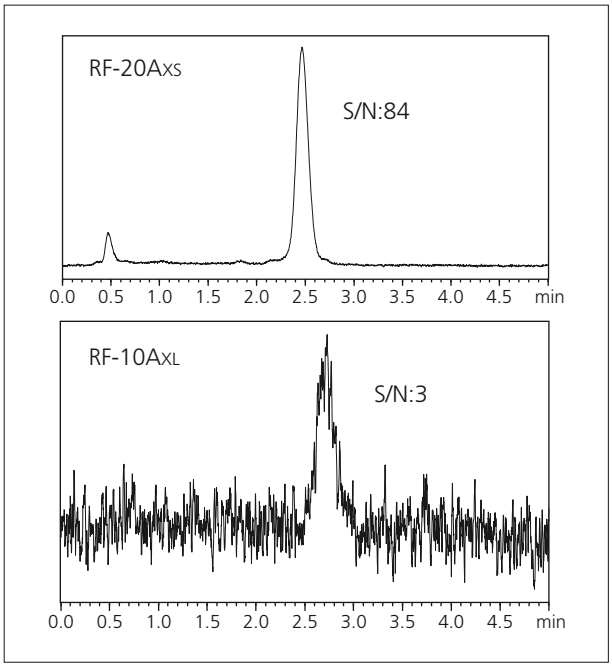

Fig. 1 Chromatograms of 10 fmol PA-Glycan

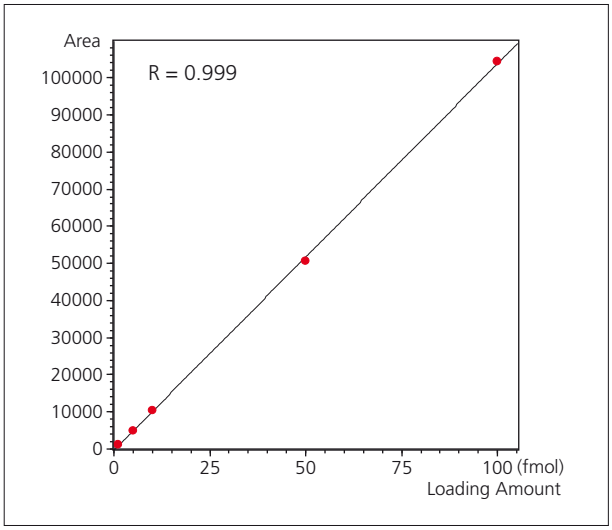

Fig. 2 Calibration Curve (1 – 100 fmol Injected)

*Mobile Phase Preparation
1.26 g (20 mmol) ammonium formate (M.W.: 63.026) was dissolved in 1 L of distilled water or methanol, and 95 µL of formic acid was added.

Analysis of Glycans in Antibody Drugs

According to the pretreatment procedure of Fig. 3, the glycans were extracted from 2 types of antibody drugs, and following purification, were subjected to fluorescent derivatization by PA (pyridylamination).
Fig. 4 shows the chromatograms of PA-glycans from antibody drugs, and Table 2 shows the analytical conditions used. Comparing the peaks in the vicinity of 50 minutes for the drugs A and B, respectively, the quantity of glycans associated with that peak in antibody drug A is much greater than that in drug B. The peak response is quite different for many other peaks, which illustrates the formulation differences between drug manufacturers.

Table 2 Analytical Conditions

Instrument	: Nexera X2
Column	: Aeris™ PEPTIDE XB-C18 (150 mm L. × 2.1 mm I.D., 1.7 µm)
Mobile Phases	: A) 20 mmol/L Ammonium Formate 0.0095 % (v/v) Formic Acid-Water (pH 4.5)
	B) 20 mmol/L Ammonium Formate 0.0095 % (v/v) Formic Acid-Methanol
Time Program	: B Conc. 0 % (0 min) → 5 % (60 min) → 10 % (70 min) → 100 % (70.01 min → 80 min) → 0 % (80.01 min → 90 min)
Flowrate	: 0.4 mL/min
Column Temp.	: 40 ˚C
Detection	: RF-20Axs (Ex = 320 nm, Em = 400 nm)
Injection Vol.	: 3 µL

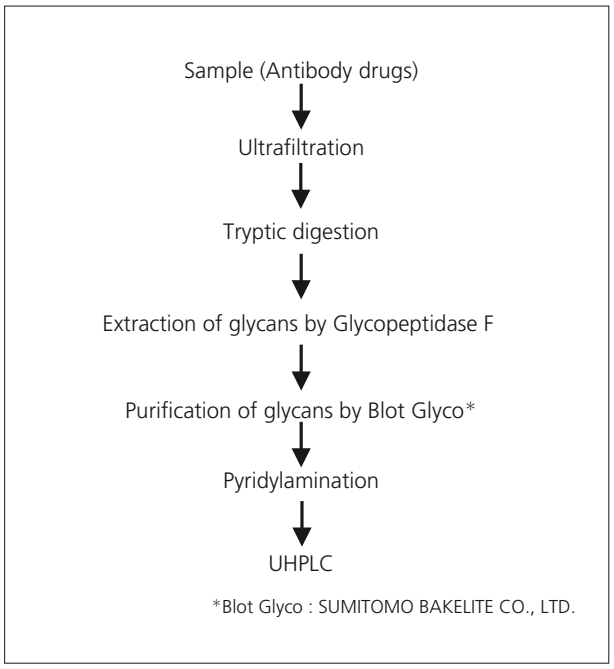

Fig. 3 Sample Preparation

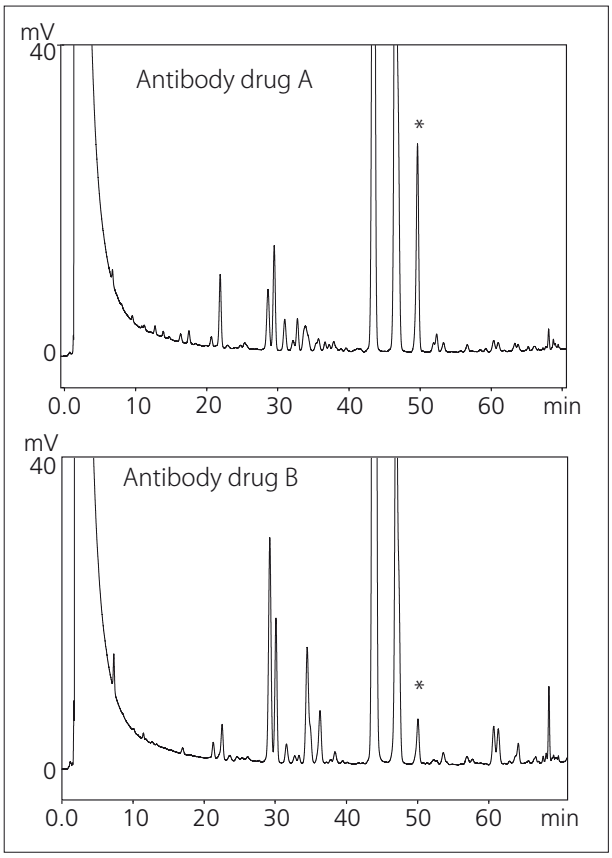

Fig. 4 Chromatograms of PA-Glycans from Antibody Drugs

Analysis of the glycans in the antibody drugs was conducted with the kind cooperation of Kenichiro Todoroki, Ph.D. of the Laboratory of Analytical and Bio-Analytical Chemistry, School of Pharmaceutical Sciences, University of Shizuoka.

For further information regarding the Aeris™ column, please contact
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First Edition: Jan. 2014

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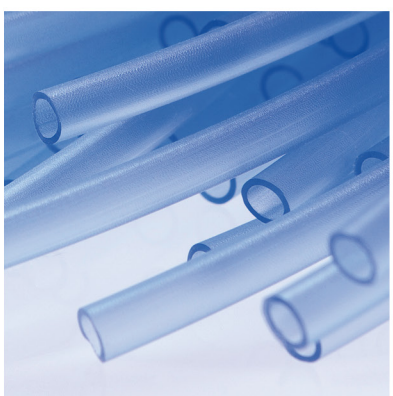
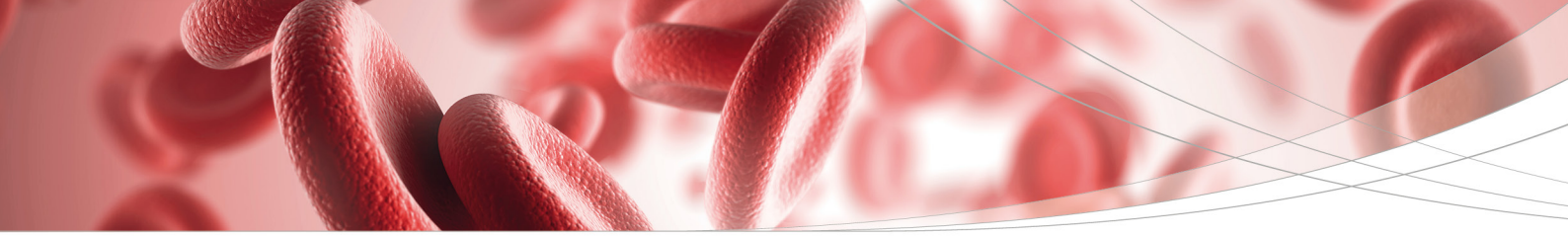
2. Mass Spectrometry





2. Mass Spectrometry

2.1 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a hyphenated technique combining the separating power of gas chromatography (GC) with the detection power of mass spectrometry to identify different substances within a sample. Mass spectrometry is a wide-ranging analytical technique which involves the production, subsequent separation and identification of charged species according to their mass to charge (m/z) ratio. It is well known for analysis of drug of abuses.

LAAN-J-MS-E090	Analysis of impurities in tests for residual solvents in pharmaceuticals using GC-MS
SSI-GCMS-1403	Analysis of blood alcohol by headspace with simultaneous GC-FID and MS detection
SSI-GCMS-1401	Analysis of opioids using isotope dilution with GCMS-TQ8030 GC/MS/MS
C146-E175A	Automatic identification and semi-quantitative analysis of psychotropic drugs in serum using "GC/MS forensic toxicological database"
LAAN-J-MS-E100	Analysis of toxicological substances in whole blood using smart forensic database (1)
LAAN-J-MS-E104	Multicomponent analysis of metabolites in human plasma using GC-MS/MS
C146-E217	Effectiveness of metabolomics research using gas chromatograph/quadrupole mass spectrometer with high-sensitivity and high-speed scanning
SCA_280_082	The metabolites analysis of serum using fast-GCMS/MS with tandem column

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Application Data Sheet

No.90

GC-MS
Gas Chromatograph Mass Spectrometer

Analysis of Impurities in Tests for Residual Solvents in Pharmaceuticals Using GC-MS

The HS-GC-FID method is adopted for tests of residual solvents in pharmaceuticals, but GC-MS is effective for confirming any unknown peaks (impurities) detected in such tests.
United States Pharmacopoeia (USP) 467 is well known as a test method for residual solvents in pharmaceuticals. In this method, analysis is performed using analysis columns with an internal diameter of 0.32 mm or 0.53 mm, and a length of 30 m, at a linear velocity of 35 cm/s. When GC-MS is used to confirm unknown peaks in chromatograms obtained by GC-FID analysis using large bore columns, approximating the retention times in both chromatograms is essential. If the linear velocity is set at 35 cm/s with GC-MS, the retention times are roughly approximated. However, using such large bore columns and a linear velocity of 35 cm/s in GC-MS will prevent analysis under the designated USP conditions for several reasons. For example, the flowrate will be large, which will have an impact on sensitivity, and the inlet pressure will exceed the control range (decompression region) due to the vacuum in the MS section.
With its differential vacuum system, the GCMS-QP2010 Ultra features high vacuum efficiency, enabling analysis using the same 0.53 mm analysis columns as in GC-FID. The linear velocity of 35 cm/s designated in USP 467 can be achieved by connecting the optimal resistance tube to the column outlet.
This Data Sheet presents an example of the analysis of unknown peaks in a test for residual solvents in pharmaceuticals, using analysis columns that complies with USP467 Procedure A for residual solvents, utilizing the Shimadzu HS-20 Trap headspace sampler, which features a trapping function, and the GCMS-QP2010 Ultra.

Analysis Conditions

The analysis conditions are shown in Table 1.
To achieve control at low linear velocities with a large bore column, a resistance tube was connected to the column outlet. The optimal resistance tube size was configured by referring to the backflush setting software.

Table 1 Analysis Conditions

Headspace Sampler:	HS-20 Trap		
GC-MS:	GCMS-QP2010 Ultra		
[HS-20]			
Mode:	Loop (1 mL)		
Vial Warming:	80 °C	Vial Warming Time:	60 min
Vial Agitation:	Off		
Vial Pressurization:	75 kPa		
Vial Pressurization Time:	3.0 min	Pressure Equilibration Time:	0.1 min
Load Time:	0.5 min	Load Equilibration Time:	0.1 min
Injection Time:	45 min	Needle Flash Time:	45 min
Sample Line Temp.:	150 °C	Transfer Line Temp.:	150 °C
[GC]			
Columns:	Rxi®-624sil MS, 30 m × 0.53 mm I.D., d.f. 3.0 µm + resistance tube 0.82 m × 0.18 mm I.D. *1 Rxi®-624sil MS, 30 m × 0.32 mm I.D., d.f. 1.8 µm + resistance tube 1.45 m × 0.18 mm I.D.		
Column Temp.:	40 °C (20 min) – 10 °C/min – 240 °C (20 min)		
Control Mode:	Pressure (He)	0.53 mm column: 25.0 kPa (20 min) – -0.8 kPa/min – 9.0 kPa (5 min) 0.32 mm column: 69.8 kPa (20 min) – 3.0 kPa/min – 129.8 kPa (5 min)	
Injection Mode:	Split (split ratio 5)		
[MS]			
Ion Source Temp.:	200 °C	Interface Temp.:	250 °C
Measurement Mode:	Scan	Scan Range:	29 - 250 *m/z*
Event Time: 0.2 sec			
Ionization Voltage:	70 V		

*1: A resistance tube (PN 10046, Shimadzu GLC) was cut at the optimal length, and then connected using a capillary column press-tight connector (PN 221-38102-91). The length of the resistance tube shown here is only an example, and may change depending on the analysis column lot.

(1) Sample Analysis using a 30 m Column with an Internal Diameter of 0.53 mm

Figs. 1 and 2 show the GC-MS results of measuring a Class 2 Residual Solvents Standard Solution (water-soluble articles) using a 30 m column with an internal diameter of 0.53 mm, to compare them to the results obtained from separate measurements by GC-FID. With a 30 m × 0.53 mm I.D. column, the column outlet pressure will be negative, so the flowrate designated in Procedure A cannot be set. Here, the linear velocity of 35 cm/s designated in Procedure A was achieved by connecting an 82 mm × 0.18 mm I.D. resistance tube to the column outlet, and a chromatogram pattern approximating the GC-FID results was obtained. The column flowrate with GC-MS was calculated at 4.86 mL/min, but measurements were not a problem for the GCMS-QP2010 Ultra, with its high vacuum efficiency.

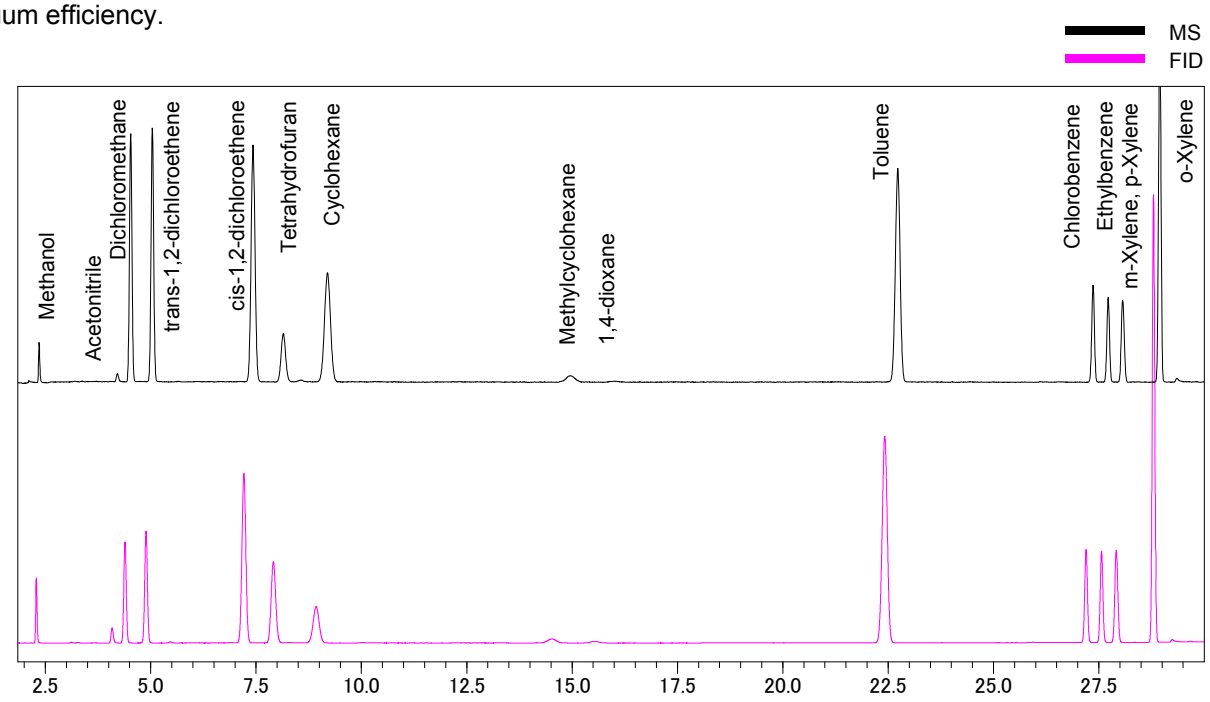

Fig. 1 Chromatogram Comparison (Internal Diameter 0.53 mm, Class 2A)

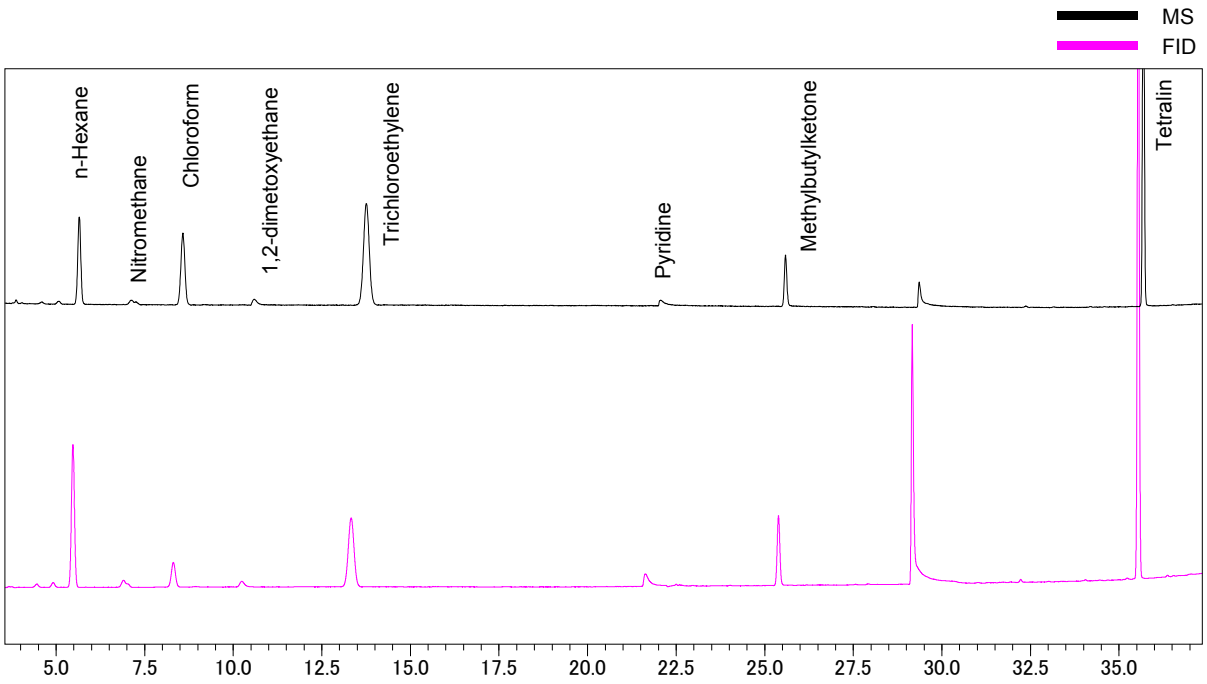

Fig. 2 Chromatogram Comparison (Internal Diameter 0.53 mm, Class 2B)

(2) Sample Analysis Using a 30 m Column with an Internal Diameter of 0.32 mm

Figs. 3 and 4 show the results of measuring a Class 2 Residual Solvents Standard Solution (water-soluble articles) using a 30 m column with an internal diameter of 0.32 mm. With the 0.32 mm I.D. column, measurements can be performed with GC-MS if the linear velocity is set at 40 cm/s or higher. However, the retention time will differ significantly from that for GC-FID measured under the designated USP conditions. Here, the linear velocity of 35 cm/s designated in Procedure A was achieved by connecting an 145 mm × 0.18 mm I.D. resistance tube to the column outlet, and a chromatogram pattern approximating the GC-FID results was obtained.

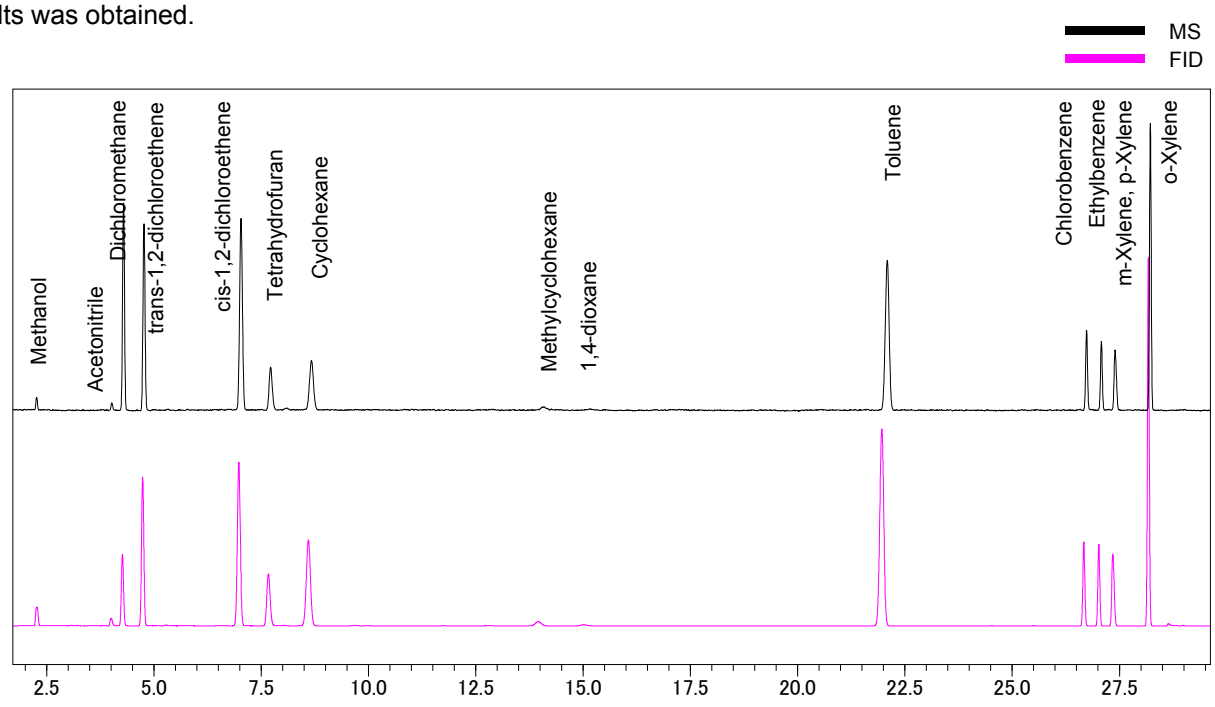

Fig. 3 Chromatogram Comparison (Internal Diameter 0.32 mm, Class 2A)

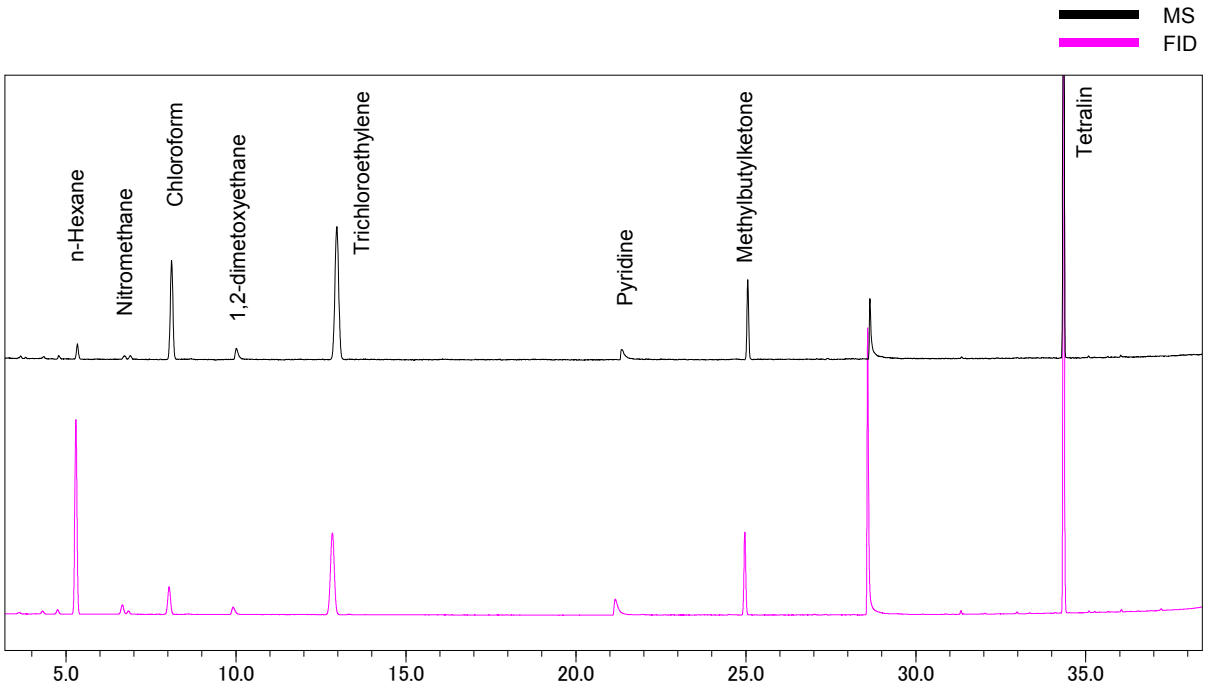

Fig. 4 Chromatogram Comparison (Internal Diameter 0.32 mm, Class 2B)

(3) A Sample Impurity Analysis

Fig. 5 shows an example of the measurement of a commercially available pharmaceutical drug test solution using a 30 m × 0.53 mm I.D. column. The peaks ((1) and (2)) not contained in Standard Solutions Class 1, 2A, and 2B were confirmed in both the FID and MS results. They were inferred from the mass spectra to be n-Butanol and Butylacetate, target components in Class 3. When trap injection (5 cycle extraction) was performed, peak A, which was essentially undetectable with loop FID and GCMS analysis, was detected with high sensitivity, and was identified as Ethyl Acetate (Fig. 6). Accordingly, use of the HS-20 and GCMS-QP2010 Ultra evidently simplifies the analysis of unknown peaks in tests for residual solvents in pharmaceuticals.

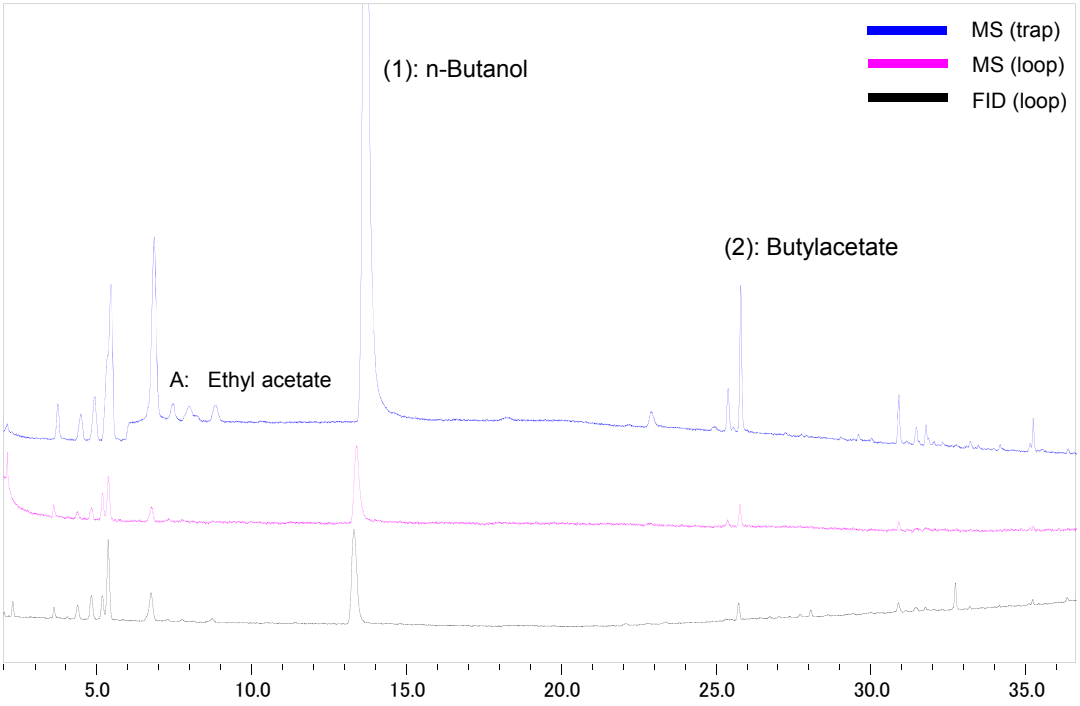

Fig .5 Test Solution Chromatograms

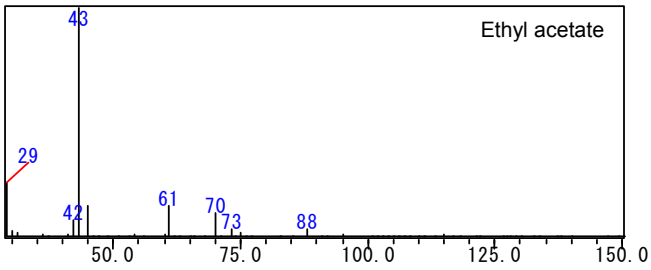

Fig. 6 Mass Spectrum for Peak A

First Edition: November, 2013

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Application News

Gas Chromatograph Mass Spectrometer

No. GCMS-1403

Analysis of Blood Alcohol by Headspace with Simultaneous GC-FID and MS Detection

■ Introduction

Determination of Blood Alcohol Content (BAC) has been a standard analytical method in criminal labs for many years. The typical instrument configuration consists of a static headspace instrument for sample introduction, followed by gas chromatography (GC) with two dissimilar capillary columns for separation, and two Flame Ionization Detectors (FIDs) for detection and quantitation. Two sets of data are obtained simultaneously, and the quantitative results from the two FIDs are compared for confirmation of the reported BAC levels.

With the BAC method, compound identification is done by comparing the retention time (RT) of blood alcohol in the unknown sample to the RT obtained from analysis of an analytical standard. Recently however, additional compound identification provided by matching the ethanol mass spectrum to a library spectrum, in addition to RT, has proven to offer an additional level of confirmation. This application note describes BAC analysis using a GC-FID in parallel with a mass spectrometer (MS) for positive compound identification.

■ Experimental

Instrument Configuration

The Shimadzu HS-20 Loop headspace sampler (Figure 1) was used in the static-loop headspace mode for sample introduction. Effluent from the HS-20 was split 20-to-1, and then divided to two identical columns using a 3-way "T" fitting. The outlet ends of the two columns were connected to the FID and MS detectors. Because the MS detector was under vacuum, RTs for the two columns were different and the exact split ratio between the FID and the MS was not determined. Instrument configuration and operating parameters are outlined in Table 1.

Figure 1: Shimadzu HS-20 Loop Headspace Sampler with GCMS-QP2010 SE

Table 1: Instrument Operating Conditions and Method Parameters

Head Space	**HS-20 Loop Model**
Operation Mode	Static headspace with loop
Sample	1-mL sample volume 10-mL headspace vial
Equilibration	15 minutes at 65 °C Agitation level 3 (of 9 levels)
Sample Loop	1-mL loop Vial pressurization 0.5 min, equilibration 0.1 min Loop load time 0.5 min, equilibration 0.1 min Injection time 0.5 min
Sample Pathway Temperature	150 °C
Transfer Line Temperature	150 °C

Gas Chromatograph	**GC-2010 Plus**
Injection	Split injection from HS-20, with 20:1 split ratio to inlet side of SGE SilFlow pre-column splitter ("T" fitting) Nominal 50:50 division to two capillary columns
Column	Pre-column "T" fitting splitter to two columns Rtx-BAC1, 30 m x 0.32 mm x 1.8 µm film (x2) Helium carrier gas Constant linear velocity, 40 cm/second (each column)
Oven Program	Isothermal at 40 °C Total GC run time 5.0 minutes Total cycle time 6.0 minutes

Detector #1	**GCMS-QP2010 SE**
Operating Mode	Scan mode 30-150 m/z
Ion Source	200 °C, EI mode, 70 eV
Solvent Cut Time	0.9 min
MS Interface	200 °C
Detector #2	**Flame Ionization Detector**
FID Temperature	240 °C
FID Gas Flow Rates	H_2 = 40 mL/min Air = 400 mL/min Makeup (He) = 30 mL/min

Sample Preparation

Forensic ethanol solutions were purchased commercially with concentrations of 0.01, 0.05, 0.2, and 0.4 g/dL. An internal standard (IS) solution of n-propanol was prepared at 0.2 g/dL in TOC-grade water. Finally, a control standard (CS) was prepared by mixing methanol, ethanol, acetone, and isopropanol in TOC-grade water at 0.05 g/dL. Aliquots for analyses were prepared by mixing 1.0 mL of the IS solution with 100 µL of the individual calibration or control standard in a 10-mL headspace vial, and sealing immediately with a crimper prior to analysis.

■ Results and Discussion

Chromatography

The FID was at atmosphere and the MS was under vacuum, so the Retention Times (RT) for the 4 target compounds were different in the two chromatograms. The different RTs are inconsequential, since all compounds were individually calibrated on each of the two detectors, and RTs using the standard procedure (i.e., dissimilar columns and two FIDs) would also have been different. No effort was made to adjust the RTs for this project, but this can be done quite easily by adding a restriction to the outlet of the FID column. The FID and MS chromatograms are shown in Figure 2 with the target compounds and internal standard labeled.

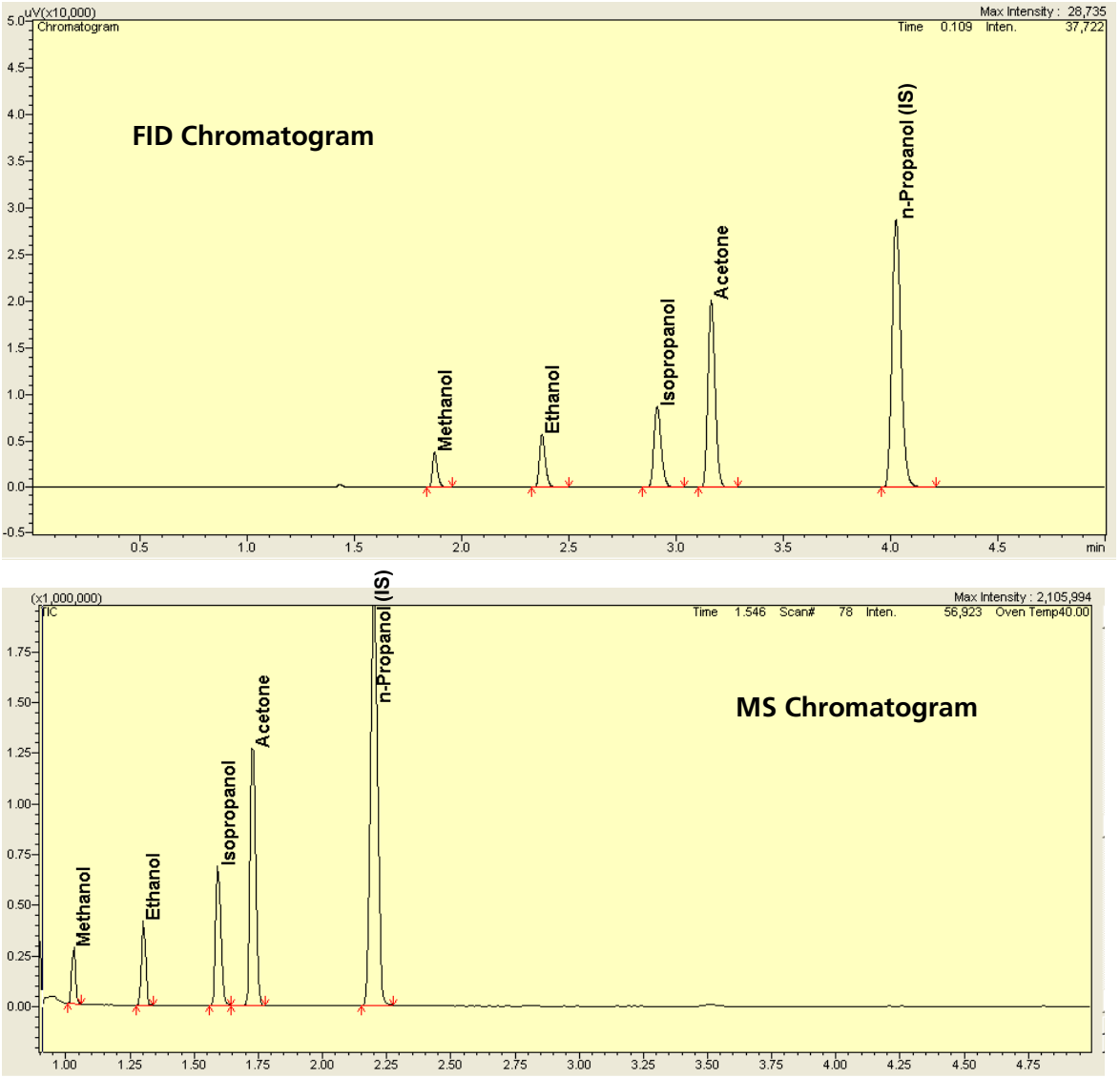

Figure 2: Chromatograms from the FID and MS with Compound Peaks Labeled

Ethanol Confirmation

Identity of the ethanol was confirmed in the MS chromatogram by matching the mass spectrum for the ethanol peak to the standard spectrum in the NIST Library. In all cases the identity of ethanol was confirmed through library matching with a similarity index of 98 or better. Figure 3 illustrates the NIST Library matching and confirmation of ethanol.

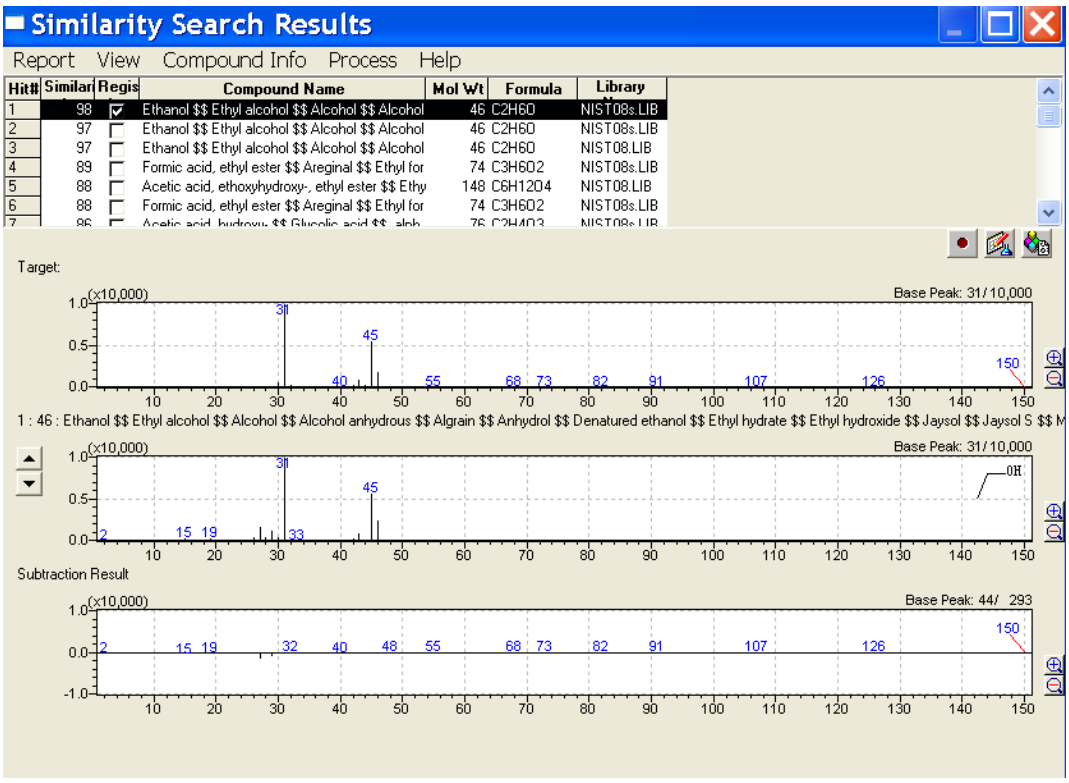

Figure 3: Mass Spectral Library Search Using the NIST11 Library to Confirm the Identity of Ethanol

Calibration

A 4-point calibration curve was generated by analyzing 3 individual aliquots at each calibration level. Data were collected on both the FID and the MS, and individual curves plotted using the internal standard technique. Calibration curves were created using the average of the data collected for the 3 individual standards at each concentration level. Figure 4 is the plotted calibration curves for ethanol on the FID and MS detectors. Table 2 shows the linearity for all 4 compounds in the FID and MS detectors.

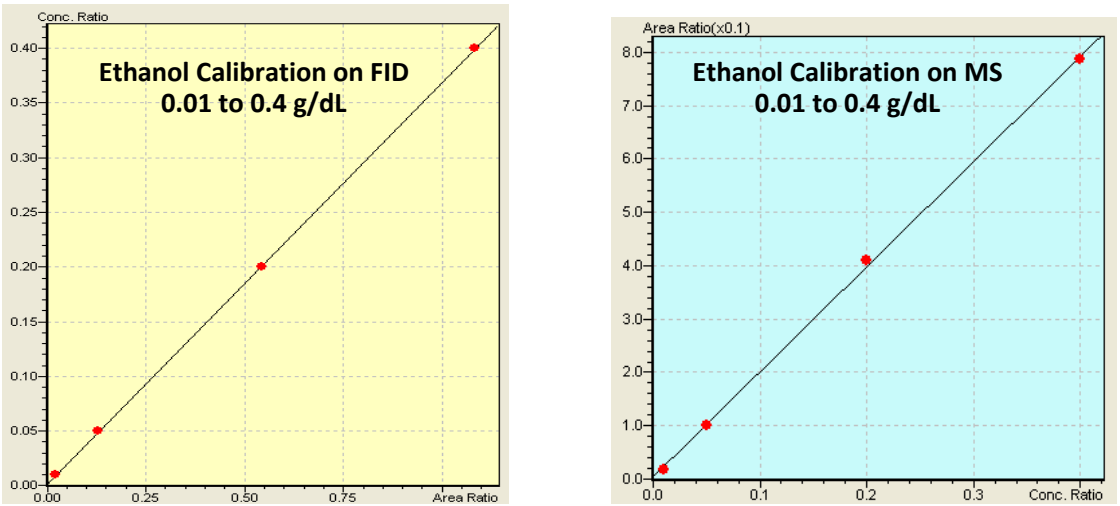

Figure 4: Calibration Curves for Ethanol on the FID and MS Detectors

Table 2: Linearity of Calibration Compounds on the FID and MS Detectors over Range of 0.01 to 0.4 g/dL

Compound	R^2 on FID	R^2 on MS
Methanol	0.9999	0.9995
Ethanol	0.9999	0.9998
Isopropanol	0.9999	0.9991
Acetone	0.9999	0.9992

Precision

Six replicate aliquots of the CS (0.05 g/dL) were prepared and analyzed using the conditions outlined in Table 1 to measure the analytical precision of the system. Overlaid chromatograms from the FID and MS are shown in Figure 5. Table 3 lists the precision results for all 4 target compounds.

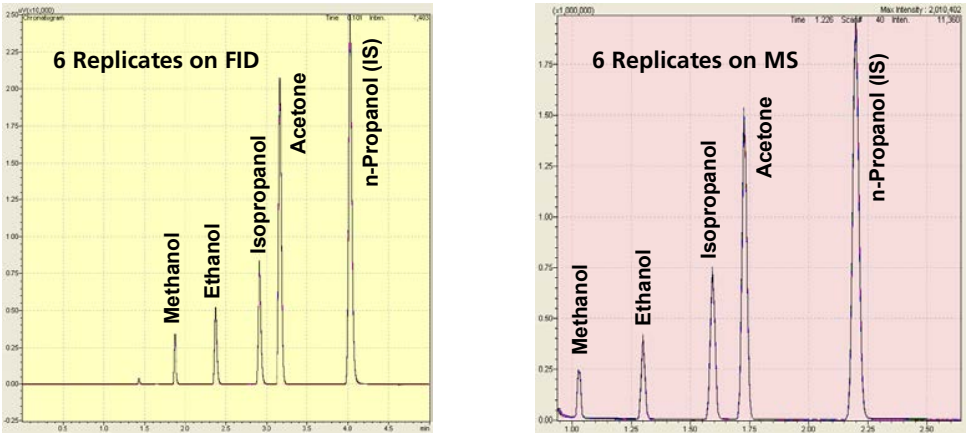

Figure 5: Overlaid Chromatograms from 6 Replicate Analyses of the Control Standard Run on the FID and the MS

Table 3: Precision Results for 6 Replicate Analyses of the Control Standard at 0.05 g/dL

Compound	RSD on FID (n = 6)	RSD on MS (n = 6)
Methanol	1.6%	1.0%
Ethanol	1.4%	0.9%
Isopropanol	1.1%	1.5%
Acetone	0.8%	1.7%

■ Summary and Conclusions

When a mass spectrometer is used in parallel with a GC-FID for analysis of blood alcohol content, the additional compound identification provided by matching the alcohol mass spectrum to an industry-standard library spectrum provides unambiguous, defensible confirmation of the ethanol. Calibration over the target concentration range is linear on both detectors, and precision is demonstrated below 2% for analysis of six replicate standards at the concentration range of interest.

First Edition: January 2014

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Application News

Gas Chromatograph Mass Spectrometer

No. GCMS-1401

Analysis of Opioids Using Isotope Dilution with GCMS-TQ8030 GC/MS/MS

■ Introduction

Development of methods for analysis of drugs of abuse has become a high priority for both forensic toxicology and law enforcement. The large numbers of individual drugs and new "designer drugs" has made method development for these compounds a significant undertaking.

Gas chromatography mass spectrometry (GCMS) has been used extensively for analysis of drug residues and trace-level drugs in biological fluids. The most significant challenges have been matrix interference and achievement of meaningful detection limits for the compounds of interest. Triple quadrupole GC/MS/MS has emerged as a powerful technique for trace-level analysis in these complex biological matrices. Operation of the triple quadrupole GC/MS/MS in the Multiple Reaction Monitoring (MRM) mode provides exceptional sensitivity, selectivity, and specificity for detection and quantitation of targeted drugs in the presence of background interferences.

The isotope dilution technique, using isotopically-labeled analogs of target compounds as internal standards, is a widely used analytical approach for precise quantitation in drug assays. However, in many cases, when using deuterium-labeled analogs the mass spectra differ only slightly from the corresponding unlabeled compounds. The challenge is complicated when the native and labeled compounds completely or partially co-elute, as they often do, and the spectra overlap. Combining the specificity of unique MRM transitions for close-eluting native and labeled analogues, with the sensitivity of triple quadrupole MRM transitions is a powerful technique for unambiguous, quantitative determination of this important compound class.

This application note presents instrument configuration, operating parameters, and analytical results for analysis of a common narcotic, hydrocodone, using the isotope dilution technique paired with the specificity of the MRM analysis mode of the Shimadzu GCMS-TQ8030 triple quadrupole GC/MS/MS (Figure 1). Internal standard calibration of codeine and oxycodone was also included in the study.

Figure 1: Shimadzu GCMS-TQ8030 Triple Quadrupole GC/MS/MS

■ Experimental

The analyses were conducted using a Shimadzu GCMS-TQ8030 triple quadrupole GC/MS/MS operated in the multiple reaction monitoring (MRM) mode, with optimized collision energy (CE) for each MRM transition providing ultimate sensitivity. The instrument configuration and operating conditions are shown in Table 1.

Table 1: Instrument Configuration and Operating Conditions for Analysis of Opioid Drugs

Instrument	GCMS-TQ8030
Inlet	270 °C Splitless liner with glass wool (Shimadzu 221-48876-02) Splitless injection, sampling time 1 minute
Column	RXI-5MS, 30 m x 0.25 mm x 0.25 µm (Restek 13423) Helium carrier gas Constant linear velocity 37 cm/second
Oven Program	100 °C, hold 1.0 minute 20 °C/minute to 250 °C, hold 3.0 minutes 10 °C/minute to 300 °C, (no final hold) MS interface 250 °C Analysis time 20 minutes
Ion Source	200 °C Electron ionization (EI) mode, 70 eV
Operation Mode	Multiple Reaction Monitoring (MRM) Argon gas, 200 kPa
Detector	Electron multiplier 1.0 kV

Six MRM transitions were selected for both hydrocodone-d_3 and hydrocodone, most of which had unique precursor ions paired with common product ions. (Refer to Table 2.) This approach allowed evaluation of any potential mass spectral interference, or cross-talk, between the transition pairs of these two co-eluting compounds. Three transitions were selected for codeine and oxycodone, since they were chromatographically resolved from the other compounds, and there were no isotopically labeled internal standards used.

Table 2: MRM Transition Details with Optimized Collision Energies (CE)

Compound	Transition #1 (CE)	Transition #2 (CE)	Transition #3 (CE)	Transition #4 (CE)	Transition #5 (CE)	Transition #6 (CE)
Hydrocodone-d_3 (IS)	302 > 242 (11V)	302 > 214 (19V)	302 > 185 (27V)	302 > 273 (19V)	302 >245 (27V)	302 > 231 (27V)
Hydrocodone	299 > 242 (11V)	299 > 214 (19V)	299 > 185 (27V)	299 > 270 (19V)	299 > 242 (23V)	299 > 228 (23V)
Codeine	299 > 162 (11V)	299 > 229 (19V)	299 > 280 (15V)			
Oxycodone	315 > 258 (11V)	315 > 230 (19V)	315 > 201 (19V)			

Calibration standards were prepared in methanol, and data for a 5-point calibration were acquired over the range of 25-200 ng/mL (parts-per-billion, ppb). The calibration curve for hydrocodone was generated using the isotope dilution technique. The concentration of the internal standard, hydrocodone-d_3 was held constant at 100 ng/mL. The concentration range of the calibration was sufficient to satisfy the requirements of the specific application. The chromatographic conditions chosen were intended to fit into a larger scheme for analysis of numerous drug classes, so optimization of the chromatographic conditions for efficiency was not considered in this study.

■ Results and Discussion

Chromatography

The total ion chromatogram (TIC) acquired in the MRM mode for the opioid drug mix is shown in Figure 2. The chromatographic peaks for hydrocodone-d_3 and hydrocodone partially overlap, with the deuterium labeled analog eluting first. In the MRM mode, the TIC is the sum of the signal for each MRM transition for that particular analyte, so the appearance of the chromatogram is slightly different than the typical TIC chromatogram from full scan analysis.

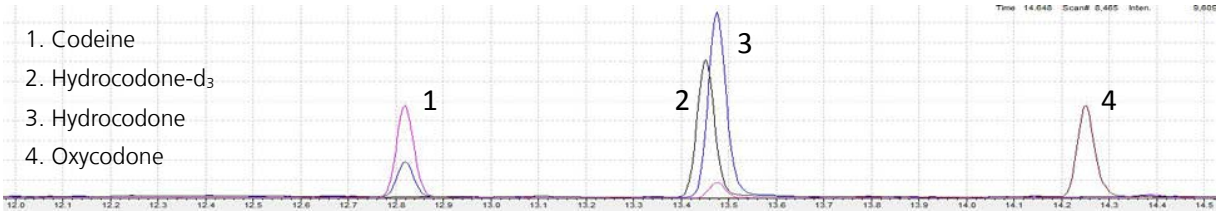


Figure 2: Total Ion Chromatogram (TIC) of Opioid Standard

Mass Spectral Results in the Full Scan ("Q3 Scan") Mode

The full scan mass spectra of hydrocodone-d_3 and hydrocodone are shown in Figures 3A and 3B. Notable features of these mass spectra are the prominent molecular ions for the labeled and unlabeled compounds at *m/z* 302 and 299, respectively, with the difference of 3 *m/z* units associated with the isotopically labeled n-methyl group on hydrocodone-d_3.

Common fragment ions are present in both spectra at *m/z* 242, 214, 199, 185, and 115 (indicated with an ↓ in figures 3A and 3B). These fragments represent loss of a fragment which includes the labeled n-methyl group from hydrocodone-d_3, and the corresponding unlabeled n-methyl group from hydrocodone, to form identical fragment ions from the two compounds.

Fragment ion pairs in the spectra for the labeled/unlabeled compounds can be seen at *m/z* 287 and 284, 273 and 270, 231 and 228, 99 and 96, 62 and 59 (indicated with an * in figures 3A and 3B). In this case, the corresponding fragments are offset by a difference of 3 *m/z* units (e.g. 287 and 284), and represent the loss of the same non-labeled group from hydrocodone-d_3 and hydrocodone, respectively.

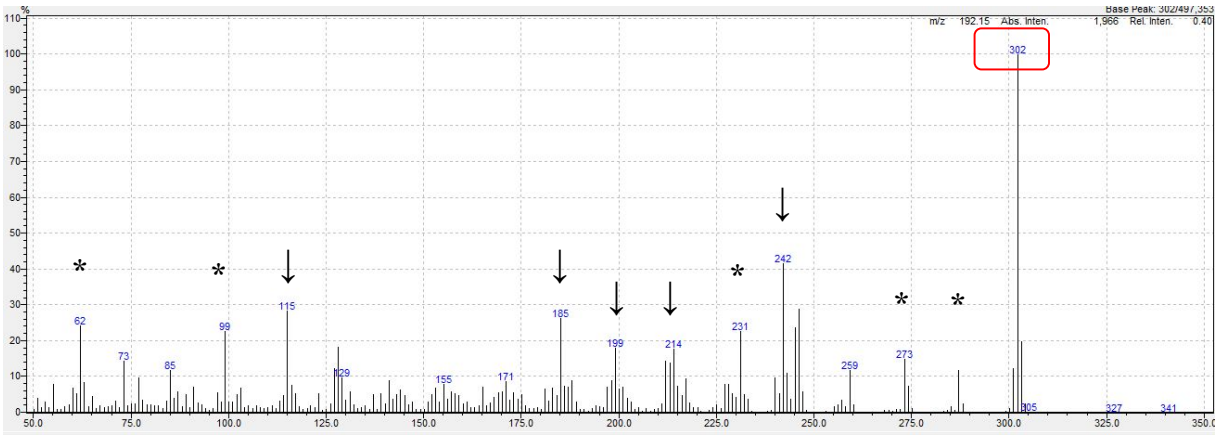


Figure 3A: Total Ion Mass Spectrum of Hydrocodone-d_3

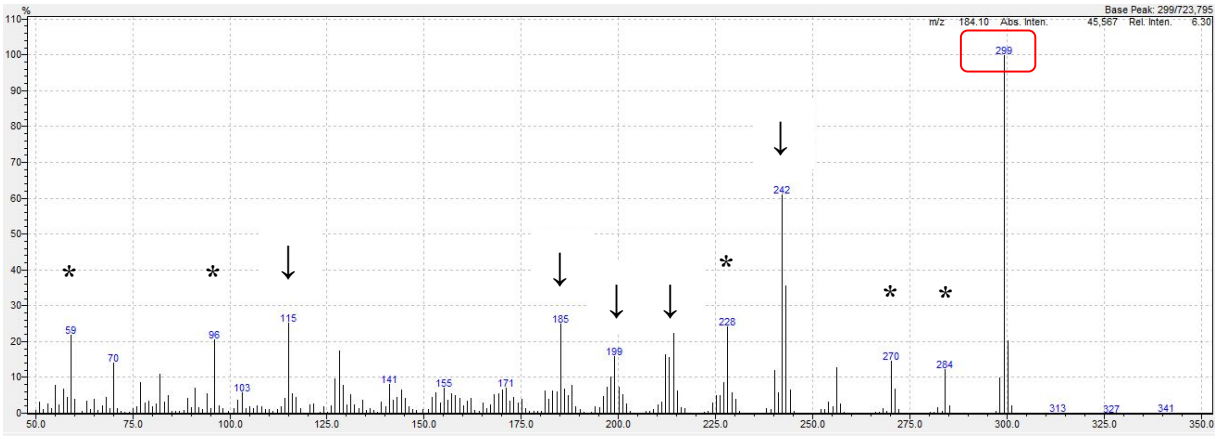


Figure 3B: Total Ion Mass Spectrum of Hydrocodone

The full scan spectra of hydrocodone-d_3 and hydrocodone were used to select precursor and product ions for the MRM transitions. Three transitions were selected for each compound based on their unique molecular ions and common product ions (e.g. 302 → 242 and 299 → 242). To illustrate the unique specificity of the MRM mode, a second set of three transitions was defined using the molecular ions and unique product ions for hydrocodone-d_3 and hydrocodone (e.g. 302 → 273 and 299 → 270). The ions selected for MRM transitions are tabulated in Table 2 above.

Mass Spectral Results in the Multiple Reaction Monitoring (MRM) Mode

Operation of the GCMS-TQ8030 in the MRM mode provides enhanced selectivity for analysis of trace-level contaminants in complex matrices, such as drugs of abuse in biological fluids, because the co-extracted matrix interferences are significantly minimized. The compound specificity that can be achieved by using unique MRM transitions for each compound, even when they have common product ions, is illustrated in Figure 4. Figure 4 includes six overlaid MRM chromatograms for hydrocodone-d_3 and six for hydrocodone, as described above. Note that the chromatograms corresponding to the MRM transitions for hydrocodone-d_3 and hydrocodone are uniquely defined for each of the analytes and do not interfere with one another, even for the three transitions that have common MRM product ions. The non-interfering chromatograms illustrate the power of the MRM mode, and the specificity that can be achieved when unique transitions are selected for close-eluting compounds with similar mass spectra.

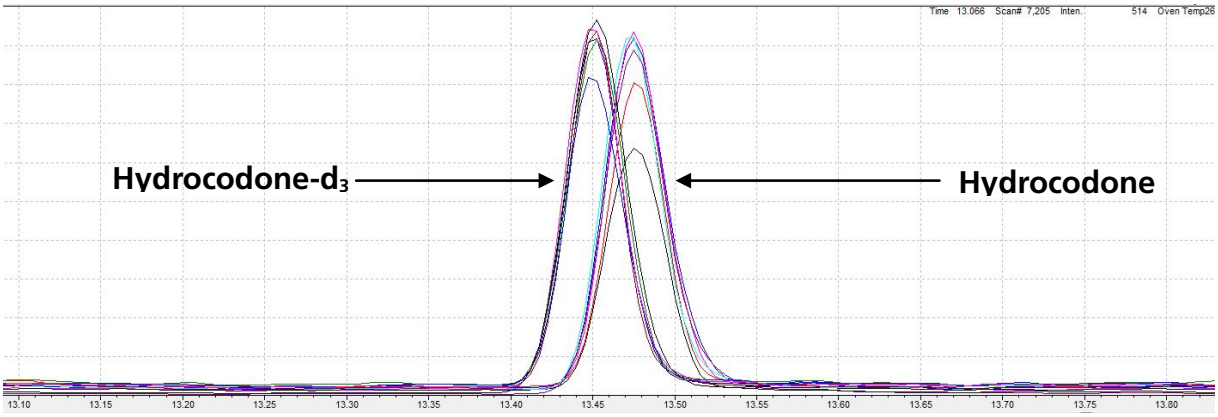


Figure 4: Six Overlaid MRM Chromatograms for Hydrocodone-d_3 and Six for Hydrocodone

Cross-Talk

"Cross-talk" is a phenomenon unique to triple quadrupole mass spectrometry. It occurs when residual ion fragments are not fully swept from the collision cell at the end of a cycle; they remain in the collision cell and are detected as "ghost fragments" in subsequent transitions. Cross-talk is depicted graphically in Figures 5A and 5B below. Figure 5A depicts slowing down of product ions in the collision cell, which results from interactions with the CID gas. In some cases, a small portion of the residual product ions have slowed down, and may not be completely swept from the collision cell during the transition, resulting in cross-talk. Figure 5B illustrates the results of cross-talk as "ghost" mass spectral fragment peaks that can appear in subsequent transitions.

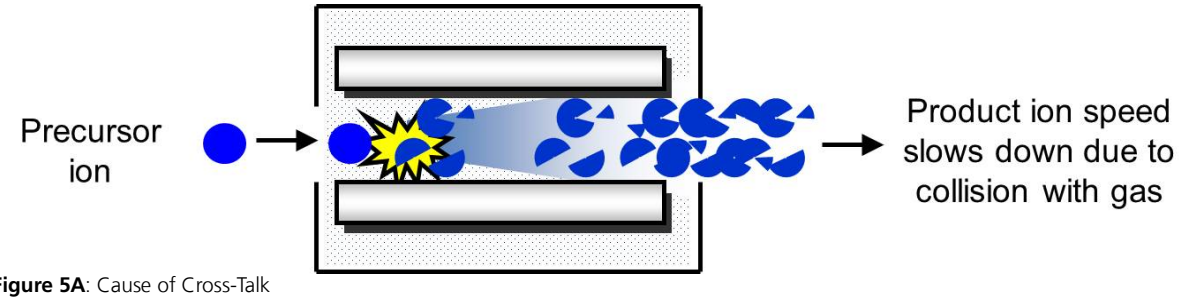


Figure 5A: Cause of Cross-Talk

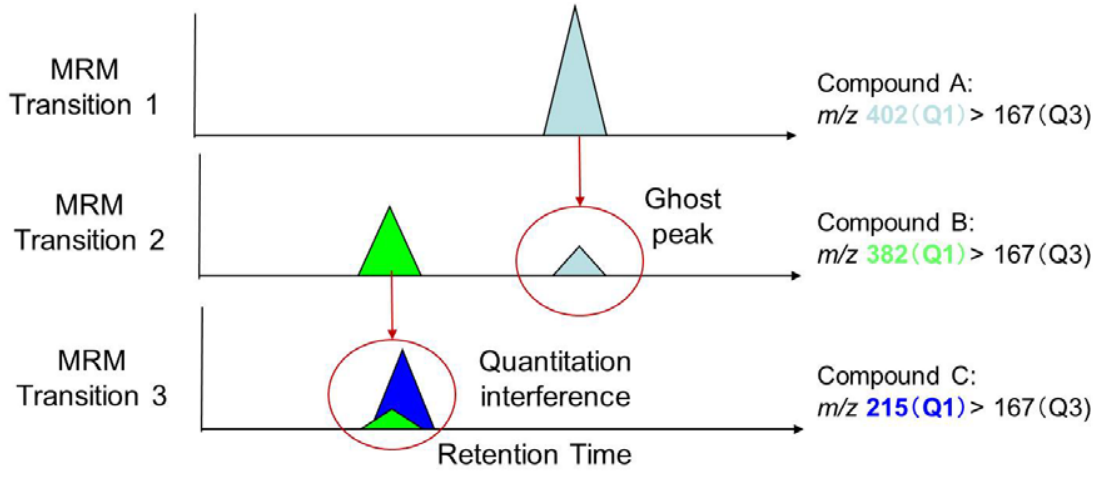
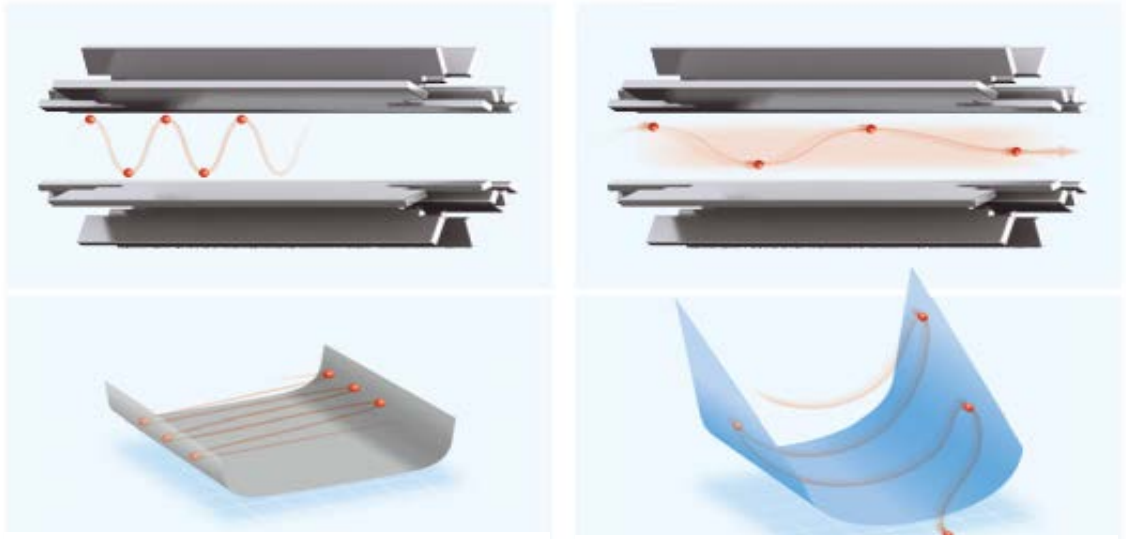


Figure 5B: Results of Cross-Talk

Cross-talk is virtually eliminated in the GCMS-TQ8030 using UFsweeper® technology. The UFSweeper design alters the pseudo-potential surface within the collision cell, shortening the path which ions must travel and accelerating them through the cell and toward Q3. This process completely clears the collision cell with each transition, and eliminates cross-talk from one transition to the next. The pseudo-potential surface of the GCMS-TQ8030 UFsweeper® technology is illustrated in Figure 6 below. The overlaid chromatograms in Figure 4 clearly show that there was no indication of cross-talk present.



With conventional technology, ions lose momentum and have a longer travel path due to collision with CID gas

The UFSweeper® pseudo-potential surface shortens the pathway and accelerates ions through the collision cell without losing momentum

Figure 6: Graphic Depiction of UFSweeper® Technology

Calibration Results

Five calibration standards were prepared for the opioids over the range of 25-500 ng/mL (ppb) and transferred to autosampler vials with limited-volume inserts for analysis; hydrocodone-d_3 was used as the the internal standard and was held at a constant concentration of 100 ng/mL. The calibration standards were analyzed using the instrument conditions outlined above. The electron multiplier was adjusted to give acceptable response at the lowest calibration level and avoid saturation at the highest calibration level.

Response factors were calculated and percent relative standard deviation (%RSD) determined using the GCMSsolution software. The precision of the calibration is evaluated using the %RSD of the response factors and the correlation coefficient (r) for each of the calibration analytes. The %RSD and correlation coefficient values for the multi-point calibration are shown in Table 3. The linear, multi-point calibration curve for hydrocodone is illustrated in Figure 7. Calibration results demonstrate linearity for each of the analytes.

Table 3: Results of the 5-Point Calibration for Three Opioids From 25 to 200 ng/mL using the MRM Analysis Mode

Compound	Calibration Type	Mean RF	RF %RSD	r
Codeine	Internal Standard	0.643	12.1	0.9995
Hydrocodone	Isotope Dilution	1.011	15.6	0.9999
Oxycodone	Internal Standard	0.376	14.8	0.9999

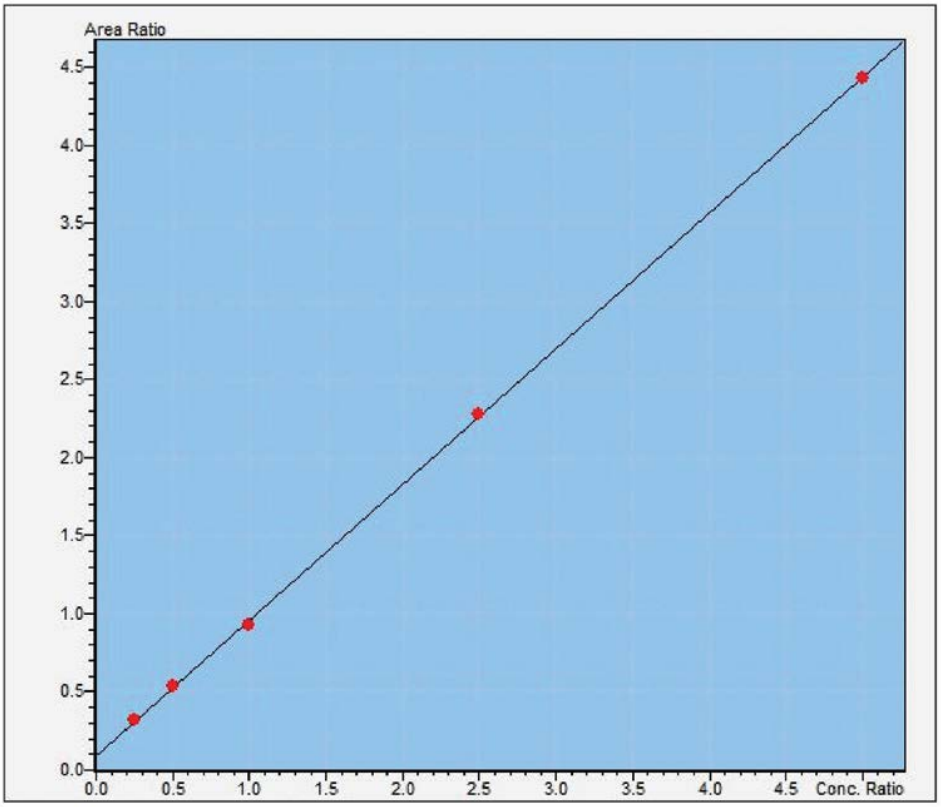


Figure 7: Linear, Multi-point Calibration for Hydrocodone from 25 to 200 ng/mL

■ Summary and Conclusion

The results demonstrate the power and specificity of the MRM analysis mode when using unique transitions for close-eluting compounds such as hydrocodone-d_3 and hydrocodone, even when they have similar mass spectra and common product ions. This experiment also illustrates the effectiveness of the Shimadzu GCMS-TQ8030 fast scanning and UFsweeper technologies for completely clearing the collision cell with each transition and eliminating any cross-talk. The multi-point calibration for hydrocodone was linear and passes thru zero, further supporting that there was no interference from cross-talk or the close-eluting deuterium-labeled internal standard.

■ Acknowledgement

The authors wish to acknowledge the collaboration of chemists from the Niagara County Sherriff's Department Laboratory, Lockport, NY for suggesting the experiment and for providing analytical standards described in this application note.

First Edition: January 2014

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Technical Report

Automatic Identification and Semi-quantitative Analysis of Psychotropic Drugs in Serum Using "GC/MS Forensic Toxicological Database"

Hitoshi Tsuchihashi[1]

Abstract:

A sample consisting of serum extract from a patient administered with psychotropic drugs was analyzed by GC/MS; identification and semi-quantitation of the substances were conducted using the "GC/MS Forensic Toxicological Database". All three administered substances were identified, and their semi-quantitative values were calculated. The results indicated that it is possible to qualitatively and semi-quantitatively determine drugs using this database.

Keywords: GC/MS, psychotropic drug, phenobarbital, semi-quantitation, forensic medicine, toxicology

Introduction

Accidental poisoning due to the abuse and excessive intake of stimulants and other illegal drugs or psychotropic drugs continues to be a troublesome social issue. In particular, when death occurs due to acute drug poisoning, identifying the specific drug responsible and determining its concentration in the blood are essential subjects in university forensic classrooms and critical activities in prefectural police department forensic laboratories.

Identification and quantitative analysis of a drug substance in the blood can be very time-consuming, requiring a calibration curve for verification of retention times using a standard drug sample, and determination of the appropriate preparation and pretreatment of the actual sample. In addition to the automatic search algorithm built into the GC/MS Forensic Toxicological Database, a semi-quantitative analysis feature is also included which uses relative response factors for drugs that often lead to poisoning. These features allow identification of drugs for which standard samples are difficult to obtain, and evaluation based on estimated quantitation values (via semi-quantitation) for these substances.

In this Technical Report, we utilized the substance identification and semi-quantitation features included in the GC/MS Forensic Toxicological Database to identify 3 substances, including the barbiturate, Phenobarbital (used to treat epilepsy), the antipsychotic, chlorpromazine, and the antihistamine antiemetic, promethazine (used to treat Parkinson's disease), in a spiked sample of actual serum. In addition, we verified the results using a serum sample from an actual patient administered these 3 substances.

Experiment

Reagents

The phenobarbital sodium, chlorpromazine hydrochloride, and promethazine hydrochloride were obtained from Wako Chemicals, and each was adjusted to a free-state concentration of 10 mg/mL (by using methanol for phenobarbital sodium and distilled water for chlorpromazine hydrochloride and promethazine hydrochloride). After preparing a mixed solution of these, each at a concentration of 100 µg/mL, the mixture was added to blank serum, and the concentration was adjusted to 10 µg/mL. This was then used as the analytical sample (spiked serum). In addition, after receiving informed consent from a psychiatric patient who had been administered these 3 substances, we used the received blood serum as the actual sample. In addition, two custom standard solutions were obtained from Shimadzu GLC, one an n-alkane C7 – C33 sample (Custom Retention Time Index Standard, Restek Corp.) for retention time correction, and the other an internal standard (Custom Internal Standard, Restek Corp.) sample necessary for the semi-quantitation.

1 Department of Legal Medicine, Osaka Medical College

Pretreatment Procedure

Into 500 µL of each serum sample (spiked serum and actual sample), 20 µL of 10% hydrochloric acid was titrated to acidify, 1000 µL of a chloroform-isopropanol mixture (3:1, v/v) was added, and after vigorously mixing, centrifuging was conducted for 15 minutes, and the organic layer (acidic fraction) was collected. After conducting this operation twice in succession, 20 µL of 28% aqueous ammonia was added to the aqueous layer to make the mixture basic. Then, 1000 µL of a chloroform-isopropanol mixture (3:1, v/v) was added, and after conducting the mixing / centrifuging process described above, the organic layer (basic fraction) was collected.

After combining the acidic and basic fractions, dewatering of the mixture was conducted using anhydrous sodium sulfate, and evaporative drying was performed at 40°C under nitrogen. The obtained residue was dissolved in 250 µL ethyl acetate, and this was used as the sample for GC/MS analysis.

Equipment

For the GC/MS analysis, the GCMS-QP2010 Ultra was used, and GCMSsolution software was used for data processing. Table 1 shows the analytical conditions that were used for the analyses. For retention time adjustment, the AART (Automatic Adjustment of Retention Time) feature included in GCMSsolution Postrun Analysis was used to calculate the retention times of the 162 substances (included in the free-state substance analytical method for psychiatric drugs) from the retention indices, and these were used as the standard retention times for identification. The retention time windows were set to ±0.2 minutes, and the substances included in the samples were identified using the automatic identification feature. In addition, the internal standard used for quantitation was introduced automatically into the GC injection port simultaneously with the sample using the internal standard automatic addition feature of the AOC-20i+s.

Table 1 Analytical Conditions

Instruments	
GC-MS	: GCMS-QP2010 Ultra
Auto-injector	: AOC-20i + s
Column	: Rxi®-5Sil MS (30 m × 0.25 mm I.D. df=0.25 µm, Restek Corporation)
GC condition	
Column Temp.	: 60°C (1 min)-10°C/min-320°C (10 min)
Carrier Gas	: He (Constant Linear Velocity Mode)
Carrier Gas Velocity	: 45.6 cm/sec
Injection Mode	: Splitless
Sample injection volume	: 1 µL
IS injection volume	: 1 µL
MS condition	
Interface Temp.	: 280°C
Ion Source Temp.	: 200°C
Scan Interval	: 0.3 sec
Monitor ion for semi-quantitation	: *m/z* 204 for phenobarbital
	: *m/z* 318 for chrolpromazine
	: *m/z* 72 for promethazine

Results and Discussion

Semi-Quantitation Results of Spiked Serum

The total ion current chromatogram (TIC) obtained from analysis of the spiked serum using the abovementioned procedure is shown in Fig. 1. the 3 added substances were detected within ±0.03 minutes of the expected calculated retention time, and accurate identification was achieved based on the retention times (Fig. 2). Since each substance is detected based on the extracted ion chromatogram (EIC) of multiple *m/z* values set beforehand, the EIC chromatogram can be detected even at trace concentrations or when a discrete chromatographic peak in the TIC cannot be detected due to matrix interferences. The detected chromatographic peak can be accurately identified automatically through confirmation of the degree of similarity with the standard mass spectrum (Fig. 3). In addition, the quantitation values were calculated from the obtained peak intensity ratio of each target substance and internal standard substance, and the relative response factor.

Thus, utilizing the automatic search feature of the GC/MS Forensic Toxicological Database, phenobarbital, chlorpromazine, and promethazine were all detected automatically, and semi-quantitation values were obtained for these 3 substances. The semi-quantitation values obtained using the semi-quantitation feature of the GC/MS Forensic Toxicological Database and the relative response factors (obtained from analysis of the standard solution) are shown in Table 2.

As shown in Table 2, fairly good quantitative results were obtained for chlorpromazine and promethazine, but the quantitative results for phenobarbital indicate a value 1.8 times that of the added amount. The semi-quantitation feature of the GC/MS Forensic Toxicological Database generates a semi-quantitation value which is a rough estimate of "the drug concentration in the final sample" based on the response factor obtained from the previously-analyzed standard sample. Therefore, the semi-quantitation value can vary considerably depending on the drug recovery ratio and sample concentration during sample pretreatment, as well as the matrix effect. Especially, in the case of a serum sample, the recovery of a drug with high lipid solubility will decrease greatly, increasing the likelihood that the difference between the obtained semi-quantitation value and the true value will widen considerably. Thus, since the calculated quantitation value can be expected to vary depending on the pretreatment procedure, the GC injection port, and the column condition, it is necessary to regard it only as an approximate estimated value. For quantitative analysis requiring great accuracy, standard samples must be used.

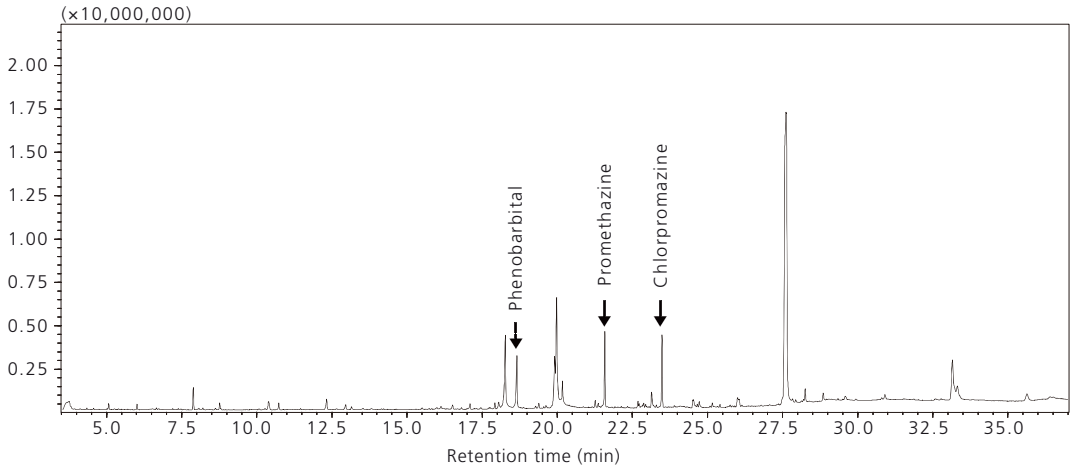


Fig. 1 Total Ion Current Chromatogram Obtained from Spiked Serum

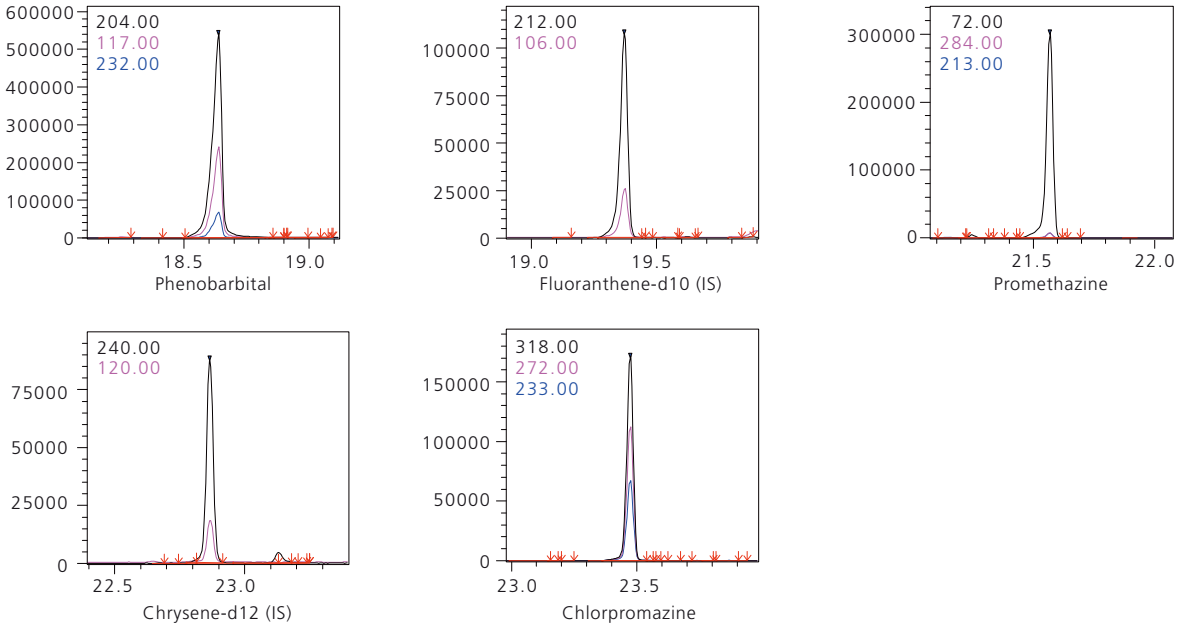


Fig. 2 Mass Chromatograms of 3 Added Substances and Principal Internal Standard Samples

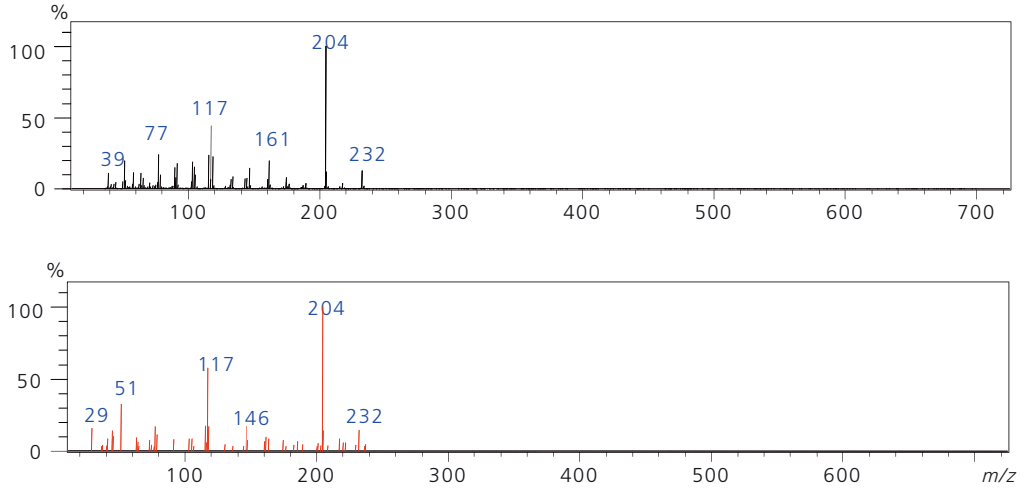


Fig. 3 Comparison of Measured Mass Spectrum of Phenobarbital (above) and Standard Mass Spectrum (below)

Table 2 Semi-Quantitated Results of Spiked Plasma

Compounds	Additive Amount (μg/mL)	Semi-quantitated (μg/mL)	Response Factor
Phenobarbital	10.0	17.6	0.175
Chlorpromazine	10.0	11.3	0.144
Promethazine	10.0	11.4	1.782

Semi-Quantitation Results for Actual Sample

In the case of the actual sample, just as with the spiked serum, the 3 substances were automatically detected by the automatic search algorithm, and the semi-quantitative results were obtained. The chromatogram obtained from analysis of the actual sample is shown in Fig. 4. Also, with respect to the actual sample, the quantitation values obtained using the internal standard method were compared with the semi-quantitation values calculated from the GC/MS Forensic Toxicological Database. Those data are shown in Table 3.

As with the spiked serum, the semi-quantitation values obtained for chlorpromazine and promethazine were relatively close to the values obtained from the calibration curve, but the semi-quantitation value for phenobarbital indicated a value that was about 3 times higher.

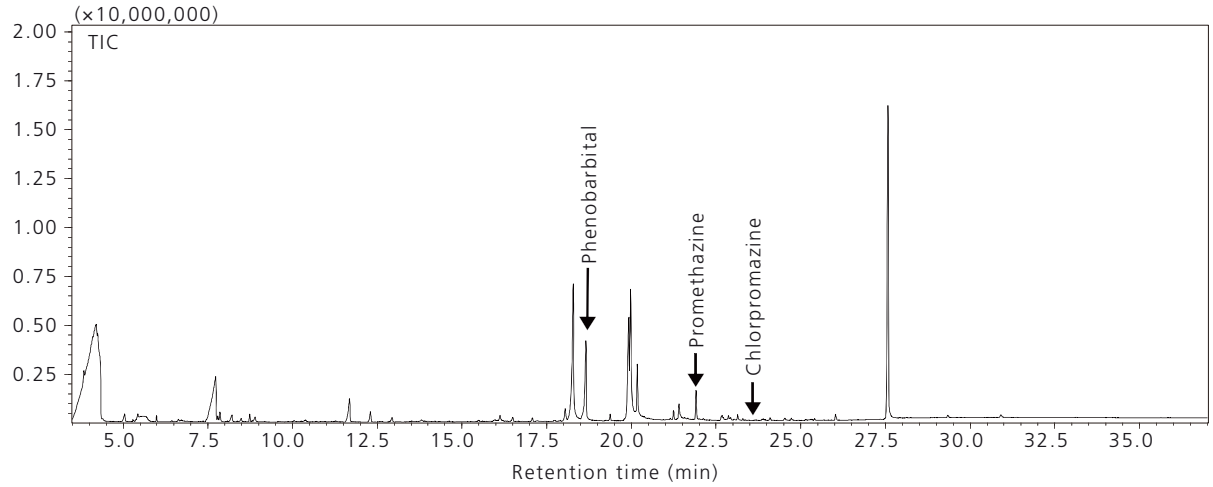


Fig. 4 Total Ion Current Chromatogram Obtained Using Actual Sample

Table 3 Comparison of Quantitated Results and Semi-Quantitated of Real Specimen

Compounds	Quantitation* (µg/mL)	Semi-Quantitation (µg/mL)
Phenobarbital	11.6	33.2
Chlorpromazine	0.03	0.02
Promethazine	0.13	0.15

* Determined from calibration curve generated using spiked serum.

Conclusion

Automatic qualitative and semi-quantitative analyses were conducted for 3 psychotropic drug substances (phenobarbital, chlorpromazine, and promethazine) in serum using the GC/MS Forensic Toxicological Database. Using a method which incorporated information on 162 psychiatric drugs, automatic identification of phenobarbital, chlorpromazine, and promethazine was possible using retention time correction via the AART feature of the GCMSsolution software. Relatively accurate quantitative results were obtained for chlorpromazine and promethazine, but the results obtained for phenobarbital tended to be 2 to 3 times higher than the spiked levels. Since the semi-quantitative values obtained using this feature of the GC/MS Forensic Toxicological Database are just quantitative estimates of the concentrations in the final sample, they should be considered as values subject to great variation, depending on such factors as the error in the drug recovery ratio or sample concentration in pretreatment, matrix effects, and instrument condition. However, since automatic quantitative analysis using this database can be conducted simultaneously with an automatic database search, it can certainly be useful for obtaining a rough estimate of a drug's concentration while conducting qualitative analysis, or for quickly estimating concentration of a specific drug when there is no time for preparing calibration standards.

Gas Chromatograph Mass Spectrometer

GCMS-QP2010 Ultra

Features

1. High sensitivity
2. Easy maintenance
3. Identification of compounds using retention indices

The Shimadzu GCMS-QP2010 series has optimum functions and performance for forensic toxicology.

1. The Shimadzu GCMS-QP2010 series features an extremely high sensitivity and capability to measure forensic toxicology-related compounds down to low concentrations.
2. Urine and blood samples contain lots of contaminants. When samples such as these are measured by GC/MS, contamination of the ion source becomes problematic.The GCMS-QP2010 series is less likely to become dirty, and, moreover, can be easily cleaned even if the ion source is contaminated.
3. It is difficult to obtain standard samples for forensic toxicology-related compounds. However, in the GC/MS Forensic Toxicological Database for the GCMS-QP2010 series, the information of more than 500 medicinal toxicants is registered to method files together with optimum analysis conditions.

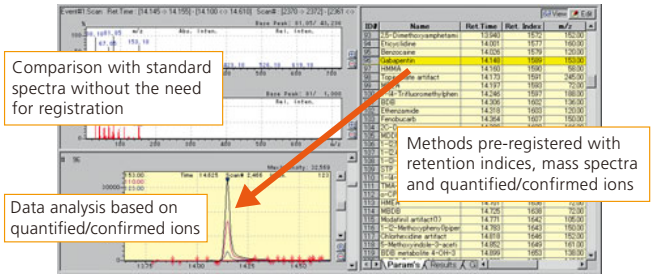
GC/MS Forensic Toxicological Database (Drugs of Abuse / Medicines / Pesticides)

The "GC/MS Forensic Toxicological Database" is exclusively for the GCMSsolution workstation software for GCMS-QP2010 series gas chromatograph mass spectrometers. It is pre-registered with 1011 mass spectra including free-, TMS- and TFA- body types for 502 compounds that are required in forensic toxicological analysis of drugs of abuse, drugs for psychiatric and neurological disease, and other medicines and pesticides.

This database comprises the following: method files pre-registered with analytical conditions, mass spectra, retention indices, etc., compound information including CAS numbers, etc., libraries containing mass spectra and retention indices, and a handbook (printed version of library information).

Spectra for 591 drugs of abuse, 274 drugs for psychiatric and neurological disease, 110 medicines, and 36 pesticides are registered to the methods and libraries.

Use of this database enables high-precision identification of compounds based on the AART (Automatic Adjustment of Retention Time) that uses retention indices, and based on mass chromatograms compared with standard mass spectra and quantified/confirmed ions.



First Edition: December, 2010
(Second Edition: June, 2012)

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Printed in Japan 3655-05218-10ANS

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Application Data Sheet

No.100

GC-MS
Gas Chromatograph Mass Spectrometer

Analysis of Toxicological Substances in Whole Blood Using Smart Forensic Database (1)

By providing mass separation in two stages, GC-MS/MS is capable of separating out interferences in biological samples and toxicological substances. Therefore, it is simple to determine whether toxicological substances are present, significantly reducing the time required for data analysis. In order to analyze toxicological substances in MRM mode, however, MRM transitions and collision energies (CE) must be optimized, which is very labor intensive.

Smart Forensic Database is an MRM database containing retention indices, MRM transitions, collision energies, and quantitation/confirmation ion ratios for 201 toxicological substances often involved in poisonings. The retention times for the registered toxicological substances are accurately estimated simultaneously from low-boiling point components to high-boiling point components, using measurement data from a standard n-alkane mixture via the GCMSsolution AART function. Smart MRM, which is provided with the GCMS-TQ8040, can then create MRM analysis methods automatically using the database.

This article introduces an example of applying Smart Forensic Database to the analysis of toxicological substances in a whole-blood sample.

Experimental

Liquid-liquid extraction via EXtrelut® NT3 was used to pretreat the whole-blood sample. The collected whole-blood sample was measured into 1 mL portions for acidic fractionation and basic fractionation, and each portion was diluted with 1 mL of Milli-Q water. The acidic fraction was adjusted to a pH 5 using 10 % hydrochloric acid, and the basic fraction was adjusted to a pH 9 using 10 % ammonia water. The respective solutions were added to the EXtrelut® NT3 columns and left to stand for 30 minutes, after which each was eluted with a 10 mL chloroform:isopropanol (3:1) mixture. The extracted solutions of acidic fraction and basic fraction were then mixed, and after dessication with silica gel and drying in a nitrogen airflow, the sample solution was re-dissolved in a 200 μL chloroform:isopropanol (3:1) mixture. To check the MRM sensitivity, the sample obtained was spiked with promethazine, phenobarbital, chlorpromazine, and triazolam so that the concentration of each compound becomes 50 ng/mL in whole blood.

Analytical Conditions

The conditions registered in Smart Forensic Database were used as the GC-MS/MS analysis conditions. For the compounds subject to MRM measurement, a simultaneous Scan/MRM analysis method was created, in which the 201 components registered in the database were set.

Table 1: Analytical Conditions

GC-MS:	GCMS-TQ8040
Column:	Rxi® -5SilMS (Length 30 m, 0.25 mm I.D., df=0.25 μm)
Glass liner:	Splitless insert with wool (PN: 221-48876-03)

[GC]	
Injection temp.:	260 °C
Column oven temp.:	60 °C (2 min) → (10 °C /min) → 320 °C (15 min)
Injection mode:	Splitless
Flow control mode:	Linear velocity (45.6 cm/sec)
Injection volume:	1 μL

[MS]	
Interface temp.:	280 °C
Ion source temp.:	200 °C
Acquisition mode:	Scan/MRM
Scan event time:	0.1 sec
Scan mass range:	*m/z* 43 – 600
Scan speed:	10,000 u/sec
MRM event time:	0.3 sec
Total loop time:	0.4 sec

Results

The extracted whole-blood sample was spiked with four toxicological substances so that the concentration of each substance becomes 50 ng/mL in whole blood, and then measured using Scan/MRM mode. Fig. 1 shows the mass chromatograms obtained, and Fig. 2 shows the repeatability obtained by repeating analyses five times. With the Scan mode analysis, confirmation ions were not detected, there was an overlap with cholesterol, and the peak for triazolam could not be confirmed. With the MRM mode, however, each component was clearly detected, and favorable repeatability results of 4.29 % max. were obtained.

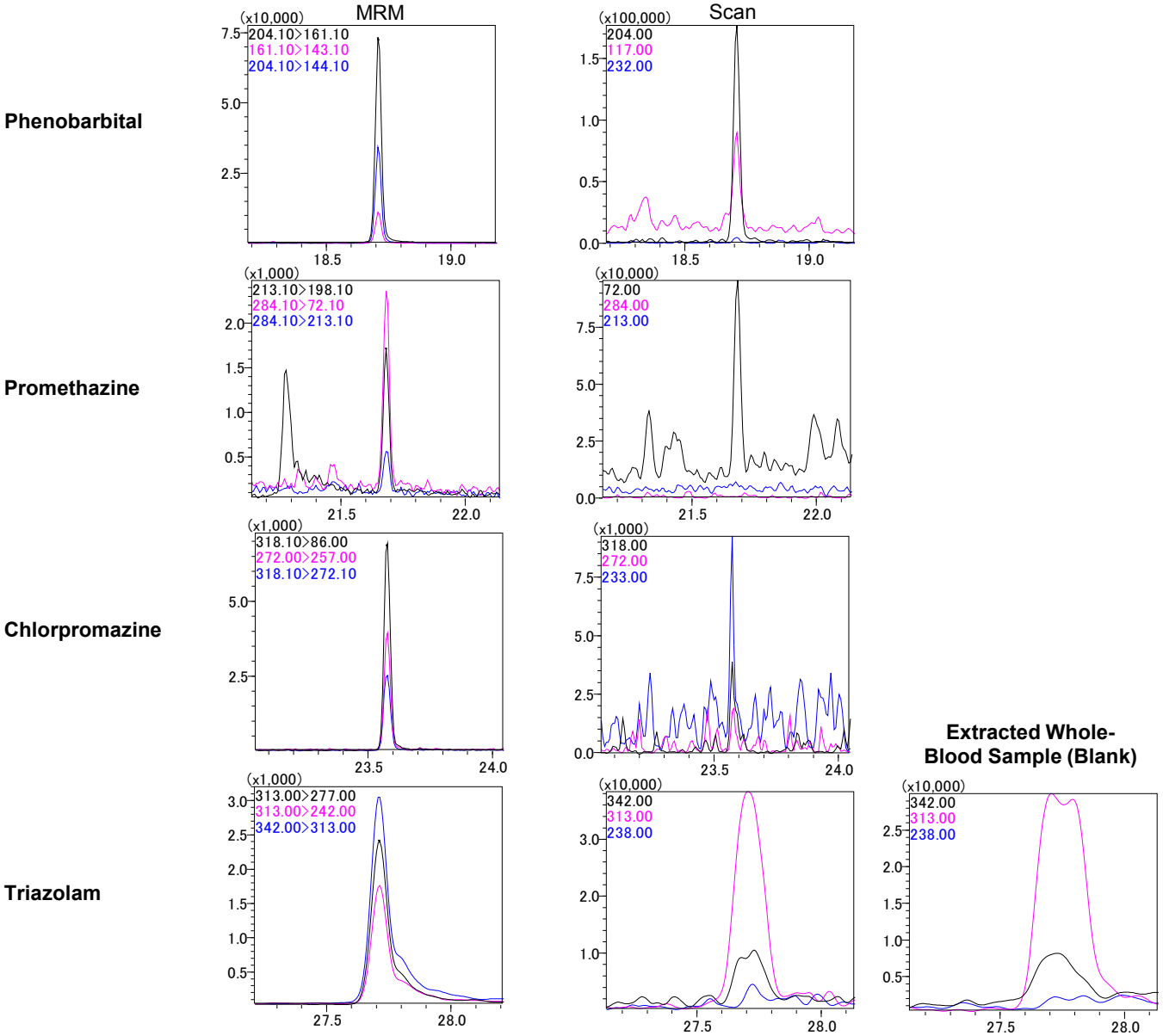

Fig. 1: Scan and MRM Mass Chromatograms for Four Toxicological Substances in Whole-Blood Samples (L: MRM; R: Scan)

Table 1: Area Repeatability at Five Replicates (Concentration in Whole Blood: 50 ng/mL)

	Data 1	Data 2	Data 3	Data 4	Data 5	Average	SD	%RSD
Phenobarbital	131,876	133,119	137,359	136,480	133,656	134,498	2323.7	1.73
Promethazine	2,756	2,873	2,742	2,885	2,829	2,817	65.7	2.33
Chlorpromazine	12,832	12,899	12,657	13,484	14,024	13,179	565.3	4.29
Triazolam	10,909	10,315	10,704	10,838	10,701	10,693	229.5	2.15

The data evaluated in this article was obtained from a sample that was spiked with the substances after extraction. There is no guarantee that a favorable recovery ratio will be obtained with the pretreatment method described above.

This data was provided by Associate Professor Kei Zaitsu in the Department of Legal Medicine & Bioethics, Nagoya University Graduate School of Medicine.

First Edition: September, 2014

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Application Data Sheet

No.104

GC-MS
Gas Chromatograph Mass Spectrometer

Multicomponent Analysis of Metabolites in Human Plasma using GC-MS/MS

The analysis of metabolomes, such as when searching for disease biomarkers, is performed in many areas in the medical field, whether it be for fundamental research or for clinical studies. Single quadrupole GC-MS provides excellent chromatographic resolution and enables stable measurements, and is therefore widely utilized for metabolome analyses involving the comprehensive analysis of in vivo metabolites. However, biological samples contain many metabolites and various matrices, so separation with single quadrupole GC-MS can be difficult. With triple quadrupole GC-MS/MS MRM, MS separation is performed twice, with Q1 and Q3. This helps remove the impact of overlapping peaks due to interfering components in comparison with scan mode and SIM mode, in which MS separation is performed with a single quadrupole, and thus enables the acquisition of accurate quantitative results with high-sensitivity detection.
Smart Metabolites Database registers MRM information of 475 metabolites mainly contained in biological samples such as blood, urine and cells. It enables simultaneous measurement of 475 metabolites using MRM mode. This application data sheet presents an analysis of metabolites in standard human plasma using the scan and MRM methods included in the Smart Metabolites Database, as well as a comparison of the results.

Analysis Conditions

In the pretreatment process, 2-isopropylmalic acid was added as an internal standard to 50 μL of standard human plasma, after which metabolites were extracted with a methanol/water/chloroform (2.5:1:1) solution. Methoxime and trimethylsilyl derivatives were then formed to obtain the samples[1]. The respective samples were measured each in scan and MRM modes using methods included in the Smart Metabolites Database. Table 1 shows the analysis conditions.

Table 1: Analysis Conditions

GC-MS:	GCMS-TQ8040		
Column:	BPX-5 (Length 30 m; 0.25 mm I.D.; df = 0.25 μm) (SGE, P/N:054101)		
Glass insert:	Split insert with wool (P/N: 225-20803-01)	[MS]	
		Interface temp.:	280 °C
[GC]		Ion source temp.:	200 °C
Sample injection unit temp.:	250 °C	Measurement mode:	Scan
Column oven temp.:	60 °C (2 min) → (15 °C/min) → 330 °C (3 min)	Mass range:	*m/z* 45-600
Injection mode:	Split	Event time:	0.2 sec
Split ratio:	30		
Carrier gas control:	Linear velocity (39.0 cm/sec)	Measurement mode:	MRM
Injection volume:	1 μL	Loop time:	0.25 sec

Analysis Results

Fig.1 shows the resulting total ion current chromatogram (TIC) of MRM measurement.

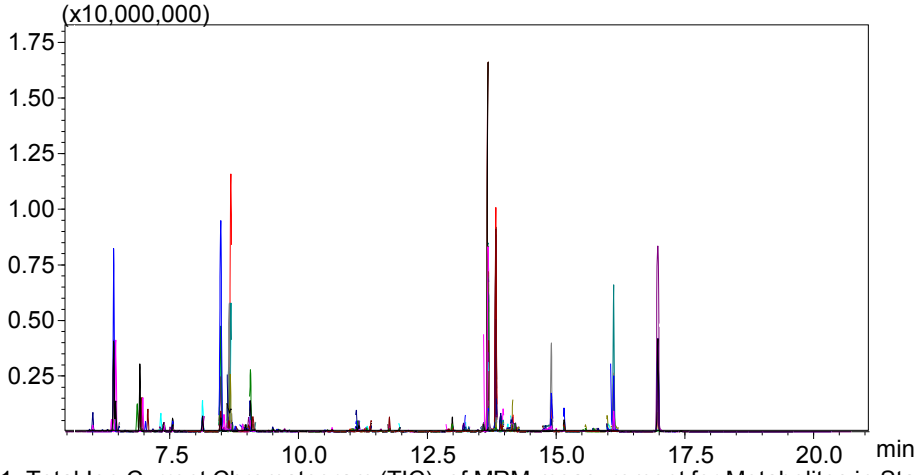

Fig. 1: Total Ion Current Chromatogram (TIC) of MRM measurement for Metabolites in Standard Human Plasma

[1] S. Nishiumi, M. Shinohara, A. Ikeda, T. Yoshie, N. Hatano, S. Kakuyama, S. Mizuno, T. Sanuki, H. Kutsumi, E. Fukusaki, T. Azuma, T. Takenawa, M. Yoshida, Metabolomics 6 (2010) 518-528

Fig. 2 shows mass chromatograms for plasma metabolites obtained in scan and MRM modes. In scan mode, some of the metabolites shown were not detected due to interfering components and insufficient sensitivity. In contrast, favorable results were obtained with MRM, which eliminated the impact of interfering components, enabling high-sensitivity measurements

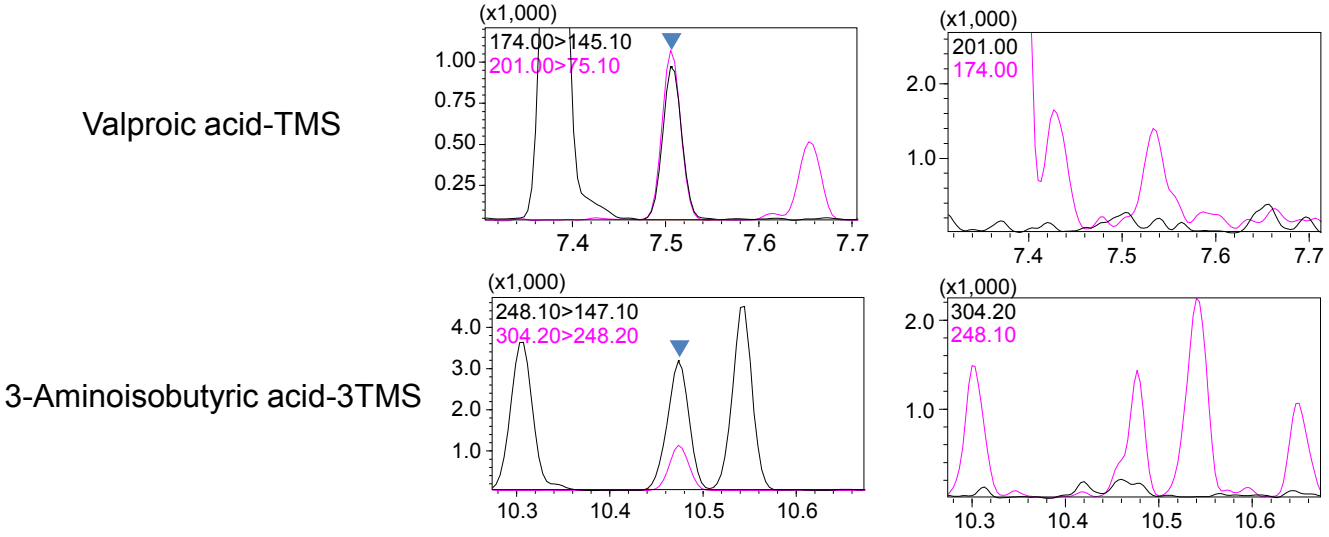

Fig. 2: Comparison of MRM (Left) and Scan (Right) Mass Chromatograms for Metabolites in Standard Human Plasma

Table 2 lists the metabolites detected using MRM measurement. From the standard human plasma, it was possible to detect 221 TMS derivatized metabolites, including 2-isopropylmalic acid, added as an internal standard.

Table 2: List of TMS Derivatized Metabolites Detected using MRM measurement *

1	Acetylglycine-TMS	33	Cystamine-nTMS	65	Glutamine-3TMS
2	Aconitic acid-3TMS	34	Cysteine-3TMS	66	Glutamine-4TMS
3	Adipic acid-2TMS	35	Cystine-4TMS	67	Glutaric acid-2TMS
4	Alanine-2TMS	36	Decanoic acid-TMS	68	Glyceric acid-3TMS
5	Allantoin-4TMS	37	5-Dehydroquinic acid-5TMS	69	Glycerol 2-phosphate-4TMS
6	Allose-meto-5TMS(1)	38	5-Dehydroquinic acid-meto-4TMS	70	Glycerol 3-phosphate-4TMS
7	2-Aminoadipic acid-3TMS	39	2-Deoxy-glucose-4TMS(1)	71	Glycerol-3TMS
8	2-Aminobutyric acid-2TMS	40	2-Deoxy-glucose-4TMS(2)	72	Glycine-2TMS
9	2-Aminoethanol-2TMS	41	2'-Deoxyuridine-3TMS	73	Glycine-3TMS
10	2-Aminoethanol-3TMS	42	Dihydrouracil-TMS	74	Glycolic acid-2TMS
11	3-Aminoisobutyric acid-3TMS	43	Dihydroxyacetone phosphate-meto-3TMS(1)	75	Glycyl-Glycine-4TMS
12	2-Aminopimelic acid-3TMS	44	Dihydroxyacetone phosphate-meto-3TMS(2)	76	Glyoxylic acid-meto-TMS
13	3-Aminopropanoic acid-3TMS	45	Dihydroxyacetone-2TMS	77	1-Hexadecanol-TMS
14	5-Aminovaleric acid-3TMS	46	Dimethylglycine-TMS	78	Histidine-3TMS
15	1,5-Anhydro-glucitol-4TMS	47	Dopamine-4TMS	79	Homocysteine-3TMS
16	1,6-Anhydroglucose-3TMS	48	Elaidic acid-TMS	80	Homoserine-2TMS
17	Arabitol-5TMS	49	Erythrulose-meto-3TMS(2)	81	Hydroquinone-2TMS
18	Arginine-3TMS	50	Ethylmalonic acid-2TMS	82	3-Hydroxyanthranilic acid-3TMS
19	Ascorbic acid-4TMS	51	Fructose-meto-5TMS(2)	83	2-Hydroxybutyric acid-2TMS
20	Asparagine-3TMS	52	Fucose-meto-4TMS(2)	84	3-Hydroxybutyric acid-2TMS
21	Asparagine-4TMS	53	Fumaric acid-2TMS	85	2-Hydroxyglutaric acid-3TMS
22	Aspartic acid-3TMS	54	Galactitol-6TMS	86	3-Hydroxyglutaric acid-3TMS
23	Azelaic acid-2TMS	55	Galacturonic acid-meto-5TMS(2)	87	2-Hydroxyhippuric acid-2TMS
24	Benzoic acid-TMS	56	Glucaric acid-6TMS	88	2-Hydroxyisobutyric acid-2TMS
25	Cadaverine-3TMS	57	Gluconic acid-6TMS	89	3-Hydroxyisobutyric acid-2TMS
26	Caproic acid-TMS	58	Glucosamine-5TMS(1)	90	2-Hydroxyisocaproic acid-2TMS
27	Catechol-2TMS	59	Glucose 6-phosphate-meto-6TMS(1)	91	2-Hydroxyisovaleric acid-2TMS
28	Cholesterol-TMS	60	Glucose-meto-5TMS(1)	92	3-Hydroxyisovaleric acid-2TMS
29	Citramalic acid-3TMS	61	Glucose-meto-5TMS(2)	93	Hydroxylamine-3TMS
30	Citric acid-4TMS	62	Glucuronic acid-meto-5TMS(1)	94	5-Hydroxymethyl-2-furoic acid-2TMS
31	Citrulline-3TMS	63	Glucuronic acid-meto-5TMS(2)	95	4-Hydroxyphenylacetic acid-2TMS
32	Creatinine-3TMS	64	Glutamic acid-3TMS	96	4-Hydroxyphenyllactic acid-3TMS

Table 2: List of TMS Derivatized Metabolites Detected using MRM measurement (continued)

97	4-Hydroxyproline-3TMS	139	2-Methyl-3-hydroxybutyric acid-2TMS(2)	181	Quinolinic acid-2TMS
98	3-Hydroxypropionic acid-2TMS	140	3-Methylglutaric acid-2TMS	182	Rhamnose-meto-4TMS(2)
99	Hypotaurine-3TMS	141	7-Methylguanine-2TMS	183	Ribonolactone-3TMS
100	Hypoxanthine-2TMS	142	Methylsuccinic acid-2TMS	184	Ribose-meto-4TMS
101	Indol-3-acetic acid-2TMS	143	Monostearin-2TMS	185	Ribulose-meto-4TMS
102	Inositol-6TMS(2)	144	Myristic acid-TMS	186	Sarcosine-2TMS
103	Isocitric acid-4TMS	145	N6-Acetyllysine-2TMS	187	Sebacic acid-2TMS
104	Isoleucine-2TMS	146	N-Acetylglutamine-3TMS	188	Serine-2TMS
105	Isoleucine-TMS	147	N-Acetylmannosamine-meto-4TMS(1)	189	Serine-3TMS
106	Isomaltose-meto-8TMS(2)	148	N-Acetylneuraminic acid-6TMS	190	Sorbitol-6TMS
107	2-Isopropylmalic acid-3TMS	149	N-Acetyl-Ornithine-4TMS	191	Sorbose-meto-5TMS(2)
108	Isovalerylglycine-TMS	150	N-Acetylserine-2TMS	192	Stearic acid-TMS
109	2-Ketobutyric acid-meto-TMS(1)	151	Niacinamide-TMS	193	Suberic acid-2TMS
110	2-Ketoglutaric acid-3TMS	152	Nicotinic acid-TMS	194	Succinic acid-2TMS
111	2-Ketoglutaric acid-meto-2TMS	153	Nonanoic acid-TMS	195	Sucrose-8TMS
112	2-Ketoisocaproic acid-meto-TMS(1)	154	O-Acetylserine-2TMS	196	3-Sulfinoalanine-3TMS
113	2-Ketoisocaproic acid-meto-TMS(2)	155	Octadecanol-TMS	197	Tagatose-meto-5TMS(1)
114	2-Keto-isovaleric acid-meto-TMS	156	Octanoic acid-TMS	198	Threitol-4TMS
115	Kynurenine-3TMS	157	Oleamide-TMS	199	Threonic acid-4TMS
116	Lactic acid-2TMS	158	Oleic acid-TMS	200	Threonine-3TMS
117	Lactitol-9TMS	159	O-Phosphoethanolamine-4TMS	201	Trehalose-8TMS
118	Lactose-meto-8TMS(1)	160	Ornithine-3TMS	202	Triethanolamine-3TMS
119	Lactose-meto-8TMS(2)	161	Ornithine-4TMS	203	Tryptamine-2TMS
120	Lauric acid-TMS	162	Oxalic acid-2TMS	204	Tryptophan-3TMS
121	Leucine-2TMS	163	5-Oxoproline-2TMS	205	Tyramine-3TMS
122	Linoleic acid-TMS	164	Palmitic acid-TMS	206	Tyrosine-3TMS
123	Lysine-4TMS	165	Palmitoleic acid-TMS	207	Uracil-2TMS
124	Maleic acid-2TMS	166	Pantothenic acid-3TMS	208	Urea-2TMS
125	Malic acid-3TMS	167	ParaXanthine-TMS	209	Uric acid-4TMS
126	Maltose-meto-8TMS(1)	168	Phenylacetic acid-TMS	210	Uridine-3TMS
127	Mannitol-6TMS	169	Phenylalanine-2TMS	211	Uridine-4TMS
128	Mannose-meto-5TMS(2)	170	3-Phenyllactic acid-2TMS	212	Urocanic acid-2TMS
129	Margaric acid-TMS	171	3-Phosphoglyceric acid-4TMS	213	Valine-2TMS
130	Mesaconic acid-2TMS	172	Phosphoric acid-3TMS	214	Valproic acid-TMS
131	meso-Erythritol-4TMS	173	Proline-2TMS	215	Vanilmandelic acid-3TMS
132	Methionine sulfone-2TMS	174	2-Propyl-5-hydroxy-pentanoic acid-2TMS	216	Xanthine-3TMS
133	Methionine-2TMS	175	Psicose-meto-5TMS(2)	217	Xanthosine monophosphate-6TMS
134	3-Methoxy-4-hydroxybenzoic acid-2TMS	176	Putrescine-4TMS	218	Xylitol-5TMS
135	5-Methoxytryptamine-2TMS	177	Pyridoxal-meto-2TMS(1)	219	Xylose-meto-4TMS(1)
136	3-Methyl-2-oxovaleric acid-meto-TMS(1)	178	Pyridoxamine-4TMS	220	Xylose-meto-4TMS(2)
137	3-Methyl-2-oxovaleric acid-meto-TMS(2)	179	Pyrogallol-3TMS	221	Xylulose-meto-4TMS
138	2-Methyl-3-hydroxybutyric acid-2TMS(1)	180	Pyruvic acid-meto-TMS		

∗ : TMS and meto indicate trimethylsilylation and methoximation, respectively.

First Edition: February, 2015

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Technical Report

Effectiveness of Metabolomics Research Using Gas Chromatograph / Quadrupole Mass Spectrometer with High-Sensitivity and High-Speed Scanning

Hiroshi Tsugawa[1,*], Eiichiro Fukusaki[1]

Abstract:

Gas Chromatography/Mass Spectrometry (GC/MS) has been recognized as a core technology for metabolomics research for the comprehensive analysis of low-molecular weight compounds in living organisms, and is widely used for biomarker discovery and quality assessment. To fulfill the demand for a high-throughput, high-sensitivity analytical system for GC/MS metabolomics research, we developed a high-speed quadrupole mass spectrometer (Q/MS), and conducted studies to determine the effectiveness of this instrument. In an experiment using a standard sample consisting of 10 amino acids, acquisition was possible over a wide quantitation range of 3.5–4.5 orders of magnitude. In analysis of the plasma from a mouse with acute inflammation, identification and quantitation of 168 compounds were possible, and using common inflammation markers, we were able to characterize the convergence process of the acute inflammatory response, which previously was not possible from the perspective of the metabolome. With the advent of this GC-Q/MS, further development in the study of metabolomics can be expected.

Keywords: metabolomics, gas chromatography, quadrupole mass spectrometer, scan speed

1. Introduction

Due to the high stability of the gas chromatograph/mass spectrometer (GC/MS), and the high repeatability of the quantitative data that is generated, GC/MS is recognized as a core analytical technique in metabolomics research. In metabolomics studies using GC/MS, comprehensive analysis of hydrophilic low-molecular weight compounds is primarily conducted for quality evaluation and biomarker discovery.

GC/MS metabolomics studies have been frequently conducted using a gas chromatograph connected to a time-of-flight (TOF) mass spectrometer (GCTOF/MS). This provides the major advantage of high speed data acquisition obtained with a time-of flight instrument, enabling (1) high-throughput analysis, in this case, Fast GC, and (2) acquisition of abundant data consisting of more than 30 points for each peak, permitting accurate data processing using such features as peak-top discrimination and deconvolution to ensure acquisition of highly repeatable qualitative and quantitative data. For this reason, the GC-TOF/MS, with its narrow gas chromatography 1 – 3 second peak width, has become an indispensable instrument for metabolomics research. However, due to the high cost of time-of-flight instruments and their narrow quantitation range (dynamic range), there has been adequate incentive to develop a more practical analytical technique.

Here, along with the newly released Shimadzu GCMS-QP2010 Ultra quadrupole mass spectrometer capable of high-speed scan measurement, we investigated the usefulness of this instrument (below, GC-Q/MS). In this report, we verified the quantitation range of the instrument using a standard sample containing 10 types of amino acids, and also report the results of a metabolomics analysis of the blood plasma of a mouse in the state of acute inflammation.

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*Present: RIKEN Plant Science Center (Concurrently visiting researcher of above division)

2. Experiment

Sample Preparation of Standard Solution of 10 Amino Acids

Ten types of amino acids were used, including asparagine, glycine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, and valine. The solutions were prepared so that the respective concentrations were 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM, 50 µM, 100 µM, 500 µM, 1 mM, 5 mM, 10 mM, and 50 mM. Then, three 150 µL aliquots of each of these solutions were dispensed into separate vials, and then lyophilized. In addition, 10 µL ribitol (0.2 mg/mL) was added to each sample as an internal standard. Then, 100 µL of methoxyamine (20 mg/mL, pyridine) was added to the post-lyophilized dried samples, which were then incubated for 90 minutes at 30 °C while centrifuging at 1,200 rpm. In addition 50 µL of MSTFA was added, and incubated for 30 minutes at 37 °C while centrifuging at 1,200 rpm.

Sample Preparation of Acute Inflammation-Affected Mouse Plasma

The mice were obtained from CLEA Japan. To induce inflammation, Lipopolysaccharide (LPS) derived from *Escherichia coli* O111:B4 (L2630; Sigma-Aldrich Co, St. Louis, Missouri, USA) was administered intraperitoneally into 20 mice at a dosage of 1 mg/kg body weight. One hour after administration of LPS, all of the blood was collected from 10 of the 20 mice, and 24 hours after LPS administration, all of the blood was collected from the remaining 10 mice. As a negative control, 8 mice were administered 200 µL phosphate buffered saline (PBS) intraperitoneally, and 1 hour later, all the blood was collected from the 8 mice. The collected blood was immediately treated with heparin (sodium heparin, Mochida Pharmaceutical, Japan, 5,000 units/5 mL), and after centrifuging at 3,000 × *g* at 4 °C for 10 minutes, the supernatant plasma was transferred to 1.5 mL Eppendorf tubes, then frozen in liquid nitrogen and stored at –80 °C until experimentation was conducted.

Then, in preparation for GC/MS analysis, 50 µL of plasma was transferred to a 1.5 mL Eppendorf tube, and 250 µL of a mixed solution ($MeOH/H_2O/CHCl_3$, 2.5/1/1) was added. In addition, 10 µL of 0.5 mg/mL 2- isopropylmalic acid was prepared as an internal standard, and added to the tube. Incubation was then conducted for 30 minutes at 37 °C while centrifuging at 1,200 rpm, which was followed by separation centrifuging for 3 minutes at 4 °C while centrifuging at 16,000 rpm. After transferring 225 µL of the supernatant to a newly prepared 1.5 mL Eppendorf tube, 200 µL Milli-Q water was added, and vortexing was conducted. After centrifuging at 16,000 rpm at 4 °C for 3 minutes, 125 µL of each supernatant was transferred to a newly prepared 1.5 mL Eppendorf tube. To remove any remaining methanol, centrifuge concentration was conducted for 1 hour and the sample was freeze-dried overnight.

Derivatization was conducted by adding 80 µL Methoxyamine solution (20 mg/mL, pyridine), and after thoroughly vortexing, sonication was conducted for 20 minutes. Then, incubation was conducted for 90 minutes at 37 °C while centrifuging at 1,200 rpm. Next, 40 µL MSTFA was added, and incubation was conducted for 30 minutes at 37 °C while centrifuging at 1,200 rpm. Finally, after centrifuging at 16,000 rpm at 4 °C for 3 minutes, 80 µL of supernatant was transferred to a vial for analysis, and analysis was conducted by GC/MS.

Analytical Conditions

The GC/MS analytical conditions are shown in Table 1.

Table 1 GC/MS Analytical Conditions

Instruments	
GC-MS	:GCMS-QP2010 Ultra
Autoinjector	:AOC-20i + s
Column	:CP-SIL 8 CB low bleed MS (30 m × 0.25 mm I.D. df = 0.25 µm, Agilent)
Analytical Conditions	
GC	
Injection Temp.	:230 °C
Column Temp.	:80 °C (2 min) – (15 °C/min) – 330 °C (6 min)
Injection Mode	:Split
Carrier Gas	:He (Constant Linear Velocity)
Linear Velocity	:39 cm/sec
Purge Flow	:5 mL/min
Injection Volume	:1 µL
MS	
Ion Source Temp.	:200 °C
Interface Temp.	:250 °C
Acquisition Mode	:Scan
Scan Range	:*m/z* 85 – 500
Event Time	:0.05 sec
Scan Speed	:10000 u/sec (ASSP™)

3. Results

We identified the compounds from the obtained analytical data based on the retention times and a mass spectrum database of standard compounds. In addition, we prepared a data matrix containing the compound name, retention time, quantitation ion, and peak height for each sample peak detected (standardized based on the peak height of the internal standard substance). The results of GC-Q/MS analysis of the amino acid sample mixture demonstrated the acquisition of all 10 amino acids within a wide quantitation range of 3.5–4.5 orders of magnitude. (Fig. 1)

All 168 compounds were identified from the mouse plasma analysis data. The results obtained from subjecting the acquired data matrix to principle component analysis are shown in Fig. 2. Also, Table 2 lists the 35 metabolites detected with significant differences among all of the identified compounds.

Principal component analysis indicated that the PBS processed group (control), the group treated 1 hour after LPS administration, and the group treated 24 hours after LPS could be distinguished from one another from their metabolic profile. Regarding the convergence process of the acute inflammatory response, this can be seen as an important process in the recovery from tissue inflammation, but presently, that molecular mechanism remains largely unknown. In particular, while a variety of changes have been observed in the process of convergence, such as the decrease of neutrophils and the increased influx of macrophages into various tissues such as the lungs, up to now, from a clinical standpoint, the convergence mechanism with respect to inflammation markers in blood has not been elucidated. These results suggest that the convergence process in acute late stage inflammation in mice, which previously could not be measured with frequently used inflammation markers, can be measured using blood plasma. Further, as shown in Table 2, abundant metabolite information can be acquired using this instrument. In particular, in plasma metabolome analysis using GC/MS, except for glucose, the intensity of all the metabolite peaks is low, making it difficult to conduct peak identification and quantitation. Therefore, there is a need for a high-sensitivity analytical system. The results presented here suggest that the newly released GC-Q/MS can provide high-resolution phenotypic analysis of metabolites.

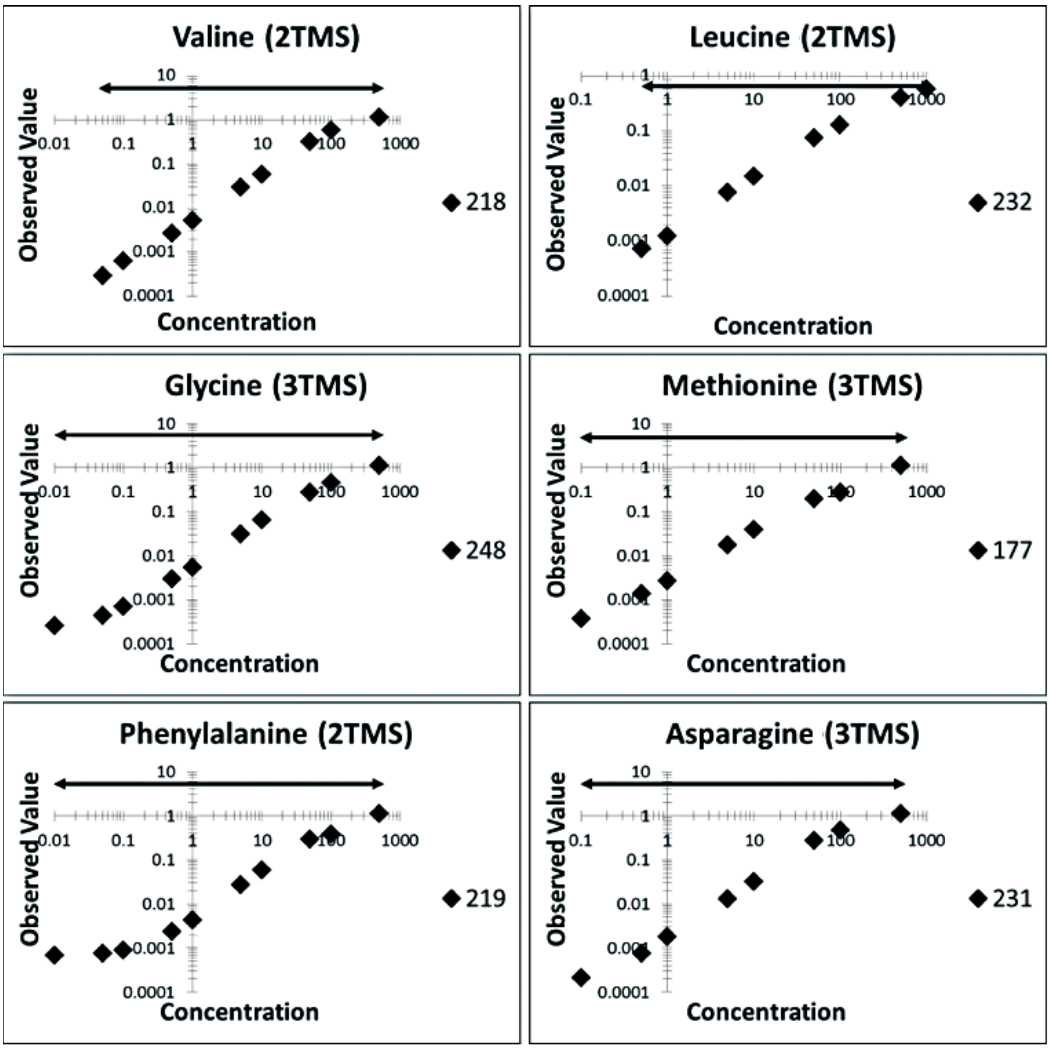

Fig. 1 Example of Quantitation Range of Analyzed Amino Acid Mixture

The Y-axis of the quantitation range in the amino acid mixture analysis data represents the relative peak intensity of the ribitol internal standard substance, and the X-axis represents the concentration submitted to analysis. The legend shows the *m/z* value used for quantitation of the compound.

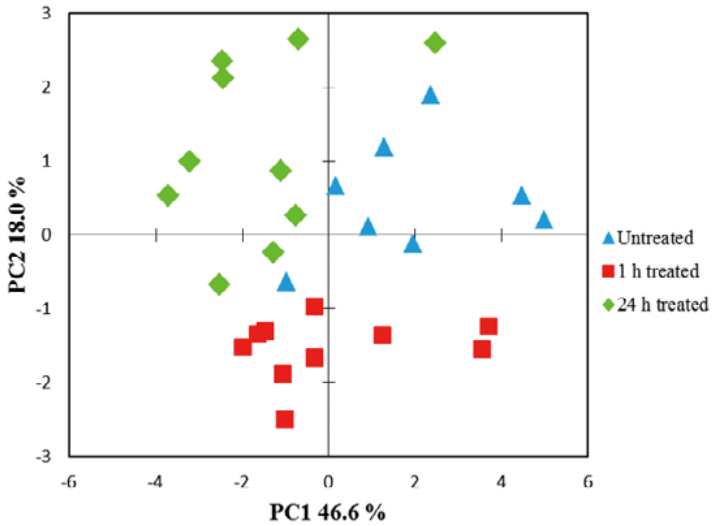

Fig. 2 Principal Component Analysis of Plasma in Mouse with Acute Inflammation

Table 2 Compounds Detected with Significant Difference Among 3 Groups

	Mean ± SD			*P* value		
Compound Name	A (n = 8)	B (n = 10)	C (n = 10)	AB	BC	CA
2-Aminoadipic acid	6.8 ± 3.1 × E-03	1.0 ± 0.2 × E-02	2.3 ± 1.2 × E-02	0.0593[w]	0.0181[w]	0.0028[w]
2-Hydroxybutyric acid	2.4 ± 0.5 × E-03	1.6 ± 0.3 × E-03	3.5 ± 0.8 × E-03	0.0027	0.0023	0.7742
3-Hydroxybutyric acid*	7.0 ± 2.3 × E-01	3.4 ± 0.8 × E-01	5.2 ± 1.0 × E-01	0.0156	0.0104[w]	0.0027[w]
Acetylsalicylic acid	2.9 ± 1.2 × E-03	2.3 ± 0.9 × E-03	4.0 ± 0.8 × E-03	0.5794	<0.0001	<0.0001
Aconitic acid	1.2 ± 0.1 × E-03	9.1 ± 1.9 × E-04	1.3 ± 0.3 × E-03	0.4878	<0.0001	<0.0001
Alanine (2TMS)*	1.8 ± 0.3 × E+00	1.2 ± 0.1 × E+00	2.1 ± 0.5 × E+00	0.0002	<0.0001[w]	0.007
Arabinose	5.2 ± 1.2 × E-03	3.5 ± 0.4 × E-03	5.4 ± 1.3 × E-03	0.0729	0.0001	<0.0001[w]
Arabitol	5.5 ± 2.1 × E-02	9.1 ± 2.3 × E-02	2.5 ± 0.9 × E-01	0.0026[w]	0.0004	0.0711[w]
Asparagne (3TMS)	2.6 ± 0.8 × E-02	1.4 ± 0.3 × E-02	2.2 ± 0.4 × E-02	0.0064	0.0052	0.6297
Behenic acid	6.6 ± 1.4 × E-04	4.9 ± 2.4 × E-04	1.1 ± 0.3 × E-03	0.0012	<0.0001	0.4514
Citrulline	4.1 ± 1.0 × E-03	3.4 ± 0.5 × E-03	4.8 ± 0.7 × E-03	0.0006	<0.0001	0.5445
Cystathionine	1.2 ± 0.2 × E-01	7.5 ± 1.7 × E-02	1.1 ± 0.1 × E-01	0.0018	0.0004	0.1968
DethioBiotin	1.1 ± 0.3 × E-03	1.7 ± 0.9 × E-03	3.6 ± 2.0 × E-03	0.0248	<0.0001	0.0083
Glycine (3TMS)	1.1 ± 0.2 × E+00	8.3 ± 1.1 × E-01	1.1 ± 0.1 × E+00	0.2472	0.0004	0.0392
Heptadecanoic acid*	1.3 ± 0.1 × E-02	1.3 ± 0.1 × E-02	1.9 ± 0.1 × E-02	0.0004	<0.0001	0.0003[w]
Homoserine	1.3 ± 0.1 × E-03	1.2 ± 0.3 × E-03	2.0 ± 0.2 × E-03	0.0013	0.0184	<0.0001
Icosanoic acid	4.5 ± 1.0 × E-03	5.5 ± 1.2 × E-03	9.8 ± 2.5 × E-03	0.0129	<0.0001	0.0384
Inositol	3.8 ± 0.8 × E-01	2.9 ± 0.4 × E-01	3.7 ± 0.6 × E-01	0.0028	0.004	0.5411
Isoleucine (2TMS)	3.1 ± 0.7 × E-01	2.0 ± 0.5 × E-01	3.3 ± 0.3 × E-01	0.0003	0.0001[w]	0.1287
Leucine (2TMS)*	9.3 ± 2.2 × E-02	5.6 ± 1.5 × E-02	9.8 ± 1.4 × E-02	0.005	0.0002[w]	<0.0001[w]
Methionine (3TMS)	1.9 ± 0.3 × E-02	1.0 ± 0.5 × E-02	2.3 ± 0.7 × E-02	0.0043	0.0043[w]	0.0794[w]
N-Acetyl-Valine	2.5 ± 0.5 × E-03	1.8 ± 0.5 × E-03	3.4 ± 0.6 × E-03	0.0177	<0.0001	0.0713
Phenylalanine (2TMS)	1.3 ± 0.1 × E-01	9.9 ± 1.8 × E-02	1.9 ± 0.3 × E-01	0.0046[w]	0.001[w]	0.7157
Prolinamide	1.8 ± 1.2 × E-03	5.6 ± 2.5 × E-03	8.5 ± 2.4 × E-03	0.0305[w]	0.0006	0.1121
Proline (2TMS)	4.2 ± 1.2 × E-02	2.9 ± 0.7 × E-02	5.4 ± 1.2 × E-02	0.0597	<0.0001	0.0027
Rhamnose	3.4 ± 1.2 × E-03	5.5 ± 1.4 × E-03	1.4 ± 0.4 × E-02	0.1749[w]	0.002	0.0006
Sarcosine*	3.2 ± 0.7 × E-03	2.1 ± 0.6 × E-03	4.6 ± 2.1 × E-03	0.003[w]	<0.0001	<0.0001
Serine (3TMS)*	2.4 ± 0.7 × E-01	1.7 ± 0.5 × E-01	2.9 ± 0.4 × E-01	0.0036	0.0002[w]	<0.0001[w]
Threonic acid	1.4 ± 0.3 × E-02	1.1 ± 0.1 × E-02	1.6 ± 0.3 × E-02	<0.0001	<0.0001	0.5716
Threonine (3TMS)	1.4 ± 0.4 × E-01	1.1 ± 0.3 × E-01	2.1 ± 0.4 × E-01	0.0085[w]	0.0003	0.3221
Thymine*	7.7 ± 3.0 × E-04	9.6 ± 2.5 × E-04	1.7 ± 0.5 × E-03	0.0011	<0.0001	0.0101
Tryptophan (3TMS)	6.8 ± 1.9 × E-02	9.6 ± 1.6 × E-02	2.2 ± 0.4 × E-01	0.1005	<0.0001	0.0005
Tyrosine (3TMS)	5.1 ± 0.8 × E-01	2.5 ± 0.5 × E-01	5.3 ± 1.0 × E-01	0.0846	0.0001	0.0854
Valine (2TMS)	6.5 ± 1.1 × E-01	4.4 ± 1.0 × E-01	7.9 ± 1.0 × E-01	0.0001	0.0002	0.2049
Xylitol	6.9 ± 1.8 × E-03	8.6 ± 2.0 × E-03	2.2 ± 0.7 × E-02	0.0855	0.0003[w]	0.0001[w]

Compounds detected with a significant difference in at least one pair among the 3 groups are listed. For cases in which a significant difference was detected among all of the three groups, an asterisk (*) is appended to the compound name. The Welch's t-test results are indicated using a W superscripted to the p-value. Correction of the p-value in multiple comparisons was conducted using the Bonferronimethod ($p < 0.017$).

4. Conclusion

Use of the newly released high-speed scanning quadrupole mass spectrometer GCMS-QP2010 Ultra (Shimadzu) permits high-throughput analysis and data point acquisition equivalent to that of a time-of-flight mass spectrometer. Further, the results of this study using a mixture of amino acids and mouse plasma demonstrate the high sensitivity and high dynamic range of this instrument, and suggest that the time-course changes in the state of acute inflammation can be measured using the metabolome. Here, by demonstrating the high sensitivity and high throughput metabolomics analysis that is possible with this extremely versatile quadrupole mass spectrometer, metabolomics studies can be expected to become more widespread using the GC-Q/MS.

References

1) Tsugawa H, Bamba T, Shinohara M, Nishiumi S, Yoshida M, Fukusaki E. Practical Non-targeted Gas Chromatography/Mass Spectrometry-based Metabolomics Platform for Metabolic Phenotype Analysis. *J Biosci Bioeng.* 2011 Sep;112 (3) :292-8.

Shimadzu GC/MS and the Quantitation of Biomolecules

Because biomolecules such as amino acids, organic acids, and fatty acids do not possess many volatile polar functional groups, they are difficult to measure directly by gas chromatography/mass spectrometry (GC/MS). It is therefore necessary to first convert these substances to volatile compounds by derivatizing these polar groups. Due to the considerable time and effort required for these tasks, this analysis tends to be avoided.

However, due to the advantages of GC/MS over other types of chromatography, such as the high resolution as compared with liquid chromatography, increased attention to GC/MS has led to its applicability for measurement of biological substances. In particular, high resolution is easily achieved with GC/MS by using a capillary column, and even if there is overlapping of contaminant and analyte peaks, appropriate selection of the fragment ion that is generated using the electron ionization (EI) method permits quantitation of the target substance without any adverse effects from the contaminant. In addition, since ion suppression, which is a problem with LC/MS/MS, is less likely to occur, this method is applicable for quantitation of samples containing large amounts of contaminants.

The Shimadzu GCMS-QP2010 Series offers excellent features and performance for bioanalysis.

1. The GCMS-QP2010 Series has the sensitivity required to permit measurement of biomolecules at very low concentrations.
2. Biological samples contain large amounts of contaminants. Measurement of such samples by GC/MS is a problem due to contamination of the ion source. The GCMS-QP2010 Series is quite resistant to contamination, but even when the ion source becomes contaminated, it is easily cleaned.
3. Batch analysis of biological samples typically involves a compli cated parameter setting procedure, however, the GCMS-QP2010 Series GC/MS metabolite database includes method files that incorporate the optimal analytical conditions and quantitation parameters.

GCMS-QP2010 Ultra

Gas Chromatograph Mass Spectrometer

GCMS-QP2010 Ultra Features

1. High sensitivity
2. Easy maintenance
3. Compound identification using retention indices

GC/MS Metabolite Database

(Amino Acids, Fatty Acids, Organic Acids)

The GC/MS Metabolite Database is a library designed for the GCMSsolution Workstation, the software package used with the GCMS-QP2010 Series gas chromatograph mass spectrometer. The mass spectrum library with retention indices significantly reduces the number of candidate compounds, thereby improving the reliability of results.

This database contains 4 types of method files including information such as analytical conditions, mass spectra, and retention indices, 4 types of libraries including compound information along with CAS numbers, mass spectra, and retention indices, as well as a handbook (published library information).

The methods and libraries contain spectra for a wide range of metabolite-related substances, including amino acids, fatty acids, and organic acids. There are 261 spectra that were obtained using the electron ionization method, and 50 spectra that were obtained using the chemical ionization method.

First Edition: January, 2013

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Printed in Japan 3655-12212-30ANS

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Application News

No. SCA_280_082

GCMS

The metabolites analysis of serum using fast-GC-MS/MS with tandem column

[1]Shuichi Kawana, [1]Yukihiko Kudo, [1]Katsuhiro Nakagawa, [2]Rebecca Kelting, [3]Alan Northage, [4]Kenji Hara, [1]Haruhiko Miyagawa
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[4]Fukuoka University, Fukuoka, Japan

Introduction

Current situation in metabolic profiling using GC/MS:

- Many samples need to be analyzed in metabolomics research.
- Samples should be analyzed within 24 hours of derivatization due to degradation.

Requirement of shortening analysis time in GC-MS.

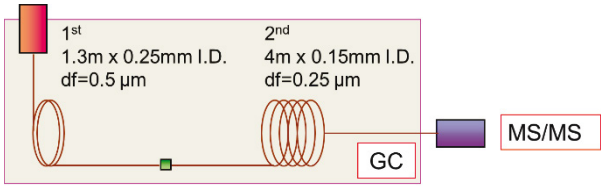
Problems of shortening analysis time in GC-MS:

- Narrow- bore (0.10 and 0.18mm I.D.) columns are not applied to the analysis due to the limited sample capacity.
- Chromatographic resolution of medium-bore columns causes peak overlapping.

Developed system to solve the problems:
Fast GC–MS/MS with a combination of two short columns (tandem column).

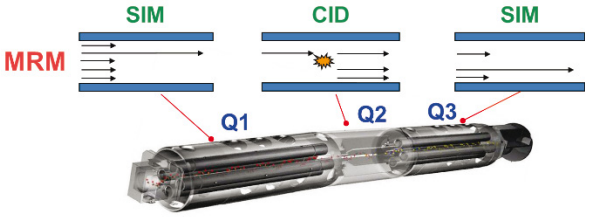
Fast-GC/MS/MS System

Tandem column



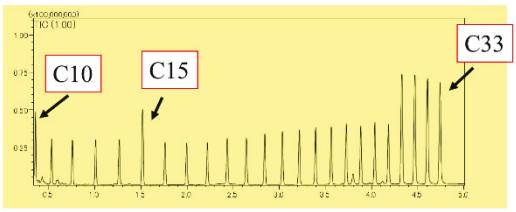
1st column : High sample capacity
2nd column : High chromatogram separation

GC-MS/MS (High separation)



Validation results (Standard sample)

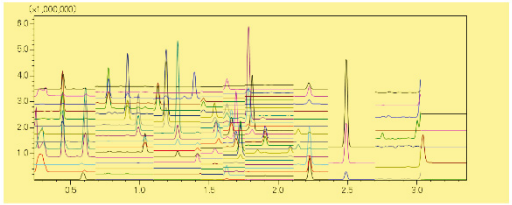
n-alkane analysis with fast-GC/MS



0.3 min **5 min**

Standard sample with fast-GC/MS/MS

(25 compounds metabolites (10ug/ml))



0.3 min **5 min**

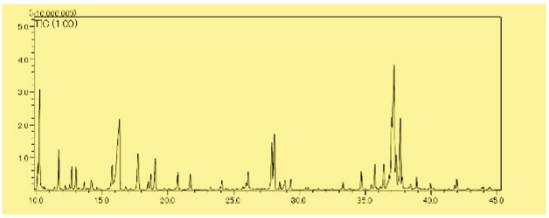
Fast-GC/MS/MS (MRM) Reproducibility (n=6, 10ug/ml), Calibration curve (0-10ug/ml*)

ID	Compound name	R.T. (min)	%RSD	Contribution
1	Lactic acid-2TMS	0.437	0.52	0.995
2	pyruvate-meto-TMS	0.447	0.50	1.000
3	Sarcosine-2TMS	0.595	0.63	0.994
4	Valine-2TMS	0.771	0.55	1.000
5	Leucine-2TMS	0.91	0.71	1.000
6	Proline-2TMS	0.988	1.31	0.999
7	Succinate-2TMS	1.051	1.25	1.000
8	Fumaric acid-2TMS	1.141	0.42	0.998
9	Threonine-3TMS	1.192	0.38	1.000
10	Glutaric acid-2TMS	1.276	0.54	0.999
11	Malic acid-3TMS	1.468	0.56	0.998
12	Aspartic acid-3TMS	1.543	0.37	1.000
13	Methionine-2TMS	1.551	0.54	0.999
14	Cysteine-3TMS	1.631	1.23	0.999
15	2-Isopropylmalic acid-2TMS (I.S.)	1.662	-	-
16	alpha-ketoglutarate-meto-2TMS	1.708	0.56	0.996
17	Glutamic acid-3TMS	1.787	0.56	0.998
18	Phenylalanine-2TMS	1.809	0.96	0.999
19	Asparagine-3TMS	1.909	1.97	0.995
20	Aconitate-3TMS	2.092	0.62	0.994
21	Glutamine-3TMS	2.146	2.52	0.998
22	Citric acid-4TMS	2.22	0.54	0.998
23	Isocitric acid-4TMS (I.S)	2.228	0.48	0.998
24	Tyrosine-3TMS	2.495	1.05	0.996
25	Tryptophan-3TMS	3.043	1.64	0.996

* 0, 0.05, 0.1, 0.5, 1, 5, 10 ug/ml

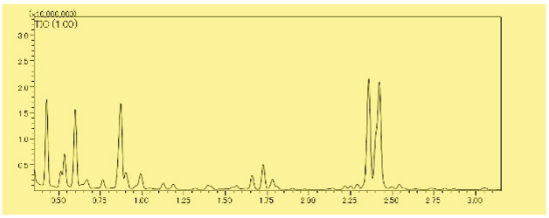
Serum sample analysis

Conventional GC/MS



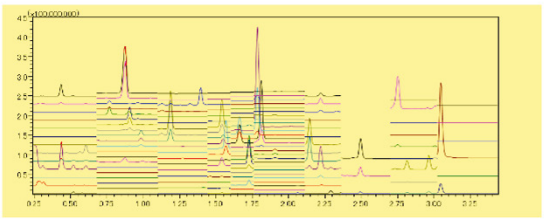
45 min

Fast-GC/MS



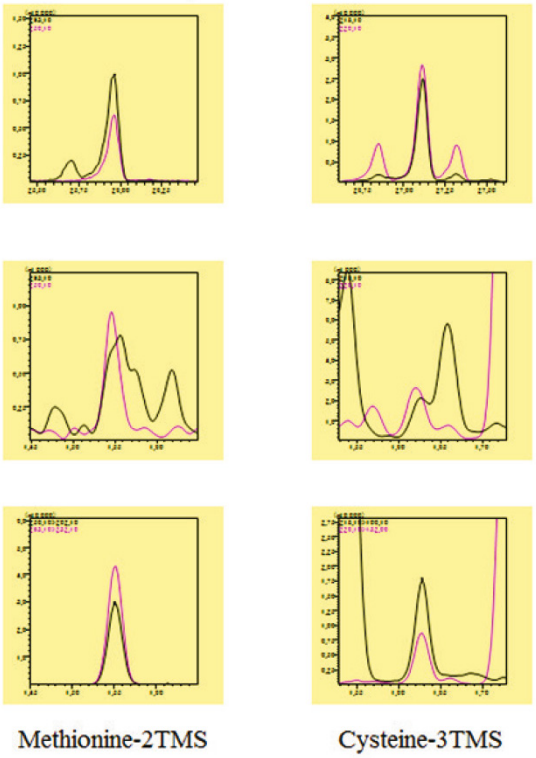
3 min

Fast-GC/MS/MS



3 min

Mass chromatogram



The reproducibility (n=6) and concentration in serum with fast-GC/MS/MS

ID	Compound name	Transition	%RSD	Con. (ug/ml)
1	Lactic acid-2TMS	219.00>191.10	0.77	81.75
2	pyruvate-meto-TMS	174.00>89.00	3.16	0.06
3	Sarcosine-2TMS	190.10>147.10	1.27	13.20
4	Valine-2TMS	218.10>100.10	0.86	9.37
5	Leucine-2TMS	102.10>73.00	0.41	7.89
6	Proline-2TMS	216.10>147.10	0.54	7.54
7	Succinate-2TMS	172.10>128.00	2.95	1.12
8	Fumaric acid-2TMS	245.00>217.10	2.21	0.18
9	Threonine-3TMS	291.00>101.10	0.88	5.98
10	Glutaric acid-2TMS	261.00>147.10	1.32	0.12
11	Malic acid-3TMS	307.00>263.10	3.35	0.30
12	Aspartic acid-3TMS	232.10>188.20	0.48	6.77
13	Methionine-2TMS	250.10>202.10	2.32	1.43
14	Cysteine-3TMS	218.10>100.10	2.87	0.34
15	2-Isopropylmalic acid-2TMS (I.S.)	349.10>259.10	-	-
16	alpha-ketoglutarate-meto-2TMS	198.10>73.00	3.37	0.17
17	Glutamic acid-3TMS	128.10>73.00	0.54	22.75
18	Phenylalanine-2TMS	218.10>100.10	0.70	7.00
19	Asparagine-3TMS	231.10>132.10	3.03	2.29
20	Aconitate-3TMS	285.10>147.10	8.52	0.39
21	Glutamine-3TMS	245.10>156.10	1.55	54.67
22	Citric acid-4TMS	347.10>183.10	1.44	14.87
23	Isocitric acid-4TMS (I.S)	245.10>191.10	1.17	0.39
24	Tyrosine-3TMS	218.10>100.10	0.71	5.55
25	Tryptophan-3TMS	202.10>201.10	0.91	9.49

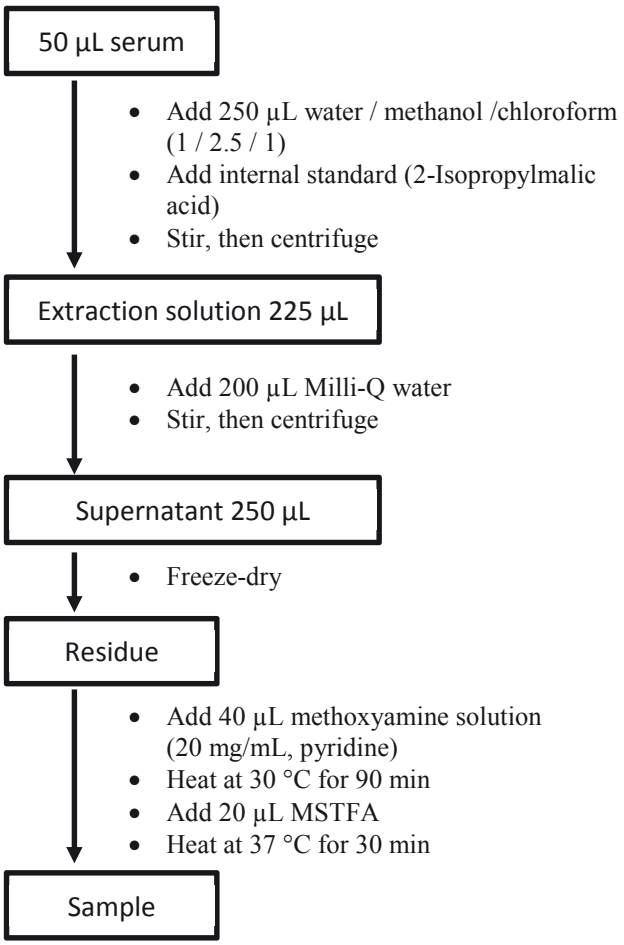
Mean %RSD: 0.41 - 8.52 %.

Experimental

Sample:

- 25 standard solution (0, 0.05, 0.1, 0.5, 1, 5, 10, 50 µg/ml)
- Human serum

Sample treatments[1)]



1) Nishiumi S et. al. Metabolomics. 2010 Nov;6(4):518-528

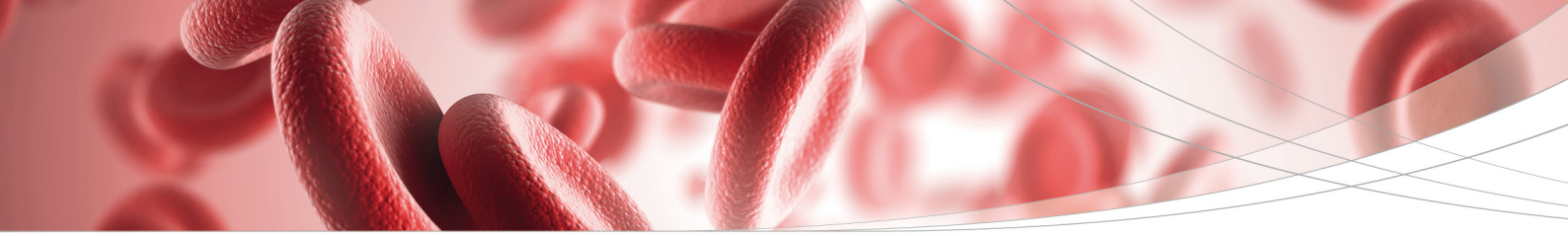
Analytical Conditions

GC-MS: GCMS-TQ8040 (SHIMADZU)
Data analysis: GCMSsolution Ver. 4.3
Column: Conv. DB-5 (Length 30 m, 0.25 mm I.D., df = 1.00 µm) (Agilent, U.S.A.)
Fast BPX5 (1.3 m x 0.25 mm I.D., df = 0.5 µm) + BPX5 (4 m x 0.15 mm I.D., df = 0.25) (SGE, Australia)

[GC]
Inj. Temp.: 280 °C
Column Oven Temp.:
Conv. 100 °C (4 min) → (4 °C/min) → 320 °C (0 min)
Fast 100 °C (0.35 min) → (50 °C/min)→ 340 °C (0.35 min)
Flow Control :Conv. 39.0 cm/sec, Fast 250 kPa
Split ratio: 5
Injection Volume: 1 µL

[MS]
Interface Temp.: 280 °C
Ion Source Temp.: 200 °C
Data acquisition: Scan or MRM
Scan *m/z*: 50 - 600 (0.06s) 10,000 u/sec

Method creation using Smart Metabolites Database (SHIMADZU).



2. Mass Spectrometry

2.2 Liquid Chromatography-Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry (MS). It is a powerful technique that brings together very high sensitivity and high selectivity. Its application is oriented towards the separation, general detection and potential identification of compounds of particular masses in the presence of other chemicals (e.g. complex mixtures like blood, serum, plasma, urines). It is now spreading in clinical field (research and routine) as a replacement of immunoassays thanks to the capability of multiplexing analysis and also to reduce risk of cross-contamination in immuno-assays.

SCA_210_008	Analysis of immunosuppressants in whole blood using the LCMS-8050 and the MagnaMedics MagSi-TDM^{PREP} immunosuppressants kit
SCA_210_006	Analysis of immunosuppressants in whole blood using the LCMS-8040 and the RECIPE ClinMass® LC-MS/MS complete kit, advanced MS1100
SCA_210_019	Quantification of 16 anti-HIV drugs from human plasma
SCA_210_010	Analysis of 25-OH vitamin D2 / D3 in plasma and serum by LCMS-8050 using RECIPE ClinMass® LC-MS/MS complete kit MS7000
SCA_210_017	Fast, sensitive and simultaneous analysis of multiple steroids in human plasma by UHPLC-MS/MS
AD-0067	Ultra sensitive LC/MS/MS method for quantitative determination of ethinyl estradiol in human plasma
SCA_210_003	Determination of methylmalonic acid in serum, plasma and urine by LCMS-8050 using RECIPE ClinMass complete kit, advanced MS5100
LAAN-A-LM-E061	Measurement of adenosine deaminase activity in urine with LCMS-8040
LAAN-A-LM-E062	Measurement of fumarylacetoacetate activity in DBS (Dried Blood Spot) with LCMS-8040
SCA_210_005	Determination of Homocysteine in plasma/serum by LCMS-8050 using RECIPE ClinMass complete kit, MS2000
LAAN-A-LM-E063	Measurement of homocysteine in plasma with LCMS-8040
LAAN-A-LM-E064	Measurement of methylmalonic acid, 3-OH propionic acid and succinic acid in DBS (Dried Blood Spot) with LCMS-8040
LAAN-A-LM-E065	Simultaneous analysis of amino acids and acylcarnitines in DBS (Dried Blood Spot) with LCMS-8040
SCA_210_022	Analysis of ethylglucuronide and ethylsulfate in urine, plasma and serum by LCMS-8050 using RECIPE ClinMass® LC-MS/MS complete kit MS8000
PO-CON1446E	Determination of Δ^9-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation
LAAN-A-LM-E085	High-sensitivity determination of catecholamines in plasma using the LCMS-8060 Triple Quadrupole LC/MS/MS
SCA_210_020	A novel fast quantification method for amino acids in human plasma by LC-MS/MS, without ion pairing of derivatization

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Application News

No. SCA_210_008

LCMS

Analysis of Immunosuppressants in whole blood using the LCMS-8050 and the MagnaMedics MagSi-TDM^{PREP} Immunosuppressants kit

Sven Goethel (MagnaMedics Diagnostics B.V.), Vincent Goudriaan (Shimadzu Benelux B.V.)

Introduction

Immunosuppressants are an important class of compounds which are commonly used by transplant recipients to avoid organ rejection. In addition, they are used for the treatment of immune mediated diseases or disorders of the immune system and non-autoimmune inflammatory reactions such as heavy allergic asthma. The therapeutic concentration range of these compounds, typically narrow, requires careful monitoring from whole blood to ensure the correct dosage.
In these research experiments on-line separation of whole blood was performed, analyzing the immunosuppressant compounds Tacrolimus, Sirolimus, Everolimus and Cyclosporin A (CsA) by liquid chromatography tandem mass spectrometry LC-MS/MS.

Materials and methods

The LCMS-8050 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system equipped with a 6-port-2-position switching valve. Compounds were measured using a commercially available test kit MagSi-TDM^{PREP} Immunosuppressants kit (MagnaMedics Diagnostics B.V., De Asselen Kuil 20a, 6161 RD Geleen, The Netherlands). Calibrators 0-6 and control samples Level I, II and III were obtained from Recipe (9933 and 8833 respectively), analytical column was obtained from Phenomenex (Kinetex 50x2.1 mm ID, 2.6 µm) and mobile phases were manually prepared (A: 2 mM ammonium formate + 0.1% formic acid in water; B: 2 mM ammonium formate + 0.1% formic acid in methanol). Sample preparation was automated by using a Primadiag-MPS liquid handling system (PrimaDiag S.A.S.). The following protocol was used: 25 µL whole blood sample, calibrator or control sample is transferred into a microtiter plate well which is positioned above a magnet. 60 µL Lysis solution (MagnaMedics) is added to the well, mixed and incubated for one minute. 10 µL internal standard (Recipe MS1412) is added and mixed. 50 µL Premixed Bead solution (10 µL 2M ZnSO4 and 40 µL MagSi-TDM^{PREP} particle mix prepared during lysis) is added and mixed. Proteins were precipitated by addition of acetonitrile followed by intense aspiration and dispensing of the mixture. After magnetic separation, 80 µL of the supernatant was transferred to an HPLC vial. Full plate sample preparation time took less than 20 minutes. 5 µL was injected for LC-MS/MS analysis. Column effluent was diverted to waste between injection and 2.0 minutes to exclude salts which were used during sample prep.
The LC-MS instrument was equipped with an electrospray ionization source (ESI).

Analytical conditions

UHPLC:	Nexera X2 UHPLC
Column flow:	0.4 mL/min
Gradient:	50 – 100% B (in 2 min) – 50% B (in 1 min)
Column temperature:	40 °C
Injection volume:	5 µL
Mass spectrometer:	LCMS-8050
Source conditions:	
Nebulizer Gas:	3 L/min
Heating Gas:	10 L/min
Interface temperature:	300 °C
Desolvation Line:	220 °C
Heat Block temperature:	400 °C
Drying Gas:	10 L/min
Interface voltage:	4.5 kV
Dwell time:	34 msec
Pause time:	3 msec
Ionization:	ESI, positive mode
Scan Type:	MRM

Compound	Target (T)/ Internal std (I)	MRM	RT (min)
Tacrolimus	T	821.6>768.4	2.52
Sirolimus	T	931.6>864.5	2.55
Everolimus	T	975.8>908.6	2.57
Cyclosporin A	T	1219.9>1202.6	2.80
$^{13}Cd_2$-Tacrolimus	I	824.6>771.5	2.52
$^{13}Cd_3$-Sirolimus	I	935.7>864.5	2.55
$^{13}C_2d_4$-Everolimus	I	981.7>914.5	2.56
d_{12}-Cyclosporin	I	1232.0>1214.8	2.80

Table 1 Immunosuppressants with optimized MRM transitions and retention times (RT).

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Fig. 1 Nexera X2 UHPLC–LCMS-8050 LC-MS/MS system.

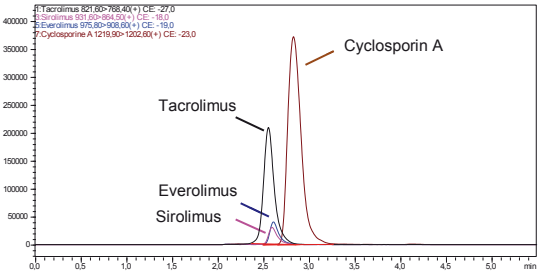

Fig. 2 LC-MS/MS separation of Tacrolimus, Sirolimus, Everolimus and Cyclosporin A.

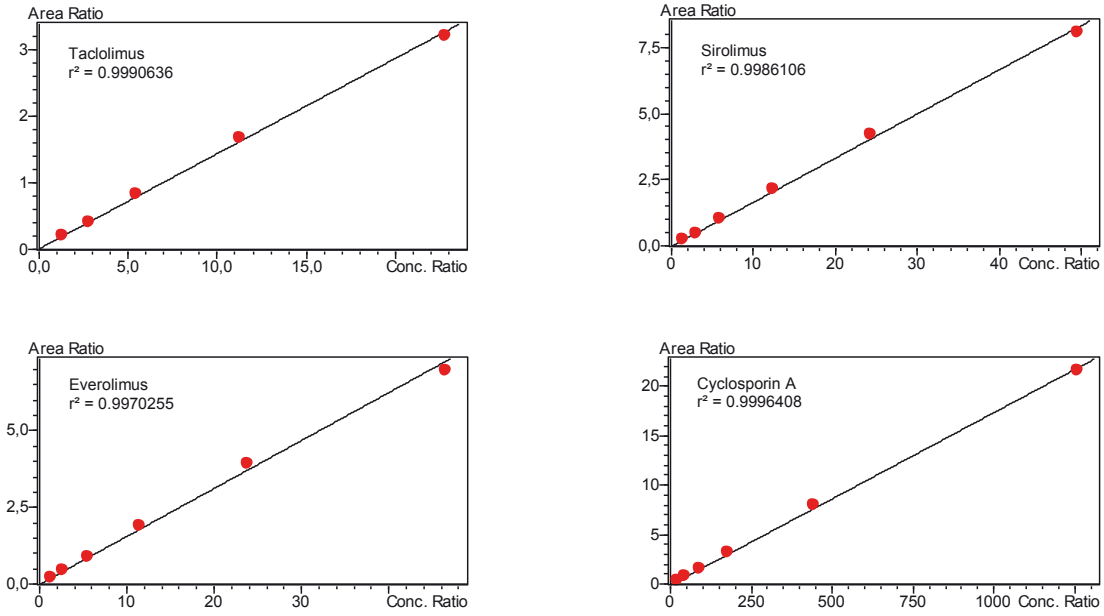

Fig. 3 Calibration curves for Tacrolimus, Sirolimus, Everolimus and Cyclosporin A.

	Tacrolimus quality controls			Sirolimus quality controls		
	Level I	**Level II**	**Level III**	**Level I**	**Level II**	**Level III**
Control 1	3.65	8.37	14.90	3.92	13.60	22.34
Control 2	3.35	7.73	15.04	3.75	14.13	24.37
Control 3	3.44	8.12	14.55	3.73	13.02	23.21
Mean conc (µg/L)	**3.48**	**8.07**	**14.83**	**3.80**	**13.58**	**23.31**
Stand Dev	**0.15**	**0.32**	**0.25**	**0.10**	**0.56**	**1.02**
%RSD	**4.4**	**4.0**	**1.7**	**2.7**	**4.1**	**4.4**
Theor. Value (µg/L)	**3.71**	**7.66**	**15.2**	**4.18**	**12.6**	**21.0**
Accuracy in %	**93.8**	**105.4**	**97.6**	**90,9**	**107.8**	**111.0**

Table 2 Accuracy for Tacrolimus and Sirolimus quality controls.

	Everolimus quality controls			Cyclosporin A quality controls		
	Level I	Level II	Level III	Level I	Level II	Level III
Control 1	3.50	12.86	20.31	58.23	134.4	237.9
Control 2	3.86	11.34	19.35	57.33	125.5	237.1
Control 3	3.17	11.73	18.77	58.21	126.1	230.6
Mean conc (µg/L)	**3.51**	**11.98**	**19.48**	**57.92**	**128.7**	**235.2**
Stand Dev	**0.35**	**0.79**	**0.78**	**0.51**	**4.97**	**4.02**
%RSD	**9.8**	**6.6**	**4.0**	**0.9**	**3.9**	**1.7**
Theor. Value (µg/L)	**3.89**	**12.3**	**20.3**	**58.7**	**116.0**	**228.0**
Accuracy in %	**90.2**	**97.4**	**95.9**	**98.7**	**111.0**	**103.1**

Table 3 Accuracy for Everolimus and Cyclosporine A quality controls.

	Everolimus	Cyclosporine A	Sirolimus	Tacrolimus
Process efficiency %	106.7	103.3	112.0	69.9
Matrix effect %	87.4	80.6	84.8	56.1
Recovery %	122.1	128.1	132.0	124.0

Table 4 Process efficiency, matrix effect and recovery.

Measurement	Sample 1	Sample 2	Sample 3	Sample 4
#1	5.21	6.68	8.70	7.08
#2	5.08	6.47	10.18	7.77
#3	4.80	7.08	10.07	7.42
#4	4.93	6.48	9.06	8.08
#5	4.84	7.03	10.78	7.11
Average conc (µg/L)	**4.97**	**6.75**	**9.76**	**7.49**
stand. dev.	**0.17**	**0.29**	**0.86**	**0.43**
RSD %	**3.44**	**4.34**	**8.77**	**5.76**

Table 5 Everolimus real sample data.

Results

The calibration curve determined in triplicate showed good linearity over a clinically relevant concentration range of 1.37-65 µg/L for Tacrolimus, 1.45-66.8 µg/L for Sirolimus, 1.62-71.7 µg/L for Everolimus and 25.8-1746 µg/L for Cyclosporin A (Fig. 3).

Three control samples covering the calibration curve concentration range were analyzed three times to assess analytical reproducibility. The percentage relative standard deviation was between 0.9% and 9.8% for these measurements.

Real sample data on Everolimus showed relative standard deviations between 3.4 and 8.8%. This is well below the threshold for immunosuppressant analysis.

Conclusion

Magnetic bead based sample preparation in an SPE-free workflow showed good sensitivity, linearity and suitability for immuno-suppressants measurements and for quantitative determination of Everolimus in whole blood samples.

For Research Use Only. Not for use in diagnostic procedures.

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Application News

No. SCA_210_006

LCMS

Analysis of Immunosuppressants in whole blood using the LCMS-8040 and the RECIPE ClinMass® LC-MS/MS Complete Kit, advanced MS1100

Silvia Bächer (RECIPE Chemicals + Instruments GmbH), Anja Grüning (Shimadzu Europa GmbH)

Introduction

Immunosuppressants are an important class of compounds which are commonly used by transplant recipients to avoid organ rejection. In addition, they are used for the treatment of immune mediated diseases or disorders of the immune system and non-autoimmune inflammatory reactions such as heavy allergic asthma. The therapeutic concentration range of these compounds, typically narrow, requires careful monitoring from whole blood to ensure the correct patient dosage.

In these experiments on-line separation of whole blood was performed, analyzing the immunesuppressant compounds Tacrolimus, Sirolimus, Everolimus and Cyclosporin A (CSA) by liquid chromatography tandem mass spectrometry LC-MS/MS.

Analytical conditions

UHPLC:	Nexera X2 UHPLC
Column temperature:	60 °C
Injection volume:	50 µL
Mass spectrometer:	LCMS-8040
Source conditions:	
Nebulizer Gas:	3 L/min
Desolvation Line:	200 °C
Heat Block temperature:	450 °C
Drying Gas:	15 L/min
Interface voltage:	2.5 kV
Dwell time:	30 msec
Pause time:	3 msec
Ionization:	ESI, positive mode
Scan Type:	MRM

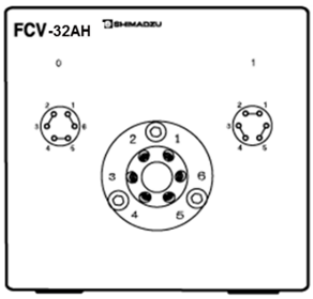

Fig. 1 The FCV-32AH six port valve was configured to switch between sample loading (pump SPE) and isocratic sample elution (pump MP).

Starting conditions:
Pump SPE: 0.1 mL/min
Pump MP: 0.5 mL/min
FCV: position 1

Materials and methods

The LCMS-8040 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system. This was equipped with the switching valve FCV-32AH and configured for isocratic sample loading onto the SPE column (pump SPE) and isocratic compound elution (pump MP). Compounds were measured using a commercially available test kit ClinMass® LC-MS/MS Complete Kit, advanced for Immunosuppressants, MS1100 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany). Calibrators, control samples, SPE column, analytical column and mobile phase solvents were provided by the kit. Sample preparation consists of protein precipitation by adding 220 µL of precipitant which includes the internal standard to 100 µL whole blood sample. Following incubation and centrifugation, 50 µL of supernatant was injected for analysis.

The LC-MS instrument was equipped with an electrospray ionization source (ESI).

Time (min)	Pump / FCV	Action	Setting
0.00	FCV	position 1	loading
0.00	Pump SPE	flow rate	0.1 mL/min
0.00	Pump MP	flow rate	0.5 mL/min
0.1	Pump SPE	flow rate	2.5 mL/min
0.50	FCV	position 0	elution
0.5	Pump SPE	flow rate	2.5 mL/min
0.51	Pump SPE	flow rate	0.1 mL/min
1.30	Pump MP	flow rate	0.5 mL/min
1.35	Pump MP	flow rate	1.0 mL/min
1.49	Pump SPE	flow rate	0.1 mL/min
1.50	Pump SPE	flow rate	2.5 mL/min
1.55	Pump MP	flow rate	1.0 mL/min
1.65	Pump MP	flow rate	0.5 mL/min
1.65	FCV	position 1	conditioning
1.99	Pump SPE	flow rate	2.5 mL/min
2.00	Pump SPE	flow rate	0. 1 mL/min
2.00	Pump MP	flow rate	0.5 mL/min

Table 1 LC parameters for rapid compound elution and fast analysis time.

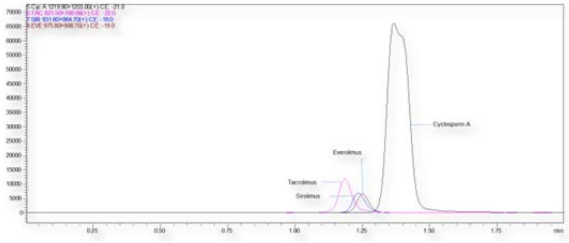

Fig. 2 LC-MS separation of Tacrolimus, Sirolimus, Everolimus and CSA in less than two minutes by on-line SPE followed by isocratic chromatography.

Compound		MRM1	MRM2	RT
Tacrolimus	T	821.5>768.6	821.5>576.6	1.18
Sirolimus	T	931.6>864.7	931.6>882.7	1.24
Everolimus	T	975.8>908.7	975.8>926.8	1.26
Cyclosporin A	T	1219.9>1203	1219.9>1184.9	1.37
$^{13}Cd_2$-Tacrolimus	I	824.6>772.3	824.6>771.3	1.18
$^{13}Cd_3$- Sirolimus	I	935.7>864.7	935.7>882.7	1.24
$^{13}C_2d_2$- Everolimus	I	981.5>914.8	981.5>932.8	1.26
d_{12}-Cyclosporin	I	1232.1>1215.1	1232.1>1197.0	1.37

Table 2 Immunosuppressants optimized MRM transitions, retention times (RT).
T/I = target or internal standard.

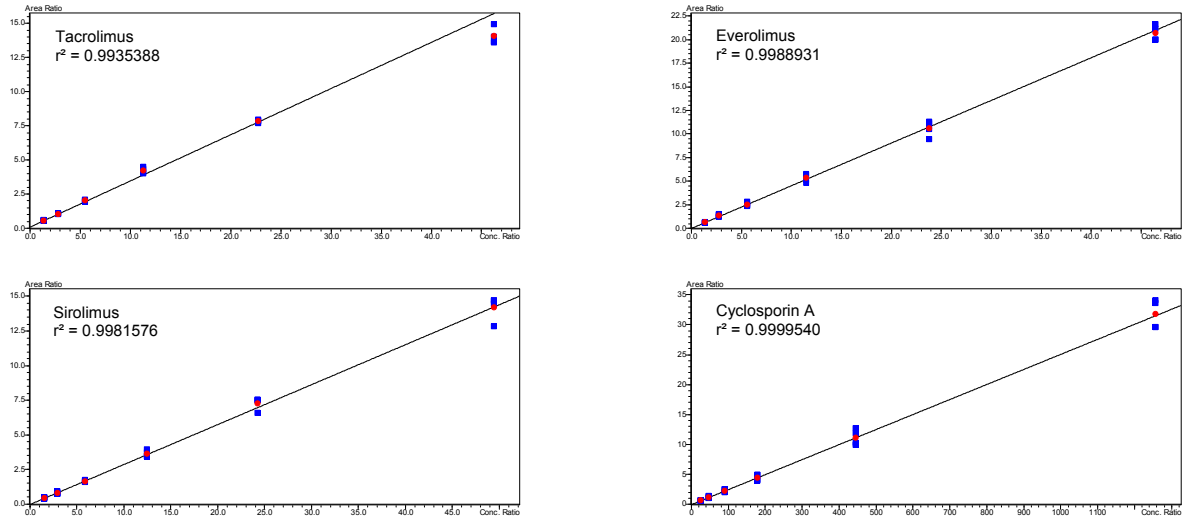

Fig. 3 Calibration curves for Tacrolimus, Sirolimus, Everolimus and Cyclosporin A.

Results

The rapid elution of Immunosuppressants after on-line solid phase extraction followed by isocratic chromatography produced excellent peak shape and accuracy with elution in less than two minutes (Fig. 2).

The calibration curve determined in duplicate showed good linearity over a clinically relevant concentration range of 1.36-46.3 µg/L for Tacrolimus, 1.52-49.5 µg/L for Sirolimus, 1.35-46.5 µg/L for Everolimus and 25.2-1258 µg/L for Cyclosporin A (Fig. 3)

Conclusion

The application of the ClinMass® Complete Kit, advanced for Immunosuppressants in whole blood – on-line analysis proved easy to implement and showed good sensitivity and linearity in a clinically relevant concentration range.

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Application News

LCMS-8050

No. SCA_210_019

Quantification of 16 anti-HIV drugs from human plasma

Introduction

This Application News describes a simple LC-MS/MS analytical method for the simultaneous quantification of new agents dolutegravir, elvitegravir, rilpivirine and other thirteen anti-HIV drugs from human plasma by Shimadzu Nexera X2 coupled with Triple Quadrupole Mass Spectrometer LCMS-8050.

Sample preparation

Protein precipitation step

A simple protein precipitation solution of 600 µL (acetonitrile:methanol 50:50 v/v) and 40 µL of IS working solution (quinaxoline 5 µg/ml in H_20:MeOH 50:50) were added to 100 µL of plasma samples in a PTFE microfuge tube. After vortexing for 30 seconds, the mixture was centrifuged at 12000 rpm (7200 g) for 10 minutes at 4 °C. Then the supernatant was diluted 1:1 with water in vial for the injection.

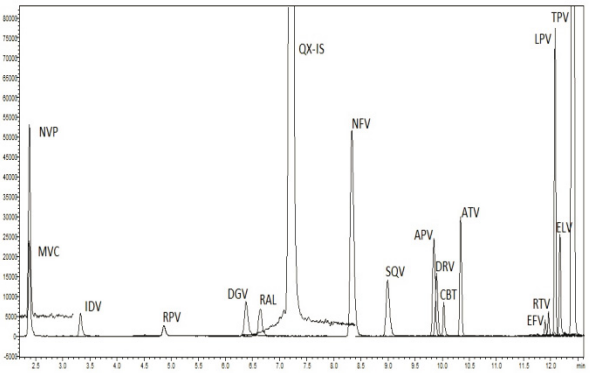

Figure 1. Chromatogram of a CAL1/LOQ

Table 1. Experimental Condition

HPLC conditions (Nexera X2)	
Analytical Column	AQUITY UPLC HSS T3 1.8 µm (2.1 x 150 mm)
Solvent A	H_2O + Formic Acid 0.05%
Solvent B	ACN + Formic Acid 0.05%
Flow rate	0.500 mL/min
Injection Volume	2 µL
Autosampler temp.	15 °C
Column oven temp.	45 °C

GRADIENT	
Time (min)	Solvent B%
0,0	30
8,0	43
9,0	55
10,0	60
11,0	75
11,1	95
13,0	95
13,1	30
15,0	30

MS settings (LCMS-8050)	
Ionization mode	ESI (positive-negative)
Interface voltage	4 kV
Nebulizer gas	3.0 L/min
Drying gas	10 L/min
Heating gas	10 L/min
Interface temp.	300 °C
DL temp.	250 °C
Heat block temp.	400 °C
CID Gas	270 kPa

Table 2. MRM Transitions

Compounds	Tag	Polarity	Precursor (m/z)	Product (m/z)
Maraviroc	MVC	+	514.15	388.95
Nevirapine	NVP	+	267.10	225.90
Indinavir	IDV	+	614.15	421.00
Rilpivirine	RPV	+	367.15	128.15
Dolutegravir	DGV	+	420.15	277.10
Raltegravir	RAL	+	445.00	109.05
Nelfinavir	NFV	+	568.10	135.10
Saquinavir	SQV	+	671.20	570.10
Darunavir	DRV	+	548.05	391.95
Cobicistat	CBT	+	776.15	606.00
Amprenavir	APV	+	506.00	245.00
Atazanavir	ATV	+	705.20	168.05
Ritonavir	RTV	+	721.10	295.90
Elviltegravir	ELV	+	447.80	344.00
Lopinavir	LPV	+	629.15	155.20
Efavirenz	EFV	-	313.85	243.95
Tipranavir	TPV	+	603.05	411.00
Quinoxaline	QX-IS	+	312.85	245.95

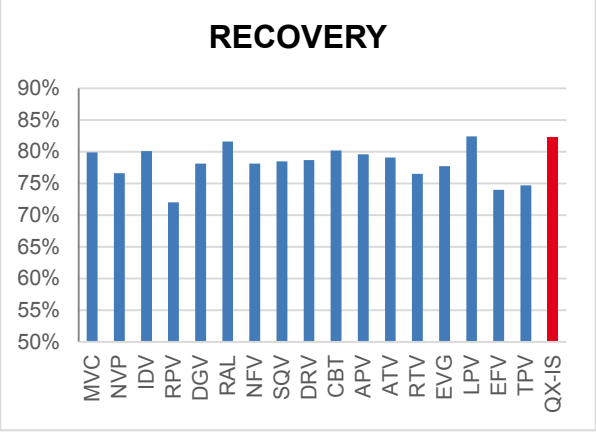

Figure 2. Recovery for all drugs was above 70% with a matrix effect below 10%

The high performance and sensitivity of the instruments allows to utilize only few microliters of plasma ensuring in any case precision and accuracy also for very low concentrations.

Table 3. Accuracy and precision

Drugs	QCs Conc. ng/mL	Accuracy (%)	Intraday Precision RSD% Assay n°=6	Interday Precision RSD%
MVC	2000	101,3	3,7	4,7
	500	103,5	2,5	3,1
	50	99,5	4,0	4,7
NVP	6400	101,5	2,8	4,2
	1600	102,4	3,1	3,4
	160	99,2	2,8	3,4
IDV	6400	101,9	3,7	5,2
	1600	103,8	2,6	3,0
	160	101,0	3,5	6,2
RPV	2000	106,8	5,2	10,5
	500	107,5	7,3	9,5
	50	98,7	4,2	5,4
DGV	4800	101,5	2,6	3,6
	1200	103,2	2,8	3,8
	120	101,5	2,7	4,9
RAL	2400	101,5	2,4	3,4
	600	102,0	2,7	2,8
	60	98,9	3,0	5,2
NFV	6400	102,1	4,1	5,8
	1600	104,5	2,6	3,0
	160	97,5	3,7	7,0
SQV	5600	102,5	4,2	5,4
	1400	107,4	2,7	3,1
	140	101,4	3,8	9,9
DRV	8000	101,4	3,6	4,8
	2000	103,5	2,5	3,1
	200	96,8	2,9	4,6
CBT	2400	102,9	4,3	6,0
	600	106,0	3,1	3,3
	60	101,1	4,8	7,3
APV	8000	101,7	3,3	4,7
	2000	102,8	2,5	3,0
	200	96,3	2,7	4,3
ATV	4800	101,9	4,2	5,8
	1200	105,2	2,7	3,2
	120	101,4	3,7	7,3
RTV	2000	100,6	5,0	7,7
	500	104,5	3,2	3,9
	50	99,8	5,2	5,9
ELV	2400	101,8	2,9	5,6
	600	103,1	2,8	4,1
	60	99,4	5,2	7,0
LPV	12000	104,1	4,5	6,3
	3000	104,1	2,9	4,7
	300	98,8	3,1	6,9
EFV	6400	103,9	3,8	5,4
	1600	104,4	3,1	3,9
	160	97,5	4,1	5,9
TPV	144000	105,2	7,7	9,7
	360000	104,6	3,9	4,4
	3600	100,4	3,8	10,7

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Application News

No. SCA_210_010

LCMS

Analysis of 25-OH Vitamin D2 / D3 in Plasma and Serum by LCMS-8050 using RECIPE ClinMass® LC-MS/MS Complete Kit MS7000

Dr. Johannes Engl (RECIPE Chemicals + Instruments GmbH), Anja Grüning (Shimadzu Europa GmbH)

Introduction

Vitamin D measurement has become an important component in clinical assays largely because deficiency is associated with a number of disorders such as rickets, osteomalacia and osteoporosis. When serum concentration falls below 20 ng/mL osteoporosis can result, with normal levels ranging from 20-50 ng/mL. Developments in high pressure fast chromatography LC-MS/MS have now enabled on-line sample preparation methods and analysis times in less than 3 minutes.
In these experiments tandem mass spectrometry was used to quantify 25-OH Vitamin D2 / D3 using a kit containing standard compounds and pre-optimised method parameters.

Materials and methods

The LCMS-8050 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system. This was equipped with the switching valve FCV-32AH and configured for sample loading onto the column (pump SPE) and isocratic compound elution (pump MP). Compounds were measured using a commercially available test kit ClinMass® LC-MS/MS Complete Kit for 25-OH-Vitamin D2 / D3, MS7000 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany). Calibrators, control samples, analytical column and mobile phase solvents were provided by the kit. Sample preparation involved taking 50 µL of sample, adding to it 150 µL of precipitant solution (containing internal standard). Following incubation and centrifugation, 10 µL of supernatant was injected for analysis.
The LC-MS instrument was equipped with an atmospheric pressure chemical ionization source (APCI).

Analytical conditions

UHPLC:	Nexera X2 UHPLC
Column temperature:	40°C
Injection volume:	10 µL
Mass spectrometer:	LCMS-8050
Source conditions:	
Nebulizer Gas:	1.5 L/min
Interface temperature:	375 °C
Desolvation Line:	225 °C
Heat Block temperature:	250 °C
Drying Gas:	Off
Interface voltage:	4.5 kV
Dwell time:	25 msec
Pause time:	3 msec
Ionization:	APCI, positive mode
Scan Type:	MRM

Fig. 1 The FCV-32AH six port valve was configured to switch between sample loading (pump SPE) and isocratic sample elution (pump MP).

Starting conditions:
Pump SPE: 0.1 mL/min
Pump MP: 0.5 mL/min
FCV: position 1

Time (min)	Pump / FCV	Action	Setting
0.00	FCV	position 1	loading
0.00	Pump SPE	flow rate	0.1 mL/min
0.00	Pump MP	flow rate	0.5 mL/min
0.01	Pump SPE	flow rate	5 mL/min
0.75	FCV	position 0	elution
0.75	Pump SPE	flow rate	5 mL/min
0.85	Pump SPE	flow rate	0.1 mL/min
2.15	Pump SPE	flow rate	0.1 mL/min
2.20	FCV	position 1	conditioning
2.20	Pump SPE	position 1	2 mL/min
2.85	Pump SPE	flow rate	2 mL/min
2.90	Pump SPE	flow rate	0.1 mL/min
3.00	Pump SPE	flow rate	0. 1 mL/min
3.00	Pump MP	flow rate	0.5 mL/min

Table 1 LC parameters were chosen for rapid compound elution and fast analysis time.

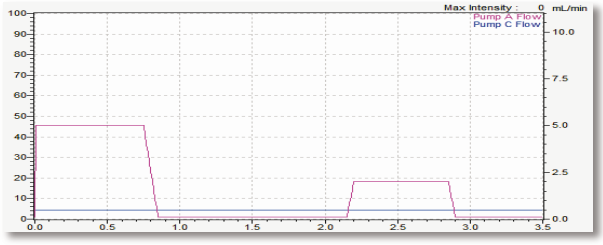


Fig. 2 LC time program during sample loading and elution.

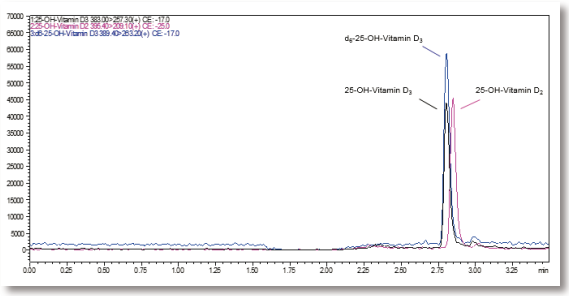
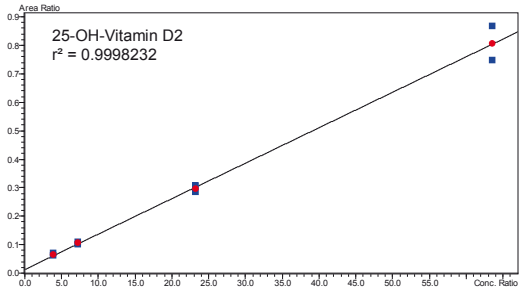


Fig. 3 LC-MS separation of 25-OH Vitamin D2 / D3 and deuterated standard in less than three minutes by isocratic chromatography.

Compound		Formula	MRM1	MRM2	RT
Vitamin D3	T	C27H44O	383>257	383>211	2.80
Vitamin D2	T	C28H44O	395>209	395>269	2.84
d6-Vitamin D3	I	C27H38D6O	389>263	389>211	2.80

Table 2 25-OH-Vit D optimized MRM transitions, retention times (RT). T/I = target or internal standard.



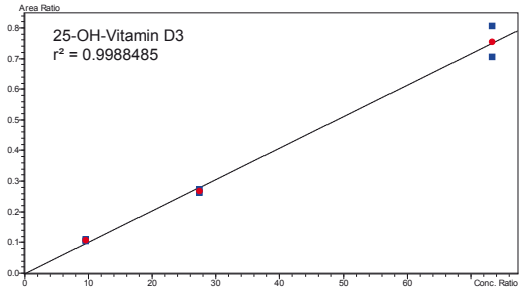


Fig. 4 Calibration curves for 25-OH Vitamin D2 and 25-OH Vitamin D3.

25-OH-Vitamin D2	Control Level I (16.3 µg/L)	Control Level II (36.6 µg/L)
	Conc.	Conc.
Control 1	16.631	40.649
Control 2	16.259	39.991
Control 3	16.437	39.770
Control 4	17.802	39.087
Control 5	17.159	41.588
Control 6	18.246	41.662
Control 7	16.450	39.186
Control 8	16.709	36.783
Mean	16.962	39.840
SD	0.717	1.576
%RSD	4.23	3.96

Table 3 Reproducibility for 25-OH-Vitamin D2

25-OH-Vitamin D3	Control Level I (20.5 µg/L)	Control Level II (44.3 µg/L)
	Conc.	Conc.
Control 1	21.553	48.244
Control 2	22.094	48.852
Control 3	20.529	45.402
Control 4	20.752	45.208
Control 5	21.786	49.444
Control 6	21.559	46.223
Control 7	20.902	47.329
Control 8	20.876	43.416
Mean	21.256	46.765
SD	0.563	2.064
%RSD	2.65	4.41

Table 3 Reproducibility for 25-OH-Vitamin D3

Results

The rapid elution of vitamin D3 and D2 by isocratic chromatography produced excellent peak shape and accuracy with elution in less than three minutes (Fig. 3).

The calibration curve determined in duplicate showed good linearity over a clinically relevant range of 3.94-63.6 µg/L for 25-OH-Vitamin D2 and 9.61-73.4 µg/L for 25-OH-Vitamin D3 (Fig. 4)

Two control samples at high and low concentration were analyzed in eightfold to measure analytical reproducibility. The percentage relative standard deviation was typically lower than 5% from these measurements.

Conclusion

The application of the clinical ClinMass® Complete Kit, for 25-OH-Vitamin D2/D3 in Plasma and Serum proved easy to implement and showed good sensitivity and linearity in a clinically relevant concentration range.

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Application News

LCMS-8050

No. SCA_210_017

Fast, sensitive and simultaneous analysis of multiple steroids in human plasma by UHPLC-MS/MS

■ Introduction

Accurate steroid measurement is essential to diagnose many disorders like sexual differentiation or gonadal function. Steroid metabolism is also important to study for many diseases (e.g. congenital adrenal hyperplasia, Cushing's disease, primary aldosteronism, endometriosis...).
Furthermore, since steroid metabolism is regulated by several mechanisms, a panel analysis may be of great advantage to get a complete picture or fingerprint. Method based on immunoassays suffer from a lack specificity, throughput and cannot be applied to multiple steroids in one sample. The last point is particularly important when only low sample volume are available like in pediatric diagnosis. In regard to these requirements, LC-MS/MS is gaining attention for steroid analysis since it offers specificity (LC separation of steroids and their conjugates), precision, sensitivity, low sample volume, automation and high throughput and profiling.
A simultaneous highly sensitive analysis method for 15 steroids by UHPLC-MS/MS was developed to support research or clinical studies.

■ Materials and methods

Chemicals

The used water and methanol were of ULC/MS quality (Biosolve). Dichloromethane was of LV-GC quality (Biosolve). Certified steroid standard solutions and stable isotope labelled steroid solutions were provided by Cerilliant (Sigma-Aldrich)

Calibration Standards and Quality Controls

A double charcoal stripped human plasma pool (6 donors, 3 males, 3 females) from whole blood collected on EDTA-K3 (Seralab, UK) was used to prepare the calibration standards. Plasma aliquotes were spiked using the same spiking volume at all levels representing not more than 0.5% of the total volume.
A calibration curve ranging from 0.5 to 15000 pg/mL for all compounds was prepared. The lowest limit of quantification was determined a posteriori for each compound with a signal to noise ratio of 10.

Sample Preparation

Sample preparation was performed using supported liquid extraction (SLE) Standards, QCs or samples (550 µL) were spiked with 10 µL of internal standard solution (each at a final concentration of 1 ng/mL) and diluted with 550 µL of ultrapure water. Diluted sample (1000 µL) was directly loaded on a SLE cartridge (1 mL, Biotage). After complete loading the sample was let in contact with sorbent for at least 5 minutes. Then compounds were eluted with 5 mL of dichloromethane. After evaporation to dryness under a stream of nitrogen at room temperature, the extracts were reconstituted in 50 µL of water/methanol 1/1 and transferred to a vial with glass insert and stored at 5 °C prior to analysis

■ UHPLC-MS/MS conditions

Two MRMs were selected for target compounds and one MRM for ISTDs.

Table 1: Analytical conditions

LC	
LC system	Nexera X2 (Shimadzu, Japan)
Column	Restek Raptor Biphenyl, 2.7 µm, 50 x 3 mm
Mobile Phase A	Water + additive
Mobile Phase B	Methanol * additive
Gradient Program	70 to 73% B in 1 min, 0.5% min at 73% B, 73 to 100% B in 2.5 min, 0.5 min at 100% B Total run time 5.8 min
Flow rate	0.8 mL/min
Column temp.	30 °C
Injection Volume	30 µL
MS	
MS system	LCMS-8050 (Shimadzu, Japan)
Ionization	Heated ESI (positive / negative)
Dwell time	8 to 73 ms, depending on the number of concomitant MRMs in order to get at least 30 points per peak/per MRM
Pause time	1 ms
Polarity switching	5 ms
Temperatures	Interface: 400 °C
	Heater block: 500 °C
	Desolvation line: 150 °C
Gas flows	Heated gas (Air): 10 L/min
	Drying gas (N_2): 10 L/min
	Nebulising gas (N_2): 3 L/min

■ Results

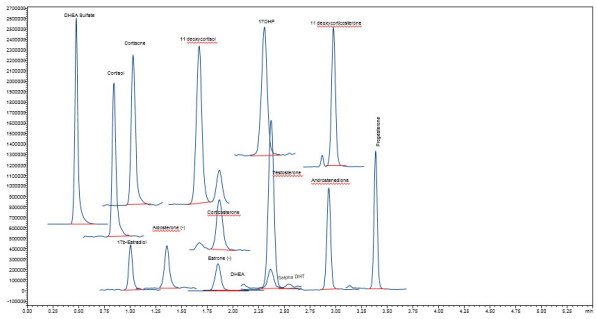

Figure 1: Plasma standard at 10 pg/mL (DHEA sulfate at 1 ng/mL)

Table 2: LOQ and Calibration ranges

Steroid	ISTD	LOQ	Linear Range
Aldosterone	D7-Aldosterone	2 pg/mL	0.002 – 15 ng/mL
Estradiol	D5-Estradiol	1 pg/mL	0.001 – 15 ng/mL
Testosterone	^{13}C3-Testosterone	2 pg/mL	0.002 – 15 ng/mL
5a-Dihydrotestosterone	D3-5a-DHT	25 pg/mL	0.025 – 15 ng/mL
11-Deoxycortisol	D5-11-Deoxycortisol	1 pg/mL	0.001 – 15 ng/mL
11-deoxycorticosterone	D5-11-Deoxycortisol	1 pg/mL	0.001 – 7.5 ng/mL
Corticosterone	D5-11-Deoxycortisol	1 pg/mL	0.001 – 7.5 ng/mL
Cortisol	D4-Cortisol	1 pg/mL	0.001 – 15 ng/mL
Cortisone	D4-Cortisol	5 pg/mL	0.005 – 15 ng/mL
17-Hydroxyprogesterone	^{13}C3-17-OHP	1 pg/mL	0.001 – 7.5 ng/mL
Androstenedione	^{13}C3-Androstenedione	1 pg/mL	0.001 – 15 ng/mL
Progesterone	D9-Progesterone	0.5 pg/mL	0.0005 – 7.5 ng/mL
Estrone	D5-Estradiol	0.5 pg/mL	0.0005 – 15 ng/mL
DHEA	D5-DHEA	10 pg/mL	0.01 – 15 ng/mL
DHEA Sulfate	D5-DHEA Sulfate	100 pg/mL	0.1 –1 500 ng/mL

Table 3: QC results for testosterone, estradiol and aldosterone

	QC A (50 pg/mL)			
	Calc. Conc.	Mean	Accuracy	%RSD
Testosterone	58.9 55.4	57.1	114%	4.3%
Estradiol	53.1 53.7	53.4	107%	0.7%
Aldosterone	55.2 58.5	56.8	114%	4.1%
	QC B (100 pg/mL)			
	Calc. Conc.	Mean	Accuracy	%RSD
Testosterone	112 96.9	104	104%	10.0%
Estradiol	102 94.8	98.6	98.6%	5.4%
Aldosterone	111 102	106	106%	6.3%
	QC C (750 pg/mL)			
	Calc. Conc.	Mean	Accuracy	%RSD
Testosterone	653 700	676	90.2%	4.8%
Estradiol	676 689	683	91.0%	1.4%
Aldosterone	677 714	695	92.7%	3.8%
	QC D (7500 pg/mL)			
	Calc. Conc.	Mean	Accuracy	%RSD
Testosterone	6573 6904	6739	89.8%	3.5%
Estradiol	7026 7111	7069	94.2%	0.8%
Aldosterone	6290 7167	6728	89.7%	9.2%

■ Conclusion

A method for panel steroid analysis was set up. A high sensitivity was reached in order to be able to manage low sample volumes or to monitor deficiency disease levels. The simple sample preparation and the fast analysis enable to assay large sample numbers to support research or clinical studies.

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Application News

No. AD-0067

LCMS-8040 UFMS

Ultra sensitive LC/MS/MS Method for Quantitative Determination of Ethinyl Estradiol in Human Plasma

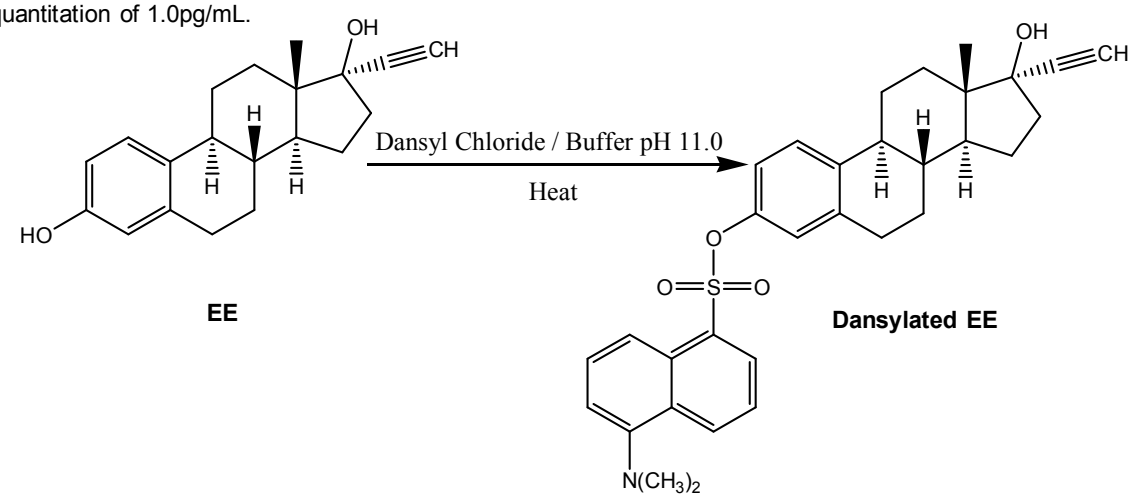
Manikandan Venkateswaran, Vinod Pillai. S.L, Venkat Manohar and Arvind Thyagarajan
*Indian Institute of Chromatography & Mass Spectrometry (IICMS)**

Abstract

An ultra sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for quantitative analysis of oral contraceptive Ethinyl estradiol (EE) has been developed in human plasma using UHPLC NEXERA coupled to LCMS-8040 triple quadrupole mass spectrometer and described here. A two stage sample clean-up and also off-line derivitisation using Dansyl chloride enabled ultra sensitivity up to 1.0 pg/mL using 750µL of human plasma. The method was found to be linear in the range of 1 -200pg/mL.

❑ Introduction

Ethinyl estradiol (EE) is a potent synthetic estrogen that is widely used therapeutically as an oral contraceptives primarily because of its high estrogenic activity. It is also used for treatment of menopausal and post menopausal symptoms, treatment of female hypogonadism, osteoporosis and as a palliative care treatment in malignant neoplasms of breast and prostrate. A very sensitive method for estimation of EE from human plasma using the UHPLC Nexera connected to a LCMS8040 triple quadrupole mass spectrometer is described here. Ethinyl estradiol (EE) and the internal standard (Ethinyl estradiol-D4) were extracted from plasma matrix using Hexane: Methyl-Tert.Butyl Ether (MTBE) mixture (50:50 v/v), derivitised with Dansyl chloride, then further cleaned up using Sola CX (10mg/1mL) SPE cartridge and injected onto LC/MS/MS system. Chromatographic separation of EE from other related estrogenic peaks were achieved using a Purospher star RP18, 100 x 2.1mm, 2.0µ column with a 0.1% Formic acid in water and ACN as mobile phase. The offline derivitisation procedure using the Dansyl chloride lead to the introduction of easily ionizable tertiary amino group in the EE moiety that greatly improved the analyte sensitivity in the electrospray ionization and enabled achieving the desired lower limit of quantitation of 1.0pg/mL.



EE **Dansylated EE**

***Figure1**: Chemical structures of Ethinyl Estardiol(EE) and its dansylated product*

❑ Experimental

Preparation of aqueous standards: Stock solutions of EE was prepared separately at 1.0mg/mL concentration in methanol. The solution was further diluted with water : methanol (50:50) mixture to get intermediate standards at various concentrations ranging from 20-4000pg/mL. Similarly, stock solution of Ethinyl estradiol-D4 (IS) was prepared at 1.0mg/mL concentration in methanol. This solution was further diluted with water : methanol (50:50) mixture to get intermediate standard at concentration of 2000pg/mL.

Preparation of Plasma calibration standards (CC): 713µL of human plasma was spiked with 37.5 µL of each aqueous EE standard solution and 50 µL of IS solution in a polypropylene (PP) tube with cap, vortexed for 15 seconds to obtain plasma calibration standard whose concentration ranged from 1.0 – 200.0pg/mL. Each of these samples were then extracted according to the procedure as described under sample preparation. Care was taken to make sure that each vial was immediately capped to avoid contamination.

** Address: No. 7, Chakrapani Road, Guindy, Chennai - 600 032, Tamil Nadu, India*

Preparation of plasma quality control standards (QC): The Quality control standard solutions were prepared at three intermediate concentrations of that of CC standards namely 3.0, 90.0 and 180.0pg/mL (LQC, MQC and HQC respectively). Six individual preparations of each of the QC standards were prepared to evaluate precision and recovery. Each of these sample preparation were then extracted according to the procedure as described under sample preparation.

Sample preparation: Ethinyl estardiol as such has only a limited ionization in the ESI source. Hence in order to enhance ionization, a derivitisation with dansyl chloride has been suggested in the literature. A liquid-liquid extraction was used first to extract the drug from the plasma matrix followed by derivitisation with dansyl chloride in a water bath. After dansylation, the sample is cleaned up through an additional SPE step and then introduced onto the LC/MS/MS system. By this way of a two stage sample cleanup, most of the phospholipids from the plasma matrix are removed and other closely related estrogenic compounds that are present are either removed or well resolved from the EE.

To 750µL of plasma sample in a PP vial, added 100µL of 0.1M hydrochloric acid and briefly mixed for 10s. Added 2.5mL of mixture of Hexane and MTBE in the ratio 50:50 v/v. The drug was extracted by vortexing for 15 minutes followed by centrifugation for 5 minutes at 4000 rpm. Two mLs of the top organic layer was removed and evaporated using an Xcelvap solvent evaporator at 45˚C under stream of nitrogen. To the residue added 100µL of sodium carbonate solution(100mM) followed by 150µL of dansyl chloride solution (0.1mg/mL in acetone). The mixture was heated in a water bath at 60˚C for 15 minutes. The extracts were mixed with 500µL of water and then transferred to a Sola CX (10mg/1mL) cartridge that has been already pre-conditioned and equilibrated with 1000µL each of methanol and water. The cartridge was washed with 1000µL each of 5% methanol in water (two times) followed by 20% methanol in water and eluted with 2 x 200µL of Acetonitrile and 2-propanol mixture in the ratio 90:10 v/v. The eluate was directly injected into the LC/MS/MS system.

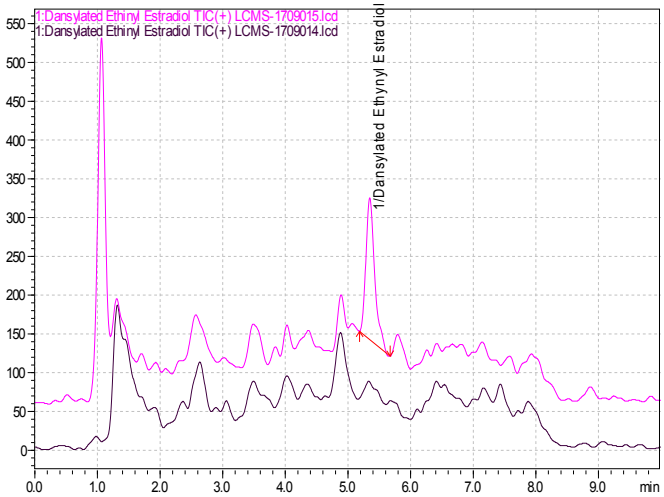


***Figure 2**: Representative overlay chromatograms of EE at LLOQ with blank*

The LC-MS conditions are as summarized as in Table-1.

***Table 1:** Analytical conditions*

Column	: Puropsher star RP18, 100 x 2.1mm, 2.0µm		
Mobile phase-A	: 0.1% v/v Formic acid in water		
Mobile phase-B	: Acetonitrile		
Gradient	: 65%B initial to 85%B in 2 min, , ramp to 90%B in 2.5 min, , ramp to 95%B in 1.0 min, hold at 95%B for 0.5 min, back to 65% in 0.1 min, equilibrate at 65%B for 3.9 minutes.		
Flow rate	: 300 µL/min	DL temp	: 250 ^{0}C
Column temp	: 35 ^{0}C	Heat block	: 400 ^{0}C
Drying gas	: 20 L/min	Interface	: ESI
Nebulizing gas	: 3.0 L/min	Interface volt	: 4.5 kV
Injection volume	: 15.0 µL	Run time	: 10 min.
For EE			
MRM	: 529.90 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: - 39.0V
Q1 pre-bias	: -20.0V	Q3 pre-bias	: -32.0V
For IS			
MRM	: 534.00 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: - 41.0V
Q1 pre-bias	: -40.0V	Q3 pre-bias	: -32.0V

❑ Results and Discussion

LLOQ

The concentration of EE at lower limit of quantitation (LLOQ) was determined to be 1.0pg/mL. This was confirmed from the coefficient of variance (%CV) of 15.08% for the six replicate injections of EE at this concentration. A representative mass chromatogram of EE at its LLOQ concentration and overlaid with blank is as presented in Figure-2.

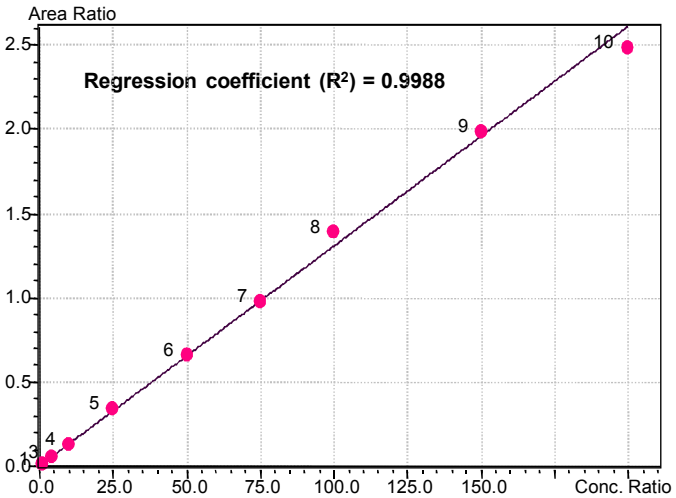


***Figure-3** : Calibration curve of EE*

Linearity

The CC standards were used to construct a calibration curve by plotting the area ratio of EE with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). A linear dynamic range of 1.0 to 200.0pg/mL was achieved for EE with a R^2 value of 0.9988 that meets the acceptance criteria. Figure-3 shows a representative calibration curve of EE in plasma using Ethinyl estradiol-D4 as internal standard. Table-2 summarizes the back calculated concentrations obtained for the calibration standards.

***Table -2**: Accuracy of EE in CC samples*

Nominal Concentration (pg/mL)	Measured Concentration (pg/mL)	Accuracy*
1.0	1.0	100.5
4.0	4.0	98.8
10.0	9.7	97.2
25.0	25.5	102.0
50.0	49.9	99.8
75.0	74.3	99.1
100.0	106.5	106.5
150.0	151.9	101.3
200.0	189.8	94.9

* Expressed as Bias = (mean concentration / nominal concentration) x 100

Precision & Accuracy of QC samples

Low, middle and high QC samples containing EE were prepared at concentrations of 3.0, 90.0 and 180.0pg/mL in plasma. The precision (%CV, n=6) for the QCs for EE varied from 3.3 to 9.8 % while the average percent accuracy for QC samples were 96.6% (Table-3). A representative mass chromatogram of MQC and HQC are shown in Figure 4 and 5.

***Table -3**: Precision and accuracy of EE in QC samples*

Nominal Conc. (pg/mL)	Measured conc. (pg/mL)	Accuracy*	Precision (n=6)
3.0	2.7	90.0	9.8
	3.4	113.3	
	2.7	90.0	
	2.9	96.7	
	2.7	90.0	
	2.7	90.0	
90.0	89.6	99.6	3.3
	86.8	96.4	
	82.3	91.4	
	87.7	97.4	
	86.8	96.4	
	83.1	92.3	
180.0	183.0	101.7	3.6
	186.8	103.8	
	176.5	98.0	
	176.4	98.0	
	180.3	100.2	
	168.2	93.4	

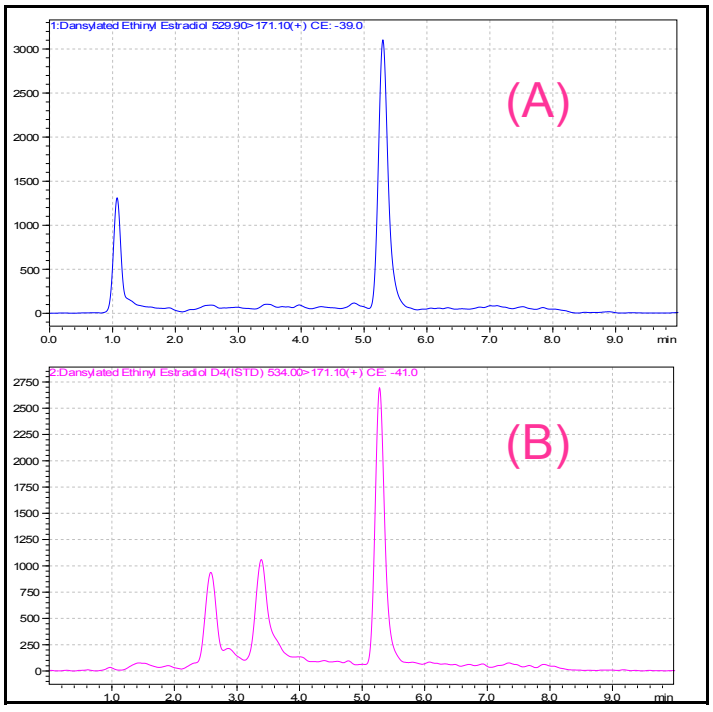


***Figure-4**: Mass chromatogram of EE (A) and IS (B) at MQC*

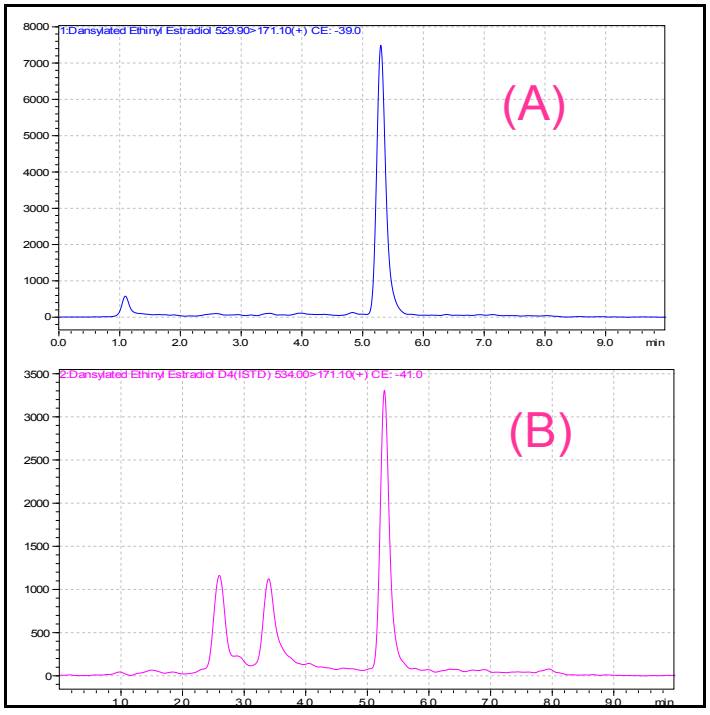


***Figure-5** : Mass chromatogram of EE (A) and IS (B) at HQC*

Recovery of QC samples

The recovery of EE was calculated by comparing the peak area obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. The Average recovery across QC concentration is 64.5% and is found to be consistent across all the three levels.

❑ References

1. *Drug Discov Ther* 2007; 1(2): 108-118
2. *Rapid Commun. Mass Spectrom.* 2004; 18: 1621-1628
3. *Biomed. Chromatogr*. 2004; 18: 414 - 421
4. *J Chrom B*. 2005; 825: 223 - 232

❑ Conclusions

An ultra sensitive method capable of detecting Ethinyl Estradiol to as low as 1.0pg/mL has been developed and evaluated using the LCMS-8040 triple quadrupole mass spectrometer. An important aspect of this method is the sample preparation which involves a liquid-liquid extraction, derivitisation followed by solid-phase extraction. The method focuses on the clean up of the analyte from the biological matrix whereby the sensitivity of the method is enhanced greatly. A linear range of 1.0 – 200.0pg/mL has been established with a regression value of 0.9988. The average percent accuracy for EE was 100.0% for the standard curve samples and 96.6 for the QC samples. No interference peak was observed in the plasma blank demonstrating effective removal of all biological matrix and other related endogenous compounds during extraction. Hence the developed method shows significant promise for applications that need ultra-low level detection of EE in plasma samples.

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Application News

No. SCA_210_003

LCMS

Determination of Methylmalonic Acid in Serum, Plasma and Urine by LCMS-8050 using RECIPE ClinMass Complete Kit , advanced MS5100

Irene Doering (RECIPE Chemicals + Instruments GmbH), Anja Grüning (Shimadzu Europa GmbH)

Introduction

Measurement of methylmalonic acid (MMA) is used as a specific diagnostic marker for the group of disorders known collectively called as methylmalonic acidemias.
The metabolic pathway involves methylmalonyl-coenzyme A (CoA) being converted into succinyl-CoA. Vitamin B_{12} is also needed for this conversion. Therefore measurement of MMA can be used to diagnose a number of genetic disorders in this pathway and is elevated in 90-98% of patients with B_{12} deficiency.
Typically the concentration of MMA is low and normally requires off-line extraction before analysis, however in these experiments a sensitive method was developed requiring minimal pre-treatment with just 3 µL sample injection to achieve suitable detection.

Analytical conditions

UHPLC:	Nexera X2 UHPLC
	0.7 mL/min (start 0% B)
Mixer:	180 µL
Column temperature:	25 °C
Injection volume:	3 µL
Mass spectrometer:	LCMS-8050
Source conditions:	
Nebulizer Gas:	3 L/min
Heating Gas:	12 L/min
Interface temperature:	400 °C
Desolvation Line:	175 °C
Heat Block temperature:	325 °C
Interface voltage:	-1.5 kV
Dwell time:	50 msec
Pause time:	3 msec
Ionization:	Electrospray ionization (ESI) negative mode
Scan Type:	MRM

Materials and methods

The LCMS-8050 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system. MMA was measured using a commercially available test kit ClinMass® Complete Kit, advanced for Methylmalonic Acid in Serum, Plasma and Urine, MS5100 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany). Calibrators, control samples, analytical column and mobile phase solvents were provided by the kit. 100 µL of sample was added to 400 µL of precipitant solution (containing internal standard). Following centrifugation 3 µL of supernatant was analysed.
For analysis the $[M-H]^-$ ion was measured and used as the precursor ion (negative electrospray ionization).

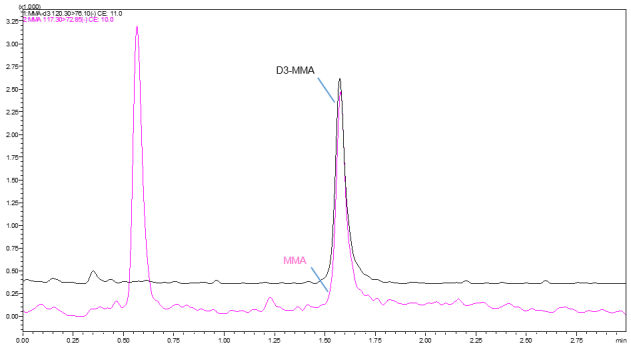

Fig. 1 LC-MS separation of MMA and deuterated standard in under three minutes by fast chromatography.

Table 1 MMA optimized MRM transitions, retention time (RT). T/I = target or internal standard

Compound		Formula	MRM1	MRM2	RT
MMA	T	$C_4H_6O_4$	117.3>72.8	117.3>55.0	1.57
D3-MMA	I	$C_4H_3D_3O_4$	120.3>76.1	120.3>58.0	1.57

Table 2 LC parameters were chosen for rapid compound elution and fast analysis time.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (mL/min)
0.00	100	0	0.7
0.30	100	0	0.7
0.31	70	30	0.7
0.60	70	30	0.7
0.61	40	60	0.7
1.30	40	60	0.7
1.31	0	100	0.7
1.40	100	0	0.7
3.00	100	0	0.7

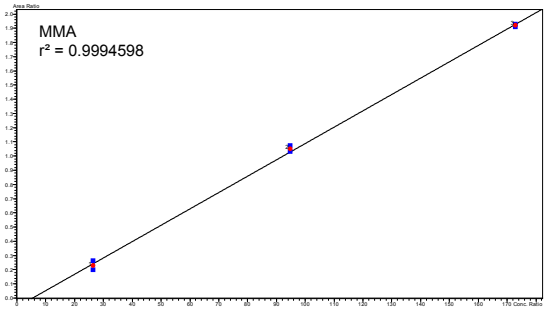

Fig 2 Calibration curve for methylmalonic acid (concentration range 26.4-173 µg/L)

Results

The rapid separation of MMA produced good peak shape and was eluted in less than two minutes.
The calibration curve determined in duplicate showed good linearity over a clinically relevant range 26.4-173 µg/L (Fig. 2).

Conclusion

The application of the clinical ClinMass® Complete Kit, advanced for Methylmalonic Acid in Serum, Plasma and Urine proved easy to implement and showed good sensitivity and linearity in a clinically relevant concentration range.

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Application News

No.C90

Liquid Chromatography Mass Spectrometry

Measurement of Adenosine Deaminase Activity in Urine with LCMS-8040

Adenosine deaminase (ADA) is an enzyme involved in the metabolism of nucleic acid within the cell, and converts the nucleic acid bases adenosine (ADO) and deoxyadenosine (dADO) into inosine and deoxyinosine, respectively (Fig. 1). We analyzed for adenosine and deoxyadenosine as indicators in checking for ADA enzyme activity.
Here we describe an example analysis performed using an LCMS-8040 high-performance liquid chromatograph-triple quadrupole mass spectrometer and employing an analytical protocol used by the Mass Spectrometry, Clinical Chemistry and Pharmacology Lab. of Meyer Children's Hospital (Florence, Italy).

Sample Preparation and Analytical Conditions

Samples for analysis were extracted from urine in accordance with the preparation method shown in Fig. 2. Samples extracted from plasma and a dried blood spot (DBS) can also be analyzed, and the relevant preparative methods are shown in Fig. 2 for reference. LC and MS conditions are shown in Table 1. Multiple reaction monitoring (MRM) was performed with adenosine and deoxyadenosine as the target compounds and using ^{13}C-adenosine and $^{13}C_5$-deoxyadenosine as internal standards.

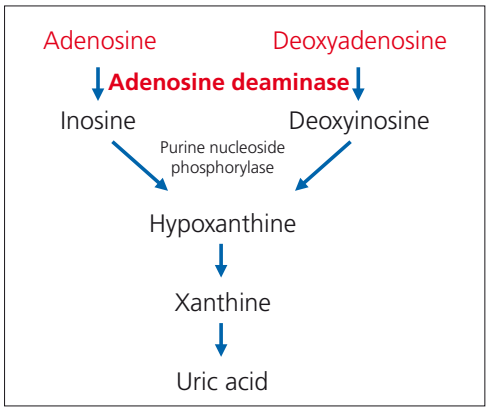

Fig. 1 Metabolic Pathway

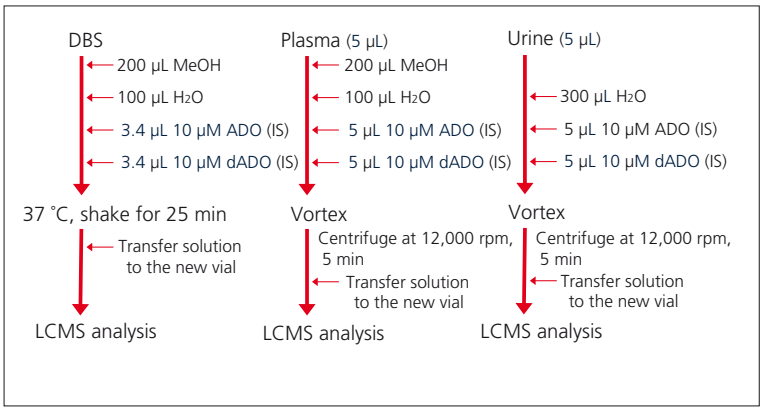

Fig. 2 Preparation Protocol

Table 1 Analytical Conditions

Column	: Synergi fusion RP (150 mm L. × 2 mm I.D., 4 µm)	Ionization Mode	: ESI(+)
Mobile Phase A	: 0.1 % HCOOH-H_2O	Probe Voltage	: +4.5 kV
Mobile Phase B	: 0.1 % HCOOH-CH_3CN	Nebulizing Gas Flow	: 3.0 L/min
Ratio	: 60 %B	Drying Gas Fow	: 15.0 L/min
Flowrate	: 0.2 mL/min	DL Temperature	: 200 °C
Column Temperature	: 30 °C	Block Heater Temperature	: 400 °C
Injection Volume	: 3 µL	MRM	: Adenosine (267.80>136.05)
Analysis Time	: 5 min		Deoxyadenosine (251.80>136.05)
MS	: LCMS-8040		Adenosine IS (268.80>136.05)
			Deoxyadenosine IS (256.80>136.05)

Analysis Results

Results of analysis are shown in Fig. 3. The "Sample" plot shows when there is no ADA enzyme activity in the sample, and the "Control" plot shows when ADA enzyme activity is present in the sample. A peak representative of deoxyadenosine was detected in the "Sample" plot results, and no deoxyadenosine peak was detected in the "Control" plot results. This analytical system can be used to check for enzyme activity.

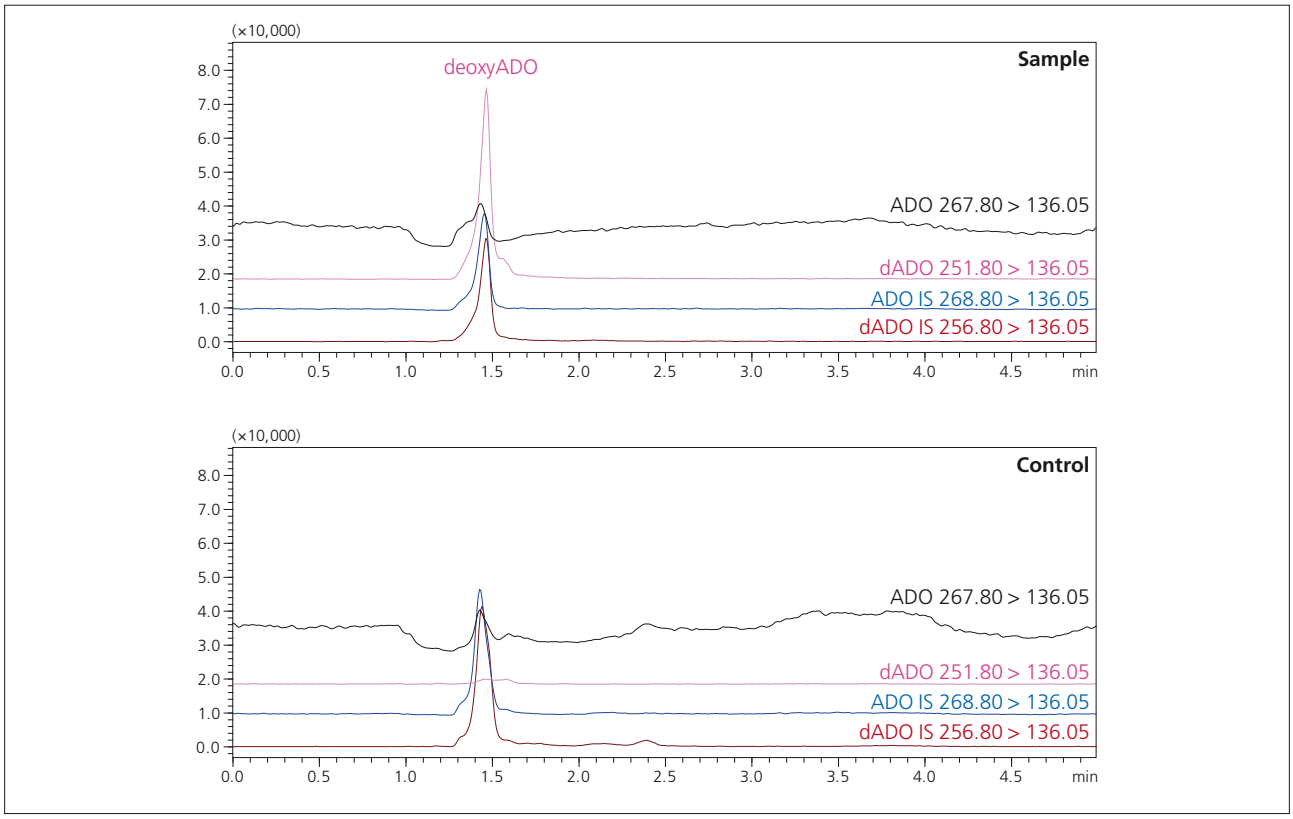

Fig. 3 Extracted-Ion Chromatograms of Target Compounds

[References]
G la Marca et al. The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 88 (2014) 201-206
G la Marca et al. Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase defficiency. J ALLERGY CLIN IMMUNOL VOLUME 131, NUMBER 6 (2013) 1604-1610

[Acknowledgement]
The present Application News was prepared with the assistance of materials and guidance provided by Dr. G. la Marca (Mass Spectrometry, Clinical Chemistry and Pharmacology Lab., Meyer Children's Hospital, Florence, Italy). We are sincerely grateful for his assistance.

Note: This analytical system may only be used for research applications, and may not be used for clinical diagnosis.

First Edition: Apl. 2014

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Application News

No.C91

Liquid Chromatography Mass Spectrometry

Measurement of Fumarylacetoacetate Activity in DBS (Dried Blood Spot) with LCMS-8040

The intermediate metabolites fumarylacetoacetate and succinylacetone (SuAC) are used as indicators when analyzing for fumarylacetoacetate hydrolase activity, which is an enzyme involved in amino acid metabolism (Fig. 1).
Here we describe an example analysis for succinylacetone performed using an LCMS-8040 high-performance liquid chromatograph-triple quadrupole mass spectrometer and employing an analytical protocol used by the Mass Spectrometry, Clinical Chemistry and Pharmacology Lab. of Meyer Children's Hospital (Florence, Italy).

Sample Extraction from DBS and MS Analysis

Filter paper blotted with blood (dried blood spot, DBS) was used to prepare the analytical sample. After cutting a 3.2-mm diameter disk from a DBS, samples were extracted in accordance with the protocol described in Fig. 2. Samples extracted from plasma and urine can also be analyzed, and the relevant preparative methods are shown for reference.
LC and MS conditions are shown in Table 1. Multiple reaction monitoring (MRM) was performed with succinylacetone as the target compound and using $^{13}C_4$-succinylacetone as an internal standard.

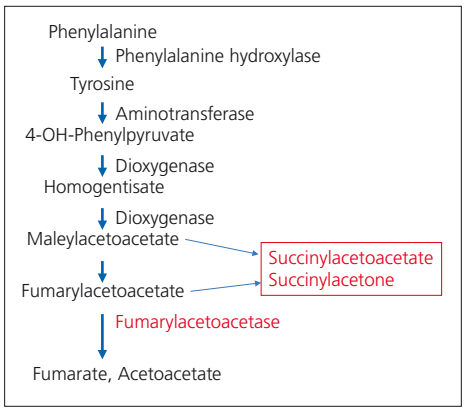

Fig. 1 Metabolic Pathway

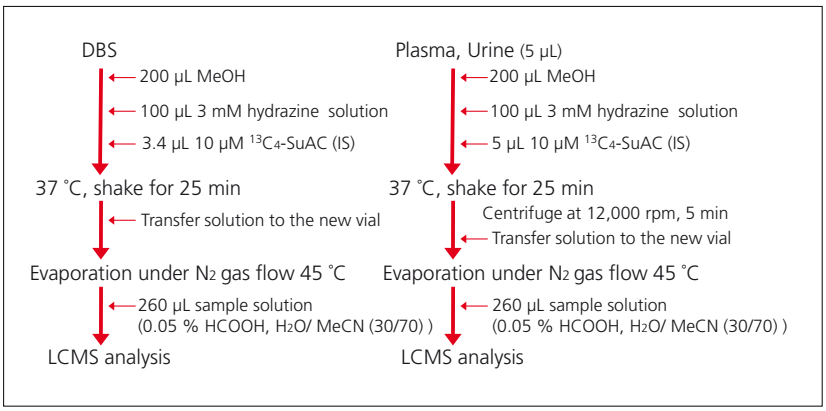

Fig. 2 Preparation Protocol

Table 1 Analytical Conditions

Column	: Synergi POLAR-RP (150 mm L. × 2.0 mm I.D., 4 µm)	Ionization Mode	: ESI (+)
Mobile Phase A	: 0.1 % HCOOH-H_2O	Probe Voltage	: +4.5 kV
Mobile Phase B	: CH_3OH	Nebulizing Gas Flow	: 2.5 L/min
Ratio	: 80 %B	Drying Gas Flow	: 15.0 L/min
Flowrate	: 0.2 mL/min	DL Temperature	: 250 °C
Column Temperature	: 30 °C	Block Heater Temperature	: 400 °C
Injection Volume	: 5 µL	MRM	: Succinylacetone (154.8 > 136.9)
Analysis Time	: 4 min		$^{13}C_4$-Succinylacetone (158.9 > 141)

Analysis Results

Results of analysis are shown in Fig. 3. The "Sample" plot shows when there is no fumarylacetoacetate hydrolase activity present in the sample, and the "Control" plot shows when fumarylacetoacetate hydrolase activity is present in the sample. A DBS where the filter paper was blotted with blood spiked with succinylacetone was used as a positive control. A peak representative of succinylacetone was detected in the "Sample" plot results. This analytical system can be used to check for enzyme activity.

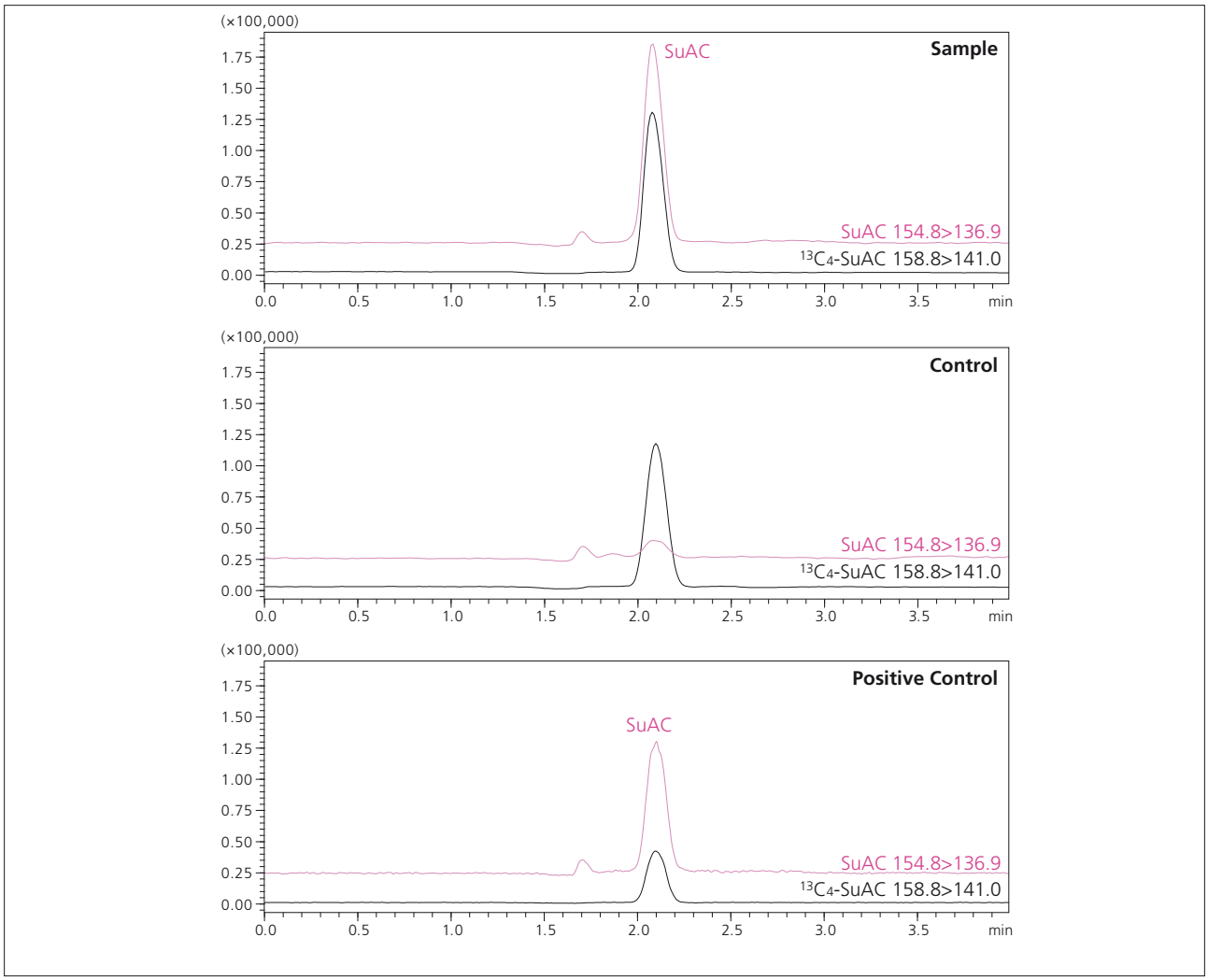

Fig. 3 Extracted-Ion Chromatograms of Target Compounds

[References]
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la Marca G, et al. The successful of succinylacetone as a marker of tyrosinaemia type I in Tuscany newborn screening program.. Rapid Commun. Mass Spectrom. 23 (2009) 3891-3893

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Note: This analytical system may only be used for research applications, and may not be used for clinical diagnosis.

First Edition: Apl. 2014

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Application News

No. SCA_210_005

LCMS

Determination of Homocysteine in Plasma / Serum by LCMS-8050 using RECIPE ClinMass Complete Kit, MS2000

Irene Doering (RECIPE Chemicals + Instruments GmbH), Anja Grüning, Ute Potyka (Shimadzu Europa GmbH)

Introduction

Homocysteine is a sulfurous α-amino acid of the methionine metabolism. It is biosynthesized from methionine and a homologue of the amino acid cysteine, differing by an additional methylene bridge (-CH2-). An enhanced blood homocysteine concentration is a medical condition which is termed as hyperhomocysteinemia. It is considered as an independent risk factor for degenerative vascular diseases, which may lead to heart attack, stroke and thromboses.

In the healthy cell metabolism homocysteine is a short-living intermediate product, which is either transformed or removed by various metabolic pathways. The malfunction of the methionine metabolism (see Fig. 1) therefore causes an increase of the cellular homocysteine concentration and the release of homocysteine into the blood.

Hereby, the central metabolic position of homocysteine at the intersection between the catabolic transsulfuration and the remethylation cycle plays an important role. During this catabolic degradation of homocysteine, vitamins B12 and B6, as co-factors of the involved enzymes, play a crucial role. Weak and moderate hyperhomocysteinemias are often caused by a vitamin B deficiency (B6, B12, folic acid).

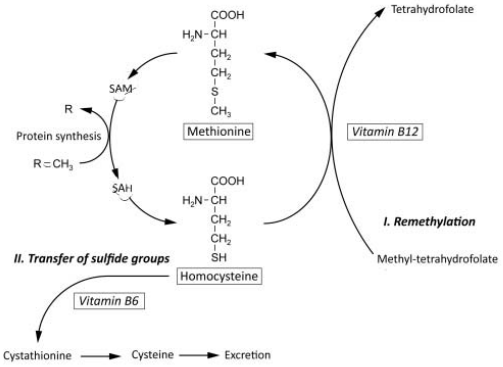

Figure 1: Metabolic pathway of Methionine

Materials and methods

The LCMS-8050 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system. Homocysteine was measured using the commercially available ClinMass® Complete Kit for Homocysteine (total homocysteine) in Plasma and Serum, MS2000 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany). Calibrators, control samples, analytical column and mobile phase solvents were provided by the kit. Prior to the LC-MS/MS analysis a sample preparation is performed. The samples are spiked with an internal standard (50 µl), the bound homocysteine is released (reduction) and after a precipitation step, 1 µl of the supernatant can be injected.
The samples were analysed in MRM-Mode. The optimized MRM transitions are listed in Table 1.

Analytical conditions

UHPLC:	*Nexera X2 UHPLC*
	0.7 mL/min (start 100% mobile phase, isocratic)
Column temperature:	*25°C*
Injection volume:	*1 µL*
Mass spectrometer:	*LCMS-8050*
Source conditions:	
Nebulizer Gas:	*3 L/min*
Heating Gas:	*10 L/min*
Drying Gas:	*10 L/min*
Interface temperature:	*350 °C*
Desolvation Line:	*200 °C*
Heat Block temperature:	*400 °C*
Interface voltage:	*2 kV*
Dwell time:	*100 msec*
Pause time:	*3 msec*
Ionization:	*Electrospray ionization (ESI) positive mode*
Scan Type:	*MRM*

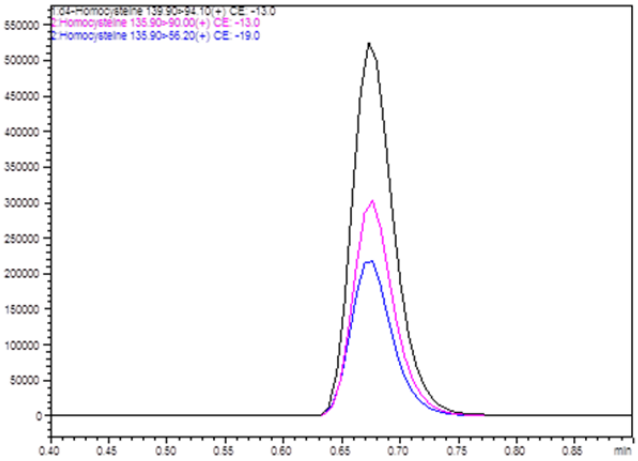

Figure 2: Chromatogram of Homocysteine (pink and blue line) and Internal Standard D4-Homocysteine (black line)

Table 1: optimized MRM transitions of Homocysteine and Internal Standard

Compound	Formula	MRM Quant.	MRM Qual.	Retention time
Homocysteine	$C_4H_9NO_2S$	136.1 > 90.1	136.1 > 56.1	0.68 min
D4-Homocysteine	$C_4D_4H_5NO_2S$	140.1 > 94.1		0.68 min

Results

Using the ClinMass® Complete Kit of Recipe analysis of Homocysteine in Plasma and Serum can be performed quite easily and very fast, in less than 1 minute, showing a good peak shape (Fig. 2). The presented calibration curve demonstrates good linearity over a range of 0.75 to 6.84 mg/L (Fig. 3).

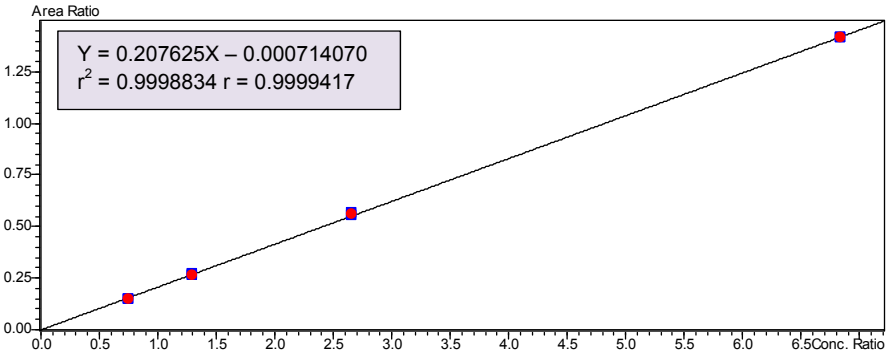

Figure 3: Calibration curve of Homocysteine with some statistical details

Conclusion

The application of the clinical ClinMass® Complete Kit for Homocysteine in Serum and Plasma proved to be easy to implement and showed good sensitivity and linearity in a clinically relevant concentration range.

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Application News

No.C92

Liquid Chromatography Mass Spectrometry

Measurement of Homocysteine in Plasma with LCMS-8040

Homocysteine is used as an indicator when analyzing for activity of enzymes involved in the methionine metabolism pathway, such as methionine synthase and methylenetetrahydrofolate reductase (Fig. 1).
Here we describe an example analysis for homocysteine performed using an LCMS-8040 high-performance liquid chromatograph-triple quadrupole mass spectrometer and employing an analytical protocol used by the Mass Spectrometry, Clinical Chemistry and Pharmacology Lab. of Meyer Children's Hospital (Florence, Italy).

Sample Preparation and Analytical Conditions

Samples for analysis were extracted from plasma specimens in accordance with the preparation method shown in Fig. 2. LC and MS conditions are shown in Table 1. Multiple reaction monitoring (MRM) was performed with homocysteine as the target compound and using d8-homocysteine as an internal standard.

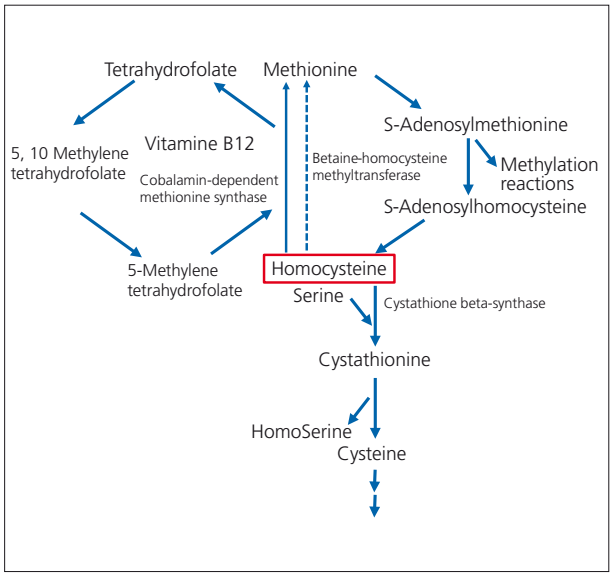

Fig. 1 Metabolic Pathway

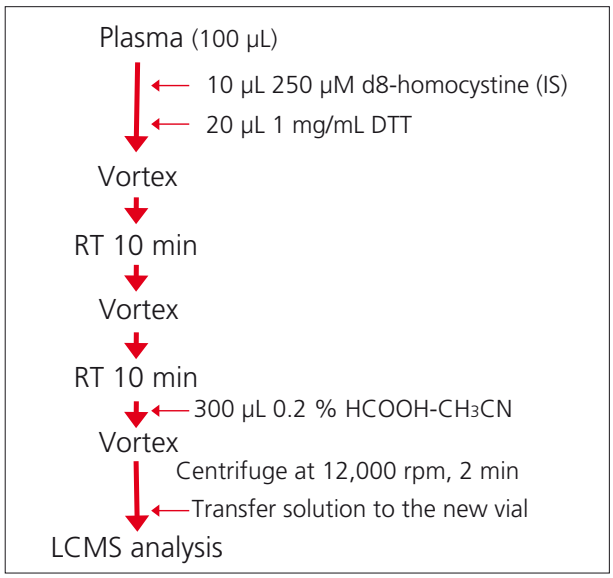

Fig. 2 Preparation Protocol

Table 1 Analytical Conditions

Column	: SUPELCO SIL LC-CN (33 mm L. × 3.0 mm I.D., 3 µm)	Ionization Mode	: ESI (+)
Mobile Phase A	: 0.1 % HCOOH-H_2O	Probe Voltage	: +4.5 kV
Mobile Phase B	: CH_3CN	Nebulizing Gas Flow	: 3.0 L/min
Ratio	: 70 %B	Drying Gas Flow	: 15.0 L/min
Flowrate	: 0.45 mL/min	DL Temperature	: 200 °C
Column Temperature	: 30 °C	Block Heater Temperature	: 350 °C
Injection Volume	: 1 µL	MRM	: Homocysteine (135.8 > 90.1) d4-Homocysteine (IS) (139.8 > 94.1)
Analysis Time	: 5 min		

Analysis Results

Results of analysis are shown in Fig. 3. The "Sample" plot shows when there is no methionine synthase or methylenetetrahydrofolate reductase activity present in the sample. The "Control" plot shows when these enzymes are active and present in the sample. The "Positive Control" plot shows when homocysteine is added to the plasma specimen in advance. A peak specific to homocysteine was detected in the "Sample" plot results. This analytical system can be used to check for enzyme activity.

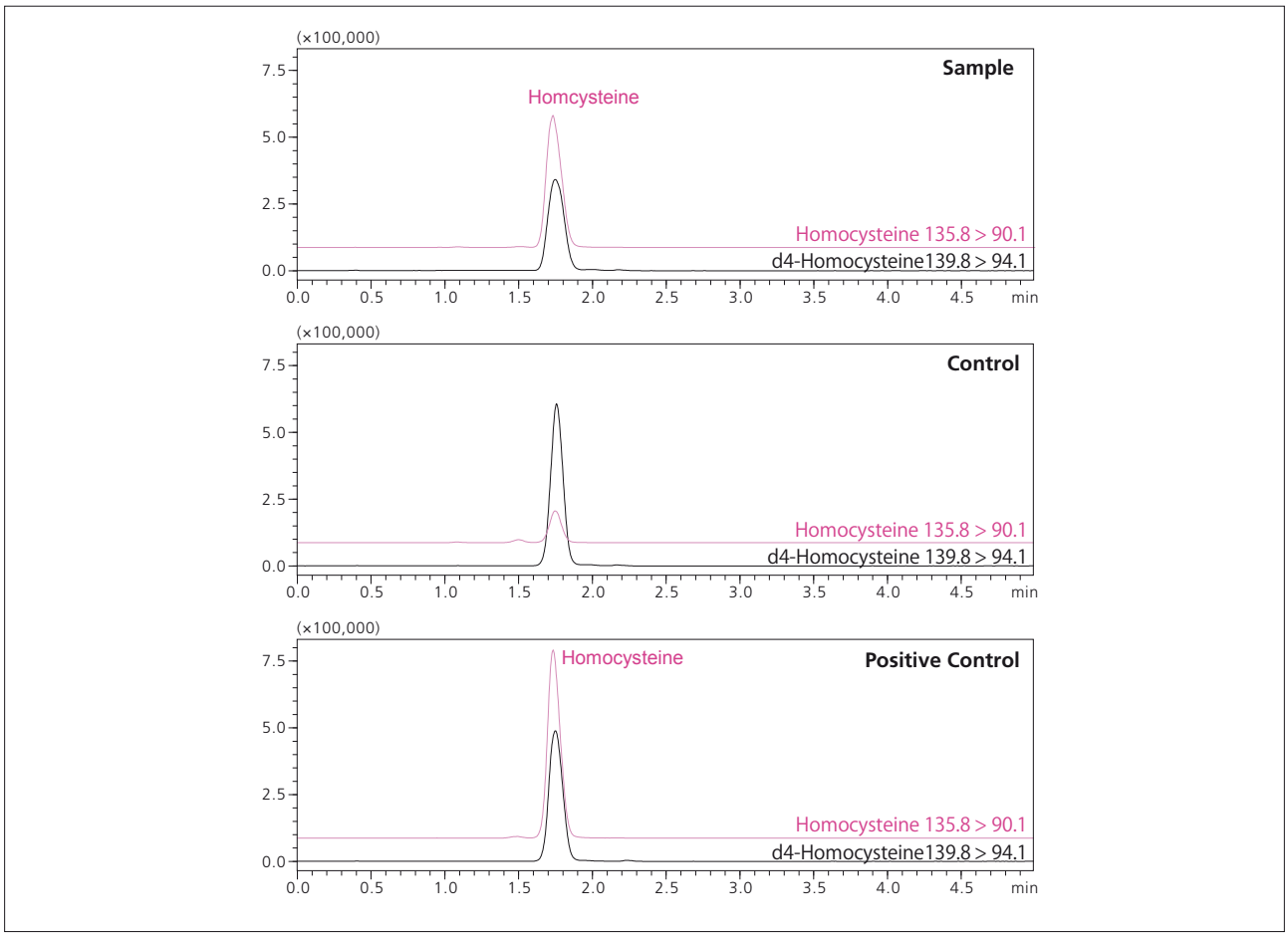

Fig. 3 Extracted-Ion Chromatograms of Target Compounds

[References]
la Marca G, et al. Progress in expanded newborn screening for metabolic conditions by LC-MS/MS in Tuscany: Update on methods to reduce false positive. JIMD Short report #127 (2008)
J. Magera M, et al, Method for the determination of total homocysteine in plasma and urine by stable isotope dillution and electrospray tandem mass spectrometry. Clinical Chemistry 45:9 (1999) 1517-1522

[Acknowledgement]
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First Edition: May. 2014

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No.C93

Liquid Chromatography Mass Spectrometry

Measurement of Methylmalonic Acid, 3-OH Propionic Acid and Succinic Acid in DBS (Dried Blood Spot) with LCMS-8040

Compounds such as Methylmalonic acid (MMA), propionylcarnitine, 3-OH propionic acid (3-OH PA), and succinic acid (SA) can be used as indicators when analyzing for methylmalonyl-CoA mutase and propionyl-CoA carboxylase activity, which are enzymes involved in amino acid, cholesterol, and fatty acid metabolism (Fig. 1).
Here we describe an example analysis performed using an LCMS-8040 high-performance liquid chromatograph-triple quadrupole mass spectrometer and employing an analytical protocol used by the Mass Spectrometry, Clinical Chemistry and Pharmacology Lab. of Meyer Children's Hospital (Florence, Italy).

Sample Extraction from DBS and MS Analysis

Filter paper blotted with blood (dried blood spot, DBS) was used to prepare the analytical samples. After cutting a 3.2-mm diameter disk from a DBS, samples were extracted in accordance with the preparation method shown in Fig. 2. Samples extracted from plasma and urine may also be analyzed, and the relevant preparative methods are shown for reference.
LC and MS conditions are shown in Table 1. Multiple reaction monitoring (MRM) was performed with methylmalonic acid, 3-OH propionic acid, and succinic acid as the target compound and using 13C-methylmalonic acid as an internal standard.

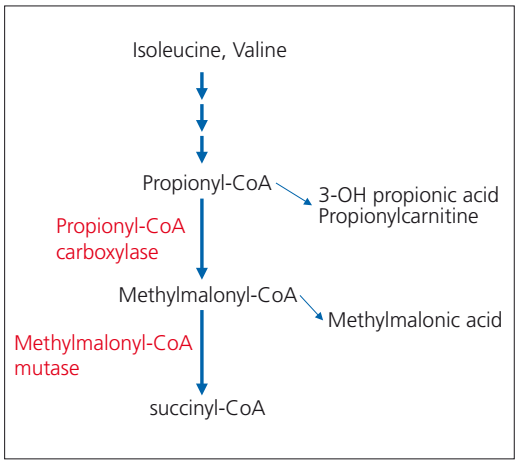

Fig. 1 Metabolic Pathway of Amino Acid

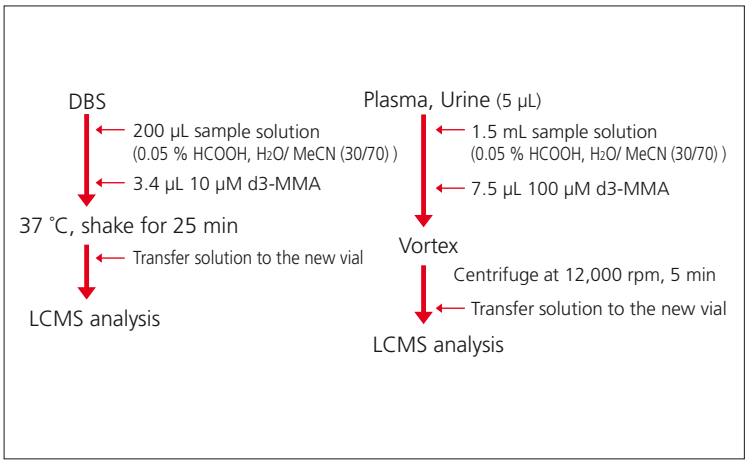

Fig. 2 Preparation Protocol

Table 1 Analytical Conditions

Column	: Gemini C6-Phenyl (100 mm L. × 2.0 mm I.D., 3 µm)	Probe Voltage	: +4.5 kV
Mobile Phase A	: 0.1 % HCOOH-H_2O	Nebulizing Gas Flow	: 2.5 L/min
Mobile Phase B	: 0.1 % HCOOH-CH_3CN	Drying Gas Flow	: 15.0 L/min
Ratio	: 60 %B	DL Temperature	: 250 °C
Flowrate	: 0.2 mL/min	Block Heater Temperature	: 400 °C
Column Temperature	: 30 °C	MRM	: Succinic acid (116.9 > 73.2)
Injection Volume	: 5 µL		MMA (116.9 > 73.1)
Analysis Time	: 5 min		d3-MMA (119.9 > 76.1)
Ionization Mode	: ESI (+)		Lactic acid (89 > 58.9)
			3-OH Propionic acid (89 > 58.9)

Analysis Results

Results of analysis are shown in Fig. 3. The "Sample A" and "Sample B" plots show when there is no methylmalonyl-CoA mutase activity and no propionyl-CoA carboxylase activity present in the sample, respectively. The "Normal" plot shows when both these enzymes are active and present in the sample. Peaks representative of methylmalonic acid, 3-OH propionic acid, and succinic acid were detected in Sample A, and peaks representative of 3-OH propionic acid and succinic acid were detected in Sample B. Lactic acid (LA) and 3-OH propionic acid have the same molecular weight and the same MRM transition, but on separating the two compounds, a peak specific to 3-OH propionic acid was detected. This analytical system can be used to check for enzyme activity.

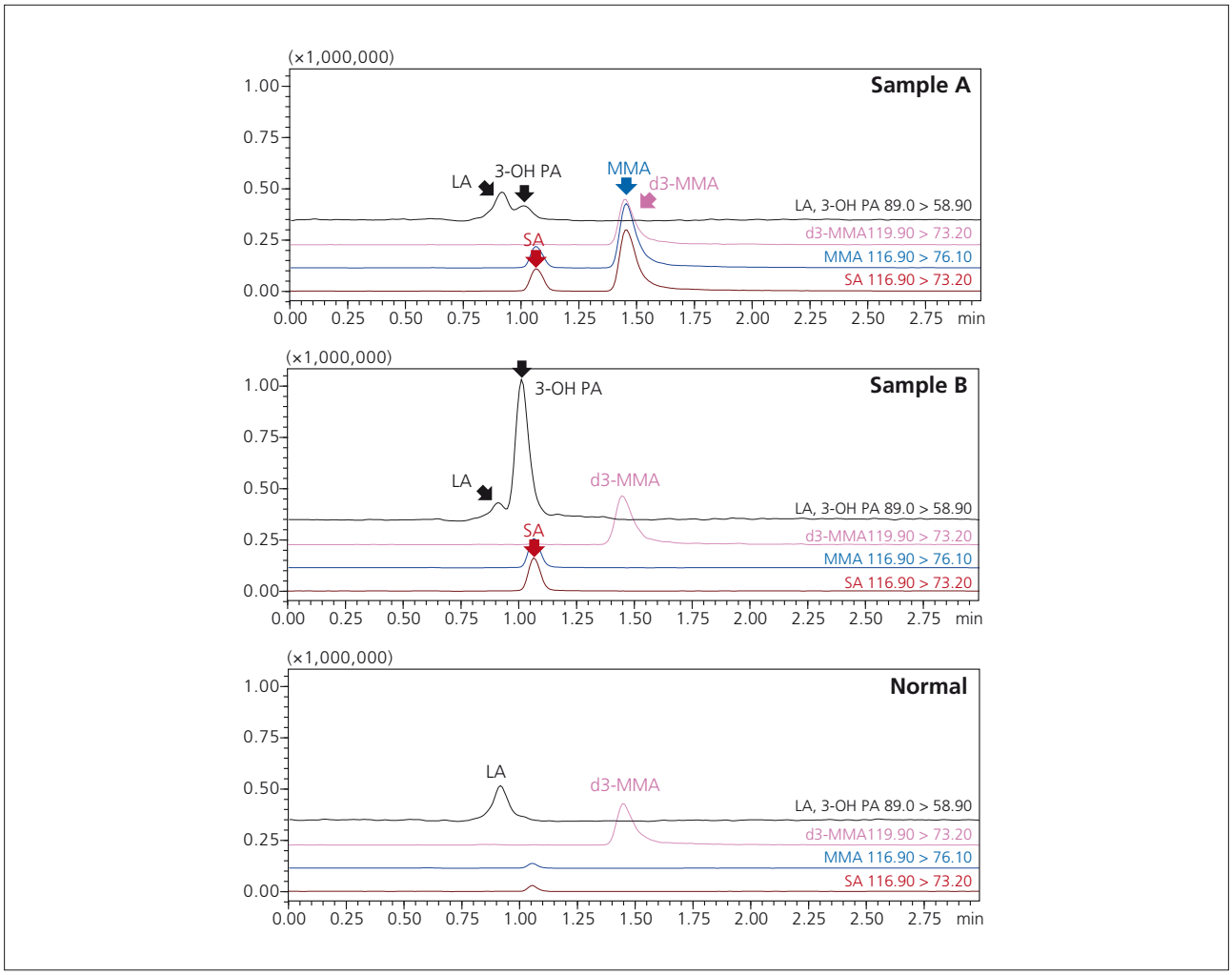

Fig. 3 Extracted-Ion Chromatograms of Target Compounds

[References]
la Marca G, et al. Progress in expanded newborn screening for metabolic conditions by LC-MS/MS in Tuscany: Update on methods to reduce false positive. JIMD Short report #127 (2008)
la Marca G, et al. Rapid 2nd-Tier Test for Measurement of 3-OH-Propionic and Methylmalonic Acids on Dried Blood Spots: Reducing the False-Positive Rate for Propionylcarnitine during Expanded Newborn Screening by Liquid Chromatography–Tandem Mass Spectrometry. Clinical Chemistry 53:1364–1369 (2007)

[Acknowledgement]
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Note: This analytical system may only be used for research applications, and may not be used for clinical diagnosis.

First Edition: May. 2014

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Application News

No.C94

Liquid Chromatography Mass Spectrometry

Simultaneous Analysis of Amino Acids and Acylcarnitines in DBS (Dried Blood Spot) with LCMS-8040

LC-MS/MS can be used to perform rapid analysis of multicomponent samples of amino acids, which are indicators of amino metabolism, and acylcarnitines, which are indicators of fatty acid metabolism. LC-MS/MS can also be used to detect amino acids and acylcarnitines in the blood at high sensitivity with uncomplicated sample preparation, which is why LC-MS/MS shows increasing promise for application in future metabolic research.
Solution that was extracted from dried blood spot (DBS) was added to a 96-well plate and an autosampler used to perform automatic screening by LC-MS/MS.
Here we describe an example analysis performed using an LCMS-8040 high-performance liquid chromatograph-triple quadrupole mass spectrometer and employing an analytical protocol used by the Mass Spectrometry, Clinical Chemistry and Pharmacology Lab. of Meyer Children's Hospital (Florence, Italy).

Sample Extraction from DBS and MS Analysis

The sample preparation method is shown in Fig. 1. Filter paper blotted with blood (DBS) was used to prepare the sample. After cutting 3.2-mm diameter disks from the DBS for the 96-well plate, sample preparation was performed in accordance with the protocol. After sample preparation, flow injection analysis (FIA) was performed with the extracted solution. Stable isotopes such as adenosine and deoxyadenosine mixed with stable isotopes of amino acids, carnitine, and acylcarnitine (manufactured by Cambridge Isotope Laboratories, Inc.) were used as internal standards for standard extraction.
Analysis was performed by neutral loss scanning of 46 Da for amino acids, precursor ion scanning of 85 *m/z* for acylcarnitines, and multiple reaction monitoring (MRM) for some amino acids, succinylacetone, adenosine, and deoxyadenosine. Information on the target compounds is shown in Table 1. Quantitation of target compounds was performed using internal standards. LC and MS conditions are shown in Table 2.

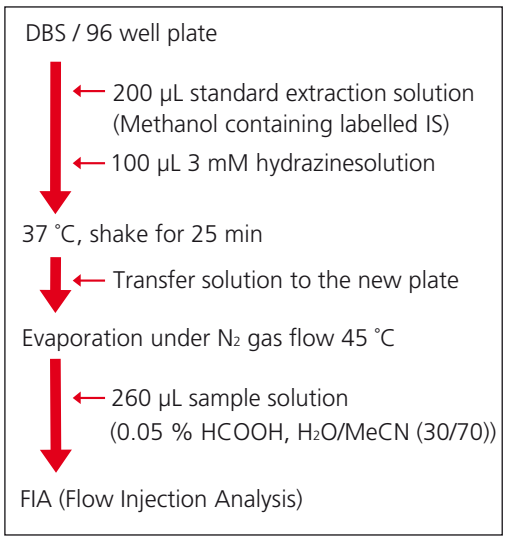

Fig. 1 Preparation Protocol

Table 1 Target Compounds

Compound	type	*m/z*	event	Compound	type	*m/z*	event	Compound	type	*m/z*	event
C0	Target	162.1	2:Precursor(+)	C3DC	Target	248.1	2:Precursor(+)	Ala	Target	90	3:NLS(+)
C0 IS	ISTD	171.2	2:Precursor(+)	C3DC IS	ISTD	221.1	2:Precursor(+)	Ala IS	ISTD	94	3:NLS(+)
C5	Target	246.2	2:Precursor(+)	C4OH	Target	248.1	2:Precursor(+)	Val	Target	118.1	3:NLS(+)
C5 IS	ISTD	255.2	2:Precursor(+)	C4OH IS	ISTD	235.2	2:Precursor(+)	Val IS	ISTD	126.1	3:NLS(+)
C6	Target	260.2	2:Precursor(+)	C5:1	Target	244.2	2:Precursor(+)	Xleu	Target	132.1	3:NLS(+)
C8	Target	288.2	2:Precursor(+)	C5:1 IS	ISTD	255.2	2:Precursor(+)	Xleu IS	ISTD	135.1	3:NLS(+)
C8 IS	ISTD	291.2	2:Precursor(+)	C5DC	Target	276.1	2:Precursor(+)	Met	Target	150.1	3:NLS(+)
C8:1	Target	286.2	2:Precursor(+)	C5DC IS	ISTD	291.2	2:Precursor(+)	Met IS	ISTD	153.1	3:NLS(+)
C8:1 IS	ISTD	291.2	2:Precursor(+)	C5OH	Target	262.2	2:Precursor(+)	Tyr	Target	182.1	3:NLS(+)
C6 IS	ISTD	255.2	2:Precursor(+)	C5OH IS	ISTD	255.5	2:Precursor(+)	Tyr IS	ISTD	188.1	3:NLS(+)
C2	Target	204.1	2:Precursor(+)	C12OH	Target	360.3	2:Precursor(+)	Asp	Target	134.1	3:NLS(+)
C2 IS	ISTD	207.1	2:Precursor(+)	C12OH IS	ISTD	381.3	2:Precursor(+)	Asp IS	ISTD	137.1	3:NLS(+)
C3	Target	218.1	2:Precursor(+)	C14OH	Target	388.3	2:Precursor(+)	Glu	Target	148.1	3:NLS(+)
C3 IS	ISTD	221.1	2:Precursor(+)	C14OH IS	ISTD	381.3	2:Precursor(+)	Glu IS	ISTD	151.3	3:NLS(+)
C4	Target	232.2	2:Precursor(+)	C16OH	Target	416.3	2:Precursor(+)	Phe	Target	166.1	3:NLS(+)
C4 IS	ISTD	235.2	2:Precursor(+)	C16OH IS	ISTD	403.3	2:Precursor(+)	Phe IS	ISTD	172.1	3:NLS(+)
C10	Target	316.1	2:Precursor(+)	C18OH	Target	444.4	2:Precursor(+)	Gly	Target	76.00>30.10	1:MRM(+)
C10 IS	ISTD	291.2	2:Precursor(+)	C18OH IS	ISTD	403.3	2:Precursor(+)	Gly IS	ISTD	78.00>32.10	1:MRM(+)
C10:1	Target	314.1	2:Precursor(+)	C18:1OH	Target	442.4	2:Precursor(+)	Cit MRM	Target	176.10>113.10	1:MRM(+)
C10:1 IS	ISTD	291.2	2:Precursor(+)	C18:1OH IS	ISTD	403.4	2:Precursor(+)	Cit MRM IS	ISTD	178.10>115.10	1:MRM(+)
C12	Target	344.3	2:Precursor(+)	C14:2	Target	368.3	2:Precursor(+)	Arg MRM	Target	175.10>116.10	1:MRM(+)
C12 IS	ISTD	381.3	2:Precursor(+)	C14:2 IS	ISTD	381.3	2:Precursor(+)	Arg MRM IS	ISTD	180.10>121.10	1:MRM(+)
C12:1	Target	342.3	2:Precursor(+)	C16:1OH	Target	414.3	2:Precursor(+)	Arg Succ MRM	Target	291.10>176.20	1:MRM(+)
C12:1 IS	ISTD	381.3	2:Precursor(+)	C16:1OH IS	ISTD	403.3	2:Precursor(+)	Arg Succ MRM IS	ISTD	180.10>121.10	1:MRM(+)
C14	Target	372.3	2:Precursor(+)	C10:2	Target	312.2	2:Precursor(+)	Orn	Target	133.10>70.10	1:MRM(+)
C14 IS	ISTD	381.3	2:Precursor(+)	C10:2 IS	ISTD	291.2	2:Precursor(+)	Orn IS	ISTD	135.10>72.10	1:MRM(+)
C14:1	Target	370.3	2:Precursor(+)	C4DC	Target	262.1	2:Precursor(+)	Met MRM	Target	150.10>104.10	1:MRM(+)
C14:1 IS	ISTD	381.3	2:Precursor(+)	C4DC IS	ISTD	235.2	2:Precursor(+)	Met MRM IS	ISTD	153.10>107.10	1:MRM(+)
C16	Target	400.3	2:Precursor(+)	C6DC	Target	290.2	2:Precursor(+)	SuAC	Target	155.00>137.20	1:MRM(+)
C16 IS	ISTD	403.3	2:Precursor(+)	C6DC IS	ISTD	255.2	2:Precursor(+)	SuAC IS	ISTD	159.00>141.20	1:MRM(+)
C16:1	Target	398.3	2:Precursor(+)	C10 OH	Target	332.2	2:Precursor(+)	ADO	Target	268.20>136.10	1:MRM(+)
C16:1 IS	ISTD	403.3	2:Precursor(+)	C10 OH IS	ISTD	291.2	2:Precursor(+)	ADO IS	ISTD	269.20>136.10	1:MRM(+)
C18	Target	428.4	2:Precursor(+)	C8DC	Target	318.2	2:Precursor(+)	Deoxi ADO	Target	252.20>136.10	1:MRM(+)
C18 IS	ISTD	403.3	2:Precursor(+)	C8DC IS	ISTD	381.3	2:Precursor(+)	Deoxi ADO IS	ISTD	257.20>136.10	1:MRM(+)
C18:1	Target	426.4	2:Precursor(+)	C18:2 OH	Target	440.4	2:Precursor(+)	Guanosina	Target	284.10>152.00	4:MRM(+)
C18:1 IS	ISTD	403.3	2:Precursor(+)	C18:2 OH IS	ISTD	403.4	2:Precursor(+)	Guanosina IS	ISTD	289.10>156.90	4:MRM(+)
C18:2	Target	424.3	2:Precursor(+)					Deoxy Guanosina	Target	268.00>152.00	4:MRM(+)
C18:2 IS	ISTD	403.3	2:Precursor(+)					Deoxy Guanosina IS	ISTD	270.10>151.90	4:MRM(+)
								Inosina	Target	269.00>137.10	4:MRM(+)
								Inosina IS	ISTD	273.10>140.90	4:MRM(+)
								Deoxy Inosina	Target	253.10>137.10	4:MRM(+)
								Deoxy Inosina IS	ISTD	273.10>140.90	4:MRM(+)

Table 2 Analytical Conditions

Mobile Phase A	: 0.1 % HCOOH-H_2O	Ionization Mode	: ESI (+)
Mobile Phase B	: 0.1 % HCOOH-CH_3CN	Probe Voltage	: +4.5 kV
Ratio	: 70 %B	Nebulizing Gas Flow	: 3.0 L/min
Flowrate	: 0.07 mL/min	Drying gas Flow	: 20.0 L/min
Injection Volume	: 40 µL	DL Temperature	: 300 °C
Analysis Time	: 2.2 min	Block Heater Temperature	: 500 °C

Example of Analysis Results

Results from measuring a control sample are shown in Table 3. The Neonatal Solution amino acid/acylcarnitine analytical support software was used to calculate the concentration (μM) of target compounds automatically, and to show whether the concentration was within the range of criteria that were set in advance. The results for the control sample shows that the concentrations were within the range of the criteria set in advance.

Table 3 Analysis Results for Control Samples

Compound	C0	C2	C3	C3DC	C4	C4OH	C4DC	C5
Criteria Upper Limit (Caution)	-	-	-	-	-	-	-	-
Criteria Upper Limit (Notice)	45.00	48.00	3.30	0.70	0.92	0.50	0.54	0.56
Criteria Lower Limit (Notice)	5.50	6.30	0.20	0.01	0.01	0.01	0.04	0.01
Criteria Lower Limit (Caution)	-	-	-	-	-	-	-	-
test_001	8.32	19.84	1.47	0.20	0.14	0.18	0.19	0.07
test_002	9.99	19.36	2.43	0.20	0.15	0.17	0.23	0.06
test_003	7.64	21.79	1.20	0.17	0.12	0.15	0.16	0.05
test_004	7.16	15.14	1.48	0.18	0.27	0.17	0.27	0.09
test_005	8.12	21.15	1.30	0.21	0.11	0.20	0.25	0.07
test_006	11.70	24.70	1.63	0.23	0.19	0.22	0.30	0.04
test_007	12.31	12.52	1.66	0.10	0.14	0.09	0.24	0.04
test_008	12.37	18.24	1.57	0.16	0.13	0.14	0.16	0.06
test_009	12.46	16.08	1.41	0.11	0.14	0.10	0.20	0.07
test_010	6.93	17.85	1.23	0.25	0.15	0.21	0.21	0.06
test_011	15.53	24.85	2.62	0.24	0.21	0.23	0.27	0.08
test_012	8.67	10.11	0.56	0.17	0.12	0.15	0.12	0.06
test_013	5.83	12.29	0.99	0.15	0.17	0.14	0.26	0.06
test_014	5.70	13.08	1.14	0.23	0.44	0.23	0.26	0.07
test_015	7.33	14.44	1.61	0.28	0.15	0.26	0.38	0.05
test_016	10.18	20.75	1.08	0.33	0.17	0.29	0.13	0.05
test_017	10.42	15.67	2.14	0.13	0.12	0.13	0.16	0.05
test_018	11.79	21.11	1.54	0.18	0.16	0.16	0.27	0.05

Analysis Results for Prepared Samples

Results from measuring 7 samples are shown in Table 4. Samples A through G were analyzed, and target compounds were detected that did not meet the set criteria (yellow cells in Table 4). The chromatogram profiles of each target compound are compared with that of the control sample in Fig. 2. Target compounds that did not meet the criteria are shown to have a profile that differs from the control sample.

Table 4 Analysis Results for 7 Samples

Sample A: ArgSuc, Sample B: VLCAD, Sample C: MCAD, Sample D: Cit I, Sample E: GAL I, Sample F: Tyr I, Sample G: PA

Compound	C3	C5DC	C6	C8	C14	C14:1	C18:1	Tyr	Cit	Arg Succ
Criteria Upper Limit (Caution)	-	-	-	-	-	-	-	-	-	-
Criteria Upper Limit (Notice)	3.30	0.15	0.25	0.40	0.57	0.44	2.43	200.00	30.00	1.00
Criteria Lower Limit (Notice)	0.20	0.01	0.01	0.01	0.01	0.01	0.39	0.01	3.00	0.01
Criteria Lower Limit (Caution)	-	-	-	-	-	-	-	-	-	-
Sample A_ArgSuc	0.33	0.01	0.02	0.09	0.11	0.03	0.37	50.93	71.01	57.85
Sample B_VLCAD	0.59	0.02	0.04	0.04	0.91	3.54	3.16	68.26	38.28	0.78
Sample C_MCAD	0.32	0.01	0.44	2.49	0.10	0.02	0.63	57.79	5.57	0.04
Sample D_Cit I	0.91	0.01	0.01	0.01	0.06	0.02	0.32	57.02	116.82	0.44
Sample E_GAL I	0.28	1.66	0.01	0.01	0.08	0.03	0.68	87.22	7.86	0.67
Sample F_Tyr I	0.23	0.00	0.01	0.05	0.06	0.08	1.09	344.21	31.90	0.74
Sample G_PA	8.58	0.00	0.02	0.02	0.03	0.03	0.54	23.28	13.74	0.29

Compound	SuAC	C14:1/C4	C3/C0	C3/C4	C3/C16	C5DC/C4	C5DC/C8	C5DC/C12	C8/C10
Criteria Upper Limit (Caution)	-	-	-	-	-	-	-	-	-
Criteria Upper Limit (Notice)	2.00	2.14	0.30	25.80	1.60	0.56	0.75	1.07	2.42
Criteria Lower Limit (Notice)	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01
Criteria Lower Limit (Caution)	-	-	-	-	-	-	-	-	-
Sample A_ArgSuc	1.71	0.21	0.01	2.56	0.31	0.10	0.15	0.32	1.01
Sample B_VLCAD	1.23	23.85	0.05	3.94	0.22	0.12	0.43	0.13	0.32
Sample C_MCAD	1.12	0.21	0.03	2.72	0.28	0.12	0.01	0.33	12.17
Sample D_Cit I	1.07	0.25	0.04	13.05	0.65	0.13	0.67	0.75	1.36
Sample E_GAL I	0.97	0.40	0.03	3.95	0.25	23.60	223.72	59.59	0.16
Sample F_Tyr I	10.43	1.19	0.01	3.44	0.28	0.04	0.05	0.05	0.67
Sample G_PA	0.96	0.57	0.33	154.82	12.51	0.07	0.21	0.30	0.73

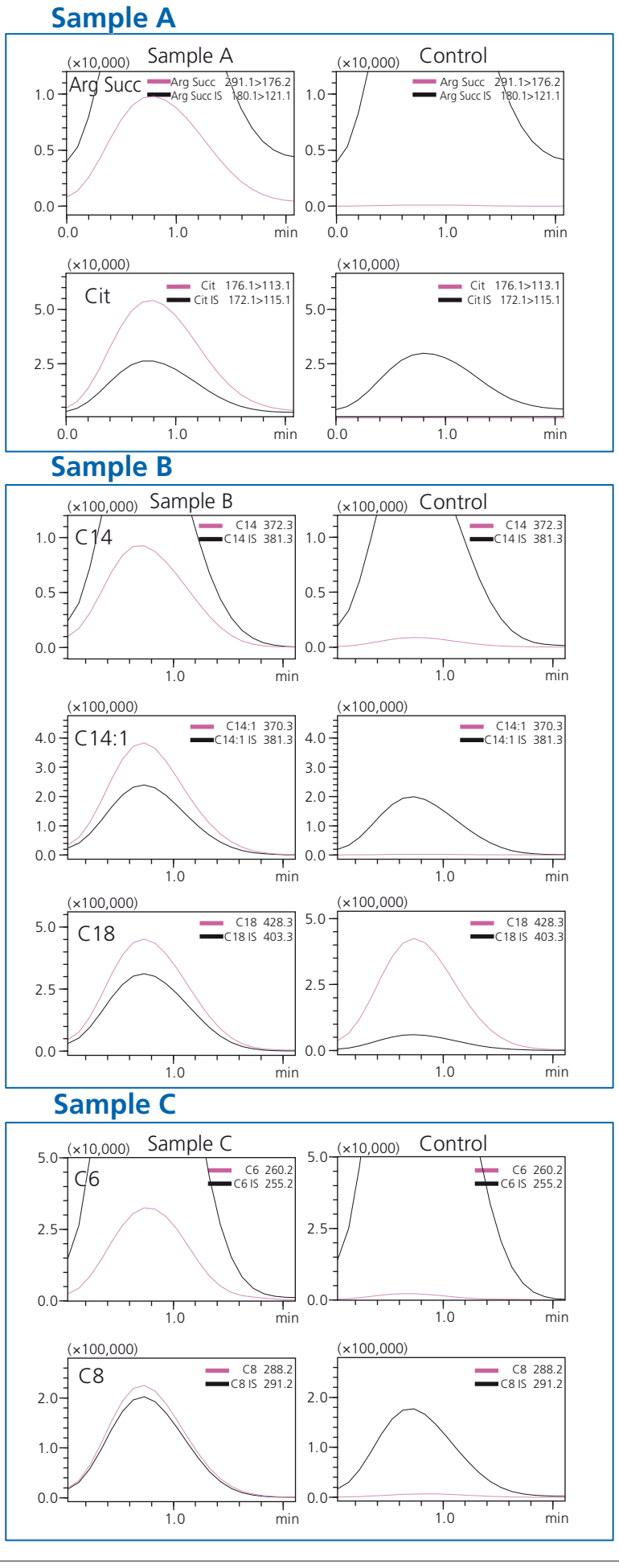

Fig. 2-1 Extracted-Ion Chromatograms of Target Compounds

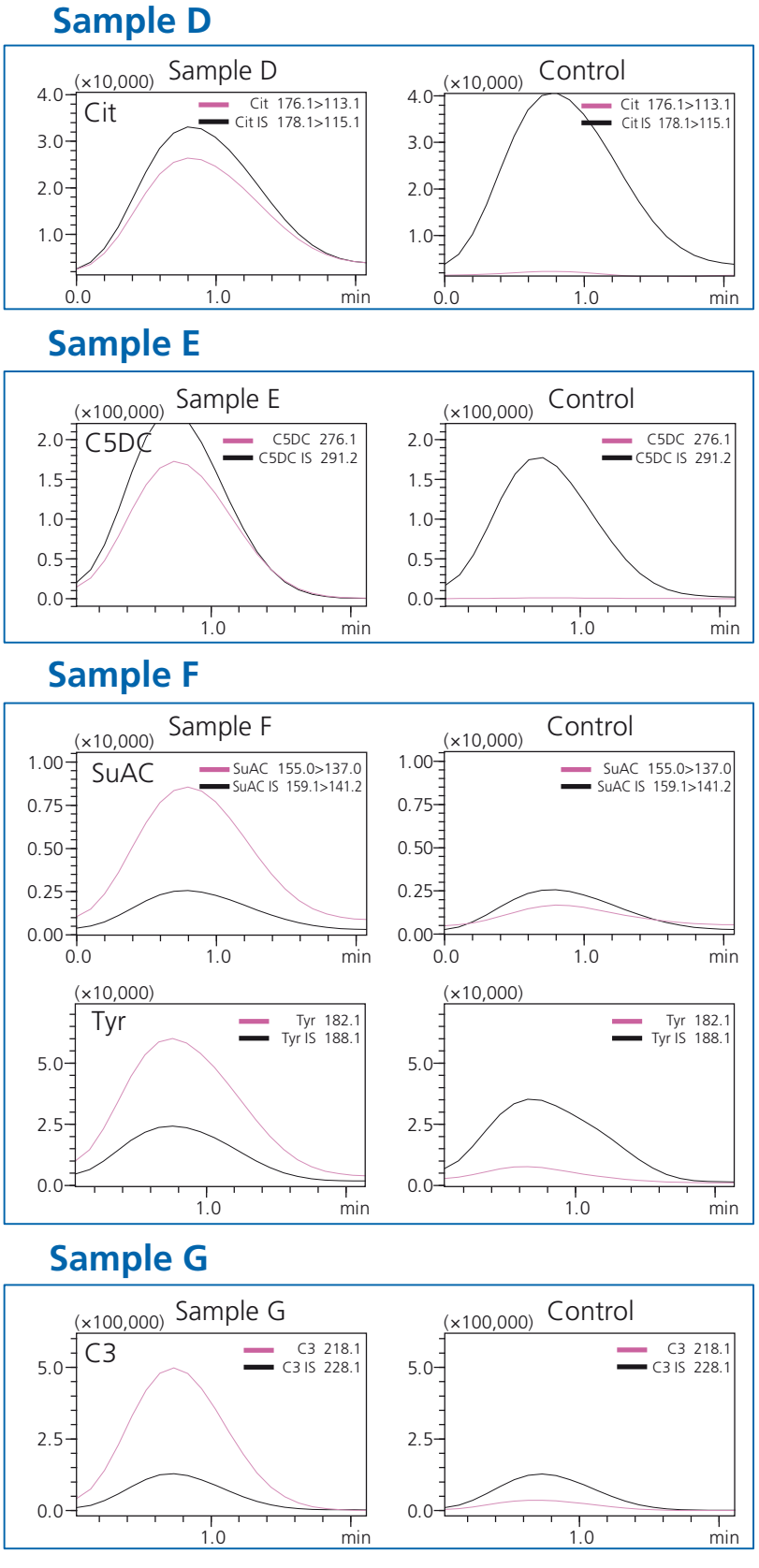

Fig. 2-2 Extracted-Ion Chromatograms of Target Compounds

Note: This analytical system may only be used for research applications, and may not be used for clinical diagnosis.

First Edition: May. 2014

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Application News

No. SCA_210_022

LCMS-8050

Analysis of Ethylglucuronide and Ethylsulfate in Urine, Plasma and Serum by LCMS-8050 using RECIPE ClinMass® LC-MS/MS Complete Kit MS8000

Dr. Johannes Engl (RECIPE Chemicals + Instruments GmbH), Anja Grüning (Shimadzu Europa GmbH)

■ Introduction

In many societies around the world alcohol abuse represents a serious social and economic problem. For alcohol misuse several parameters of laboratory diagnostics are available. They are used for the assessment of acute drinking, chronic abuse (alcoholics), abstinence control and relapse diagnosis.
Acute alcohol abuse, which dates back several hours, is mainly determined by ethanol in the respiratory air (alcohol breath test) and by the blood alcohol level (short-term marker). For the investigation of a long-term, chronic abuse the determination of CDT (Carbohydrate Deficient Transferrin) has been established for routine analysis.
Ethylglucuronide (EtG) and Ethylsulfate (EtS) are formed in the ethanol metabolism and therefore serve in addition to the short-term marker ethanol and the longterm marker CDT for the verification of alcohol abuse. They can be determined in urine in a time range up to 80 h after excessive consume of alcohol. Even in case of low to mid uptake of alcohol, Ethylglucuronide and Ethylsufate can be detected up to 24 h respectively 48 h.

■ Materials and methods

The LCMS-8050 triple quadrupole mass spectrometer was coupled to a Nexera X2 UHPLC system. EtG and EtS were measured using a commercially available test kit ClinMass® Complete Kit for Ethylglucuronide and Ethylsulfate in Urine, Plasma and Serum, MS8000, MS8100 (RECIPE Chemicals + Instruments GmbH, Dessauerstraße 3, 80992 München, Germany). Calibrators, control samples, analytical column and mobile phase solvents were provided by the kit. 50 µL of urine sample was added to 1000 µL of internal standard solution and mixed for 5 sec. 10 µL of the sample was analysed.
For analysis the [M-H]- ion was measured and used as the precursor ion (negative electrospray ionization).

■ Analytical conditions

UHPLC:	Nexera X2 UHPLC
	0.2 mL/min (0.0 - 2.5 min)
	0.5 mL/min (2.6 - 4.8 min)
	0.2 mL/min (4.9 - 5.0 min)
Column temperature:	40 °C
Injection volume:	10 µL
Mass spectrometer:	LCMS-8050
Source conditions:	
Nebulizer Gas:	3 L/min
Heating Gas:	10 L/min
Drying Gas:	5 L/min
Interface temperature:	200 °C
Desolvation Line:	200 °C
Heat Block temperature:	200 °C
Interface voltage:	-2.5 kV
Dwell time:	50 msec
Pause time:	3 msec
Ionization:	Electrospray ionization (ESI) negative mode
Scan Type:	MRM

Table 1 EtG/EtS MRM transitions, retention time (RT). T/I = target or internal standard

Compound		Formula	MRM1	MRM2	RT
EtG	T	$C_8H_{14}O_7$	221>75	221>85	2.08
D5-EtG	I	$C_8H_9D_5O_7$	226>75	226>85	2.07
EtS	T	$C_2H_6O_4S$	125>97	125>80	3.95
D5-EtS	I	$C_2HD_5O_7S$	130>98	130>80	3.92

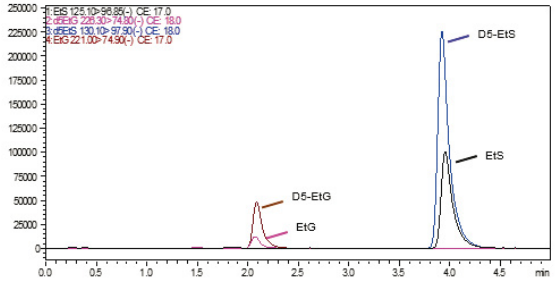
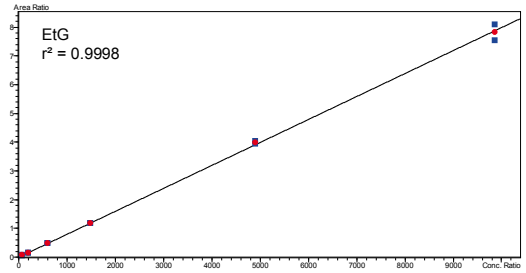


Figure 1 LC-MS separation of EtG / EtS and deuterated standard in five minutes by isocratic chromatography.



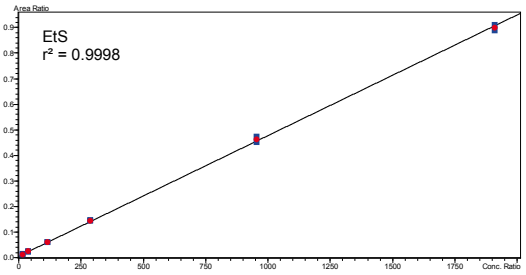


Figure 2 Calibration curves for EtG and EtS.

EtG	Control Level I (111 µg/L)	Control Level II (518 µg/L)	Control Level III (2052 µg/L)
	Conc.	Conc.	Conc.
Control 1	108.055	523.773	2124.918
Control 2	94.895	529.889	2005.968
Control 3	118.378	509.452	2137.082
Control 4	104.611	503.697	2071.541
Mean	106.485	516.703	2084.877
SD	9.691	12.187	59.814
%RSD	9.10	2.36	2.87

Table 2 Reproducibility for EtG

EtS	Control Level I (48 µg/L)	Control Level II (201 µg/L)	Control Level III (799 µg/L)
	Conc.	Conc.	Conc.
Control 1	49.174	209.118	781.053
Control 2	50.413	199.111	772.997
Control 3	46.561	212.100	790.709
Control 4	44.721	200.284	775.448
Mean	47.717	205.153	780.052
SD	2.563	6.434	7.864
%RSD	5.37	3.14	1.01

Table 3 Reproducibility for EtS

■ Results

The rapid elution of EtG and EtS by isocratic chromatography produced excellent peak shape and accuracy with elution in five minutes (Fig. 3).

The calibration curve determined in duplicate showed good linearity over a clinically relevant range of 78.6-9860 µg/L for EtG and 15.3-1910 µg/L for EtS (Fig. 2)

Three control samples at high, mid and low concentration were analyzed in fourfold to measure analytical reproducibility. The percentage relative standard deviation was typically lower than 10% from these measurements.

■ Conclusion

The application of the clinical ClinMass® Complete Kit, for Ethylglucuronide and Ethylsulfate in Urine, Plasma and Serum proved easy to implement and showed good sensitivity and linearity in a clinically relevant concentration range.

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Determination of Δ^9-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation

ASMS 2014 ThP600

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Determination of Δ⁹-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation

Introduction

In France, as in other countries, cannabis is the most widely used illicit drug. In forensic as well as in clinical contexts, Δ⁹-tetrahydrocannabinol (THC), the main active compound of cannabis, and two of its metabolites [11-hydroxy-Δ⁹-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (THC-COOH)] are regularly investigated in biological fluids for example in Driving Under the Influence of Drug context (DUID) (figure 1).
Historically, the concentrations of these compounds were determined using a time-consuming extraction procedure and GC-MS. The use of LC-MS/MS for this application is relatively recent, due to the low response of these compounds in LC-MS/MS while low limits of quantification need to be reached. Recently, on-line Solid-Phase-Extraction coupled with UHPLC-MS/MS was described, but in our hands it gave rise to significant carry-over after highly concentrated samples. We propose here a highly sensitive UHPLC-MS/MS method with straightforward QuEChERS sample preparation (acronym for Quick, Easy, Cheap, Effective, Rugged and Safe).

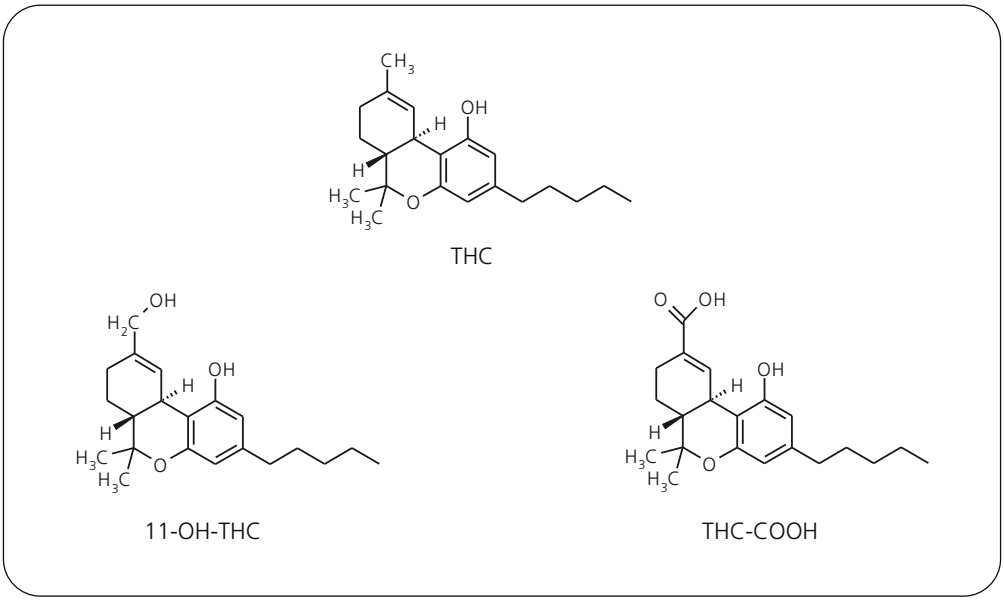


Figure 1: Structures of THC and two of its metabolites

Methods and Materials

Isotopically labeled internal standards (one for each target compound in order to improve method precision and accuracy) at 10 ng/mL in acetonitrile, were added to 100 µL of sample (urine, whole blood or plasma) together with 50 mg of QuEChERS salts ($MgSO_4$/NaCl/Sodium citrate dehydrate/Sodium citrate sesquihydrate) and 200 µL of acetonitrile. Then the mixture was shaken and centrifuged for 10 min at 12,300 g. Finally, 15 µL of the upper layer were injected in the UHPLC-MS-MS system. The whole acquisition method lasted 3.4 min.

Determination of Δ⁹-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation

UHPLC conditions (Nexera MP system)

Column	: Kinetex C18 50x2.1 mm 2.6 µm (Phenomenex)
Mobile phase A	: 5mM ammonium acetate in water
B	: CH_3CN
Flow rate	: 0.6 mL/min
Time program	: B conc. 20% (0-0.25 min) - 90% (1.75-2.40 min) - 20% (2.40-3.40 min)
Column temperature	: 50 °C

MS conditions (LCMS-8040)

Ionization	: ESI, negative MRM mode
Ion source temperatures	: Desolvation line: 300°C Heater Block: 500°C
Gases	: Nebulization: 2.5 L/min Drying: 10 L/min

MRM Transitions:

Compound	MRM	Dwell time (msec)
THC	313.10>245.25 (Quan)	60
	313.10>191.20 (Qual)	60
	313.10>203.20 (Qual)	60
$THC-D_3$	316.10>248.30 (Quan)	5
	316.10>194.20 (Qual)	5
11-OH-THC	329.20>311.30 (Quan)	45
	329.20>268.25 (Qual)	45
	329.20>173.20 (Qual)	45
$11-OH-THC-D_3$	332.30>314.40 (Quan)	5
	332.30>271.25 (Qual)	5
THC-COOH	343.20>245.30 (Quan)	50
	343.20>325.15 (Qual)	50
	343.20>191.15 (Qual)	50
	343.20>299.20 (Qual)	50
$THC-COOH-D_3$	346.20>302.25 (Quan)	5
	346.20>248.30 (Qual)	5

Pause time	: 3 msec
Loop time	: 0.4 sec (minimum 20 points per peak for each MRM transition)

Results

Chromatographic conditions

A typical chromatogram of the 6 compounds is presented in figure 1.

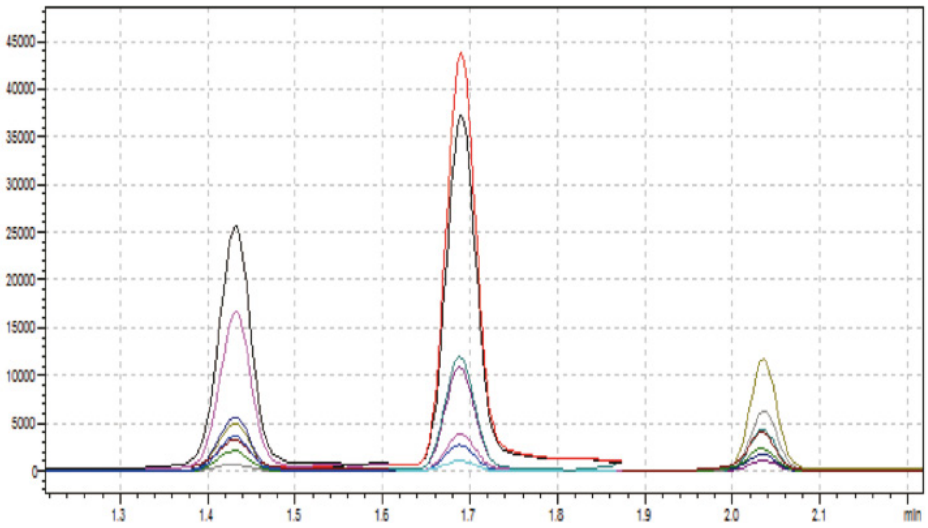


Figure 1: Chromatogram obtained after an injection of a 15 µL whole blood extract spiked at 50 µg/L

Extraction conditions

As described by Anastassiades et al. J. AOAC Int 86 (2003) 412-31, the combination of acetonitrile and QuEChERS salts allowed the extraction/partitioning of compounds of interest from matrix. This extraction/partitioning process is not only obtained with whole blood and plasma-serum where deproteinization occurred and allowed phase separation, but also with urine as presented in figure 2.

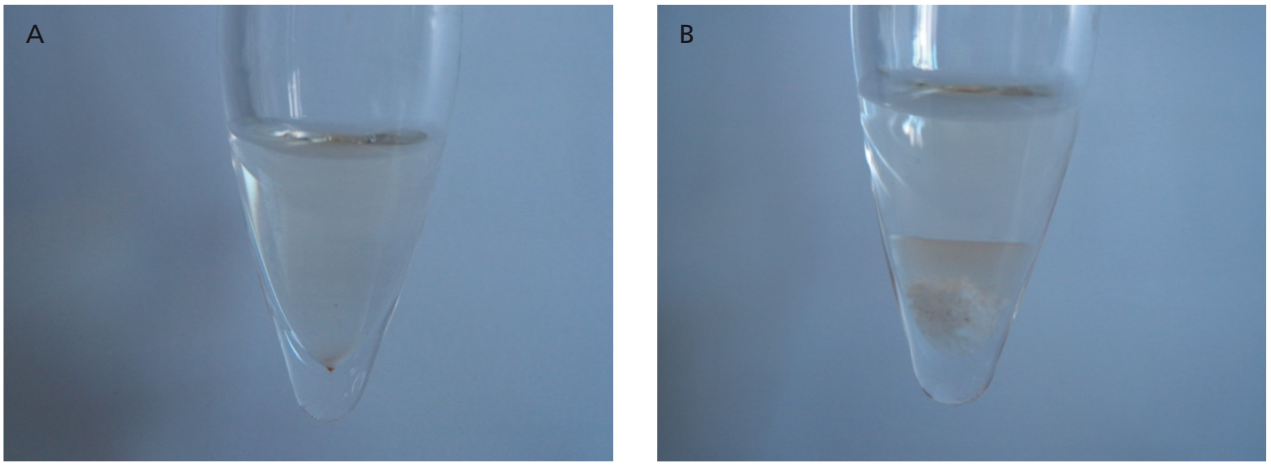
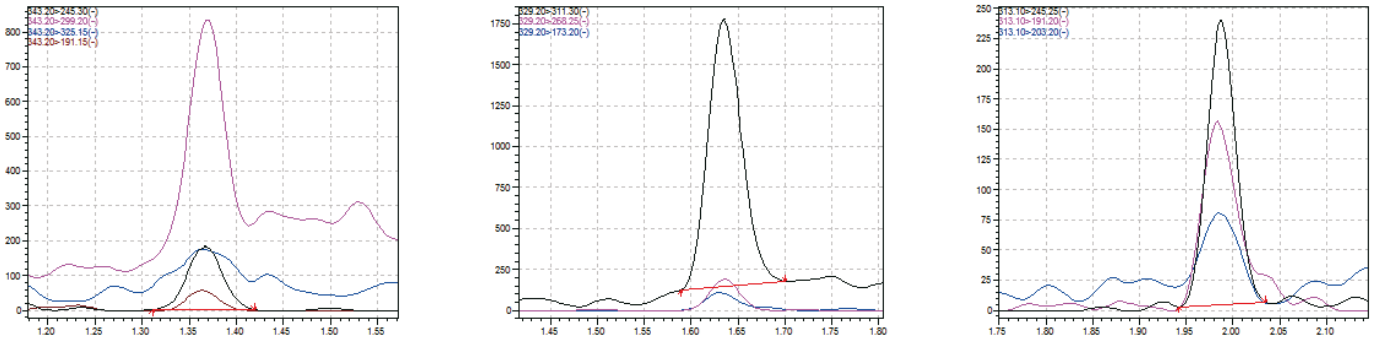


Figure 2: influence of QuEChERS salts on urine extraction/partitioning: A: acetonitrile with urine sample lead to one phase / B: acetonitrile, QuEChERS salts and urine lead to 2 phases.

Determination of Δ⁹-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation

Validation data

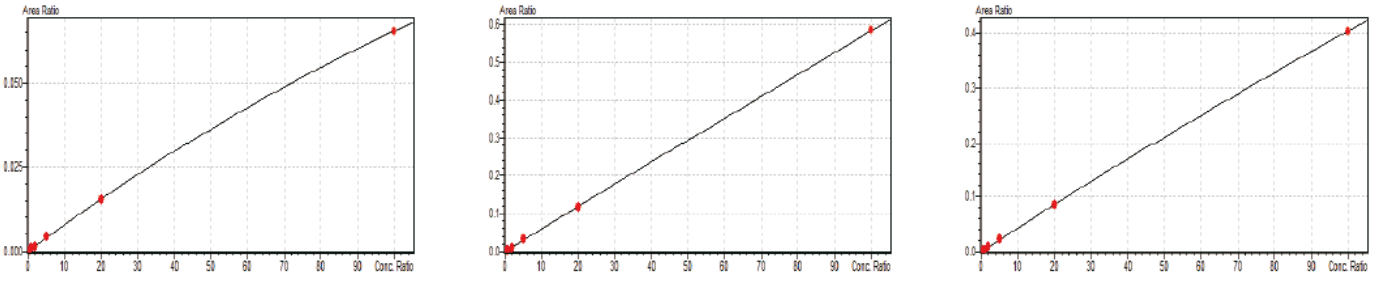
One challenge for the determination of cannabinoids in blood using LC-MS/MS is the low quantification limits that need to be reached. The French Society of Analytical Toxicology proposed 0.5 µg/L for THC et 11-OH-THC and 2.0 µg/L for THC-COOH. With the current application, the lower limit of quantification was fixed at 0.5 µg/L for the three compounds (3.75 pg on column). The corresponding extract ion chromatograms at this concentration are presented in figure 3.



THC-COOH | 11-OH-THC | THC

Figure 3: Chromatogram obtained after an injection of a 15 µL whole blood extract spiked at 0.5 µg/L (lower limit of quantification).

The upper limit of quantification was set at 100 µg/L. Calibration graphs of the cannabinoids-to-internal standard peak-area ratios of the quantification transition versus expected cannabinoids concentration were constructed using a quadratic with 1/x weighting regression analysis (figure 4).



THC-COOH | 11-OH-THC | THC

Figure 4: Calibration curves of the three cannabinoids

Contrary to what was already observed with on-line Solid-Phase-Extraction no carry-over effect was noted using the present method, even when blank samples were injected after patient urine samples with concentrations exceeding 2000 µg/L for THC-COOH.

Determination of Δ^9-tetrahydrocannabinol and two of its metabolites in whole blood, plasma and urine by UHPLC-MS/MS using QuEChERS sample preparation

Conclusions

- Quick sample preparation based on QuEChERS salts extraction/partitioning, almost as short as on-line Solid Phase Extraction.
- Low limit of quantification compatible with determination of DUID.
- No carry over effect noticed.

First Edition: June, 2014

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Application News

No.C114

Liquid Chromatography Mass Spectrometry

High-Sensitivity Determination of Catecholamines in Plasma Using the LCMS-8060 Triple Quadrupole LC/MS/MS

Catecholamines are a family of signaling molecules found in brain, adrenal medulla and other nervous systems. Catecholamines in plasma, namely norepinephrine (NE), epinephrine (EP) and dopamine (DA), are commonly measured in clinical research. Analysis of catecholamines in plasma requires both high sensitivity and high throughput.
Presented here is a platform designed to demonstrate the capability to detect catecholamines in plasma, comprising multiplexed plasma sample preparation by Biotage EVOLUTE WCX solid phase extraction followed by high-sensitivity quantitation by LCMS-8060.

MRM Analysis of Deuterated Standards to Estimate LLOQ

Three catecholamine compounds (NE, EP and DA) were separated by Shimpack MAqC-ODS Ⅰ, a reversed-phase column that contains metal ions on the particle surface. The result shown in Fig. 1 demonstrates that the cation-exchange property of MAqC-ODS retained NE well, which is very poorly retained by conventional C18 columns.
Since plasma samples contain endogenous catecholamines, it is difficult to evaluate the LLOQ in plasma matrix. Here we used deuterated catecholamine compounds as standards to estimate the LLOQ in plasma matrix, rather than as internal standards for quantitation. A neat standard curve was prepared by serial dilution in HPLC solvent, whereas a matrix-matched standard curve was prepared by dilution with pooled plasma sample treated with WCX-SPE as described below. Table 1 summarizes the quantitation results, which convincing demonstrate the capability of LCMS-8060 to detect catecholamines at ultra-high sensitivity without matrix interference.

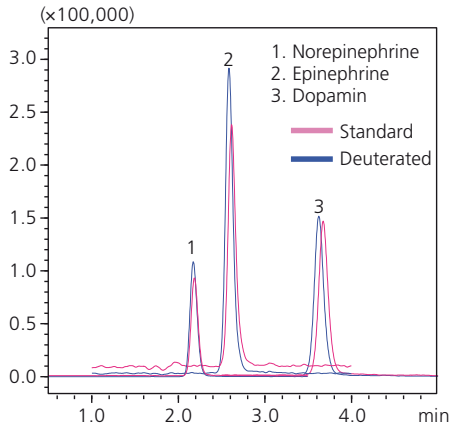

Fig.1 Representative MRM Chromatograms of 3 Catecholamines

Table 1 Quantitative Range of Neat and Matrix-Matched Calibration Curves

Compound name	Neat standard curve		Matrix-matched	
	Range (pg/mL)	Linearity (r^2)	Range (pg/mL)	Linearity (r^2)
Noepinephrine-d6 (158.1 > 111.1)	2.5 – 2000	0.9999	2.5 – 2000	0.9997
Epinephrine-d6 (190.1 > 172.1)	10 – 2000	0.9999	10 – 2000	0.9994
Dopamine-d4 (158.1 > 95.1)	5 – 2000	0.9999	10 – 2000	0.9995

In the actual quantitation assay, deuterated catecholamines are spiked as internal standard at 500 pg/mL in plasma, which is then treated with WCX-SPE and analyzed by LCMS-8060.
Fig. 2 shows the MRM chromatograms of spiked and endogenous catecholamines in plasma.

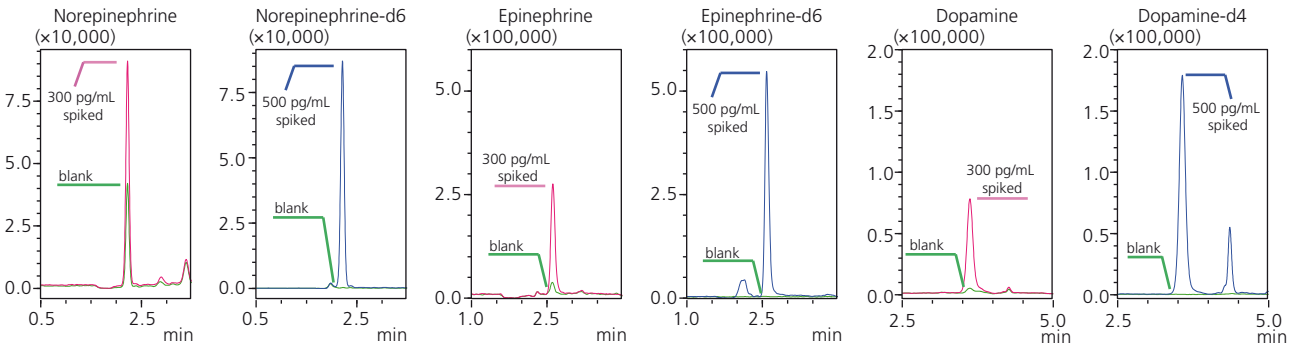

Fig. 2 Detection of NE, EP and DA and Their Deuterated Internal Standards in Plasma

Analytical Performance of QC Samples

We evaluated the analysis performance of catecholamine determination by a series of QC samples that were prepared by spiking to control plasma a fixed amount of deuterated compounds as internal standard and varying amounts of normal catecholamine compounds.

All QC samples were pretreated with EVOLUTE WCX and analyzed by LCMS-8060. A matrix calibration curve was plotted with blank-subtracted area ratio to determine catecholamine concentration in plasma sample, and its performance was evaluated as summarized in Table 2.

Table 2 Catecholamine Determination in QC Samples

Compound name	Spiked Conc. (pg/mL)	Measured IS Area Ratio	Linearity (r^2)	Theoretical Conc. (pg/mL)	Determined Conc. (pg/mL)	Accuracy	Repeatability (n=2)
Norepinephrine (152.1 > 107.1)	0 18.1 72.5 300 600 1200	0.4969 0.4862 0.6023 1.0176 1.5652 2.6390	0.9994	292.2 310.3 364.7 592.2 892.2 1492.2	- 282.0 346.6 577.3 881.5 1478.1	- 92.3 % 95.8 % 97.2 % 97.9 % 97.6 %	0.6 % 4.5 % 3.4 % 0.5 % 1.4 % 0.1 %
Epinephrine (184.1 > 166.1)	0 18.1 72.5 300 600 1200	0.1590 0.2056 0.3947 1.1140 2.0267 4.2128	0.9986	52.2 70.3 124.7 352.2 652.2 1252.2	- 66.3 123.7 341.8 618.5 1281.3	- 94.4 % 99.2 % 97.0 % 94.8 % 102.3 %	1.2 % 2.4 % 3.7 % 0.7 % 3.9 % 2.7 %
Dopamine (154.1 > 91.1)	0 18.1 72.5 300 600 1200	0.0223 0.0511 0.1260 0.4281 0.8436 1.6754	0.9999	13.5 31.6 86.0 313.5 613.5 1213.5	- 34.6 89.4 310.5 614.5 1223.2	- 109.4 % 104.0 % 99.0 % 100.2 % 100.8 %	6.0 % 4.3 % 4.7 % 1.7 % 0.2 % 0.1 %

High-throughput Plasma Preparation

In order to detect plasma catecholamines with high sensitivity and accuracy, and minimize instrument maintenance intervals, plasma samples need preparation to remove interfering molecules such as proteins. Such routine work is best multiplexed and automated to maximize efficiency. Fig. 3 describes a preparation protocol using EVOLUTE WCX extraction plate.

Fig. 3 Plasma Sample Preparation by Biotage EVOLUTE WCX Plate

HPLC Conditions

Analytical column	: Shimpack MAqC-ODS I (150 mm × 2.0 mm, 5 µm)
Mobile phase A	: 0.1 % Formic acid in water
Mobile phase B	: 0.05 % Formic acid in methanol
Time program	: 1 %B. (0 - 0.5 min) → 50 %B. (3 min) → 99 %B. (3.1 - 7 min) → 1 %B. (7.1 - 12 min)
Flowrate	: 0.2 mL/min.
Injection volume	: 5 µL
Post-column addition	: Mobile phase B at 0.2 mL/min

MS Conditions (LCMS-8060, ESI positive)

Interface voltage	: +0.6 kV
Nebulizer gas flow	: 2.2 L/min
Drying gas flow	: 3 L/min
Heating gas flow	: 17 L/min
DL temp.	: 250 ˚C
Interface temp.	: 250 ˚C
Heat block temp.	: 400 ˚C

*For Research Use Only. Not for use in diagnostic procedures.

Table 3 Sample Preparation Protocol

Plasma prep	Commercially available human plasma was aliquoted, spiked with deuterated IS compounds (500 pg/mL) and normal standard compounds. Mix 300 µL of plasma with equal volume of 50 mM ammonium acetate (pH 7.0).
Step 1	To each well of EVOLUTE WCX, add 900 µL of methanol and wash.
Step 2	Add 900 µL of ammonium acetate (pH 7.0) and equilibrate the well.
Step 3	Add diluted plasma samples (600 µL) to wells. Discard flow-through
Step 4	Add 300 µL of 10 % methanol and discard flow-through
Step 5	Add 300 µL isopropanol to wells and discard flow-through
Step 6	Add 1000 µL of 5 % formic acid 95 % methanol to elute target compounds. Collect eluate.
Step 7	Evaporate the eluate by N_2 purge at 40 ˚C
Step 8	Reconstitute with 150 µL of 0.1 % formic acid, ready for LCMS analysis.

First Edition: Jun. 2015

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Application News

No. SCA_210_020

LCMS-8040

A novel fast quantification method for amino acids in human plasma by LC-MS/MS, without ion pairing or derivatization

■ Introduction

Amino acids are routinely assayed to diagnose inherited metabolism disorders. As they are highly polar compounds, they are hardly retained onto reverse phase columns. Derivatization or addition of ion pairing reagents in the mobile phase is required. For easier and rugged analysis of amino acids, there was need for a new solution, not using pre-mentioned reagents. In this context, a new LC-MS/MS method was developed, for the simultaneous high sensitive quantification of 47 amino acids, using a mixed mode column (taking advantage of both hydrophilic and ion exchange interactions) and typical volatile mobile phase suitable for LC-MS. Sample preparation was very simple. Plasma was precipitated and supernatant was directly injected. All amino acids were separated in 15 minutes. Particular attention was paid to separate isobaric amino acids chromatographically or by using specific MRM transitions. Good linearity was obtained in the range 2.5 – 250 µmol/L, along with a good repeatability and stability.

■ Materials and methods

The new method was developed on Shimadzu LCMS-8040 triple quadrupole mass spectrometer coupled to the Nexera X2 high performance liquid chromatograph. A mix of acetonitrile, tetrahydrofuran and 25 mM ammonium formate buffer with 0.1% formic acid was used as mobile phase A, a mix of acetonitrile and 100 mM ammonium formate buffer was used as mobile phase B, in gradient mode.

Injection volume was 1 µL, reducing the potential matrix effect.
The plasma sample preparation consisted of a protein precipitation step with strong acid followed by centrifugation (Figure 1).

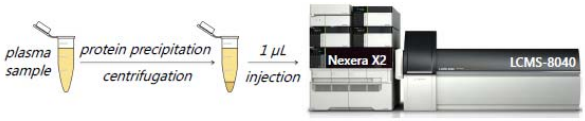

Figure 1: Sample workflow overview

■ Chromatographic Separation

A total of 47 amino acids were quantified in an analysis time of 15 min (Figure 2).

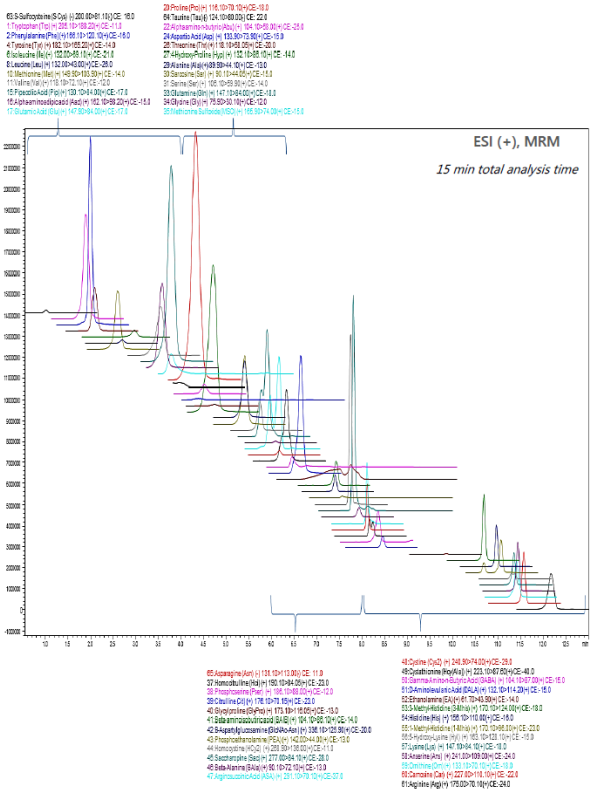

Figure 2: Typical MRM chromatograms for the analysis of 47 amino acids by LC-MS/MS without derivatization

■ Specific MRM transitions for isobaric amino acids

MRM transitions were optimized and specific MRM were chosen for isobars, so that they do not need to be fully resolved by chromatography, as shown for example for leucine and isoleucine (Figure 3).

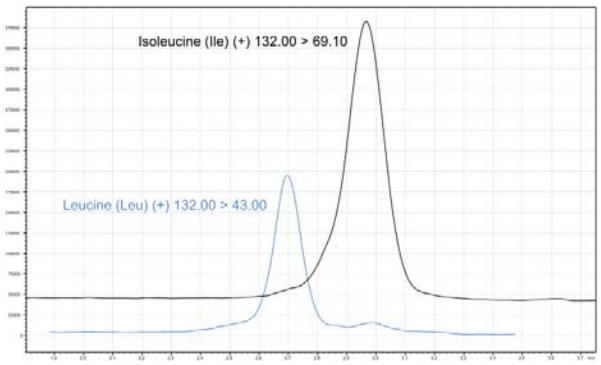

Figure 3: Leucine and Isoleucine typical MRM chromatograms. Specific MRM transitions were chosen so that they do not need to be fully resolved by chromatography.

■ Performance Evaluation

Plasma control injections show a good repeatability (n=20) and stability (5h), even without internal standard correction (Figure 4).

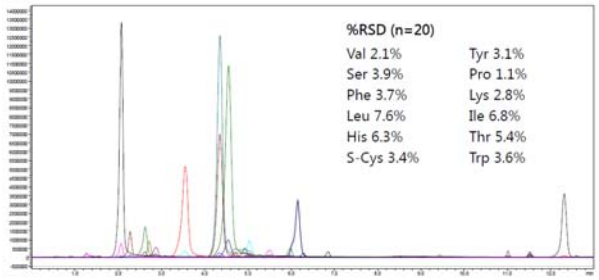

Figure 4: Typical plasma sample MRM chromatogram and RSD values (n=20).

Good linearity is obtained in the range 2.5-250 µmol/L (Figure 5).

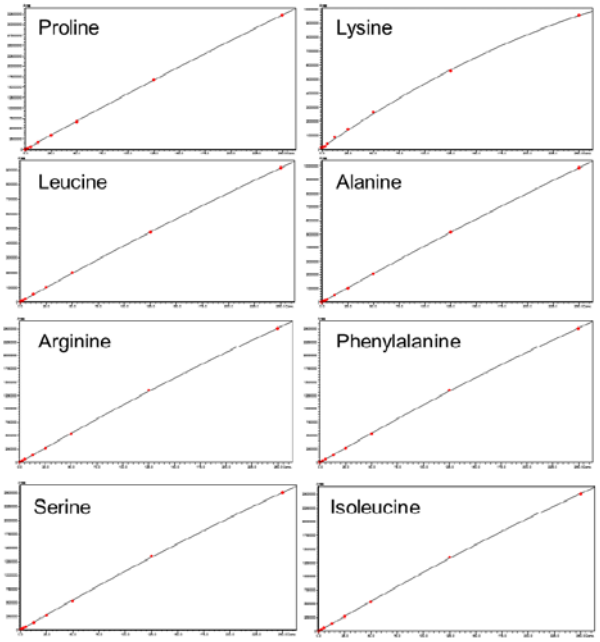

Figure 5: Typical calibration curves obtained in the range 2.5 – 250 µmol/L.

■ Conclusion

A novel LC-MS/MS method was developed for the simultaneous and high sensitive quantification of 47 amino acids in human plasma, with no need of derivatization or ion pairing agent and a very simple sample preparation. The total average cost per sample is reduced by a 6-fold factor per sample compared to current methods with derivatization. Amino acids were quantified in a total analysis time of 15 min with a good distinction between isobaric ones. No carryover was observed and good linearity was obtained in the range 2.5 – 250 µmol/L. The method proved its fits for pupose to support diagnosis and may be used also in clinical studies to monitor drug effects on metabolism.

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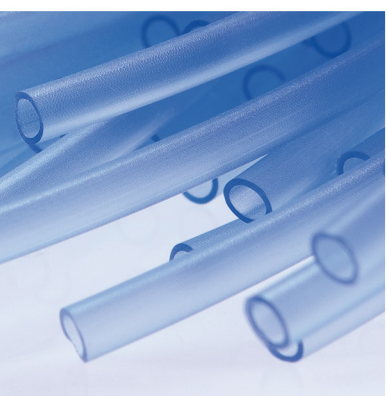
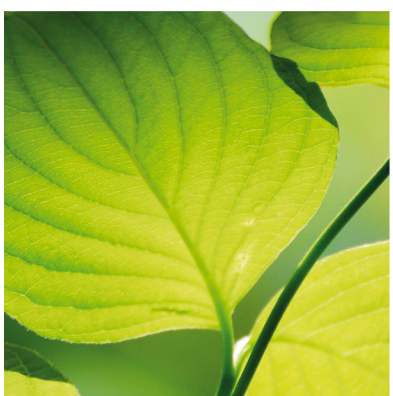
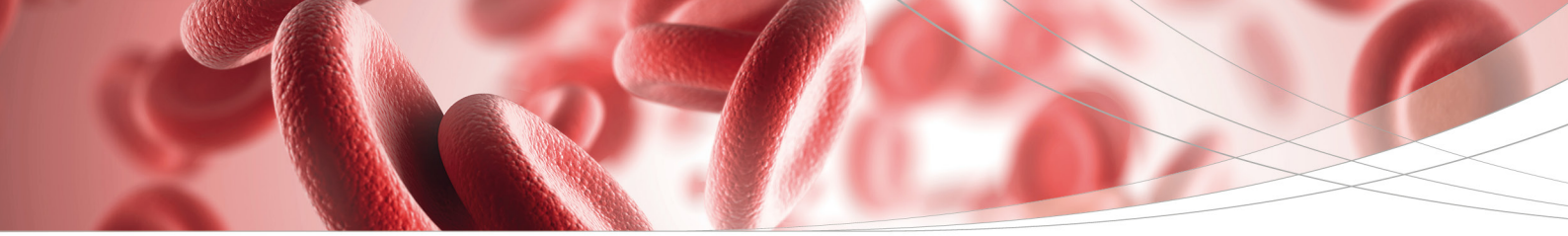
3. Spectroscopy





3. Spectroscopy

3.1 Atomic Spectroscopy

3.1.1 Atomic Absorption Spectroscopy (AAS)

AAS quantitates concentrations of elements in a vapor, when a ground state atom absorbs light energy of a specific wavelength and is elevated to an excited state. The amount of light energy absorbed at this wavelength is increased when the number of atoms of the selected element in the light path increases. The relationship between the amount of light absorbed and the concentration of the element present in known standard solutions can be used to determine unknown sample concentrations by measuring the amount of light they are absorbing.

SCA_120_023 Determination of Al in Serum using GFA-7000 with Omega Platform tube

3.1.2 Energy Dispersive X-Ray Fluorescence (EDX)

XRF allows analysis of element composition of samples in a wide variety of applications. This technique provides non-destructive and fast measurements of liquid and solid samples and is best suited for analyzing the elemental range from sodium/carbon to uranium, which covers the majority of the metallic elements.

SCA_125_011 EDXRF analysis of Cd, Hg, and Pb in blood

SCA_125_012 EDXRF analysis of P, Cl, K and Ca in blood

3.1.3 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) is the measurement of light emitted by all elements present in a sample introduced into an ICP source. The emission intensities measured are then compared with the intensities of standard samples of known concentration to obtain the elemental concentrations in the unknown samples. The argon plasma is generated by an RF field and ionized argon gas. The advantage of the plasma in comparison to other energy sources is the high temperature of 10,000 °K, enabling complete atomization of the elements in a sample while minimizing interferences.

LAAN-A-CP-E012 Analysis by ICP atomic emission spectrometry in accordance with the ICH Q3D guideline for elemental impurities using ICPE-9820

LAAN-A-CP-E013 Analysis of residual catalysts in pharmaceuticals using organic solvent dilution method by ICPE-9800 series

LAAN-A-CP-E014 Analysis of harmful elements in herbal medicines by ICPE-9800 series

SHIMADZU

Application News

Spectroscopy - AAS

Determination of Al in Serum using GFA-7000 with Omega Platform tube

No. SCA-120-023

Even the smallest amounts of harmful substances can have detrimental effects on the human body. These substances pose additional risks to persons who are already suffering from a disease. It is therefore essential to be able to detect even the lowest concentrations of harmful residues in body fluids.
Biomonitoring via state-of-the-art, highly sensitive analytical methods, enables the detection of very low concentrations of harmful substances absorbed by the human body. Atomic absorption spectrophotometers, which can detect many metals such as aluminium, copper and lead, are suitable for routine biomonitoring.
In the case of aluminium, human-influenced factors as well as natural exposure play a significant role. These include, for instance, aluminium intoxication in already weak dialysis patients who may receive large doses of aluminium as a result of contaminated dialysis fluids and certain medications. Impairment of the renal function slows down elimination of the metal significantly.

Aluminium is generally determined in serum or plasma. The values in whole blood and serum correlate closely. The reference value for aluminium in serum at a concentration of approximately 5 µg/L typically lies within the measuring range of the AAS graphite furnace[1].

■ Standard system Configuration

In this case, determination of aluminium in serum was carried out using Shimadzu's new AA-7000G in combination with the highly sensitive GFA-7000 graphite furnace and the fully automatic ASC-7000 sample preparation station.

The system includes high-performance optics with a Czerny-Turner monochromator and operates in a wavelength range from 185 up to 900 nm. Two methods are available for background compensation: the deuterium method which compensates for interferences by molecular absorption and particulate caused scattering and the high-speed self-reversal method which eliminates spectral interferences caused by direct or indirect neighbouring line overlap in the entire wavelength range.

The graphite furnace with digital gas and temperature control excels with its high sensitivity and excellent long-term stability. For the fully automatic determination of aluminium in serum, the sample material is injected into the graphite tube via the ASC-7000 sample preparation station. Atomisation occurs on the new Omega platform tube, which is distinguished by its uniform temperature distribution when compared with conventional graphite tubes (figure 1).

Figure 1: Cross section through the Omega platform tube

■ Method and parameters

The calibration curve was established using an aluminium stock standard solution of 20 µg/L and exhibits excellent linearity in the required measuring range (Figure 2).

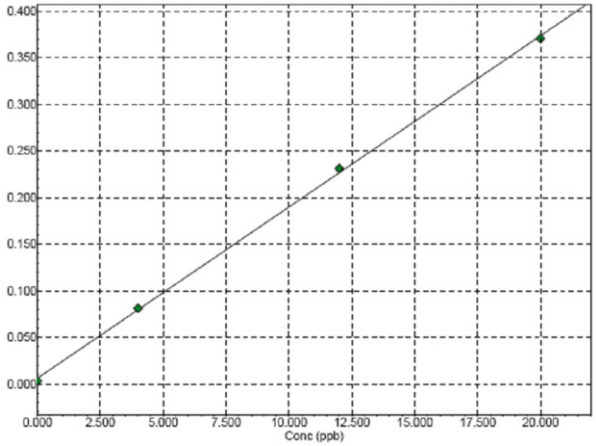


Figure 2: Aluminium calibration curve

The heating program has been optimized using the temperature search function and is prepared for a smooth heating of the serum sample and then optimized ashing at 1400°C and atomization at a temperature of 2600°C, as shown in table 1.

No.	Temp. [°C]	Time [sec.]	Heat Mode
1	95	20	RAMP
2	105	10	RAMP
3	140	20	RAMP
4	380	10	RAMP
5	420	20	RAMP
6	800	10	RAMP
7	800	5	STEP
8	1400	10	RAMP
9	1400	10	STEP
10	1400	5	STEP
11	2600	4	STEP
12	2800	3	STEP

Table 1: Aluminium heating program

Ultra-pure water with 0.1% (v/v) HNO_3 and 0.1% Triton X-100 was used as a rinsing solution for the autosampler. A reference serum (Seronorm™ 201405) was measured as a control sample. The recorded signal of the reference material is shown in Fig. 3. The results measured correspond well with the certified values. The recovery rate was 103%.

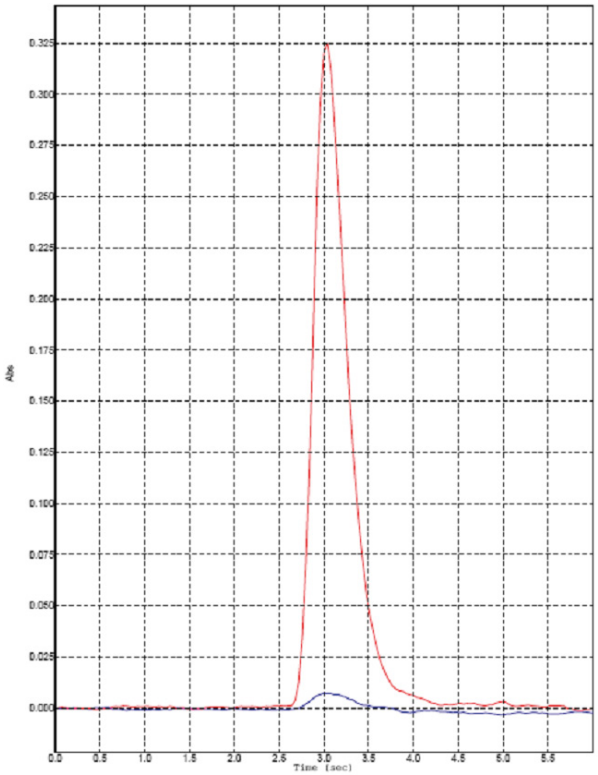


Figure 3: Recorded signal of reference serum

■ Conclusion

The new AA-7000 with GFA-7000 graphite furnace and ASC-7000 sample preparation station represents a reliable system configuration for the fully automatic determination of aluminium in serum. This system configuration, suitable for samples with complex matrices, features an Omega platform tube exhibiting excellent stability and is an ideal addition to “state of the art” graphite furnace technology.

[1] Bundesgesundheitsblatt, Bd. 41 (6), 1998

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Application News

No. SCA_125_011

EDX

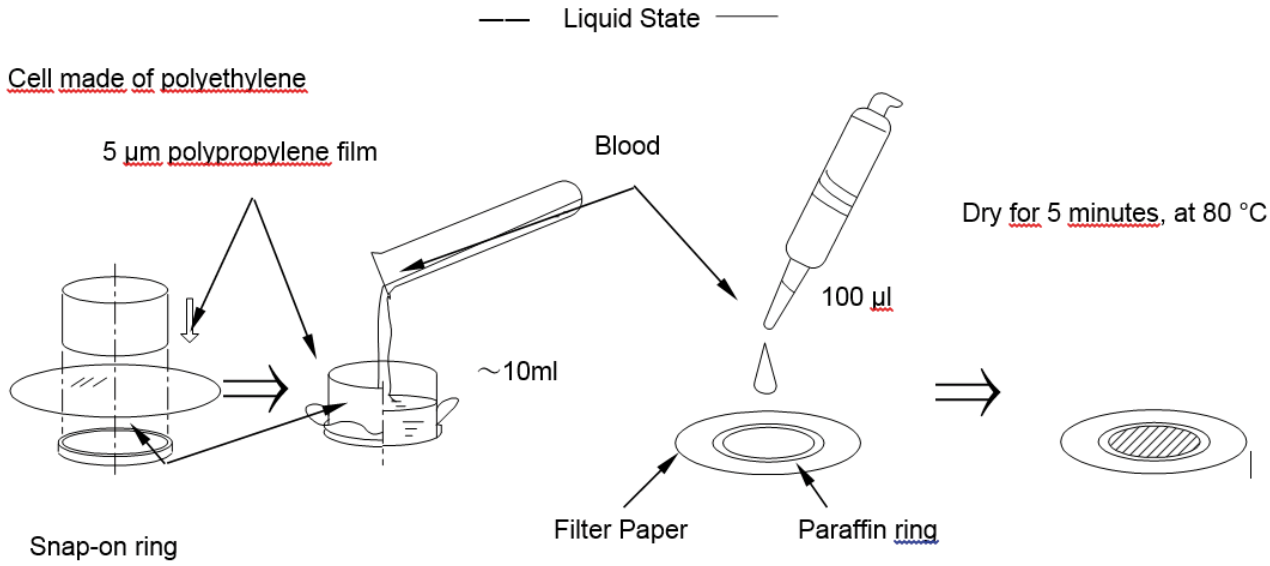
EDXRF Analysis of Cd, Hg, and Pb in Blood

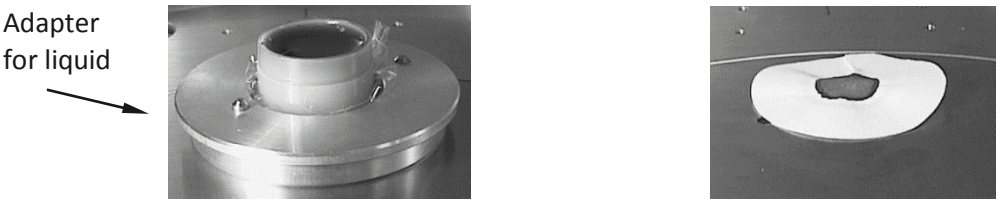
There is a need nowadays for the rapid estimation of the various type of poisons in medicine that have combined with metals in order to be able to give emergency treatment through the administering of an antidote. EDX-700/800 can identify various types of metals after a simple preparation of the sample.
Furthermore, the testing is non-destructive so the sample can be analyzed with a different analyzer after testing. Shown below are examples of qualitative analysis with the sample in liquid form and with the sample dried on filter paper.

Sample Preparation

1) Liquid Cell Method
Approximately 10 ml of the sample was poured into the liquid sample receptacle sealed with 5 µm polypropylene film.

2) Filter Method
After dropping 100 µl of the sample onto filter paper, the paper was dried for 5 minutes at 80 °C



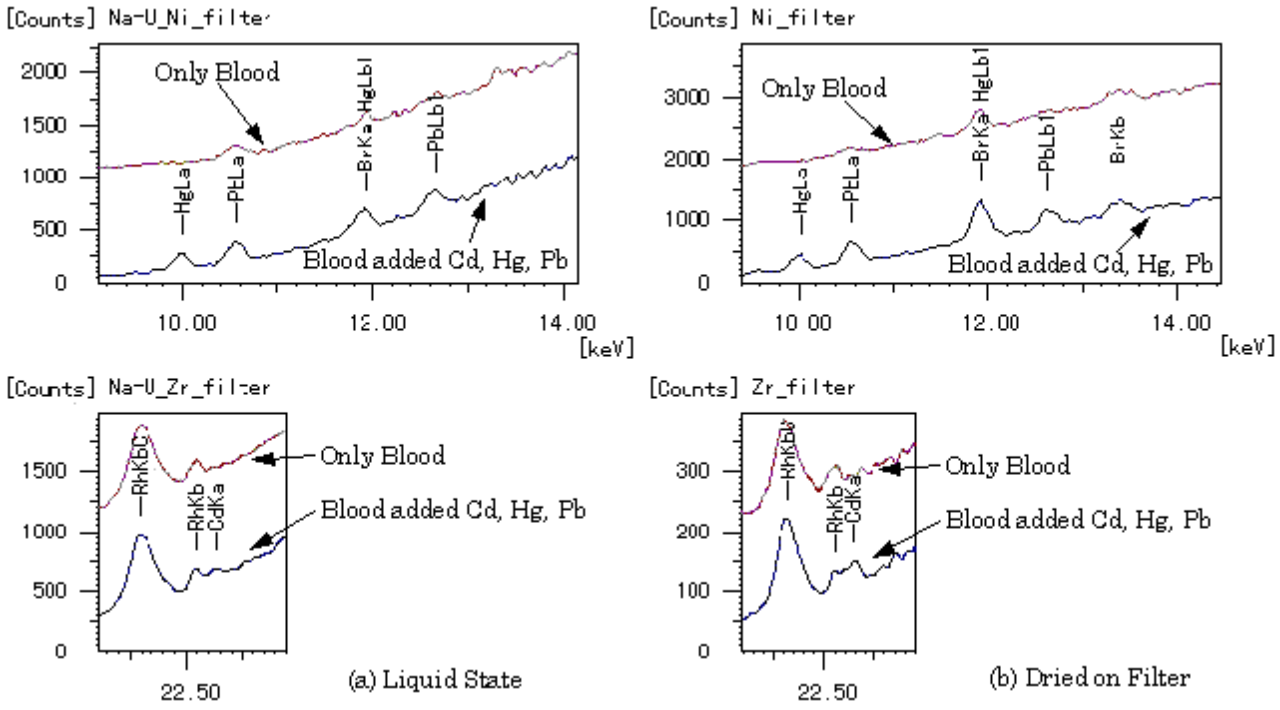


Sample loading (2) Filter method Sample loading

After dropping 100 µl of the sample onto filter paper, the paper was dried for 5 minutes at 80 °C.

Result of the Qualitative Analysis of Blood

The sample of blood and the sample with 10 ppm of Cd, Hg, and Pb were qualitatively analyzed with the liquid cell method and the filter method. The comparative results are shown on Fig. 1 In addition the difference in profile between the blood and that of the sample with the added Cd, Hg, and Pb is compared.



Only Blood" Profile has offset (Backgrounds are same between "Only Blood" and "Blood added Cd, Hg, Pb ")

Fig.1 Qualitative Analysis of Cd, Hg, Pb 10 ppm in Blood

Lower Limit of Detection(L.L.D)

The lower limit of detection of Cd, Hg, and Pb in blood calculated from the results of the qualitative analysis is shown in Table 1.

Element	Liquid State	Dried on Filter
Cd	9.9 ppm	8.0 ppm
Hg	3.4 ppm	1.9 ppm
Pb	2.2 ppm	1.6 ppm

Table 1 L.L.D of Cd, Hg, Pb in Blood

Analytical Conditions

Instrument: EDX-700
X-ray Tube: Rh target
Filter: Ni, Zr
Voltage - Current: 50kV-24-500 μA (Auto)
Atmosphere: Air
Measurement Diameter: 10 mm
Measuring Time: 1000 sec

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Application News

No. SCA_125_012

EDX

EDXRF Analysis of P, Cl, K and Ca in Blood

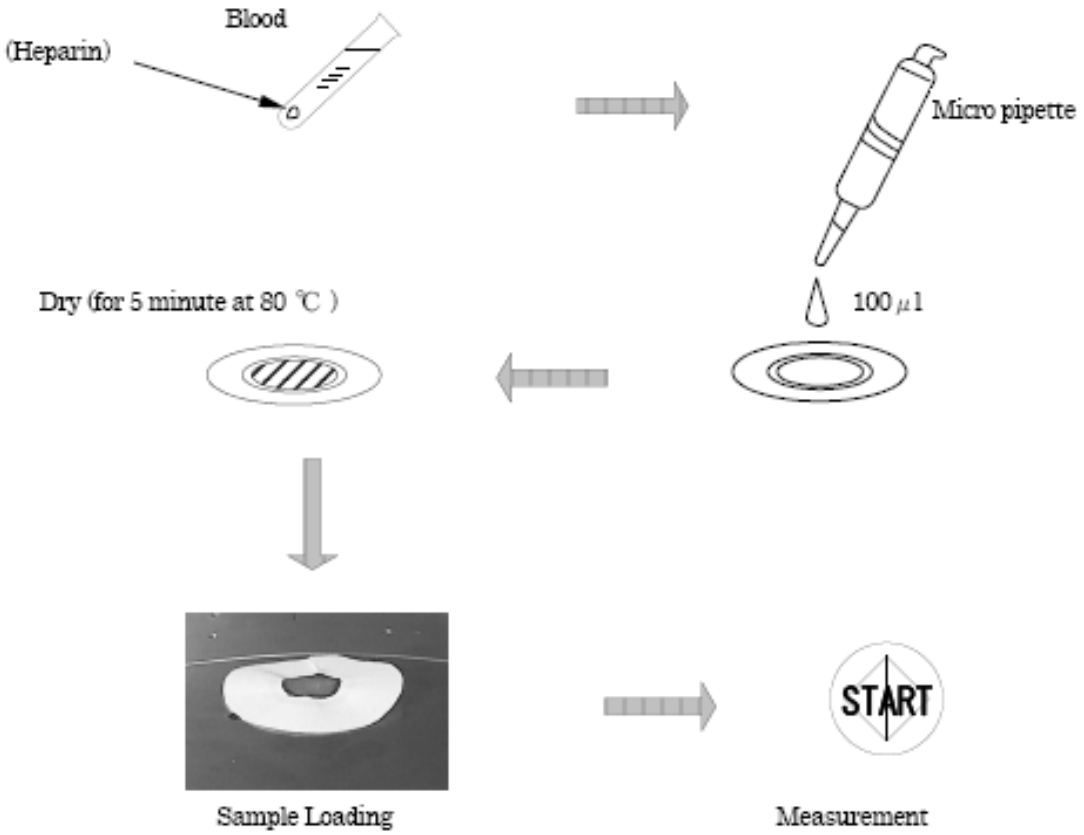
EDX is effective for quick screening analysis of tests for electrolytes (such as P, Cl, K, Ca) in blood. Carrying out the analysis in air with these light elements results in the X-ray fluorescence from the sample being absorbed by the air, consequently lowering the sensitivity. The analysis therefore needs to be carried out either in helium or a vacuum. Though it is impossible to directly analyze a liquid such as blood in a vacuum, the liquid can be dropped onto filter paper, dried, and then analyzed in a vacuum. Shown below is an example of such a qualitative analysis.

Sample

Blood to which the anticoagulant Heparin has been added (Heparin is unnecessary if the sample is prepared as shown below).

Sample Preparation

After 100 µl of the sample has dropped onto the filter paper, the paper is dried for 5 minutes at 80 °C (If heparin is not added the paper is dried at normal temperature). This is described in the diagrams below.



The Result of the Qualitative Analysis of Electrolytes in Blood

The Results of the qualitative analysis of electrolytes such as P, Cl, K, and Ca present in the blood is shown in Fig.1. P-Ca have been detected (S has been detected as well, but it is assumed that this is from the Heparin). The reference values of these constituent elements for an adult male are shown in Table 1.

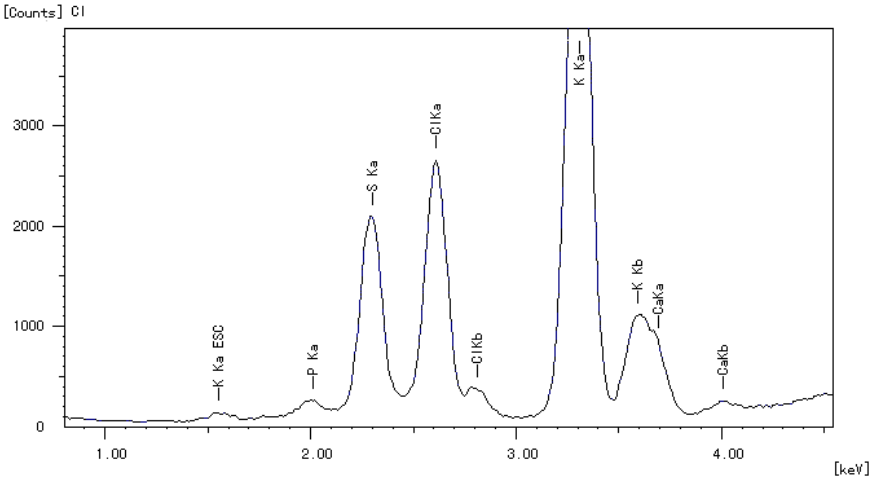


Fig.1 Qualitative Analysis of Electrolytes in Blood

P (Inorganic)	Cl	K	Ca
2.6-4.4 mg/dl (26-44 ppm)	97.8-102.6 mEq/l (0.35-0.36 %)	3.5-4.8 mEq/l (137-187 ppm)	8.6-10.4 mg/dl (86-104 ppm)

Table 1 Reference of Electrolyte in Blood[1)]

Comparison of the Atmosphere in which Analysis was Carried Out

The various sensitivities and methods are compared and summarized in Table 2 for the liquid and filter methods in air, vacuum and helium.

	Air	Vacuum	He Atmosphere
Filter Paper	● Simple, fast, drying not necessary	● Drying necessary	● Drying not necessary
Liquid	● Simple, fast, feasible	× Not possible	● Possible
Sensitivity	● Reduces with elements lighter than Ti	● Good	● Good

Table 1 Reference of Electrolyte in Blood[1)]

Analytical Conditions

Instrument: EDX-700
X-ray Tube: Rh target
Filter: Al
Voltage - Current: 15kV-1000 µA (Auto)
Atmosphere: Vacuum
Measurement Diameter: 10 mm
Measuring Time: 500 sec
Dead Time: 21 %

Reference
Extensive Blood/Urine Chemistry Tests, Immunological Tests – How to Interpret the Values – (First Volume)
Nihon Rin-rin Special Autumn Issue 1985, Nihon Rin-rin sha.

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Application News

No.J99

Inductively Coupled Plasma Atomic Emission Spectrometry

Analysis by ICP Atomic Emission Spectrometry in Accordance with the ICH Q3D Guideline for Elemental Impurities Using ICPE-9820

Introduction

Analysis of elemental impurities is one of the safety assessments required in the field of pharmaceuticals. In Japan, residual metal catalysts are classified as inorganic impurities according to the guidelines for Impurities in New Drug Substances (No. 1216001, issued by the Evaluation and Licensing Division, the Pharmaceutical and Food Safety Bureau, the Japanese Ministry of Health, Labour and Welfare), and are to be detected appropriately according to the method specified in the Japanese Pharmacopoeia, and evaluated at the stage of drug development. At the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: ICH, various guidelines were established and harmonized between Japan, Europe, and the US, including guidelines for elemental impurities in pharmaceuticals, referred to as the ICH Q3D, Guideline for Elemental Impurities.

For the analysis of elemental impurities, the methods specified for use as general analytical methods in the First Supplement of the Sixteenth Edition of the Japanese Pharmacopoeia include inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS), and atomic absorption spectrometry. Of these, ICP-AES is the most convenient, offering quick and easy multi-element analysis, and low running costs.

Here, we conducted analysis of 24 elements according to the ICH Q3D guidelines using the Shimadzu ICPE-9820 multi-type ICP atomic emission spectrometer. The ICPE-9820 offers simultaneous all element analysis with high sensitivity and high precision, while delivering high throughput. Low running costs are achieved by a unique combination of the reduced-flow mini-torch and vacuum optics, thereby reducing the overall consumption of argon.

Outline of the ICH Q3D Guideline for Elemental Impurities

In the ICH Q3D Guideline for Elemental Impurities, 24 elemental impurities were identified as elements of concern due to their toxicity, and permitted daily exposure limits (PDE) were established. The elements include lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As), referred to as the "big four," as well as residual metal catalysts added intentionally in the synthesis of a drug substance. Table 1 shows the ICH Q3D Guideline (STEP4 draft).

As permitted exposure values for the elemental impurities have been set as PDE values, the PDE values must be converted to concentrations to evaluate the elemental impurity components in the formulations or their component substances. As calculation methods, options 1, 2a, 2b, and 3 are available. Therefore, as long as the formulation is appropriate for the PDE value of the elemental impurity, any of the methods may be selected. Calculation examples for the respective options are shown in Table 2 to Table 5.

Table 1 Permitted Daily Exposure for Elemental Impurities of ICH Q3D (STEP4 draft)

Class	Element	Oral µg/day	Parenteral µg/day	Inhalation µg/day	Class	Element	Oral µg/day	Parenteral µg/day	Inhalation µg/day
1	As	15	15	2	2B	Pt	1000	10	1
	Cd	5	2	2		Se	150	80	130
	Hg	30	3	1		Rh	100	10	1
	Pb	5	5	5		Ru	100	10	1
2A	Co	50	5	3		Tl	8	8	8
	Ni	200	20	5	3	Ba	1400	700	300
	V	100	10	1		Cr	11000	1100	3
2B	Ag	150	10	7		Cu	3000	300	30
	Au	100	100	1		Li	550	250	25
	Ir	100	10	1		Mo	3000	1500	10
	Os	100	10	1		Sb	1200	90	20
	Pd	100	10	1		Sn	6000	600	60

Table 2 Calculation by Option 1: Maximum Permitted Common Concentration Limits of Elemental Impurities Across Drug Product Components for Products with Daily Intake of Not More Than 10 Grams

Component Substance	Max. Daily Intake of Each Substance (g)	PDE (µg)		Max. Permitted Concentration Assuming a 10 g Max. Daily Intake of Formulation (µg/g)		Max. Intake from Each Component (µg)	
				PDE/10 g		Max. Daily Intake (g) of Each Component × Max. Permitted Concentration (µg/g) of Each Component	
		Pb	As	Pb	As	Pb	As
Drug substance	0.2	5	15	0.5	1.5	0.1	0.3
MCC	1.1	5	15	0.5	1.5	0.55	1.65
Lactose	0.45	5	15	0.5	1.5	0.225	0.68
Calcium phosphate	0.35	5	15	0.5	1.5	0.175	0.53
Crospovidone	0.265	5	15	0.5	1.5	0.133	0.4
Magnesium stearate	0.035	5	15	0.5	1.5	0.018	0.05
HPMC	0.06	5	15	0.5	1.5	0.03	0.09
Titanium oxide	0.025	5	15	0.5	1.5	0.013	0.04
Iron oxide	0.015	5	15	0.5	1.5	0.008	0.02
Max. Daily Intake (Total)	2.5					1.25	3.75
PDE (µg/day)						5.0	15

Table 3 Calculation by Option 2a: Maximum Permitted Common Concentration Limits Across Drug Product Component Materials for a Product with a Specified Daily Intake (Assuming That Concentration Remains Constant)

Component Substance	Max. Daily Intake of Each Substance (g)	PDE (µg)		Max. Permitted Concentration (µg/g)		Max. Intake from Each Component (µg)	
				PDE/Max. Daily Intake of Actual Drug (e.g. 2.5 g)		Max. Daily Intake (g) of Each Component × Max. Permitted Concentration (µg/g) of Each Component	
		Pb	As	Pb	As	Pb	As
Drug substance	0.2	5	15	2	6	0.4	1.2
MCC	1.1	5	15	2	6	2.20	6.6
Lactose	0.45	5	15	2	6	0.9	2.7
Calcium phosphate	0.35	5	15	2	6	0.7	2.1
Crospovidone	0.265	5	15	2	6	0.53	1.59
Magnesium stearate	0.035	5	15	2	6	0.07	0.21
HPMC	0.06	5	15	2	6	0.12	0.36
Titanium oxide	0.025	5	15	2	6	0.05	0.15
Iron oxide	0.015	5	15	2	6	0.03	0.09
Max. Daily Intake (Total)	2.5					5.0	15
PDE (µg/day)						5.0	15

Table 4 Calculation by Option 2b: Maximum Permitted Common Concentration Limits Across Drug Product Component Materials for a Product with a Specified Daily Intake (Arbitrary Setting of Maximum Concentration Possible from Actual Value)

Component Substance	Max. Daily Intake of Each Substance (g)	PDE (µg)				Measured Concentration Value (µg)				Arbitrary Setting of Max. Concentration Possible from Actual Value (µg/g)				Max. Daily Intake of Each Componen (µg)			
		Pb	As	Pd	Ni	Pb	As	Pd	Ni	Pb	As	Pd	Ni	Pb	As	Pd	Ni
Drug substance	0.2	5	15	100	200	**	0.5	20	50	**	5	500	200	**	1	100	40
MCC	1.1	5	15	100	200	0.1	0.1	*	**	0.5	5	*	**	0.55	5.5	*	**
Lactose	0.45	5	15	100	200	0.1	0.1	*	**	0.5	5	*	**	0.225	2.3	*	**
Calcium phosphate	0.35	5	15	100	200	1	1	*	5	5	5	*	200	1.75	1.8	*	70
Crospovidone	0.265	5	15	100	200	0.1	0.1	*	**	0.5	5	*	**	0.132	1.3	*	**
Magnesium stearate	0.035	5	15	100	200	0.5	0.5	*	0.5	5	10	*	50	0.175	0.4	*	1.75
HPMC	0.06	5	15	100	200	0.1	0.1	*	**	2.5	5	*	**	0.15	0.3	*	**
Titanium oxide	0.025	5	15	100	200	20	1	*	**	40	20	*	**	1	0.5	*	**
Iron oxide	0.015	5	15	100	200	10	10	*	50	20	100	*	200	0.3	1.5	*	3
Max. Daily Intake (Total)	2.5													4.3	14.5	100	115
PDE (µg/day)														5	15	100	200

*: Since it has been determined that there is no possibility of Pd being present, a quantitative result is not obtained.
**: Below the detection limit

Table 5 Calculation by Option 3: Finished Product
Concentration (µg/g) = PDE (µg/day)/Daily intake of drug product (g/day)

		PDE (µg)				Maximum Permitted Concentration (µg/g)			
	Daily Intake (g)	Pb	As	Pd	Ni	Pb	As	Pd	Ni
Drug Product	2.5	5	15	100	200	2	6	40	80

Sample

· Ophthalmic solution
· Tablet (Daily intake: 1 tablet (0.2 g))

Sample Preparation

1. Pretreatment of sample (ophthalmic solution)

To 2 mL of sample (approximately 2 g), add 0.5 mL hydrochloric acid, 0.5 mL nitric acid and internal standard element Y (0.5 mg/L based on measurement solution concentration). Adjust the volume to 10 mL using distilled water to use as the measurement solution (5-fold dilution). A spike-and-recovery test solution was prepared using a similarly prepared solution spiked with a standard solution of the measurement element.

2. Pretreatment of tablet sample

Two tablets (daily dosage of 1 tablet per day (0.20 g)) were dissolved with 3 mL hydrochloric acid and 2 mL nitric acid using a microwave sample preparation system and a sample pretreatment quartz vessel.
After conducting microwave digestion, the solution volume was adjusted to 20 mL with distilled water to use as the measurement solution (50-fold dilution). At this time, the internal standard elements Y and In (Y at 0.5 mg/L and In at 1.0 mg/L) were added to the solution. Also, prior to digestion, the measurement element was added to prepare a spike-and-recovery test solution.

Instrument and Analytical Conditions

Measurement was conducted using the Shimadzu ICPE-9820 multi-type ICP atomic emission spectrometer. The measurement conditions are shown in Table 6.
The ICPE-9820 is a spectrometer that uses the latest CCD, permitting simultaneous measurement of all elements and all wavelengths, while its high-sensitivity axial observation permits high-throughput measurement. Further, the high-temperature plasma generated by the mini torch assures high sensitivity with low ionization interference to provide acquisition of accurate values. In addition, the mini-torch plasma produced by low-flowrate argon gas, the Eco mode and the vacuum spectrometer greatly reduce running costs.

Table 6 Analytical Conditions

Instrument	: ICPE-9820
Radio frequency power	: 1.2 kW
Plasma gas Flowrate	: 10 L/min
Auxiliary gas Flowrate	: 0.6 L/min
Carrier gas Flowrate	: 0.7 L/min
Sample introduction	: Nebulizer 10
Misting chamber	: Cyclone chamber
Plasma torch	: Mini-Torch
Observation	: Axial (AX) / Radial (RD)

Analysis

Quantitative analysis of the 24 elements subject to the ICH Q3D guidelines was conducted using the calibration curve-internal standard method, and spike-and-recovery testing was also conducted.

Analytical Results

Table 7 shows the results of analysis of the ophthalmic solution. The PDE value of the ophthalmic solution was used as the parenteral value. Table 8 shows the results of the tablet analysis. Good results were obtained in the spike-and-recovery testing for each of the samples (Tables 7 and 8[*1]). In addition, the detection limit calculated as the concentration in the sample (Tables 7 and 8[*2]) adequately satisfied the permitted concentrations (Tables 7 and 8[*3]).

Conclusion

Use of the ICPE-9820 permits quick, accurate analysis of the 24 elements specified in the ICH Q3D guideline.

[References]
1) Impurities in New Drug Substances (No. 1216001, issued by the Evaluation and Licensing Division, the Pharmaceutical and Food Safety Bureau, the Japanese Ministry of Health, Labour and Welfare)
2) First Supplement of the Sixteenth Edition of the Japanese Pharmacopoeia
3) ICH Q3D: Guideline for Elemental Impurities (STEP4 draft)

Table 7 Analytical Results of Eye Drop

Element	PDE value for parenteral	*3 Permitted concentration	Post-treatment concentration	Spike concentration	Measured concentration (Eye drop)	*1 Spike-and-recovery rate	*2 Converted detection limit (3σ) in ophthalmic solution
	μg	μg/mL	μg/mL	μg/mL	μg/mL	%	μg/mL
As	15	15	3	1	<DL	104	0.04
Cd	2	2	0.4	0.4	<DL	101	0.0006
Hg	3	3	0.6	0.3	<DL	105	0.007
Pb	5	5	1	0.3	<DL	102	0.01
Co	5	5	1	0.3	<DL	95	0.001
Ni	20	20	4	0.5	<DL	104	0.003
V	10	10	2	0.5	<DL	98	0.0008
Ag	10	10	2	0.5	<DL	104	0.0008
Au	100	100	20	0.5	<DL	99	0.006
Ir	10	10	2	0.5	<DL	101	0.01
Os	10	10	2	0.5	<DL	103	0.006
Pd	10	10	2	0.5	<DL	102	0.004
Pt	10	10	2	0.5	<DL	99	0.02
Se	80	80	16	0.5	<DL	103	0.02
Rh	10	10	2	0.5	<DL	95	0.007
Ru	10	10	2	0.5	<DL	103	0.003
Tl	8	8	1.6	0.5	<DL	95	0.02
Ba	700	700	140	0.5	<DL	96	0.0006
Cr	1100	1100	220	0.5	<DL	97	0.002
Cu	300	300	60	0.5	<DL	96	0.002
Li	250	250	50	0.5	<DL	99	0.01
Mo	1500	1500	300	0.5	<DL	100	0.003
Sb	90	90	18	0.5	<DL	103	0.01
Sn	600	600	120	0.5	<DL	100	0.01

PDE value for parenteral
Permitted concentration : When 1 mL of the ophthalmic solution is used per day (Option 3 is used when calculating the conversion to the PDE concentration)
Post-treatment concentration : The permitted concentration in the measurement sample after pretreatment of the sample
Spike concentration : Concentration of spiking solution in spike-and-recovery testing
Converted detection limit (3σ) in ophthalmic solution: Detection limit (3σ) in measurement solution × Dilution factor (5)
<DL: Below the detection limit (3σ)

Table 8 Analytical Results of Tablet

Element	PDE value for oral	*3 Permitted concentration	Post-treatment concentration	Spike concentration	Measured concentration (Tablet)	*1 Spike-and-recovery rate	*2 Tablet converted detection limit (3σ)
	μg	μg/g	μg/mL	μg/mL	μg/g	%	μg/g
As	15	75	1.5	0.5	<DL	107	0.5
Cd	5	25	0.5	0.1	<DL	100	0.007
Hg	30	150	3	1	<DL	101	0.1
Pb	5	25	0.5	0.1	<DL	98	0.07
Co	50	250	5	1	<DL	101	0.01
Ni	200	1000	20	1	0.1	100	0.03
V	100	500	10	1	<DL	103	0.01
Ag	150	750	15	1	<DL	104	0.02
Au	100	500	10	1	<DL	105	0.03
Ir	100	500	10	1	<DL	100	0.09
Os	100	500	10	1	<DL	85	0.04
Pd	100	500	10	1	<DL	106	0.05
Pt	1000	5000	100	1	<DL	102	0.3
Se	150	750	15	1	<DL	108	0.3
Rh	100	500	10	1	<DL	101	0.1
Ru	100	500	10	1	<DL	100	0.03
Tl	8	40	0.8	0.1	<DL	103	0.2
Ba	1400	7000	140	1	<DL	102	0.003
Cr	11000	55000	1100	1	<DL	101	0.02
Cu	3000	15000	300	1	<DL	105	0.05
Li	550	2750	55	1	<DL	104	0.1
Mo	3000	15000	300	1	<DL	101	0.03
Sb	1200	6000	120	1	<DL	105	0.1
Sn	6000	30000	600	1	<DL	100	0.03

PDE value for oral
Permitted concentration : Permitted concentration in daily intake (0.2 g) (Option 3 is used for calculation of conversion from PDE to concentration)
Post-treatment concentration : Permitted limit concentration in measurement solution following sample pretreatment
Spike concentration : Concentration of the added spike-and-recovery test solution
Tablet converted detection limit (3σ): Detection limit (3σ) in measurement solution Dilution factor (50)
<DL: Below the detection limit (3σ)

First Edition: Sep. 2014

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Application News

No.J100

Inductively Coupled Plasma Atomic Emission Spectrometry

Analysis of Residual Catalysts in Pharmaceuticals Using Organic Solvent Dilution Method by ICPE-9800 Series

Introduction

Analysis of metal impurities is one of the safety assessments required in the field of pharmaceuticals. In Japan, residual metal catalysts are classified as inorganic impurities according to the guidelines for Impurities in New Drug Substances (No. 1216001, issued by the Evaluation and Licensing Division, the Pharmaceutical and Food Safety Bureau, the Japanese Ministry of Health, Labour and Welfare), and are to be detected appropriately according to the method specified in the Japanese Pharmacopoeia, and evaluated at the stage of drug development. In addition, in February 2008, the European Medicines Agency (EMA) issued a guideline to regulate the residual amounts of metal reagents and metal catalysts originating from the drug manufacturing process, and set permissible limit values for 14 elements. Table 1 shows the details of that guideline.

For the analysis of elemental impurities, the methods specified for use as general analytical methods in the First Supplement of the Sixteenth Edition of the Japanese Pharmacopoeia include inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS), and atomic absorption spectrometry. Of these, ICP-AES is the most convenient, offering quick and easy multi-element analysis, and low running costs.

Pretreatment of the samples is typically conducted using either the solvent dilution method or the acid decomposition method. However, solvent dilution is the preferred method, as it offers simple and quick processing of samples, and less contamination during pretreatment.

Here, using a Shimadzu ICPE-9800 series multi-type ICP atomic emission spectrometer, we conducted analysis of the 14 types of metals subject to the EMA guidelines as a drug substance model. In the analysis of organic solvent samples such as DMSO, DMF, and ethanol, the ICPE-9800 series provides stable analysis results thanks to the vertical orientation of the plasma torch, which to a large extent prevents sample residuals, such as carbon deposits, from forming inside the torch.

Table 1 EMA Guideline Classification and Permissible Levels for the Various Elements

		Oral exposure		Parenteral exposure		Inhalation exposure
		PDE (µg/day)	Concentration (ppm)	PDE (µg/day)	Concentration (ppm)	PDE (ng/day)
Class1A	Pt, Pd	100	10	10	1	Pt: 70
Class1B	Ir, Rh, Ru, Os	100	10	10	1	
Class1C	Mo, Ni, Cr, V	250	25	25	2.5	Ni: 100 Cr (VI): 10
Class2	Cu, Mn	2500	250	250	25	
Class3	Fe, Zn	13000	1300	1300	130	

Samples

- Tosufloxacin tosilate
- Benazepril hydrochloride
- Captopril

Sample Preparation

The organic solvent, dimethyl sulfoxide (DMSO), was used for dissolution of the sample due to its high solubility. After weighing out 0.5 g of the powdered sample, it was dissolved in the DMSO so as to bring the total volume to 5 mL. At this time, yttrium (Y) was added at 0.1 mg/L as the internal standard element. As for the tosufloxacin tosilate, when conducting the dilution operation, a spike-and-recovery test solution was prepared by adding the single element standard sample solution so as to obtain a concentration of 1.0 mg/L.

As for the calibration curve sample, the single element standard solution was diluted appropriately with DMSO, and the internal standard element Y was added so as to obtain a concentration of 0.1 mg/L.

Instrument and Analytical Conditions

For measurement, the Shimadzu ICPE-9800 series multi-type ICP atomic emission spectrometer was used. The analytical conditions that were used are shown in Table 2.

In most ICP-AES instruments, oxygen must be introduced into the plasma torch when conducting analysis of organic solvents to suppress the precipitation of carbon at the tip of the torch. With the Shimadzu ICPE-9800 series, however, vertical orientation of the plasma torch is adopted to reduce the likelihood of carbon precipitation, and reduce the amount of carbon precipitation from the sample to an extremely low level. Therefore, in the analysis of the organic solvent samples such as DMSO, DMF, or ethanol, introduction of oxygen is unnecessary, thereby permitting stable analysis to be conducted.

Table 2 Analytical Conditions

Instrument	: ICPE-9800 Series
Radio frequency power	: 1.3 kW
Plasma gas Flowrate	: 18 L/min
Auxiliary gas Flowrate	: 1.4 L/min
Carrier gas Flowrate	: 0.70 L/min
Sample introduction	: Nebulizer 10
Misting chamber	: Co-axial chamber
Plasma torch	: Torch
Observation	: Axial

Analysis

Quantitative analysis of the 14 elements specified in the EMA guidelines was conducted using the calibration curve-internal standard method.

Analytical Results

Table 3 shows the analytical results obtained for each of the pharmaceutical substances. Table 4 shows the spike-and-recovery test results for tosufloxacin tosilate. Using the internal standard method, excellent recoveries of 98 to 101 % were obtained for all of the elements without any adverse effects due to the viscosity of the measurement sample.

Fig. 1 shows the results of stability testing over two hours using a calibration curve sample (concentration 1.0 mg/L). Stable results were obtained with a relative standard deviation (RSD) of less than 1 % for each element.

Table 5 shows the results of repeatability testing over three days using a tosufloxacin tosilate spike-and-recovery test solution (1 mg/L spike concentration). Good repeatability was obtained with an RSD in the range of 1 %.

Fig. 2 shows the spectral profile of each of the elements. It is clear that verification is possible even at one-tenth the permissible concentration level (STD 0.1 mg/L).

Conclusion

Residual metals in pharmaceutical samples dissolved in organic solvent can be stably analyzed with high sensitivity using the ICPE-9800 series, and further, measurement throughput including sample preparation is significantly improved.

[References]

1) Impurities in New Drug Substances (No. 1216001, issued by the Evaluation and Licensing Division, the Pharmaceutical and Food Safety Bureau, the Japanese Ministry of Health, Labour and Welfare)
2) First Supplement of the Sixteenth Edition of the Japanese Pharmacopoeia
3) COMMITTEE FOR MEDICAL PRODUCTS FOR HUMAN USE (CHMP): GUIDELINE ON THE SPECIFICATION LIMITS FOR RESIDUES OF METAL CATALYSTS OR METAL REAGENT (Doc. Ref. EMEA/CHMP/SWP/4446/2000), London (21 February 2008)

Table 3 Analytical Results for Pharmaceutical Substances (3 types) (Concentrations in Reagent Powders: µg/g)

<: Less than Lower Limit of Detection

Element	Cr	Cu	Fe	Ir	Mn	Mo	Ni	Os	Pd	Pt	Rh	Ru	V	Zn
Tosufloxacin Tosilate	0.11	0.05	4.37	<	0.03	<	<	<	<	<	<	<	<	0.06
Benazepril Hydrochloride	0.36	0.52	5.17	<	0.07	<	<	<	<	<	<	<	<	1.12
Captopril	0.37	0.26	0.52	<	0.10	<	<	<	<	<	<	<	<	14.4
Detection Limit (*)	0.03	0.04	0.007	0.07	0.007	0.03	0.07	0.08	0.02	0.3	0.1	0.04	0.007	0.04
	0.003	0.004	0.0007	0.007	0.0007	0.003	0.007	0.008	0.002	0.03	0.01	0.004	0.0007	0.004

(*) Lower Limit for Detection (3σ) in Pharmaceutical Substance Analysis
(Top Row: Concentrations (µg/g) in Reagent Powders, Lower Row: Concentrations (mg/L) in Solution)

Table 4 Addition and Recovery Results for Tosufloxacin Tosilate (Concentrations in Solution: mg/L)

<: Less than Lower Limit of Detection

Element	Cr	Cu	Fe	Ir	Mn	Mo	Ni	Os	Pd	Pt	Rh	Ru	V	Zn
Pre-addition	0.011	0.005	0.437	<	0.003	<	<	<	<	<	<	<	<	0.006
Post-addition	0.997	0.994	1.423	0.978	0.987	1.010	0.991	1.003	0.981	0.996	0.978	0.977	1.000	0.989
Recovery (%)	99	99	99	98	98	101	99	100	98	100	98	98	100	98

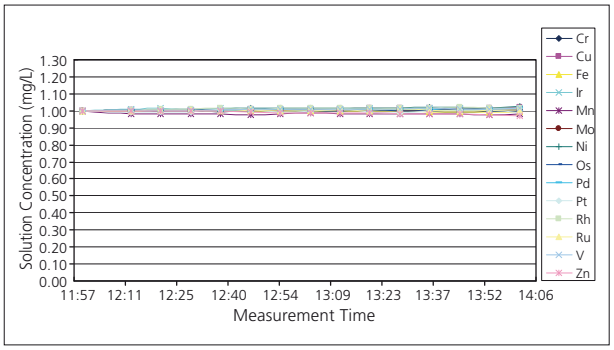

Fig. 1 Variation with Time over 2 Hours

Table 5 Reproducibility Results over 3 Days (units mg/L)

Element	1ST Day	2ND Day	3RD Day	RSD (%)
Cr	1.00	1.00	1.02	1.18
Cu	0.99	1.00	0.99	0.46
Fe	1.42	1.43	1.43	0.19
Ir	0.98	0.98	0.97	0.60
Mn	0.98	0.99	0.97	0.60
Mo	1.01	1.01	1.01	0.26
Ni	0.99	0.99	0.99	0.24
Os	1.00	1.00	1.01	0.48
Pd	0.98	0.98	0.99	0.85
Pt	1.00	1.00	1.02	0.96
Rh	0.98	0.97	0.97	0.55
Ru	0.98	0.98	1.00	1.35
V	1.00	1.00	1.00	0.23
Zn	0.99	0.98	0.97	0.87

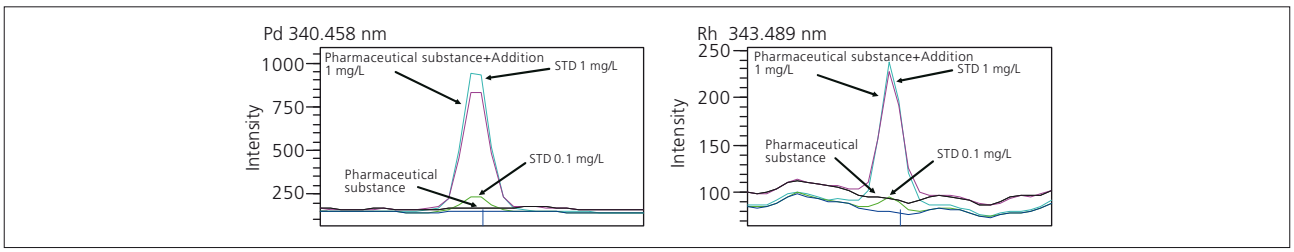

Fig. 2 Spectral Profiles for Pd and Rh (Calibration Solution (0, 0.1, 1.0 mg/L), Pharmaceutical Substance, Standard Sample Addition (Pharmaceutical Substance + Standard Sample 1.0 mg/L))

First Edition: Sep. 2014

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Application News

No.J101

Inductively Coupled Plasma Atomic Emission Spectrometry

Analysis of Harmful Elements in Herbal Medicines by ICPE-9800 Series

Introduction

The growing use of herbal medicines as alternative medicines is beginning to heighten concern regarding their quality and safety.
Herbal medicines, products consisting of animals and plants, fungi, and minerals with naturally occurring efficacy, are used without purification as drugs (over-the-counter drugs), foods, functional foods, and dietary supplements. Safety standards for harmful elements used in pharmaceuticals are provided for in each country. Table 1 shows examples of regulations regarding the permissible levels of harmful elements in herbal medicines, including the levels suggested by WHO and the import/export reference values of China.
Here, using the Shimadzu ICPE-9800 series multi-type ICP atomic emission spectrometer, we conducted an analysis of an herbal medicine. The ICPE-9800 series, with its mini-torch plasma and spectrometer which permits all element/all wavelength simultaneous analysis, provides high sensitivity, high precision, and high throughput at low cost.

Table 1 Harmful Element Regulation of Herbal Medicines (mg/kg)

Element	As	Cd	Cu	Hg	Pb
WHO recomended value		0.3			10
China Import/Export Herbal Medicine Reference Value	2	0.3	20	0.2	5

Sample

Herbal medicines distributed in Japan.

Sample Preparation

Low boiling point-elements such as arsenic (As), mercury (Hg), etc. are subject to loss due to volatilization during operations such as heating and acid addition. Therefore, a pretreatment process is required that will keep element loss to a minimum, while at the same time be efficient. Here, we conducted sample digestion using a microwave sample preparation system.
To 0.5 g of dry sample, 7.5 mL of concentrated nitric acid and 0.5 mL of concentrated hydrochloric acid were added, and digestion was conducted using a microwave sample preparation system. After digestion, distilled water was added to the process liquid to bring the total volume to 25 mL, and this was used as the analytical sample. Separately, after preparing another sample in the same manner, a standard solution containing the target elements was added, and this served as the spike-and-recovery test solution.

Instrument and Analytical Conditions

For measurement, the Shimadzu ICPE-9800 series multi-type ICP emission spectrometer was used. The analytical conditions used are shown in Table 2. The adoption of an echelle spectrometer and a CCD detector permits simultaneous analysis of all the elements at all the wavelengths, in addition to high-throughput measurement even with many target elements and samples. Also, the mini-torch plasma, Eco mode, and vacuum-housed spectrometer serve to greatly reduce running costs due to gas consumption.
Herbal medicines often contain large amounts of coexisting elements, including calcium (Ca), potassium (K), and magnesium (Mg). Typically, when a sample contains many coexisting elements, some level of error may affect the analysis value due to ionization interference. However, the ICPE-9800 series mini-torch produces a high-temperature plasma which in addition to providing high sensitivity, also suppresses the effect of ionization interference.

Table 2 Analytical Conditions

Instrument	: ICPE-9800 series
Radio frequency power	: 1.2 kW
Plasma gas Flowrate	: 10 L/min
Auxiliary gas Flowrate	: 0.6 L/min
Carrier gas Flowrate	: 0.7 L/min
Sample introduction	: Nebulizer 10
Misting chamber	: Cyclone chamber
Plasma torch	: Mini Torch
Observation	: Axial (AX)
Measurement time	: 2.5 min/sample (Including rinse time)

Analysis

Quantitative analysis of As, Cd, Cr, Cu, Hg, Pb, and Sn was conducted using the calibration curve method.

[References]

1) WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues (Japan Self-Medication Industry, published March 2009)
2) Green Trade Standards of Importing & Exporting Medicinal Plants & Preparations (Ministry of Foreign Trade and Economic Cooperation, People's Republic of China, effective July 1, 2001)

Analytical Results

Table 3 shows the semi-quantitative results for the principal components using qualitative analysis. Semi-quantitative results are calculated automatically by the database built into the software. With the ICPE-9800 series, qualitative data for all elements are acquired and saved at the same time that quantitative analysis is conducted. This feature, even after quantitative analysis, makes it possible to identify the principal elements and their concentrations, making it possible to consider the impact on the analyte elements.
Table 4 shows the analytical results, and Fig. 1 shows the spectral profiles. As for the recoveries, good results were shown for all elements, and it is clear that accurate quantitation is possible without adverse influence from coexisting elements such as Ca and K present at high concentrations. Further, the detection limit is below the WHO recomended value as well as the China import and export reference values, indicating sufficient sensitivity.

Conclusion

Use of the ICPE-9800 series offers high sensitivity, as well as accurate and low-cost measurement of harmful elements in herbal medicines.

Table 3 Semi-Quantitative Results for Herbal Medicines by Qualitative Analysis (wt%)

	Ca	K	Mg	S	Al	P	Si	Fe	Mn	Ba	Sr	Na
Horny goat weed	2.6	1.0	0.4	0.3	0.2	0.2	0.14	0.13	0.05	0.02	0.01	0.01
Fang feng (Saposhnikoviae Radix)	1.2	0.5	0.4	0.2	0.2	0.2	0.08	0.10	0.004	0.01	0.02	0.08

Table 4 Analytical Results for Herbal Medicines (µg/g)

Element / Sample Name	As	Cd	Cr	Cu	Hg	Pb	Sn
1. Cardamom	<	0.07	<	5.5	<	0.2	<
2. Cinnamon	0.3	<	0.5	7.0	<	0.5	<
3. Horny goat weed	0.5	0.14	3.0	4.7	<	1.4	0.1
Recovery Rate in Spike/Recovery Test (%)	101	99	99	95	98	95	100
4. Carrot	<	0.03	0.04	5.2	<	<	<
5. Rehmanniae Radix	<	<	0.3	3.9	<	0.2	<
6. Paeoniae radix	<	<	0.2	4.3	<	<	0.1
7. Fang feng (Saposhnikoviae Radix)	<	<	0.4	7.0	<	0.2	<
Recovery Rate in Spike/Recovery Test (%)	101	102	99	100	97	98	100
8. Turmeric (Curcumae Radix)	<	0.05	0.1	2.2	<	4.5	<
Detection Limit	0.2	0.005	0.02	0.04	0.1	0.1	0.05
WHO Recommended Value		0.3				10	
China Import/Export Herbal Medicine Reference Value	2	0.3		20	0.2	5	

Detection limit: 3-fold the concentration standard deviation obtained from 10 repeated measurements of the calibration curve blank × Dilution factor (50)
<: Below the limit of detection

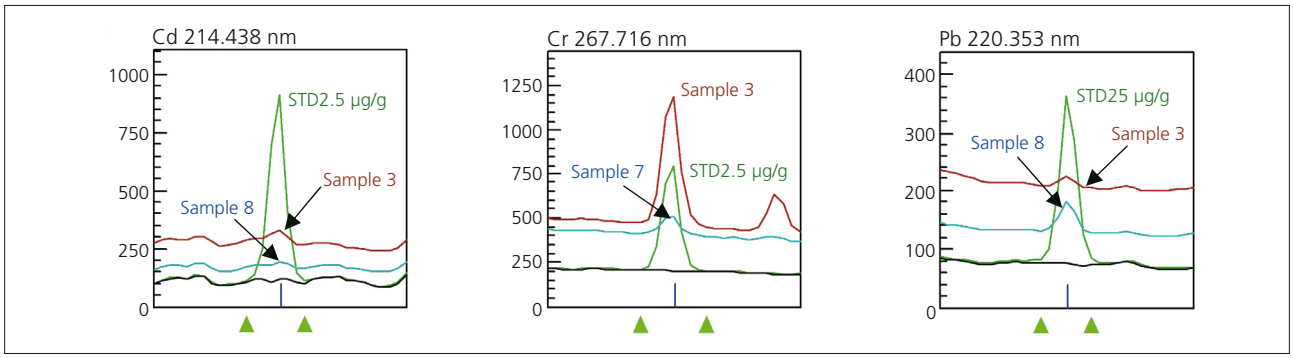

Fig. 1 Spectral Profiles of Cd, Cr, and Pb in Herbal Substances
*** The concentrations in the figures refer to the concentrations in the samples (solid)**

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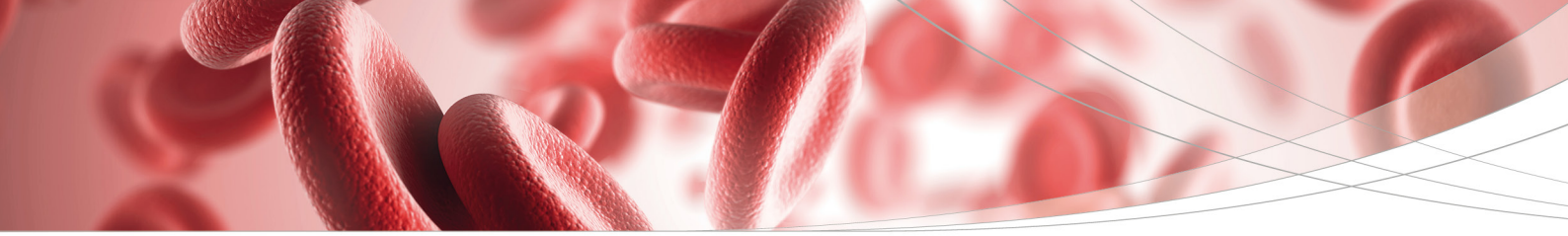

3. Spectroscopy

3.2 Molecular Spectroscopy

3.2.1 Ultraviolet Visible Spectroscopy and Near Infrared (UV-Vis NIR)

Analysis of metals, ions, colors and molecules
The ultraviolet and visible range of the light spectrum is sensitive on color determination and carbon hydrogen bonding determination. Color reactions, DNA and protein methods are easily to solve in low concentrations.

SCA_100_020	Bilirubin determination in cerebrospinal fluid with UV-Spectroscopy

3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

Quantification and identification of substances
The infrared spectroscopy can analyze all materials which react with heat. The physical vibration correlated to this heat is a identification tool for each material.

SCA-110d-094	Analysis of stones – urea, kidney, gall with infrared spectroscopy

3.2.3 Fluorescence Spectroscopy

Quantitative and qualitative analysis of substances
Fluorescence spectroscopy provides low detection limits for the determination of chemo- and bio-luminescences and fluorescences from diverse substances.
Furthermore, this analytical method enables to detect selective DNA or a cocktail of markers in tissue analysis.
Also, fluorescence spectroscopy can be applied for kinetics as well as overview scans in high speed 3D-technique.

LAAN-A-RF-E002	Fluorescence spectrum measurement of fluorescence dye indocyanine green in long wavelength region

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Application News

Spectroscopy – UV

Bilirubin Determination in cerebrospinal fluid with UV-Spectroscopy

No. SCA-100-020

Subarachnoid hemorrhage is a pathological event in the central nervous system. It is characterized by bleeding into the subarachnoid space, which is filled with cerebrospinal fluid (CSF, liquor cerebrospinalis). Bilirubin determination, such as the determination of bilirubin in CSF*, is an analytical method used in clinical analysis. In CSF-bilirubin determination, cerebrospinal fluid is analyzed for blood fragments. The UV spectroscopic application presented here is based on the 'National Guidelines for analysis of Cerebrospinal Fluid for Bilirubin in suspected Subarachnoid Hemorrhage' according to Beetham [1].

The causes of cerebral hemorrhage can be diverse, such as a stroke or an aneurysm. For treatment of a patient, it is important to determine which type of blood (old, fresh or none) is present in the cerebrospinal fluid. Red blood cells will initially enter the cerebrospinal fluid. These cells contain oxyhemoglobin, which is converted to bilirubin by enzymatic processes. In addition to bilirubin, methemoglobin may also be formed. As the process is time-dependent, the bilirubin concentration and the presence of oxidation products, such as oxyhemoglobin provide information on the state of the patient. In addition, it is possible to estimate the duration of the oxidation process.

■ Multi-component measurement required

The color pigments mentioned above are suitable for spectroscopic analysis in the visible range. Quantitative but also qualitative, as well as color representation is the domain of UV-VIS spectroscopy. Cerebrospinal fluid is a mixture that is not only represented by hemoglobin and, therefore, requires a multi-component measurement, as all spectra of the individual components overlap (superposition). In this case, a straight baseline as would be expected in the analysis of liquids is not measured, but rather a rising baseline extending into the UV range. This effect occurs due to substances that respond to UV irradiation. This shifting baseline presents a challenge for the automated analysis of very small maxima or shoulders in a signal pattern.

Beetham has defined the measuring procedure according to which measurement should be carried out in the visible range from 350 to 600 nm. The analytical wavelengths for the hemoglobin color pigments are listed in table 1. The method requires corrected signal heights, which is realized via the baseline/tangents under the signals/bands. Net bilirubin absorption is determined using a fixed wavelength of 476 nm.

Pigment	Absorption range
Oxyhemoglobin	between 410 and 418 nm
Bilirubin	broad bands of 450 to 460 nm or as a shoulder in the oxyhemoglobin signal
Methemoglobin	more rarely occurring pigment; when present it occurs as a broader signal between 403 to 410 nm

Table 1: Analytical wavelengths for hemoglobin.

■ Discussion of the results

Beetham specifies the evaluation of the spectra. One specification is that the baseline under the signal should never intersect the signal itself. This strict instruction infers that automated evaluation cannot lead to the correct result. Manual evaluation is called for, in which the base points under the signal can be specifically adjusted/corrected.

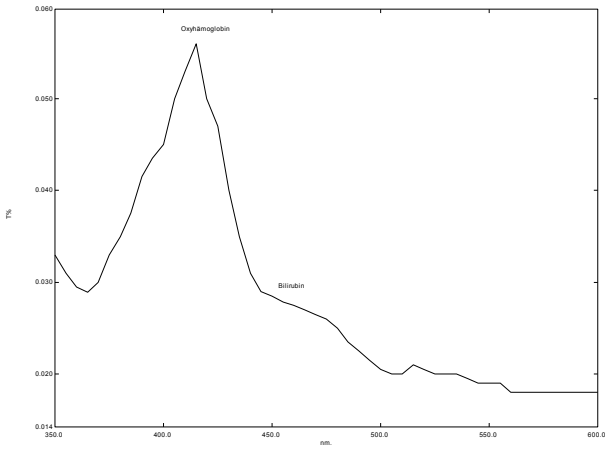

Figure 1: Representation of a UV-VIS absorption spectrum of a bilirubin sample that exhibits an oxyhemoglobin signal at 415 nm and a bilirubin shoulder at 450 to 460 nm, recorded using the UVProbe Software.

To solve this problem, an Excel spreadsheet was developed, in which it is possible to measure, to represent the spectrum, and to manually shift the baseline. Within the evaluation range, all required values of the sample could be represented.

A representation of the spectrum from the Excel sheet is shown in figure 2.

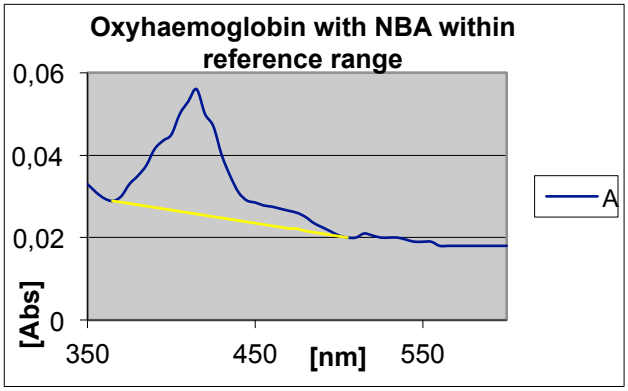

Figure 2: Representation of an absorption spectrum of a bilirubin sample (blue) and the representation of the baseline (yellow) used for the correction of the bands.

The evaluation is presented in table 2.

Base point A = 350 - 400		Base point B = 430 - 530	
365	0.0289	476	0.021
NBA at 475 nm		Limit value	Evaluation
0.00385714		< = 0.007	OK

Table 2: Calculation of the net bilirubin content (NBA) after graphical evaluation.

Shimadzu's UV-1800 spectrophotometer was used for the analysis. The spectrum was measured in the range of 350 to 600 nm. The sample was measured using a 1 cm cuvette. The limit value in this example was below 0.007 absorption units.

■ Summary

Using the Excel method, the calculation of the net bilirubin concentration can be easily determined. Using a macro simplifies the adjustment to local conditions, such as different output formats and adaptation to individual forms. On the other hand, it is easy to extend the macro with other applications. The direct instrument control of the UV-1800 allows for direct data evaluation. It is also possible to import the results from the UV-Probe software, which is automatically supplied with the UV-1800.

■ Abbreviations:

*cerebrospinal fluid (CSF) bilirubin

■ Literature

[1] 'National Guidelines for Analysis of Cerebrospinal fluid for Bilirubin in Suspected Subarachnoid Haemorrhage', Draft1; R.Beetham, M. N. Fahie-Wilson, I. Holbrook, I.D. Watson, P.R. Wenham, P.A.E. White, P. Thomas, A.M. Ward, A. Cruickshank, G. Keir, W.Egner, K. Allen

[2] Shimadzu News 3/2014, Shimadzu Europa GmbH

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Application News

Spectroscopy - FTIR

Analysis of stones – Urea, Kidney, Gall With Infrared Spectroscopy

No. SCA-110d-094

Apatite, Struvite, Weddelite – these names sound nice, but as kidney stones they are very harmful to the patients. The compounds of the stones give hint about the reason of the illness and appropriate steps against a relapse in the future can be taken.
At the Bosch Medicentrum, a hospital in the center of s´-Hertogenbosch, the Netherlands, an FTIR spectral library has been developed consisting of lots of kidney stones with a known composition. Since most of the stones are also analyzed by XRF the concentration of the components is known too. The FTIR library is a helpful tool to analyze the patients kidney stones. At all the infrared spectroscopy is a very well-known technique doing this style of analysis [1-3]. A more new aspect using it, is the reduction of sample preparation and analysis time due to high speed FTIR technology and accessory selection. In the past the KBr pellet technique taking 15 to 20 minutes was (Figure 1) investigated. Nowadays, it is possible to use the more comfortable and easy to handle ATR technique, and the sample preparation is reduced to nearly zero. The instrument used at the Bosch Medicentrum is a Shimadzu with LabSolutions software and a Golden Gate with KRS-5 lenses. With those optics the range is extended to 350 cm^{-1}.
The sample preparation itself is reduced to the following activities. At first, the stone is grinded in a mortar to obtain a fine powder. This is necessary because the differences in particle size may affect the IR spectrum. Then, a very small part of this powder (only a few milligrams) is put on the diamond-crystal of the Golden Gate and the following spectrum is measured (Figure 2). When this spectrum is searched in the library the result will be as shown in figure 4. The result is, that the determined stone material consists of 75 % Whewellite, and 25 % Apatite. In this way the chemical content and the concentration of the components can be determined quickly and easy[4].

■Spectra Analysis

For the comparison are spectra measured with classical KBr pellet and as well with ATR technique. In figure 1 and 2 the two stone materials Brushite and Whewellite are available. The following example shows the Brushite and Whewellite infrared spectrum. The spectra are from two different sources.

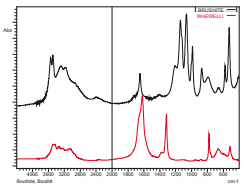

Fig. 1: Infrared spectra from urea stones which were prepared with KBr Pellet technique

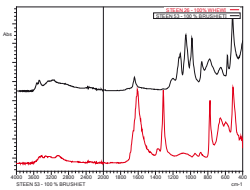

Fig. 2. Infrared spectra from urea stones which were prepared for ATR

The KBr pellet spectra are from a German hospital and the ATR spectra from the Bosch Medicentrum in the Nederlands. In both cases life material was in use. The spectra in figure 1 and 2 show clearly same spectra profiles. In detail are little differences regards the structure of the each signal, in the ratio of

signals and in the intensity (figure 3).

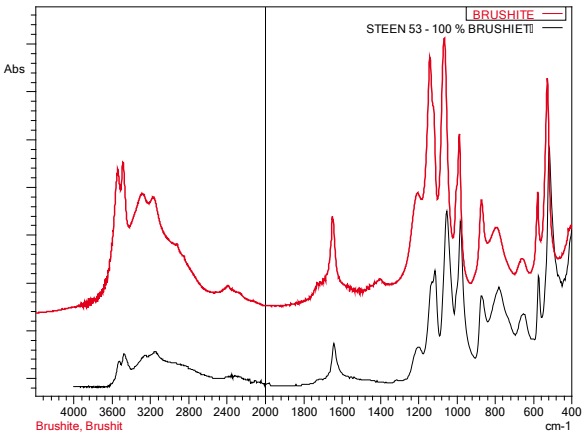

Figure 3: Display of two infrared Spectra made from Brushite with different measurement technique “KBr” (red) and “ATR” (black)

These variations are result from the measurement technique. KBr pellet is a transmission and the ATR a reflection technique. Both styles are depending on the particle size, concentration, softness and amount of water implemented. Water is part of KBr powder (hygroscopic salt), part of the stone as ad on and bounded as chemical – OH-bridge in the inorganic salt clusters. For this application the material was dried, grinded and transferred into powder form.

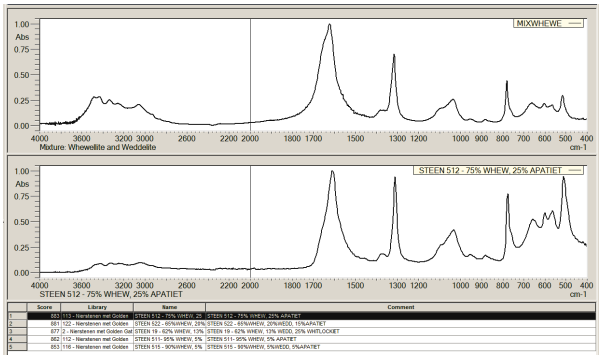

Figure 4: The infrared ATR spectrum of an unknown urea stone was compared using the Urea library, and the search result showed 75%wheddelite plus 25%apatite.

■Conclusion

The infrared spectroscopy is able to identify the right things in natural and complex systems as samples from the field are. The investigated measurement technique will be comparable to the next. The recommendation is to create libraries for each technique. Doing this the comparison includes the accessory characteristics which causes little differences in the spectra. Natural a common library can be created when using for ATR measurements the ATR correction. The correction function will convert the view of ATR spectra into the view of transmission spectra.

■Acknowledgement

Many thanks to Bosch Medicentrum, Hertogenbosh, the Nederlands for the Urea library.

■Literature

[1] Analysis of urinary calculi by infrared spectrophotometry, Menendez Gutierrez, R., Med. Segur. Trab., 36 (146), 53-62, 1989.
[2] Technique for the analysis of urinary calculi by infrared spectroscopy, Hesse, A., Molt, K., J. Clin. Chem., Biochem., 20 (12), 861-73, 1982.
[3] Analysis of urinary calculiby infrared spectroscopy, Laurence, C., Dubreil, D., Lustenberger,P., Ann. Chim. (Paris), 1 (1), 55-63.
[4]Shimadzu News, Issue 2/2003, page 8 -3

■Instrumentation

- Shimadzu FTIR
- LabSolutions IR software
- Single reflection unit from company Specac – Golden Gate
- Shimadzu Libraries and Urea library

■Instrumentation

- **Shimadzu FTIR**

Typical FTIR instrument with Michelson interferometer is the Shimadzu IRAffinity-1S. it contains a completely sealed interferometer with an optical window made from coated KBr. Same as the heart of the interferometer is a coated germanium beam splitter. Due to the r behavior of the implemented KBr parts the instrument is equipped with an automated dehumidifier which is patented by Shimadzu. The instrument is designed for the mid infrared range. Auto alignment functions keep the instrument in a stable performance, proofing functions keep the interferogram in same quality.

Shimadzu was the first company to offer dynamic auto-alignment as a standard feature in an FTIR spectrometer in its price range. With state-of-the-art technology, the Shimadzu dynamic alignment system continuously maintains optimal alignment during data acquisition, ensuring consistently reproducible spectra without the need for tedious mechanical adjustments.

Fig. 5: Shimadzu Fourier transform infrared spectrometer IRAffinity-1S, best performance in its class´

Features:

- ✓ S/N 30,000:1 with apodization triangular squared
- ✓ Automatic dryer for interferometer part
- ✓ Advanced Dynamic Alignment
- ✓ Broad range of applications
- ✓ Easy-to-use with advanced Labsolutions IR software

- **LabSolutions IR software**
  Available as file edition, database or client server version
- **Accessories**
  - Single reflection units from company Specac – Golden Gate With monolithic diamond and KRS-5 lenses.
  - Shimadzu DRS-8000
  - Shimadzu KBr pellet accessories
- **Costfree Libraries**
  - Urea Golden Gate
  - Urea Drift
  - KBr pellet

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Application News

No.A491

Spectrophotometric Analysis

Fluorescence Spectrum Measurement of Fluorescence Dye Indocyanine Green in Long Wavelength Region

In the medical and biochemical fields, the use of fluorescent dye for labeling specific molecules is a commonly used technique for the observation of tissue lesions. Indocyanine Green fluorescent dye displays fluorescence in the near infrared region in the vicinity of 810 nm, and because light of this wavelength easily passes through the living body, this dye is often used for such observation.
Measurement in wavelength regions greater than 750 nm has been difficult using conventional spectrofluorophotometers, but the newly-developed Shimadzu RF-6000 spectrofluorophotometer permits measurement over a wide wavelength range from 200 to 900 nm. Furthermore, the automatic spectral correction feature of this instrument makes it possible to automatically obtain comparable data from different instruments. Here, we introduce an example of measurement of Indocyanine Green using the RF-6000.

Instrument and Three-Dimensional Spectrum Measurement

Fig. 1 shows a photograph of the RF-6000. The RF-6000 features automatic spectrum correction, which permits the acquisition of spectra (fluorescence/excitation) in which instrument-specific characteristics are eliminated automatically. This elimination of instrument-related data anomalies is conventionally conducted as a data-processing function, but the RF-6000 eliminates the need for such a post-acquisition correction. Such corrected spectral data can thus be compared among different instruments.
Indocyanine Green powder was dissolved in pure water to prepare a 1.3 mg/L aqueous solution. First, to determine the optimal excitation wavelength, the 3D Spectrum feature of the LabSolutions RF software was used to conduct a three-dimensional spectrum measurement. A three-dimensional spectrum is a spectrum that is displayed as a mapping image which is generated by conducting fluorescence spectrum measurements while sequentially changing the excitation wavelength. The measurement results are shown in Fig. 2, and the analytical conditions that were used are shown in Table 1. The horizontal axis is used to plot the fluorescence wavelength Em, and the vertical axis is used to plot the excitation wavelength Ex. This three-dimensional spectrum is generated using the corrected spectrum.
Fluorescence is observed in the region of the peak shown in Fig. 2. The peak position is defined by the emission and excitation coordinates (Em 808 nm, Ex 780 nm), indicating that the greatest degree of fluorescence associated with this sample occurs at the excitation wavelength of 780 nm. It is possible to extract either the fluorescence or excitation spectrum for isolated viewing at any of the excitation or emission coordinates of the three-dimensional spectrum. Each of the spectra acquired at the peak position is shown in Fig. 3. The peak associated with the excitation line A is the generated fluorescence spectrum, and that associated with emission line B is the extracted excitation spectrum. As is evident from these results, the fluorescence peak wavelength is 808 nm, and the optimal excitation wavelength is 780 nm.

Fig. 1 RF-6000 Spectrofluorophotometer

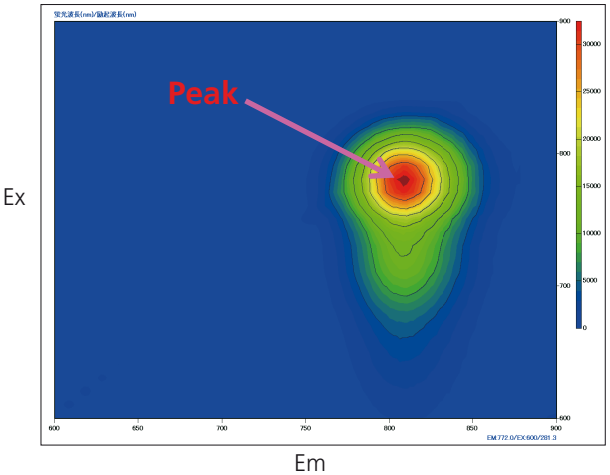

Fig. 2 Three-Dimensional Spectrum for Indocyanine Green

Table 1 Analytical Conditions Used for Three-Dimensional Spectrum

Instrument	: RF-6000 spectrofluorophotometer
Spectrum Type	: 3-dimensional spectrum
Measurement	
Wavelength Range	: Em: 600 - 900 nm, Ex: 600 - 900 nm
Scan Speed	: 2000 nm/min
Wavelength Interval	: Ex 10 nm, Em 2 nm
Bandwidth	: Ex 5 nm, Em 5 nm
Sensitivity	: High

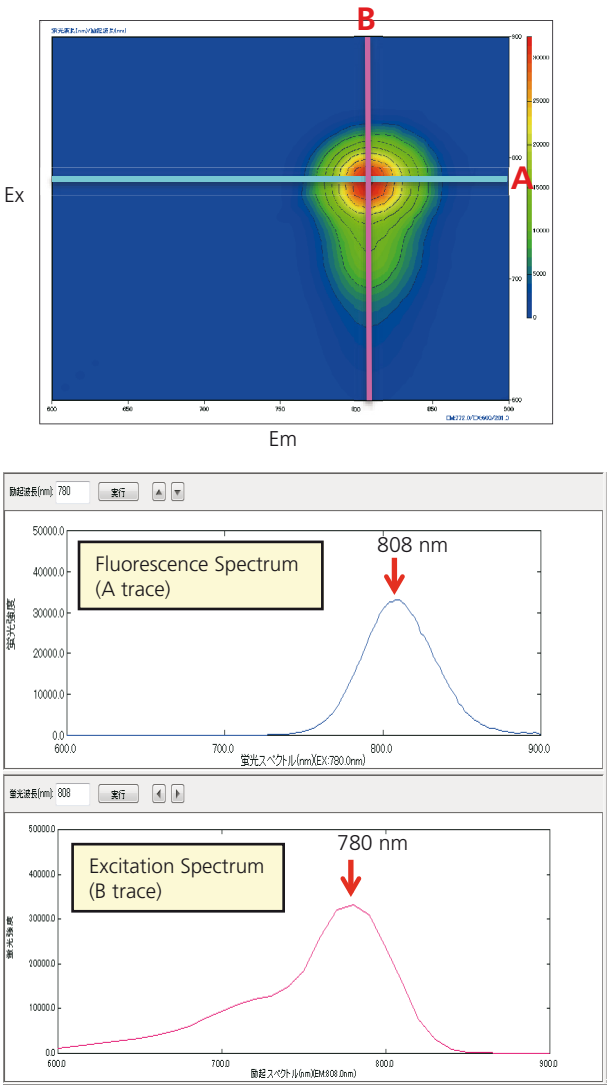

Fig. 3 Fluorescence Spectrum and Excitation Spectrum Extracted from Three-Dimensional Spectrum

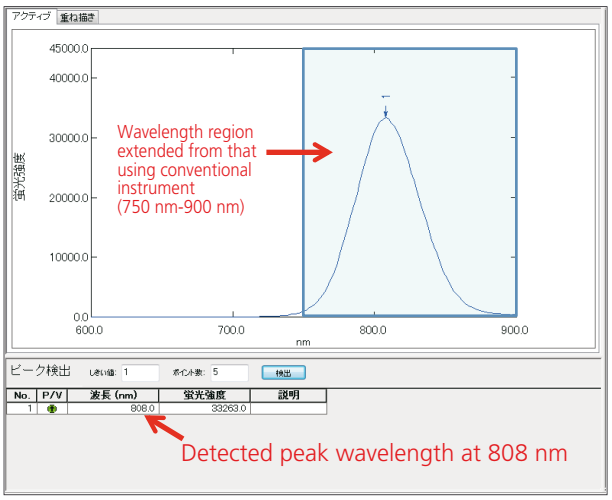

Fig. 4 Fluorescence Peak Detection

Table 2 Analytical Conditions for Fluorescence Spectrum Measurement

Instrument	: RF-6000 spectrofluorophotometer
Spectrum Type	: Fluorescence spectrum
Excitation Wavelength	: 780 nm
Measurement	
Wavelength Range	: 600 - 900 nm
Data Interval	: 2.0 nm
Scan Speed	: 200 nm/min
Bandwidth	: Ex 5 nm, Em 5 nm
Sensitivity	: High

Fluorescence Spectrum Measurement

Using the Spectrum function of the LabSolutions RF software, the results obtained from measurement of the fluorescence spectrum with a scan speed of 200 nm/min together with the peak detection results are shown in Fig. 4. The measurement conditions are shown in Table 2. Measurement with good sensitivity is clearly obtained up to the long wavelength region. The light blue region in Fig. 4 is the wavelength region (750 nm to 900 nm) in which measurement has now become possible.

Conclusion

The RF-6000 is an instrument that permits measurement over a wide wavelength range, from 200 nm to 900 nm. Using the RF-6000, we were able to conduct measurement of Indocyanine Green with its fluorescence peak in the vicinity of 810 nm with good sensitivity, a type of measurement that has been difficult with conventional instruments. Further, the newly included automatic spectrum correction function makes it possible to obtain corrected spectra in real time. The RF-6000 with its extended measurement range will permit its use in a wider range of new applications.

First Edition: Jul. 2015

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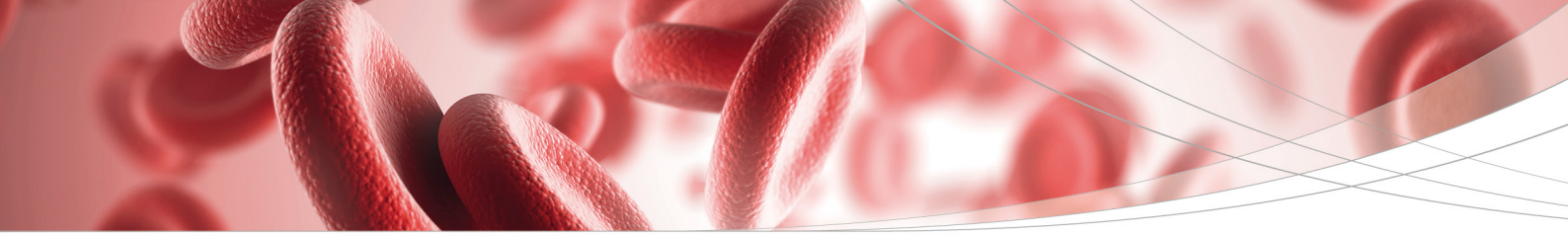
4. Life Science Lab Instruments



4. Life Science Lab Instruments

4.1 Matrix Assisted Laser Desorption Ionization (MALDI)

The MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time of Flight) technology offers multiple options for profiling of proteins. This enables identification of contamination, differentiation of bacteria, fungi, yeasts and much more. MALDI-TOF is now widely used in microbiology for bacteria identification. It is a robust and easy technology that fits in routine labs for quick control.

MO361	Structural elucidation of complex lipids using MALDI-TOF with high-energy CID

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Application News

AXIMA *Performance* MALDI-TOF/TOF

No.MO361 Structural Elucidation of Complex Lipids Using MALDI-TOF with High-Energy CID

- Characterisation of positional isomers of neutral lipids
- True high-energy CID (20 KeV) was performed on triacylglycerols
- High m/z region of CID spectrum is dominated by abundant charge-remote fragmentation of the fatty acid substituent
- Low mass fragment ions provide important structural information
- The presence of double bonds or hydroxyl groups within the fatty acid chain can be determined
- High mass accuracy measurements

Introduction

Historically, double-focussing or tandem sector instruments were used to generate the most comprehensive structural characterisation of triacylglycerols through high-energy CID (4-5 keV) of sodiated species.

With this approach all the important structural features of triacylglycerols species, with the exception of sn-2 chirality, could be determined:

- The acyl carbon number: corresponding to the total number of carbon atoms within the fatty acid substituents (excluding the three carbon atoms of the glycerol moiety)
- The position of substituents: distinguishing between sn-1/sn-3 and the sn-2 substituents
- The number of double bonds. The observed molecular mass is reduced by 2 Da per additional double bond in the molecule.

With the declining use of (tandem) sector instruments for biological applications in mass spectrometry, only a combination of MALDI with an appropriate TOF/TOF analyser accommodates the combined need for high resolution, good precursor ion selection and high collision energy for the structural analysis of triacylglycerols.

Previously, MALDI-TOF MS analysis of lipids primarily relied on spontaneous fragmentation through PSD, yielding product ion spectra containing only two types of fragment ions (B- and C-type ions)[1]. Only a single publication reported CID fragmentation of triacylglycerols, however these were of relatively poor quality[2].

In this work, the complete structural analysis of $[M+Na]^+$ ions of synthetic as well as naturally occurring triacylglycerols mixtures (cocoa butter) is performed. The AXIMA *Performance* MALDI-TOF/TOF[3] is an ideal platform for this type of analysis. Taking full advantage of the true high energy CID conditions (20 keV with helium gas) and the unique curved-field reflectron offered by the AXIMA *Performance*, these results are the first reported use of high-energy CID MALDI-TOF/TOF MS for the characterisation of positional isomers of neutral lipids[4].

Experimental

All triacylglycerol samples (synthetic compounds and cocoa butter) were dissolved in methanol/chloroform. MALDI mass measurements were carried out using an AXIMA *Performance* MALDI-TOF/TOF mass spectrometer. 2,4,6-trihydroxyacetophenone was used as MALDI matrix in all experiments, this was doped with sodium chloride to promote the formation of sodiated species $[M+Na]^+$. Fragmentation spectra were acquired under PSD and high-energy CID conditions (20 keV with helium gas).

Results

Triacylglycerols could be readily desorbed and ionised by MALDI as sodiated adducts without significant in-source decay. Only B- and C-type ions were observed in the PSD spectrum of the $[M+Na]^+$ adduct ion of 1,2-dipalmitoyl-3-oleoyl-glycerol. These are formed through the loss of one free fatty acid and one sodium fatty acid carboxylate residue as shown in Figure 1. These ions do not allow the distinction between the two outer fatty acid-substituents (sn-1, sn-3) and the middle position (sn-2). Therefore only structural information regarding the fatty acid composition of this triacylglycerol could be obtained using PSD conditions.

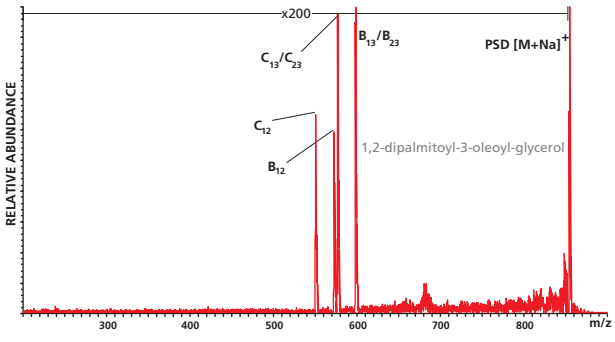

Figure 1: PSD analysis of 1,2-dipalmitoyl-3-oleoyl-glycerol

In contrast to the PSD spectrum, the 20 keV CID spectrum of the same triacylglycerol yields a rich array of various structurally diagnostic product ions such as E-, F-, G- and J-type ions[1] in the low mass region in addition to the ions observed under PSD conditions (Figure 2).

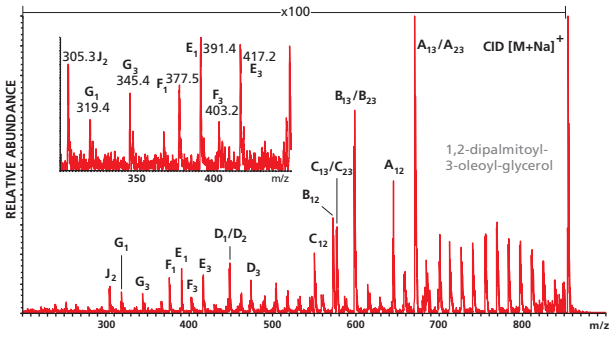

Figure 2: High-energy CID analysis of 1,2-dipalmitoyl-3-oleoyl-glycerol. Inset: low mass region showing diagnostic E-, F-, G- and J-ions

The strong simultaneous charge-remote fragmentation of the three fatty acids substituents observed in the high mass region of the high-energy CID spectra is thought to be the result of a formal 1,4-elimination of molecular hydrogen (H_2) and loss of neutral alkenes.

Additionally, in the high-energy CID spectrum only, the low mass region displays D-type product ions and more significantly the structurally important ions $E_{1/3}$-, $F_{1/3}$- and $G_{1/3}$-type ions only present in sn-1 and sn-3 substituents and the J_2-type ion characteristic of the sn-2 substituent. These low mass product ions can be used to accurately differentiate structural isomers of triacylglycerols.

In particular $E_{1/3}$ (m/z 417.2 and m/z 391.4), $F_{1/3}$ (m/z 403.2 and m/z 377.5), $G_{1/3}$ (m/z 345.4 and m/z 319.4) and J_2 (m/z 305.3) indicate that, in the case shown, the palmitic acid substituent is linked to the middle position (sn-2) of the glycerol backbone.

Figure 3 shows the high-energy CID spectrum of a structural isomer of the triacylglycerol shown in Figure 2, namely 1,3-dipalmitoyl-2-oleoyl-glycerol.

In this case the outer two fatty acids are identical in mass, yielding an $E_{1/3}$ ion at m/z 391.4, an $F_{1/3}$ ion at m/z 377.4 and $G_{1/3}$ ion at m/z 319.3. The J_2 ion now appears at m/z 331.2, indicating the oleoyl-substituent to be linked to the sn-2 position. Three additional synthetic triacylglycerols were considered, to further demonstrate the value of high-energy CID in isomer differentiation. Figure 4 illustrates the high-energy CID spectra of the sodiated adducts of 1,3-dioleoyl-2-palmitoyl-glycerol, 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol and 1,2-distearoyl-3-oleoyl-glycerol.

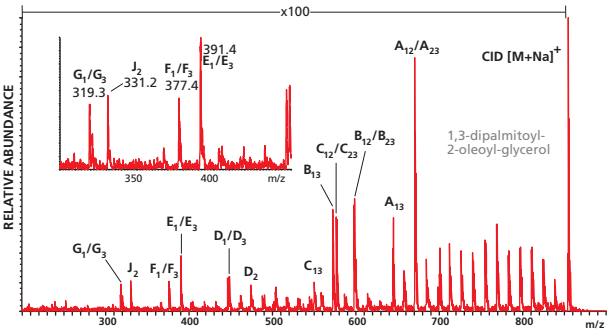

Figure 3: High-energy CID analysis of 1,3-dipalmitoyl-2-oleoyl-glycerol. Inset: low mass region showing diagnostic E-, F-, G- and J-ions

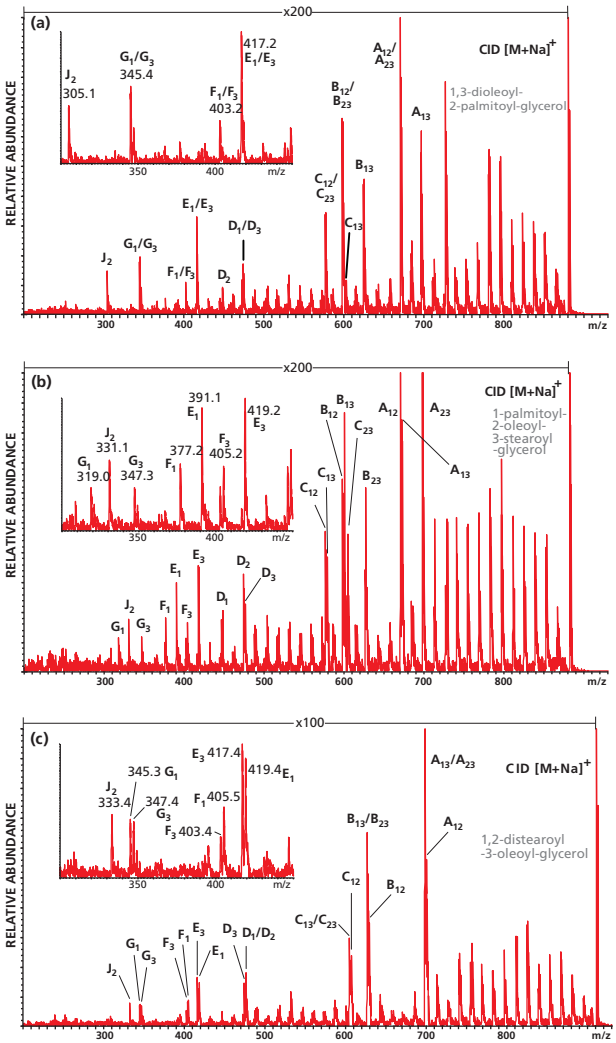

Figure 4: High-energy CID spectra of the sodiated adducts of (a) 1,3-dioleoyl-2-palmitoyl-glycerol, (b) 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol and (c) 1,2-distearoyl-3-oleoyl-glycerol

Again, in all three of these cases the positions of the various fatty acid substituents could be unequivocally assigned using the distinctive $E_{1/3}$-, $F_{1/3}$-, $G_{1/3}$- and J_2-type ions. Closer inspection reveals additional information regarding the lipid. In particular in the case of 1,3-dioleoyl-2-palmitoyl-glycerol , the mass difference of 54 Da in the high mass region (m/z 727.7 and m/z 781.6) indicates the position of the double bond within the fatty acid. Furthermore the resolving power in MS^2 mode is demonstrated in the spectrum of 1,2-distearoyl-3-oleoyl-glycerol, where the observed $E_{1/3}$-type ions are separated by 2 Da (m/z 417.4 and m/z 419.4). In this case, the product ions from other charge-remote fragmentation coincide with those defining the position of the double bond in the mono-unsaturated fatty acid substituent.

This technique was applied to the differentiation of triacylglycerols positional isomers in plant oils, in particular cocoa butter. The three principal triacylglycerol components of cocoa butter (m/z 855.8, m/z 883.8 and m/z 911.9) are displayed in Figure 5.

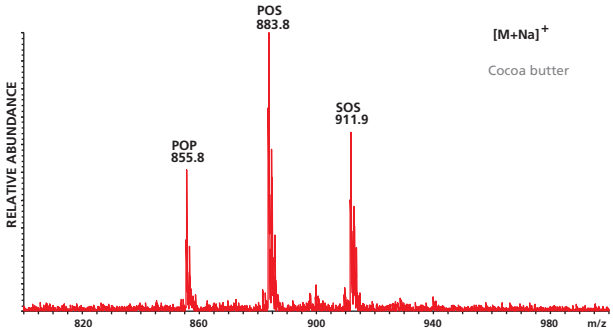

Figure 5: MALDI-TOF mass spectrum of the triacylglycerols in cocoa butter. Abbreviations used: O = oleic acid, P = palmitic acid, S = stearic acid

High-energy CID of precursor ions m/z 855.8 and 883.8 produced identical spectra in the low mass diagnostic region to those shown in Figures 3 and 4b indicating they are 1,3-dipalmitoyl-2-oleoyl-glycerol and 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol respectively (Figures 6a and b).

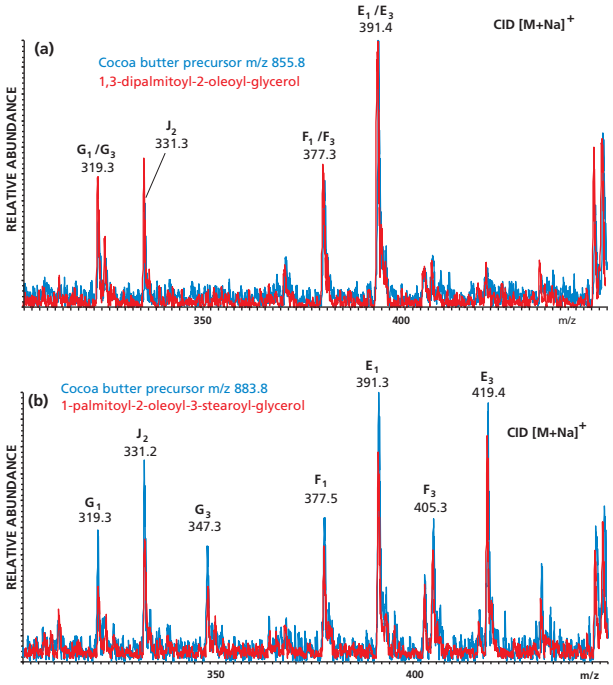

Figure 6: Comparison of the low m/z region of the high-energy CID spectra of cocoa butter components m/z 855.8 and 883.8 (blue traces) and synthetic triacylglycerols (red traces): 1,3-dipalmitoyl-2-oleoyl-glycerol and 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol

More significant is the case of the final main triacylglycerol component of cocoa butter. The low mass region of the high-energy CID of m/z 911.9 is displayed in Figure 7 and overlaid with that of 1,2-distearoyl-3-oleoyl-glycerol (previously shown in Figure 4c).

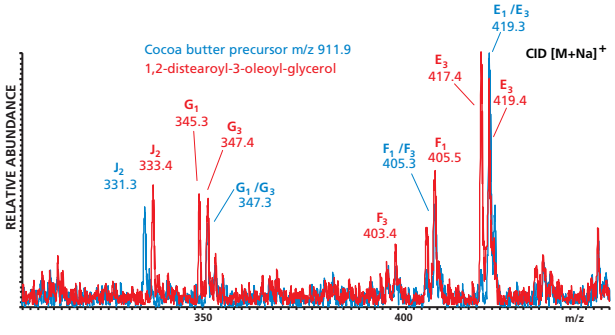

Figure 7: Comparison of the low m/z region of the high-energy CID spectra of cocoa butter component m/z 911.9 and synthetic triacylglycerol: 1,2-distearoyl-3-oleoyl-glycerol

It is evident from these spectra that there are significant differences between the two CID spectra. Of particular interest is the 2 Da shift in the mass of the diagnostic J_2-type ion (m/z 333.4 for 1,2-distearoyl-3-oleoyl-glycerol and m/z 331.3 for the cocoa butter component). This confirms that the fatty acid-substituent linked to the hydroxy group of the sn-2 position of the glycerol backbone was oleic acid in case of the cocoa butter triacylglycerol. Therefore the two triacylglycerols (1,2-distearoyl-3-oleoyl-glycerol and 1,3-distearoyl-2-oleoyl-glycerol) are simply positional isomers, the natural one corresponding to 1,3-distearoyl-2-oleoyl-glycerol.

Conclusion

MALDI-MS of sodiated triacylglycerols using true high-energy CID TOF/TOF conditions (ELAB = 20 keV) has proven a valid alternative to sector-field analyser based experiments. More significantly, the AXIMA *Performance* has been shown to allow accurate differentiation of positional isomers within this class of lipids.

References

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(2) Al-Saad KA et al. MALDI TOF MS of lipids: ionization and prompt fragmentation patterns. *RCM* 2003; **17**: 87-96.

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First Edition: May, 2012

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