SHIMADZU
Excellence in Science

S-PHM-B22

The Ultimate Guide:

Redefining Pharma Roadmap With Shimadzu's End-To-End Solutions

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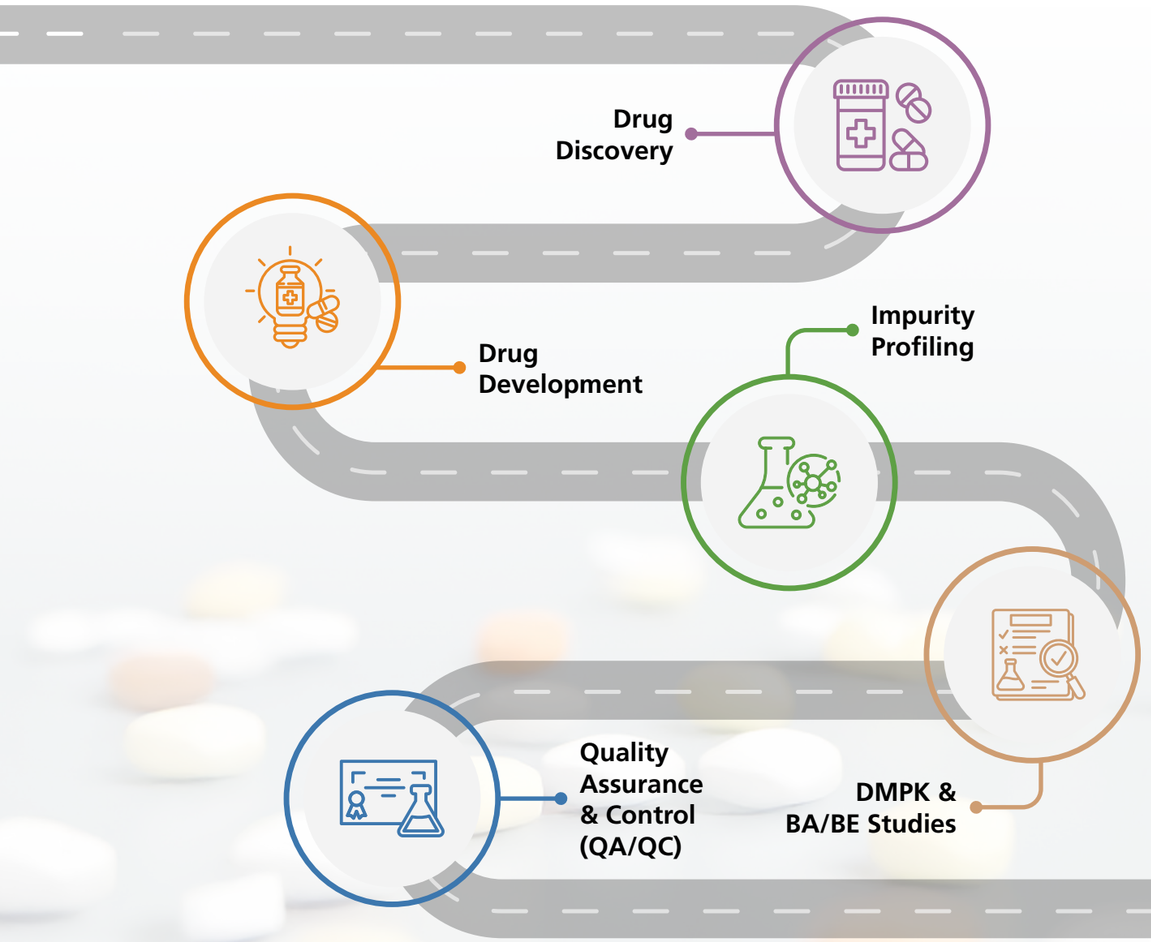
OVERVIEW

Analytical method development begins early in drug discovery and evolves throughout the drug lifecycle. As a drug candidate advances through development stages, it is crucial to ensure its safety, efficacy, and stability. This includes identifying potential by-products, impurities that may arise during manufacturing, and extractables and leachables from plastics and packaging, all before the drug reaches commercialization.

At the core of the pharmaceutical roadmap are five critical pillars:

Pharma Roadmap



While the requirements for a drug candidate evolve throughout its lifecycle, the need for advanced chromatography and detection techniques remains constant. Leading-edge analytical solutions are essential at every stage to ensure that the pharmaceutical products meet stringent safety and quality standards.

Shimadzu solutions leverage proprietary technologies like Analytical Intelligence™ and Ultra-Fast Mass Spectrometry (UFMS™) to enable effective pharmaceutical analysis with accuracy, efficiency, and automation. These advanced systems simplify complex workflows, delivering seamless integration across all stages of drug development while significantly enhancing productivity.

In this latest eBook, explore how Shimadzu is driving the future of pharmaceutical research, providing the in-demand tools to address today's challenges and unlock tomorrow's opportunities.

01

Drug Discovery

In the rapidly evolving field of modern drug discovery, medicinal chemistry plays a crucial role from the initial stages of hits screening to leads identification and preparative purification. This branch of chemistry is crucial for synthesizing novel compound entities that possess the desired pharmacological properties. Often, the goal is to achieve a target compound with high purity and reasonable yield, while maintaining a high throughput to accelerate the drug discovery process.

Analysis Requirements	Shimadzu's Analytical Capabilities
1. Initial Hits Screening - Screen large compound libraries for biological activity - Separate and identify diverse compounds - Analyze complex mixtures efficiently	**Nexera UC/UC Prep | Online SFE-SFC System**  • Efficiently extract target compounds with high selectivity • Rapidly process large sample libraries
2. Lead Identification - Detect and identify high-potential leads - Accurate mass measurement and structural analysis - Integrate with databases for compound characterization	**LCMS-9050 QTOF | LabSolutions Insight Explore | LabSolutions Insight Biologics**  • Accurate mass measurement for reliable identification • LabSolutions Insight Explore for complete data management and analysis • LabSolutions Insight Biologics for oligonucleotide characterization to identify 0.5% impurities with 99.5% accuracy
3. Method Optimization - Develop robust methods for lead refinement - Optimize analytical conditions for high-resolution separation	**Nexera LC/SFC Switching System**  • LC/SFC offers switching capability for flexibility • Supports diverse chiral and achiral separations for stereochemistry investigations
4. Automated Scale-Up - Purify and isolate optimized leads - Automated workflows for faster lead refinement	**Nexera Prep with LCMS-2050 | LabSolutions MD, Open Solution & ASAPrep**  • Efficient purification with accurate structural identity confirmation • Automates method development using LabSolutions MD • Automated scale up using Open Solution and ASAPrep
5. Preparative Purification - Isolate high-purity compounds for preclinical/clinical studies - Remove impurities for regulatory standards - Scalable purification solutions	**Nexera UFPLC + Purification Solution**  • Automated purification process for target compounds in short time • Efficient compound recovery with minimized waste • Combines UFPLC with software for complete processing control

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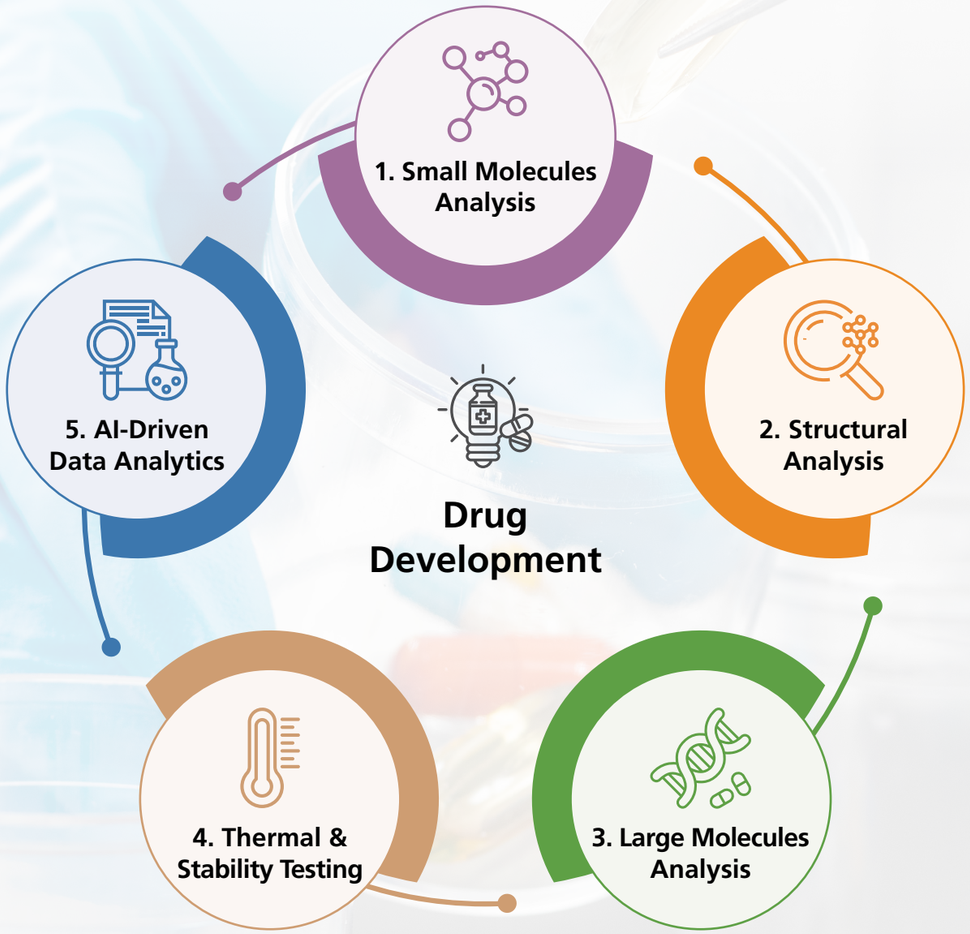
02

Drug Development R&D

Once the promising drug candidates are identified, they will advance to the research and development (R&D) phase, during which extensive preclinical trial tests in animals and cells are conducted to assess the safety, efficacy, and potential side effects of the compounds. This phase is resource-intensive and focuses on optimizing the selected candidates for further evaluation in human subjects.



Analysis Requirements	Shimadzu's Analytical Capabilities
1. Small Molecules Analysis - Detects and quantifies small molecules like APIs or impurities - Separates structurally similar compounds or isomers	**Nexera UC | LabSolutions MD**  • Efficient separation of small, low-polarity molecules and challenging isomers using SFC • Automated method development using LabSolutions MD
2. Structural Analysis - Determines molecular structure, stereochemistry, and functional groups to confirm identification	**MALDImini-1 | MALDI-8030**  • Mass and structural analysis for small molecule identification using MALDImini-1 • Dual-polarity ionization for characterization using MALDI-8030
3. Large Molecules Analysis - Bioanalysis of sensitive biomolecules e.g., mAbs and peptides - Characterizes post-translational modifications (PTMs)	**Nexera XS Inert & LabSolutions MD | LCMS-9050 QTOF | LabSolutions Insight Biologics | Shim-pack Bio-Diol Columns, Shim-pack Scepter Claris**  • Elevated pressure tolerance of 105 MPa in UHPLC • Excellent inertness, corrosion resistance, and wide pH range compatibility
4. Thermal & Stability Testing - Determines melting temperatures and transitions for DNA, RA and protein stability - Simulates real-world storage conditions to assess product stability	**TMSPC-8i | Integrated LabSolutions UV/Vis Tm**  • Measures protein melting points and denaturation for stability • LabSolutions UV-Vis Tm ensures high data integrity with LabSolutions DB/CS
5. AI-Driven Data Analytics - Manages and interprets large datasets from high-throughput instruments - Utilizes AI tools for consistent, error-free processing	**i-PDeA | Peakintelligence™** • Resolves overlapping chromatogram peaks for better interpretation • Peakintelligence™ reduces data processing time by 1/3

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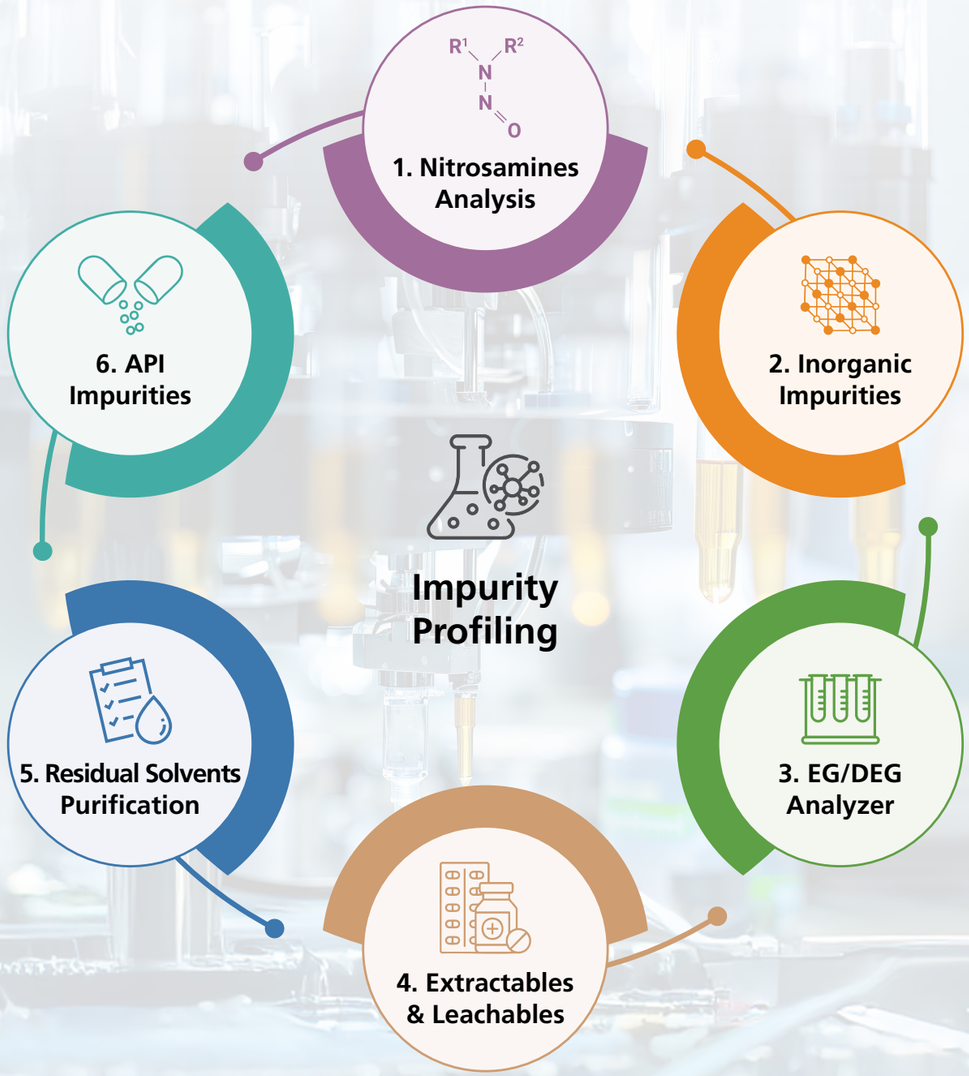
03

Impurity Profiling

Impurity profiling is a critical aspect of drug development, involving the identification, characterization, and quantification of undesired substances within pharmaceutical products. These impurities can originate from various sources, such as raw materials, synthesis pathways, degradation processes, or contamination during manufacturing. The rigorous assessment of these substances is essential for ensuring the drug's safety and efficacy.



	Analysis Requirements	Shimadzu's Analytical Capabilities
1. Nitrosamines Analysis	- Ultra-high sensitivity for trace-level analysis - High specificity to distinguish nitrosamine varieties - Rapid quantification for faster turnaround time	**LCMS-8060 RX | GCMS-TQ8050 NX**  • Utilizes UFMS™ for ultra-fast scanning and high-resolution trace detection
2. Inorganic Impurities	- Complies with ICH Q3D for elemental impurities at ppm/ppb - Multi-elemental analysis in a single run for efficiency - Quick screening and identification of inorganic impurities	**ICPMS-2040/2050 | AIRsight IR/Raman Microscope**  • Trace-level elemental analysis with compliance • Spectroscopy solutions for contaminants analysis with minimal preparation
3. EG/DEG Analyzer	- High-throughput analysis - Reliable detection amid complex sample matrices - Quick screening and identification of inorganic impurities'	**Brevis GC Analyzer | Multi Data Report (MDR)**  • EG/DEG analysis in glycerin, propylene glycol, and sorbitol solution via GC-FID (USP Monographs) • Automated calculation and reporting with LabSolutions DB/CS Multi-Data Report
4. Extractables & Leachables	- Profiling of E&L in drug packaging and manufacturing - Identify volatile, semi-volatile, and non-volatile compounds	**LCMS-9050 | TD-GCMS-QP2020 NX | HS Trap-GCMS-QP2020 NX**  • UFstabilization™ in LCMS-9050 ensures reliable analysis • HS-GCMS and TD-GCMS offer flexibility for high-sensitivity analysis
5. Residual Solvents Purification	- Identifies and reports Class I-III solvent residues accurately - Boosts compliance testing efficiency for productivity	**Nexis GC + HS-20 NX | GCMS-TQ8050 NX**  • Offers high sensitivity and excellent reproducibility • Integrates with LabSolutions CS for compliance e.g., FDA 21 CFR Part 11
6. API Impurities	- Enhances API safety and efficacy through impurity profiling - Robust methods to identify structurally-similar impurities	**LCMS-2050 + LabSolutions MD**  • Compact, high-performance MS for fast API impurity screening • Use of LabSolutions MD to visualize resolution of API and impurities by design space for efficient method development

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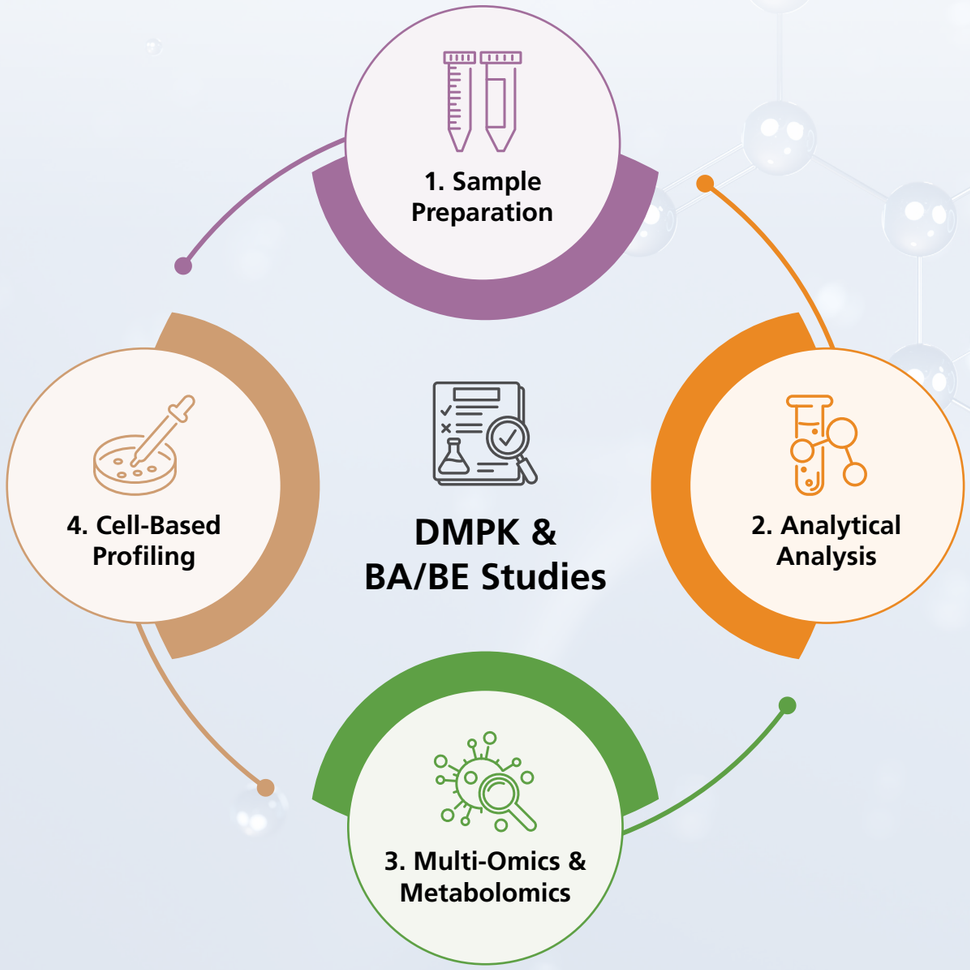
04

DMPK & BA/BE Studies

Bioavailability (BA) / Bioequivalence (BE) studies, popularly known as BA/BE studies, are essential to verify that a test product is comparable to a reference product in pharmaceutical term, thus ensuring similar therapeutic effectiveness and patient safety. Contract Research Organizations (CROs) play a vital role in conducting these studies by providing the expertise, infrastructure, and compliance knowledge required.



Analysis Requirements	Shimadzu's Analytical Capabilities
1. Nitrosamines Analysis - Ensures consistent, reproducible biological sample handling - Reduces contamination and manual errors in preparation	**GCMS-TQ8050 NX**  • Automates sample preparation • Seamlessly integrates with Shimadzu LCMS systems • Supports high-throughput workflows
2. Analytical Analysis - Trace level detection in complex matrices with high sensitivity and precision - Accurate profiling of metabolites, drugs, and impurity for ADME evaluation - High-speed data acquisition to handle simultaneous analysis	**LCMS-8060 RX with LabSolutions Insight LCMS DM Kit | GCMS-TQ8050 NX**  • Robust hardware with regulatory-compliant reporting as a complete solution • Reliable GCMS analysis using GCMS-TQ 8050 NX complements LCMS for holistic profiling
3. Multi-Omics & Metabolomics - Drug metabolites profiling for pharmacokinetics studies - Accurately quantifies primary metabolites affecting drug efficacy - Support for large-scale studies with robust databases	**Multi-omics Analysis Package for LCMS and GCMS**  • GCMS database for 600+ compounds and LCMS methods for 200 compounds to accelerate metabolomics analysis
4. Cell-Based Profiling - Assesses cell culture metabolites for pharmaceutical responses - Analyzes culture media to identify bioequivalent interactions - Tracks metabolic shifts to optimize drug formulations	**Cell Culture Profiling (CCP) Method Package**  • Analyze 144 compounds in less than 20 minutes • Simultaneous analysis of intracellular and extracellular metabolites in one run

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05

Quality Assurance & Control (QA/QC)

Quality Assurance (QA) and Quality Control (QC) are critical in the pharmaceutical industry, as they ensure patient safety, maintain regulatory compliance, uphold product quality consistency, and enhance production efficiency. However, due to the diversity of product forms and the complex and fluid regulatory environment, advanced expertise in analysis is required to apply QA/QC.

Analysis Requirements	Shimadzu's Analytical Capabilities
1. Compliance - Streamlined workflows and reduce manual intervention/errors. - Real-time monitoring and automated adjustments to enhance accuracy	**LabSolutions CS | LabSolutions BiX** • LabSolutions CS Network connects all type of PC-Based instruments for regulatory compliance and effective data management • Automated workflows from analysis to data processing and final reports
2. Spectroscopic Analysis - Identification and functional group analysis of raw materials - Reproducible spectroscopic analysis for routine and advanced applications	**IRSpirit-X series | UV-1900i Plus**  • Intuitive analysis of API, impurity, and degradation products using UV-1900i Plus • Space-saving and expandable, IRSpirit-X is ideal for raw material identification and structural analysis
3. Assay & Impurity Testing - Detects and quantifies impurities, APIs, and degradation products - Ensures uniformity in drug formulations	**Advanced i-Series**  • Enables automation and remote operation/monitoring • Advanced features include real-time mobile phase monitoring and FlowPilot
4. Residual Solvent Analysis - Trace-level analysis to meet regulatory standards - High sensitivity and quick analysis for diverse solvent matrices	**Brevis GC and HS-20 NX**  • Best-in-class performance with automatic remote operation and energy savings • HS-20 NX offers ultra-low carryover for maximized reliability
5. Dissolution Testing - Accurately monitors dissolution rates for batch consistency - High sample throughput for efficient QC - Comply with regulatory requirements for drug release studies	**Nexera FV**  • Automates processes, from media dispensing to data analysis • Can also be used as a standard UHPLC system for high throughput
6. Total Organic Carbon (TOC) - Reliably measures organic contaminants in pharmaceutical-grade water - Supports cleaning validation and monitors water systems for compliance	**TOC Analyzer**  • Can oxidize hard-to-decompose organic compounds • Ultra-wide sample range from 4 µg/L to 30,000 mg/L

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06

Shimadzu Informatics Solutions For Pharmaceutical Manufacturing

The pharma Industry recognizes Pharma 4.0 initiatives as a way to enhance efficiency, improve product quality, and ensure regulatory compliance. To accelerate towards Pharma 4.0, Shimadzu Informatics Solutions supports Pharmaceutical Laboratories in adopting end-to-end digitalization. This includes automating laboratory workflows for higher productivity, ensuring data integrity and compliance with regulatory requirements such as 21 CFR Part 11, and enabling real-time data sharing and remote access across the global workforce. With these strategic approaches in place, Shimadzu effectively ensures enhanced efficiency in the pharma industry.

Analysis Requirements	Shimadzu's Analytical Capabilities
1. Data management - Centralized data & user management - Data integrity & security - Audit trails & validation - Backup & retrieval - Regulatory compliance	**LabSolutions CS** • One compliant network system for entire lab instruments; chromatography, spectroscopy & others • Data management of balance to mass spectrometry instruments • End-to-end operational workflows for data interpretation, reporting & electronic signatures & approvals
2. Sample Management - Lab information management - Instruments & calibrations - Inventory control - Sample life cycle	**CaliberLIMS** • Pharma specific LIMS software that covers entire QC lab operations • Instrument interface for automatic results capturing • Controlled system driven workflow for error free operations • Data analytics & advanced reporting
3. Lab Analytics & Asset Management - Instrument utilization statistics - User performance indicators - Sample & batch counts - Instrument reservation for analysis	**LabSolutions BiX** • Web based system for efficient lab asset management • Graphical dashboards for instrument utilization, user performance, system administration & more • Analysis planner & instrument planner • Audit trail review & lab overview
4. Quality Assurance - Controlled quality processes for deviation management, CAPA, change control and others to ensure quality compliance - Document life cycle management & document control - Efficient competency management	**Enterprise Platform for Integrated Quality** • Integrated digital system for quality assurance, document management and training management • AssureIQ: Digitalised quality workflow for all types of quality processes from deviation to vendor management • DocsIQ: Efficient document life cycle management with digital control • LearnIQ: Digitalised competency management as per pharmaceutical regulations
5. Batch Records Management - Accurate batch records & safe market release - Eliminate product recalls & warnings - Efficient and faster batch releases - Integrated systems & data transfers	**Electronic Batch Record Management systems** • CaliberBRM is an electronic batch record software that ensures fully compliant & seamless manufacturing digitalization • Provides traceable, easy-to-comprehend batch records, automated logbooks, and consistent in-process quality checks, warehouse & dispensing, punches & dyes and more • Interface with process equipment and production systems to achieve paperless shop floor

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01

Shimadzu's Solutions For

Drug Discovery

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Application News

Preparative Purification Liquid Chromatograph – Nexera™ Prep

Seamless Purification for Drug Discovery with Screening, Preparative, and Purity check

Kosuke Nakajima[1,2]
[1]Shimadzu Corporation, [2]Shimadzu (Asia Pacific) Pte Ltd.

User Benefits

- Automated seamless purification can be performed, which includes screening, preparative, and purity checks.
- Open access software provides simple operation without complex settings.
- Nexera Prep achieves a compact footprint superior to a typical preparative LC system by LCMS-2050.

■ Introduction

In a drug discovery laboratory, the synthesis, screening and purification of target compounds are performed. These steps take significant time thus, a total workflow improvement is required. This article introduces the novel automation in Drug Discovery workflow for target screening, preparative, and purity check with Nexera Prep, which is integrated LC/MS analytical & preparative switching system.

■ Drug Discovery workflow

In drug discovery, target compound screening, purification, and purity check is performed as conventional workflow. Each task requires manual operations that include sample setting, up-scaling to prep LC, fractionation and purity check. Nexera Prep "LH-40" can change analysis and preparative mode and it achieves fully automated workflow for whole preparative tasks. (Fig.1 and Fig.2)

■ Nexera Prep with LCMS-2050

The LCMS-2050 single quadrupole mass spectrometer combines the user-friendliness of an LC detector with the excellent performance of MS to provide a complete package of easy-to-use high-level performance and compactness. The small footprint of the LCMS-2050 creates the flexibility to adapt to different needs. As with other LC detectors, it can be integrated into any Shimadzu LC architecture, whether it is a high throughput analytical system or a preparative LC with fraction collection. (Fig. 3)

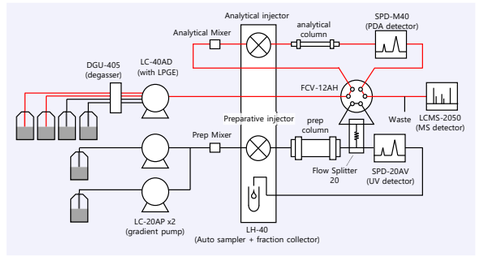

Fig. 1 Flow diagram of Nexera Prep during screening and purity check

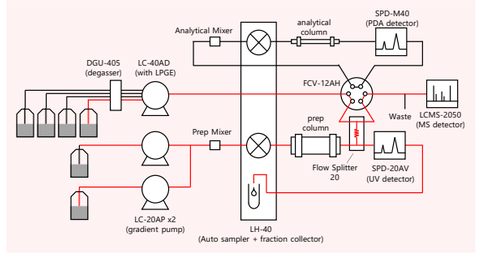

Fig. 2 Flow diagram of Nexera Prep during preparative

■ Seamless purification by Open Solution™

Open Solution creates a scale-up method using ASAPrep™ algorithm (Automated Scale-up from Analytical to Preparative). This algorithm automatically creates the "focused gradient" which is dedicated gradient profile for preparative scale utilizing information from screening results. (Application note: AD-0261) Open Solution can manage Nexera Prep's analytical & preparative mode. Users can achieve a seamless purification workflow from LC/MS screening, preparative LC/MS, and purity check with one system. (Fig. 4)

Fig. 3 Nexera Prep with LCMS-2050

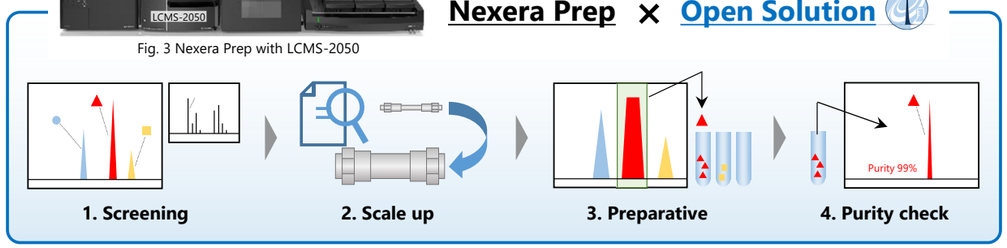

Fig. 4 Seamless purification by Nexera Prep and Open Solution

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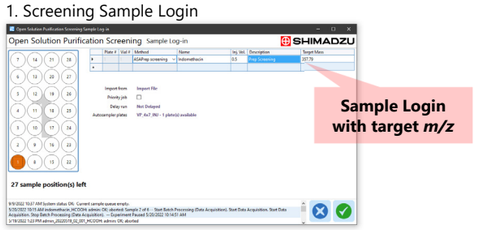
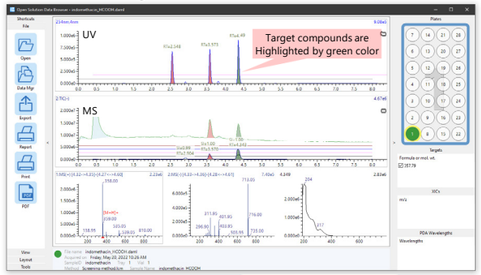
■ Purification of crude Drug compound

1. Screening Sample Login



2. LC/MS Screening



ASAPrep
Scale up algorithm

3. Auto Purification

Re-inject function

4. Purity Check

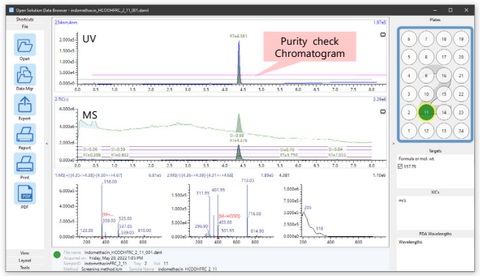


Fig. 5 Operation windows of Open Solution

We performed screening, purification, and purity check against Indomethacin which is a well-known drug compound. Table 1 shows the analytical conditions for the screening and purity check, and Table 2 shows the analytical conditions for the preparative step. The software operation windows are shown in Fig. 5 and obtained chromatograms are shown in Fig. 6.

Table 1 Analysis conditions for screening and purity check step

Column	: Shim-pack™ GISS C18 *1 (50 mm × 3 mm I.D., 1.9 µm)
Mobile phase	: A) 0.1 % formic acid in water (LPGE-A) B) 0.1 % formic acid in acetonitrile (LPGE-B)
Flow rate	: 1.5 mL/min
Time program	: B conc. 10 % (0 min)→ 90 % (6-8 min)
Column temp.	: 40℃
Injection volume	: 0.5 µL (screening: 20 mg/mL in DMSO)
Vial	: 4 mL vial kit *2
Detection	: 254 nm (SPD-M20A) Pos./Neg., Scan *m/z*: 100-1000 (LCMS-2050)

*1 P/N: 227-30049-01 *2 P/N: : 221-34269-91

Table 2 Analysis conditions for preparative step

Column	: Shim-pack GISS C18 *3 (100 mm × 20 mm I.D., 5 µm)
Mobile phase	: A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Flow rate	: 20 mL/min
Make-up	: 1.5 mL/min (methanol, LPGE-D)
Time program	: B conc. XX*4 % (0 min)→ XX+20 % (8-12 min)
Column temp.	: Ambient
Injection volume	: 200 µL (20 mg/mL in DMSO)
Vial	: 4 mL vial kit *2
Detection	: 254 nm (SPD-20AV) Pos./Neg., Scan *m/z* 100-1000 (LCMS-2050)

*3 P/N: 227-30066-02

*4 The value of XX means Initial B conc. obtained by ASAPrep algorithm.

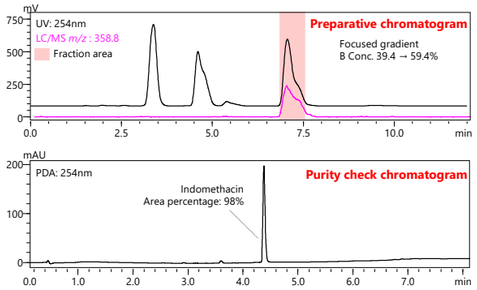


Fig. 6 Chromatograms of screening, preparative, and purity check analysis

■ Conclusion

This article describes seamless workflow for drug discovery from screening to preparative, and purity check by one system. Nexera Prep with LCMS-2050 and Open Solution software provide easy and labor-saving operations by fully automated purification workflow.

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Application News

Software for Efficient Method Development "LabSolutions™ MD"
Preparative Purification Liquid Chromatograph "Nexera™ Prep / LH-40 / LCMS-2050"

Seamless Purification Workflow from Analytical to Preparative in Single LC-MS System

Shinichi Fujisaki, Yusuke Masuda

User Benefits

- Scaling-up from analytical column to preparative column and purity/recovery check can be completed in a single system.
- LabSolutions MD efficiency optimizes separation conditions by automatically creating analysis schedules.
- Single quadrupole mass spectrometer LCMS-2050 provides *m/z* information for target compounds.

■ Introduction

Preparative HPLC is utilized in various fields, such as pharmaceuticals, food, and chemical engineering, for purifying target compounds from mixed samples, searching for active ingredients in natural products, and analyzing the structures of impurities and unknown compounds. To achieve high purity and recovery rate for fractionation of target compounds, it is crucial to establish analytical conditions that separate these compounds from other co-existing components. Due to the significant sample and mobile phase consumption associated with preparative HPLC conditions, the optimization of separation conditions is typically performed on an analytical scale to minimize these consumptions. During this optimization, various HPLC conditions, including gradient profiles, are adjusted to find the optimal separation. This is a time-consuming process for creating each analysis schedule. Also, confirmation of purity and recovery rate after scaling-up is followed by transferring fractions from fraction tubes to an autosampler manually. This article presents an efficient preparative purification workflow (Fig. 1), which includes investigating separation conditions at analytical scale, scaling-up for fractionation, and confirming purity and recovery rate. All of these processes are carried out using single analytical/preparative convertible LC-MS system.

Optimization of separation conditions in analytical scale

Optimization of loadability on column

Fractionation of target compounds

Confirmation of purity/recovery

Fig. 1 Workflow of Preparative Purification

■ Overview of LC-MS System

The flow path diagram of analytical/preparative convertible LC-MS system is shown in Fig. 2. The analytical flow path (the upper of Fig. 2) is used to optimize separation conditions, loadability on column, and purity/recovery check, while the preparative flow path (the lower of Fig. 2) is used for the preparative separation of target compounds. The liquid handler (LH-40), which has both analytical and preparative flow paths and can inject fractions from fraction tubes directly into the analytical flow path, allows a complete workflow of preparative purification with this system. In addition, LCMS-2050 provides not only mass information of target compounds when optimizing separation conditions, but also a combination trigger of UV and MS signals during fractionation. Therefore, target compounds can be recovered with high purity. To do so, this system is configured to split the preparative flow path and introduce a portion of the mobile phase into MS with a make-up solvent to achieve both fractionation and MS detection.

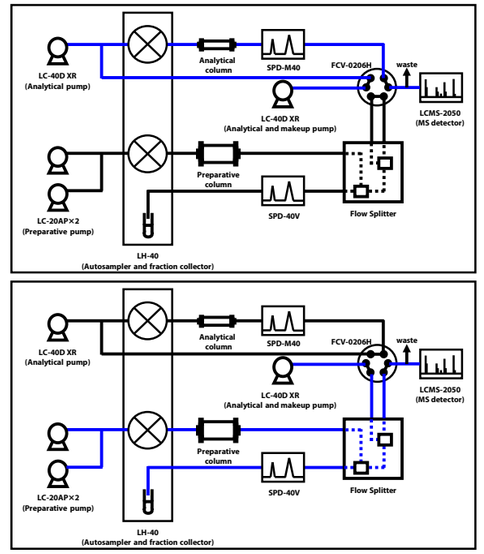


Fig. 2 Analytical Flow Path (Upper), Preparative Flow Path (Lower)
*blue colored flow path is in operation

The following section describes an example of using this analytical/preparative convertible LC-MS system to provide an efficient preparative purification workflow with a mixture of seven pharmaceutical standards (target compound for fractionation : Hydrocortisone) as model compounds.

■ Optimization of Separation Conditions in Analytical Scale

The separation conditions of Hydrocortisone are optimized in analytical scale. HPLC chromatogram (analytical conditions : Table 1) before optimization is shown in Fig. 3, in which Salicylic acid is eluted very close to Hydrocortisone. Increasing loadability under these conditions may cause further deterioration of the separation of these two compounds and the reduction of the purity at the recovery. Consequently, improvement of the separation is essential. The separation is optimized by varying the gradient conditions in nine different profiles (initial concentration and gradient slope in three different levels each). LabSolutions MD, a dedicated software for supporting method development, was used for automatic analysis schedule generation to improve the efficiency of the optimization (Fig. 4). The obtained chromatograms are shown in Fig. 5. The separation of Hydrocortisone and Salicylic acid was optimized at an initial concentration of 15% and a gradient slope of 20 minutes (③ in Fig. 5). Then, the next optimization of loadability was conducted based on these conditions. During optimizing separation conditions, mass information (Hydrocortisone : *m/z* 363.3) was simultaneously obtained by LCMS-2050.

Link to Content Page →

Table 1 Analytical Conditions

Mobile Phase	: Pump A : 0.1% formic acid in water
	: Pump B : Acetonitrile
Column	: Shim-pack Scepter™ C18-120
	(150 mm × 4.6 mm I.D., 5 µm)*1
Sample	: (A) Hydrocortisone, (B) Salicylic acid,
	(C) Metoclopramide, (D) Lidocaine,
	(E) Furosemide, (F) Papaverine, (G) Quinidine
Sample Concentration	: 100 mg/L (Hydrocortisone), 10 mg/L (others)
Injection Volume	: 10 µL
LC Conditions	
Time Program	: B Conc. 25%(0 min)→45%(20 min)
	→25%(20.01-25 min)
Column Temp.	: Ambient
Flow rate	: 1 mL/min
Sample loop size	: 500 µL
Syringe size : 500 µL	
Detection (PDA)	: 245 nm (SPD-M40, conventional cell)
MS conditions	
Ionization	: ESI/APCI (DUIS™), positive and negative
Mode	: SCAN (*m/z* 100-500)
Nebulizing Gas Flow	: 2.0 L/min （N_2）
Drying Gas Flow	: 5.0 L/min （N_2）
Heating Gas Flow	: 7.0 L/min （N_2）
DL Temp.	: 200 ℃
Desolvation Temp.	: 450 ℃
Interface Voltage	: 3.0/-2.0 kV (positive/negative)

*1 P/N : 227-31020-05

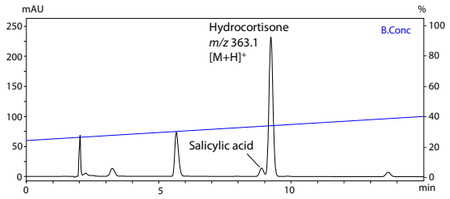
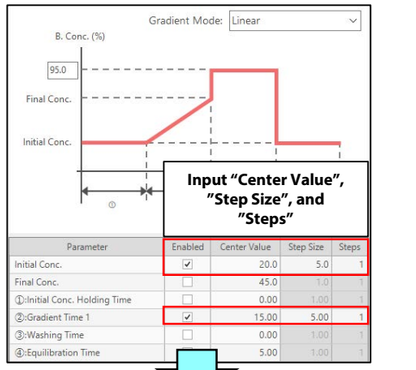
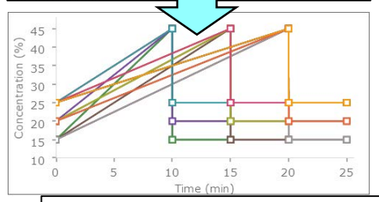


Fig. 3 HPLC Chromatogram before Optimizing the separation





Analysis schedules (three initial Conc. and three Gradient Time1 : nine patterns) including column equilibration are automatically generated

Fig. 4 Automatic Generation of Analysis Schedules by LabSolutions MD

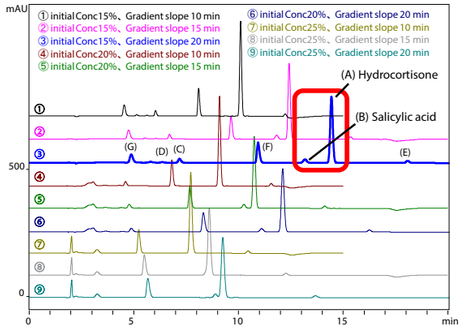


Fig. 5 Result of Optimizing Separation Conditions with LabSolutions MD
*blue colored chromatogram ③ shows the best separation of Hydrocortisone and Salicylic acid

■ Optimization of Loadability on Column

Fig. 6 shows the result of optimization of loadability conducted at injection volumes of 10, 20, 30, 40, 50, and 100 µL using Hydrocortisone (10,000 mg/L) under the optimized conditions at the analytical scale (③ in Fig. 5). At an injection volume of 100 µL, the separation of Hydrocortisone and Salicylic acid was not sufficient (inside the blue oval in Fig. 6). In addition, a small peak (circled in red in Fig. 6), which seemed to be an impurity, was not clearly separated at the base of the peak. Up to an injection volume of 50 µL, Hydrocortisone is well separated from the neighboring peaks. Consequently, scaling-up was implemented using 50 µL injection volume.

Fig. 6 Result of Optimization of Sample Loadabily

■ Fractionation of Target Compounds

Hydrocortisone was fractionated using a UV trigger. The preparative conditions are presented in Table 2 (only the settings differing from those in Table 1 are listed), and the resulting preparative HPLC chromatogram is shown in Fig. 7 (the blue area represents the fractionation interval). Based on the cross-sectional area ratio (approximately 20-fold) between the preparative column (20 mm I.D.) and the analytical column (4.6 mm I.D.), the flow rate was increased to 20 mL/min (with a constant linear velocity before and after scaling-up), and the injection volume was set at 1 mL. As a result, similar separation profiles were achieved before and after scaling-up, allowing for effective fractionation of Hydrocortisone while maintaining adequate separation from Salicylic acid."

Table 2 Preparative Conditions

Column	: Shim-pack Scepter™ C18-120
	(150 mm × 20 mm I.D., 5 µm)*1
Sample Concentration	: 10000 mg/L (Hydrocortisone),
	1000 mg/L (others)
Injection Volume	: 1 mL
LC Conditions	
Flow rate (Prep)	: 20 mL/min
Flow rate (Makeup)	: 1.5 mL/min (Methanol)
Sample loop size	: 2 mL
Syringe size : 5 mL	
Detection (UV)	: 245 nm (SPD-40V, preparative cell)
MS conditions	
Desolvation Temp.	: 100 ℃

*1 P/N : 227-31102-03

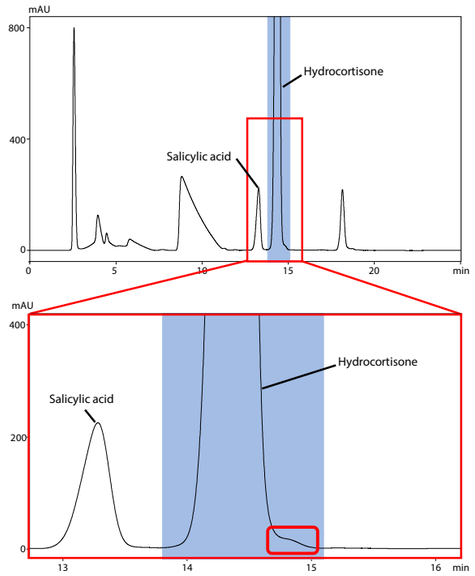


Fig. 7 UV triggered Preparative Chromatogram
*fractionation interval is colored blue

■ Confirmation of Purity/Recovery

Fig. 8 shows the chromatogram obtained when a fractionated Hydrocortisone was re-injected into analytical flow path, as well as the chromatogram of a standard mixture (as a reference for calculating recovery) prepared to be the same concentration as fractionated Hydrocortisone. The purity and recovery rate of Hydrocortisone are shown in Table 3. The purity was more than 99% using area normalization, and the recovery rate was also excellent.

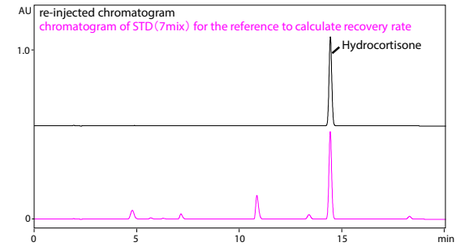


Fig. 8 Re-injected Chromatogram (Upper)
Chromatogram of STD (Lower)

Table 3 Purity and Recovery Rate of Fractionated Hydrocortisone
(n=3, average value)

	Purity (Area %)	Recovery Rate (%)
Hydrocortisone	99.7	101.2

In the lower part of Fig. 7, an enlarged image of the interval where Hydrocortisone was eluted is presented. A very small peak, likely an impurity, is observed at the base of the Hydrocortisone (inside the red frame). The fractionation results demonstrate a purity of 99.7% (Table 3) with UV trigger only. However, the preparative LC-MS system also supports MS triggered fractionation, enabling the separation of target compounds with even higher purity by avoiding impurities, thanks to the high identification performance of the MS. More information on the MS triggered preparative LC-MS system can be found in Application News "01-00651-EN".

■ Conclusion

The analytical/preparative convertible LC-MS system is employed to efficiently complete the preparative purification workflow which consists of the optimization of separation conditions at an analytical scale, scaling-up for fractionation, and confirmation of purity and recovery rate. LabSolutions MD, equipped to automatically generate analysis schedules with various HPLC parameters, facilitates the efficient optimization of separation conditions. Also, LCMS-2050 provides mass information simultaneously. Moreover, the liquid handler LH-40 allows for the direct injection of obtained fractions into the analytical flow path, ensuring seamless confirmation of purity and recovery rate without transferring fractions from a fraction tubes to an autosampler manually.

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Application News

Preparative Purification Liquid Chromatograph – Nexera™ Prep

Workflow Enhancement for Purification of New Synthetic Compounds in Drug Discovery Process

Kosuke Nakajima[1,2], Devadiga Navin[3]
[1]Shimadzu Corporation, [2]Shimadzu (Asia Pacific) Pte Ltd., [3]Shimadzu Analytical (India) Pvt Ltd.

User Benefits

- Automatic screening against synthetic crude compounds provides a labor-saving operation.
- The easy scale-up algorithm achieves smooth transfer from a screening step to a purification step.

■ Introduction

In a drug discovery laboratory, the synthesis, screening and purification of target compounds are performed. These steps take significant time thus a total workflow improvement is required. This article introduces a novel automation for target screening and preparative purification by LC/MS using a dedicated, designed software.

■ Conventional Workflow

Fig. 1 shows a conventional workflow of target compound screening and purification in drug discovery. First, crude compounds from the synthesis step are analyzed by LC-MS, and the operator checks each result. Then, the preparative method is developed manually through the conventional scale-up method. Finally, the crude compounds are purified by preparative LC.

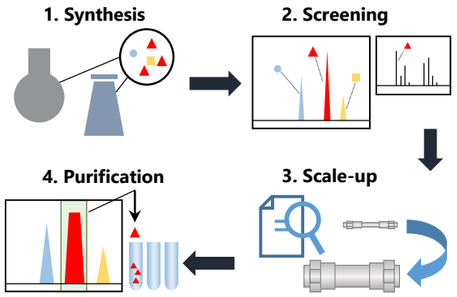


Fig. 1 Conventional workflow of target screening and purification

■ Advanced Workflow

► Automatic Screening by Open Solution

Typical screening steps require manual checking for each crude sample. Open Solution™ is an open-access software that assists the whole drug discovery workflow from screening to purification. The software automatically classifies crude samples into three situations using target *m/z* information and MS spectrometry (Fig. 2).

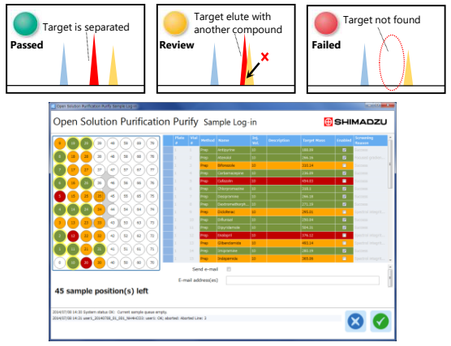


Fig. 2 Open Solution classify crude compounds to three situations

► Automatic Scale-up by ASAPrep

Open Solution creates a scale-up method using ASAPrep™ algorithm (Auto Scale-up from Analytical to Preparative). This algorithm creates the focused gradient profile for preparative scale utilizing information from screening results. (Fig. 3) The only need for the user is to choose passing samples (green colored) and start the purification step by just one click.

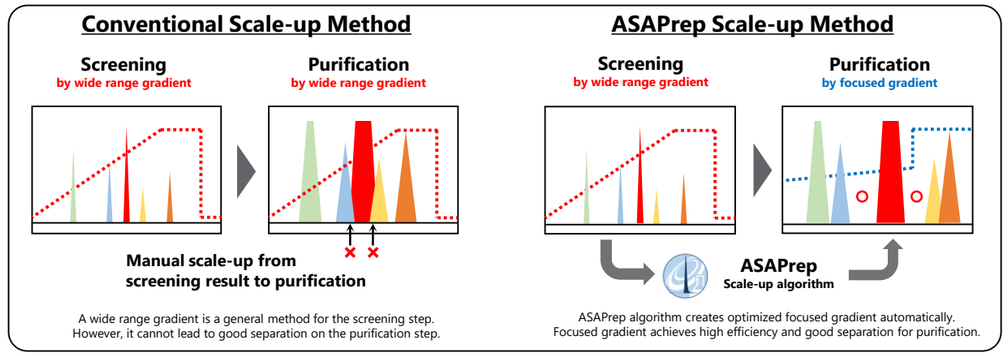


Fig. 3 Comparison of workflow between conventional and ASAPrep algorithm

■ Screening and Purification of Crude Medical Compounds

We performed screening, scale-up, and purification against three types of medical compounds, containing impurities. Table 1 shows the analytical conditions for the screening step, and Table 2 shows the analytical conditions for the purification step. All these results are shown in Table 3. LC-MS analysis (LCMS-2020) finds three target compounds at the screening step. Then, the ASAPrep algorithm calculated the dedicated, focused gradient profile by the retention time of each target compound through the screening result. The focused gradient that followed the ASAPrep algorithm showed good separation at the purification step and identified each compound by LCMS-2020.

Table 1 Analytical conditions for screening step

Column	: Shim-pack GISS C18 *1 (100 mm × 2.1 mm I.D., 1.9 µm)
Mobile phase	: A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Flow rate	: 0.5 mL/min
Time program	: B conc. 5 % (0 min)→ 95 % (3-4 min) →5 % (4.01-5 min)
Column temp.	: 40℃
Injection volume	: 1 µL
Vial	: SHIMADZU LabTotal™ for LC 1.5 mL, Glass*2
Analytes	: Target compound 2 mg/mL in DMSO
Detection	: 254 nm (SPD-M20A) Pos./Neg., Scan *m/z*: 50-1000 (LCMS-2020)

*1 P/N: 227-30048-02 *2 P/N: 227-34001-01

Table 2 Analytical conditions for purification step

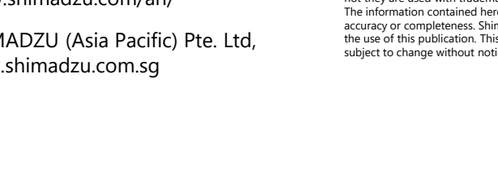
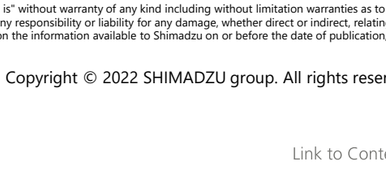
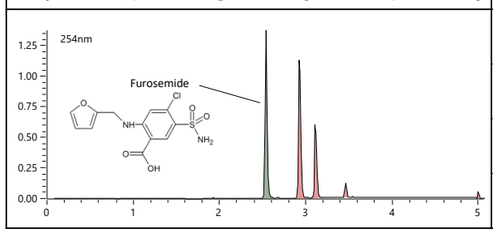
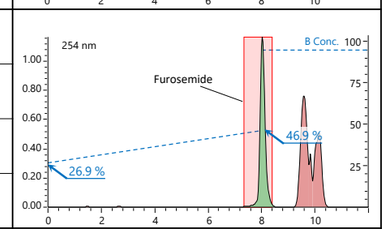
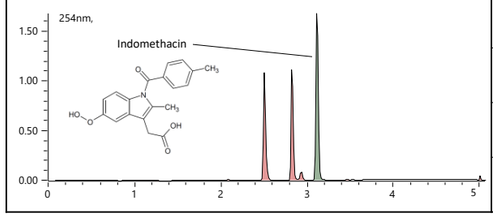
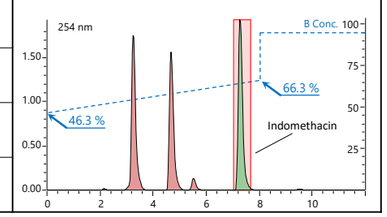
Column	: Shim-pack GISS C18 *3 (100 mm × 20 mm I.D., 5 µm)
Mobile phase	: A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Flow rate	: 20 mL/min
Time program	: B conc. XX*4 % (0 min)→ XX+20 % (8-12 min)
Column temp.	: Ambient
Injection volume	: 400 µL
Vial	: 10 mL screw vial *5
Analytes	: Target compound 20 mg/mL in DMSO
Detection	: 254 nm (SPD-20AV) Pos./Neg., Scan *m/z* 50-1000 (LCMS-2020)

*3 P/N: 227-30066-02 *5 P/N: 220-97331-09
*4 XX: Initial B conc. of focused gradient

■ Conclusion

This article introduces workflow enhancement for drug discovery from screening to purification. Open Solution software and ASAPrep algorithm provide easy and labor-saving operations to check screening results and preparative method development. Automatic workflow for purification is expected to increase productivity and reduce labor costs.

Table 3 Results of screening and purification steps

Screening Chromatogram	ASAPrep Algorithm	Purification Chromatogram
	Target mass 254.28 Retention time 2.83 min Focused gradient Int. B Conc. 36.9 % Screening result PASS	
	Target mass 330.74 Retention time 2.53 min Focused gradient Int. B Conc. 26.9 % Screening result PASS	
	Target mass 357.79 Retention time 3.11 min Focused gradient Int. B Conc. 46.3 % Screening result PASS	

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Application News

Preparative Purification Liquid Chromatograph – Nexera™ Prep

High Throughput Preparative LC with Auto Switching Dilution Injection Function

Kosuke Nakajima[1,2], Devadiga Navin[3]
[1]Shimadzu Corporation, [2]Shimadzu (Asia Pacific) Pte Ltd., [3]Shimadzu Analytical (India) Pvt Ltd.

User Benefits

- Large volume injection enables to improve the throughput of preparative LC.
- Dilution injection shows a better peak shape with strong elution dissolution solvents.
- The auto switching setup makes the system which is suitable for typical injection as well as for large volume injection with strong elution dissolution solvents.

■ Introduction

Preparative LC is a widely used purification technique. High throughput purification is required in various industries. However, large volume injection which is required to achieve high throughput purification, causes peak broadening. This article introduces a large amount of injection and a high throughput preparative LC using dilution injection.

■ Importance of large volume injection to improve the throughput of purification

Loading amount is one of the critical parameters to determine the throughput of preparative LC. In most cases, the amount of crude sample to purify is larger than the loading capacity of conventional preparative LC. Therefore, multiple preparative operations are necessary to purify target quantity of sample. Increasing the loading amount leads to a less number of repetitions of the process and improves the purification throughput. The combination between highly concentrated sample and injection volume determines the loading amount of each preparative process. (Table 1) Especially, large volume injection is required because making high concentrate sample causes precipitation in the preparative LC.

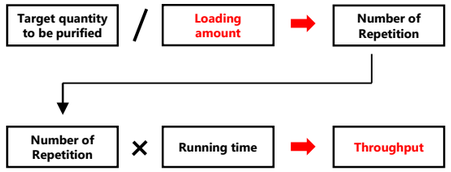

Fig. 1 The relationship between loading amount and throughput

Table 1 Loading amount in the sample

	Loading amount	
Sample Concentration	**500 µL injection**	**2000 µL injection**
1 mg/mL	0.5 mg	2 mg
5 mg/mL	2.5 mg	10 mg
20 mg/mL	10 mg	40 mg

■ Limitations of large volume injection

Large volume injection enables to improve the purification throughput. However, large volume injection affects peak shapes due to the lack of mixing between dissolution solvent and mobile phase. Especially, large volume injection causes peak broadening when the dissolution solvent has a higher eluotropic strength than the starting mobile phase of preparative LC (Fig. 2). Table 2 describes the analysis condition for typical injection. The effect of large volume injection with high elution strength solvent on the peak shape can be seen in Fig. 3. The peak shape has become worse in 2000 µL injection due to dissolution solvent.

Fig. 2 Effect of injection volume on the peak shape

Table 2 Analysis conditions for typical injection

Column	: Shim-pack™ GISS C-18 *1 (100 mm × 20 mm I.D., 5 µm)
Mobile phase	: A: Water, B: Acetonitrile
Flow rate	: 20 mL/min
Time program	: B conc. 5 % (0 min)→ 90 % (4.5-6.5 min) →5 % (6.51-8 min)
Column temp.	: Ambient
Sample Solvent	: Dimethyl sulfoxide (DMSO)
Analytes	: 5 mg/mL caffeine, 50 mg/mL sulfamethazine
Vial	: 10 mL screw vial *2
Detection	: PDA 250 nm

*1 P/N: 227-30066-02 *2 P/N: 220-97331-09

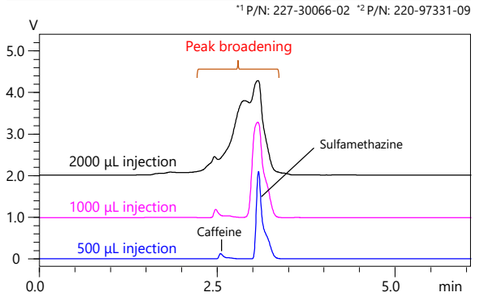

Fig. 3 Chromatograms of two compounds with different injection volume

■ Dilution injection for large volume injection

Dilution injection is a technique to improve peak shape for large volume injection. Dissolution solvent is diluted by diluent with a lower eluotropic strength than starting mobile phase, and the target compounds are concentrated at the tip of the columns. Fig. 4 shows the improvement of peak shape by dilution injection. Fig. 5 shows the flow diagrams of the switching system between typical injection and dilution injection. Fig. 5a shows the flow path for typical injection whereas Fig. 5b shows the flow path for dilution injection.

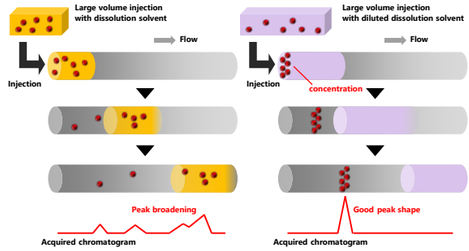

Fig. 4 Improvement of peak shape by dilution injection

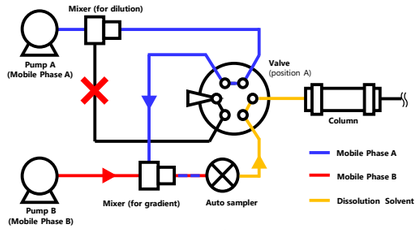

Fig. 5a Flow path diagram for typical injection

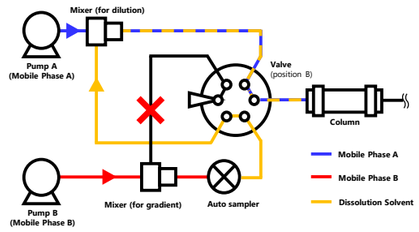

Fig. 5b Flow path diagram for dilution injection

■ Large volume injection of caffeine and sulfamethazine with dilution injection

In this study, we used caffeine and sulfamethazine as target compounds with dilution injection. Table 3 shows the analysis condition for dilution injection. The acquired chromatograms are as shown in Fig. 6. Typical injection showed peak broadening, however, dilution injection leads good peak shapes and separation between the two analytes.

Table 3 Analysis conditions for dilution injection

Column	: Shim-pack GISS C-18 (100 mm × 20 mmI.D., 5 µm)
Mobile phase	: A: Water, B: Acetonitrile
Flow rate	: 20 mL/min
Dilution	: B conc. 5 % (0-2.2 min, diluent: water)
Time program	: B conc. 5 % (0 min)→ 50 % (4.5 min) →5 % (4.51-6 min)
Column temp.	: Ambient
Sample Solvent	: Dimethyl sulfoxide (DMSO)
Analytes	: 5 mg/mL caffeine, 50 mg/mL sulfamethazine
Vial	: 10 mL screw vial
Detection	: PDA 250 nm

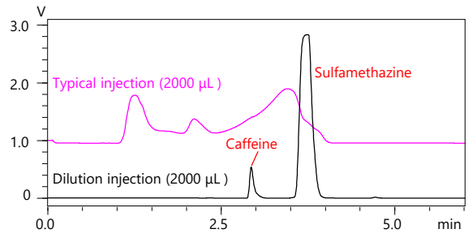

Fig. 6 Comparison between the typical injection and dilution injection

■ Consecutive dilution injection

In the dilution process, another capability of the system is to achieve large loading amount by consecutive injections as described in Fig. 7. Consecutive injections for mixture of caffeine and sulfamethazine was performed with the same analysis condition as shown in Table 3. In this process, more than 500 mg of target compounds were introduced into the separation column by five times consecutive injections. The acquired results showed achievement of higher loading amount with good separation and peak shape, as shown in Fig. 8.

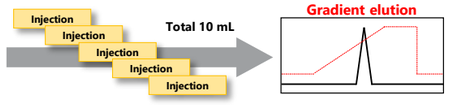

Fig. 7. Schematic representation of consecutive dilution injection

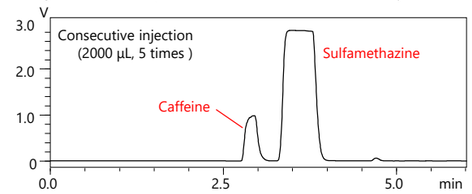

Fig. 8 Chromatogram of consecutive dilution injection

■ Conclusion

This article is an example of the large volume injection. In most cases, a large volume injection affects the peak shape and separation. Shimadzu Nexera prep system with switching system for both typical injection and dilution injection enables better chromatographic performance for samples with large loading amount.

04-AD-0248-EN First Edition: Sep. 2021

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Preparative Purification Liquid Chromatograph "Nexera™ Prep / LH-40 / LCMS-2050"
Software for Efficient Method Development "LabSolutions™ MD"

Application News

High Purity Preparative Purification Enabled by UV/MS Trigger on LC-MS System

Shinichi Fujisaki, Yusuke Masuda

User Benefits

- Scaling-up from analytical column to preparative column and purity/recovery check can be completed in a single system.
- High identification performance of MS enables high purity fractionation of target compounds.
- Utilizing software simulation to optimize parameters for fractionation reduces the labor required.

■ Introduction

Preparative HPLC is utilized in various fields, such as pharmaceuticals, food, and chemical engineering, for purifying target compounds from mixed samples, searching for active ingredients in natural products, and analyzing the structures of impurities and unknown compounds. In Application News "01-00650-EN", an introduction was made to analytical/preparative convertible LC-MS system designed to manage the entire preparative purification workflow (Fig. 1). This workflow consists of the optimization of separation conditions at analytical scale, scaling-up, fractionation, and confirmation of purity/recovery. The process began with the efficient optimization of separation conditions, achieved using LabSolutions MD, a dedicated software for supporting method development, to ensure adequate separation between the target compound for fractionation (Hydrocortisone) and the nearby co-eluted peaks. Then followed by loadability optimization, scaling-up, and fractionation with UV signal as a trigger. This article introduces the achievement of higher purity in the fractionation of Hydrocortisone by effectively eliminating impurities through the use of MS signal, which offers enhanced identification performance in addition to UV signal.

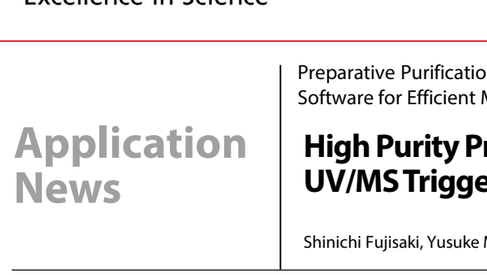


Fig. 1 Workflow of Preparative Purification

■ Overview of LC-MS System

The flow path diagram of analytical/preparative convertible LC-MS system is shown in Fig. 2. The analytical flow path (the upper of Fig. 2) is used to optimize separation conditions, loadability on column, and purity/recovery check, while the preparative flow path (the lower of Fig. 2) is used for the preparative separation of target compounds. The liquid handler (LH-40), which has both analytical and preparative flow paths and can inject fractions from fraction tubes directly into the analytical flow path, allows a complete workflow of preparative purification with this system. A combination of UV and MS signals can also be used as triggers for fractionation. Since MS detects compounds after spraying and volatilizing the mobile phase, introduced compounds cannot be recovered. Therefore, the preparative flow is split to introduce a portion of the mobile phase eluted from the preparative column into MS with make-up solvent, enabling both MS-triggered fractionation and MS detection. This system, equipped with both analytical and preparative functions, can be controlled by LabSolutions. The software features fractionation simulation

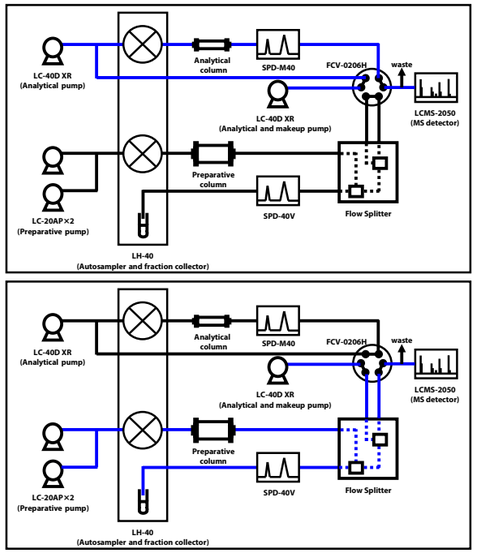
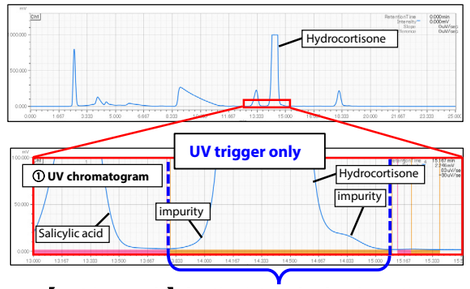


Fig. 2 Analytical Flow Path (Upper), Preparative Flow Path (Lower)
*blue colored flow path is in operation

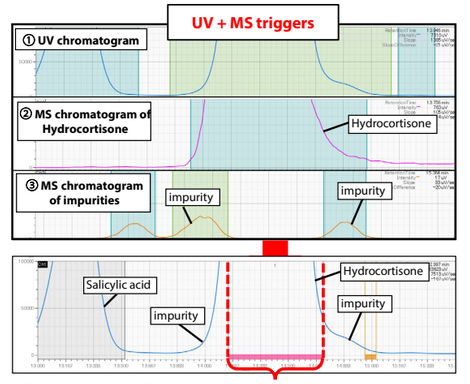
that simplifies parameter setting for fractionation by just selecting the target peaks in the chromatogram displayed.

■ High Purity Fractionation Triggered by UV and MS Signals

Fig. 3 displays the results of fractionation simulations of Hydrocortisone using two settings: "UV trigger only" and "UV + MS triggers." In the case of fractionation using only the UV trigger (① in the upper part of Fig. 3), there was a risk to fractionate co-eluted impurities at the beginning and/or the end of the Hydrocortisone peak. On the other hand, by combining MS and UV triggers (①, ②, and ③ in the lower part of Fig. 3), impurity information from the MS chromatogram can be used for fractionation. Thus, the intervals where two impurities were eluted were eliminated from the fractionation, resulting in a higher purity of the recovered solution. The actual results of the fractionation using these settings are shown in Fig. 4 (fractionation conditions: Table 1). As demonstrated in the fractionation simulations in Fig. 3, in the case of "UV trigger only," the blue interval was fractionated, and the recovered solution contained very few impurities. However, in the case of "UV + MS triggers," the MS chromatogram of impurities (③ in Fig. 3) indicated that impurities were eluted at two locations: the beginning and the end of the Hydrocortisone peak. Consequently, the fractionation was able to eliminate these impurity intervals.



【UV trigger only】 fraction intervals of Hydrocortisone



【UV+MS triggers】 fraction intervals of Hydrocortisone

Fig. 3 Fractionation Simulation

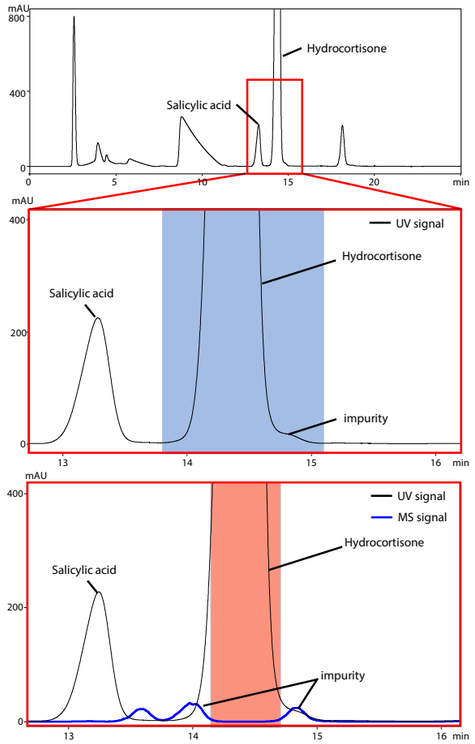


Fig. 4 Comparison of Fractionation Results between "UV trigger only" (blue colored interval) and "UV + MS triggers" (red colored interval)

Table 1 Preparative Conditions

Mobile Phase	: Pump A : 0.1% formic acid in water
	: Pump B : Acetonitrile
Column	: Shim-pack Scepter™ C18-120 (150 mm × 20 mm I.D., 5 µm)*1
Sample	: Hydrocortisone, Salicylic acid, Metoclopramide, Lidocaine, Furosemide, Papaverine, Quinidine
Sample Concentration	: 10000 mg/L (Hydrocortisone), 1000 mg/L (others)
Injection Volume	: 1 mL
LC Conditions	
Time Program	: B Conc. 25%(0 min)→45%(20 min) →25%(20.01-25 min)
Column Temp.	: ambient
Flow rate (Prep)	: 20 mL/min
Flow rate (Makeup)	: 1.5 mL/min (Methanol)
Sample loop size	: 2 mL
Syringe size : 5 mL	
Detection (PDA)	: 245 nm (SPD-40V, preparative cell)
MS conditions	
Ionization	: ESI/APCI (DUIS™), positive and negative
Mode	: SCAN (*m/z* 100-500)
Nebulizing Gas Flow	: 2.0 L/min (N_2)
Drying Gas Flow	: 5.0 L/min (N_2)
Heating Gas Flow	: 7.0 L/min (N_2)
DL Temp.	: 200 ℃
Desolvation Temp.	: 100 ℃
Interface Voltage	: 3.0/-2.0 kV (positive/negative)

*1 P/N : 227-31102-03

■ Confirmation of Purity/Recovery

Fig. 5 shows the re-injected chromatograms of Hydrocortisone fractionated using "UV trigger only" and "UV + MS triggers" into the analytical flow path of this system, as well as the chromatogram of the mixed standard solution (used as a reference for calculating recovery). The purity and recovery rate of each fractionation are shown in Table 2. In the "UV + MS triggers" chromatogram, the re-injected chromatogram (red line in Fig. 5) indicates that no impurity peak was detected compared to the "UV trigger only" chromatogram (blue line in Fig. 5) because the target compound was fractionated in an impurity-free interval. However, since the amount of impurities was negligible, both the purity (area %) and recovery rate of the fractions obtained using "UV trigger only" and "UV + MS triggers" were close to 100%, which is a favorable result.

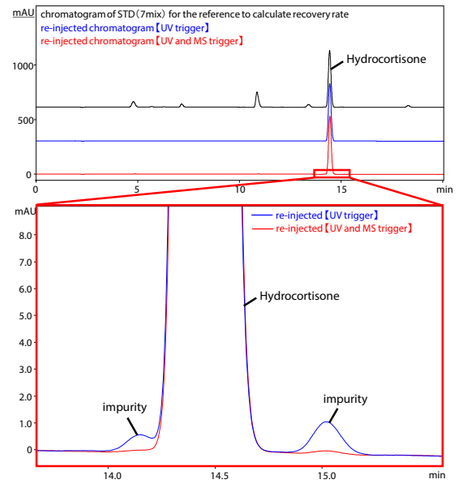


Fig .5 Chromatograms for Purity/Recovery Confirmation and STD (7mix)

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Table 2 Purities and Recovery Rates of Hydrocortisone
(n=3, average value)

	Purity (Area %)	Recovery Rate(%)
UV trigger only	99.7	101.2
UV＋MS triggers	100.0	100.7

■ Conclusion

High-purity fractionation was introduced through the combined use of UV and MS signals as triggers. The utilization of an MS trigger enables the preparative purification of target compounds with enhanced purity, as it allows for the exclusion of co-eluted compounds, aside from the targets, from the fraction intervals using MS chromatogram. The analytical/preparative convertible LC-MS system employed in this article features two flow paths, one designed for analytical use and the other for preparative use, offering an efficient preparative purification workflow with this single system. For further details, please refer to Application News "01-00650-EN".

01-00651-EN First Edition: Nov. 2023

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Application News

Semi-Preparative Supercritical Fluid Chromatograph – Nexera™ UC Prep

Highly Effective Purification of Drug Compounds

Kosuke Nakajima[1,2]
[1]Shimadzu Corporation, [2]Shimadzu (Asia Pacific) Pte Ltd.

User Benefits

- Preparative SFC provides significant reduction of fraction drying time comparing with conventional preparative HPLC
- Preparative SFC provides notable reduction of organic solvent consumption comparing with conventional preparative HPLC
- The stacked injection function provides consecutive purification and reduces run time

■ Introduction

High throughput purification is required in various fields. Preparative LC is widely used for purification of compounds., but it takes a long time. Additionally, high consumption of organic solvents leads to ineffective running cost. Here, we introduce a high throughput and low running cost preparative SFC purification.

■ Purification by Preparative LC system

Reverse phase mode is one of the most popular separation modes that is widely used in analytical HPLC and preparative HPLC. HPLC with reverse phase mode has high resolution and is suitable for purification of various compounds. On the other hand, water is often used as mobile phase in reverse phase mode and can cause extensive drying time. Table 1 and Fig. 1 show a practical example for ketoprofen and indomethacin of preparative LC purification.

Table 1. Preparative LC Conditions

Column	: Shim-pack™ Scepter C18-120 *1 (50 mm× 20 mm I.D., 5 µm)
Mobile phase	: A: Water (containing 0.1 % (v:v) formic acid) B: Acetonitrile
Flow rate	: 20 mL/min
Time program	: B conc. 10 %(0-1 min)→ 90 % (7-9 min) →10 %(9.01-10 min)
Column temp.	: Ambient temperature
Injection vol.	: 500 µL (10 mg/mL for each compound in acetonitrile)
Vial	: 10 mL screw vial *2
Detection	: 250 nm (PDA)

*1 P/N: 227-31102-01
*2 P/N: 220-97331-09

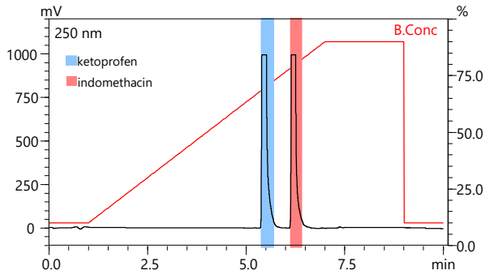

Fig. 1 Preparative LC chromatogram of drug compounds

■ Features of Preparative SFC

Supercritical Fluid Chromatography (SFC) is one of the chromatography using supercritical fluid (i.e., supercritical carbon dioxide) as mobile phase. The low cost of carbon dioxide (purity: 99.9 %) can significantly trim the total running cost of preparative SFC. Moreover, fraction drying time is shorter than conventional preparative LC because carbon dioxide naturally evaporates under atmospheric pressure.

■ Retention time modulation by gradient mode in Preparative SFC

In SFC analysis, organic solvent , called as "modifier" is used to modulate the retention time and/or selectivity. By continuously changing the amount of modifier through gradient mode, retention of compounds can be modulated. Table 2 and Fig. 2 show preparative SFC conditions and preparative SFC chromatogram of drug compounds that are the same compounds analyzed by conventional preparative LC in figure 1. A good separation was achieved on Shim-pack UC Diol column.

Table 2. Preparative SFC conditions (gradient mode)

Column	: Shim-pack UC Diol II *3 (250 mm × 20 mmI.D., 5 m)
Mobile phase	: CO_2 B: Methanol
Flow rate	: 60 mL/min
Time program	: B conc. 0 %(0-1 min)→ 40 % (7-9 min) →0 %(9.01-10 min)
Column temp.	: 40℃
Injection vol.	: 500 µL (10 mg/mL for each compound in *n*-Heptane/2-propanol = 2:1, v:v)
Vial	: 10 mL screw vial
BPR setting	: 10 MPa
Detection	: 250 nm (PDA)

*3 P/N: 227-32606-04

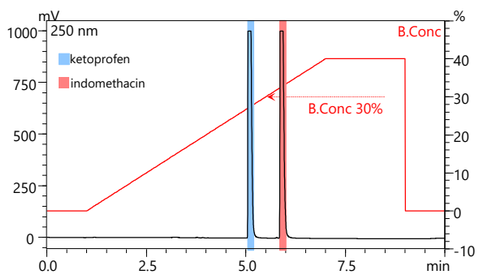

Fig. 2 Preparative SFC chromatogram of drug compounds (Gradient mode)

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■ Improved efficiency of purification with stacked injection mode

"stacked injection" is a technique to improve the efficiency of preparative operations by consecutively injecting a sample in a queue and utilizing the buffer/waiting time for peak elution (Technical Report C190-E247). An isocratic program was developed for stacked injection mode based on results of gradient mode. Table 3 and Fig. 3 show Preparative SFC conditions and chromatogram by isocratic mode separation and with stacked injections. Repeated injections of for ketoprofen and indomethacin (n=5) were set and shown in Fig. 4 and Fig. 5.

Table 3. Preparative SFC conditions by (Isocratic mode)

Column	: Shim-pack UC Diol II (250 mm× 20 mmI.D., 5 µm)
Mobile phase	: A: CO_2 B: Methanol
Flow rate	: 60 mL/min
Time program	: B conc. 30 %(0-4 min)
Column temp.	: 40 ℃
Injection vol.	: 500 µL (10 mg/mL of each compound in *n*-Heptane/ 2-propanol = 2:1)
Vial	: 10 mL screw vial
BPR setting	: 10 MPa
Detection	: 250 nm (PDA)

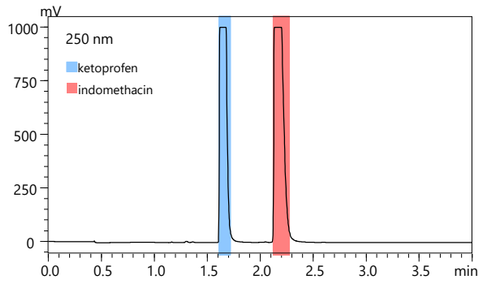

Fig. 3 Preparative SFC chromatogram (Isocratic mode)

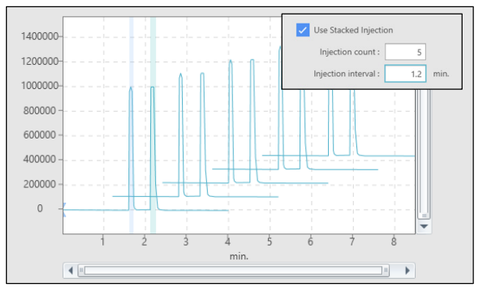

Fig. 4 Setting in Prep Solution *4 for stacked injection mode

*4 Prep Solution: Dedicated software for Nexera UC Prep

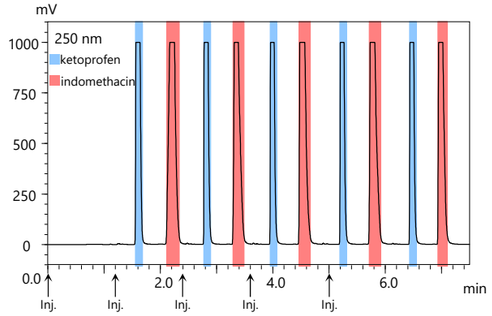

Fig 5. Preparative SFC chromatogram "stacked Injection mode"

■ Comparison of fraction drying time by Preparative LC and Preparative SFC

Comparison of mobile phase consumption and drying time of the collected fraction from Preparative LC and Preparative SFC is shown in Table 4 and Fig. 6. Preparative LC utilized a massive amount of water and organic solvents. By Preparative SFC, significant reduction of running cost was achieved due to the usage of carbon dioxide instead of them. Moreover, cost reduction is supported by shorter preparative time from stacked injection.

Fig. 6 shows the comparison of the fraction drying time collected by Preparative LC and Preparative SFC. Preparative SFC significantly shortened the drying time by 20 times or greater.

Table 4 Comparison of organic solvents consumption

	Mobile Phase	Volume	Mobile Phase	Volume	Total time
Preparative HPLC	Water	630 mL	Acetonitrile	470 mL	55 min
Preparative SFC	CO_2	315 mL	Methanol	135 mL	7 min

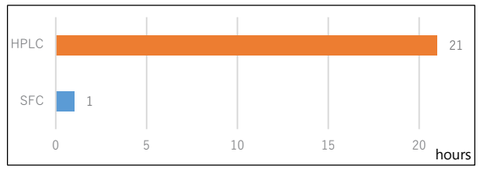

Fig. 6 Comparison of solvent drying time

■ Conclusion

This article introduced purification process of ketoprofen and indomethacin by Preparative SFC as an example of practical drug compounds purification. Preparative SFC provides superior advantages compared to Preparative LC in terms of process time length, drying time and running cost. Nexera UC Prep is expected to be a game changer for preparative workflow.

01-00134-EN First Edition: June 2021

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Ultra Fast Preparative and Purification LC System Nexera™ UFPLC

Application News

Preparative Purification of Ibuprofen and Related Substances by Nexera UFPLC

Yusuke Masuda and Masataka Nikko

User Benefits

- Fully automated purification process (Fractionation, purification, concentration, recovery, etc.) significantly reduces the operator workload and improves the overall analytical efficiency.
- Target compounds can be recovered after removing the salt contained in the mobile phase solvent.
- The time required for the drying process of the fraction can be reduced because the fraction with a high content of organic solvent can be recovered.

■ Introduction

Preparation and purification by liquid chromatography is a widely-used technique in the pharmaceutical, food, and chemical industries for drug synthesis, finding effective compounds in natural products, and for structural analysis of unknown trace compounds. Nexera UFPLC*1 enables substantial labor savings in preparative purification by automating not only the fractionation of the target compound but also related processes such as concentration, purification, and recovery. This article describes an example of preparative purification of a mixed sample of the pharmaceutical ibuprofen and its analogues using Nexera UFPLC (Fig. 1).

*1 UFPLC: Ultra Fast Preparative and Purification Liquid Chromatograph

Fig. 1 Ultra Fast Preparative and Purification LC system Nexera™ UFPLC

■ Preparative Purification by UFPLC

UFPLC automatically performs the various processes related to preparative isolation of target compounds using a combination of preparative LC and trapping columns. The details of those processes are as follows.

1. Separate target compounds in a complex sample by preparative LC and introduce them into trapping columns (fractionation and concentration)
2. Wash the trapping columns online to remove impurities and counter ions (purification)
3. Elute target compounds from trapping columns using organic solvent (elution)

An outline of the respective processes is shown in Fig. 2, and a simplified flow chart of UFPLC is shown in Fig. 3.

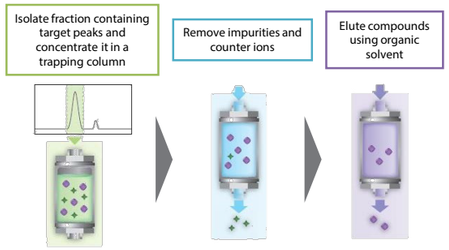


Fig. 2 Flow of Fractionation, Concentration, Purification, and Elution by UFPLC

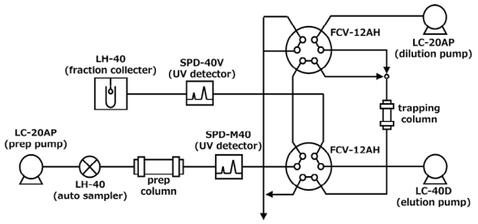


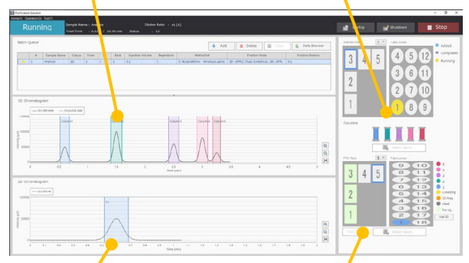
Fig. 3 A simplified flow chart of UFPLC
(Display the flow path while introducing the target compound into the trapping column)

■ UFPLC Dedicated Software Purification Solution™

For preparation and purification by UFPLC, the dedicated Purification Solution software is used. Purification Solution facilitates the entire process, from setting the preparative conditions to the liquid recovery of the target compound on a simple user interface. The Purification Solution operation screen is shown in Fig. 4. The chromatogram of preparative separation, the trapping column into which fractions were introduced, and the elution chromatogram after purification are displayed on a single screen. It allows the user to confirm the entire process of preparative purification for the target compounds at a glance.

Preparative LC Chromatogram
The peaks corresponding to each fraction are color-coded

Trapping Column
Trapping column is displayed in the same color as fractionated peak



Elution Chromatogram
Color-coded display of the area collected in the fraction collector in the same color as the trapping column

Fraction Collector
Shows location of final recovery solution on fraction collector

Fig. 4 Purification Solution™ Operation Screen

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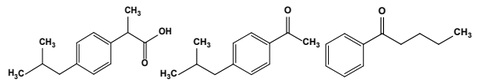
■ Preparative Purification of Ibuprofen and its Analogues

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) used as a fever-reducing drug and analgesic. The United States Pharmacopeia (USP) provides analytical methods for ibuprofen and its analogue, 4-isobutylacetophenone, using valerophenone as an internal standard. Each compound was separately purified to high purity from a mixed solution of these three compounds using Nexera UFPLC.

Fig. 5 shows the structural formulas of ibuprofen and its analogues, and Table 1 shows the preparative purification conditions. The mixed solution was prepared by dissolving the three target compounds in the mobile phase to adjust the content of each compound to 10 mg/mL.



Ibuprofen　4-Isobutylacetophenone　Valerophenone

Fig. 5 Structural Formulas of Ibuprofen and its Analogues

Table 1 Preparative and Purification LC

System:	Nexera UFPLC
Preparative LC Conditions	
Column:	Shim-pack™ Scepter C18-120*1 (150 mm × 20 mm I.D., 5 µm)
Mobile Phase:	A) 1 % (wt/v) Chloroacetic acid in water (pH3*2) B) Acetonitrile A/B = 40 : 60 (v/v)
Flowrate:	20 mL/min
Column Temp.:	Ambient
Injection Vol.:	200 µL (Fig. 5) 2000 µL (Fig. 8)
Detection:	230 nm (SPD-M40, High pressure preparative flow cell)
Rinsing Conditions	
Column:	Shim-pack UFPLC 20×30*3 (30 mm × 20 mm I.D., 20-30 µm)
Rinse Solvent:	A) Acetonitrile/water = 2 : 98 (v/v) B) 0.2 % Formic acid in water C) Water
Time Program:	A 40 mL/min (0.01-3 min) → B 15 mL/min (3.01-11 min) → C 40 mL/min (11.01-15.9 min)
Elution Conditions	
Eluent:	Acetonitrile
Flowrate:	9 mL/min
Detection:	230 nm (SPD-40V, Preparative flow cell)

*1 P/N: 227-31102-03　*2 pH 3.0 adjusted with ammonium hydroxide
*3 P/N: 228-80220-41

■ Verification of Purity of Ibuprofen and its Analogues

Fig. 6 shows the preparative LC chromatogram of the mixed solution. The injection volume was 200 µL. The fractions of ibuprofen, valerophenone and 4-isobutylacetophenone collected by Nexera UFPLC were analyzed by Nexera XR to verify the purity of the compounds. Table 2 shows the analytical conditions, and Fig. 7 shows the chromatograms. Table 3 shows the purities of the target compounds in each fraction by area percentage (peak detection range: 2.5-15 min).

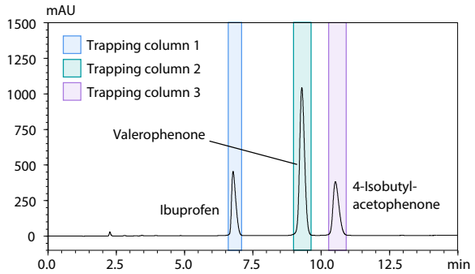


Fig. 6 Preparative LC Chromatogram of Ibuprofen and its Analogues (Nexera UFPLC)

Table 2 Analytical Conditions for Purity Verification

System:	Nexera XR*1
Column:	Shim-pack Scepter C18-120*2 (150 mm × 4.6 mm I.D., 5 µm)
Mobile Phase:	A) 1 % (wt/v) Chloroacetic acid in water (pH 3*3) B) Acetonitrile A/B = 40 : 60 (v/v)
Flowrate:	0.8 mL/min
Column Temp.:	30 °C
Injection Vol.:	10 µL
Detection:	230 nm (SPD-40V, Standard flow cell)

*1 600 mm × 0.3 mm I.D. tubing was used to connect the SIL-40C XR autosampler to the column inlet
*2 P/N: 227-31020-05
*3 pH 3.0 adjusted with ammonium hydroxide

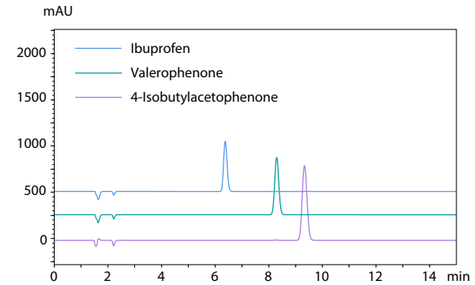


Fig. 7 Chromatograms of Fraction Obtained by Nexera UFPLC (Nexera XR)

Table 3 Purities of Target Compounds Contained in Collected Fractions (Area Percentage, Peak detection range : 2.5-15 min)

Compound	Area %
Ibuprofen	99.5
Valerophenone	99.8
4-Isobutylacetophenone	99.6

■ Concentration and Drying of Ibuprofen Fraction

When a complex sample is separated and collected by preparative LC, the solvent may be dried in order to use the collected fraction in the next step. However, the fraction obtained in reversed-phase mode contains water, so drying takes a long time. Moreover, if the mobile phase contains non-volatile compounds, these compounds may be precipitated together with the target compound during drying, resulting in a decrease in the purity of the recovered target compound. In preparative purification using UFPLC, the fraction containing the target compound is introduced and concentrated into a trapping column and then purified on the trapping column by washing the mobile phase components during preparation process. In addition, the drying time is shortened substantially due to the use of an organic solvent for sample recovery from the trapping columns.

UFPLC can also concentrate target compounds in a trapping column by repeatedly injecting the sample and introducing the fraction containing the target compound into the same trapping column. The trapping column used in this paper, Shim-pack UFPLC 20×30, has a maximum loading capacity of 100 mg.

The concentration, purification, and recovery processes can be carried out efficiently by introducing the target compound repeatedly into this trapping column, even if the concentration of the target compound in the analysis sample solution is low.

Here, after injecting 2000 µL of the mixed solution and separating ibuprofen by preparative LC under the conditions shown in Table 1, the ibuprofen fraction was introduced into a Shim-pack UFPLC 20×30. The procedure was repeated 5 times, and after concentrating and purifying the sample in the trapping column, the target compound was eluted with an organic solvent (sample load to the trapping column: 100 mg). The preparative purification flow is shown in Fig. 8, and the preparative chromatogram of the mixed solution is shown in Fig. 9.

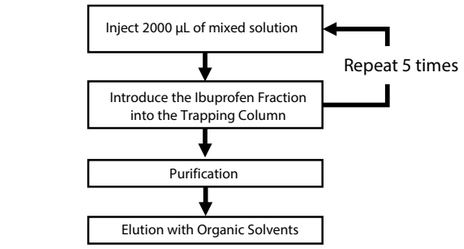


Fig. 8 Flow of Recovery of High Concentration Fraction of Ibuprofen

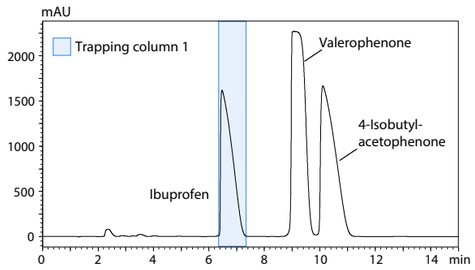


Fig. 9 Preparative Chromatogram of Ibuprofen (2000 µL Injection, Nexera UFPLC)

To confirm the reduction in drying time, we compared the time taken to dry the ibuprofen fractions collected from the standard preparative LC and Nexera UFPLC using a centrifugal concentrator. The comparison results are shown in Table 4. Drying of the standard preparative LC fraction required approximately 260 min, whereas the UFPLC fraction was dried in about 120 min. When the respective dried yields were checked, compounds derived from the mobile phase were found in the standard preparative fraction. Consequently, it could not be used as-is for the next step. In the case of the UFPLC fraction, just ibuprofen was confirmed after drying due to purification on the trapping column (Fig. 10).

Table 4 Comparison of Fractions of Standard Preparative LC and Nexera UFPLC

Method	Sample loading amount (mg)	Drying time (min)
Standard Preparative LC	20	260
Nexera UFPLC	100*1	120

*1 Introducing a Fraction of Ibuprofen into the Trapping Column was repeated 5 times

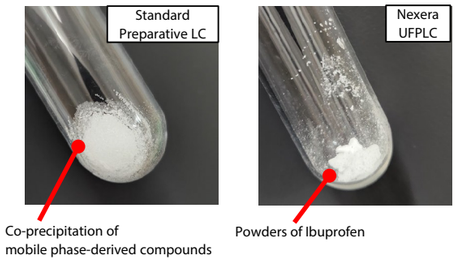


Fig. 10 Conditions of Ibuprofen Fractions after Drying

■ Conclusion

This article introduced an example of preparative purification of a mixed sample of the pharmaceutical ibuprofen and its analogues by using UFPLC. By concentrating, purifying, and recovering the fractionation with UFPLC, the drying time of the highly concentrated fraction of ibuprofen was reduced to about half compared with standard preparative LC.

Automating the concentration and purification processes online, enables labor and time savings in the preparative purification process.

Moreover, the Nexera UFPLC is extremely flexible and can be used for both standard preparative LC and UFPLC.

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Application News

Software for Efficient Method Development "LabSolutions™MD"
Preparative Purification Liquid Chromatograph "Nexera™Prep / LH-40 / LCMS-2050"

Efficient Preparative Purification Workflow of Synthetic Peptide Using Analytical/Preparative Switching LC-MS System

Yuki Suzuki, Yusuke Masuda

User Benefits

- A multi-step seamless preparative purification workflow of synthetic peptides can be performed within a single LC-MS system setup.
- LabSolutions MD can provide easy investigation for optimal separation conditions for target compounds.
- Target compounds can be purified from crude samples with high purity and high recovery based on the excellent identification capability of LCMS-2050.

■ Introduction

In the 2000s, biopharmaceuticals such as antibody drugs have emerged but there are many challenges to overcome because of their manufacturing process using genetic technology. Consequently, middle molecule drugs are paid attention, peptide therapeutics, one of middle molecule drugs which have advantages of being manufactured at low cost, easily taken into cells because of their small molecular weight, and prevented from degradation by adopting a specific three-dimensional structure when they are taken into human body. Since such peptides are produced by chemical synthesis like small molecule drugs, it is essential to purify, fractionate, and confirm the purity of the final synthesized product. In this article, we present a case study of multi-step seamless preparative purification workflow (optimizing separation conditions, scaling-up, fractionation, and confirming purity/recovery) of a peptide using preparative purification liquid chromatograph Nexera Prep (Fig. 1) based on Application News 01-00650-EN and 01-00651-EN.

Fig.1 System setup of Nexera™ Prep

■ Overview of analytical/preparative switching LC-MS

In this article, an analytical/preparative switching LC-MS system equipped with both analytical and preparative flow paths was used. The analytical flow path was used to evaluate separation conditions, loadability, and purity/recovery, while the preparative flow path was used for fractionation of a target peptide. LCMS-2050 provides not only mass information of target compounds when optimizing separation conditions but can also be effectively used for sample collection (MS triggered fraction collection). Therefore, target compounds can be recovered with high purity. Refer to Application News 01-00650-EN for detailed information.

■ Optimization of separation conditions in analytical scale

Separation conditions for a crude synthetic sample containing the target synthetic peptide (parathormone (1-34): PTH) were investigated in an analytical scale. Fig. 2 ① shows a UV chromatogram (analytical conditions: Table 1) of the sample before separation conditions were optimized. Under these conditions, the separation between PTH and co-existing impurities was not sufficient, and increased loading amount would deteriorate the separation from impurities furthermore, so improving the separation was essential to recover PTH with high purity.

LabSolutions MD, which enables comprehensive investigation for HPLC separation conditions using different parameter settings (twenty-five patterns of gradient profiles and five patterns of combination of initial and final concentrations of organic solvent) was employed for this study.

The best result for the separation of PTH (blue arrow) and impurities in the synthesized crude sample were obtained under conditions of 25% initial concentration and 35% final concentration of organic solvent (Fig. 2 ③).

Table 1 Analytical conditions

Mobile Phase	: Pump A : 0.1% TFA in water Pump B : 0.1% TFA in acetonitrile
Column	: Shim-pack Scepter™ C18-120 (150 mm × 4.6 mm I.D., 5 µm)*1
Sample Concentration	: 2 mg/mL in N-methylpyrrolidone
Injection Volume	: 10 µL
LC Conditions	
Time program (%B)	: B Conc. X%(0 min)→Y%(10 min) →90%(10.01-15 min)→X%(15.01-20 min) X : 10, 15, 20, 25, 30 Y : 30, 35, 40, 45, 50
Column Temp.	: Ambient
Flow rate	: 1 mL/min
Sample loop size	: 500 µL
Syringe size	: 500 µL
Detection (PDA)	: 220 nm (SPD-M40, conventional cell)
MS Conditions	
Ionization	: ESI/APCI (DUIS™), positive mode SCAN (m/z 500-2000)
Nebulizing gas Flow	: 2.0 L/min
Drying gas Flow	: 5.0 L/min
Heating gas Flow	: 7.0 L/min
DL Temp.	: 200 ℃
Desolvation Temp.	: 250 ℃
Interface Voltage	: 0.5 kV

*1 P/N：227-31020-05

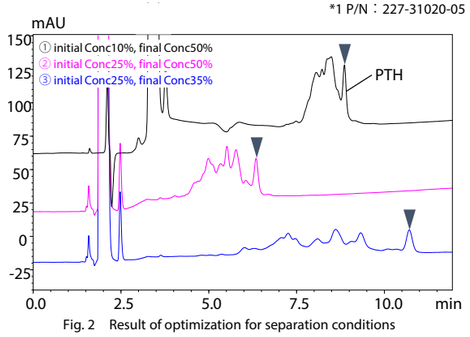

Fig. 2 Result of optimization for separation conditions

■ Evaluation of loading capacity

Under the conditions optimized in analytical scale (Fig. 2 ③), the loading amount was investigated at injection volumes of 5, 10, 20, and 50 µL using a synthetic sample (10 mg/mL) (Fig. 3). Since the separation of PTH did not deteriorate as the injection volume was increased, it was decided to scale up and perform preparative analysis with 50 µL of injection volume.

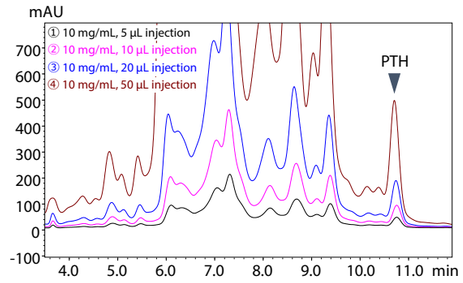

Fig. 3 Result of evaluation for loading amount

■ Fractionation of PTH

PTH was fractionated using UV and MS triggers. The preparative conditions are shown in Table 2 (only parameters different from Table 1 are listed), and the resulting LC chromatogram is shown in Fig. 4 (blue area is the fractionated interval). Based on the ratio of the cross-section area (approximately 20-fold) of the preparative column (20 mm i.d.) to the analytical column (4.6 mm i.d.), the flow rate was scaled up to 20 mL/min (linear velocity was constant before and after scaling-up) and the injection volume to 1 mL.

Similar separation patterns were obtained before and after scaling-up, and separation from impurities was maintained; the combination use of MS and UV triggers resulted in highly selective PTH fractionation.

Table 2 Analytical condition

Column	: Shim-pack Scepter C18-120 (150 mm × 20 mm I.D., 5 µm)*1
Sample Concentration	: 10 mg/mL in N-methylpyrrolidone
Injection Volume	: 1000 µL
LC Conditions	
Flow rate (Prep)	: 20 mL/min
Flow rate (Makeup for MS)	: 1.5 mL/min (0.1% propionic acid in water/methanol = 90/10)
Sample loop size	: 2 mL
Syringe size	: 5 mL
Detection (PDA)	: 220 nm (SPD-40V, preparative cell)

*1 P/N：227-31102-03

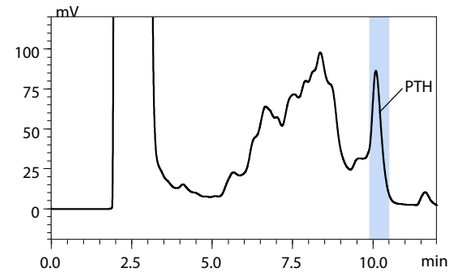

Fig. 4 Preparative chromatogram with UV+MS triggers
*Blue band indicates fractionated interval

■ Confirming purity of collected fraction

Fig. 5 shows the chromatogram obtained by reinjecting the fractionated PTH into the analytical path and the chromatogram of a synthetic sample before fractionation, which was prepared to have the same theoretical concentration as that of collected PTH fraction. Comparison of the chromatograms indicates that the target PTH was successfully purified.

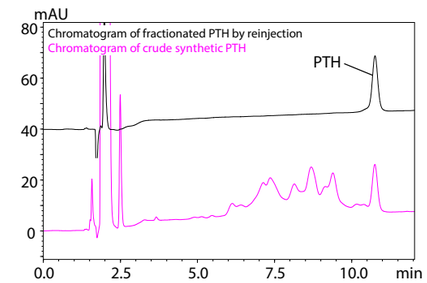

Fig 5 Purity confirmation of fractionated PTH

■ Evaluation of purity and recovery using standard PTH

The preparative performance (purity and recovery) of this system setup was evaluated using a standard solution of angiotensin I. Fig. 6 shows the chromatogram obtained when the fractionated angiotensin I was reinjected into the analytical path and the chromatogram of the standard solution prepared to have the same theoretical concentration as that of the collected angiotensin I fraction. The purity and recovery are shown in Table 3. The purity was 100% in terms of area-normalization, and the recovery calculated from the comparison of peak areas was 97.9%, indicating that the reliable fractionation was able to be performed.

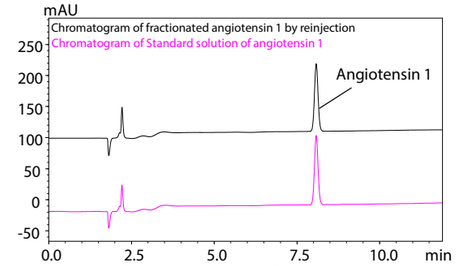

Fig 6 Purity and recovery confirmation of Angiotensin I

Table 3 Purity and recovery of fractionated angiotensin 1

	Purity (area%)	Recovery (%)
Angiotensin I	100.0	97.9

■ Conclusion

The seamless preparative purification workflow can be executed using analytical/preparative switching LC-MS system. In addition, LabSolutions MD can automatically create an analytical batch schedule in which various HPLC parameter settings are examined, resulting in efficient optimization of separation conditions.

Furthermore, LCMS-2050 enables highly selective fractionation of target compounds based on MS trigger. The analytical/preparative switching LC-MS used in this article has both analytical and preparative flow paths, enabling an efficient preparative purification workflow in the preparative purification of peptide including its synthesis confirmation process.

<Related Applications>

1. Seamless Purification Workflow from Analytical to Preparative in Single LC-MS System, 01-00650-EN
2. High Purity Preparative Purification Enabled by UV/MS Trigger on LC-MS System, 01-00651-EN

01-00758-EN First Edition: Jun. 2024

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Application News

Supercritical Fluid Chromatograph – Nexera™ UC

Rapid Extraction of Various Compounds from Natural Products

Kosuke Nakajima[1,2]
[1]Shimadzu Corporation, [2]Shimadzu (Asia Pacific) Pte Ltd.

User Benefits

- Manual operation can be reduced against typical extraction method.
- Automated multiple extractions for many samples can be achieved.
- Organic solvent consumption can be reduced against typical liquid extraction.

■ Introduction

Natural products include various compounds used as drug substances or aromatic oil. Generally, liquid-phase extraction is widely used to extract valuable compounds from natural products. However, it requires a lot of complex operations and time.

This article introduces the application of Supercritical Fluid Extraction (SFE) from natural products by Nexera UC SFE fraction system.

■ Features of Supercritical Fluid Extraction

Supercritical Fluid Extraction (SFE) is one of the extraction methods using supercritical fluid carbon dioxide ($SFCO_2$) as extraction liquid. $SFCO_2$ has unique features that lead to high-throughput and high recovery rate extraction. (Fig. 1) Moreover, the low cost of carbon dioxide (purity: 99.9%) can significantly reduce the total running cost of the extraction phase.

Fig. 1 Unique features of SFE (Supercritical Fluid Extraction)

■ Nexera UC SFE Fraction System

Nexera UC SFE fraction system has an extraction module and fraction collector. （Fig. 2） The extraction module, storing up to 48 extraction vessels, achieves automated multiple extractions for many samples. Fig. 3 shows the flow diagram of the system.

Fig. 2 Nexera UC SFE fraction system

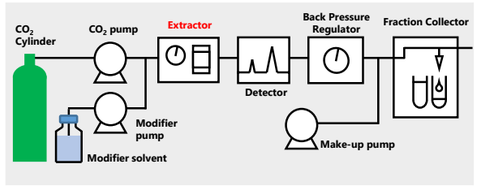

Fig. 3 Flow path diagram for Nexera UC SFE system

■ Extraction Flow

Fig. 4 shows the sample preparation protocol for SFE. The samples are homogenized and packed in a SFE vessel. Nexera UC SFE works with the combination of two types of extraction modes. (Fig. 5) After the extraction phase, $SFCO_2$ delivers the target compounds, and they are collected at fraction collector with make-up solvent.

Nexera UC SFE fraction system achieves high recovery rate collection by new Gas-Liquid Separator (GLS) “Lotus Stream”. Fig. 6 shows GLS working that the liquid is separated appropriately from the CO_2.

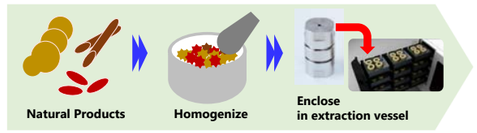

Fig. 4 Pretreatment overview of SFE

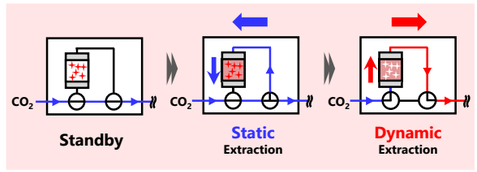

Fig. 5 SFE extraction mode

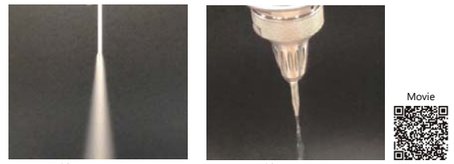

Fig. 6 Gas-Liquid Separator “Lotus Stream”

■ SFE procedure against natural products

We performed SFE extraction against three types of natural products. (Tea leaves, Ginger, Nutmeg) Fig. 7 shows the sample preparation protocol for these natural products. Table 1 shows the extraction conditions, and Fig. 8 shows the extraction results. The PDA detector installed behind the extracting module can confirm the results of the extraction process.

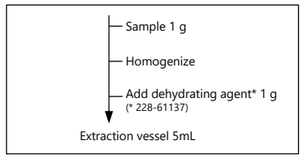

Fig. 7 Sample preparation protocol

Table 1 Extraction conditions

Extraction solvent	: CO_2/Methanol = 7:3
Flow rate	: 5 mL/min
Time program	: Static mode (0-2 min)→Dynamic mode (2.01-7 min) → Wash(7,01-10 min)
Vessel temp.	: 50℃
Back pressure	: 15 MPa
Fraction time	: 2-7 min
Make-up	: 2 mL/min Tetrahydrofuran
Detection	: PDA (250 nm, 280nm, 300 nm), Prep-Cell

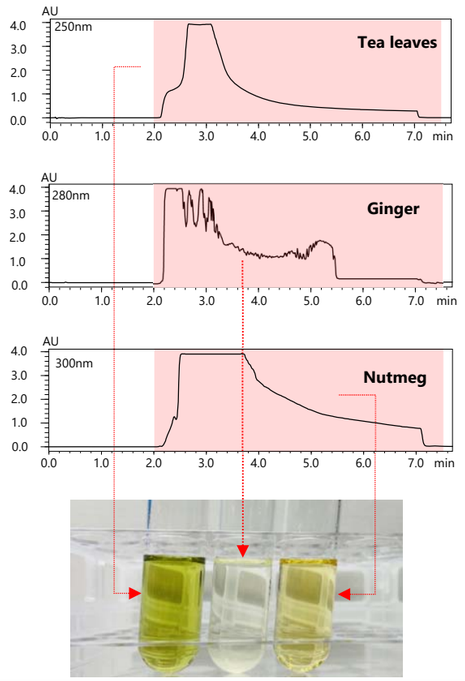

Fig. 8 Extraction chromatograms from natural products

■ Confirmation of extract fractions by analytical HPLC

These obtained fractions were analyzed by analytical HPLC. Table 2 shows the analytical conditions, and Fig. 9 shows obtained chromatograms. As a result, it was possible to find various compounds contained in the SFE fractions from each natural product.

Table 2 Analytical conditions

Column	: Shim-pack™ XR-ODS II*1 (100 mm× 2 mm I.D., 2.2 µm)
Mobile phase	: A: Water B: Acetonitrile
Flow rate	: 0.5 mL/min
Time program	: B conc. 2% (0 min) → 98% (7-8 min) → 2% (8.01-10 min)
Column temp.	: 40℃
Injection vol.	: 1 µL
Vial	: SHIMADZU LabTotal™ for LC 1.5 mL, Glass*2
Detection	: PDA (250 nm, 280 nm, 300 nm)

*1 P/N: 228-41623-92 *2 P/N: 227-34001-01

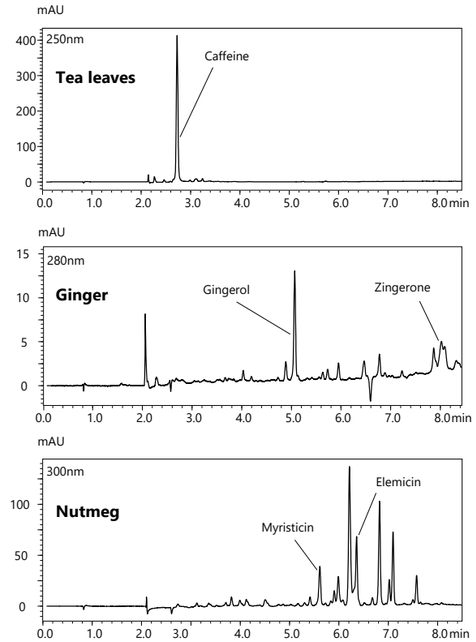

Fig. 9 Analytical chromatograms of extracts from natural products

■ Conclusion

This article introduced the extraction process from natural products by Nexera UC SFE Fraction system. SFE can be expected to provide superior advantages compared to typical liquid extraction in terms of process time length and running cost.

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Application News

HRAM for ID / LCMS™-9030 / LabSolutions Insight Explore™

A Demonstration of HRMS with CE Spread Function for Identification of Cytotoxic Contaminant Present in Marine Bacteria Extract for Natural Product Discovery

Zhaoqi Zhan[1] , Zhe Sun[1], Doralyn S. Dalisay[2], Jonel P. Saludes[2], Jon Andre Gonzalvo[2]
[1] Shimadzu Asia Pacific, Singapore; [2] University of San Agustin, Philippines

User Benefits

- The flexible CE spread feature on LCMS™-9030 Q-TOF enriches the fragment ions of precursors, which enables to enhance the identification reliability of compounds.
- The LabSolutions Insight Explore s/w facilitates the identification workflow by automatically formula prediction, database search and fragment annotation for easy structural elucidation.

■ Introduction

Natural products from marine bacteria are the important source of new therapeutics in drug discovery in recent years. However, raw materials such as crude microbial extracts from where these natural products come from may contain contaminants that can cause harm to human health or give a false positive result in cytotoxicity assays. Hence, detection and identification of bioactivity compounds and contaminant analysis are crucial steps towards resolving suspected contamination of raw materials. Among various analytical instruments and technologies, mass spectrometry with fragmentation has been used to provide evidences in structural elucidation and identification of unknown compounds by GC-MS and LC-HRMS for decades. In this article, an easy-to-use and reliable approach for identification of compounds by LC-Q-TOF is described.

■ Experimental

A Shimadzu LCMS-9030 Q-TOF was employed for the sample analysis. Details of the analytical conditions are compiled in Table 1.

Analysis approach

Two factors are critical for unambiguous structural elucidation and identification of compounds by LC-Q-TOF: the number of fragments obtained in MS/MS and the annotation of fragment ions. The number of fragments of a precursor ion depends on the collision energy (CE). The traditional design of collision cell with a fixed CE during MS/MS measurement generates usually lesser number of fragments, which is a main obstacle in structural analysis. An ultra fast CE spread feature is introduced on the LCMS-9030, which can generate more fragments due to a wider range of CE applied on each MS/MS measurement. For tedious fragment annotation, the LabSolutions Insight Explore software enables fully automated assigning of the mass peaks via *in-silico* fragmentation. An easy data analysis approach as illustrated in Figure 1 involves obtaining formula from accurate mass of a precursor (< 1ppm), public database search (PubChem or ChemSpider) and fragment assigning (peak annotation) [1].

Table 1 Analytical conditions

LC Conditions	
Column	Shim-pack™ GIST C18 (2.1 X 100 mm, 2 µm)
Flow Rate	0.4 mL/min
Mobile Phase	A: Water with 0.1% formic acid B: Methanol with 0.1% formic acid
Elution mode	Isocratic 80% B – 20% A
Oven Temp.	40°C
Injection Vol.	1 µL
Interface Conditions (LCMS-9030)	
Interface	HESI 4.5 kV
Interface Temp.	300°C
DL Temp.	250°C
Heat Block Temp.	400°C
Nebulizing Gas Flow	2 L/min
Heating Gas Flow	10 L/min
Drying Gas Flow	10 L/min
Data acquisition (Q-TOF)	
MS mode (TOF)	Positive, *m/z* 100-600
MS/MS (Q-TOF)	6 precursors, *m/z* 50-500, CE: -25V spread (+/-)17V
Dwell time	0.05 sec / event
Loop time	0.35 sec / data point

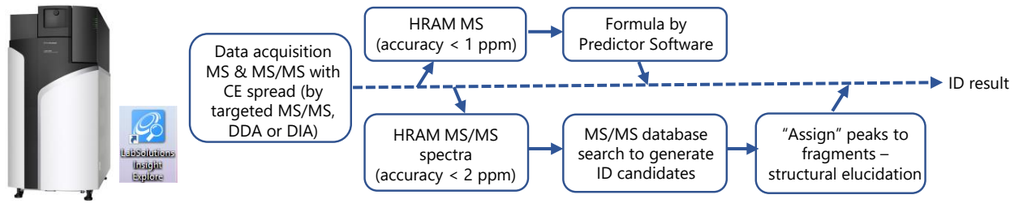

Figure 1 LCMS™-9030 with LabSolutions Insight Explore for identification & structural elucidation of unknown compounds

■ Results and Discussion

Results of MS and MS/MS of extract sample

An HPLC purified extract from marine bacteria (MBE) prepared in University of San Agustin [2] was used in this study. The sample contains a main compound and small amounts of other components as shown in MS TIC in Figure 2. The major compound P3 and the three minor components P1, P2 and P4 were detected in MS TIC firmly. The main ions of every peak were selected as the targeted precursors for MS/MS measurement. Details of the MS/MS parameters are shown in Table 2.

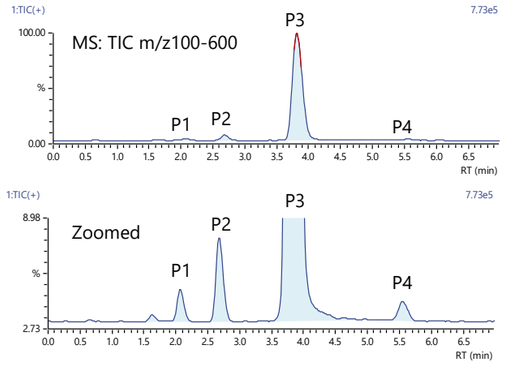

Figure 2 TIC of MBE sample on LCMS-9030

Table 2 MS & MS/MS parameters with CE spread settings

Event#	Type (+)	Precursor Ion (*m/z*)	TOF (*m/z*)	Targeted peak	CE & CE Spread
1	MS	N.A	100-600	All	N.A.
2	MS/MS	361.1861	50-500	P1	25±17
3	MS/MS	361.2223	50-500	P2	25±17
4	MS/MS	403.2338	50-500	P3	25±17
5	MS/MS	329.1598	50-500	P3	25±17
6	MS/MS	343.2117	50-500	P4	25±17
7	MS/MS	269.1382	50-500	P4	25±17

Prediction of Empirical formula

The mass accuracy of the TIC MS measurement was well maintained within 1 ppm with external standard mass calibration method [3]. The exact mass of the measured elution peaks as shown in Figure 2 were applied to the "Formula Predictor", which generates candidates of molecular formula with element settings including H, C, O, N, P and S. The formula (with highest score) that are most likely to indicate each component are listed in Table 3. As illustrated in Figure 1, these formulas are used as references to confirm the subsequent identification via MS/MS database search.

MS/MS spectra obtained with CE spread

In MS/MS measurements on Q-TOF, the fragmentation pattern of precursor ion depends on the CE value and other relevant factors such as collision gas and design of the collision cell. The most important variable factor is the CE voltage, which determines the numbers of fragments and their intensity. An ultrafast CE spread feature available on the LCMS-9030 allows to set range of CE for every MS/MS measurements. In this analysis method, CE of 25 V and CE spread of (+/-)17 V were used, which corresponds to an ultrafast ramping of CE from 8 V to 42 V for every collision process. As a result, more fragments that require different CE are obtained and combined into the same MS/MS spectrum. This CE spread collision (Table 2) has obvious advantages over the fixed CE instrument in qualitative analysis of unknown compounds because of more structural information being revealed. As can be seen in Figure 3, the MS/MS spectrum of P3 precursor ion (403.23) shows numerous fragments under the CE spread conditions (8~42 V). It is worth to note that the spectrum pattern can be optimized through settings of appropriate CE and CE spread values.

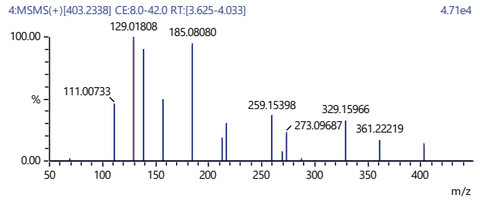

Figure 3 MS/MS spectrum of P3 main precursor (*m/z* 403.23) with CE spread setting of 8~42 V

Identification of compounds via database search and structural elucidation

The MS/MS spectrum of a targeted precursor contains structural information carried by the fragments and their accurate masses. The LabSolutions Insight Explore program has the function to send the MS/MS spectrum to database search such as ChemSpider or PubChem generating possible identification results with matching scores (Table 3, right portion). For each MS/MS database search, several structures are usually generated and listed down as candidates. In general, a higher Assign score indicates the higher probability for the best

Table 3 Summary of identification results of four components in MBE sample by HRAM on LCMS-9030 (error <1 ppm)

Peak	MS (TOF) Spectrum (error <1 ppm)						MS/MS TOF Spectra (error <2 ppm)		
	RT (min)	Area (%)	Measured [M+H]+ (*m/z*)	Formula	Diff. (ppm)	DBE*	No. of fragments	ID Result via DB search (ChemSpider)	Assign score (%)
P1	2.064	1.7	361.1858	$C_{17}H_{28}O_8$	0.415	4	11	Triisopropyl 2-acetoxy-1,2,3-propanetricarboxylate	100
P2	2.674	3.4	361.2223	$C_{18}H_{32}O_7$	0.712	3	8	Tributyl citrate	92.3
P3 (main)	3.809	94.4	403.2328	$C_{20}H_{34}O_8$	0.486	4	11	Tributyl acetyl citrate	91.7
P4	5.546	1.1	343.2116	$C_{18}H_{30}O_6$	0.162	4	5	Tributyl (E)-aconitate	87.5

** DBE: double bond equivalence*

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Table 4 Annotation of fragment ions of P3 precursor using the Assign function in LabSolutions Insight Explore

#	m/z	Int. (%)	Formulae (M)	Charge	+/- ppm
0	403.2328	13.2	(precursor)		0.4
1	361.2222	15.8	C18H33O7	[+H]+	0.3
2	329.1597	34.5	C16H25O7	+	0.5
3	273.0969	21.4	C12H17O7	+	0.0
4	269.1383	6.9	C14H21O5	[+H]+	0.0
5	259.1540	34.3	C13H23O5	+	-0.1
6	217.0343	26.6	C8H9O7	+	-0.1
7	213.0757	17.2	C10H13O5	+	-0.3
8	185.0808	96.5	C9H13O4	[+H]+	-0.2
9	157.0129	43.2	C6H5O5	+	-1.6
10	139.0024	85.2	C6H3O4	+	-1.0
11	129.0181	100.0	C5H5O4	[+H]+	-1.3
12	111.0073	39.4	C5H3O3	+	-3.0

matching structure if the mass errors of the fragments are sufficiently small (e.g., < 2 ppm). The most-likely ID results of the four precursors of P1, P2, P3 and P4 are shown in Table 3 (right portion). It is worth to note that the identification result by MS/MS database search must be in accordance with the empirical formula obtained from formula predictor using the MS spectrum (Table 3, left portion).

Furthermore, the Assign function of the program annotates automatically all the fragment ions of every candidate to interpret the corresponding structure. The fragment annotations for P3 precursor (m/z 403.2328) are shown in Table 4 and few selected fragments are shown in Figure 4. This *in-silico* fragmentation provides an easy tool for the structural elucidation which is usually a very tedious and skill-required process.

The identification result for the main component (P3) by the current approach is tributyl acetyl citrate ($C_{20}H_{34}O_8$), which is supported by ^1H and ^{13}C NMR analysis [2]. However, the ID results for the low abundant P1, P2 and P4 are reported for reference. As shown in Figure 5, the P2 and P4 are likely the structural analogues of the main compound P3. The accurate masses of P1 and P2 are very closed (m/z 361.1858 and m/z 361.2223). However, their structures are substantially different.

■ Conclusion

An easy approach for impurity identification by HRAM on LCMS-9030 is demonstrated with an extract from marine bacteria (MBE). The flexible CE spread feature allows MS/MS producing more fragments, which is critical for ID and structural elucidation. The LabSolutions Insight Explore provides a powerful tool to facilitate the data analysis from formula prediction on MS (<1 ppm) to MS/MS (<2 ppm) database search and fragment annotations for structural elucidation.

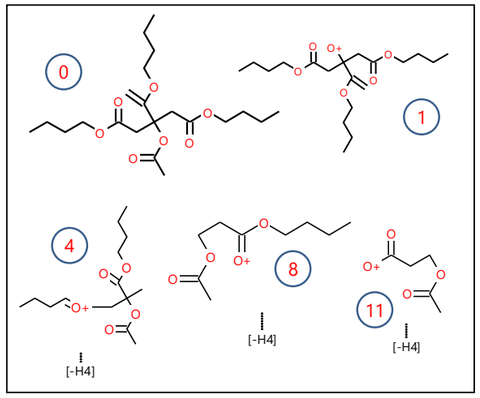

Figure 4 Representative fragment ions interpreted by the Assign function (structure # refers to Table 4)

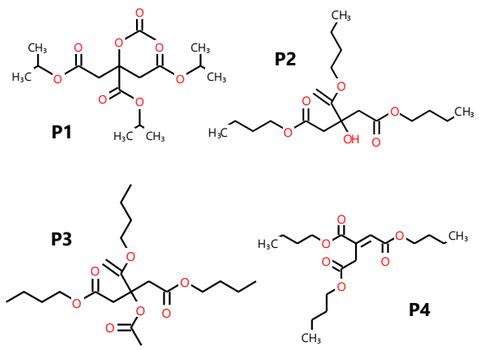

Figure 5 Identification results of MBE sample. P3 is confirmed as tributyl acetyl citrate. (Refer to Table 3 for others)

■ References

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■ Acknowledgement

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Liquid Chromatograph Mass Spectrometer LCMS™-9030

Application News

Data-Dependent Analysis Approach in LC/HRMS: Annotation of Natural Product Components

Udi Jumhawan[1], Chua Chun Kiang[1], Chia Shao Hua[1], Iida Tetsuo[2]
[1]Shimadzu (Asia Pacific) Pte Ltd, [2]Shimadzu Corporation Japan

User Benefits

- LCMS-9030 Q-TOF provides high quality and high purity MS/MS spectra by employing data-dependent analysis approach for component annotation of natural product
- Combination of Shimadzu's powerhouse software, LabSolutions™ LCMS and LabSolutions Insight Explore™, demonstrates efficient data acquisition and processing workflow of complex HRMS data

■ Introduction

LC/HRMS is currently the most prominent analytical tool for untargeted analysis. Combination of soft ionization technique and versatile mass analyser, working in tandem or hybrid configuration, enables aid for component annotation by providing highly-resolved and accurate MS/MS spectra.

Resourceful data acquisition approaches have been developed to further facilitate a more time-efficient for data collection including data-dependent analysis (DDA) and data-independent analysis (DIA). Both approaches are commonly used and proved to be complementary. DDA approach has been recognized for providing a higher purity MS/MS spectrum. It includes survey scan followed by automated fragmentation for precursor ions above a pre-set abundance threshold.

Chemists have made tremendous efforts to annotate components in natural products especially herbal medicines. These components may exhibit therapeutic effects and thus it is important to carry out complete characterization. In this article, a simplified DDA-based LC/HRMS workflow was demonstrated for component annotation of herbal medicine, *Aconitum carmichaeli.*

■ Measurement Conditions and Samples

A 500 mg of *Aconitum carmichaeli* was cut into small pieces and vortexed in 70% methanol for 30 mins. The extract was filtered using 0.22 µm PTFE filter and subsequently injected into LCMS-9030 Q-TOF (Fig. 1).

Analysis was performed on LCMS-9030 Q-TOF. Analytical conditions (liquid chromatography and mass spectrometry) are described in Table 1. Data acquisition and processing were carried out by using LabSolutions LCMS and LabSolutions Insight Explore, respectively.

Fig. 1 LCMS™-9030 Q-TOF

Table 1 Analytical conditions for analysis of natural product by LCMS-9030

Column	C18 column (100 mm x 2.1 mm x 2.7 µm)
Mobile phase	A : 0.1% formic acid in water B : acetonitrile
Gradient program	30 min gradient program
Flow rate	0.4 mL/min
Oven temperature	40˚C
Injection volume	10 µL
Interface	Heated ESI
MS mode	Full scan (MS^1) and DDA, positive
Mass range	100-1000 *m/z*
CE spread	15-55 eV
No. of dependent events	20
Heat block temperature	400˚C
DL temperature	250˚C
Interface temperature	300˚C
Nebulizing gas	N_2, 3 L/min
Drying gas	N_2, 10 L/min
Heating gas	Zero air, 10 L/min

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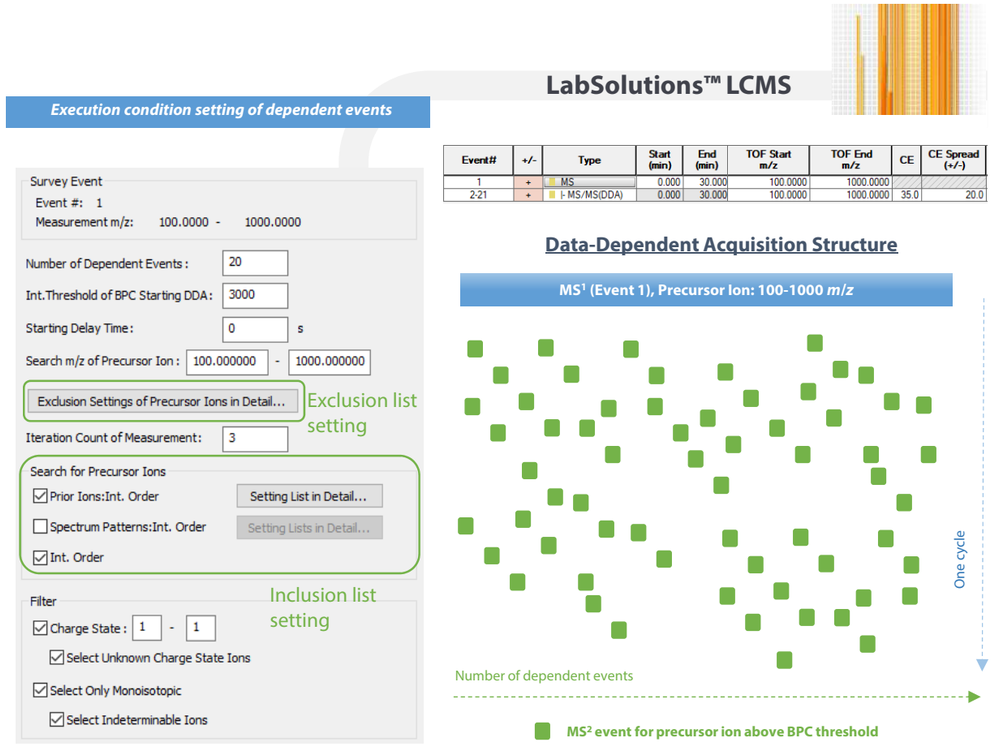


Fig. 2 LabSolutions™ LCMS software dialog and the structure of DDA data collection mode for LCMS-9030 Q-TOF

■ Results and Discussion

DDA Data Collection

DDA data acquisition was controlled by the LabSolutions LCMS software. Generic data collection was established using TOF survey scan (MS^1) ranged from 100 to 1000 *m/z*. Base peak chromatogram (BPC) intensity threshold (BPC > 3000) was applied to execute MS/MS fragmentation using collision energy spread (15-55 eV). Twenty dependent (MS/MS) events were set to allow sufficient MS/MS data collection (Fig.2). Ion exclusion and inclusion settings are available in the LabSolutions LCMS software dialog to automatically exclude background ions and include ions of interest, respectively.

DDA Data Analysis

Analysis of DDA data is very straightforward. Since MS/MS data is automatically executed based on intensity threshold, data deconvolution is not needed. In comparison to DIA approach, DDA method should enable high quality and high purity MS/MS spectra. LabSolutions Insight Explore was utilized for data processing from component detection to library searching and *in-silico* fragmentation (Fig.3).

Component detection was carried out using the Analyze module. Components or precursor ions are detected by searching every scan for ions that behave as a chromatographic peak with a take-off, apex and landing. Ion filtering is then conducted based on peak width, peak response and signal-to-noise ratio.

Detected precursor ions can be compared against suspect screening list to expedite identification workflow. Prediction of chemical formula and overview of isotopic pattern by the Formula Predictor module will provide visual confirmation. Isotopic pattern of the acquired MS^1 data highly matched to that of theoretical pattern (Iso Score: 99.75). Iso score represents how well the masses of the isotopic peaks match the theoretical isotopic pattern.

Both precursor and fragment (MS/MS) ions are used for library searching in the Library Search module (under Edit function) against local library or public domains such as PubChem and ChemSpider through the Assign module. The Assign module is designed to perform *in-silico* fragmentation of detected component based on the acquired MS/MS data and the MOL file from public libraries. This will improve confidence level of component annotation.

LabSolutions Insight Explore™

Analyze

- **Component Detection**
  - The **Analyze** module automatically performs component detection (based on MS^1 or MS/MS)

Detected Components

RT	m/z	Response	Precursor Ion
7.965	616.31248	12126	616.31248
12.412	177.12709	12132	177.12709
12.387	195.13796	12196	195.13796
12.387	195.13796	12196	195.13796

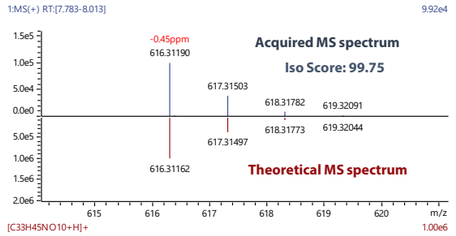
Screening Targets

Target Name	Target Formula	Target m/z
Available		
Hypaconitine	C33H45NO10	616.31218
2,2',4'-trimethylpropiophenone	C12H16O	177.12795
Cnidilide	C12H18O2	195.13851
Neocnidilide	C12H18O2	195.13851

- **Suspect Screening**
  - Screening against a target list enables quick detection of suspected targets

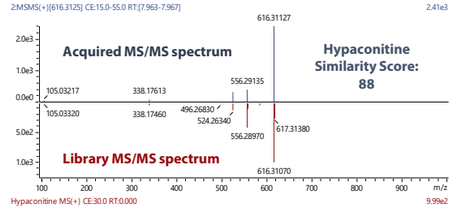
Formula Predictor

- **Isotope Pattern**
  - The **Formula Predictor** module provides scores as a metric to shortlist the correct chemical formula for unknown compounds
  - Mirror plot displaying acquired and theoretical isotopic patterns provides fast visual confirmation



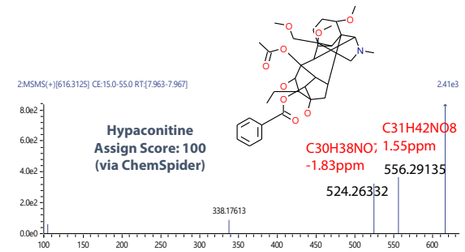
Library Search

- **Local Library Search**
  - The **Library Search** module enables matching of MS/MS spectrum to a curated local library for identification of unknown compounds



Assign

- **Public Library Search**
  - The **Assign** module enables searching and scoring against public libraries such as PubChem and ChemSpider



- ***In-silico* Fragmentation**
  - Assign scores are provided based on *in-silico* fragmentation analysis on the MOL files automatically retrieved from public libraries to help analysts annotate unknown compounds

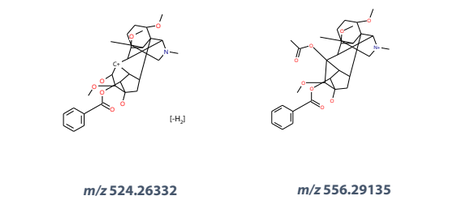


Fig. 3 DDA data processing workflow with LabSolutions Insight Explore™

LabSolutions Insight Explore™

Precursor

- **Precursor**
  - The **Precursor** module populates MS¹ and MS/MS data in a single window
  - Heat map provides overview of precursor ions' intensity distribution
  - It is designed to locate MS/MS spectra data for specific searches

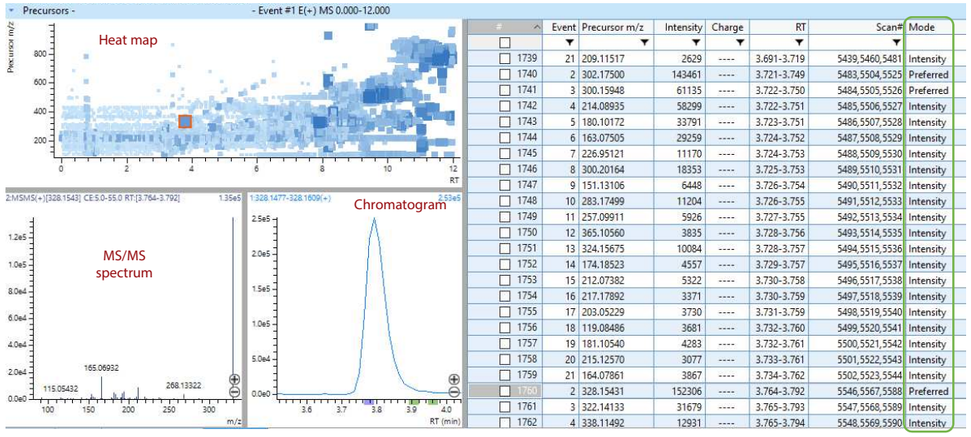


Fig. 4 Precursor module in LabSolutions Insight Explore™

To populate precursor ions and locate specific data for specific search, the Precursor module is available in the Insight™ Explore (Fig.4). It is equipped with heat map to show precursor ion's intensity distribution as well as individual pane for chromatogram and MS/MS spectrum. Especially for DDA data, selection mode of precursor ion will also be displayed in the last column (precursor ions can be selected based on intensity threshold or inclusion/preferred list). Library search and *in-silico* fragmentation can be performed from the Precursor module.

A total of 441 precursor ions were extracted from 7707 components found in the extract of *Aconitum carmichaeli*. Hypaconitine was one of the annotated components. Annotation was carried out based on screening against suspect list as well as local (similarity score: 88) and public databases (Assign score: 100). *In-silico* fragmentation provided substantial affirmation for Hypaconitine annotation.

■ Conclusion

DDA approach provides significant aids for component annotation in natural product studies as it enables high quality and high purity MS/MS spectra. DDA approach would greatly benefit from improvements in data acquisition and data processing. With the effortless performance of LCMS-9030 Q-TOF and simplicity of Insight Explore, Shimadzu offers solution for component annotation and transforms it into a seamless routine work.

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High Performance Liquid Chromatograph Mass Spectrometer
LCMS-2050 and LCMS-9030

Application News

Rapid Screening of Herbal Medicine Using Single Quadrupole LC-MS

Takanari Hattori, Yasuko Shibayama, and Yoshiyuki Watabe

User Benefits

- Rapid screening of herbal medicine can be conducted using single quadrupole LC-MS. Direct injection analysis without separation column enables high-throughput analysis of less than one minute.
- Data analysis of the obtained mass spectrum with eMSTAT Solution™ allows easy classification and characterization of herbal medicine samples, as well as discriminant analysis to determine the group in which an unknown sample should be categorized.

■ Introduction

It is known that the medicinal properties of crude drugs, which are natural products, vary depending on their growing environment, such as the place of origin and weather, and the time of harvest. Therefore, an equivalence evaluation of a new crude drug with a present crude drug is important. Since crude drugs are composed of hundreds to thousands of components, a multifaceted component analysis is required in the chemical evaluation. In recent years, the chemical evaluation of crude drugs has also been conducted using metabolomics technology, which is a comprehensive analysis of metabolites.

In this Application News, we will present a case study of screening of Kampo medicine, so called herbal medicine, using flow injection analysis (FIA) and single quadrupole LC-MS. Although Kampo medicine is manufactured by blending multiple crude drags and has complex matrix, it can be subjected to simple screening analysis for classification and characterization by using a novel method described here.

■ Sample and pretreatment

Six commercially available Kakkonto extract granules were analyzed. 15 mL of 50% methanol aqueous solution was added to 1.5 g of Kakkonto extract granules and stirred for 15 minutes. Ultrasonic extraction was then performed for 15 minutes, and the supernatant was collected by centrifugation (12,000 rpm, 20 minutes). The supernatant was diluted 10-fold with a 50% methanol aqueous solution containing internal standards (5 ppm reserpine, 20 ppm chloramphenicol) and used as the sample for measurement.

■ Instrumentation and analytical conditions

Fig.1 shows the combination setup of Nexera™ series HPLC and LCMS-2050, a single quadrupole LC-MS. It is compact, easy to use, and provides excellent performance. FIA, in which sample is injected directly into the mass spectrometer without analytical column, generally contaminates mass spectrometer frequently, but LCMS-2050 can be applied to FIA because of its robustness and easy maintenance feature in case of contamination recovery. Table 1 shows the analytical conditions for rapid screening.

Fig.1 Nexera™/ LCMS-2050 Setup

Table 1 Analytical Conditions for Rapid Screening

[Flow Injection Conditions] (Nexera XR)		
Flow Rate	:	0.1 mL/min (0 min)→0.05 mL/min (0.1 min) →0.1 mL/min (0.65 min) →1 mL/min (1 min)
Mobile Phase	:	Water/Methanol=50/50
Injection Volume	:	1 µL
[MS Conditions] (LCMS-2050)		
Ionization	:	ESI/APCI (DUIS™), Positive and Negative Mode
Mode	:	Scan (*m/z* 50-2000)
Interface Voltage	:	+3.0 kV / -2.0 kV
Nebulizing Gas Flow	:	2.0 L/min
Drying Gas Flow	:	5.0 L/min
Heating Gas Flow	:	7.0 L/min
Desolvation Temp.	:	450˚C
DL Temp.	:	200˚C

The combination setup of Nexera series HPLC and LCMS-9030, a quadrupole time-of-flight (QTOF) LC-MS, shown in Fig. 2 was employed for detected peak identification. Table 2 shows the analytical conditions for peak identification.

Fig. 2 Nexera/ LCMS-9030 Setup

Table 2 Analytical Conditions for Peak Identification

[Flow Injection Conditions] (Nexera XR)		
Flow Rate	:	0.1 mL/min
Mobile Phase	:	Water/Methanol=50/50
Injection Volume	:	1 µL
[MS Conditions] (LCMS-9030)		
Ionization	:	ESI, Positive or Negative Mode
Mode	:	MS and MS/MS
Nebulizing Gas Flow	:	3.0 L/min
Drying Gas Flow	:	10.0 L/min
Heating Gas Flow	:	10.0 L/min
Interface Temp.	:	300˚C
DL Temp.	:	250˚C
HB Temp.	:	400˚C

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■ Data analysis

The obtained mass spectral data were converted to JCAMP format using LabSolutions™ LCMS and analyzed by eMSTAT Solution, which is equipped with a statistical analysis mode and a discriminant analysis mode, allowing even those that are not so familiar with statistical procedures to easily perform everything from statistical analysis to discriminant analysis (Fig. 3). For each sample, four consecutive analyses were performed to create a data set for multivariate analysis. Data obtained in positive mode were corrected by reserpine, and data obtained in negative mode were corrected by chloramphenicol.

■ Rapid Screening

Scan analysis of the five Kakkonto extracts A-E revealed 91 peaks in the positive mode and 199 peaks in the negative mode. The results of principal component analysis (PCA) of the data obtained in positive mode are shown in Fig. 4. Kakkonto B and E were plotted in proximity, indicating similar characteristics. Kakkonto A was found to have significantly different characteristics compared to the other Kakkonto samples. Fig. 5 shows the results of PCA of the negative mode data. Kakkonto A and C were plotted in proximity, indicating that they had similar characteristics.

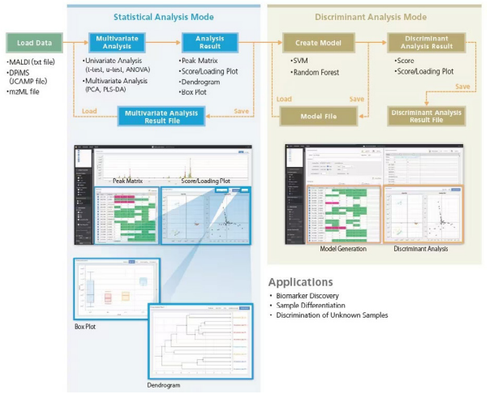


Fig. 3 Workflow on eMSTAT Solution™

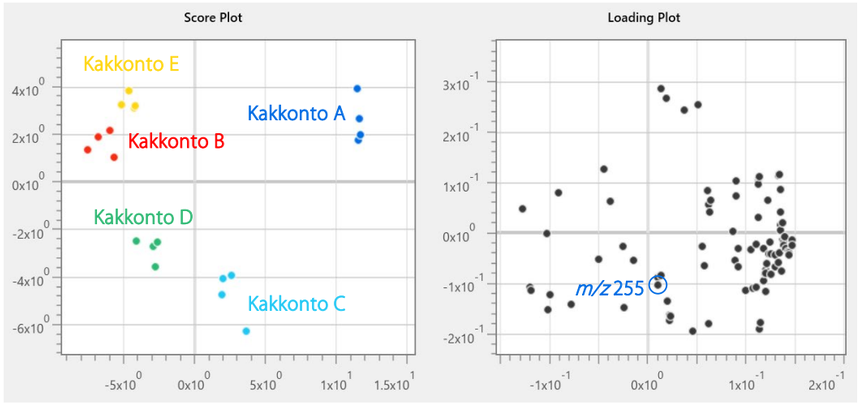


Fig. 4 Results of PCA (Positive Mode)

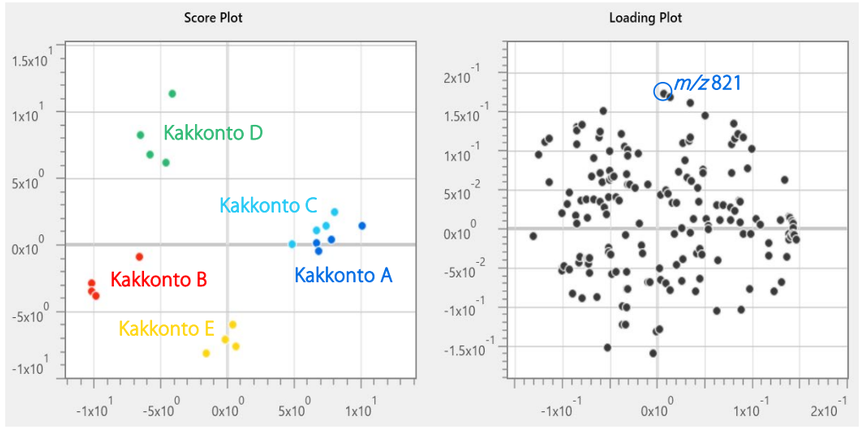


Fig. 5 Results of PCA (Negative Mode)

Kakkonto B and E, which showed similar characteristics in the positive mode PCA, were grouped into one and subjected to PCA again. Based on the results, a discriminant model (algorithm: RandomForest) was created to determine which present Kakkonto-type the unknown Kakkonto belongs to. As a result of discriminant analysis of the separately analyzed Kakkonto F using this discriminant model (Fig. 6), Kakkonto F was plotted in proximity to Kakkonto B and E and was determined to be the type similar to Kakkonto B and E (average score: 88.5).

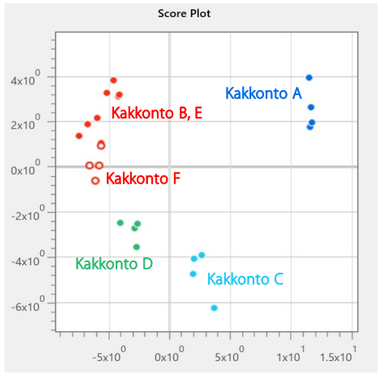


Fig. 6 Results of Discriminant Analysis

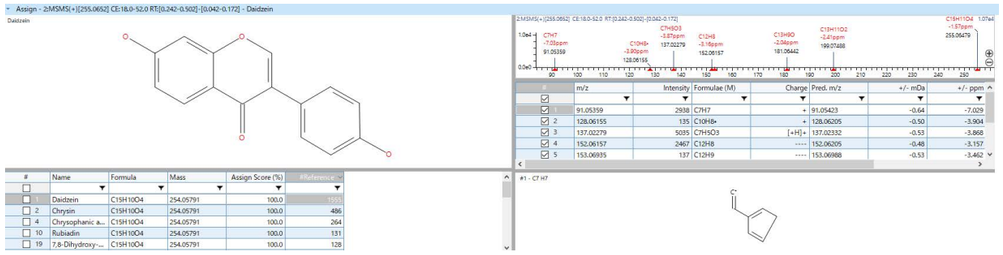
■ Peak Identification by QTOF LC-MS

Among the peaks detected by single quadrupole LC-MS, some peaks were thought to be active componets in Kakkonto. To identify these peaks with high accuracy, MS and MS/MS analyses were performed using QTOF LC-MS. The obtained data were analyzed with the analysis software LabSolutions Insight Explore™, and MS/MS spectral information was searched online based on the ChemSpider database. As a result, as shown in Fig. 7, the peaks at *m/z* 255 and *m/z* 821 detected in positive and negative mode respectively were estimated to be daidzein and glycyrrhizin, for example. The difference between the theoretical *m/z* and measured *m/z* was less than 1 mDa and was able to be estimated with high accuracy.

■ Conclusion

Using Kakkonto extract granules as samples, we introduced a simple screening analysis of Chinese herbal medicine (Kampo medicine). Using FIA and single quadrupole LC-MS, 290 peaks including active medicinal components were detected in the samples, which were able to be classified and characterized by PCA. The results were also used for discriminant analysis to determine which present Kakkonto-type the unknown Kakkonto was in proximity to. This method provides improved efficiency to screening analyses for a large number of Kampo medicines and crude drugs owning to the high throughput performance of one minute analysis time.

• Identification Result of Peak at *m/z* 255 (+)



• Identification Result of Peak at *m/z* 821 (-)

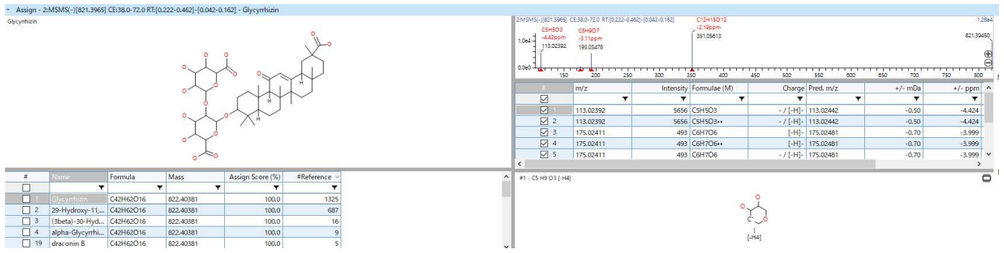


Fig. 7 Identification Results using QTOF LC-MS

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Application News

Liquid Chromatograph Mass Spectrometer LCMS™-9030

Simplifying Natural Product Analysis with Data-Independent Acquisition Workflow

Chua Chun Kiang[1], Udi Jumhawan[1], Chia Shao Hua[1], Doriane Toinon[2]
[1]Shimadzu (Asia Pacific) Pte Ltd., [2]Shimadzu Corporation Japan

User Benefits

- Data-independent acquisition mode on Shimadzu LCMS-9030 Q-TOF enables comprehensive coverage of chemical compounds for natural product research
- LabSolutions™ LCMS simplifies the setup of data-independent acquisition mode
- LabSolutions Insight Explore™ delivers seamless data-processing tools to analyze data-independent acquisition data

■ Introduction

Natural products are intensively studied because they are a rich source of chemical diversity capable of delivering pharmacological benefits. High-resolution mass spectrometry (HRMS) instrument such as quadrupole time-of-flight (Q-TOF) is frequently used to aid the identification of unknown natural products. In addition, natural product chemists are equally concerned with obtaining a full coverage of chemical compounds to reduce false-negative identifications.

The data-independent acquisition (DIA) method performed on the HRMS instrument is thus designed to maximize the unknown compounds coverage and their identification. DIA method acquires MS/MS scans systematically without prior knowledge of target precursor information. Due to the independence between precursors and fragment ions in DIA analysis, it is necessary to have a powerful data analysis solution that allows a rigorous and exploitable analysis of data.

In this article, we will demonstrate the acquisition and data processing workflow solutions for DIA method on Shimadzu LCMS-9030 Q-TOF. A traditional herb sample, *Aconitum carmichaeli*, will be used to illustrate the workflow.

■ Measurement Conditions and Samples

Sample Preparation. The extract of *Aconitum carmichaeli* was prepared by agitating 500 mg of *Aconitum carmichaeli* chunks in 70% methanol for 30 mins. The extract was filtered with a 0.2 µm syringe filter and directly injected into the LCMS-9030 Q-TOF.

Analytical System. Chromatographic analysis was performed using the Shimadzu Nexera™ X2 system equipped with two binary pumps, degasser, autosampler, and a thermostatically controlled column unit. Chromatographic separation was carried out on a Shimadzu Velox C18 column (2.1 mm x 100 mm, 2.7 µm particle size). The column temperature was maintained at 40 °C. The mobile phase were consisted of (A) H_2O with 0.1% formic acid and (B) acetonitrile. The gradient elution program of the mobile phase was 0-8 min (5-40% B), 8-10 min (40% B), 10-17.5 min (40-60% B), 17.5-21.5 min (60-95% B), 21.5-23.5 min (95% B), 23.5-24.5 min (95-5% B), 24.5-30 min (5%B). The flow rate was set at 0.4 mL/min. The sample volume injected was 10 µL.

The mass spectrometer used was LCMS-9030 Q-TOF, a quadrupole time-of-flight system. Data-independent acquisition mode was performed with a precursor ion range of

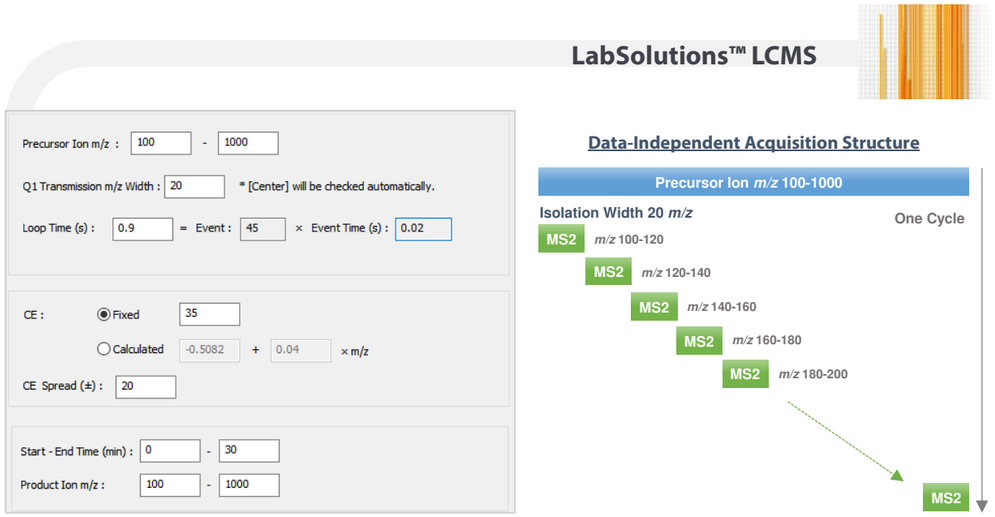


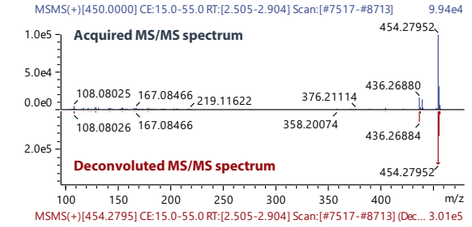
Fig. 1 LabSolutions™ LCMS software dialog for data-independent acquisition mode parameter settings and the structure of DIA data collection for LCMS-9030.

LabSolutions Insight Explore™

Analyze

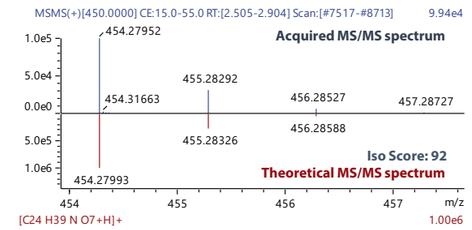
- **Deconvolution**
  - The **Analyze** tool automatically performs component detection and MS/MS deconvolution
  - Fragment ions are correlated to their respective precursor ions
- **Suspect Screening**
  - Screening against a target list enables quick detection of suspected targets

Deconvoluted Components				Screening Targets		
RT	m/z	Response	Precursor Ion	Target Name	Target Formula	Target m/z
2.689	454.27952	2684546	454.27952	Fuziline	C24H39NO7	454.27993
2.780	378.26359	14172	378.26359	Karaconline/aco...	C22H35NO4	378.26389
2.888	438.28464	1444335	438.28464	Bullatine B/10-h...	C24H39NO6	438.28502



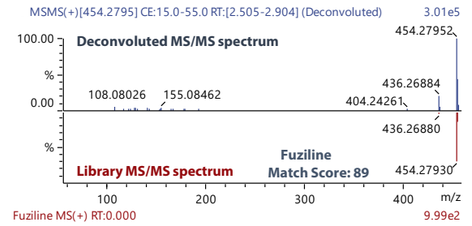
Formula Predictor

- **Isotope Pattern**
  - The **Formula Predictor** tool provides scores as a metric to shortlist the correct chemical formula for unknown compounds
  - Mirror plot displaying acquired and theoretical isotopic patterns provides fast visual confirmation



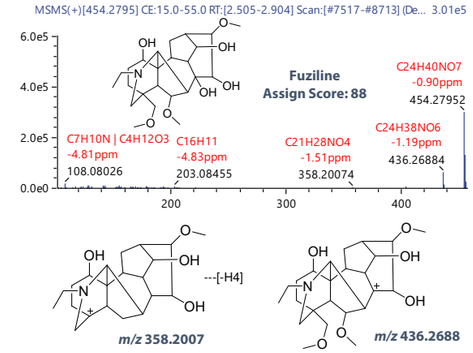
Library Search

- **Local Library Search**
  - The **Library Search** tool enables matching of MS/MS spectrum local libraries for seamless identification of unknown compounds



Assign

- **Public Library Search**
  - The **Assign** tool enables searching and scoring against public libraries such as PubChem and ChemSpider
- ***In-silico* Fragmentation**
  - Assign scores are provided based on *in-silico* fragmentation analysis on the MOL files automatically retrieved from public libraries to help analysts annotate unknown compounds



Scheme 1. DIA data processing workflow with LabSolutions Insight Explore™.

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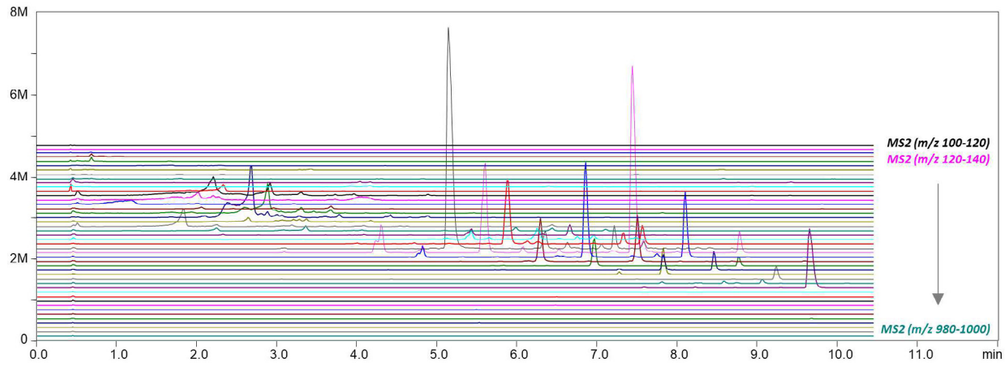

Fig. 2 Total ion chromatograms of DIA-MS/MS events for the analysis of *Aconitum carmichaeli* extract.

m/z 100-1000, an isolation window width of 20 *m/z*, a collision energy spread of 15-55 eV, and a product ion range of *m/z* 100-1000 in positive mode. The conditions of the heated ESI source were as follows: drying gas (N_2) flow rate 10.0 L/min, nebulizing gas (N_2) flow rate 3.0 L/min, heating gas (zero air) flow rate 10.0 L/min, heat block temperature 400 °C, DL temperature 250 °C, and interface temperature 300 °C. Data processing was performed with Shimadzu LabSolutions LCMS v5.99 and LabSolutions Insight Explore software. A suspected screening list containing compound name, accurate mass and chemical formula was used for targeted screening. NIST 2020 MS/MS database entry was used for library match.

■ Results and Discussion

DIA Data Collection

The collection of DIA data is greatly simplified in the LabSolutions™ LCMS v.5.99 software. With the LCMS-9030 Q-TOF, DIA data is collected by isolating and fragmenting a defined mass-to-charge (*m/z*) window and acquiring the MS/MS data within a cycle. Fig. 1 shows the software interface for setting the DIA parameters. The interface systematically guides users to setup precursor ion *m/z* range, isolation window width, expected loop/cycle time, collision energy spread, acquisition time, and product ion *m/z* range. It is noteworthy that high-speed data acquisition up to 100 Hz and wide collision energy spread can be applied for DIA analysis. A generic DIA method was used for the analysis of the *Aconitum carmichaeli* extract. Fig. 2 shows a segment of total ion chromatograms from retention time 0-12 min for DIA-MS/MS events.

DIA Data Processing

The analysis of DIA data is usually more rigorous than conventional MS data analysis. Hence, the LabSolutions Insight Explore software is specifically designed to simplify the qualitative analysis of Q-TOF data. This saves time and avoid a tedious data process.

LabSolutions Insight Explore is consisted of a series of tools like Analyze, Formula Predictor, Library Search, and Assign, which support the qualitative data analysis. The DIA data analysis workflow is shown in Scheme 1.

The Analyze tool incorporates enhanced component detection and deconvolution algorithms for DIA data. The deconvolution process hence re-establishes the link between precursors and fragment ions. Precursor ions of deconvoluted components can then be compared with target list to locate suspected targets. The Formula Predictor tool can be subsequently used to predict chemical formulas for results generated by the Analyze tool. Isotopic scores are calculated for each predicted formula to increase screening confidence. Furthermore, the isotopic pattern of acquired and predicted mass peaks can visually compared. The deconvoluted MS/MS spectra can be matched against a local MS/MS database using the Library Search tool to aid the identification of unknown compounds. In the event that the local MS/MS database has limited entries, the Assign tool can be used to search against public libraries such as PubChem and ChemSpider using accurate mass or chemical formulas. An *in-silico* fragmentation algorithm built within the Assign tool annotates MS/MS fragment ions based on the chemical structure of the suspected compound. It further helps to identify unknown compounds by providing scores on the goodness of fragment ions annotation.

For the analysis of this *Aconitum carmichaeli* extract, a total of 5150 components were detected within the retention time of 0-12 min. 260 unique precursor ions were found. Amongst them, a deconvoluted component with precursor ion of *m/z* 454.2797 was annotated as fuziline, a steroid alkaloid (Scheme 1). It was successfully matched against the NIST 2020 MS/MS database entry with a match score of 89. Furthermore, the Assign tool shortlisted fuziline as possible target with an Assign score of 88. Based on *in-silico* fragmentation calculation, the fragment ions from the deconvoluted MS/MS spectrum could be structurally annotated to support the identification of the unknown component.

■ Conclusion

Natural product research greatly benefits from the availability of DIA mode. This analysis mode increases the coverage of chemical compounds discovered and simplifies the discovery efforts. The LCMS-9030 Q-TOF system enables the analysis of even the most complex natural products. Supported by LabSolutions Insight Explore as a dedicated software solution to deliver data processing solutions for DIA data, the workflow of natural product discovery can be greatly simplified.

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Application News

Software for Efficient Method Development "LabSolutions™ MD"

Efficient Method Development Using Single Quadrupole Mass Spectrometer -Automatic Detection of Co-eluted Peaks-

Shinichi Fujisaki

User Benefits

- LCMS-2050 single quadrupole mass spectrometer provides not only accurate peak tracking based on *m/z* but also automatic detection of co-eluted peaks for efficient method development.
- LabSolutions MD enables easy searching for method operatable design region that satisfies the criteria of resolution for multiple compounds.

■ Introduction

In the process of separation optimization in LC method development, optimal analytical conditions are searched by varying parameters such as mobile phase composition, gradient curve, and column oven temperature. However, accurate peak tracking among obtained chromatograms is generally challenging due to variations in elution times and co-eluted compounds caused by different analytical conditions through optimization. A PDA (photodiode array) detector can provide peak tracking using differences in UV spectra, but for co-eluted peaks and related substances such as impurities and degraded products, accurate identifications by UV spectra may be difficult. On the other hand, the use of a mass spectrometer in addition to a PDA detector is expected to improve the accuracy of determining co-eluted peaks and tracking related substances based on *m/z*. This article describes how to improve the efficiency of separation optimization in method development through accurate peak tracking and automatic detection of co-eluted peaks by utilizing LCMS-2050 and LabSolutions MD, a dedicated software for supporting method development.

■ Analytical Conditions

Table 1 shows the analytical conditions used to optimize the simultaneous analysis of six small molecular pharmaceuticals. Gradient elution was applied using a 0.15% formic acid aqueous solution as the aqueous mobile phase and a mixture of acetonitrile and methanol as the organic mobile phase. The separation of individual compounds was investigated comprehensively by varying the mobile phase composition of organic solvent and column oven temperature. Specifically, the acetonitrile ratio in the organic mobile phase was varied from 0% to 100% in 10% increments (eleven levels), and the column oven temperature was varied from 30 °C to 40 °C in 5 °C increments (three levels).

Table 1 Analytical Conditions and Target Compounds

System	: Nexera™ X3 (Method Scouting System)
Sample	: Quinidine, Lidocaine, Metoclopramide, Papaverine, Dibucaine, Amitriptyline
Mobile phase	: Pump A : 0.15% formic acid in water
	: Pump B : Acetonitrile/Methanol = X : (100 - X)
	*X = 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100
Column	: Shim-pack Scepter™ C18-120 (100 mm × 3.0 mm I.D., 1.9 µm)*1
Injection Vol.	: 0.5 µL (80 mg/L)
LC Conditions	
Time program	: B Conc. 20%(0 min)→75%(12min) →90%(12.01-14min)→20%(14.01-17 min)
Column Temp.	: 30, 35, 40 °C
Flow rate	: 0.7 mL/min
Detection (PDA)	: 254 nm (SPD-M40, UHPLC cell)

*1 P/N: 227-31013-03

MS Conditions	
System	: LCMS-2050
Ionization	: ESI/APCI (DUIS™), positive and negative mode
Mode	: SCAN (*m/z* 150-400)
Nebulizing gas flow	: 2.0 L/min
Drying gas flow	: 5.0 L/min
Heating gas flow	: 7.0 L/min
DL Temp.	: 200 °C
Desolvation Temp.	: 450 °C
Interface voltage	: +3.0 kV / -2.0 kV
Qarray voltage	: +20 V

■ Peak Tracking based on m/z by LCMS-2050

Fig. 1 displays chromatograms obtained by varying the acetonitrile ratio (from 0% to 100% in 10% increments) in the organic mobile phase and the column oven temperature (from 30 °C to 40 °C in 5 °C increments). In the chromatogram at 60% acetonitrile (highlighted in red in Fig. 1), five peaks were detected. Notably, two combinations of impurities, Quinidine (②)/Lidocaine (③) and Dibucaine (⑥)/Amitriptyline (⑦), were co-eluted, and these un-separated peaks might have gone unnoticed. When co-elution of peaks is suspected, LabSolutions MD highlights the target peak in orange (as indicated by the black circle in Fig. 1) by using mass information from LCMS-2050. It also provides *m/z* for multiple co-eluted compounds. For instance, the peak suspected of co-elution at the acetonitrile ratio of 60% contained compounds each with m/z values of 344.25 and 278.23 respectively (Fig. 2), suggesting co-elution of Dibucaine and Amitriptyline. Peak tracking using *m/z* revealed the presence of up to seven compounds in the sample. It showed that impurities of Quinidine (②) and Lidocaine (③), as well as Dibucaine (⑥) and Amitriptyline (⑦), were eluted in a different order depending on the variation in the acetonitrile ratio in the organic mobile phase. In this way, LabSolutions MD can eliminate the possibility of missing un-separated peaks and provide a more efficient workflow for searching for optimal separation conditions, thanks to accurate peak tracking using *m/z*, as well as automatic determination and highlighting of co-eluted peaks.

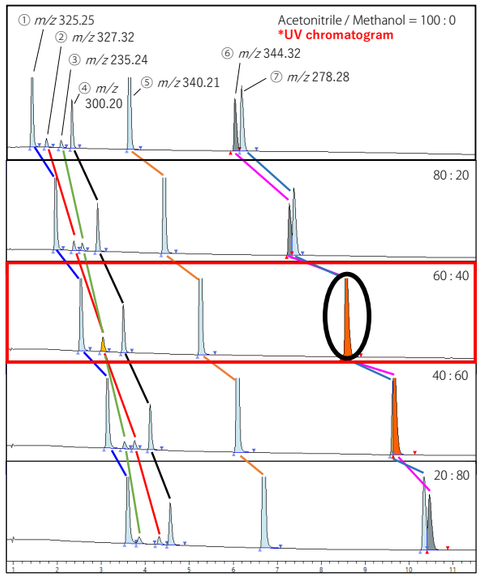

Fig. 1 Peak Tracking based on *m/z*

①Quinidine, ②Impurity of Quinidine ③Lidocaine, ④Metoclopramide, ⑤Papaverine, ⑥Dibucaine, ⑦Amitriptyline

* Acetonitrile ratios in organic solvent are 100, 80, 60, 40, and 20% from the top
* 30 ℃ of column temperature for all the chromatograms

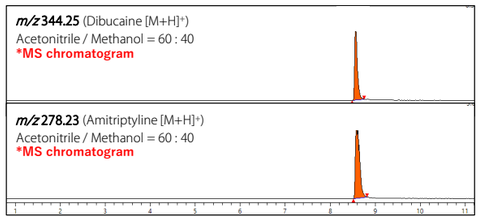

Fig. 2 MS Chromatograms and *m/z* of Peaks Suspected to be Co-eluted (black circled in Fig. 1).

■ Visualization of Resolution by Design Space

LabSolutions MD provides an efficient workflow for searching optimal separation conditions by visualizing resolutions for each compound through design space. The lower limits of resolution (criteria) for the targeted compounds are shown in Table 2. The Method Operatable Design Regions (MODRs), considered to have higher resolutions than the respective criteria, are shown as black hatched area in Fig. 3. The vertical axis represents the acetonitrile ratio in the organic mobile phase, and the horizontal axis represents column oven temperature. The colored area is considered to have lower resolutions than criteria and the remaining MODR is expected to provide higher resolutions than criteria. The conditions with higher acetonitrile ratio and lower column oven temperature (point A in Fig. 3) are expected to offer optimal separation. The visualization of resolution by design space enables easy and quick identification of MODRs that meet the criteria for individual peaks. This allows efficient optimization and an understanding of the relationship between various parameters and resolutions without depending on user experience.

Table 2 Criteria of Resolution for Each Compound

No.	Compound	Lower limit of resolution
①	Quinidine	3.0
②	Impurity of Quinidine	1.5
③	Lidocaine	1.5
④	Metoclopramide	1.5
⑤	Papaverine	1.5
⑥	Dibucaine	1.0
⑦	Amitriptyline	1.0

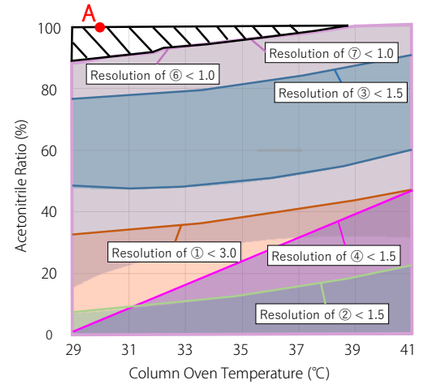

Fig. 3 Design Space of Resolution for Each Compound
* MODR is a black hatched area (upper left).

■ Chromatogram at Optimized Conditions

Fig. 4 shows a chromatogram at point A (acetonitrile ratio : 100%, column oven temperature : 30 °C), indicating the optimal separation conditions identified within the design space. It was confirmed that the resolutions for respective compounds under the optimized conditions met the criteria in Table 2.

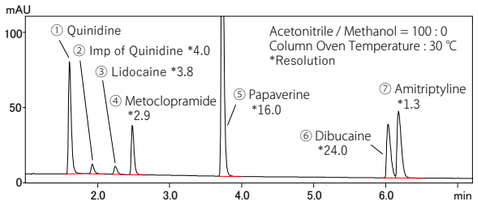

Fig. 4 Chromatogram at Optimized Conditions

■ Conclusion

The efficient optimization of separation conditions with LabSolutions MD and LCMS-2050 was introduced. Employing the mass information of LCMS-2050 can contribute to eliminate the possibility of missing co-eluted peaks and performing accurate peak tracking. Additionally, the visualization of resolutions using design space facilitates the easy identification of MODR that meets the resolution criteria for multiple compounds.

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Application News

LabSolutions™ MD : efficient method development based on Analytical Quality by Design

Efficient Method Development through Design Space Evaluation on Different Brand of Columns

S. Fujisaki, Y. Zhou

User Benefits

- Shim-pack Arata™ C18 can improve separation that is difficult to do with other C18 columns because of its unique selectivity.
- By using LabSolutions MD it is possible to easily identify the difference of selectivity of various columns by evaluating the design space.
- LabSolutions MD makes it efficient to search for the best column by comparing the design spaces of resolution.

■ Introduction

Since the column selection in method development has large effect on separation patterns of analytes, an efficient screening of various columns is often required. LabSolutions MD, a new Shimadzu software for method development, supports efficient method development based on Analytical Quality by Design (AQbD). AQbD-based analysis method development consists of different phases: initial screening, optimization and robustness evaluation. This article introduces an example of column screening using the design space evaluation concept. The selectivity of six brands of C18 column was visualized by design spaces considering different mobile phases composition and different gradient program. This makes it possible to understand the impact of the parameters on separation, enabling to search for the best column with a smaller number of analyses. In this article, it was found that the Shimadzu Shim-pack Arata C18 column has unique selectivity in comparison with the other C18 columns.

■ Analysis Conditions

The differences of selectivity of six brands of C18 column were evaluated through visualization of design spaces (Table 1 shows analysis conditions). In order to evaluate the difference of selectivity between the columns, a basic compound (amitriptyline), an acidic compound (benzoic acid), and a neutral compound (phenol) were used as the target compounds. The analyses were carried out by isocratic separation using a 0.1% formic acid in water as the aqueous mobile phase, and a mixed solution of acetonitrile and methanol as the organic solvent. The ratio of acetonitrile in the organic solvent was varied from 30% to 70% in increments of 20% (3 levels), and the initial concentration of organic solvent was also varied from 40% to 50% in increments of 5% (3 levels), and design spaces for minimum resolution were built for each column based on the results of total of 9 analyses (3 × 3 combinations).

Table 1 Analysis Conditions

System : Nexera™ X3 (Method Scouting System)	
Compound : Amitriptyline Benzoic acid Phenol	
Mobile Phase: A) 0.1% formic acid in water B) Acetonitrile/Methanol = 30 : 70, 50 : 50, 70 : 30 (3 levels)	
Column :	
1 : Shim-pack Arata C18	(100 mm × 3.0 mm I.D., 2.2 µm)*1
2 : Column A (C18 column)	(100 mm × 3.0 mm I.D., 1.8 µm)
3 : Column B (C18 column)	(100 mm × 3.0 mm I.D., 2.5 µm)
4 : Column C (C18 column)	(100 mm × 3.0 mm I.D., 3 µm)
5 : Column D (C18 column)	(100 mm × 3.0 mm I.D., 3 µm)
6 : Column E (C18 column)	(100 mm × 3.0 mm I.D., 3 µm)

Analytical Conditions (Isocratic):	
B Conc.	: 40, 45, 50% (3 levels)
Column Temp.	: 40 ˚C
Flow Rate	: 0.5 mL/min
Injection Vol.	: 1 µL (80 mg/L)
Detection	: 254 nm (SPD-M40, UHPLC cell)

*1 P/N 227-32802-03

■ Visualization of Resolution by Design Space

The design spaces of minimum resolution between the three types of compounds, amitriptyline, benzoic acid, and phenol, were drawn for the six brands of C18 columns, as shown in Fig. 1, where the vertical line shows the initial concentration of organic solvent and the horizontal line shows the ratio of acetonitrile in the organic solvent. In these figures, the red and blue regions represent high resolution and small resolution, respectively.

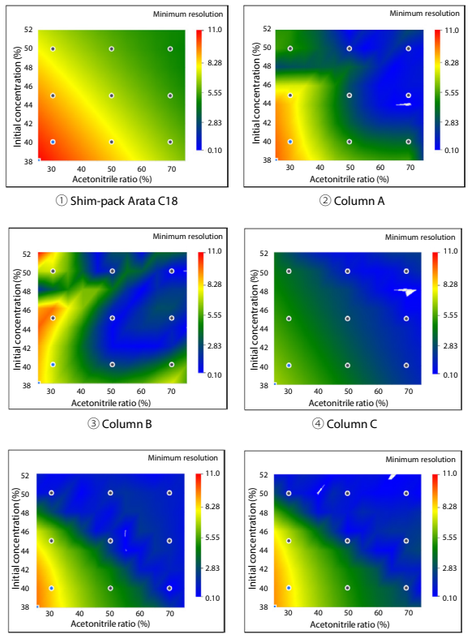

Fig. 1 Design Space for Minimum Resolution of Columns (6 Brands)
* The black dots (total 9 points) in the figure are points where analyses were conducted.
* The white regions (②, ④, ⑥) in the figure are regions where resolution is below the lower limit (0.1).

The design space of ① Shim-pack Arata C18 has a larger red region (higher resolution) compared to other columns, and there is no blue region (lower resolution) in the entire design space. This suggests that Shim-pack Arata C18 provides excellent resolution, as it has different selectivity compared to the other columns over the entire region. As demonstrated by this experiment, the difference of selectivity of each column can be understood easily by visualizing its pattern through design spaces, enabling higher efficiency when searching for the optimum column and analysis condition.

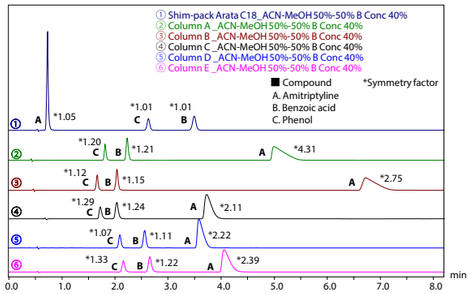

Fig. 2 Chromatograms Obtained with Each Column
Organic solvent: acetonitrile/methanol = 50 : 50,
initial concentration of organic solvent: 40%

Fig. 2 shows the chromatograms obtained with the conditions of acetonitrile ratio in organic solvent as 50% and initial concentration of organic solvent as 40%. With Shim-pack Arata C18, amitriptyline (Peak A) has an excellent peak shape (symmetry factor: 1.05), but with the other columns, deterioration of peak symmetry (symmetry factors: 2.11 to 4.31) can be seen due to interaction with the silanol on the surface of packing material. Moreover, Shim-pack Arata C18 also shows superior peak symmetry to those of the other columns for both the benzoic acid (Peak B) and phenol (Peak C). This experiment also revealed that Shim-pack Arata C18 column shows different elution pattern between acidic and basic compounds, and thus has different selectivity in comparison with the other columns. Because Shim-pack Arata C18 has this unique selectivity, it is possible to maintain better resolution, even in cases where the ratio of acetonitrile in the organic solvent is increased to shorten the analysis time (Fig. 3).

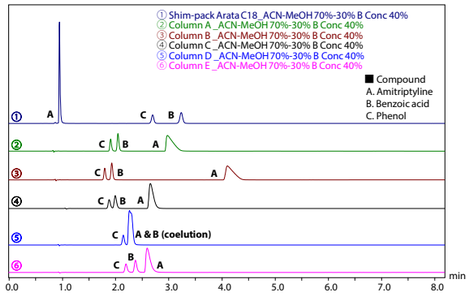

Fig. 3 Chromatograms Obtained with Each Column
Organic solvent: acetonitrile/methanol = 70 : 30,
initial concentration of organic solvent: 40%

Fig. 3 shows the chromatogram for the analyses with a 70% acetonitrile ratio in organic solvent and 40% initial concentration of organic solvent. When compared with Fig. 2, due to the increase of acetonitrile ratio, resolution of benzoic acid (Peak B) and phenol (Peak C) gets worse in chromatograms obtained with columns ② to ⑥, but Shim-pack Arata C18 column keeps enough resolution. Thus, with Shim-pack Arata C18, an excellent peak shape of basic compound and improved separation due to its unique selectivity can be expected in comparison with the other C18 columns.

■ Simplify the Search for Optimum Condition

LabSolutions MD can simplify the search for optimum analysis condition by overlapping design spaces. Fig. 4 shows the area of analysis condition that meets both minimum resolution ≥6 and resolution for amitriptyline ≥18 for each column. In the case of ① Shim-pack Arata C18, the region enclosed by the blue line in the figure is the region where minimum resolution <6 and the region enclosed by the red line is the region where resolution for amitriptyline is <18, and the remaining region (shown by the black hatching) is the region of conditions that satisfies both criteria. Among the six brands of columns, the results show that Shim-pack Arata C18 has the largest region that satisfies both criteria. As shown here, overlapping design spaces enables a quick and simple search for the region that satisfies the criteria of resolution set freely for multiple peaks.

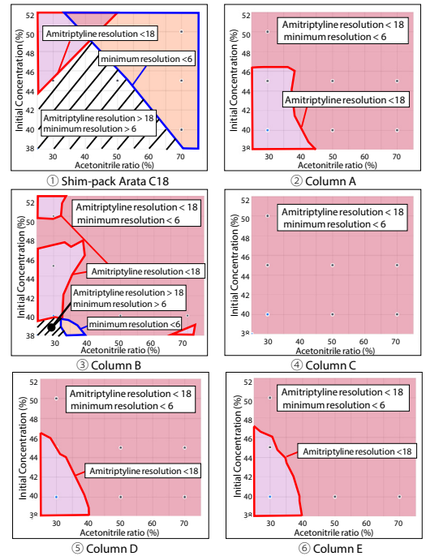

Fig. 4 Overlap of Design Spaces
of Minimum Resolution <6 and Resolution for Amitriptyline <18

■ Conclusion

LabSolutions MD makes it possible to achieve high efficiency in searches for the optimum column and analysis condition, without relying on the user experience, by visualizing and comparing the difference of selectivity between multiple columns through design space.

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Application News

Supercritical Fluid Chromatography System Nexera™ UC
Software for Efficient Method Development LabSolutions™ MD

Efficient Scouting of Chiral Separation Conditions Using LabSolutions MD

Yusuke Masuda

User Benefits

- Supercritical fluid chromatography offers quicker separation of chiral compounds.
- LabSolutions MD offers more efficient scouting of analytical conditions for new compounds.
- Peak shapes of acidic and basic analytes can be improved by adding suitable additives to the modifier.

■ Introduction

Chiral compounds contain at least one asymmetric carbon, so they cannot be superimposed on their mirror image. Although HPLC is the dominant chromatographic technique used to separate chiral compounds, there is growing interest in chiral separation that uses supercritical fluid chromatography (SFC). SFC is normally performed using a mobile phase of supercritical carbon dioxide, which offers low polarity, low viscosity and high diffusivity. Polar organic solvents (modifiers) are then added to this mobile phase to control interactions between the analytes and the stationary phase. Chiral compounds are typically separated by HPLC under normal phase conditions, but the unique properties of SFC mentioned above offer advantages, such as higher separation speeds and lower organic solvent consumption, which reduce costs and lower the environmental impact.

Therefore, the pharmaceutical sector uses SFC because it can provide rapid optical resolution in the synthesis of novel drugs. Finding the best column and mobile phase (modifier) combination for a given analyte species from the wide variety of chiral columns available is a very time-consuming and labor-intensive process, so there is demand for faster and more streamlined methods for scouting chiral separation conditions.

This article describes a workflow for developing separation conditions for chiral compounds that uses the Nexera UC chiral screening system and LabSolutions MD analytical method development software.

■ Nexera UC Chiral Screening System

The Nexera UC chiral screening system consists of an SFC system, a solvent switching valve, and a column switching valve. It can automatically and continuously switch the modifiers between up to 12 columns, enabling the comprehensive collection of analytical data. Because it can switch between up to seven different modifiers and investigate a variety of separation conditions, the Nexera US chiral screening system can identify high-resolution separation conditions for chiral compounds quicker and with less work than other systems.

Fig. 1 Nexera™ UC Chiral Screening System

■ Analyte Compounds

This article describes a process for optimizing the analytical conditions for three analyte compounds: chlormezanone (neutral compound), flurbiprofen (acidic compound), and disopyramide (basic compound) (Fig. 2). Each of these compounds has one asymmetric carbon and two isomers.

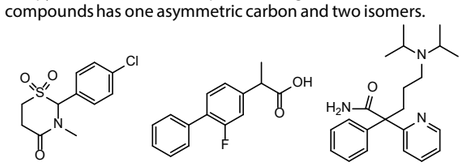

Fig. 2 Structural Formula of Analyte Compounds
(From Left: Chlormezanone, Flurbiprofen, and Disopyramide)

■ Creating Column Screening Schedules

LabSolutions MD offers excellent ease of use and can create multiple analytical conditions automatically in a single operation and then screen these conditions efficiently. The modifier and column conditions can also be set by simply selecting from the modifiers and columns that are pre-registered in the software database (Fig. 3). Scouting for analytical conditions manually is a labor-intensive process that involves manually switching between modifiers and columns and manually preparing analytical methods and batch schedules. However, LabSolutions MD can configure and automate these tasks with ease.

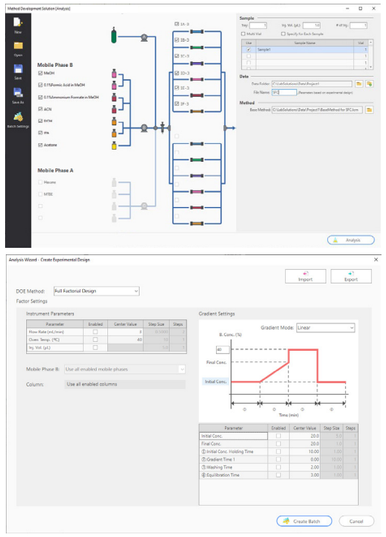

Fig. 3 Analysis Schedule Preparation Window

LabSolutions MD was used to scout 42 analytical conditions for each analyte compound from a combination of six columns and seven modifiers. The screening conditions are shown in Table 1. The screening results for chlormezanone are shown in Fig. 4.

Table 1 Modifier and Column Scouting Conditions

System:	Nexera UC Chiral Screening System
Column:	CHIRALPAK® IA-3 (100 mm x 3.0 mmI.D., 3 µm) CHIRALPAK® IB-3 (100 mm x 3.0 mmI.D., 3 µm) CHIRALPAK® IC-3 (100 mm x 3.0 mmI.D., 3 µm) CHIRALPAK® ID-3 (100 mm x 3.0 mmI.D., 3 µm) CHIRALPAK® IE-3 (100 mm x 3.0 mmI.D., 3 µm) CHIRALPAK® IF-3 (100 mm x 3.0 mmI.D., 3 µm)
Mobile Phase A:	CO_2
Mobile Phase B:	Methanol Acetonitrile Ethanol 2-Propanol Acetone 0.1 % Formic acid in methanol 0.1 % Ammonium formate in methanol
Flowrate:	3.0 mL/min
Time Program:	B. Conc. 20 % (0-8 min) → 40 % (8.01-10.0 min) → 20 % (10.01-12.0 min)
Column Temp.:	40 °C
Injection Volume:	2 µL in ethanol
BPR Pressure:	10 MPa
Detection:	220 nm (for chlormezanone) 245 nm (for flurbiprofen) 260 nm (for disopyramide) (PDA with a high-pressure flow cell)

■ Rapid Identification of Optimized Conditions from Screening Results

The column screening stage yields as many chromatograms as the conditions that are screened (Fig. 4). All these chromatograms must then be assessed to determine which conditions offer the best separation. This assessment step is typically very labor intensive and requires experience in chromatography. However, LabSolutions MD can evaluate separation conditions that are based on the resolution between the target peaks. This removes the need for intuition and experience to identify the optimal conditions, so this task can now be performed by anyone quickly and easily (Fig. 5).

When analyte compounds have multiple asymmetrical carbons and are expected to separate into many peaks, LabSolutions MD can rank separation conditions that are based not just on the resolution between the target peaks but also on the number of detected peaks or a score (Equation 1) that is calculated from the number of detected peaks (P) and the resolution (Rs).

$$(\text{Score}) = P \times (Rs1 + Rs2 + \ldots + RsP - 1) \qquad \text{(Equation 1)}$$

The chromatogram with the highest score in this analysis is outlined in red in Fig. 4. In this analysis, LabSolutions MD showed that using methanol as a modifier with the CHIRALPAK® IC-3 column offered the best chiral separation of chlormezanone isomers.

			Response
Sample Name	MPB Nick Name	Column Nick	Resolution
Chlormezanone	MeOH	IA-3	11.049
Chlormezanone	0.1%Ammo···	IF-3	10.872
Chlormezanone	0.1%Ammo···	IA-3	10.727
Chlormezanone	EtOH	IF-3	10.629
Chlormezanone	0.1%Formi···	IA-3	10.628
Chlormezanone	EtOH	IA-3	10.445
Chlormezanone	MeOH	ID-3	10.193
Chlormezanone	0.1%Ammo···	ID-3	10.005
Chlormezanone	0.1%Formi···	ID-3	9.928
Chlormezanone	MeOH	IF-3	8.102

Fig. 5 Separation Conditions Ranked by Score
(Top 10 Scoring Separation Conditions are Shown.)

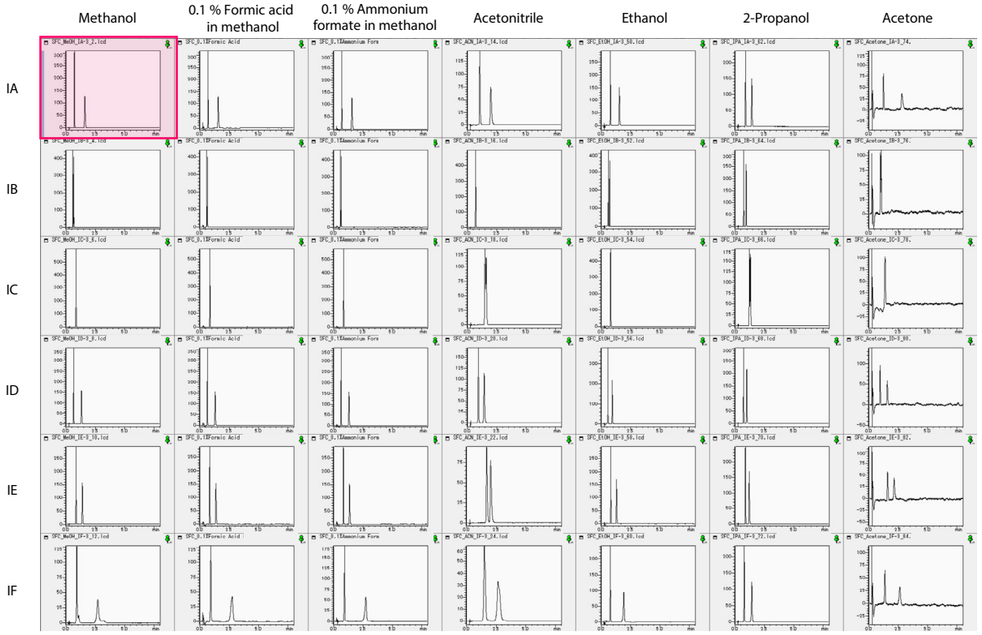

Fig. 4 Chlormezanone Chromatograms Obtained during Screening
(Highest Scoring Chromatogram Outlined in Red)

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■ Tuning Separation and Improving Peak Shapes with Additives

With HPLC, buffers and ion-pair reagents are sometimes added to the mobile phase to tune the selectivity of a separation or to improve peak shapes. Additives are also added for similar purposes with SFC, but they are added to the modifier and not the supercritical carbon dioxide mobile phase. Adding acids, such as formic acid, and bases, such as amines, in SFC can improve peak shapes by preventing the ionization of target components and masking secondary functional groups in the stationary phase.

■ Optimum Separation Conditions for Flurbiprofen and Disopyramide

The optimum conditions for chiral separation of the acidic compound flurbiprofen and the basic compound disopyramide were scouted by a method that is also used for the neutral compound chlormezanone.

Chromatograms that were obtained from flurbiprofen and disopyramide during this scouting process are shown in Fig. 6.

Good peak shapes were obtained from the acidic compound flurbiprofen even without adding an acidic additive to the modifier. This is because unlike LC, SFC uses a slightly acidic mobile phase (carbon dioxide), so good peak shapes can be obtained from many acidic compounds without additives. Peak shapes from some acidic compounds can also be improved by adding additives, such as formic acid and trifluoroacetic acid.

For the basic compound disopyramide, adding a basic additive to the modifier reduced nonspecific absorption by preventing ionization of the target components and masking secondary functional groups in the stationary phase, which improved separation and peak shapes.

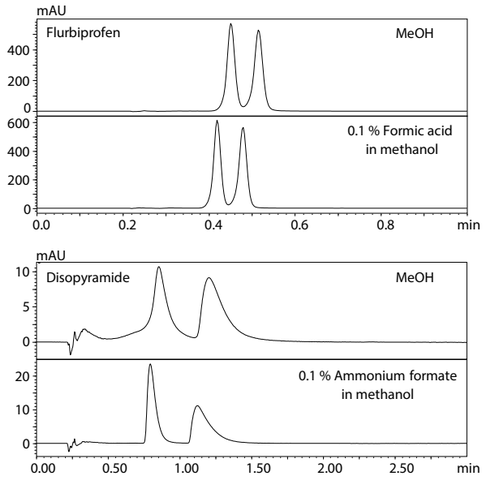

Fig. 6 Chromatograms from Flurbiprofen (Top) and Disopyramide (Bottom)

■ Conclusion

This article describes a workflow for finding separation conditions for chiral compounds using the Nexera UC chiral screening system and LabSolutions MD analytical method development software. LabSolutions MD allowed analytical conditions to be investigated with less work and greater efficiency.

Because SFC uses a slightly acidic mobile phase (carbon dioxide), it can produce good peak shapes from many acidic compounds without using additives. This article also shows that SFC can produce good separation of even basic compounds when suitable additives are added to the modifier. The Nexera UC chiral screening system can switch between up to seven different modifiers in an analysis sequence, enabling the efficient investigation of additives that are not just organic solvents.

Furthermore, the carbon dioxide used by SFC is cheaper than the organic solvents commonly used by HPLC and reduces disposal costs, which promises lower running costs for SFC. SFC can also be used for preparative purification and it offers significant time and labor savings in chiral purification.

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Liquid Chromatograph Mass Spectrometer LCMS-9050

Application News

An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software

Noriko Kato and Junna Nakazono

User Benefits

- LabSolutions Insight Biologics software offers a simple workflow for the characterization of oligonucleotides and oligonucleotide impurities.
- Allows comprehensive characterization of oligonucleotide impurities.
- Fragment coverage is shown in a simple graphical format that highlights missing and modified nucleotides.

■ Introduction

Oligonucleotide therapeutics have undergone rapid development in recent years and are attracting interest as a new modality in drug discovery. Such development has created a demand for the comprehensive detectin and identification of impurities in oligonucleotide therapeutics to ensure their safety and efficacy. This Application News describes an impurity analysis workflow for oligonucleotides based on the LCMS-9050 quadrupole time-of-flight mass spectrometer system and LabSolutions Insight Biologics software.

■ Samples

An unrefined, phosphorothioate-modified 20-mer oligonucleotide with the nucleotide sequence CTG CTA GCC TCT GGA TTT GA (unrefined PS 20-mer) was analyzed.

■ Analytical Conditions

Analysis was performed with the Nexera™ XS inert system and LCMS-9050 system in data dependent acquisition (DDA) mode. The LC conditions used are shown in Table 1 and the MS conditions used are shown in Table 2.

Table 1 LC Conditions

[HPLC Conditions] (Nexera XS inert)

Column: Shim-pack Scepter™ Claris C18-120, 100 mm x 2.1 mm I.D., 1.9 µm*1

Mobile Phases: A) Aqueous solution of 100 mM HFIP and 10 mM TEA
B) 50 % Methanol solution of 50 mM HFIP and 5 mM TEA

Gradient Program:

Time (min)	Flowrate (mL/min)	A. Conc	B. Conc
0.00	0.3	95	5
1.00	0.3	95	5
26.00	0.3	60	40
26.10	0.3	10	90
30.00	0.3	10	90
30.10	0.3	95	5
34.00	0.3	95	5

Column Temp.: 60 °C
Injection Volume: 2 µL

*1: P/N: 227-31210-02

Table 2 MS Conditions

[MS Conditions] (LCMS-9050)

Ionization:	ESI (Negative mode)
Mode:	MS scan (*m/z* 550 to 2500), DDA
Interface Voltage:	-3.0 kV
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
Heating Gas Flow:	10.0 L/min
DL Temp.:	250 °C
Block Heater Temp.:	400 °C
Interface Temp.:	350 °C

■ Configuring the Analysis Parameters

LabSolutions Insight Biologics is data analysis software that characterizes oligonucleotides and oligonucleotide impurities. First, the user creates an oligonucleotide sequence in the parameter configuration window using the nucleobases, linkers, ribose and modifications provided by the software. Nucleobases, linkers, ribose, and base modifications can be added and removed in each tab as required. Once an oligonucleotide sequence is entered, the software displays the molecular structure, monoisotopic mass, and structural formula (right side) of that oligonucleotide (Fig. 1). The structural formula is also updated in real time based on the entered sequence, allowing for easy visual identification of entry errors.

As shown in Fig. 2, nucleobases can be displayed with various colors for easy identification, and fragmentation sites can be also shown.

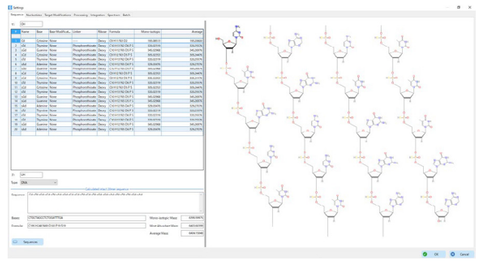

Fig. 1 Parameter Configuration Window

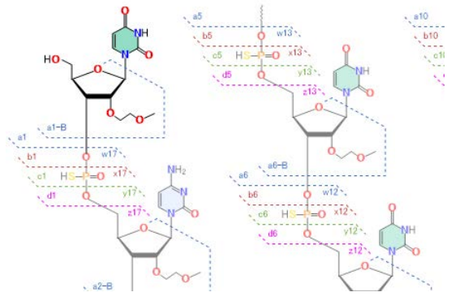

Fig. 2 Colored Display of Fragmentation Sites

A "Target Modifications" tab is also used to select the anticipated impurities. In addition to impurities such as different strand lengths, missing nucleobases, depurination/depyrimidination, deamination, and protecting groups, as well as additional ions, unknown modifying groups, the software can also search for molecular changes added by the user.

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■ Results

The unrefined PS 20-mer was detected by photodiode array and mass spectrometry. The chromatograms obtained by each method are shown in Fig. 3. The LC chromatogram (top) and MS chromatogram (bottom) are aligned to allow a direct comparison between the peaks.

The mass spectrum has been displayed as a component chromatogram, which is based on MS1 spectra and combines signals from different valences and isotopes. Generation of a component chromatogram is a unique feature of this software, whereby all individual signals contributing to the component have been summed into a single XIC chromatogram. Fig. 4 shows the multivalent ion mass spectrum and deconvoluted mass spectrum of the PS 20-mer. The spectra of oligonucleotide impurities were also deconvoluted and used to search for impurities.

An exhaustive search for impurities based on MS1 data identified over 30 impurities with different strand lengths, missing bases, and additional ions.

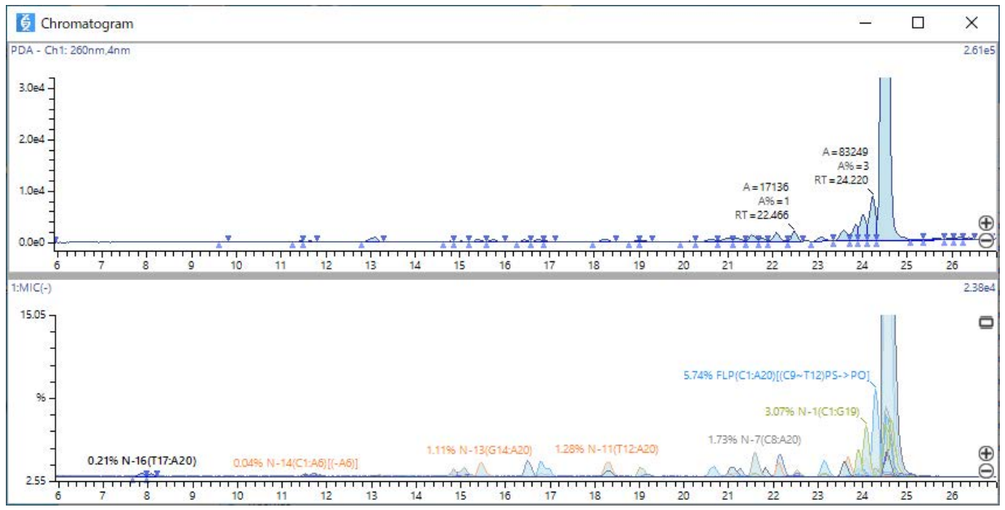
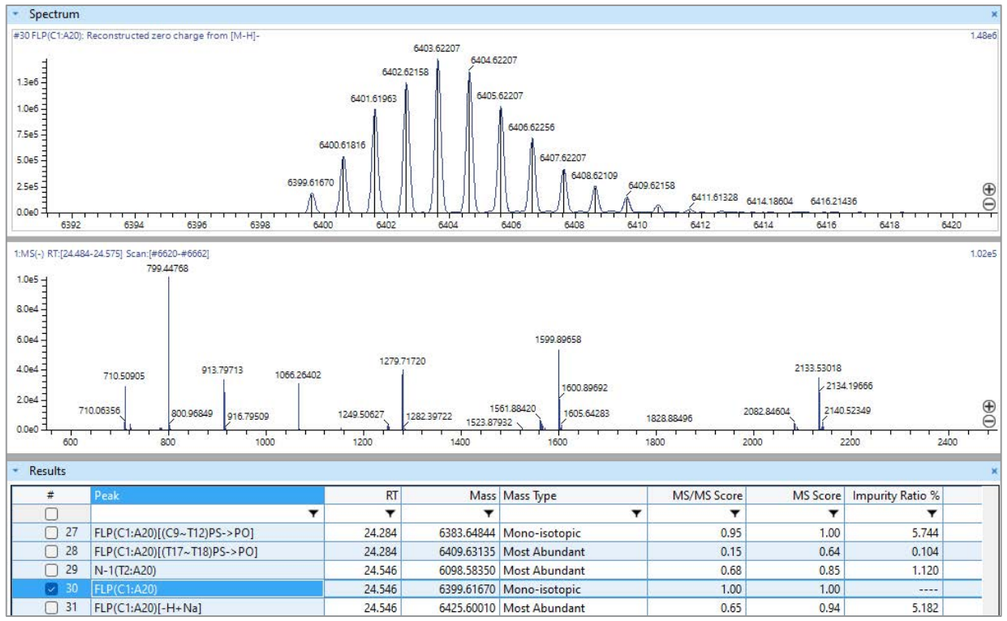


Fig. 3 Unrefined PS 20-mer UV Chromatogram (Top) and Component Chromatogram (Bottom)



#	Peak	RT	Mass	Mass Type	MS/MS Score	MS Score	Impurity Ratio %
27	FLP(C1:A20)[(C9~T12)PS->PO]	24.284	6383.64844	Mono-isotopic	0.95	1.00	5.744
28	FLP(C1:A20)[(T17~T18)PS->PO]	24.284	6409.63135	Most Abundant	0.15	0.64	0.104
29	N-1(T2:A20)	24.546	6098.58350	Most Abundant	0.68	0.85	1.120
30	FLP(C1:A20)	24.546	6399.61670	Mono-isotopic	1.00	1.00	----
31	FLP(C1:A20)[-H+Na]	24.546	6425.60010	Most Abundant	0.65	0.94	5.182

Fig. 4 PS 20-mer Mass Spectrum (Top) and Deconvoluted Mass Spectrum (Bottom)

The software also displays sequence coverage based on the MS2 fragmentation spectra.

The results obtained from aligning the fragments against the PS 20-mer sequence are shown in Figs. 5 and 6. The fragment ions generated by the analysis are cleaved at every nucleobase on the PS 20-mer and have sequences that match the PS 20-mer.

The software displays sequence coverage in two modes, and users can switch between these modes depending on the information of interest: a fill mode that shows signal information and the completeness of the aligned fragment ions, and a branch mode that shows the fragment sequence.

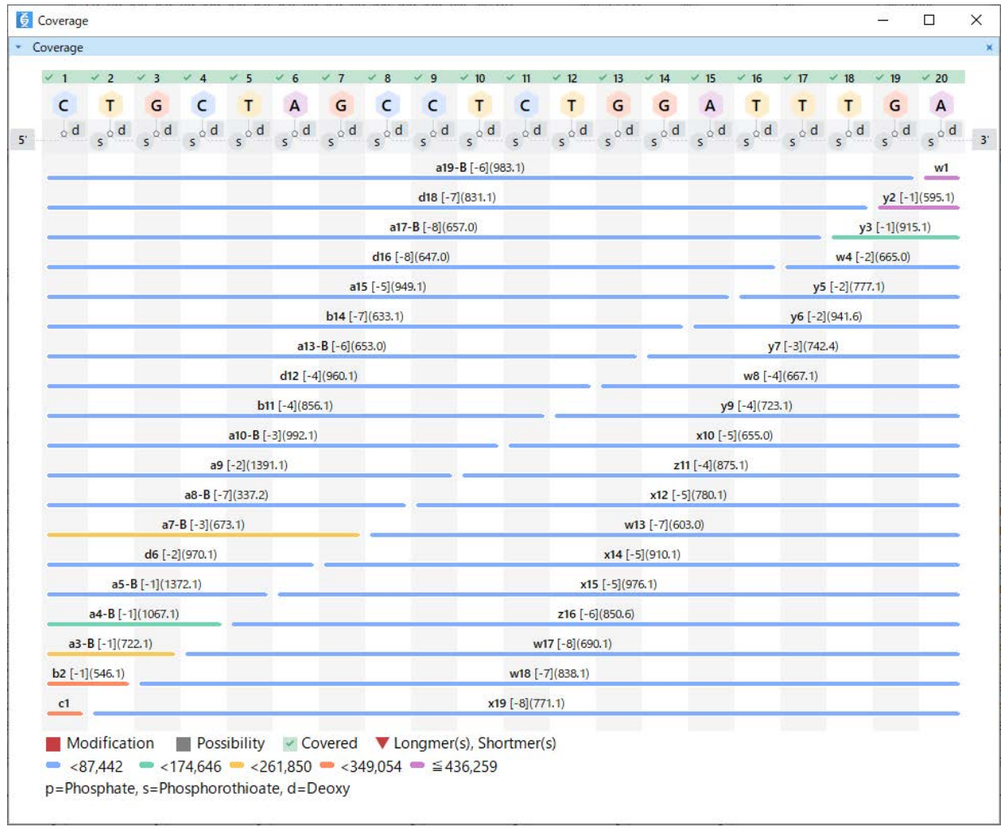


Fig. 5 PS 20-mer Sequence Coverage (Fill Mode)

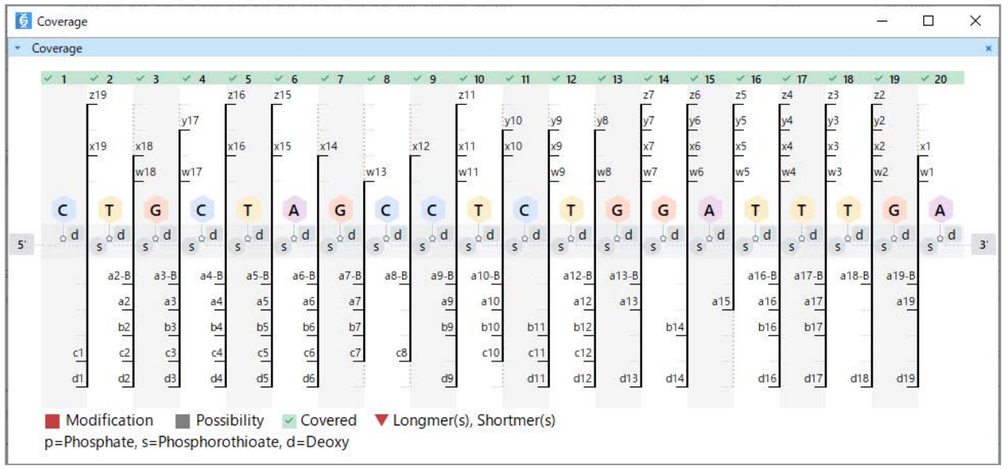


Fig. 6 PS 20-mer Sequence Coverage (Branch Mode)

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■ Examining the Sequence of an Impurity

The sequence of an impurity missing 14 nucleotides from the 5′ end (hereinafter N-14) and present as 0.5 % of the main sample component was examined. The validity of the data is shown by the high percentage sequence coverage, as indicated by the green tick marks above the verified nucleobases in Fig. 7.

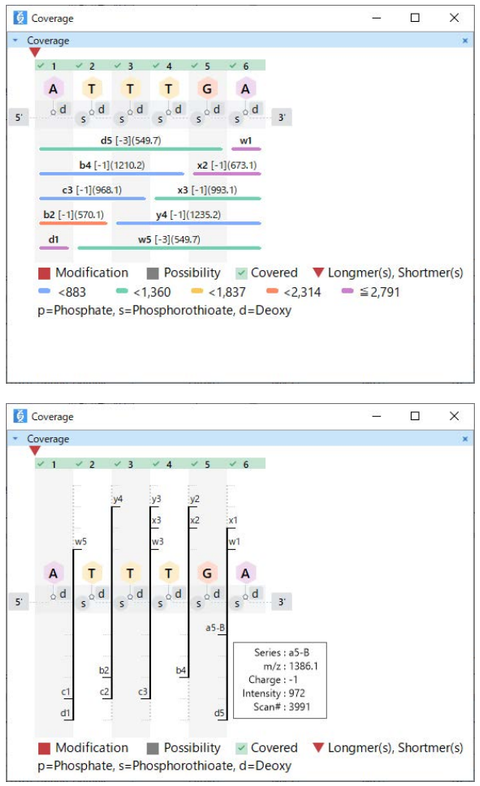

Fig. 7 N-14 Sequence Coverage

■ Conclusion

LabSolutions Insight Biologics software can comprehensively characterize and identify the sequences of oligonucleotide impurities.

The analysis workflow described in this article achieved complete sequence coverage not only for the main sample component, but also for an impurity with a relative abundance of 0.5 % compared with the main component.

<Acknowledgements>
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MALDI-DIT-MS

Application News

Oligonucleotide Analysis Using the Compact MALDImini™-1 MALDI Digital Ion Trap Mass Spectrometer

Yuko Fukuyama

User Benefits

- Enables molecular weight analysis and sequence analysis of oligonucleotides, including modification sites.
- MS^n enables reliable end sequence analysis.
- The compact benchtop design does not take up much space.

■ Introduction

Due to rapid progress in practical applications of oligonucleotide therapeutics in recent years, there is a growing need for corresponding analytical technologies. The application of mass spectrometry (MS) to oligonucleotide therapeutics analysis has already begun, but more accurate and rapid analytical techniques are required.

Most approved oligonucleotide therapeutics are based on single- or double-stranded synthetic oligonucleotides that are several tens of bases in length (molecular weight of about 6000 to 15000 Da). Such oligonucleotides contain a variety of chemical modifications depending on the purpose.

The MALDImini-1 matrix-assisted laser desorption/ionization-digital ion trap-mass spectrometer (MALDI-DIT-MS) (Fig. 1) is a compact benchtop instrument that can perform MS analysis for *m/z* values up to 70,000 and MS^n analysis for *m/z* values below 5,000 in positive ion mode.

This article presents an example of using the MALDImini-1 system to analyze the molecular weight and sequence of a synthetic oligonucleotide with the same sequence as mipomersen, an oligonucleotide therapeutic.

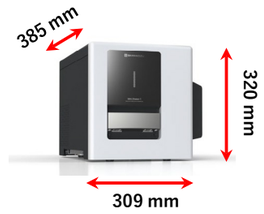

Fig. 1 MALDImini™-1 (MALDI-DIT-MS)

■ Synthetic Oligonucleotide Sample

Table 1 shows the sequence information of the synthetic oligonucleotide used as a model for the oligonucleotide therapeutic. This sequence is the same as mipomersen.

Table 1 Sequence Information of Synthetic Oligonucleotide Used in this Application News*1

MW	Sequence (20 bases)
7177	5′-MG-MC-MC-MU-MC-dA-dG-dT-dC-dT-dG-dC-dT-dT-dC-MG-MC-MA-MC-MC-3′

*1 M: 2′-O-(2-methoxyethyl) nucleoside; d: 2′-deoxynucleoside; The 5-positions of cytosine and uracil are substituted with methyl groups. Phosphodiester bonds between all nucleotides are replaced by phosphorothioate bonds.

■ Sample Preparation and Measurement Conditions

A 20 pmol/μL solution of synthetic oligonucleotide was prepared as a sample solution. As matrix solutions, 3-hydroxypicolic acid (3-HPA) and 2,4-dihydroxyacetophenone (2,4-DHAP) were each dissolved in acetonitrile/water (50/50, v/v) solution containing 70 mM diammonium hydrogen citrate to make a 40 mg/mL solution of each. A 3-HPA/2,4-DHAP matrix mixture solution was prepared by mixing the above 3-HPA and 2,4-DHAP solutions at a 1:1 (v/v) ratio. This matrix mixture improves sensitivity and mass spectral quality in the MALDImini-1 system compared to conventional methods (patent pending). After mixing the sample and matrix solutions at 1:1 (v/v), 1 μL of the mixture was dropped onto the sample plate, dried, and measured.

Measurements were performed by raster-scanning with the MALDImini-1 system. Table 2 shows the condition settings used for molecular weight analysis and sequence analysis. Using those settings for sequence analysis, a large number of fragment ions derived from the oligonucleotide can be detected by MALDI-DIT-MS specific degradation (detailed below).

Table 2 MALDImini-1 Condition Settings for Molecular Weight and Sequence Analysis*2

	LP	DV-1 (V)	DV-2 (V)	RF delay (*ms*)
M.W. Analysis	60-65	1300	7000	25
Sequence Analysis	65-75	1600	8000	15-17

*2 LP: laser power; DV-1: detector voltage; DV-2: dynode voltage. The same conditions can be used for scan ranges *m/z* 650 to 5000 and *m/z* 2000 to 18000. The values in the table are examples only and will vary depending on the system used. The DV-1 value for sequence analysis should be approximately 300 V higher than the value for molecular weight analysis.

■ Molecular Weight Analysis of Synthetic Oligonucleotide

Fig. 2 shows the mass spectrum of the synthetic oligonucleotide obtained by MALDImini-1 using a 3-HPA/2,4-DHAP matrix mixture and the settings for molecular weight analysis in Table 2.

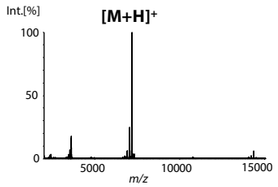

Fig. 2 Mass Spectrum of Synthetic Oligonucleotide

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■ Sequence Analysis of Synthetic Oligonucleotide

Fig. 3 shows the definition of nucleic acid fragment ions[1]. Fig. 4 shows the mass spectra of the synthesized oligonucleotide obtained using the MALDImini-1 system and the condition settings for sequence analysis indicated in Table 2. Under those conditions, many fragment ions, mainly *a*/*w*-ions, were detected. The *a*/*w*-ions are obtained in the form of a ladder, allowing easy sequence analysis.

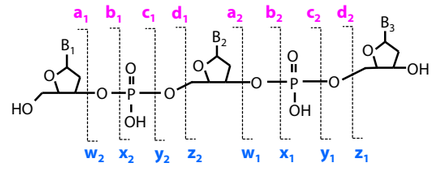

Fig. 3 Definition of Fragment Ions of Nucleic Acids[1]

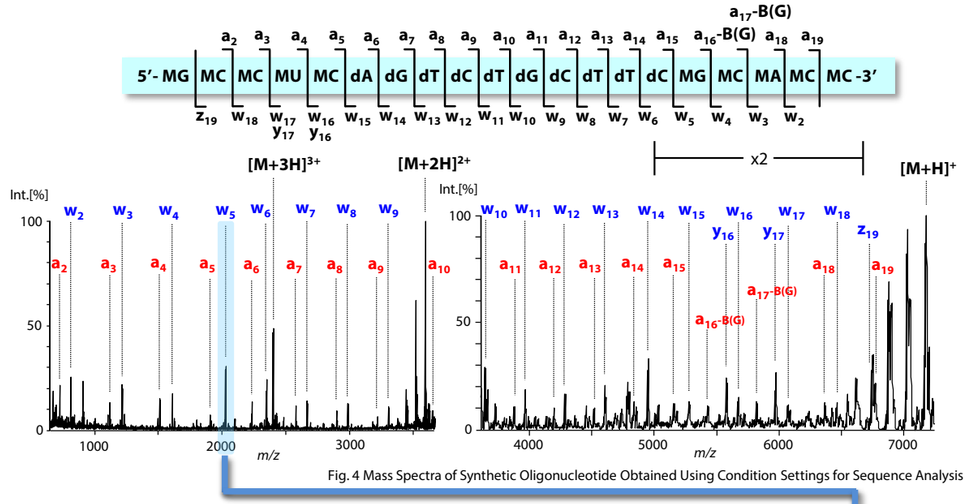

Fig. 4 Mass Spectra of Synthetic Oligonucleotide Obtained Using Condition Settings for Sequence Analysis

In Fig. 4, multiple fragment ions overlap in the vicinity of $[M+H]^+$, making it difficult to attribute the fragment ions used for sequence analysis of the terminal portion of the oligonucleotide. Fig. 5 shows the MS^2 spectrum (pseudo-MS^3 spectrum) obtained using one of the fragment ions shown in Fig. 4, the w_5-ion, as the precursor ion. The MS^2 spectrum enabled the fragment ions containing terminal sequence information to be easily attributed. By using the MS^2 spectrum in Fig. 5 in combination with the mass spectrum in Fig. 4, enabled more accurate sequence analysis.

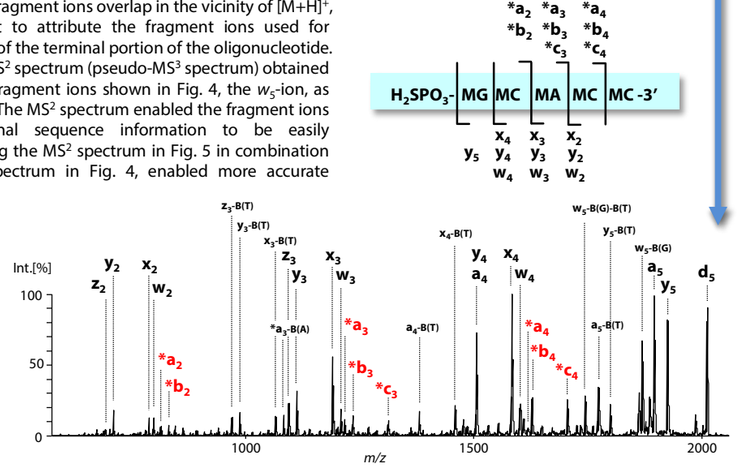

Fig. 5 MS^2 Spectrum of the w_5-ion in Fig. 4

■ Conclusion

This example confirmed that molecular weight information and base sequence information for entire synthetic oligonucleotides sequences, including modification sites, can be obtained easily and quickly by using a MALDI-DIT-MS system and adjusting the condition settings.

Consequently, the system is expected to be used in the field of nucleic acid analysis as a simple analytical device for research sites, while also offering the advantage of a compact benchtop design.

<Reference>
1) McLuckey SA, J. Am. Soc. Mass. Spectrom., 1992, 3, 60-70.

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Application News

LCMS-2050 Liquid Chromatograph Mass Spectrometer

Oligonucleotide Characterization for Quality Control on the Shimadzu Single Quad Mass Spectrometer LCMS-2050

Udara Jayasundara[1], Vikki Johnson[1], Stephen Kurzyniec[1]
[1]Shimadzu Scientific Instruments, Inc.

User Benefits

- The Shimadzu LCMS-2050 single quadrupole mass spectrometer and SPD-M30A photodiode array detector offer a straightforward workflow for quantitating oligonucleotides, streamlining the analysis process.
- Confident mass confirmation can be obtained over a wide mass range of nucleotide oligomers.
- The Shimadzu LCMS-2050 enables the detection and identification of lower levels of impurities commonly found in synthetic oligomers.

■ Introduction

Oligonucleotide therapeutics have garnered increased attention in recent years as an innovative class of treatments in immunology, virology, and RNA-based therapies. It is crucial to differentiate between full-length and truncated nucleotides, as well as modified and unmodified versions for effective quality control. Traditional hybridization techniques, such as ELISA (enzyme-linked immunosorbent assay) and qPCR (polymerase chain reaction), offer sensitivity in detection, but lack specificity for effective impurity analysis. Liquid chromatography-mass spectrometry (LC-MS) is an advanced technique with inherent specificity that can be utilized to analyze impurities, degradants, and other biological/chemical modifications that cannot be analyzed by hybridization techniques.

Mass spectrometry is commonly perceived as complex, often requiring specialized expertise for routine quality control applications. The Shimadzu single quadrupole LCMS-2050 is a user-friendly instrument capable of acquiring reliable and sensitive data with simplified operation. The automatic setting of the sampling rate from peak width and number of data points enables users of any experience level to generate quality data by streamlining method development. Developing a robust workflow for accurately determining intact mass and quantitation of synthetic oligomers is essential for maintaining high-quality products.

The LCMS-2050, with a mass range of *m/z* 2-2000, is an ideal instrument to address this challenge by facilitating data acquisition over multiple charge states. Utilization of PDA (photodiode array) detection in series with MS can enhance quantitation accuracy due to the strong absorption of nucleic acids at 260nm.

This application note outlines a workflow for confident mass confirmation across the entire range of nucleotide length oligomers (10-60-mer), along with quantitation of yield using both UV and mass spectrometry data. The acquired data from these workflows were analyzed considering impurities, including aborted sequences (N-1, N-2) and mobile phase adducts.

Shimadzu LCMS-2050 Single Quadrupole LC/MS

■ Experimental

Sample Preparation

Custom designed single-stranded DNA oligomers, with lengths ranging from 10-90-mer nucleotides, as shown in **Table 1**, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) with standard desalting purification. All oligomers were reconstituted to a stock concentration of 100 µM and stored at -20 °C when not in use.

Analytical Conditions

All oligomers were eluted with ion pairing, reversed phase conditions using HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) reagent in the mobile phase. Instrument parameters are shown in **Table 2**. A shallow gradient was used to separate the main peak of interest from other interfering peaks. Estimates of impurities were detected by MS as some impurities, such as N-1 aborted sequences, are difficult to separate by chromatography. A steep gradient was used for mass confirmation for the oligonucleotides to allow for quick elution over a large mass range. The MS source conditions (gas flow, temperature, and voltages) were optimized to obtain higher signal intensity for oligomers and to keep the common HFIP adduct abundance low.

Calibration

The 30-mer oligonucleotide calibration curve was prepared by diluting the stock in nucleus free water to 0.1, 0.2, 0.5,1, 2, 5, 10, 20, 30, 40, 50 µM concentrations to create a 10-point calibration curve. Quantitation by MS was performed by selected ion monitoring (SIM) corresponding to the -10-charge state and quantitation by UV was performed by absorbance at 260 nm.

Mass Confirmation

Mass confirmation was analyzed for all oligonucleotides. Triplicate injections of 50 pmol mass on column was performed to verify reproducibility of mass confirmation.

Impurity Analysis

Impurity analysis was performed on the 10-mer oligonucleotide at 50 pmol mass on column.

Instrumentation

Samples were analyzed in series using a photodiode array detector (SPD-M30A) and single quadrupole mass spectrometer (LCMS-2050) coupled to a Nexera UHPLC capable of pressures up to 15,000psi. A Shimadzu Scepter Claris column with bioinert surface treatment on the body and frit was used for analysis.

Table 1: Analyzed oligonucleotides.

Length	Sequence (DNA, 5' -3')	Average mass (Da)
10-mer	CACTGAATAC	2996.0
15-mer	ACCTGAATACCAATA	4529.0
20-mer	TCATCACACTGAATACCAAT	6029.0
25-mer	CTATACCGCTGAATACCAATCACTG	7570.0
30-mer	ACACTGAATACCAATCACTGAATACTACGC	9112.0
35-mer	TCACACTCATGAATACCAATCACTGAATACCAATA	10620.0
40-mer	ACACTGAATACCAATTGACACATACTACGCTGAACACTGA	12210.1
45-mer	ACAAATCTGAATACCAATCACCGCTGAATACTATGAACACTGACC	13719.0
50-mer	TCATCACACTGAATACCAATCACTGAATACCAATACACTGAATACCAATA	15211.0
60-mer	TCAACCTCAATACCAATCACTCACTGAGAATACCAATACACTGAATACCAATAGAATAAT	18293.1

Table 2: Oligonucleotide analysis instrument parameters.

Liquid Chromatography (LC) Conditions			
Column	Shimadzu Scepter-Claris C18 120 (150mm x 2.1mm I.D x 1.9µm)		
Mobile Phase A	1% (95mM) HFIP, 0.1% (4.3 mM) TEA in water		
Mobile Phase B	1% (95 mM) HFIP, 0.1% (4.3 mM) TEA in 50% MeOH		
Injection Volume	0.5 µL (mass confirmation and impurity), 1 µL (calibration)		
Column Temperature	50 °C		
Autosampler Temperature	5 °C		
Gradient (Quantitation)	Time (min)	Flow (mL/min)	B%
	0.40	0.4	10
	4.65	0.4	24
	4.90	0.4	24
	4.91	0.4	99
	4.92	0.45	99
	6.40	0.45	99
	6.41	0.45	10
	6.42	0.4	10
	8.40	0.4	10
Gradient (Mass Confirmation)	Time (min)	Flow (mL/min)	B%
	0.0	0.4	1
	0.5	0.4	1
	6.5	0.4	99
	8.0	0.4	99
	8.01	0.4	1
	10.0	0.4	1
MS Conditions			
Mode	Scan (mass confirmation) SIM (quantitation)		
Mass Range	550-2000 *m/z*		
Ionization	ESI/APCI (DUIS) (-) mode		
Event Time	0.4 sec (full scan), 0.013 sec (SIM)		
Cycle	0.5 sec		
Detector Voltage	1.3 kV		
Nebulizing Gas Flow	2.0 L/min		
Desolvation Temperature	450 °C		
Desolvation Line Temperature	200 °C		
Qarray Voltage	-20V (Auto)		
PDA Conditions			
Wavelength	260 nm		
Slit Width	1 nm		

■ Results and Discussion

Quantitation by UV and MS

The LCMS-2050 has an extended mass range (*m/z* 2-2000), proving to be beneficial in detecting a range of oligomers with multiple charge states. All the oligomers, ranging from 10 to 60-mer, were injected separately with extended gradient elution. The mass detector with SIM events and UV detector were used for quantitation and mass confirmation.

A linear relationship with an R^2 value of 0.998 was observed for the 30-mer oligonucleotide from 0.1 to 50 µM with absorbance at 260 nm (as depicted in **Figure 1A**). The MS-based calibration curve, generated using the peak area from a SIM scan event for the average mass of the -10-charge state from the intact oligonucleotide, also exhibits a strong fit with an R^2 value of 0.999 (shown in **Figure 1B**). Both the UV and MS calibration curves demonstrated accuracy levels within 30%.

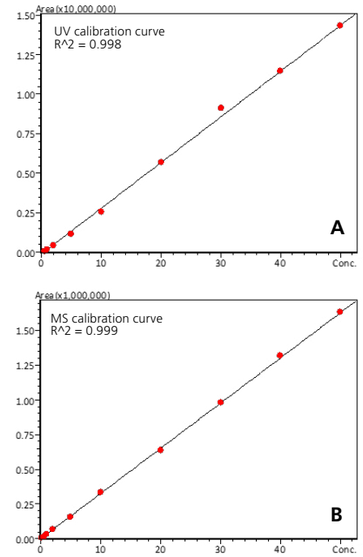

Figure 1: A) UV calibration curve with a linear dynamic range from 0.1-50 µM as measured by peak area at 260 nm. (B) MS calibration curve with a linear dynamic range from 0.5-50 µM as measured by peak area of the -10-charge state (910.2 *m/z*) from the intact oligonucleotide.

Mass Confirmation

Oligomers ranging from 10-60-mer were injected in triplicate at 50 pmol for mass confirmation. Multiple charge states were observed between *m/z* 550-2000 and the most abundant charge state was chosen for each oligomer as shown in **Table 3**. As a single quadruple mass spectrometer is a low-resolution detector, average mass was used for mass confirmation. The data shown in **Table 3** indicates that mass accuracy is ≤0.2 Da for all analyzed oligonucleotides.

The standard deviation (SD) and Relative Standard Deviation (RSD) of observed *m/z* are less than 0.080 and 0.01, respectively, for oligomers ranging from 10-45-mer in length. For longer oligomers, specifically 50-60-mer, the SD and RSD are 0.15 and 0.03, respectively. The overlay of the extraction ion chromatogram (EIC) of the most abundant charge state for each oligomer is shown in **Figure 2**.

Table 3: Mass confirmation for 10-60-mer oligonucleotides using respective high-abundance charge state.

Nucleotide Length	Most abundant charge state (*m/z*)	Theoretical *m/z*	Observed *m/z*			Mean (n=3)	Mass Accuracy	Std. Dev (n=3)	RSD (%)
			run 1	run 2	run 3				
10-mer	-3	997.7	997.6	997.6	997.6	997.6	0.1	<0.01	<0.01
15-mer	-6	753.8	753.8	753.8	753.8	753.8	0.0	<0.01	<0.01
20-mer	-9	668.9	668.9	668.9	668.9	668.9	0.0	<0.01	<0.01
25-mer	-10	756.0	755.9	755.9	756.0	755.9	0.1	0.05	0.01
30-mer	-13	699.9	699.9	699.9	699.9	699.9	0.0	<0.01	<0.01
35-mer	-14	757.6	757.5	757.6	757.6	757.5	0.0	0.04	<0.01
40-mer	-16	762.1	762.1	762.1	762.2	762.1	0.0	0.05	0.01
45-mer	-18	761.2	761.2	761.2	761.3	761.2	0.0	0.08	0.01
50-mer	-19	799.6	799.5	799.8	799.6	799.6	-0.1	0.15	0.02
55-mer	-23	724.5	724.5	724.3	724.5	724.4	0.1	0.12	0.02
60-mer	-23	794.3	793.9	794.3	794.1	794.1	0.2	0.20	0.03

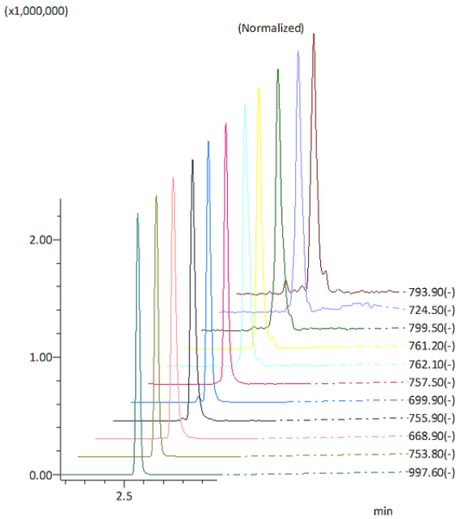

Figure 2: Overlay of replicate 1 EIC of the most abundant charge state for each oligomer from 10-60-mer, represented in **Table 3**.

Impurity Analysis

Potential impurities, such as aborted sequences (N-2, N-3) and solvent adducts, were identified using MS SCAN data from 550-2000 *m/z*. Impurities were identified using extracted ion chromatograms (EIC) with a 100-ppm width. **Figure 3** shows EICs of those impurities and solvent adducts identified for -3 charge state of the 10-mer. Only low levels of N-2 and N-3 were observed. (**Figure 3B** and **3C**, respectively). However, higher abundance of sodium salt adducts $[M+Na^{+}-3H]^{-2}$, (**Figure 3D**) and potassium salt adducts $[M+K^{+}-3H]^{-2}$, (**Figure 3E**) were identified. Trace levels of HFIP adducts from the mobile phase additive were observed (**Figure 3F**) as result of ion source optimization prior to analysis.

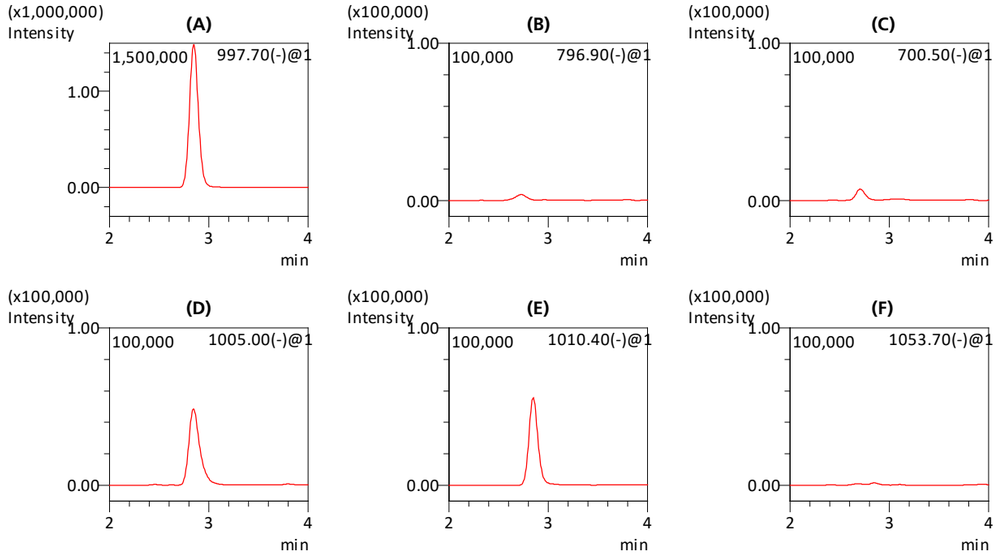

Figure 3: Observed impurities shown in EIC for - 3 charge state of 10-mer.
(A); $[M-3H]^{-3}$, m/z 997.7
(B); -3 charge state of [N-2] 5′ aborted sequence, $[M-3H]^{-3}$ m/z 796.9
(C); -3 charge state of [N-3] 5′ aborted sequence $[M-3H]^{-3}$, m/z 700.5
(D); $[M+Na+-3H]^{-2}$, m/z 1005.0
(E); $[M+K+-3H]^{-2}$, m/z 1010.4
(F); $[M+K+-3H]^{-2}$, m/z 1053.7

■ Conclusion

The Shimadzu single quadrupole LCMS-2050 coupled with a Nexera LC unit with an SPD-M30A PDA detector offers simple and reliable MS and UV-based quality control analysis for oligonucleotides.

MS data shows that a LCMS-2050 can achieve higher mass accuracy for a wide range of oligonucleotides. Additionally, excellent quantitation accuracy is achievable in both MS and UV based analysis under optimized source conditions.

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Application News

MALDI-TOF Mass Spectrometry Analysis MALDI-8020/MALDI-8030

Protein and Peptide Mass Spectrometry Imaging on the MALDI-8020 Benchtop MALDI-TOF Mass Spectrometer

Catherine Rawlins

User Benefits

- Simple imaging analysis of peptides and proteins in the rat brain on an affordable, easy-to-use benchtop MALDI-TOF system
- High quality spectra with class-leading mass resolution and sensitivity producing detailed MALDI images
- Workflow applicable to many tissue types and with different methods of matrix application

■ Introduction

MALDI mass spectrometry imaging (MSI) is a powerful technique that utilizes the capabilities of the spectrometer to collect thousands of individual spectra at various positions on a sample. Subsequently, using dedicated software, the resulting detected ions can be spatially represented as pseudo-colour images to visualize key molecules relative to their position in the sample / tissue.

The MALDI-8020 linear time-of-flight (TOF) mass spectrometer (Fig. 1) is capable of generating quality MS images, such as lipids in rat brains. The ability to rapidly exchange sample plates in the instrument (<3 mins) and the short 'instrument-ready' time mean that acquisitions can be quickly started, which is advantageous during optimisation of imaging methods. We have previously demonstrated MALDI imaging for a variety of target analytes on the MALDI-TOF benchtop system with the analysis of fingerprints, soybeans and PET films (Shimadzu Application News; 01-00389-EN and 01-00392-EN). Here, we demonstrate the capability of our imaging platform to easily achieve quality mass spectra and MS images for intact proteins from tissue sections and for peptides following on-tissue digestion, with linear mode analysis of rat brain tissue.

Fig. 1 MALDI-8020 Benchtop linear TOF mass spectrometer

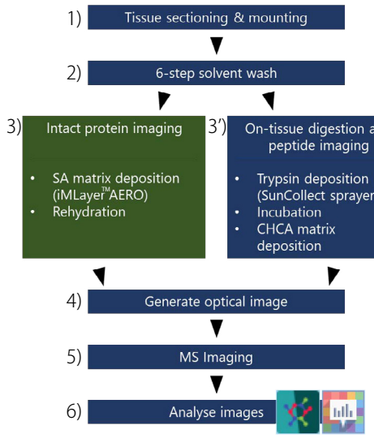

Fig. 2 Workflow for protein and peptide MS imaging with Sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) matrices, respectively

■ Measurement Conditions and Samples

Rat brain samples embedded in CMC media were prepared on FlexiVision-mini ITO Slides by AMSBIO (Oxford, UK). The slide was stored at -80 °C and vacuum desiccated to room temperature, prior to sample processing.

To prepare the tissue for peptide and protein analysis (Fig. 2-2), the lipids and salts need to be removed as they will dominate the mass spectrum and suppress the signal of the proteins. For delipidation, the slides were washed using a 6-step solvent wash protocol [1] : 1) 30 seconds in 70% ethanol, 2) 30 seconds in 100% ethanol, 3) 2 minutes in Carnoy's solution (60:30:10 of ethanol:chloroform:acetic acid), 4) 30 seconds in 100% ethanol, 5) 30 seconds in H_2O, and 6) 30 seconds in 100% ethanol. After washing, the slides were vacuum desiccated before matrix deposition.

For on-tissue digestion, porcine trypsin (67 ng/µL in 90:10 of 20 mM ammonium bicarbonate:acetonitrile (ACN)) was deposited on tissue using a SunCollect sprayer (SunChrom, Germany). The slide was then placed in a rehydration chamber with 50:50 ACN:H_2O at 37 °C for 18 hours. After the incubation, α-Cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL in 50:50 ACN:0.1 % trifluoroacetic acid (TFA)) was applied, again using the SunCollect sprayer, followed by rehydration for 2 minutes with no solvent and then 3 minutes with 5% acetic acid at 85 °C . Rehydration extracts analytes to the surface.

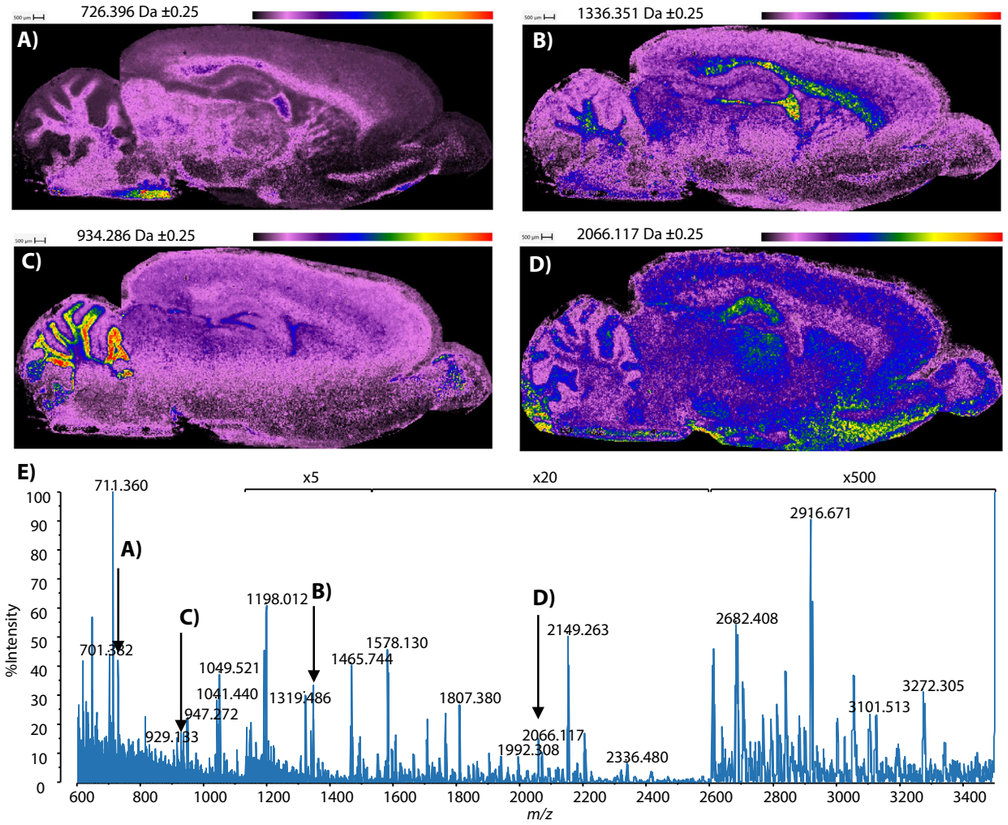

Fig. 3 MS Images of digested peptides: Tentative assignments to A) MBP peptide: HGFLPR, T160-165 (m/z 726.394), B) MBP peptide: YLATASTMDHAR, T148-159 (m/z 1336.633) localized in the white matter [4], C) & D) unknown peptides localized in the cerebellum and grey matter respectively, E) TIC spectrum of rat brain peptides

For intact protein analysis, sinapinic acid (10 mg/mL in 70:30 ACN: 0.1% TFA) was sprayed onto the tissue using an iMLayer™ AERO automated spraying device (Shimadzu, Japan). The tissue was coated with 8 layers at 50 mm/s and 4 layers at 30 mm/s. Following coating, the slide was placed in a rehydration chamber with 5% acetic acid for 3.5 minutes at 85 °C .

Samples were analysed at 50 µm spatial resolution (50 µm stage step size) in linear mode on a benchtop MALDI-TOF instrument (MALDI-8020, Shimadzu Corporation).

The acquisition method used for the peptides was 30 shots/profile, 200 Hz repetition rate, mass range 300-3000 m/z, and pulsed extraction at 2700 Da. There was a total of 67,482 profiles and the acquisition period was 2 hrs 50 mins.

The acquisition method used for the proteins was 20 shots/profile, 100 Hz repetition rate, mass range 3000-30000 m/z, and pulsed extraction at 22000 Da. There was a total of 68,836 profiles and the acquisition period was 3 hrs 47 mins.

Data was analyzed in IonView™ software (Shimadzu Corporation).

■ Results of peptide and protein imaging

In an earlier work (not shown), on-tissue digestion and protein imaging protocols were successfully validated on a MALDI-TOF-TOF (MALDI-7090, (Shimadzu Corporation)) instrument. In this work, we have transferred these methods to a benchtop linear MALDI-TOF MS instrument (MALDI-8020) .

MSI of the trypsin digestion of the rat brain showed that on the MALDI linear TOF system it was still possible to tentatively assign peptides based on mass (in the absence of MS/MS), to commonly observed rat brain proteins, e.g. myelin basic protein (MBP) is characteristically localized in the white matter, and this was consistent with the MALDI images obtained (Fig. 3) [2].

Intact protein MSI similarly revealed analytes corresponding to common proteins in the rat brain (e.g. MBP) (Fig. 4). The tentative protein identifications in Fig. 4 are based on observed mass of the detected species [3,4]. It was possible to obtain data at much higher masses compared to routine lipid MSI analysis showing suitability of this workflow for protein imaging. Signals beyond 16 kDa were low, but this was due to the embedding media used to improve the sectioning of the rat brain specimen [5].

The quick acquisition times of 2 hrs 50 mins and 3 hrs 47 mins respectively for the peptide and intact protein imaging of the full rat brains facilitated optimisation of the method.

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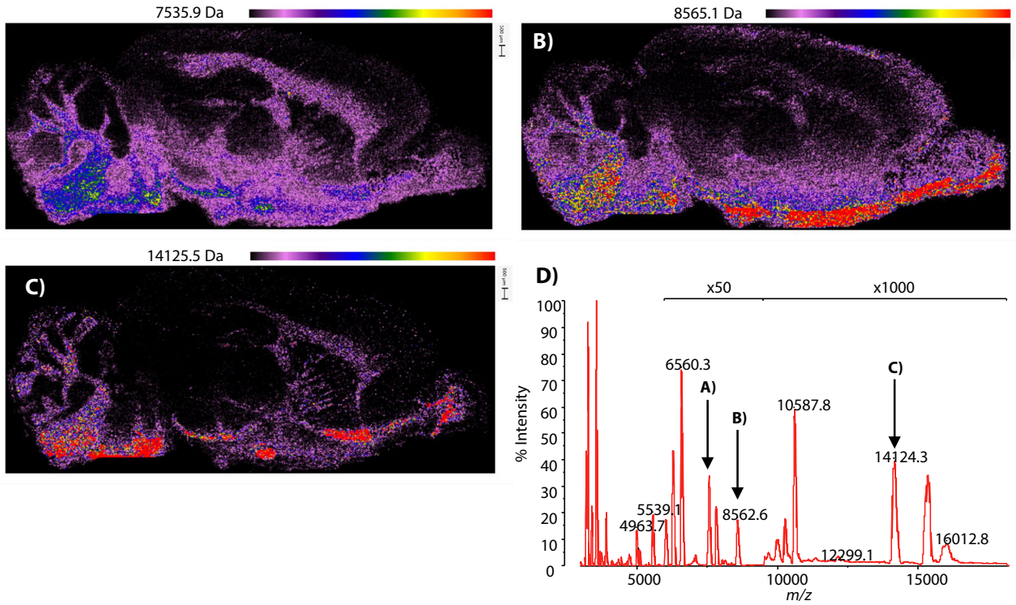

Fig. 4 MS Images of intact proteins: Tentative assignments to A) Neurogranin (m/z 7537), B) Ubiquitin (m/z 8565), C) Myelin Basic Protein (m/z 14124), D) TIC spectrum of rat brain proteins

■ Conclusion

We have demonstrated the capability of a low-cost benchtop MALDI-TOF instrument for MALDI mass spectrometric imaging, for the *in situ* analysis of peptides and proteins.

The quick sample plate exchange in the benchtop MALDI instrument (<3 mins) and fast acquisition times (2 hrs 50 mins at 200 Hz laser speed for a full rat brain spanning 67,482 profiles) facilitated development of the imaging method.

Proteins up to 16 kDa were easily detected using a simple workflow showing suitability for protein imaging.

MSI of peptides detected from the on-tissue digestion were shown on MALDI images to be consistent with characteristic localization of the corresponding proteins, showing suitability for peptide imaging and applications targeting the distribution of similar high mass species in tissue.

MS/MS identification of the peptides would be required for confirmation, but this application note suitably demonstrates protein and peptide MSI on the MALDI-8020 would be useful for screening applications.

This economical, compact, and robust instrument is ideal for those new to MALDI imaging and would represent a great resource for universities and teaching laboratories.

■ References

[1] Yang et al (2011) Matrix sublimation/recrystallization for imaging proteins by mass spectrometry at high resolution. Anal. Chem. 83, 5728-5734.

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[4] Groseclose et al (2007) Identification of proteins directly from tissue: in situ tryptic digestions coupled with imaging mass spectrometry. J. Mass Spectrom. 42, 254–262

[5] Franck & Rawlins et al (2010) MALDI mass spectrometry imaging of proteins exceeding 30000 Da. Med. Sci. Monit., 16(9): BR293 299.

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Liquid Chromatograph Mass Spectrometer LCMS-9050

Application News

Analysis of mRNA 5′ Cap Structure Using a Quadrupole Time-of-Flight Mass Spectrometer

Junna Nakazono

User Benefits

- The LCMS-9050 quadrupole time-of-flight mass spectrometer and the LabSolutions Insight™ Biologics analysis software enable characterization of the 5′ cap structure of mRNA.
- The software enables simple confirmation of the fragment coverage of nucleic acids and their impurities.
- The software enables analysis of modifications and impurities that are set by the user.

■ Introduction

There has been increased attention on the new drug discovery modality of mRNA because of its efficacy in COVID-19 vaccines, and its applications for vaccines and other pharmaceuticals are expected to grow. Currently authorized mRNA vaccines are synthesized using *in vitro* transcription to add the Cap-1 structure (m7GpppRm-) on the 5′ end. This modification contributes to mRNA recognition, better efficiency of translation, and stability of mRNA in cell, making 5′ cap structure analysis an important element of mRNA quality controls.

This Application News presents a study of mRNA 5′ cap structure using the LCMS-9050 quadrupole time-of-flight mass spectrometer and LabSolutions Insight Biologics analysis software.

■ Samples

Given that mRNA is a large molecule, LC/MS analysis is typically done by analyzing fragments generated by cleavage enzyme reactions. In this study, the model sample consisted of Cap-1 structure mRNA with 36 bases (Cap-1 groups) obtained by *in vitro* transcription using plasmid DNA as a template. The 5′ cap modified unreacted RNA (pppR-) was also provided for analysis as an impurity.

■ Analytical Conditions

Analysis was performed using the Nexera™ XS inert and LCMS-9050 systems in Data Dependent Acquisition (DDA) mode. The analytical conditions are shown in Table 1.

Table 1 Analytical Conditions

UHPLC (Nexera XS inert)	
Column:	Shim-pack Scepter™ Claris C18-120* (150 mm × 2.1 mm I.D., 1.9 µm)
Mobile Phase A:	95 mM HFIP, 5 mM DIPEA - water
Mobile Phase B:	70 mM HFIP, 5 mM DIPEA, 65% acetonitrile – water
Gradient Program:	B Conc. 5% (0-2 min) – 25% (22 min) – 90% (23-24 min) – 5% (24.1 -30 min)
Flowrate:	0.3 mL/min
Column Temp.:	60 °C
Injection Volume:	5 µL
MS (LCMS-9050)	
Ionization:	ESI negative
Mode:	MS *m/z* 550-2500, MS/MS (DDA) *m/z* 100-2500
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
Heating Gas Flow:	10.0 L/min
Interface Temp.:	250 °C
DL Temp.:	250 °C
Block Heater Temp.:	400 °C

* P/N 227-31210-03

■ Setting the Analysis Parameters

LabSolutions Insight Biologics software is used to analyze nucleic acids and their impurities. First, the user creates a nucleic acid sequence in the parameter configuration window using the software presets for nucleobases, linkers, ribose, and modifications. Nucleobases, linkers, ribose, and base modifications can be added and removed in each tab as required. Once a sequence is entered, the software displays the molecular formula, monoisotopic mass, and structural formula (Fig. 1).

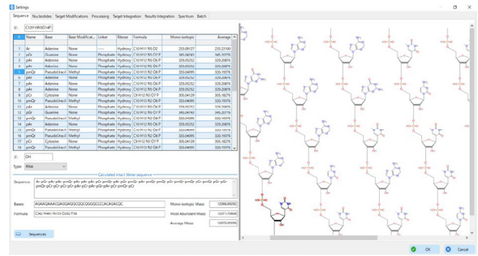

Fig. 1 Parameter Configuration Window

The Target Modifications tab is also used to select the anticipated impurities. In addition to impurities, such as different strand lengths, missing nucleobases, depurination/depyrimidination, deamination, and protecting groups, as well as adduct ions and unknown modifying groups, the software can also search for molecular changes added by the user. To enable detection of the 5′ cap modified unreacted group as the impurity in this study, "5′ uncapped" was added as the target modification (Fig. 2).

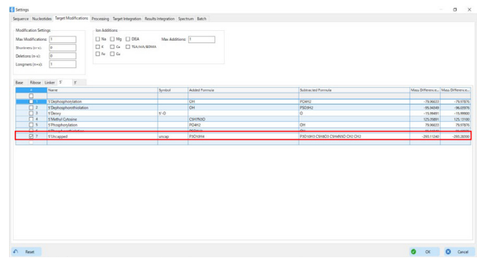

Fig. 2 Setting the Target Modification

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■ Identifying Cap-1 and Unreacted Groups

Fig. 3 shows the component chromatogram of analyzed samples obtained by mixing Cap-1 group and the unreacted group by 0.5 µg each. The mass chromatogram is displayed as a component chromatogram, based on MS1 spectra and by combining signals from different valences and isotopes. Fig. 4 shows a result of multivalent ion analysis. Metal adducts were also detected. Moreover, longmers with an additional cytidine (C) (N+1 groups) were detected in the Cap-1 group and the unreacted group at ratios of 6.6 and 7.9%, respectively (Fig. 5). These groups were putatively identified as secondary reaction products generated by *in vitro* transcription.

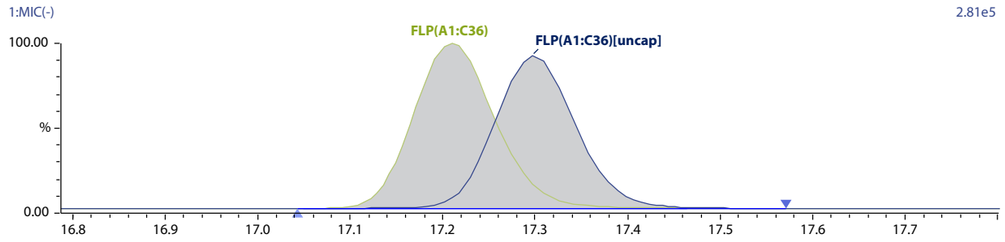

Fig. 3 Component Chromatograms of the Cap-1 Group and the Unreacted Group Mixture
FLP (A1:C36) indicates the Cap-1 group, and FLP (A1:36)[uncap] indicates the unreacted group.

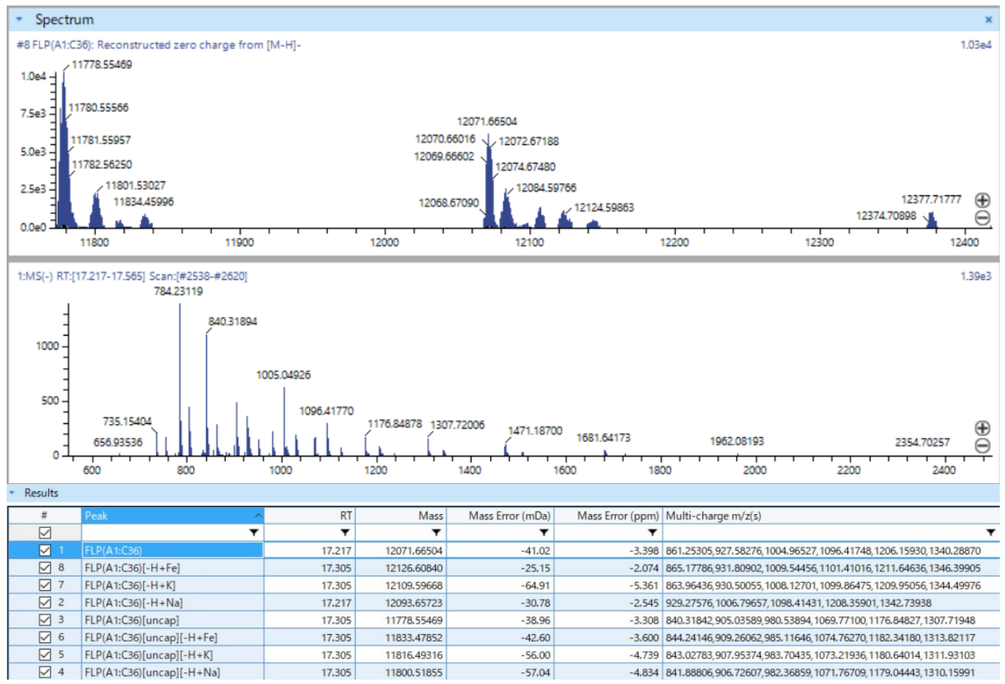

Fig. 4 Results of Multivalent Ion Analysis
Top: Deconvoluted mass spectrum; Center: Mass spectrum; Bottom: Identification results

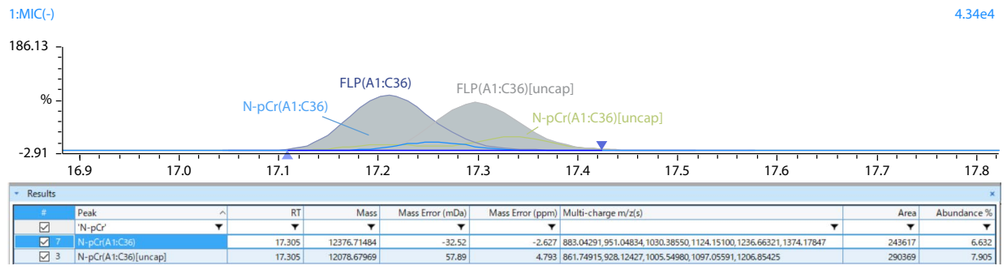

Fig. 5 Results of N+1 Group Identification in Cap-1 Group and Unreacted Groups Mixture
N-pCr (A1:C36) and N-pCr (A1:C36)[uncap] indicate the N+1 groups for the Cap-1 group and the unreacted group, respectively.

■ Sequence Identification

Fig. 6 shows the results of sequence coverage in the unreacted group, based on fragment ion information in MS/MS spectra. Upon identification of the selected target modification, the position of the modification is displayed in red. Identification of the N+1 group in the unreacted group also revealed characteristic fragment ions, including 3′ side C additions (Fig. 7). The detection of different strand lengths is indicated by the red inverse triangles.

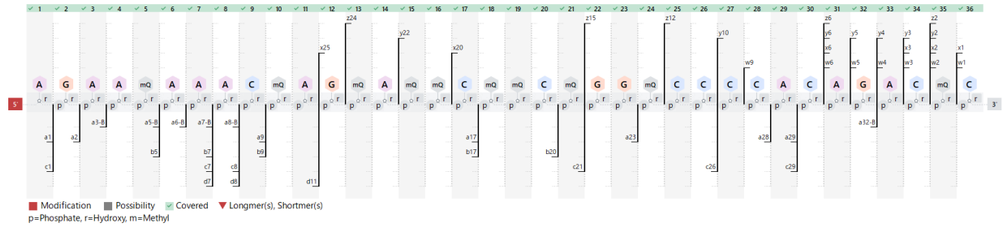

Fig. 6 Sequence Coverage of the Unreacted Group

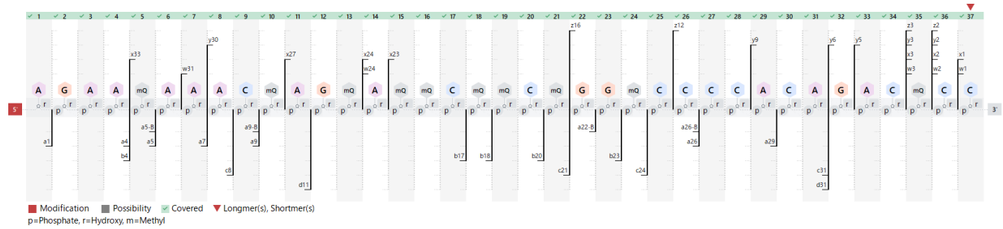

Fig. 7 Sequence Coverage of the N+1 Group in the Unreacted Group

■ Impurity Quantitation

Analysis of the samples, which were obtained by spiking the Cap-1 group with the unreacted group at concentrations of 0, 5, and 10%(w/w), was performed with the LabSolutions Insight Biologics software. The analysis showed the areas value had good linearity ($R^2 > 0.99$) (Fig. 8).

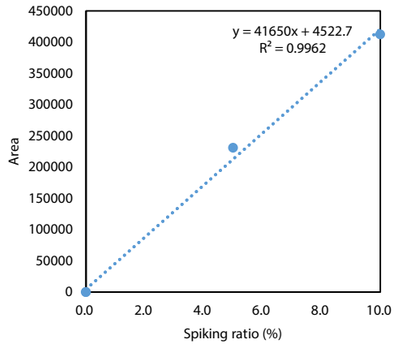

Fig. 8 Linearity of Unreacted Group (Impurity) Area Values

■ Conclusion

In this study, molecular weight identification and sequence analysis of 5′ cap modified mRNA was performed using the high-resolution LCMS-9050 mass spectrometer and LabSolutions Insight Biologics software. This instrument and software also enabled detection and sequence identification of impurities (N+1 group), in addition to detecting the Cap-1 and unreacted groups. LabSolutions Insight Biologics software simplifies the identification of modifications and deletions and additions with different strand lengths via the sequence coverage window.

The software also yielded area values of impurity with good linearity in the samples obtained by spiking the Cap-1 group with the unreacted group at concentrations of 0, 5, and 10%.

Related Applications

1. An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software
   Application News No. 01-00595A-EN
2. Simple Analysis of Impurities in Oligonucleotide Therapeutics Using a Single Quadrupole Mass Spectrometer
   Application News No. 01-00656-EN

01-00733-EN First Edition: Jun. 2024

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Application News

Liquid Chromatograph Mass Spectrometer LCMS-2050

Analysis of mRNA 5′ Cap Structure Using a Single Quadrupole Mass Spectrometer

Junna Nakazono

User Benefits

- The LCMS-2050 single quadrupole (SQ) mass spectrometer and the LabSolutions Insight™ Biologics analysis software can confirm the molecular weights of nucleic acids.
- The LCMS-2050 enables a user-friendly operation that is similar to LC systems.
- The software enables users to set analytical parameters for modifications and impurities as desired.

■ Introduction

There has been increasing attention regarding the new drug discovery modality of mRNA because of its efficacy for COVID-19 vaccines. Currently, authorized mRNA vaccines are synthesized using *in vitro* transcriptions to add the Cap-1 structure (m7GpppRm-) on the 5′ end. This modification contributes to mRNA recognition, better efficiency of translation, and mRNA stability in cells, making 5′ cap structure analysis an important element of mRNA quality controls.

The quadrupole time-of-flight mass spectrometer that was introduced in the Application News No. 01-00733 is useful for sequence coverage identification of nucleic acids that SQ cannot perform. However, SQ mass spectrometers are easy to use and similar to operate as an LC, so they are in increasing demand for confirming molecular weights, such as for quality controls. Below is an introduction to 5′ capped mRNA analysis using the LCMS-2050 SQ mass spectrometer and LabSolutions Insight Biologics analysis software.

■ Samples

Given that mRNA is a large molecule, LC/MS analysis is typically done by analyzing fragments generated by cleavage enzyme reactions. In this study, the model samples consisted of Cap-1 structure mRNA with 36 bases (Cap-1 groups) obtained by *in vitro* transcription using plasmid DNA as a template. The 5′ cap modified unreacted RNA (pppR-) was also provided for analysis as an impurity.

■ Analytical Conditions

Analysis was performed using the Nexera™ XS inert and LCMS-2050 systems. The analytical conditions are shown in Table 1. The LCMS-2050 is equipped with a heated DUIS™ ion source for ionization, which combines the advantages of both ESI and APCI.

Table 1 Analytical Conditions

UHPLC (Nexera XS inert)	
Column:	Shim-pack Scepter™ Claris C18-120* (150 mm × 2.1 mm I.D., 1.9 µm)
Mobile Phase A:	95 mM HFIP, 5 mM DIPEA – water
Mobile Phase B:	70 mM HFIP, 5 mM DIPEA, 65 % acetonitrile – water
Gradient Program:	B Conc. 5 % (0 - 2 min) – 25 % (22 min) – 90 % (23 - 24 min) – 5 % (24.1 - 30 min)
Flowrate:	0.3 mL/min
Column Temp.:	60 °C
Injection Volume:	5 µL

MS (LCMS-2050)	
Ionization:	ESI/APCI (DUIS) negative
Interface Voltage:	-2.0 kV
Mode:	MS *m/z* 550-2000
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	7.0 L/min
Desolvation Temp.:	250 °C
DL Temp.:	200 °C

* P/N 227-31210-03

■ Setting the Analysis Parameters

LabSolutions Insight Biologics software can analyze nucleic acids and their impurities. First, the user creates a nucleic acid sequence in the parameter configuration window using the software presets for nucleobases, linkers, ribose, and modifications. Nucleobases, linkers, ribose, and base modifications can be added and removed in each tab as required. Once a sequence is entered, the software displays the molecular formula monoisotopic mass, and structural formula (Fig. 1).

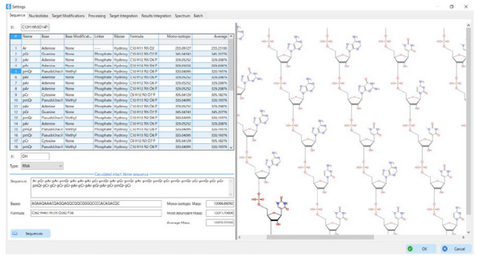


Fig. 1 Parameter Configuration Window

The Target Modifications tab is also used to select the anticipated impurities. In addition to impurities such as different strand lengths, missing nucleobases, depurination/depyrimidination, deamination, protecting groups, additional ions, and unknown modifying groups, the software can also search for molecular changes added by the user as desired. In this study, to enable detection of the 5′ cap modified unreacted group as the impurity, "5′ uncapped" was added as the target modification (Fig. 2).

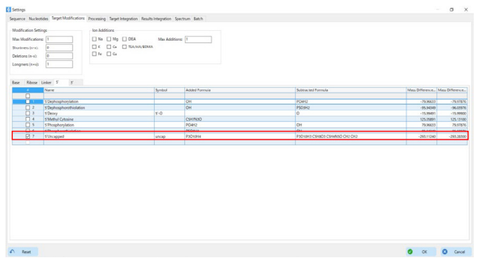


Fig. 2 Setting the Target Modification

■ Identifying Cap-1 and Unreacted Groups

Fig. 3 shows the component chromatogram of analyzed samples obtained by spiking Cap-1 group (0.5 µg) with the unreacted group at 10 % (w/w). The mass chromatogram is displayed as a component chromatogram, based on MS1 spectra and by combining signals from different valences and isotopes. Fig. 4 shows the multivalent ion analysis for the Cap-1 group. Both Cap-1 and unreacted groups were detected with a mass error of less than 1 Da compared to the theoretical molecular weight value.

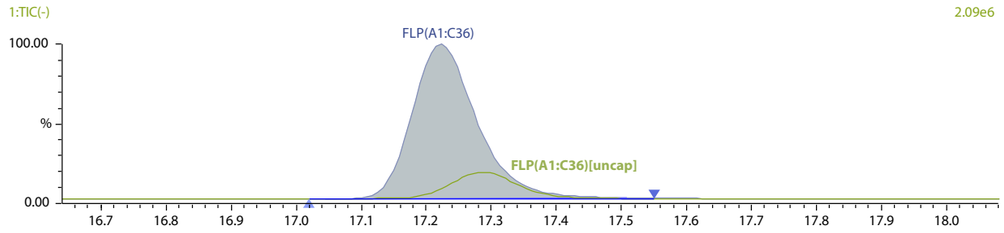
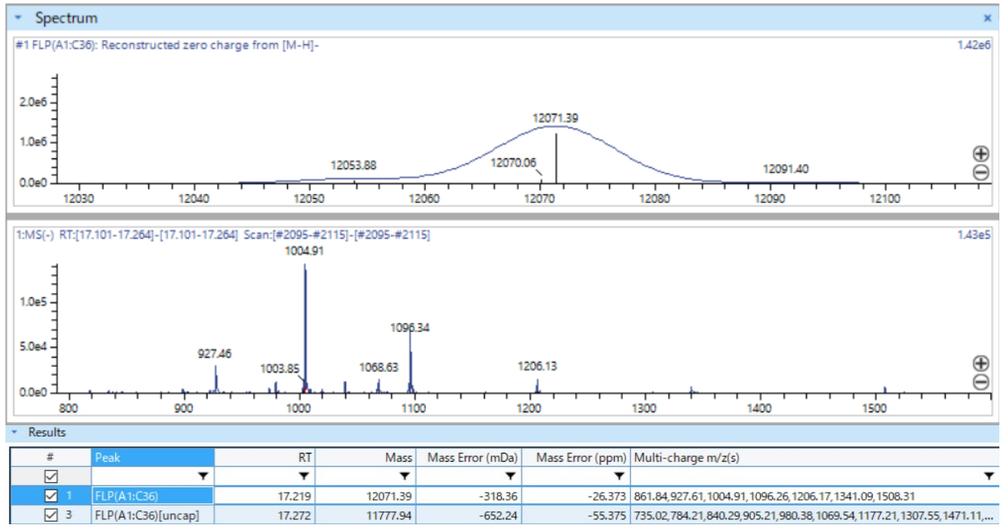


Fig. 3 Component Chromatograms of the Cap-1 Group and the Unreacted Group
FLP (A1:C36) indicates the Cap-1 group, and FLP (A1:36)[uncap] indicates the unreacted group.



#	Peak	RT	Mass	Mass Error (mDa)	Mass Error (ppm)	Multi-charge m/z(s)
☑ 1	FLP(A1:C36)	17.219	12071.39	-318.36	-26.373	861.84,927.61,1004.91,1096.26,1206.17,1341.09,1508.31
☑ 3	FLP(A1:C36)[uncap]	17.272	11777.94	-652.24	-55.375	735.02,784.21,840.29,905.21,980.38,1069.54,1177.21,1307.55,1471.11,...

Fig. 4 Results of Multivalent Ion Analysis
Top: Deconvoluted mass spectrum; Center: Mass spectrum; Bottom: Identification results

■ Conclusion

In this study, molecular weight identification of 5′ cap modified mRNA was performed using the LCMS-2050 mass spectrometer and LabSolutions Insight Biologics software. Both Cap-1 and unreacted groups were detected with a mass error of less than 1 Da compared to the theoretical molecular weight value. The LCMS-2050 demonstrated it is easy to use and similar to operate as an LC, making it suitable for confirming molecular weights such as for quality controls.

Related Applications

1. Analysis of mRNA 5′ Cap Structure Using a Quadrupole Time-of-Flight Mass Spectrometer
   Application News No. 01-00733-EN
2. Simple Analysis of Impurities in Oligonucleotide Therapeutics Using a Single Quadrupole Mass Spectrometer
   Application News No. 01-00656-EN

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UV-1900i/UV-2600i/UV-2700i UV-Vis Spectrophotometers

Application News

Thermal Stability Analysis of Nucleic acid Drugs by New Tm Analysis System

Akari Goto

User Benefits

- Easily determine the temperature at which 50 % of double-stranded nucleic acids dissociate into single strands (Tm value).
- Automate annealing (pretreatment) and Tm value analysis using the average and derivative methods.
- Achieve the industry's highest data integrity when linked to the LabSolutions™ DB/CS system.

■ Analytical Method

Nucleic acid drugs are a generic term for drugs made of oligonucleic acids consisting of a dozen or more bases of nucleic acid or modified nucleic acid linked together and manufactured by chemical synthesis. Nucleic acid drugs have been actively developed in recent years as next-generation innovative therapeutics because they can target molecules such as mRNA that conventional low-molecular-weight drugs and antibody drugs cannot.

Melting temperature (Tm value), an indicator of thermal stability, plays an important role in nucleic acid drug development and quality control. The Tm value is the temperature at which 50 % of double-stranded nucleic acids dissociate into single strands, and generally the higher the temperature, the more thermally stable it is.

In this study, we performed thermal stability analysis (Tm analysis) of nucleic acids using the UV-2600i UV-visible spectrophotometer and a new Tm analysis system. Using this system enables the Tm value of nucleic acids to be easily calculated.

■ Tm Analysis System

The Tm analysis system comprises a UV-visible spectrophotometer*1 shown in Fig. 1, a TMSPC™-8i 8-cell thermoelectrically temperature-controlled cell holder, and the LabSolutions UV-Vis Tm software shown in Fig. 2. Tm analysis is a method of analysis in which the temperature of the nucleic acids is increased, and the change in absorbance (melting curve) is measured to determine the Tm value. The procedure used to determine the Tm value is (1) check the absorption spectrum, (2) anneal the sample, (3) measure the melting curve, and (4) perform analysis as shown in Fig. 3. However, using this system, the work from annealing (2) to analysis (4) can be automated using the same software, which greatly improves efficiency. In addition, data management is easy because the absorption spectrum (1), the melting curve (3), annealing (2), and analysis conditions (4) are stored as a single file. Linking this system with the Shimadzu LabSolutions DB/CS system further improves data integrity.

*1: Fig. 1 shows UV -2600i and TMSPC-8i, but it can also be combined with UV-1900i/ 2700i.

Fig. 1 UV -2600i and 8-Series Thermoelectrically-Controlled Cell Holder TMSPC™-8i

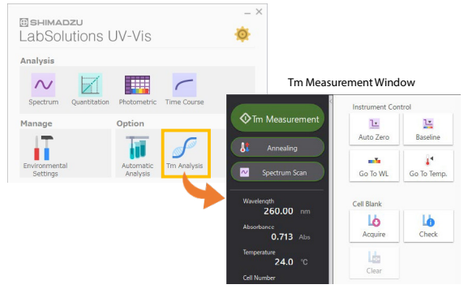

Fig. 2 LabSolutions™ UV-Vis Tm Software

Fig. 3 Tm Measurement Procedure

■ Analytical Method

This study used the nucleic acid M13 primer, prepared at 12 µM in buffer (17 mM NaCl, 10 mM phosphate buffer) as the sample. The sample solution was degassed beforehand to prevent air bubbles from forming during measurements at high temperature. Two optical pathlengths, 10 mm and 1 mm, are available with the dedicated 8-cell micro multi-cell and can be selected depending on the sample absorbance (concentration). In this experiment, a cell with a 1 mm optical pathlength was used. The samples were measured under the conditions shown in Table 1.

Table 1 Measurement Conditions

Instruments:	UV-2600i TMSPC-8i
Measuring Wavelength:	260 nm
Measuring Wavelength (for Calibration):	320 nm
Slit Width:	2.0 nm
Temperature Range:	15 – 90 °C
Acquisition Rate:	1 °C
Temperature Changing Speed:	1 °C/min

Fig. 4 shows the parameter setting window. To perform annealing, set the temperature from panel (1). Click the "Settings" button for the temperature program in panel (2) and enter parameters such as start and target temperatures and waiting time on the temperature program window (Fig. 5). This window allows the operator to simulate the temperature program and check how much time is required. To automatically analyze Tm values after measurement, the analysis method can be set in panel (3). The analysis methods available are the average method, which determines the Tm value from the intersection of the midline of the tangent to the melting curve, and the derivative method, which determines the Tm value from the maximum value of the first derivative. All the conditions set here can be saved as a template, eliminating the need to set up the conditions again the next time.

After setting the parameters, the entire process, from annealing to measurement and analysis, can be performed automatically by clicking the "Tm Measurement" button.

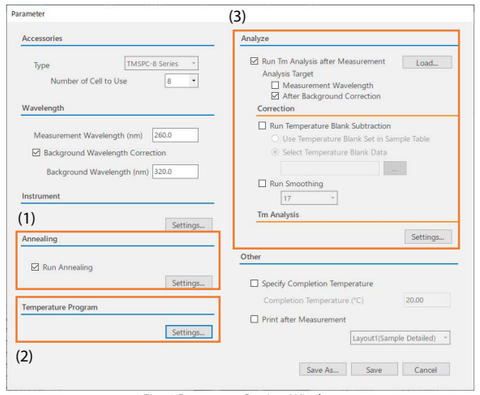

Fig. 4 Parameter Setting Window

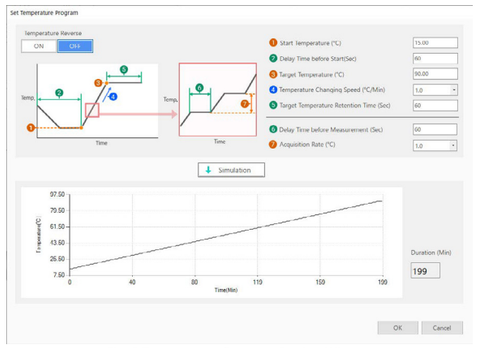

Fig. 5 Temperature Program Setting Window

■ Measurement Results

Fig. 6 shows the absorption spectrum of the sample. Tab switching ((4) in Fig. 6) allows the user to switch between the absorption spectrum and the melting curve and check them.

Fig. 7 shows the melting curves analyzed by the two methods, the average method and the derivative method, and Table 2 shows the Tm values calculated by each analysis method.

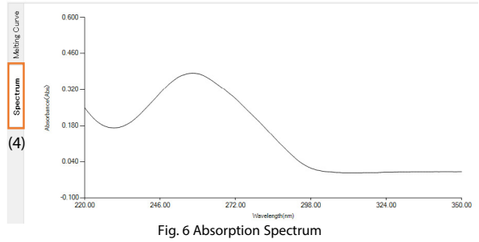

Fig. 6 Absorption Spectrum

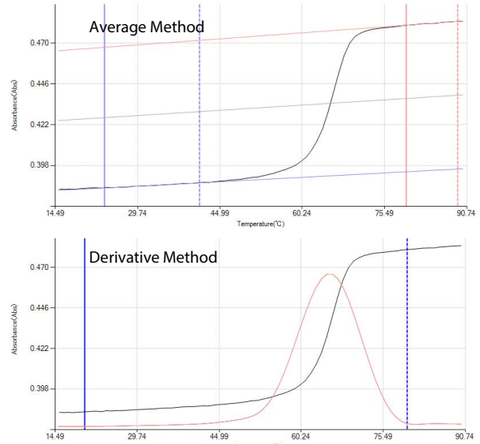

Fig. 7 Melting Curve

Table 2 Measurement Results of the Sample

Analysis Method	Tm value (°C)
Average Method	65.47
Derivative Method	65.98

As shown in Table 2, the Tm values obtained by the average method were 65.47 °C, and those obtained by the derivative method were 65.98 °C. The Tm values obtained by both methods were very close.

■ Conclusion

In this study, we performed thermal stability analysis (Tm analysis) of nucleic acids using a UV-2600i UV-vis spectrophotometer and the Tm analysis system. This system can calculate the Tm value of nucleic acid drugs efficiently and easily because the process from annealing to analysis can be performed automatically.

This system, which streamlines the procedure for determining Tm values, is expected to dramatically accelerate the development of nucleic acid drugs.

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01-00484-EN First Edition: Apr. 2023

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Application News

Nexera™ XS inert High Performance Liquid Chromatograph
LCMS-2050 Liquid Chromatograph Mass Spectrometer

Simple Analysis of Impurities in Oligonucleotide Therapeutics Using a Single Quadrupole Mass Spectrometer

Takanari Hattori, Noriko Kato, and Junna Nakazono

User Benefits

- Oligonucleotides and related impurities can be easily analyzed using a Nexera XS inert UHPLC system and a LCMS-2050 single quadrupole mass spectrometer.
- The molecular weight of impurities can be estimated with high precision by deconvoluting the resulting mass spectra.

■ Introduction

Oligonucleotide therapeutics have attracted attention in recent years as a new modality for drug discovery, because they can be used to create disease-specific therapeutic agents and can be designed easily by chemical synthesis. Typically, they are composed of oligonucleotides with about a dozen to several dozen bases (including modified bases). However, the development of analytical methods for quality assurance and standardization is still in progress. Quality control requires analyzing impurities, such as by-products, unreacted residues, and degradation products, in addition to the principal components. HPLC-UV is commonly used for purity confirmation, but if impurities are detected, they must be checked to confirm whether they are known impurities or not. Mass spectrometry, which provides molecular weight information, is a valuable analytical tool in such cases. This article describes an analysis of oligonucleotides and related impurities using an inert UHPLC system and a single quadrupole mass spectrometer.

■ Samples

A 20-mer oligonucleotide and three related impurities were synthesized as a model sample of antisense oligonucleotide. The sequences of each oligonucleotide are shown in Table 1. The full-length product (FLP) and three related impurities were mixed and analyzed. The impurities included an n-1(3′) deletion missing 1 nucleotide from the 3′ end, an n-3(3′) deletion missing 3 nucleotides from the 3′ end, and an n-10(5′) deletion missing 10 nucleotides from the 5′ end.

Table 1 Sample Information

Name	Sequence (5′→3′)	Length
FLP	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	20 mer
n-1 (3′)	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*	19 mer
n-3 (3′)	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*	17 mer
n-10 (5′)	dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	10 mer

Note: * = 2′-O-methoxyethyl, m = 5-methyl, and d = 2′-deoxy

■ Instruments and Analysis Conditions

A Nexera XS inert UHPLC system with a Shim-pack Scepter™ Claris C18-300 inert column was used to reduce sample adsorption. High-pressure gradient analysis was performed using mobile phases consisting of ultrapure water containing HFIP and triethylamine and methanol. For mass spectrometry, a compact, easy to use, and high-performance LCMS-2050 single quadrupole mass spectrometer was used. The LCMS-2050 is equipped with a heated DUIS™ ion source for ionization, which combines the advantages of both ESI and APCI. It covers a mass range of *m/z* 2 to 2,000, making it suitable for analyzing oligonucleotide therapeutics with a large molecular weight (MW). The analysis conditions are shown in Table 2.

Fig. 1 Nexera™ XS inert and LCMS-2050 Systems

Table 2 Analysis Conditions

HPLC conditions (Nexera XS inert)	
Column:	Shim-pack Scepter Claris C18-300*1 (100 mm × 2.1 mm I.D., 1.9 µm)
Flow Rate:	0.3 mL/min
Mobile Phases:	A) 100 mmol/L HFIP and 10 mmol/L TEA in water
Mobile Phase:	B) Methanol
Time Program:	10 % B (0 min) → 35 % B (15 min) → 40 % B (20 min) → 90 % B (20.1 to 22 min) → 10 % B (22.1 to 26 min)
Column Temp.:	60 °C
UV Detection:	190 to 400 nm
Injection Volume:	6 µL
MS Conditions (LCMS-2050)	
Ionization:	ESI/APCI (DUIS), negative mode
Interface Voltage:	-3.0 kV
Mode:	Scan (*m/z* 550-2000)
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	7.0 L/min
Desolvation Temp.:	450 °C
DL Temp.:	200 °C

*1 P/N: 227-31209-02

■ Results

Fig. 2 shows the UV (260 nm) and TIC chromatograms of the model oligonucleotides. Peaks were confirmed in the order of n-10 (5′), n-3 (3′), n-1 (3′), and FLP. The mass spectra of impurities and FLP are shown in Fig. 3. Multiply-charged ions (3 to 11 charges) were detected. The mass spectra of each peak were deconvoluted to estimate the molecular weights (Fig. 4). That resulted in estimated molecular weights of 3552 for n-10 (5′) (theoretical MW: 3553), 5986 for n-3 (3′) (theoretical MW: 5987), 6776 for n-1 (3′) (theoretical MW: 6776), and 7169 for the FLP (theoretical MW: 7169). These results showed small mass errors from the theoretical values, indicating high accuracy of the LCMS-2050 system.

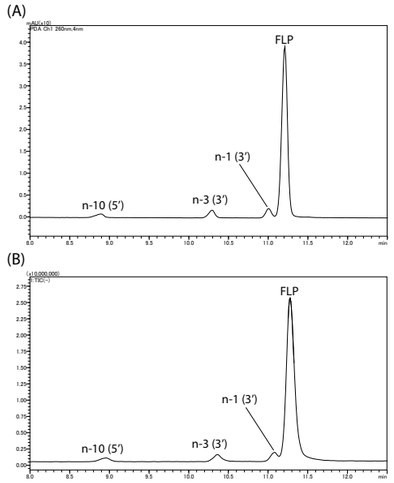

Fig. 2 Chromatograms of Model Oligonucleotide
(A) UV Chromatogram, (B) TIC Chromatogram

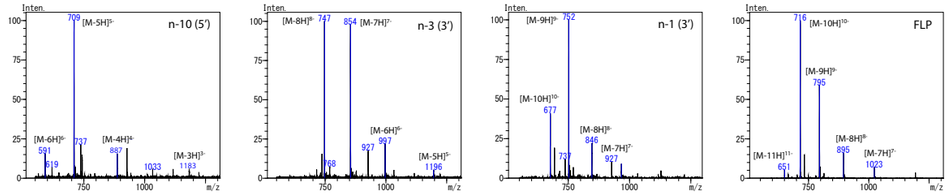

Fig. 3 Mass Spectra of Impurities and FLP

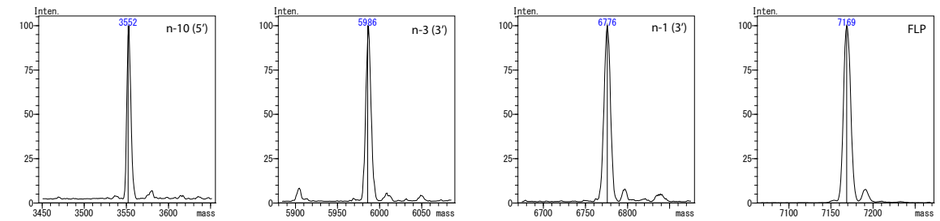

Fig. 4 Deconvolved Mass Spectra

■ Conclusion

By using the Nexera XS inert UHPLC system and the LCMS-2050 single quadrupole mass spectrometer, the principal component and related impurities of the model oligonucleotide were successfully analyzed. The deconvolution of the mass spectra of each peak allowed for accurate estimation of their molecular weights, showing small mass errors from the theoretical values. The LCMS-2050 enables fast and highly sensitive analysis across a wide mass range, while maintaining a user-friendly operability similar to LC systems. The system provides a useful analytical tool for quality control of oligonucleotide therapeutics that is accessible to users with or without prior experience in mass spectrometry. For comprehensive impurity identification and sequence analysis of oligonucleotides, analysis using a quadrupole time-of-flight (Q-TOF) LC-MS system, as introduced in Application News No. 01-00595A-EN, is recommended. With dedicated analysis software, it allows for comprehensive impurity detection and precise sequencing based on accurate mass measurements.

Related Applications

1. Efficient Method Development of Oligonucleotides by Reversed-Phase Ion-Pair Chromatography, Application News No.01-00558-EN
2. An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software, Application News No.01-00595A-EN

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Application News

High Performance Liquid Chromatography / Nexera™ XS inert

DMT-on Purification of Phosphorothioate Oligonucleotide Using SHIMSEN™ Styra HLB SPE Cartridge

Derrick Tan

User Benefits

- A simple and straightforward workflow for purification of modified phosphorothioate oligonucleotide.
- Stability of SHIMSEN Styra HLB SPE cartridge at high pH allows direct loading of deprotected oligonucleotide without removal of highly basic deprotecting agent.
- Stability of SHIMSEN Styra HLB SPE cartridge at low pH allows on-cartridge detritylation using acid.

■ Introduction

Oligonucleotide has been used in various applications, including genetic testing (e.g. primers for PCR) and therapeutics (e.g. antisense oligonucleotides and siRNA). These oligonucleotides are usually chemically synthesized using solid-phase synthesis and would require purification to remove impurities such as protecting groups and failure sequences. In this regard, the use of solid-phase extraction (SPE) cartridges offers a simple and efficient strategy for purifying oligonucleotides without the need for complex instruments or extensive processing. In this application, we present the procedure for DMT-on purification of synthetic phosphorothioate oligonucleotide using SHIMSEN Styra HLB SPE cartridge (P/N: 380-00855-10). Analysis of purified oligonucleotide was achieved on Shimadzu Nexera XS inert system using Shim-pack Scepter Claris C18-120 which feature a bioinert coating on the inner surface of the column body, preventing absorption of oligonucleotides to the column surface and increasing analysis sensitivity.

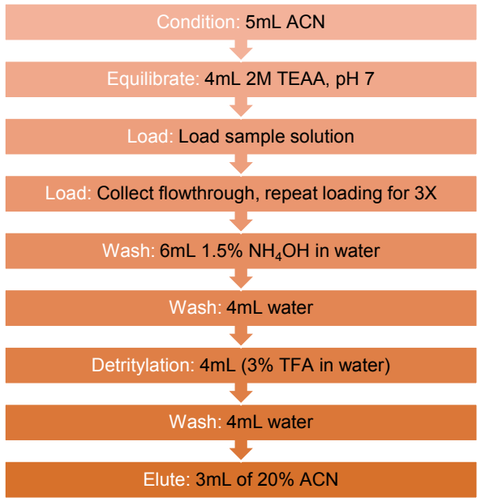

Figure 1. PS Oligonucleotide purification steps.

■ Experimental

The oligonucleotides used in this study are fully phosphorothioate gapmers comprising 10 nucleotides of DNA flanked on both ends by 3 nucleotides of locked nucleic acid (LNA). The oligonucleotides were synthesized and purified by the Phan lab at Nanyang Technological University, Singapore.

Oligonucleotide deprotection:

Oligonucleotides were synthesized on controlled pore glass (CPG) and subsequently cleaved and deprotected in 3 mL of 32% ammonium hydroxide solution at room temperature for 36h. The solution containing the deprotected oligonucleotide was extracted and the CPG was washed once with 1 mL of deionized water followed by twice with 1 mL of 2M TEAA (pH 7). The combined solution was used for purification by SHIMSEN Styra HLB SPE cartridge.

DMT-on oligonucleotide purification by SHIMSEN Styra HLB SPE:

Purification was performed according to procedure detailed in Figure 1 using SHIMSEN Styra HLB, 200mg/6mL (P/N: 380-00855-10). Samples and solvents were allowed to flow through the SPE by gravity. The purified oligonucleotide was analyzed by Nexera™ XS inert UHPLC system. Table 1 list the UHPLC analysis conditions.

Table 1. UHPLC conditions

LC system	:	Shimadzu Nexera™ XS inert
Column	:	Shim-pack Scepter™ Claris C18-120 1.9 µm, 100 × 2.1 mm *1
Column Temp.	:	55 °C
Flow rate	:	0.42 mL/min
Mobile phase A	:	15 mM TEA, 400 mM HFIP
Mobile phase B	:	Acetonitrile
Gradient program	:	10% B (0 min) → 10%B (0.5 min) → 28% B (10.5 min) → 100% B (15.5 min) → 100% B (18.5 min) → 10% B (19.5 min) →10% B (24.5 min)
Injection volume	:	2 µL
Detector	:	PDA, 260 nm

*1 P/N: 227-31209-02

■ Results and Discussion

Automated solid phase oligonucleotide synthesis is typically carried out on solid support such as controlled pore glass (CPG) or polystyrene via phosphoramidite chemistry. Figure 2 shows the typical solid phase oligonucleotide synthetic cycle. Synthesis usually starts from 3'- end, adding a single nucleoside monomer during each cycle, growing towards the 5'- end. During the synthesis, failure sequences (resulted from incomplete coupling) are being capped. Assuming that the capping reaction is efficient, only the full length sequence should contain a dimethoxytrityl (DMT) group on the 5'-end after the completion of the synthesis. Subsequent cleavage and deprotection of the synthetic oligonucleotides are usually performed in highly basic ammonium hydroxide solution. As DMT groups are highly hydrophobic, the full length sequence can be retained on a reversed phase SPE cartridge while the failure sequences are washed off. The DMT group can then be removed on-cartridge under acidic condition and the purified product can be eluted. SHIMSEN Styra HLB cartridges features wide pH stability range of 1-14, allowing purification of deprotected DMT-on oligonucleotide without the need to remove ammonium hydroxide or pH adjustment. At the same time, the stability of the cartridge under low pH allows on-cartridge detritylation reaction under acidic condition using trifluoro acetic acid (TFA) solution.

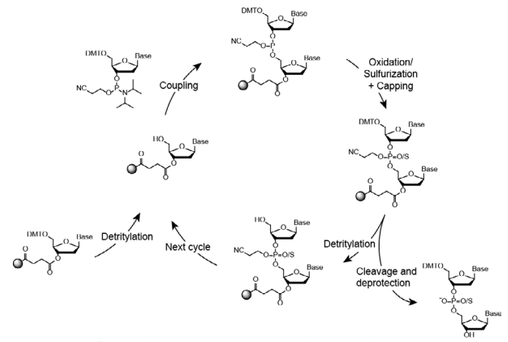

Figure 2. Solid phase oligonucleotide synthesis cycle.

The purification of a 16 nucleotides phosphorothioate gapmer oligonucleotide was successfully achieved using the SHIMSEN Styra HLB SPE cartridge. The SPE cartridge was first conditioned with acetonitrile (ACN), followed by equilibration using a 2M triethylammonium acetate (TEAA) solution. TEAA act as an ion-pairing reagent to aid in retaining the oligonucleotide. The deprotected oligonucleotide could be directly loaded onto the cartridge without the need to remove the basic ammonium hydroxide deprotection solution. The DMT-on oligonucleotide is retained in the cartridge while the impurities elutes in the flowthrough (Figure 3a, 3b).

Next, the cartridge was washed with a 1.5% ammonium hydroxide solution to elute the remaining impurities (Figure 3c), and this is followed by washing with water to remove any remaining ammonium hydroxide as residual ammonium hydroxide will affect the subsequent detritylation reaction. Detritylation was then performed by passing a 3% TFA solution through the cartridge, followed by another water wash to eliminate any residual TFA. Finally, the purified DMT-off oligonucleotide was eluted using 20% ACN and purity was determined by HPLC to be 81% (Figure 3d).

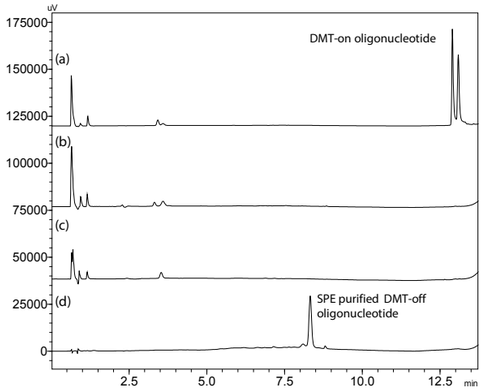

Figure 3. Chromatogram of (a) crude deprotected DMT-on oligonucleotide, (b) flowthrough after loading on SHIMSEN Styra HLB SPE cartridge, (c) flowthrough of 1.5% NH_4OH solution wash, (d) SHIMSEN Styra HLB SPE cartridge purified oligonucleotides.

■ Conclusion

This study showcases a rapid and straightforward approach, employing the SHIMSEN Styra HLB SPE cartridge for purification of synthetic phosphorothioate oligonucleotides by DMT-on strategy.

■ Acknowledgement

We thank the research group of Prof. Anh Tuan Phan at Nanyang Technological University, Singapore, in particular Kah Wai Lim, Natalie Lim, Fiona Hanindita and Apple Lim, for their generous support in this work.

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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060NX

Determination of Nitrosamine Impurities and NDSRI in Anti-diabetic Drugs on Shimadzu LCMS-8060NX

Shao Hua Chia[1], Siew Qi Yap[1], Zhi Wei Edwin Ting[1]
1 Shimadzu (Asia Pacific) Pte Ltd

User Benefits

- Simple and sensitive LC-MS/MS method to quantify ten nitrosamine impurities with simple sample pre-treatment
- Achieve good linearity of $R^2 > 0.999$ with recovery within 70 – 120 % without matrix-matched calibration

■ Introduction

Detection of nitrosamines as impurities in drugs was first reported in June 2018 where N-nitrosodimethylamine (NDMA) was found in valsartan, an angiotensin II receptor blocker (ARB). Since then, nitrosamines have been found in other ARBs and drug classes. [1,2] Nitrosamines are a group of chemical compounds that can be found in several sources such as tobacco, cured meats, cosmetics and pharmaceutical products. They are formed when an amine reacts with nitrite or nitrosating agents e.g., nitrous acid. While nitrosamine drug substance related impurities (NDSRIs) are a class of nitrosamine impurities that share similar structure with the active pharmaceutical ingredients (APIs). Figure 1 shows an example of API sitagliptin and its NDSRI.

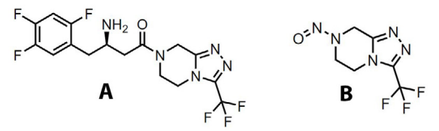

Figure 1. (A) Sitagliptin and (B) its NDSRI nitroso-sitagliptin (NTTP)

Nitrosamines have been a concern due to their potential health risks, as some studies have linked them to an increased risk of cancer. As a result, regulatory authorities have implemented strict limits on the presence of nitrosamines in pharmaceutical products. It is important for pharmaceutical manufacturers to be aware of the formation and presence of nitrosamines to ensure the safety and efficacy of their products and protect patient health.

The aim of this study is to present a simple and sensitive analytical method for ten nitrosamine impurities using APCI on Shimadzu LCMS-8060NX in MRM mode.

■ Measurement Conditions and Samples

The nitrosamine impurity standards were purchased from Toronto Research Chemicals and Restek. High concentration stock solutions were prepared in LCMS grade methanol from the purchased standards and later diluted serially for calibration levels.

Drug samples were prepared by first weighed and crushed into 50 mL centrifuge tube, then extracted with ultrapure water with an extraction ratio of API equivalent to 100 mg to 1 mL of extraction solvent. The samples were sonicated and centrifuged, and then filtered through 0.22 µm nylon filter before injecting into LCMS.

LCMS analytical conditions are described in Table 1, Table 2 and Table 3, respectively. Data were acquired with Shimadzu LabSolutions™ and analysed with LabSolutions Insight™ LCMS software.

Table 1. Analytical LC conditions for detection of nitrosamine impurities

Nexera™ XS LC-40	
Column	Shim-pack™ Scepter C18-120 (150 mm x 3.0 mm I.D., 1.9 µm) P/N: 227-31013-04
Mobile phase	A : 0.1 % formic acid in water B : 0.1 % formic acid in methanol
Gradient program	28 min elution gradient program
Flow rate	0.4 mL/min
Oven temperature	45 ˚C
Injection volume	10 µL
Switching valve	FCV-0206

Table 2. MS conditions for detection of nitrosamine impurities

LCMS-8060NX	
Interface	APCI
Acquisition Mode	MRM, positive mode
Heat block temperature	200 ˚C
DL temperature	200 ˚C
Interface temperature	400 ˚C
Nebulising gas	N_2, 4.4 L/min
Drying gas	N_2, 10 L/min

Table 3. MRM transitions for ten nitrosamine impurities

Compound	Mode	MRM Transitions	
		Quantifier	Reference
NDMA	+	75.05>43.05	75.05>58.10
NDEA	+	103.15>75.15	103.15>29.05
NMEA	+	89.10>61.10	89.10>43.10
NEIPA	+	117.05>75.10	117.05>47.10
NDIPA	+	131.05>89.10	131.05>47.10
NDPA	+	131.20>89.20	131.20>47.15
NDBA	+	159.25>57.20	159.25>41.10
NMBA	+	147.15>44.10	147.15>117.25
NPYR	+	101.10>55.15	101.10>41.10
NTTP	+	222.15>192.10	222.15>42.10

Link to Content Page →

■ Result and Discussion

Calibration and Linearity

Calibration curves for ten nitrosamines were established with mixed standards ranging from 0.1 ng/mL to 10 ng/mL (the amount concentration was back calculated to be 1 ppb to 100 ppb in API). The limit of quantitation (LOQ) for each nitrosamine is less than 10% of the acceptable limit as per EMA guidelines to justify for omission of specification.

Figure 2 shows the calibration curve for four of the nitrosamine impurities. Excellent linearity was obtained with $R^2 > 0.999$ for all target nitrosamines throughout the calibration range as described in Table 4. Figure 3 shows the MRM chromatogram of the neat standard mixture at 1 ng/mL (10 ppb) while Figure 4 shows the chromatogram of each nitrosamine at their respective LOQs.

Accuracy and Precision

The percent accuracy results for the nitrosamines are in accordance with the ICH guideline of within ± 20 %.

Meanwhile, the average precision of <10 % was determined based on the investigation at the limit of quantitation (LOQ) with n = 6 also described in Table 4.

Spiked Sample Recovery

Recovery study was performed to examine the analytical performance of the method through the spiking of nitrosamine standard mixture at respective LLOQs on two sets of pharmaceutical drug samples, D1 and D2. Table 4 describes the respective recovery for the nitrosamine impurities.

Analysis of drug samples

The analytical method was employed on the anti-diabetic drug samples as indicated in Table 5. It was revealed that certain nitrosamine impurities were detected in the drug samples.

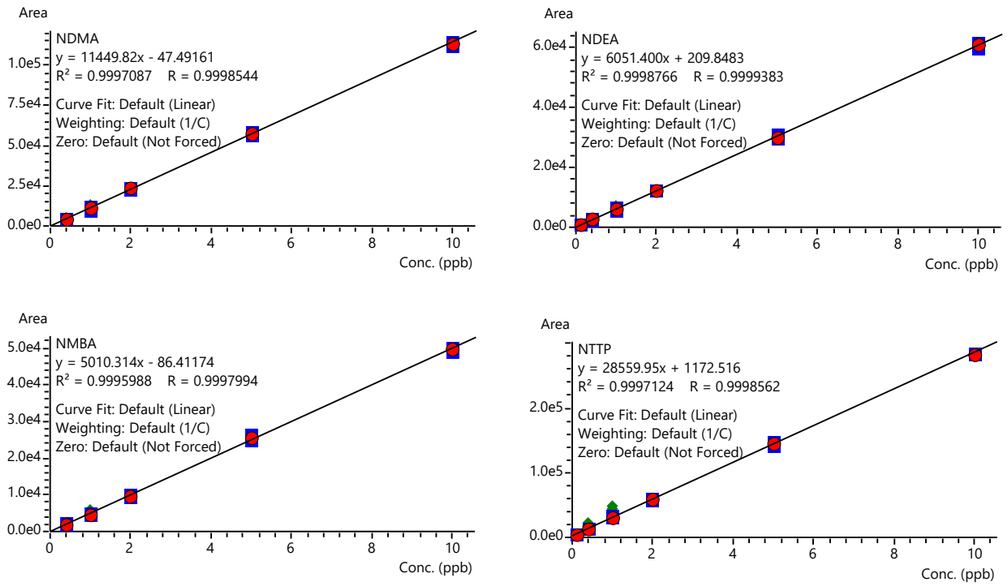

Figure 2. Calibration curves for NDMA, NDEA, NMBA and NTTP on Shimadzu LCMS-8060NX

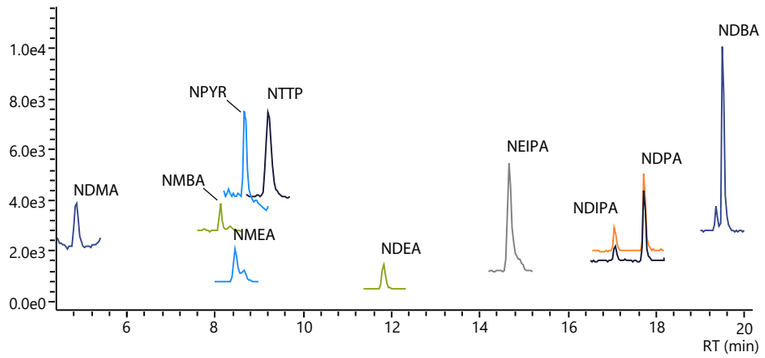

Figure 3. MRM chromatogram of ten mixed nitrosamine standards at 1 ng/mL (10 ppb)

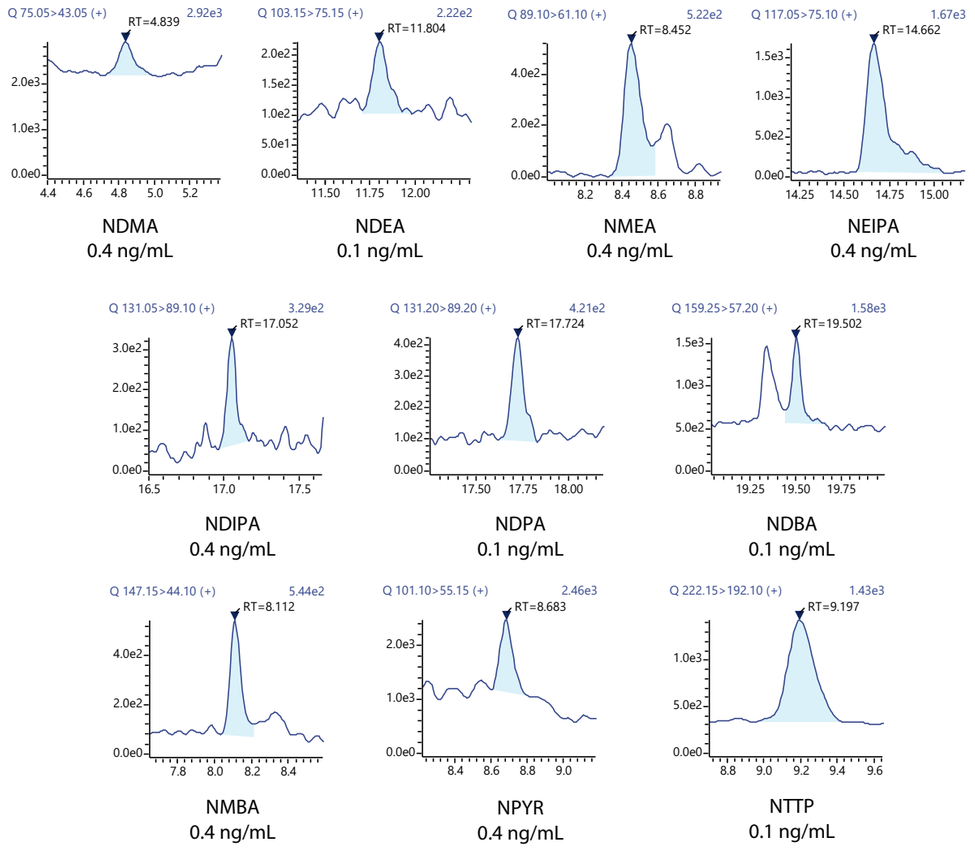

Figure 4. MRM chromatograms of each nitrosamine at respective LOQs

Table 4. Summary for nitrosamines with respective linearity, accuracy, % RSD and % recovery in spiked sample D1 and D2

Nitrosamine	R^2	Linearity range		Accuracy (%)	% RSD (Area)	% RSD (Conc.)	D1 Recovery# (%)	D2 Recovery# (%)
		(ng/mL)	(*ppb)					
NDMA	0.9997	0.4 – 10	4 – 100	97.2	6.20	6.13	110.4	74.5
NDEA	0.9999	0.1 – 10	1 – 100	97.4	5.90	8.00	80.3	85.9
NMEA	0.9996	0.4 – 10	4 – 100	100.3	7.74	8.33	120.0	102.2
NEIPA	0.9992	0.4 – 10	4 – 100	102.0	3.93	4.03	119.2	109.1
NDIPA	0.9995	0.4 – 10	4 – 100	104.4	4.22	4.35	98.6	98.8
NDPA	0.9999	0.1 – 10	1 – 100	97.0	6.78	7.78	101.9	81.2
NDBA	0.9999	0.1 – 10	1 – 100	96.3	4.69	6.76	87.1	94.2
NMBA	0.9996	0.4 – 10	4 – 100	103.7	6.84	6.56	113.9	108.5
NPYR	0.9998	0.4 – 10	4 – 100	100.5	5.77	6.19	115.6	83.5
NTTP	0.9997	0.1 – 10	1 – 100	92.3	6.25	9.03	81.7	119.4

* Concentration calculated with respect to target analytes in API

Recovery performed at respective LLOQs of nitrosamine impurities

Table 5. Results of drug samples tested for nitrosamine impurities (in ppb)

Nitrosamine	S1	S2	S3	S4	S5	S6	S7	S8	S9
NDMA	N.D.	N.D.	N.D.	N.D.	N.D.	< LOQ	N.D.	N.D.	N.D.
NDEA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NMEA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NEIPA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NDIPA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NDPA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NDBA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NMBA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NPYR	19.4	N.D.	N.D.	N.D.	N.D.	13.33	4.10	< LOQ	< LOQ
NTTP	N.D.	N.D.	N.D.	158.77	170.05	53.36	49.91	59.21	88.85

* N.D. Not detected

■ Conclusion

A highly sensitive LC-MS/MS method using the Shimadzu LCMS-8060NX was developed to detect ten nitrosamine impurities. This straightforward yet effective method allows for the quantification of analytes at concentrations as low as 0.1 ng/mL.

With a small 10 µL injection volume and simple sample pre-treatment, the method demonstrated excellent recovery, accuracy and precision, making it well-suited for pharmaceutical safety testing. Additionally, by adjusting the chromatographic conditions based on the elution pattern of the specific drug product, this method can be adapted to quantify these impurities in a range of drug substances or products.

■ Reference

[1] *Nitrosamines as Impurities in Drugs – Health Risk Assessment and Mitigation Public Workshop*, Food and Drug Administration; 2021. https://www.fda.gov/media/150932/download.

[2] *Nitrosamine impurities, European Medicines Agency*. https://www.ema.europa.eu/en/human-regulatory-overview/post-authorisation/pharmacovigilance-post-authorisation/referral-procedures-human-medicines/nitrosamine-impurities.

[3] Research, C. for D.E. and *Control of Nitrosamine Impurities in Human Drugs*. U.S. Food and Drug Administration; 2020. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/control-nitrosamine-impurities-human-drugs

[4] HSA | *Nitrosamine impurities in medicines*; 2018. https://www.hsa.gov.sg/therapeutic-products/medicines-quality-and-compliance-monitoring/nitrosamine-impurities-in-medicines (accessed 2024-02-01)

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Application News

Gas Chromatograph Mass Spectrometer GCMS-TQ8050 NX, HS-20 NX

Ultra-Sensitive Dynamic Headspace GC-MS/MS Method for Trace Level Quantitation of Nitrosamines in Deferiprone API

Hemant Kesarkar, Sanket Chiplunkar, Prashant Hase, Durvesh Sawant, Aseem Wagle, Rahul Dwivedi, Dheeraj Handique, Pratap Rasam and Jitendra Kelkar
Shimadzu Analytical (India) Pvt. Ltd.

User Benefits

- Dynamic headspace achieves high sensitivity for trace level estimation at desired quantitation levels.
- Compared to static headspace, dynamic headspace with multi-injection count option provides flexibility during method development.

■ Introduction

Overview : Regulatory bodies related to pharmaceutical industry have extensively investigated the presence of genotoxic impurities, called Nitrosamines (NSA), in many drugs. Deferiprone (Fig. 1) is an iron chelator used to treat patients with transfusional iron overload caused by thalassemia syndromes. Hence it is imperative to make Deferiprone drug available with safe levels of NSA.

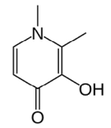

Fig. 1 Structure of Deferiprone

NSA and their Limits : NSA are organic compounds of the chemical structure $R_2N–N=O$, where R is usually an alkyl group. These are common chemicals found in water and foods including cured or grilled meats, dairy products and vegetables. Foods and drugs which are metabolized in human body, are also able to generate NSA. Thus, everyone is exposed to some level of NSA. These impurities may increase the risk of cancer if the exposure is above acceptable levels for a longer period. Regulatory counterparts around the world, have set internationally-recognized acceptable daily intake limits for NSA. If drugs contain levels of NSA above the acceptable daily intake limit, regulatory body recommends their recall by the manufacturer.
NSA can make their way into drug substance/product from varied sources. The sources of NSA can be related to the drug manufacturing process or its chemical structure or even the conditions in which they are stored or packaged.

Toxicity/Regulations/Method : The control strategy described in the USFDA industry guidance on NSA can be employed for Deferiprone Active Pharmaceutical Ingredient (API) and Finished Dosage Form (FDF) as well. These limits are applicable only if the API or FDF having maximum daily dose of 880 mg/day contains a single NSA, and lowest of which is 30 ppb. If more than one NSA is identified, the limit for total NSA determined as listed in Table 1 should not be more than 26.5 ng/day or 30 ppb.
Furthermore, Deferiprone is used to treat the thalassemia syndromes which takes quite longer time to cure. Maximum daily intake is of 99 mg/kg/day. Considering worst case scenario, calculated maximum daily dose will be 9000 mg/day when weight of the patient as 90 kg. Hence, it is imperative to determine above mentioned NSA with Limit of Quantitation (LOQ) not exceeding 0.1 ppb. Developing method for determining total NSA at such low level in API & finish drug product creates challenges in pharmaceutical industry.

Following are the Acceptable Intake (AI) limits for NSA in drug substance/drug product with Maximum Daily Dose (MDD) of 9000 mg/day (Table 1).

Table 1 AI limits for NSA

Comp.	AI limit (ng/day)	Limit in ppm for MDD 9000 mg/day
NDMA	96.0	0.011
NDEA	26.5	0.003
NEIPA	26.5	0.003
NDIPA	26.5	0.003
NDPA	26.5	0.003
NDBA	26.5	0.003

There are several regulatory methodologies available, one such is USP General Chapter <1469> procedure-2 which makes use of static headspace.
However, the results obtained here using dynamic headspace GC-MS/MS proved to be equally precise, accurate and even more sensitive as compared to static headspace GC-MS/MS.
This application note aims to provide a part validated analysis method using Shimadzu GCMS-TQ™8050 NX with HS-20 NX dynamic headspace (Fig. 2) for trace level quantitation for following NSA.

1) N-nitrosodimethylamine (NDMA)
2) N-nitrosodiethylamine (NDEA)
3) N-nitrosoethylisopropylamine (NEIPA)
4) N-nitrosodiisopropylamine (NDIPA)
5) N-nitrosodipropylamine (NDPA)
6) N-nitrosodibutylamine (NDBA)

Summary of validation parameters is shown in Table 2.

Table 2 Summary of validation parameters

Parameters	Conc. in ppb (as such)		Conc. In ppb (w.r.t. sample)	
	NDMA	5NSA*	NDMA	5NSA*
System Precision	0.36	0.1	11.0	3.0
Precision at LOQ	0.1	0.03	3.3	0.9
Linearity	0.1 to 0.54	0.03 to 0.15	3.0 to 16.5	0.9 to 4.5
Accuracy	0.1 to 0.54	0.03 to 0.15	3.0 to 16.5	0.9 to 4.5

w.r.t. sample = with respect to sample (concentration 3.3% w/v)
5NSA*= NDEA, NEIPA, NDIPA, NDPA & NDBA

Fig. 2 GCMS-TQ™8050 NX with HS-20 NX

Link to Content Page →

■ Experimental

A mixture of NDMA, NDEA, NEIPA, NDIPA, NDPA and NDBA standards (1 ppm) was analyzed using scan mode for identification. Steps such as precursor ion selection and MRM optimization at different Collision Energies (CE) were performed and method with optimum MRM and their CE in segments was generated.

The optimized MRM method was used for part method validation (As per ICH guidelines).

■ Method

The MRM transitions of 6 NSA standards are given in Table 3 and analytical conditions are in Table 4.

Table 3 MRM transitions of NSA

MRM Transitions				
Comp.	MRM-1	CE-1	MRM-2	CE-2
NDMA	74.00>44.10	5	74.00>42.10	15
NDEA	102.00>85.10	5	102.00>44.10	11
NEIPA	116.00>99.10	5	116.00>44.10	11
NDIPA	130.00>88.10	5	130.15>42.10	11
NDPA	130.10>113.10	5	130.10>43.10	13
NDBA	116.00>99.10	5	158.00>99.10	9

Table 4 Analytical conditions

Note: Trap should be properly conditioned before starting the analysis. For conditioning procedure, please contact Shimadzu representative.

GCMS System	**: GCMS-TQ8050 NX with HS-20 NX (Dynamic)**
Column	: SH-PolarD 0.32 mm I.D. × 60 m, d.f.=0.5 µm. (P/N: 227-36276-01)
Injection Mode	: Split
Flow Control Mode	: Linear Velocity
Carrier Gas	: Helium
Linear Velocity	: 44 cm/sec
Split Ratio	: 5:1
Purge Flow	: 3 mL/min
Total Flow	: 20 mL/min
Temp. Program	: 70°C (0 min), 5°C/min to 140°C (0 min), 7°C/min to 210°C (0 min). 20°C/min to 250°C (9 min)
Diluent	: 0.5% NaOH in Water (MS Grade)
Ionization Mode	: Electron Ionization (EI)
Ion Source Temp.	: 250°C
Interface Temp.	: 235°C
CID Gas	: Argon
Oven Temp.	: 110°C
Sample Line Temp.	: 120°C
Transfer Line Temp.	: 130°C
Trap Cooling Temp.	: 80°C
Trap Desorb Temp.	: 280°C
Trap Equilib. Temp.	: 80°C
Shaking Level	: 5
Multi Inj. Count	: 3
Pressurizing Gas Pressure	: 160 kPa
Equilibrating Time	: 20.00 min.
Pressurizing Time	: 0.30 min.
Pressure Equilib. Time	: 0.10 min.
Load Time	: 0.30 min.
Load Equilib. Time	: 0.10 min.
Dry Purge Time	: 0.00 min.
Injection Time	: 10.00 min.
Needle Flush Time	: 10.00 min.
GC Cycle Time	: 45.00 min.
Detecor Voltage	: Adjust detector voltage to achieve intensity of 314 m/z as per tuning result

■ Linearity Solutions

Linearity Standard solutions of all NSA's were prepared in headspace vial as mentioned in Table 5.

Table 5 Linearity standard solution preparations

Linearity Levels	Volume taken from Linearity stock solution* (mL)	Final Volume of Linearity level (mL)	As such Conc. of NDMA in (ppb)	As such Conc. of rest of five (ppb)
Level - 1	0.30	10	0.11	0.030
Level - 2	0.50	10	0.18	0.050
Level - 3	0.75	10	0.27	0.074
Level - 4	1.00	10	0.36	0.099
Level - 5	1.50	10	0.54	0.149

**Linearity stock concentration for NDMA is 3.63 ppb and same for rest five impurities is 0.99ppb*

■ Sample Analysis

Weigh 33 mg (± 10%) of Deferiprone API and add 300 mg (± 10%) of Na_2CO_3 in a 20 mL headspace vial. Add 1 ml of diluent, crimp the vial with cap septa tightly and inject.

■ Spiked Recovery Test

Weigh 33 mg (± 10%) of Deferiprone API and add 300 mg (± 10%) of Na_2CO_3 in a 20 mL headspace vial. Further add 1 mL of respective linearity solution, crimp the vial with cap septa tightly and inject.

Fig. 3 to Fig. 8 depicts the calibration curve, overlay of linearity standards, LOQ level chromatograms for NDMA, NDEA, NEIPA, NDIPA, NDPA and NDBA respectively.

NDMA

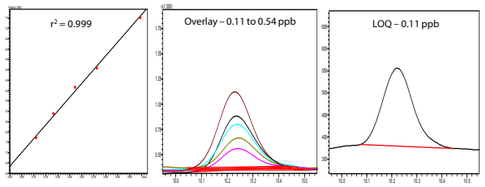

Fig. 3 Calibration curve, overlay of linearity standards and chromatogram of LOQ solution for NDMA

NDEA

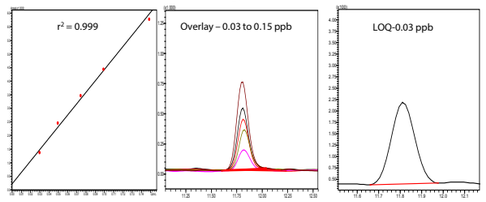

Fig. 4 Calibration curve, overlay of linearity standards and chromatograph of LOQ solution for NDEA

NEIPA

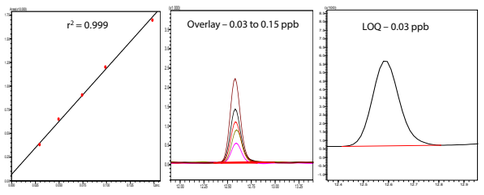

Fig. 5 Calibration curve, overlay of linearity standards and chromatogram of LOQ solution for NEIPA

NDIPA

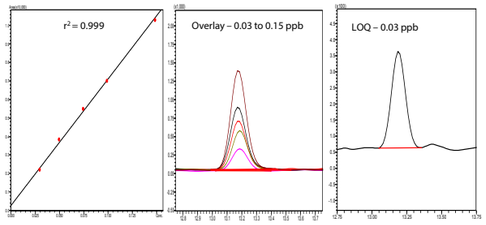

Fig. 6 Calibration curve, overlay of linearity standards and chromatogram of LOQ solution for NDIPA

NDPA

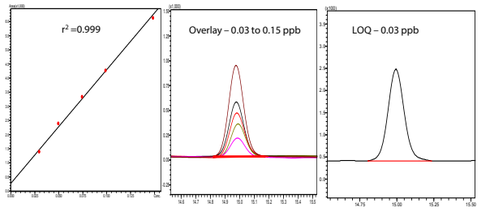

Fig. 7 Calibration curve, overlay of linearity standards and chromatogram of LOQ solution for NDPA

NDBA

Fig. 8 Calibration curve, overlay of linearity standards and chromatogram of LOQ solution for NDBA

■ Validation Parameters

System Precision :

Weigh 300 mg (±10%) of Na_2CO_3 in a headspace vial. Further, add 1 mL of level-4 linearity solution, crimp the vial with cap septa tightly and inject (Table 6).

Table 6 Summary for system precision (n=6)

Comp.	Conc. in ppb (as such)	Conc. in ppb (w.r.t sample)	% RSD* of area
NDMA	0.36	11.0	4.6
NDEA	0.099	3.0	2.6
NEIPA	0.099	3.0	3.4
NDIPA	0.099	3.0	3.0
NDPA	0.099	3.0	3.0
NDBA	0.099	3.0	3.0

Where RSD* = Relative Standard Deviation

Precision at LOQ Level :

Weigh 300 mg (±10%) of Na_2CO_3 in a headspace vial. Further, add 1 mL of level-1 linearity solution, crimp the vial with cap septa tightly and inject.

Summary for S/N and %RSD (area) at LOQ level standard solutions are shown in Table 7.

Table 7 Summary for LOQ system precision (n=6)

Comp.	Conc. in ppb (as Such)	Conc. in ppb (w.r.t sample)	%RSD of area	S/N^
NDMA	0.11	3.30	8.9	21
NDEA	0.03	0.90	3.4	98
NEIPA	0.03	0.90	3.3	178
NDIPA	0.03	0.90	5.2	50
NDPA	0.03	0.90	4.8	10
NDBA	0.03	0.90	3.8	252

^ = Peak to peak

Linearity :

Weigh 300 mg (±10%) of Na_2CO_3 in a 20 mL headspace vial. Further, add 1 mL of respective linearity solution, crimp the vial with cap septa tightly and inject.

For quantitation, five-point calibration curve for all the analyte impurities were plotted. Their concentrations were as per table no.-5

Summary of linearity standard solutions is shown in Table 8.

Table 8 Result summary for linearity (n=3)

Comp.	r^2	Conc. in ppb (as such)	Conc. in ppb (w.r.t. sample)
NDMA	0.999	0.11 to 0.54	3.3 to 16.5
NDEA	0.999	0.03 to 0.15	0.9 to 4.5
NEIPA	0.999		
NDIPA	0.999		
NDPA	0.999		
NDBA	0.994		

Accuracy :

For accuracy study, 33 mg of Deferiprone API sample was diluted with 1 mL of respective linearity solutions to get desired spike concentration in 20 mL headspace vial.

Accuracy study was performed for all the six impurities at four different concentration level including LOQ level. For Method precision, six replicates of Accuracy level-3 (100% spiked sample solution) were injected.

Results observed for the accuracy and method precision parameters was well within the criteria of 70% to 130%.

Accuracy study for level 1 to 4 for NDMA, NDEA, NEIPA, NDIPA, NDPA and NDBA is summarized in Table 9, 10, 11, and 12 respectively.

Table 9 Summary for recovery at Accuracy Level-1 (LOQ) (n=3)

Comp.	Amount spiked as such (ppb)	Amount in sample (ppb)	Amount obtained (ppb)	% Average Accuracy
NDMA	0.11	BLOQ	3.83	117
NDEA	0.03	BLOQ	0.89	97
NEIPA		BLOQ	0.82	91
NDIPA		BLOQ	0.82	92
NDPA		BLOQ	0.84	93
NDBA		BLOQ	1.14	127

BLOQ = Below Limit Of Quantitation

Table 10 Summary for recovery at Accuracy Level-2 (n=3)

Comp.	Amount spiked as such (ppb)	Amount in sample (ppb)	Amount obtained (ppb)	% Average Accuracy
NDMA	0.18	BLOQ	5.57	102
NDEA	0.05	BLOQ	1.33	87
NEIPA		BLOQ	1.27	85
NDIPA		BLOQ	1.30	88
NDPA		BLOQ	1.34	88
NDBA		BLOQ	1.60	107

BLOQ = Below Limit Of Quantitation

Table 11 Summary for recovery at Accuracy level-3 (n=6)

Comp.	Amount spiked as such (ppb)	Amount in sample (ppb)	Amount obtained (ppb)	% of Average Accuracy
NDMA	0.36	BLOQ	10.95	100
NDEA	0.10	BLOQ	2.67	87
NEIPA		BLOQ	2.58	86
NDIPA		BLOQ	2.69	90
NDPA		BLOQ	2.85	94
NDBA		BLOQ	2.92	97

BLOQ = Below Limit Of Quantitation

Table 12 Summary for recovery at Accuracy Level-4 (n=3)

Comp.	Amount spiked as such (ppb)	Amount in sample (ppb)	Amount obtained (ppb)	% of Average Accuracy
NDMA	0.54	BLOQ	12.14	93
NDEA	0.15	BLOQ	2.82	77
NEIPA		BLOQ	2.81	78
NDIPA		BLOQ	2.98	84
NDPA		BLOQ	3.08	85
NDBA		BLOQ	3.13	87

BLOQ = Below Limit Of Quantitation

Method precision was performed by injecting six replicates of 100% spiked sample solution. %RSD for content observed from those spiked sample solution was calculated. Refer summarized results in Table 13.

Table 13: Summary for Method Precision (n=6)

Comp.	Amount spiked as such (ppb)	Mean Amount obtained (ppb)	%RSD of content
NDMA	0.36	0.28	5.5
NDEA	0.10	0.08	7.3
NEIPA	0.10	0.07	7.0
NDIPA	0.10	0.08	6.2
NDPA	0.10	0.08	6.2
NDBA	0.10	0.09	11.1

■ Results

- Trace level quantification of 6 NSA impurities in Deferiprone API was successfully performed by using Shimadzu GCMS-TQ8050 NX with HS-20 NX headspace sampler (Dynamic mode).
- Precision for all 6 NSA at LOQ level was found to be less than 15.0% (Refer Table 7).
- The correlation coefficient (r^2) was greater than 0.99 for all NSA (Refer Table 8).
- Accuracy study in terms of spiked recovery was carried out at four accuracy level in which first level is LOQ level. LOQ was achieved easily at such a trace level which is difficult to achieve with static headspace technique. Further LOQ is confirmed by accuracy and precision. (Refer Table 9, 10, 11, 12 and 13 respectively).

■ Results (Cont.)

- Method precision in terms of repeatability was performed. %RSD for content observed from six replicate injections of spiked sample solution was well within the acceptance criteria of not more than 10% (Refer Table 13).

■ Conclusion

- Dynamic headspace mode, outperforms the current regulatory limits, delivering high sensitivity compared to static headspace mode for NSA analysis.
- Shimadzu GCMS-TQ8050 NX has features like a new highly efficient shielded detector and superior noise reduction technology enhances sensitivity and enables quantitation of NSA even at trace levels.

■ References

- ICH Q2 (R2): Validation of analytical procedures: Test and Methodologies
- ICH M7 (R1): Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk
- USP <1469> General chapter for Nitrosamine Impurities

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Nitrosamine impurities in drug substances / LCMS™-9030

Application News

Detection and Quantitation of Nitrosamine Impurities in Drug Substances by LC-HRMS on LCMS-9030

Zhaoqi Zhan[1], Zhe Sun[1], Deepti Bhandarkar[2], Shailendra Rane[2], Pratap Rasam[2], Jitendra Kelkar[2]
[1] Shimadzu (Asia Pacific) Pte Ltd, [2] Shimadzu Analytical (India) Pvt Ltd

User Benefits

- Simultaneous analysis of up to eight *N*-nitrosamine impurities in drug substances by LC-HRMS method on LCMS-9030.
- A targeted MS/MS (TOF) method with 2 *m/z* isolation window by the quadrupole was optimized to obtain best sensitivity.
- A mass tolerance of (±)15 ppm was adopted to produce extracted-ion chromatograms (XICs) for quantitation.

■ Introduction

Since 2018, the presences of *N*-nitrosamine impurities in some drug substances and products have been alerted by the US FDA and regulatory agencies in other countries. Nitrosamines (NSA, see Table 1) are toxic chemicals and some of them such as NDMA and NDEA are classified as probable human carcinogens. NDMA and NDEA were found presence first in drug substances and products of Angiotensin II receptor blockers (ARB) like losartan etc. NDMA was found in ranitidine and metformin drug products and recalls of the products occurred due to the content of NDMA above the Acceptable Intake limit (AI, 96 ng/day). Detection and quantitation of NDMA and other concerned nitrosamines at trace levels in drug products are established by using highly sensitive and selective mass spectrometry methods on GC-MS, LC-MS/MS [1] and LC-HRMS [2-4].

Most concerned nitrosamines (see Table 1) are small and polar compounds, which may be interfered by co-eluting species present in the testing samples in LC-MS/MS and LC-HRMS analysis. High-resolution MS methods are capable of distinguishing co-eluting interferences. An example was reported by Yang et al [4] recently, which revealed that the presence of *N*,*N*-dimethylformamide (DMF) may affect the quantitation results of NDMA in metformin products if the mass accuracy and mass resolution are not sufficient. To date, both dedicated methods for NDMA only and simultaneous analysis method for more NSA are reported and used. In this application note, an orthogonal method for detection and quantitation of up to eight nitrosamines as listed in Table 1 by LC-HRMS on LCMS-9030 is described.

Table 1 Information of eight *N*-Nitrosamine impurities

No.	Compound Name	Abbr.	CAS No	Formula	MW
1	N-Nitroso dimethylamine	NDMA	62-75-9	$C_2H_6N_2O$	74.1
2	N-nitroso-N-methyl-4-aminobutyric acid	NMBA	61445-55-4	$C_5H_{10}N_2O_3$	146.2
3	N-Nitroso diethylamine	NDEA	55-18-5	$C_4H_{10}N_2O$	102.1
4	N-Nitrosoethyl isopropylamine	NEIPA	16339-04-1	$C_5H_{12}N_2O$	116.2
5	N-Nitrosodiiso propylamine	NDIPA	601-77-4	$C_6H_{14}N_2O$	130.2
6	N-Nitrosodi-N-propylamine	NDPA	621-64-7	$C_6H_{14}N_2O$	130.2
7	N-Nitrosomethyl phenylamine-	NMPA	614-00-6	$C_7H_8N_2O$	136.2
8	N-Nitrosodi-butylamine	NDBA	924-16-3	$C_8H_{18}N_2O$	158.2

■ Experimental

Standard and sample preparation

Eight nitrosamine standards (Table 1) were prepared into individual stock solutions of 100 µg/mL (ppm) in methanol. Mixed stocks of all or selected NSA with each 100 ng/mL were prepared for making calibration series of different concentration levels in a diluent (MeOH:H2O = 15:85 (v/v) with 0.1% formic acid). Sample preparation of drug substance refers to the FDA testing method posted in 2019 and 2020 [2,3]. For metformin, 150 mg of metformin hydrochloride (solid) was dissolved 1.5 mL of the diluent in a 2 mL micro-centrifuge tube. The sample tube was vortexed for 3 min followed by shaking on a Vortex-Genie 2 mixer for 30 min at room temperature. The sample tube was centrifuged at 15,000 rpm for 5 min. Then, the supernatant was transferred and filtered with a 0.22 µm nylon syringe filter into a 1.5 mL HPLC sample vial.

Analytical conditions

A Shimadzu LCMS-9030 Q-TOF system was employed for the sample analysis. Details of the system and analytical conditions are compiled in Table 2.

Table 2 Analytical conditions on LCMS-9030

LC Conditions	
Column	Shim-pack™ Solar C18 (4.6 X 250 mm, 5 µm, P/N: 227-30600-02)
Flow Rate	0.8 mL/min
Mobile Phase	A: Water with 0.1% formic acid B: Methanol with 0.1% formic acid
Elution gradient	15% B (0-2.0 min) -> 40% B (5.0 min) -> 95% B (9.5-14.5 min) ->15% B (14.6-21 min)
Oven Temp.	45°C
Injection Vol.	40 µL
Interface Conditions (LCMS-9030)	
Interface	DUIS, ESI 4.0 kV, Corona needle 4.5 kV
Interface Temp.	400°C
DL Temp.	250°C
Heat Block Temp.	250°C
Nebulizing Gas	2.0 L/min
Heating Gas Flow	5 L/min
Drying Gas Flow	10 L/min

Link to Content Page →

■ Results and Discussion

Targeted MS/MS method

Instead of direct MS TIC method, a targeted MS/MS TIC method was established for the detection of the analytes on LCMS-9030. The quadrupole was set to target for each analyte with a mass window of ± 2 *m/z*, while full spectra were collected by TOF MS. With an optimized CE, enhanced sensitivity could be obtained as compared to direct MS TIC acquisition. The details of the acquisition parameters are compiled into Table 3.

Table 3 Key parameters of targeted MS/MS method for NSA

No.	Abbr.	$[M+H]^+$ (*m/z*)	Q Isolation Window	CE Spread (V)	TOF Mass Range (*m/z*)
1	NDMA	75.0553	2 *m/z*	4 - 8	60 – 90
2	NMBA	147.0764	2 *m/z*	2 - 6	125 – 160
3	NDEA	103.0866	2 *m/z*	4 - 8	85 – 120
4	NEIPA	117.1022	2 *m/z*	4 - 8	100 – 125
5	NDIPA	131.1179	2 *m/z*	3 - 7	115 – 150
6	NDPA	131.1179	2 *m/z*	3 - 7	115 – 150
7	NMPA	137.0709	2 *m/z*	5 - 9	127 – 147
8	NDBA	159.1492	2 *m/z*	5 - 9	140 – 170

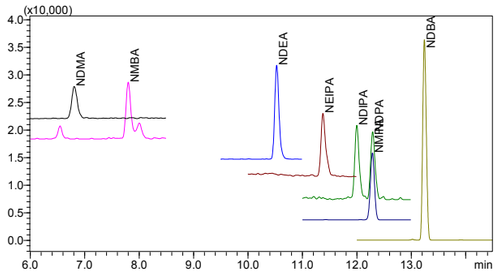

Figure 1 XICs of eight NSA mixed standards each at 5 ng/mL except NDBA at 2.5 ng/mL.

Extracted-ion chromatograms (XICs) of mixed standard are shown in Figure 1. The mass tolerance set to obtain the XICs is (±)15 ppm (NDPA (±)20 ppm), which is with reference to the recent publication by Yang et al [4]. The isotopic ion of the interference DMF (*m/z*75.0571, with ^{15}N) is very closed to the monoisotopic ion of NDMA (*m/z*75.0553), a mass tolerance of less than 20 ppm is required for distinguishing co-eluted NDMA and DMF.

Calibration curves & quantitation method

Mixed standards of the eight NSA of 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/mL (NDBA: 0.1 ~ 50 ng/mL) were used to establish calibration curves and tested for sensitivity (LOD and LOQ). Figure 2 shows the calibration curves and XIC peaks of 1 ng/mL (NDBA 0.5 ng/mL). All calibration curves are linear type with R^2 greater than 0.99 for a wide range from 0.5 ng/mL to 100 ng/mL except NDBA (0.25~10 ng/mL).

The performance parameters of the quantitation method established are summarized in Table 4. The LOQs for the eight NSA for standards in solvent achieved 1 ng/mL or lower with accuracy at 92.3% - 108.4% and repeatability at 1.9% - 15.2% for the 1 ng/mL level (NDBA 0.5 ng/mL).

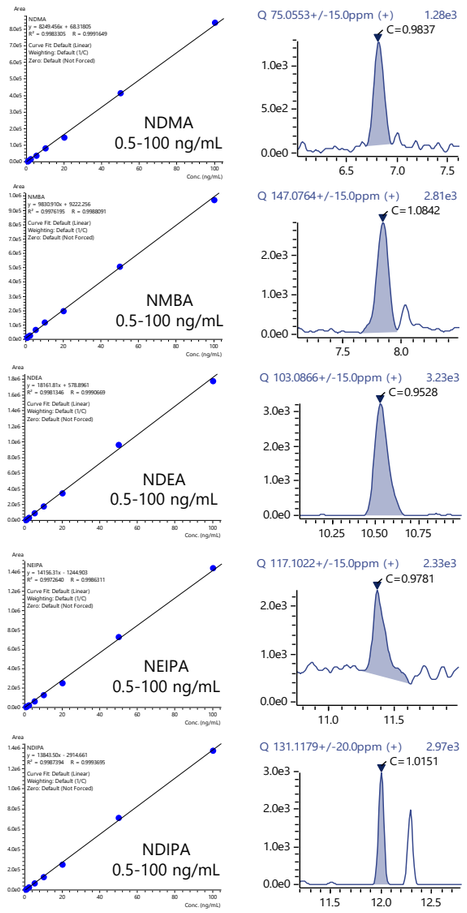

Figure 2 Calibration curves of 8 NSA mixed standards and XIC peaks of 1 ng/mL (NDBA 0.5 ng/mL)

Table 4 Calibration curves and quantitation preformation for eight *N*-nitrosamines by targeted MS/MS method on LCMS-9030

Performance Parameter	NDMA	NMBA	NDEA	NEIPA	NDIPA	NDPA	NMPA	NDBA
LOD (ng/mL)	0.5	0.5	0.2	0.5	0.5	0.5	0.1	0.1
LOQ (ng/mL)	1	1	0.5	1	1	1	0.2	0.25
Range (ng/mL)	1 ~ 100	1 ~ 100	0.5 ~ 100	1 ~ 100	1 ~ 100	1 ~ 100	0.2 ~ 100	0.25 ~ 10
R2	0.9983	0.9976	0.9981	0.9973	0.9987	0.9983	0.9971	0.9942
Accuracy at LOQ (%)	98.3	108.4	107.4	97.8	101.5	92.3	116.5	106.8
Accuracy at 1 ng/mL (%)	98.3	108.4	95.3	97.8	101.5	92.3	96.7	104
Accuracy at 5 ng/mL (%)	94.5	115.6	104.8	92.4	104.7	103.8	98.3	90.4
Accuracy at 50 ng/mL (%)	100.6	101.3	106.4	102.8	103.5	103.7	102.7	101.7 (10 ng/mL)
Conc. %RSD at 1 ng/mL (n=7)	7.39	15.18	5.87	12.27	8.20	9.95	3.19	1.89
Conc. %RSD at 5 ng/mL (n=7)	2.65	3.42	1.85	1.47	1.81	2.67	2.41	0.61

Table 5 LOD and LOQ of NSA spiked in metformin drug substance by targeted MS/MS method

	NDMA	NMBA	NDEA	NEIPA	NMPA	NDBA
LOD (ng/mL)	0.5	0.5	0.2	0.5	0.2	0.25
(ppm)	0.005	0.005	0.002	0.005	0.002	0.0025
LOQ (ng/mL)	1	1	0.5	1	0.5	0.5
(ppm)	0.01	0.01	0.005	0.01	0.005	0.005
Range (ng/mL)	1 ~ 100	1 ~ 100	0.5 ~ 100	2 ~ 100	0.5 ~ 100	0.5 ~ 10
(ppm)	0.01~ 1	0.01~ 1	0.005~ 1	0.02~ 1	0.005~ 1	0.005~ 1

Nitrosamines in metformin API

The method established was applied to metformin drug substance (API). Following the sample preparation procedure [3] by US FDA, the spiked samples of 6 nitrosamines (NDIPA and NDPA were not included) were tested for sensitivity (LOD and LOQ). As shown in Figure 3, metformin eluted as a broad peak from 2.4 min to 4.5 min (234 nm UV detector). With a programmed flow switching valve control, the eluent was diverted to the waste to avoid contamination of the extremely high concentration of metformin (100 mg/mL) in the extract samples to the interface.

The LOD and LOQ values of the six nitrosamines spiked in metformin (extract) testing solutions (100 mg/mL) are summarized in Table 5. The values in ng/mL are the concentration of testing solutions and the values in ppm are the content of NSA in metformin sample (w/w).

Nitrosamines in two ARB APIs

The method established (slight modification in elution program) was applied to losartan and candesartan drug substances. Following the sample preparation procedure [2] by US FDA, the spiked samples of 7 NSA in the drug substance extract (20 mg/mL) were tested for sensitivity (LOD and LOQ). As shown in Figures 4 and 5, losartan and candesartan eluted as a broad peak at 11.4-12.0 min and a peat at 8.6-9.0 min (254 nm UV detector), respectively. With a proper programmed flow switching valve control, the eluent was diverted to the waste to avoid contamination of the high concentration of the APIs (20 mg/mL) in the extract samples to the interface.

The LOD and LOQ values of the seven NSA in solutions (ng/mL) and in drug substances (ppm) are summarized in Table 6.

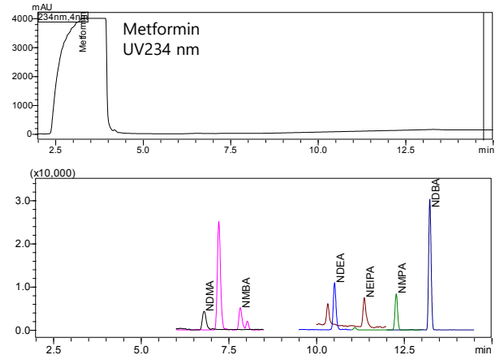

Figure 3 XICs of spiked nitrosamines (5 ng/mL mixture) with UV chromatogram (top) of metformin extract (100 mg/mL)

■ Conclusion

In this study, a targeted MS/MS (TOF) method was established for detection and quantitation of up to 8 *N*-nitrosamines on LCMS-9030 Q-TOF system. The method was evaluated in terms of LOD and LOQ, linearity, repeatability in reference to the FDA recommended method. As demonstration, the method was applied to determine nitrosamines spiked in drug substances including metformin (100 mg/mL) and two ARB, losartan and candesartan (20 mg/mL).

Table 6 LOD and LOQ of NSA spiked in losartan and candesartan drug substances by targeted MS/MS method

	NDMA	NMBA	NDEA	NEIPA	NDIPA	NDPA	NDBA
LOD (ng/mL)	0.5	0.5	0.2	0.5	0.2	0.2	0.1
(ppm)	0.025	0.025	0.01	0.025	0.01	0.01	0.005
LOQ (ng/mL)	1	1	0.5	1	0.5	0.5	0.25
(ppm)	0.05	0.05	0.025	0.05	0.025	0.025	0.013
Range (ng/mL)	1 ~ 100	1 ~ 100	0.5 ~ 100	1 ~ 100	0.5 ~ 100	0.5 ~ 100	0.25 ~ 10
(ppm)	0.05 ~ 5.0	0.05 ~ 5.0	0.025 ~ 5.0	0.05 ~ 5.0	0.025 ~ 5.0	0.025 ~ 5.0	0.0125 ~ 0.50

Note: The LOD and LOQ measured are same in the losartan and candesartan matrix (20 mg/mL)

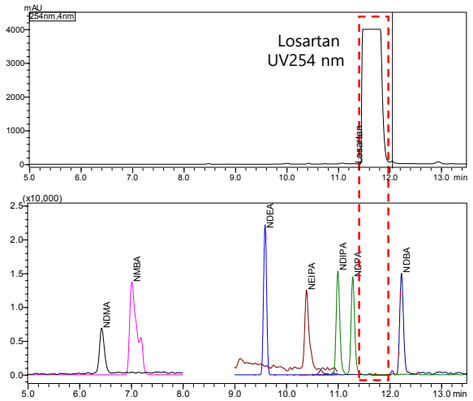

Figure 4 XICs of spiked nitrosamines (5 ng/mL mixture) and UV chromatogram of losartan extract (20 mg/mL)

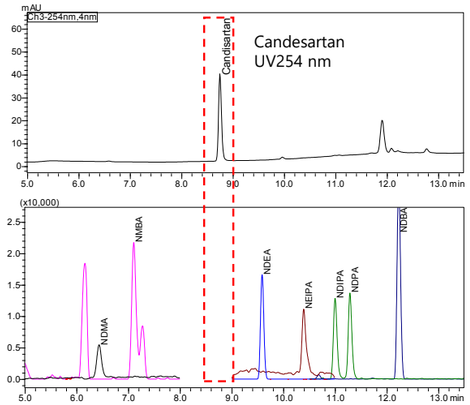

Figure 5 XICs of spiked nitrosamines (5 ng/mL) and UV chromatogram of candesartan extract (20 mg/mL)

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Application News

LabSolutions™ MD: Efficient method development based on Analytical Quality by Design

Efficient Method Development on Pharmaceutical Impurities Using Single Quadrupole Mass Spectrometer

Shinichi Fujisaki and Masataka Nikko

User Benefits

- Peak tracking using an LCMS-2050 single quadrupole mass spectrometer supports highly reliable method development by accurately tracking each impurity that has similar UV spectra.
- LabSolutions MD is efficiently able to develop method that provides both excellent resolution and shorter analysis time.

■ Introduction

Since pharmaceutical impurities require strict control to ensure safety, development of highly reliable analytical method is essential. LabSolutions MD, a new Shimadzu software for method development, supports efficient method development based on Analytical Quality by Design (AQbD). AQbD-based analytical method development consists of the phases of initial screening, optimization, and robustness evaluation. This article introduces an example of its use for the development of a robust LC method for impurities on Montelukast (a medication used in the maintenance treatment of asthma). By changing each parameter of gradient program, the resolution of Montelukast and each impurity was evaluated by visualizing through "design space." Though it was previously difficult to accurately track each impurity with similar UV spectra using photodiode array detector (PDA), LCMS-2050 can solve this problem. Furthermore, by utilizing design space of resolution and RT of last eluting peak, it is possible to efficiently develop method that provides both excellent resolution and shorter analysis time.

■ Analytical Conditions

Table 1 shows the analytical conditions used in the optimization study for separation of Montelukast and its impurities (Imp1 to 6: Fig. 1). By varying the final concentration and slope of gradient program, the resolution of Montelukast and its impurities was examined to find the optimal condition. Specifically, final concentration was varied from 75 % to 85 % in increments of 5 % (3 levels), gradient slope from 8 min to 18 min in increments of 5 min (3 levels).

Table 1 Analytical Conditions for Optimization

LC Conditions:	Nexera™ X3 (Method Scouting System)
Mobile Phase:	
Pump A: 0.15 % formic acid in water	
Pump B: 0.1 % formic acid in acetonitrile	
Column:	
Shim-pack™ Scepter Phenyl-120 (100 mm × 3.0 mm I.D., 1.9 µm)*1	
Analytical Conditions	
Initial B Conc.:	45 %
Final B Conc.:	75, 80, 85 % (3 patterns)
Gradient Slope:	8, 13, 18 min (3 patterns)
Time Program:	B Conc. 45 % (0-3 min)→75 % (11 min) →85 % (11.01-13 min) →45 % (13.01-18 min) Note: If final B Conc. is 75 %, gradient slope is 8 min
Column Temp.:	30 °C
Flowrate:	0.5 mL/min
Injection Vol.:	10 µL (1000 mg/L)
Detection (PDA):	238 nm (SPD-M40, UHPLC cell)

*1 P/N: 227-31064-03

MS Conditions:	LCMS-2050
Ionization:	ESI/APCI (DUIS™), positive and negative mode
Mode:	SCAN (*m/z* 400-800)
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	7.0 L/min
DL Temp.:	200 °C
Desolvation Temp.:	450 °C
Interface Voltage:	+3.0 kV / -2.0 kV
Qarray Voltage:	+20 V

■ Accurate Tracking of Peaks by LCMS-2050

LC chromatograms obtained at final gradient concentration of 80 % and gradient slopes of 8 min and 18 min, along with *m/z* for respective impurities (Imp1 to 6), are shown in Fig. 1. UV spectra for Imp1 to Imp6 are shown in Fig. 2. The similarity between UV spectra for Imp1 and Imp6 (similarity > 0.99), for Imp2 and Imp4 (similarity > 0.9), and for Imp3 and Imp5 (similarity > 0.999) suggests that peak tracking based on UV spectrum would be difficult. In contrast, LabSolutions MD enables peak tracking based on *m/z* with LCMS-2050 for accurate identification of impurities that have similar UV spectra (Fig. 1).

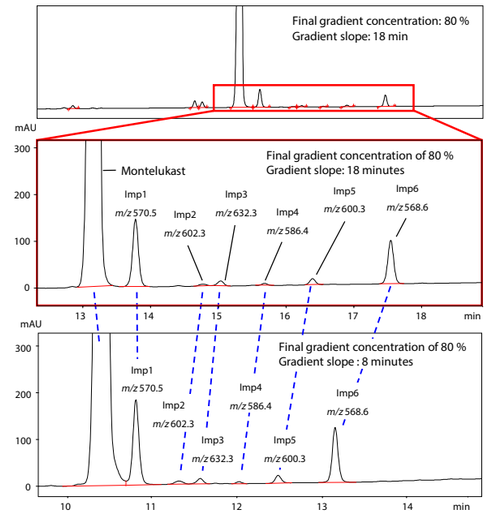

Fig. 1 LC Chromatograms with a Final Gradient Concentration of 80 % and Gradient Slopes of 8 (Lower) and 18 Minutes (Upper)

- Dashed line indicates tracking impurities based on *m/z*.
- Imp 1 to 6 are different from the impurities indicated in Japanese Pharmacopoeia.

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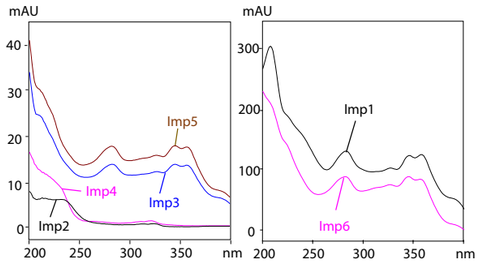

Fig. 2 UV Spectra of Montelukast Impurities (Imp1 to 6)

Next, to identify the optimal analytical condition, the resolution, when the final concentration and gradient slope are changed, is visualized by design space.

■ Design Space Evaluation for Optimal Analytical Condition

Fig. 3 shows the design space for resolution of Montelukast and Imp1. The red region indicates higher resolution, and the blue region indicates lower resolution. By visualizing resolution through design space, it was indicated that lower final concentration and longer gradient slope can achieve better separation.

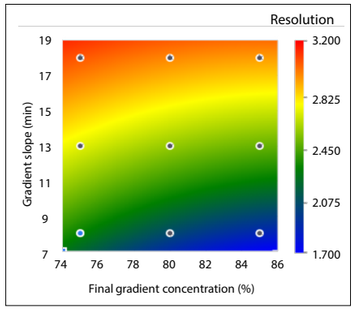

Fig. 3 Design Space for Resolution of Montelukast and Imp1

LabSolutions MD can simplify the search for optimal analytical condition by overlaying design spaces. Fig. 4 shows the area of analytical condition that meets resolution of Montelukast and Imp1 is > 2.6, minimum resolution of each compound is > 1.2, and RT of last eluting peak (Imp6) is < 17 min. The region enclosed by the green line in the figure is the region where minimum resolution is < 1.2, the region enclosed by the blue line is the region where resolution of Montelukast and Imp1 is < 2.6, the region enclosed by the pink line is the region where RT of last eluting peak is > 17 min, and the remaining region (shown by the black hatching) is the condition that satisfies all the criteria. Within the hatched area, the optimal point with the shortest analysis time is around point A, which is circled in red. Thus, by overlaying design spaces of resolution and RT of last eluting peak, optimal condition that provides enough resolution and shorter analysis time can be easily found.

Utilizing design space enables to understand how the LC parameters affect responses such as resolution and RT easily. This means a robust method can be defined without relying on the user experience.

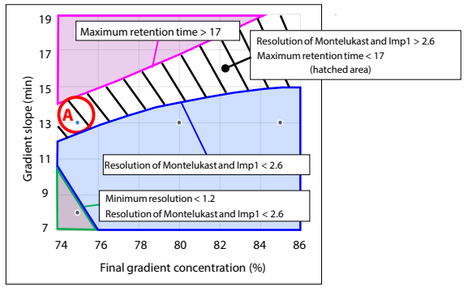

Fig. 4 Overlay of Design Spaces of Resolution and Last Eluting Peak

■ Chromatogram with Optimal Condition

The chromatogram obtained with optimal condition (point A) is shown in Fig. 5. It shows that the resolution of Montelukast and Imp1 is 2.7, minimum resolution of each impurities is 1.4 (Imp3), and RT of last eluting peak is less than 17 minutes, which successfully satisfies the criteria.

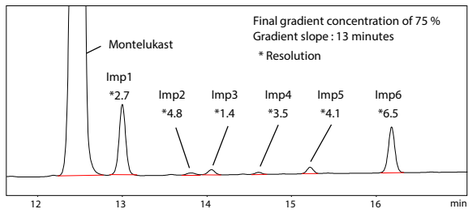

Fig. 5 LC Chromatogram Obtained with Optimized Condition
Final gradient concentration of 75 % and gradient slope of 13 minutes

■ Conclusion

This article introduces efficient method development on pharmaceutical impurities by using LabSolutions MD and LCMS-2050. Peak tracking based on *m/z* enables to identify each impurity accurately even in the case of similar UV spectra. Visualizing and overlaying the patterns of resolution for each compound and RT of last eluting peak enables a more efficient (Resolution) and fast (Analysis Time) method be easily developed regardless of the user experience.

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GC-MS GCMS-QP™2020 NX

Application News

Analysis of Extractables from Pharmaceutical Packaging Materials by Solvent Extraction-GC-MS and Headspace-GC-MS

K. Kawamura, Y. Kudo, R. Kitano, and T. Hiramatsu

User Benefits

- GC-MS can be used to analyze volatile and semi-volatile substances in pharmaceutical packaging.
- High-temperature extraction can be used with a headspace sampler to analyze samples with simple pretreatment that requires no solvent.
- Extracted additives can be identified easily and accurately using the Polymer Additives Library.

■ Introduction

Mutual interactions between the pharmaceutical and packaging are a problem with pharmaceutical packaging materials that requires particular care. Extractables are compounds generated from storage conditions that are more severe than normal, whereas leachables are compounds that can be transferred from the packaging to the pharmaceutical under normal storage conditions. They are classified as indicated in Table 1. Before pharmaceuticals can be sold, extractables and leachables must be comprehensively verified to determine packaging material risks.

Table 1 Summary of Extractables and Leachables

	Extractables	Leachables
Description	All compounds that potentially could be extracted from packaging	Compounds that leach out under normal usage conditions
Extraction Conditions	More severe conditions than for normal use or storage. High-temperature extraction or solvent extraction is used.	Under normal conditions for use or storage.
Measured Item	Packaging material	Pharmaceutical

■ Background of Extractables and Leachables in Society

Despite the variety of regulations governing extractables and leachables, still no definitive method of analysis has been specified. Most regulations indicate that complete measurement of extractables and leachables is impossible.

For example, USP 1663 says "It is not possible for a general discussion of extractables to anticipate and cover all situations where an extractables assessment might be required."

However, in July 2020, the ICH launched a new working group for evaluating and managing extractables and leachables (ICH-Q3E). The increase in biopharmaceuticals is the main reason the working group was started. The FDA summarized the potential interactions between pharmaceuticals and packaging materials in "Guidance for Industry: Container Closure Systems for Packaging Human Drugs and Biologics." That guidance indicates an increased danger to humans from parenterally administered drugs or parenterally administrable drug suspensions (Table 2). Given that biopharmaceuticals comprise macromolecules that make oral administration difficult, most are administered parenterally. As of 2020, biopharmaceuticals account for about 30 % of the overall pharmaceuticals market. Consequently, the evaluation of extractables and leachables can no longer be ignored.

Furthermore, because of the specificity of biopharmaceuticals they are often only effective for rare diseases, so the cost-effectiveness of scaling up is low. Therefore, rather than using a stainless steel culture tank or other large-scale manufacturing equipment, there is a growing trend toward successively producing a variety of small batches. Due to the plastic single-use equipment used for production, the risk of leachables being generated between the equipment and pharmaceuticals must also be taken into consideration.

Table 2 Examples of Packaging Concerns for Common Classes of Drug Products

Degree of Concern Associated with the Route of Administration	Likelihood of Packaging Component-Dosage Form Interaction		
	High	Medium	Low
Highest	Inhalation Aerosols and Solutions; Injections and Injectable Suspensions	Sterile Powders and Powders for Injection; Inhalation Powders	
High	Ophthalmic Solutions and Suspensions; Transdermal Ointments and Patches; Nasal Aerosols and Sprays		
Low	Topical Solutions and Suspensions; Topical and Lingual Aerosols; Oral Solutions and Suspensions	Topical Powders; Oral powders	Oral Tablets and Oral (Hard and Soft Gelatin) Capsules

Source: Guidance for Industry Container Closure Systems for Packaging Human Drugs and Biologics

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■ Samples and Analytical Conditions

For this article, plastic bags for liquid formulations were used as samples. The bags were made of polyvinyl chloride (PVC). GC-MS was used to identify volatile and semi-volatile extractable compounds in the samples. The extractables were pretreated by extraction using various solvents and by high-temperature extraction using a headspace sampler without any solvent.

For solvent extraction, dichloromethane (DCM) and hexane, commonly used to extract plastic components, and ethanol, commonly used in pharmaceutical manufacturing, were used as the extraction solvents. About 400 mg of the samples cut into 1 cm squares were weighed into vials containing 5 mL of respective solvents, and then the vials were sealed. After ultrasonically treating the vials for 5 hours, the vials were left at room temperature for 3 days for extraction. Then the organic solvent supernatant was analyzed by GC-MS. The analytical conditions are indicated in Table 3.

For high-temperature extraction, about 200 mg of the samples cut into 1 cm squares were weighed into headspace vials purged with nitrogen gas and then sealed. Because the vials are heated to temperatures over 200 °C for high-temperature extraction, they were sealed with heat-resistant headspace vial septa that minimize bleeding at high temperatures (Shimadzu, P/N: 225-40950-91). The analytical conditions are indicated in Table 4.

For high-temperature extraction, n-alkane was also analyzed to enable calculation of retention index values for each peak by qualitative analysis using the Polymer Additives Library. 5 µL of an n-alkane solution (1000 µg/mL hexane solvent) was sealed in a headspace vial purged with nitrogen gas and similarly analyzed.

Table 3 Analytical Conditions (Solvent Extraction: Liquid Injection-GC/MS)

Instruments	
Autosampler:	AOC-20is
GC-MS:	GCMS-QP2020 NX
Column:	SH-I-5MS (30 m × 0.25 mm I.D., df = 0.25 µm)
GC Conditions	
Injection Unit Temp.:	250 °C
Injection Mode:	Splitless
Carrier Gas:	Helium
Control Mode:	Constant Linear Velocity (36.1 cm/s)
Oven Program:	40 °C (3 min) → 10 °C/min → 330 °C (15 min)
Injection Volume:	1 µL
MS Conditions	
Interface Temp.:	300 °C
Ion Source Temp.:	230 °C
Ionization Method:	EI
Measurement Mode:	Scan (*m/z* 29 to 800)
Event Time:	0.3 sec

Table 4 Analytical Conditions (High-Temperature Extraction: Headspace-GC/MS)

Instruments	
Autosampler:	HS-20
GC-MS:	GCMS-QP2020 NX
Column:	SH-I-5MS (30 m × 0.25 mm I.D., df = 0.25 µm)
Headspace (HS) Conditions	
Mode:	Loop
Oven Temp.:	250 °C
Sample Line Temp.:	250 °C
Transfer Line Temp.:	250 °C
Vial Pressure:	150 kPa
Vial Heating Time:	10 min (1 min for n-alkane)
Vial Pressurization Time:	1 min
GC Conditions	
Injection Mode:	Split (split ratio: 10)
Carrier Gas:	Helium
Control Mode:	Constant Linear Velocity (36.1 cm/s)
Oven Program:	40 °C (3 min) → 10 °C/min → 330 °C (15 min)
MS Conditions	
Interface Temp.:	300 °C
Ion Source Temp.:	230 °C
Ionization Method:	EI
Measurement Mode:	Scan (*m/z* 29 to 800)
Event Time:	0.3 sec

■ Solvent Extraction Analytical Results

Total ion current chromatograms (TICC) obtained from analyzing hexane, DCM, and ethanol extract solutions are shown in Fig. 1. Compounds were identified by using the NIST Library (2020 version) and the Polymer Additives Library.

Compounds detected using respective extraction solvents are indicated in Table 5. They include plasticizers, such as tris(2-ethylhexyl)trimellitate (TOTM), di(2-ethylhexyl)phthalate (DEHP), and di(2-ethylhexyl)adipate (DEHA), and lubricants, such as palmitic acid and ethyl palmilate. However, there was some difference in the components detected with each extraction solvent. That difference in detected components is presumably due to differences in the polarity of respective extraction solvents. In general, hexane and other solvents with low polarity are more effective in extracting compounds with low polarity, whereas ethanol and other solvents with high polarity are more effective in extracting compounds with high polarity. In this example, most compounds were detected by extraction using ethanol.

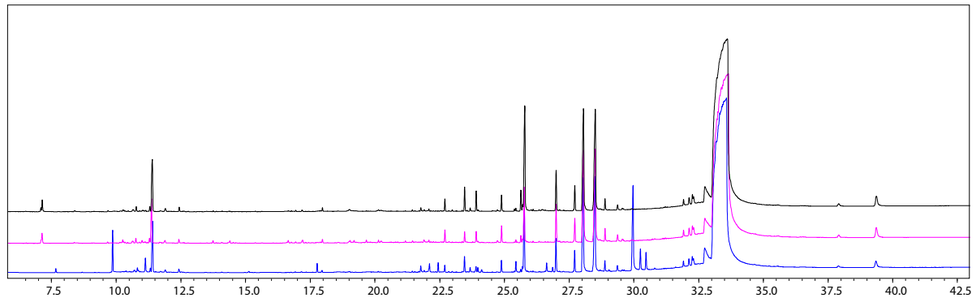

Fig. 1 TICCs for Extraction Solvents
(Black: Hexane; Pink: DCM; and Blue: Ethanol)

Table 5 Compounds Detected by Solvent Extraction

Compound	Hexane	DCM	Ethanol	Remarks
	R.T. (min)	R.T. (min)	R.T. (min)	
2-Ethylhexanol	-	-	9.86	
Isophorone	11.37	11.40	11.40	Solvent
Palmitic acid	-	21.77	21.77	Lubricant
Ethyl palmitate	-	-	22.10	Lubricant
2-Ethylhexyl methyl isophthalate	23.46	23.46	23.46	
Stearic acid	-	23.67	23.67	Lubricant
Butyl palmitate	-	23.91	23.90	Lubricant
Ethyl stearate	-	23.90	23.97	Lubricant
Terephthalic acid, ethyl 2-ethylhexyl ester	-	-	24.12	
Methyl 9,10-epoxystearate	24.89	24.89	24.88	
Butyl stearate	-	25.63	-	Lubricant
Di(2-ethylhexyl) adipate	25.75	25.78	25.78	Plasticizer (DEHA)
Ethyl stearate, 9,12-diepoxy	-	-	26.63	
Di(2-ethylhexyl) phthalate	27.00	26.99	26.99	Plasticizer (DEHP)
Bis(2-ethylhexyl) isophthalate	28.03	28.05	28.04	Plasticizer
Bis(2-ethylhexyl) terephthalate	28.48	28.51	28.50	Plasticizer
Epoxidized 2-ethylhexyl oleate	29.37	-	29.36	
Tris(2-ethylhexyl) Trimellitate	33.67	33.65	33.59	Plasticizer (TOTM)

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■ Analytical Results Using High-Temperature Extraction

Headspace-GC-MS was used for high-temperature extraction. For high-temperature extraction using a headspace sampler, samples can be analyzed after simply placing them into vials and loading the vials into the sampler, which offers the advantage of extremely simple pretreatment. First, to determine the optimal extraction temperature, the vial temperatures were varied by heating to 80, 100, 150, 200, and 250 °C to compare the results. TICCs for 80, 200, and 250 °C vial temperatures are shown in Fig. 2. In this example, a vial temperature of 250 °C was determined to provide the highest compound extraction efficiency, because it resulted in detecting the most peaks.

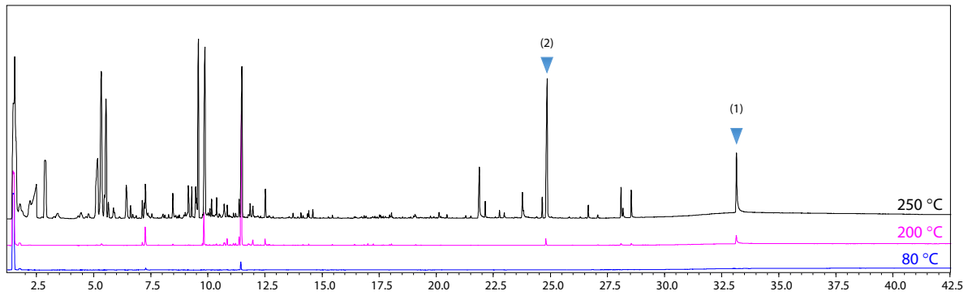


Fig. 2 TICCs for Extraction Temperatures
(Black: 250 °C; Pink: 200 °C; and Blue: 80 °C)

High-temperature extraction using headspace-GC-MS offers the advantage of simple pretreatment, but thermal decomposition of extracted components can result in more complex chromatograms that are more difficult to analyze. Therefore, the Polymer Additives Library was used in addition to the NIST Library as mass spectral libraries for compound identification. The Polymer Additives Library includes mass spectra for a wide range of additives used in polymer materials and corresponding additive decomposition products. Retention index information is registered for each compound to enable more accurate compound identification by filtering results based on retention indices.

It also includes additive classification information, so that additives can be analyzed more easily even without extensive knowledge of additives.

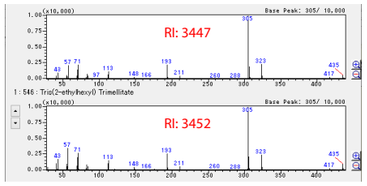
As an example of using the Polymer Additives Library for qualitative analysis, Fig. 3 shows compounds identified for peaks (1) and (2) in Fig. 2. Peak (1) was identified TOTM based on mass spectral similarity and retention indices for polymer additives. In contrast, a search for peak (2) generated no hits from the general purpose NIST Library, but by using the Polymer Additives Library it was predicted to be a decomposition product of TOTM, or in other words, a decomposition product of peak (1).

Peak (1)

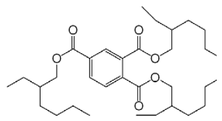
Similarity: 94

Measured spectrum

Library spectrum



Tris(2-ethylhexyl) Trimellitate



Plasticizer

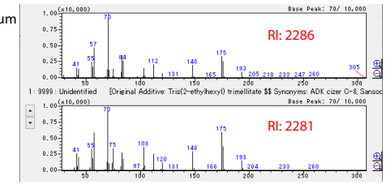
CAS#:	3319 - 31 - 1	Mol Wt:	546	Serial#:	3
Cmpd Name:	Tris(2-ethylhexyl) Trimellitate				
Formula:	C33H5406			Class Flag:	Plasticizer

Peak (2)

Similarity: 90

Measured spectrum

Library spectrum



Decomposition product of tris(2-ethylhexyl)trimellitate

CAS#:	0 - 00 - 0	Mol Wt:	9999	Serial#:	1702
Cmpd Name:	Unidentified	[Original Additive: Tris(2-ethylhexyl) trimellitate			
Formula:				Class Flag:	Plasticizer

Fig. 3 Results from Qualitative Analysis of Peaks (1) and (2) Using the Polymer Additives Library

Thus, the Polymer Additives Library can be used to easily analyze even decomposition products of extractables detected by high-temperature extraction. The compounds detected by high-temperature extraction are indicated in Table 6.

■ Conclusion

Extractables from pharmaceutical packaging materials were identified by both solvent and high-temperature extraction methods. By using a variety of solvents, solvent extraction can be used for compounds with a wide range of properties. By using a headspace sampler with high-temperature extraction, samples can be pretreated more simply and quickly than for solvent extraction. For data analysis, extractables can be identified accurately and easily by using the Polymer Additives Library. The above examples confirm that the Polymer Additives Library can be used in combination with headspace-GC-MS to easily screen extractables from pharmaceutical packaging materials.

Table 6 Examples of Compounds Detected by High-Temperature Extraction

Compound	R.T. (min)	Remarks
Benzene	2.87	Solvent
2-Ethyl-1-hexene	5.52	Decomposition products of TOTM
2-Chloro-octane	9.27	Decomposition products of PVC
3-(Chloromethyl)heptane	9.57	Decomposition products of PVC
2-Ethylhexanol	9.85	Decomposition products of TOTM
Isophorone	11.47	Solvent
Palmitic acid	21.85	Lubricant
Stearic acid	23.73	Lubricant
Butyl palmitate	23.95	Lubricant
Unidentified	24.80	Decomposition products of TOTM
Di(2-ethylhexyl) adipate	25.79	Plasticizer (DEHA)
Di(2-ethylhexyl) phthalate	27.03	Plasticizer (DEHP)
Bis(2-ethylhexyl) isophthalate	28.05	Plasticizer
2-Ethylhexyl stearate	28.14	
Bis(2-ethylhexyl) terephthalate	28.50	Plasticizer
Tris(2-ethylhexyl) Trimellitate	33.10	Plasticizer (TOTM)

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Application News

No. AD-0215

Pharmaceuticals / TD-30 & GCMS-QP2020 NX

Thermal Desorption – GC/MS Method for Screening Analysis of Extractables in Drug Packaging Materials

Cynthia Lahey[1], Elgin Ting[1], Dheeraj Handique[2], Yuvanesh Kumar[3], Yukihiko Kudo[4]
[1]Shimadzu (Asia Pacific) Pte Ltd, Singapore, [2]Shimadzu Analytical (India) Pvt Ltd, India,
[4]Shimadzu Corporation, Japan

❑ Introduction

Both extractables and leachables (E&L) from pharmaceutical packaging materials and products are of utmost concerns by authorities, since they may affect the efficacy, quality and safety [1]. Many regulatory guidance documents have been established regarding E&L approach and assessment. However, details on how to perform E&L evaluation in various packaging materials and products is still under discussion and development. Extractables are defined as the compounds that can be extracted from a drug packaging under certain conditions, e.g. in solvent and/or with heating. Meanwhile, leachables are compounds that migrate from the drug packaging into the drug under normal storage condition. Theoretically, leachables emerge from extractables, although not all leachables are extractables in practice (Figure 1) [2]. Analysis methods are needed for the detection and quantitation of extractables and leachables in pharmaceutical packaging and products. Here, we describe a screening analysis method for extractables in the packaging of ophthalmic solution by thermal desorption(TD)–GC-MS. The result is compared with leachables result of ophthalmic solution measured by GC-MS with liquid injection.

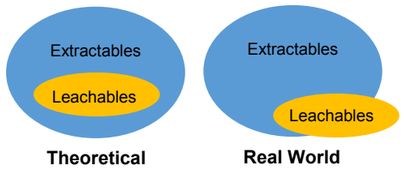

Figure 1: Relationship of Extractables and Leachables [2]

❑ Experimental

Analytical conditions

The extractables analysis was carried out using Shimadzu GCMS-QP™2020 NX coupled with thermal desorption system (TD-30). Leachables analysis was performed using the same GCMS with liquid autosampler (AOC™-20i Plus/ 20s Plus). The details of analytical conditions are shown in Table 1 and Table 2

[3]Student from Nanyang Technological University (Singapore) for internship training program

Table 1. Extractables Analytical Condition

Configuration	
Instrument	GCMS-QP2020 NX
Autosampler	TD-30
Analytical Condition	
GCMS Parameters	
Flow control mode	Linear velocity
Linear velocity	44.4 cm/s
Injection mode	Splitless
Carrier gas	Helium
Column	SH-Rxi™-5Sil MS (30 m length, 0.25 mm ID, df =0.25 μm)
Column temp program	50°C (hold time: 2 min) → rate: 10°C/min → 320°C (hold time: 6 min)
Ion source temp	200°C
Interface temp	250°C
Acquisition mode	Scan
Event time	0.3 s
m/z range	35-700 amu
TD-30 Parameters	
Tube desorb temp	150°C (15 min)
Tube desorb flow	120 ml/min
Second trap	Tenax® TA
Second trap cooling temp	-20°C
Second trap desorb temp	250°C (2 min)
Joint temp	250°C
Valve temp	250°C
Transfer line temp	250°C

Table 2. Leachables Analytical Condition

Configuration	
Instrument	GCMS-QP2020 NX
Autosampler	AOC-20i Plus/20s Plus
Analytical Condition	
GCMS Parameters	
Flow control mode	Linear velocity
Linear velocity	44.4 cm/s
Injection mode	Splitless
Carrier gas	Helium
Column	SH-Rxi™-5Sil MS (30 m length, 0.25 mm ID, df =0.25 μm)
Column temp program	50°C (hold time: 2 min) → rate: 10°C/min → 310°C (hold time: 7 min)
Ion source temp	200°C
Interface temp	250°C
Acquisition mode	Scan
Event time	0.3 s
m/z range	35-700 amu

Sample Preparation and Analysis of Extractables

In this study, we analyzed the extractables in the polymer packaging of ophthalmic solution, consisting of a bottle and a nozzle (both made of LDPE) as well as a cap (made of HDPE). These three samples were tested separately. 50 mg of each sample (cut into small pieces) was put inside an empty TD tube. Glass wool was placed on the sides of the sample to prevent it from being expelled out of the TD tube during analysis (Figure 2).

In the thermal desorption system (TD-30), the sample in the TD tube was heated to desorb its extractables. In this experiment, heating was done at 150°C desorb tube temperature. The desorbed compounds were then transferred to a second trap (containing adsorbents) for concentration and focusing. Subsequently, the extractables were released from the second trap and transferred to GCMS for analysis. These steps are illustrated in Figure 2.

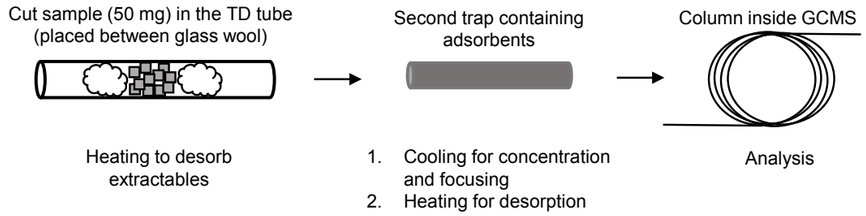

Figure 2: Schematic Diagram of Extractables Analysis on TD-GCMS

❑ Results and Discussion

Extractables Result

The chromatograms of the samples are displayed in Figure 3-5. Most of the peaks detected are hydrocarbons, which possibly came from the breakdown of lubricant wax. The bottle and nozzle samples (both LDPE) exhibit similar chromatogram profiles, while the cap sample (HDPE) has higher amount of hydrocarbons extracted.

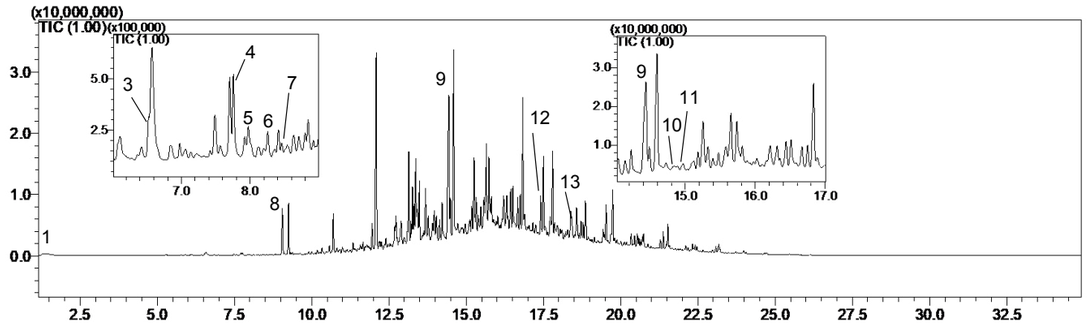

Figure 3: Total Ion Chromatogram (TIC) of Extractables in the Bottle of Ophthalmic Solution Packaging by TD-GCMS

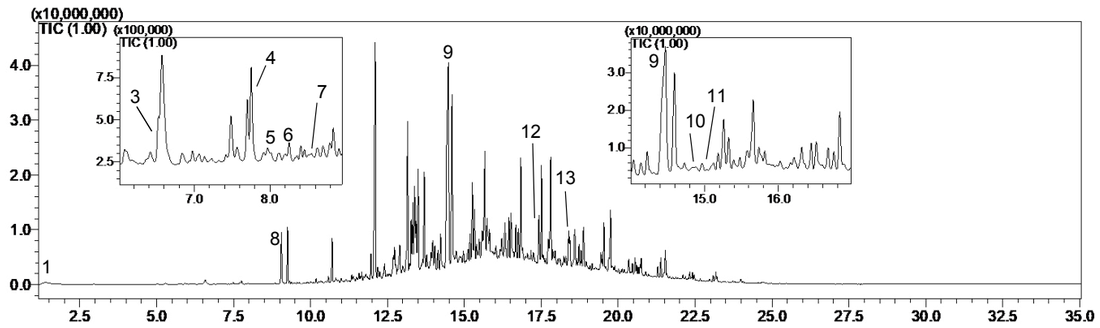

Figure 4: Total Ion Chromatogram (TIC) of Extractables in the Nozzle of Ophthalmic Solution Packaging by TD-GCMS

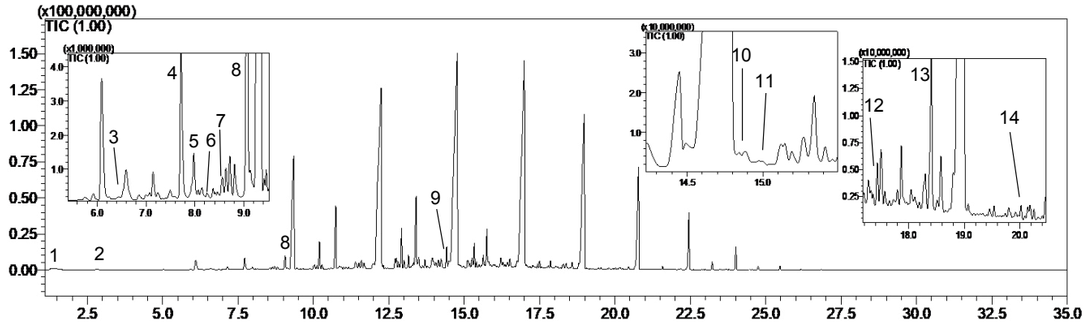

Figure 5: Total Ion Chromatogram (TIC) of Extractables in the Cap of Ophthalmic Solution Packaging by TD-GCMS

Table 3. Detection Results of Extractables in Packaging Materials by TD-GCMS (✓ Detected, × Not detected)

Peak No.	Compound	Possible source	Bottle	Nozzle	Cap
1	Acetone	Residual solvent	✓	✓	✓
2	1,3-dichloropropane		×	×	✓
3	2-Ethyl-1-hexanol	Breakdown of plasticizer or antioxidant	✓	✓	✓
4	Nonanal	Breakdown of lubricant or stabilizer	✓	✓	×
5	2-chlorobenzaldehyde		✓	✓	✓
6	Decamethylcyclopentasiloxane (D5)	Breakdown of resin modifier or lubricant	✓	✓	✓
7	Benzoic acid		✓	✓	✓
8	Naphthalene	Breakdown of fire retardant	✓	✓	✓
9	Diethyl Phthalate (DEP)	Plasticizer	✓	✓	✓
10	2,6-Bis(tert-butyl)-4-ethenylphenol	Breakdown of antioxidant	✓	✓	✓
11	Benzophenone	Breakdown of stabilizer	✓	✓	✓
12	Diisobutyl phthalate (DIBP)	Plasticizer	✓	✓	✓
13	Dibutyl phthalate (DBP)	Plasticizer	✓	✓	✓
14	Methyl stearate	Breakdown of plasticizer	×	×	✓

The results of identified extractables are presented in Table 3. Identification was carried out using NIST 14 Library and Shimadzu Polymer Additives Library. Three types of plasticizers (peak 9, 12 and 13), common additives in polymers, were detected. Various breakdown species of polymer additives (e.g. antioxidant, lubricant, fire retardant) were detected, as remarked in Table 3. Acetone, a residual solvent, was also identified in all samples.

Comparison with Leachables Result

The results of extractables obtained above are compared with that of leachables of the ophthalmic solution. The solution was stored in the complete packaging (including the bottle, nozzle and cap) under normal storage condition. The leachables analysis was performed by liquid injection of the sample to GCMS. The chromatogram profile is shown in Figure 6.

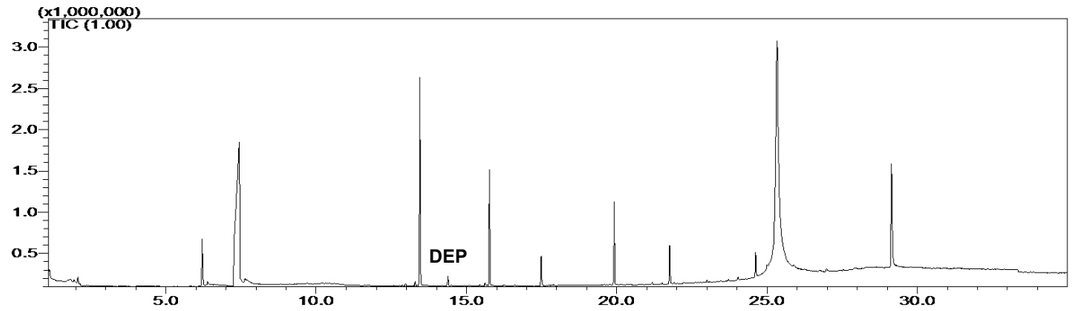

Figure 6: Total Ion Chromatogram (TIC) of Leachables in Ophthalmic Solution by Liquid Injection to GCMS

The peaks in the ophthalmic solution were mainly the content of the drug itself, except diethyl phthalate (DEP). This plasticizer was also detected in the preceding extractables analysis (peak 9, Table 3) by TD-GCMS.

❑ Conclusion

A fast and straightforward screening analysis for extractables in drug packaging was established on Thermal Desorption – GCMS system. This method is primarily suitable for qualitative screening of extractables in the drug packaging of the ophthalmic solution. Three types of plasticizers, a number of breakdowns of polymer additives, as well as other volatiles and semivolatiles were detected and identified using NIST 14 Library and Shimadzu Polymer Additives Library. As a comparison, leachables analysis of the ophthalmic solution contained in the packaging was also carried out by liquid injection of the solution to GCMS. Only one of the found extractables, i.e., DEP, was detected in the leachable analysis.

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Shimadzu GCMS-QP™2020 NX and TD-30

SHIMADZU (Asia Pacific) Pte. Ltd
79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264,
www.shimadzu.com.sg; Tel: +65-6778 6280 Fax: +65-6778 2050

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Application News

Inductively Coupled Plasma Mass Spectrometer ICPMS-2030

Extractables Analysis of Elements in Plastic Pharmaceutical Packaging

Chiho Kiriyama, Aya Urushizaki, Tadashi Taniguchi, and Takahide Hiramatsu

User Benefits

- Extracted elements can be examined based on extractables and leachables (E&L) guidelines.
- Elements extracted from packaging materials can be evaluated using ICPMS-2030.
- The ICPMS-2030 is capable of high-sensitivity analysis of multiple elements simultaneously.

■ Introduction

The extractables and leachables from packaging into pharmaceuticals is a critical issue that must be addressed. This Application News uses inductively coupled plasma mass spectrometry (ICP-MS) to perform a detailed analysis of packaging extractables that targets the elements listed in the ICH Q3D guideline on elemental impurities in pharmaceuticals. ICP-MS offers a detailed breakdown as it can simultaneously analyze an extracted solution for multiple elements with high sensitivity.

■ Extractables and Leachables (E&L)

Biopharmaceuticals and other high molecular weight pharmaceuticals are not easily absorbed into the body as oral formulations, thus they are stored in a solution such as in the case of injectables. The area of contact between a tablet formulation and its packaging is very small, while pharmaceuticals formulated in solution have a very large contact area with their packaging material, and thus leachables become an important concern. Single-use technologies play an increasingly common role in biopharmaceutical production, and leachables from plastic products used in manufacturing also pose a major issue.

Substances that transfer from packaging to a pharmaceutical product under normal conditions are typically called leachables, while substances that occur under conditions more extreme than during normal storage are called extractables. Leachables are normally identified by analyzing the pharmaceutical product itself for substances transferred under normal storage conditions. By contrast, analyzing for extractables aims to reveal potential hazards and identify which leachables occur in worst-case scenarios, thus extractables are identified by analyzing an extraction solution of the packaging material.

Standards for the measurement of extractables and leachables (E&L) include guidance issued by the FDA[1)] and other private organizations such as the PQRI[2)] and BPOG[3)], though these standards and guidelines are not harmonized. The ICH is currently developing Q3E guidelines on the assessment and control of extractables and leachables[4)], of which the current goal as of January 2023 is to reach Step 4 in 2025. The scope of ICH Q3E is anticipated to include small-molecule pharmaceuticals as well as biopharmaceutical products.

Elemental impurities must be considered among extractables and leachables. In the already-implemented ICH Q3D[5)], section 5.3 (Identification of Potential Elemental Impurities) states the following about the need for a risk assessment of the probability of elemental impurities leaching from the container closure system into the drug substance.

"For liquid and semi-solid dosage forms there is a higher probability that elemental impurities could leach from the container closure system during the shelf-life of the product. Studies to understand potential leachables from the container closure system (after washing, sterilization, irradiation, etc.) should be performed."

■ Assay of Elemental Extractables

The objective of this Application News is to measure elemental impurities in packaging extractables. The Japanese Pharmacopoeia 18th Edition, USP<233>, and Ph. Eur. Chapter 2.4.20 use ICP optical emission spectrometry (ICP-AES/OES) and ICP-MS in assay methods for the elemental impurities cited in ICH Q3D, methods that can also be used to assay elemental impurities in extractables. ICP-MS is particularly useful as it can analyze multiple elements simultaneously with high sensitivity.

The Shimadzu ICPMS-2030 (Fig. 1) used in this application is a highly sensitive and efficient analytical instrument that can operate with reduced argon gas consumption and low-purity argon gas for reduced running costs.

Fig. 1 ICPMS-2030

■ Samples for Analysis

This study performed a risk assessment of potential elemental leaching from three common infusion bags (Table 1).

Table 1 Infusion Bag Materials

Polyvinyl chloride (PVC, DEHP-free)
Polyethylene (PE)
Ethylene-vinyl acetate (EVA) copolymer

■ Experimental Method

• Preparation of Extraction Solvents

Many standards and guidelines note that the choice of extraction solvent is important for predicting which potential hazards may occur in the actual pharmaceutical product. Extraction solvents must be selected with care since E&L analyses performed under too extreme extraction conditions will not give meaningful results.

For aqueous systems, the PRQI uses water at pH 2.5 and pH 9.5 because, in general, few aqueous solvents are lower than pH 2.5 or higher than pH 9.5. The PRQI also mentions using 50 % IPA in an extraction solvent to simulate aqueous formulations containing solubilizing agents.[2)] The extraction solvents used in this study are shown in Table 2.

Table 2 Extraction Solvents and Reagents

Extraction Solvent	Reagents
pH 2.5 solution	0.01 M KCl: FUJIFILM Wako Chemicals 0.003 M HCl: Kanto Chemical
pH 9.5 solution	0.0045 M sodium dihydrogen phosphate: FUJIFILM Wako Chemicals 0.007 M disodium hydrogen phosphate: Sigma-Aldrich (pH adjusted with 1 M NaOH)
50 % IPA	High purity IPA: Kanto Chemical (diluted with pure water)

• Extraction Method

An unprinted section was cut from a transfusion bag, the surface area and weight of the section of bag were measured, and the section of bag was washed with pure water and cut into small pieces with ceramic scissors. Small pieces with a total surface area of approx. 100 cm^2 were placed into a DigiTUBE (SCP SCIENCE), 30 mL of extraction solvent was added, and the mixture was heated (70 °C for 24 H). After cooling, the liquid contents of the DigiTUBE were transferred to an empty container and used as the extraction solution. This extraction process was performed six times (n = 6) for each transfusion bag.

Extraction blanks were also created by performing the same extraction process but adding only 30 mL of extraction solvent to the DigiTUBE. This blank extraction process was performed three times (n = 3).

The extraction conditions used are shown in Table 3. This process of adding extraction solvent and sample to a test tube is based on the PQRI document, though the temperature and duration of extraction used are not identical. This is because the conditions described in this Application News represent a single trial run from a larger number of runs performed to derive a best practice for this study, an approach that is also outlined in the PQRI document[2)].

Table 3 Extraction Conditions

Total Sample Surface Area	Approx. 100 cm^2
Extraction Solvent Volume	30 mL
Heating Temperature	70 °C
Heating Time	24 hr

• Preparation of Samples for Analysis

The extraction solutions obtained from each solvent were prepared for analysis by diluting 10-fold with pure water. Nitric acid and hydrochloric acid were also added to 1 % (v/v) and 0.5 % (v/v), respectively, to stabilize the elements in the sample.

A fixed concentration of standard solution was also added to the extraction solutions to prepare samples for spike recovery analysis.

• Preparation of Standard Samples

Standard samples were prepared to create calibration curves for each targeted element. Standard samples were prepared by diluting and mixing XSTC-22 (general-purpose mixed standard solution, SPEX CertiPrep), XSTC-2071A (ICH Q3D-compatible mixed standard solution, SPEX CertiPrep), and commercially available single-element standard solutions as appropriate. Nitric acid and hydrochloric acid were also added to the standard samples to 1 % (v/v) and 0.5 % (v/v), respectively. A 50 % IPA standard sample was also prepared by adding IPA to 5 % (v/v) in the above standard solution.

The objective of this study was to primarily to target elements specified by ICH Q3D, but since no commercially available standard solution containing all these elements was available, multiple standard solutions were used to create calibration curves for all the elements.

• Analysis

The analytical conditions shown in Table 4 and calibration curves were used to simultaneously analyze extraction samples for the 24 Class 1 to 3 elements listed in ICH Q3D as well as Fe and Zn.

Be, Sc, Ga, Y, In, Te, and Bi were used as internal standard elements, and an automatic internal standard addition kit was used to add an internal standard element solution to each sample in a ratio of 1 to 9 (internal standard element solution to sample) for analysis.

The validity of results was verified by analyzing spike recovery samples and calculating spike recovery.

Table 4 ICP-MS Analytical Conditions

Instrument:	ICPMS-2030
RF Power:	1.2 kW
Plasma Gas Flowrate:	9.0 L/min
Auxiliary Gas Flowrate:	1.1 L/min
Carrier Gas Flowrate:	0.7 L/min
Nebulizer:	Nebulizer 07 UES
Pump Speed:	20 rpm
Chamber:	Electric cooled Cyclone chamber
Plasma Torch:	Mini torch
Sampling Cone/Skimmer Cone:	Cu
Collision Gas:	He
Internal Standard Element Addition Method:	Automatic addition

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■ Results of PVC Sample Analysis

• Results of PVC Extractables Analysis

Tables 5 and 6 show the extraction data obtained from PVC for the three extraction solvents. All three solvents extracted Tl and Zn. Pb and Ba were extracted with pH 2.5 and 50 % IPA, showing the amount of Pb and Ba extracted from PVC increases under acidic conditions and with 50 % IPA.

Tables 5 and 6 show the concentration of elements extracted in ng/cm^2 indicating how much of each element was extracted per 1 cm^2 of packaging material. The amount of material in contact with the drug substance must be considered when calculating the amount of elemental impurities extracted from packaging.

• Spike Recovery and Limit of Quantitation

Table 5 shows the limit of quantitation and spike recovery for measurements from the pH 2.5 extraction solution. Spike recovery results were good at between 95 and 108 %.

The packaging-adjusted PDE*1 in Table 5 is the parenteral PDE listed in ICH Q3D divided by the surface area of the inside of the transfusion bag, assuming administration of one transfusion bag per day. The measurements using ICPMS-2030 confirmed that the limit of quantitation was sufficient to measure PDE.

*1: ICH Q3D sets permitted daily exposure (PDE) limits for 24 elements in pharmaceutical products and requires valid analytical methods are used to control levels of these 24 elements.

Table 5 PDE Values and Extraction Limit of Quantitation, Spike Recovery, and Extracted Element Concentration for pH 2.5 Extraction

Class	Element	Parenteral PDE	Packaging-Adjusted PDE*2	Packaging-Adjusted Limit of Quantitation*3	Spike Recovery	Extracted Element Concentration with pH 2.5 Solvent
		µg/day	ng/cm^2	ng/cm^2	%	ng/cm^2 (n = 6)
1	Cd	2	3	0.008	100	< 0.008
	Pb	5	8	0.01	99	0.18 ± 0.04
	As	15	23	0.02	104	< 0.02
	Hg	3	5	0.05	103	< 0.05
2A	Co	5	8	0.003	101	< 0.003
	V	10	15	0.7	95	< 0.7
	Ni	20	30	0.2	102	< 0.2
2B	Tl	8	12	0.01	99	0.053 ± 0.010
	Au	300	452	0.05	95	< 0.05
	Pd	10	15	0.2	98	< 0.2
	Ir	10	15	0.01	102	< 0.01
	Os	10	15	0.02	103	< 0.02
	Rh	10	15	0.008	98	< 0.008
	Ru	10	15	0.006	102	< 0.006
	Se	80	121	0.1	95	< 0.1
	Ag	15	23	0.01	99	< 0.01
	Pt	10	15	0.03	97	< 0.03
3	Li	250	377	0.02	97	< 0.02
	Sb	90	136	0.01	101	< 0.01
	Ba	700	1056	0.008	101	0.16 ± 0.05
	Mo	1500	2262	0.009	100	< 0.009
	Cu	300	452	0.2	108	< 0.2
	Sn	600	905	0.02	101	< 0.02
	Cr	1100	1659	0.1	104	< 0.1
Other	Zn			0.2	101	148 ± 44

Table 6 Effect of Extraction Solvent on Elements Extracted from PVC

Element	Extracted Element Concentration with pH 9.5 Solvent	Extracted Element Concentration with 50 % IPA Solvent
	ng/cm^2 (n = 6)	ng/cm^2 (n = 6)
Cd	< 0.01	< 0.02
Pb	< 0.05	0.083 ± 0.02
As	< 0.03	< 0.01
Hg	< 0.1	< 0.05
Co	< 0.01	< 0.02
V	< 0.2	< 0.3
Ni	< 0.1	< 0.2
Tl	0.054 ± 0.002	0.082 ± 0.003
Au	< 0.09	< 0.03
Pd	< 0.3	< 0.2
Ir	< 0.03	< 0.03
Os	< 0.05	< 0.09
Rh	< 0.005	< 0.008
Ru	< 0.007	< 0.02
Se	< 0.2	< 0.07
Ag	< 0.006	< 0.01
Pt	< 0.05	< 0.01
Li	< 0.01	< 0.009
Sb	< 0.02	< 0.02
Ba	< 0.01	0.21 ± 0.03
Mo	< 0.006	< 0.04
Cu	< 0.1	< 0.1
Sn	< 0.02	< 0.1
Cr	< 0.2	< 0.1
Zn	70.0 ± 4.0	562 ± 95

*2: Packaging-adjusted PDE = Parenteral PDE/Internal surface area of the bag (assuming 1 bag administered per day)

*3: Packaging-adjusted limit of quantitation = Limit of quantitation in assayed sample (10σ) × 30 (extraction solvent volume) × 10 (dilution factor)/ Material surface area (100 cm^2)

*4: Extracted element concentration = (Element concentration in extraction solution - Element concentration in extraction blank) × 30 (extraction solvent volume) × 10 (dilution factor)/Material surface area

<X: Under limit of quantitation, X: Limit of quantitation

■ Results Specific to PE and EVA

Similar to PVC, almost all elements were detected in PE and EVA extraction solutions at levels below the limit of quantitation. Table 7 shows the results for elements detected in PE and EVA. The same amount of Sb was extracted from PE regardless of the solvent used and Zn was extracted from PE under basic conditions and with the 50 % IPA solvent. Fe was extracted from EVA under acidic conditions.

■ Conclusion

This study shows that the ICPMS-2030 is sensitive enough to identify and quantitate extractables at the permissible limits set by ICH Q3D. This study also reveals that the extraction behavior of different elements varies depending on the extraction solvent.

Table 7 Element Concentrations Extracted from PE and EVA and Spike Recovery

Sample	Element	pH 2.5		pH 9.5		50 %IPA	
		Extracted Element Concentration (n = 6) ng/cm^2	Spike Recovery %	Extracted Element Concentration (n = 6) ng/cm^2	Spike Recovery %	Extracted Element Concentration (n = 6) ng/cm^2	Spike Recovery %
PE	Sb	3.35 ± 0.68	102	2.48 ± 0.28	106	6.8 ± 1.3	111
	Zn	< 0.2		0.52 ± 0.29	102	0.72 ± 0.32	89
EVA	Fe	0.88 ± 0.40	107	< 0.3		< 0.5	

<X: Under limit of quantitation, X: Limit of quantitation

Packaging-adjusted limit of quantitation = Limit of quantitation in assayed sample (10σ) × 30 (extraction solvent volume) × 10 (dilution factor)/ Material surface area (100 cm^2)

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Application News

GC-MS HS-20 NX Trap/GCMS-QP 2020 NX

Analysis of Thermal Extracts from an Eye Drop Container via the Trapped Headspace (THS) Method

A. Aono

User Benefits

- With more than 20 times the sensitivity of static headspace (SHS) analysis, this system can detect extracts from pharmaceutical packaging with high sensitivity.
- With an electronic cooling trap, the system can analyze everything from low to high boiling point compounds in a single analysis cycle.
- The system can also switch between trap mode and loop mode, depending on the concentration.

■ Introduction

Compounds eluted from pharmaceutical packaging are transferred to the pharmaceuticals, and the ensuing risk to humans from continuous exposure is a focus of interest. These substances are referred to as extractables and leachables, which together are abbreviated as E&L.

Methods for measuring them are being sought at present, and various suggestions have been proposed. When screening for volatile and semi-volatile compounds, high sensitivity results are comparatively easy to obtain with the combination of GCMS and the trapped headspace (THS) method, which is why it is a focus of interest.

The HS-20 NX Trap is a trap headspace sampler equipped with an electronic cooling trap. It is optimal for thermal extraction as it can be heated up to 300 °C.

In trap mode, which uses the trapped headspace (THS) method, basically the entire volume of the headspace is concentrated into the trap. As a result, in comparison with loop mode, which uses the static headspace (SHS) method, more than 20 times the sensitivity is obtained with THS, which approaches the sensitivity of thermal desorption (TD).

This articles introduces a comparison of the measurement results for thermal extracts from an eye drop container using SHS, THS, and TD.

■ Instrument Configuration and Analysis Conditions

The analysis conditions for the thermal extracts obtained using the GCMS-QP2020 NX and the HS-20 NX Trap are shown in Table 1.

Table 1 Analysis Conditions

GCMS Analysis Conditions	
Model:	GCMS-QP2020 NX
Column:	SH-I-5Sil MS (P/N: 221-75954-30) (0.25 mm I.D. × 30 m, d.f.= 0.25 µm)
Column Temp.:	50 °C (2 min) – 10 °C/min – 320 °C (6 min) Total 35 min
Injection Mode:	Split 1 : 20
Carrier Gas Controller:	Constant Linear Velocity Mode (He)
Linear Velocity:	44.4 cm/sec
Transfer Line Temp.:	250 °C
Ion Source Temp.:	200 °C
SCAN:	*m/z* 20-600
HS Analysis Conditions	
Oven Temperature:	150 °C
Equilibration Time:	15 min
Sample Line Temp.:	250 °C
Transfer Line Temp.:	250 °C
Vial Stirring:	Off
Vial Volume:	20 mL
Vial Pressurization Time:	1.0 min
Vial Press. Equilib. Time:	0.1 min
Loading Time:	0.5 min
Load Equilib. Time:	0 min
Multi Injection:	3
Vial Pressure:	100.0 kPa (He)
Trap Cooling Temp.:	-10 °C
Trap Heating Temp.:	250 °C
Trap Adsorbent:	Tenax TA 60/80 mesh 37 mg
Injection Time:	5.0 min
Needle Flush Time:	15.0 min

■ Extractables and Leachables

Extractables are defined as compounds extracted from pharmaceutical packaging using solvents or by heating. In contrast, leachables are compounds that are transferred to the pharmaceutical agent from the pharmaceutical packaging under routine storage conditions. Theoretically, leachables are produced from extractables. However, in actuality leachables are not limited to extractables. The compounds targeted for thermal extraction in this experiment are shown in Table 2.

Table 2 Compounds Targeted for Thermal Extraction

Compound	Possible source
Nonanal	Break down of lubricant or stabilizer
Naphthalene	Break down of fire retardant
2,6-Bis(tert-butyl)-4-ethylphenol	Break down of antioxidant
DEP	Plasticizer
DiBP	Plasticizer
DBP	Plasticizer

■ Comparison of Analysis Method

With THS, the volume of gas phase concentrate is approximately 1/30 that of TD. However, the sample volume that can be filled is more than 100 times larger, so the difference in sensitivity becomes a factor of 10 (Table 3). In practice, the results indicate that the target compounds are sufficiently detected and qualified using GCMS. In addition, wide-mouth vials reduce the risk of contamination because they can be filled even if the sample has not been finely ground. (Fig. 1)

Table 3 Comparison of Analysis Methods

	THS	TD
Sample Volume	Approx. 1300 mg	Approx. 20 mg
Amount of Concentrate	Approx. 20 mL	Approx. 600 mL
Filling with the Sample	Easy	Difficult
Heating Temperature	Up to 300 °C	Up to 400 °C

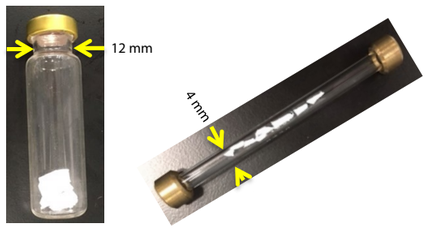
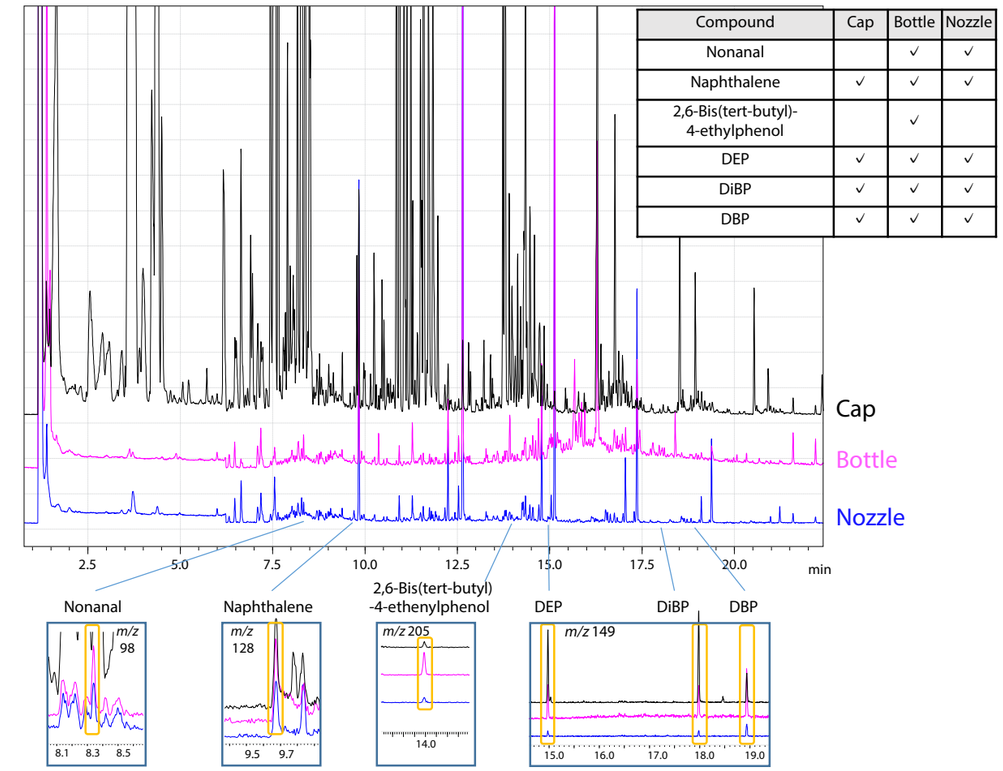


Fig. 1 Filling with the Sample (Left: Headspace Vial; Right: TD Tube)

■ Example of the Measurement of Thermal Extracts from an Eye Drop Container

From Fig. 2, it is evident that with the LDPE nozzle and bottle, the intensity of pyrolytic compounds is smaller than with the HDPE cap. However, nonanal and other target compounds were detected with sufficient sensitivity. The measurement results for the nozzle via SHS and THS are shown in Fig. 3.

Nonanal, which was basically not detected with SHS, was clearly identified with THS, at an intensity sufficient to be identified in a library search. In comparison with TD in Fig. 4, it is evident that an intensity of 1/10 or higher was obtained with THS.



Compound	Cap	Bottle	Nozzle
Nonanal		✓	✓
Naphthalene	✓	✓	✓
2,6-Bis(tert-butyl)-4-ethylphenol		✓	
DEP	✓	✓	✓
DiBP	✓	✓	✓
DBP	✓	✓	✓

Fig. 2 Thermally Extracted Compounds from an Eye Drop Container

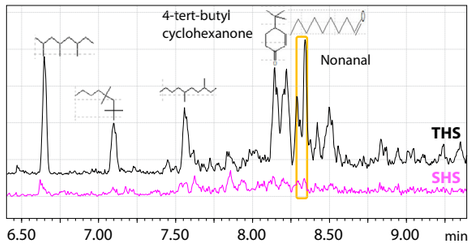


Fig. 3 Nozzle Measurement Results via SHS and THS

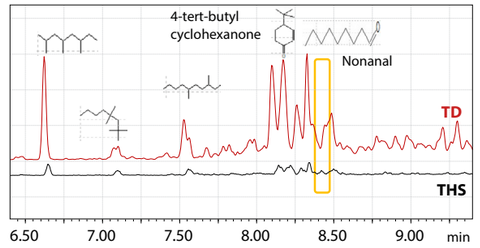


Fig. 4 Nozzle Measurement Results via THS and TD

■ Conclusion

THS can be used for measurement of thermal extracts from pharmaceutical packaging, the same as TD. In comparison with TD and other concentration systems, high sensitivity measurements can be performed inexpensively and with quick extraction. Since the system can switch between trap mode and loop mode, rather than being a dedicated E&L system, it can be utilized for a wide range of applications including the analysis of residual solvents, impurities, and diffused gases.

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ICPMS-2040/2050 Inductively Coupled Plasma Mass Spectrometer

Application News

Screening Analysis of 24 Elemental Impurity Elements in Drugs Using ICPMS-2040/2050

Kana Matsuno, Kisho Hori

User Benefits

- Controlled elements can be verified by a screening analysis of 24 elemental impurity elements.
- Use of the preset method in LabSolutions™ ICPMS makes it possible to begin the analysis simply without laborious study of conditions.
- Study of candidate elements for internal standard correction is possible.

■ Introduction

The ICH Q3D Guideline for Elemental Impurities [1] establishes permitted daily exposure (PDE) levels for 24 elements of toxicological concern.

The elements that should be considered in risk assessments differ, depending on the administration route of the drug product. For oral preparations, only seven elements of Class 1 and 2A should be considered, except for the intentional addition of elements such as catalyst utilization during synthesis. However, the origins of contamination by elemental impurities are diverse, including not only the components of the drug substance and additives, but also manufacturing equipment and implements.

Therefore, a screening analysis was carried out for all 24 elements, considering potential elemental impurities.

In addition, when internal standard correction is to be used, as in a related article [a], the content of candidate internal standard elements in the specimens must be verified in advance. In this article, a simple concentration analysis of the 24 elements in a drug product as elemental impurities was carried out using the preset method for screening analysis of drug products, which is included in the ICPMS-2040/2050 (Fig. 1). The concentration of the elements used in internal standard correction in the drug was also measured, and it was found that the elements can be used in correction.

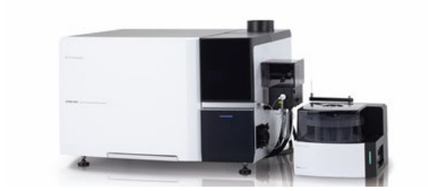

Fig. 1 ICPMS-2040/2050 and AS-20 Autosampler

Registration Elements / Mass List

	Elem	Mass	Type	Cond.	Cell Gas
1	Ag	107	QUANT	He1	He
2	As	75	QUANT	He1	He
3	Au	197	QUANT	He1	He
4	Ba	137	QUANT	He1	He
5	Be	9	QUANT	No Gas	OFF
6	Bi	209	QUANT	He1	He
7	Cd	111	QUANT	He1	He
8	Co	59	QUANT	He1	He
9	Cr	52	QUANT	He1	He
10	Cu	63	QUANT	He1	He
11	Ga	71	QUANT	He1	He
12	Hg	202	QUANT	He1	He
13	Ho	165	QUANT	He1	He
14	In	115	QUANT	He1	He
15	Ir	193	QUANT	He1	He
16	Li	7	QUANT	No Gas	OFF

Fig. 2 Analysis Elements/Mass Registration Screen

■ Flow of Analysis

First, a screening analysis of the 24 elemental impurities is conducted with a 2-point calibration curve for a simple determination of the element concentrations. The elements Sc, Ga, In, and Bi, which are frequently used in Internal standardization, were selected as the candidate internal standard elements, and the concentrations of those elements in the sample solution were measured simultaneously with the 24 elements.

A precise quantitative analysis using the selected internal standard elements is carried out for the 7 essential elements of the assessment and the elements narrowed down in the screening analysis [a].

■ Preset Method

The LabSolutions ICPMS software includes a preset method for use in screening analyses of drug products, in which the analysis conditions, measured mass, standard solution concentrations, and other conditions are registered in advance (Fig. 2 and Fig. 3). Therefore, time and trouble necessary in study of the analysis conditions and registration of a large number of measured masses and concentrations are not required.

In this article, the measurements were carried out according to the registered method.

■ Sample

Oral drug products (gastrointestinal drug, orally disintegrating (OD) tablet)

■ Sample Preparation

4 mL of pure water, 4 mL of nitric acid, and 0.5 mL of hydrochloric acid were added to approximately 0.2 g of the test sample and then digested in a microwave digestion system (200 ˚C, approximately 60 min). Hydrochloric acid was added to improve the stability of Hg and other elements in the solution.

The digestion vessel was cooled to room temperature, and the sample solution was then made up to 50 mL (250-fold dilution).

List of Calibration-Curve Standards(Check ON = Exclude Mass):

	Element	Unit	CAL1 BLK	CAL2 STD
	Ag	ug/L	0.0000000	30.00000
	As	ug/L	0.0000000	15.00000
	Au	ug/L	0.0000000	5.000000
	Ba	ug/L	0.0000000	30.00000
	Be	ug/L	0.0000000	100.0000
	Bi	ug/L	0.0000000	10.00000
	Cd	ug/L	0.0000000	5.000000
	Co	ug/L	0.0000000	30.00000
	Cr	ug/L	0.0000000	30.00000
	Cu	ug/L	0.0000000	30.00000
	Ga	ug/L	0.0000000	100.0000
	Hg	ug/L	0.0000000	5.000000
	Ho	ug/L	0.0000000	10.00000
	In	ug/L	0.0000000	10.00000
	Ir	ug/L	0.0000000	5.000000

Fig. 3 Calibration Sample Registration Screen

■ Adjustment of Standard Solution

Standard solutions containing the 24 elements that were the targets of the analysis and the candidate elements for use in correction of the internal standard, together with a blank solution, were prepared. To reduce the labor required in sample preparation, the following certified mixed standard solutions were used and prepared at the concentration registered in the method, as shown in Fig. 3. The concentrations can be modified as appropriate for the set control target value and the standard solution used.

- Standard substances
  - Certified mixed standard solutions for ICH Q3D *1 (XSTC-2071A, XSTC-2073)
  - Mercury standard solutions for ICH Q3D*2
  - Single-element standard solutions of Sc, Ga, In, and Bi *2 *3

*1 Manufactured by SPEX CertiPrep
*2 Manufactured by FUJIFILM Wako Pure Chemical Corporation
*3 Manufactured by Kanto Chemical Co., Ltd.

■ Equipment Configuration and Analysis Conditions

Table 1 shows the equipment configuration, and Table 2 shows the analysis conditions.

It should be noted that only He gas is used as the cell gas in the preset method for drug products in order to eliminate spectral interference. However, the gases that can be introduced into the cells of the ICPMS-2040 and ICPMS-2050 are as shown in Table 3. The same analysis as in this article can be carried out with either instrument.

Table 1 Equipment Configuration

Instrument	: ICPMS-2040/2050
Nebulizer	: Nebulizer DC04
Chamber	: Cyclone chamber
Torch	: Mini-torch
Skimmer cone	: Nickel
Autosampler	: AS-20

Table 2 Analysis Conditions

RF power	: 1.20 kW
Plasma gas flowrate	: 9.0 L/min
Auxiliary gas flowrate	: 1.10 L/min
Carrier gas flowrate	: 0.85 L/min
Cell gas	: He

Table 3 Available Cell Gases

Instrument	He	H_2	3rd gas (option)
ICPMS-2040	○	×	×
ICPMS-2050	○	○	○

■ Measurement Results

The sample solutions were measured using a 2-point calibration curves for the standard solutions and blank. Table 4 shows the measurement results.

It can be understood that the contents of the elemental impurities were substantially lower than the PDE concentration conversion values.

The elements Sc, Ga, In, Bi were all undetected, indicating that they can be used as internal standard elements. The amount of addition of internal standard elements should be a concentration that results in sufficiently small variations, and if the element is detected in the sample, its content should be on a negligible level.

Table 4 Results of Measurements of 24 Elemental Impurity Elements

Class	Element	PDE value for oral drug products (µg/day)	PDE concentration conversion value (µg/g)	Limit of determination in sample (µg/g)	Concentration in sample (µg/g)
1	^{111}Cd	5	0.5	0.005	<
	^{208}Pb	5	0.5	0.0005	0.007
	^{75}As	15	1.5	0.01	<
	^{202}Hg	30	3	0.001	<
2A	^{59}Co	50	5	0.004	<
	^{51}V	100	10	0.02	<
	^{60}Ni	200	20	0.05	<
2B	^{205}Tl	8	0.8	0.0008	<
	^{197}Au	300	30	0.001	0.006
	^{105}Pd	100	10	0.002	<
	^{193}Ir	100	10	0.0005	<
	^{189}Os	100	10	0.002	<
	^{103}Rh	100	10	0.0007	<
	^{101}Ru	100	10	0.003	<
	^{78}Se	150	15	0.2	<
	^{109}Ag	150	15	0.002	<
	^{195}Pt	100	10	0.002	<
3	^{7}Li	550	55	0.02	<
	^{121}Sb	1200	120	0.007	<
	^{137}Ba	1400	140	0.006	0.007
	^{95}Mo	3000	300	0.007	<
	^{63}Cu	3000	300	0.02	<
	^{118}Sn	6000	600	0.006	<
	^{52}Cr	11000	1100	0.01	0.07

PDE concentration conversion value: Option 1 was selected (maximum daily dose: 10 g).
Limit of determination (quantitative limit): 10 × σ (standard deviation of calibration curve blank) × Slope of calibration curve.
< : Below limit of determination (quantitative limit)

■ Conclusion

In this article, a simultaneous screening analysis of 24 elemental impurity elements in an oral drug product and candidate elements for internal standard correction was carried out using an ICPMS-2040/2050.

The time and work necessary in study and registration of the analysis conditions and measured masses of the large number of elements in this experiment can be greatly reduced by using the preset method of the LabSolutions ICPMS software.

<References>

1) ICH HARMONISED GUIDELINE FOR ELEMENTAL IMPURITIES Q3D(R2)

<Related Applications>

a. Application News 01-00577, Analysis of Elemental Impurities in Oral Drug Products Using ICPMS-2040/2050 –ICH Q3D–
Application News 01-00577-en

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Inductively Coupled Plasma Mass Spectrometer ICPMS-2040/2050

Application News

Analysis of Elemental Impurities in Oral Drug Products Using ICPMS-2040/2050 —ICH Q3D—

Kana Matsuno and Kisho Hori

User Benefits

- Robust quantitative analysis of control thresholds even when Option 1 (ICH Q3D) is used to convert PDEs to concentration limits
- Easily meets acceptance criteria for accuracy and precision for the analytical procedure used to quantify elemental impurities
- Preset methods eliminate the labor needed to establish analytical conditions and allow anyone to perform analysis with ease

■ Introduction

ICH Q3D (R2) Guideline for Elemental Impurities[1] establishes permitted daily exposure (PDE) levels for 24 elements of toxicological concern. The guideline requires that levels of these elements be controlled by appropriate analytical methods.

In response to this guideline, section "2.66 Elemental Impurities" [2] was included in the Japanese Pharmacopoeia (JP), sections "<232> Elemental Impurities—Limits" and "<233> Elemental Impurities—Procedures" [3] were included in the United States Pharmacopeia (USP), sections "5.20 Elemental Impurities" and "2.4.20 Determination of elemental impurities" [4] were included in the European Pharmacopoeia (EP), and inductively coupled plasma mass spectrometry (ICP-MS) was added as an analytical method.

This Application News describes using the ICPMS-2050 (Fig. 1) to analyze commercially available oral drug products (a gastrointestinal drug). Assuming that elemental impurities are measured at concentrations lower than 30 % of the PDE (control threshold), accuracy, precision, and quantitative limits were confirmed in an example analysis performed according to the quantitative procedures described in the JP, USP, and EP.

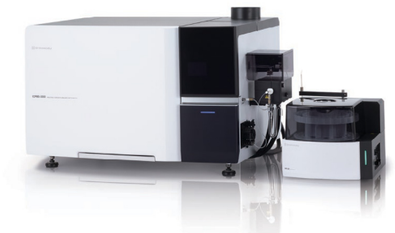

Fig. 1 ICPMS-2050 and AS-20

■ Sample

Test Sample: Oral drug products (gastrointestinal drug, orally disintegrating tablet)

Standard Solutions: Multi-element mixed standard solution for ICH Q3D oral agents (Cd, Pb, As, Co, V, and Ni)
Mercury standard solution for ICH Q3D
Single-element standard solutions of Sc, Ga, In, and Bi

■ Target Elements

The elements targeted in a risk assessment differ depending on the route of administration of the drug products. Quantities of Class 1 and 2A elements were determined as required for a risk assessment of oral drug products.

■ Sample Preparation

Test samples were prepared for analysis by the procedure shown in Fig. 2.

Approximately. 0.2 g of the test sample was added 4 mL of pure water, 4 mL of nitric acid, and 0.5 mL of hydrochloric acid, and then digested in a microwave digestion system (200 °C for approximately 60 minutes). Hydrochloric acid was added to improve the stability of Hg and other elements in the solution.

The digestion vessel was cooled to room temperature to avoid vaporization of elements with a low boiling point, and then the sample solution was made up to 50 mL for analysis (250-fold dilution).

A preparation blank solution containing no test sample was also prepared by the same procedure to verify the amounts of each element introduced as contaminants during the digestion process.

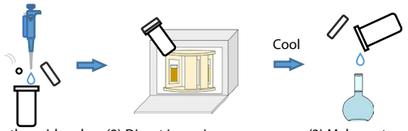

Fig. 2 Sample Preparation Procedure

■ Target Limits and Test Solution Concentrations

When evaluating the significance of elemental impurity levels, target limits can be set to 30 % of the PDE (control threshold) divided by the maximum daily dose (Equation 1). PDEs were converted into concentration limits using Option 1, which assumes a maximum total daily dose of 10 g of the drug products.

$$\text{Target Limit [µg/g]} = \frac{\text{PDE [µg/day]} \times 30\ [\%]}{10\ [\text{g/day}]} \quad \ldots[1]$$

Target limits were converted to target concentrations in the sample solution using Equation 2.

$$\text{Target concentration [µg/L]} = \frac{\text{Target Limit [µg/g]} \times \text{Sample Digestion Amount [g]}}{\text{Volume [L]}} \quad \ldots[2]$$

■ Standard Solution

- Standard Solutions
  Standard solutions containing Class 1 and Class 2A elements at 50 %, 100 %, and 150 % of the target concentrations were prepared along with a blank solution. The same volumes of nitric acid and hydrochloric acid as the sample solutions were added to the standard solutions.
- Internal Standard Solution
  Commercially available single-element standard solutions were diluted and mixed to prepare an internal standard solution containing Sc at 2000 µg/L, Ga at 1000 µg/L, and In and Bi at 100 µg/L. The same volumes of nitric acid and hydrochloric acid as the internal standard solutions were added to the sample solutions. To reduce the labor involved in sample preparations, an online internal standard kit was used to automatically add the internal standard solution to a sample at the internal standard ratio of approximately 9:1.

■ Quantitative Method

The accuracy and precision of the quantitative method were assessed by analyzing a sample solution spiked with target elements at 50 %, 100 %, and 150 % of the target concentration.

Accuracy was verified based on recovery rates from a spike and recovery test; precision was verified based on the relative standard deviation (RSD) when concentration levels were measured six times in a sample spiked with 100 % of the target concentration; and quantitative limits were verified based on spike recovery after spiking to 50 % of the target concentration.

■ Equipment Configuration and Analytical Conditions

The equipment setup is shown in Table 1, and the analytical conditions used are shown in Table 2. The analytical conditions and mass measurements were taken from a preset method available in LabSolutions™ ICPMS.

Collision mode (He gas) was used to eliminate spectral interference. The same analysis can also be performed using the ICPMS-2040, which is a dedicated collision mode system.

Table 1 Equipment Configuration

Instrument:	ICPMS-2050
Nebulizer:	Nebulizer DC04
Chamber:	Cyclone Chamber
Torch:	Mini-Torch
Skimmer Cone:	Nickel
Autosampler:	AS-20
Internal Standard Elements:	Online Internal Standard Kit (sample: internal standard = about 9 : 1)

Table 2 Analytical Conditions

RF Power:	1.20 kW
Plasma Gas Flowrate:	9.0 L/min
Auxiliary Gas Flowrate:	1.10 L/min
Carrier Gas Flowrate:	0.85 L/min
Cell Gas:	He

■ Results

The results are shown in Table 3. The concentrations in the solid test sample were calculated by subtracting the concentrations in the preparation blank from the concentrations in the sample solution and then multiplying by the dilution factor.

The concentrations of all elements in the sample solution were less than the target concentrations.

The acceptance criteria for the accuracy and precision of the JP, USP, and EP quantitative procedures are spike recoveries between 70 % and 150 % (accuracy) and RSDs of 20 % or below (precision). Each pharmacopoeia lists different quantitative limits, the most stringent of which require that the accuracy acceptance criteria are met at no more than 50 % of the target concentration. In this case, spike recovery rates between 94 % and 104 % and RSD results of 3 % or below met the acceptance criteria with sufficient margins.

Instrument detection limits (IDLs) were also verified to be sufficiently low at no more than 1/10 of 50 % of the target concentrations.

■ Conclusion

This Application News used the ICPMS-2050 to analyze oral drug products.
The ICPMS-2050 was shown to offer performance that easily meets the acceptance criteria for the accuracy, precision, and quantitative limits of the quantitative procedures described in the JP, EP, and USP.
Investigating analytical conditions and which masses to measure is time-consuming and labor-intensive; in this example analysis, such time-consuming work was avoided by using a preset method from LabSolutions ICPMS.
Based on the above, the ICPMS-2050 is an effective tool for analyzing elemental impurities in oral drug products.

< References >
1) ICH Q3D (R2) GUIDELINE FOR ELEMENTAL IMPURITIES
2) Japanese Pharmacopoeia: 2.66 Elemental Impurities
3) United States Pharmacopeia
<232> ELEMENTAL IMPURITIES-LIMITS
<233> ELEMENTAL IMPURITIES-PROCEDURES
4) European Pharmacopoeia
5.20 Elemental Impurities
2.4.20 Determination of Elemental Impurities

Table 3 Quantitative Results

Class	Element	IDL [µg/L]	Preparation Blank Concentration [µg/L]	Sample Solution Concentration [µg/L]	Concentration in Solid Sample [µg/g]	Spiked to 50 % of the Target Concentration		Spiked to 100 % of the Target Concentration			Spiked to 150 % of the Target Concentration	
						Spiked Concentration [µg/L]	Recovery [%]	Spiked Concentration [µg/L]	Recovery [%]	RSD [%]	Spiked Concentration [µg/L]	Recovery [%]
1	^{111}Cd	0.006	N.D.	N.D.	N.D.	0.3	94	0.6	97	2.2	0.9	96
	^{208}Pb	0.001	0.005	0.029	0.006	0.3	102	0.6	103	0.95	0.9	103
	^{75}As	0.01	N.D.	N.D.	N.D.	0.9	103	1.8	100	0.74	2.7	99
	^{202}Hg	0.003	N.D.	N.D.	N.D.	1.8	96	3.6	96	0.94	5.4	97
2A	^{59}Co	0.003	N.D.	N.D.	N.D.	3	100	6	100	0.73	9	100
	^{51}V	0.02	N.D.	N.D.	N.D.	6	103	12	104	0.93	18	104
	^{60}Ni	0.1	N.D.	0.2	0.05	12	104	24	104	0.60	36	104

IDL = Standard deviation upon repeated analysis of the blank solution × 3 × Calibration curve slope
N.D. = Not Detected

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Application News

Anion Suppressor Ion Chromatograph HIC-ESP

Simultaneous Analysis of Nitrate and Nitrite Ions in Pharmaceutical Additives

Ayano Tanabe[1], Chihiro Kenjo[2]
1 Shimadzu Corporation, 2 Shimadzu Techno Research

User Benefits

- Nitrite and nitrate ions contained in pharmaceutical additives can be determined with simple pretreatment procedure.
- High-sensitivity analysis on the order of µg/L can be performed using UV detector.
- Employed instrument setup can be applied to multipurpose anion analysis using suppressor-based ion chromatography.

■ Introduction

Since 2018, efforts have been underway in various countries to reduce the risk of nitrosamines contamination, originated by the detection of nitrosamines, a carcinogenic substance, in pharmaceutical products in Japan and other countries and following recall or discontinuation of certain pharmaceutical products.

Nitrosamines are known to be formed by the reaction of amines with nitrite. It has been reported that nitrous acid added for the purpose of inactivating azide compounds used in synthetic process and nitrites presenting as impurities in reagents and excipients raise a risk for the formation of nitrosamines. For this reason, the US FDA guidance[1)] and EP monograph[2)] also refer to the analysis of nitrite as an impurity.

■ Analytical Conditions

A suppressor type ion chromatograph HIC-ESP with a UV detector and Shim-pack™ IC-SA3 analytical column were employed for this study. Fig. 1 shows the flow path diagram and Table 1 shows the analysis conditions. The UV detector was used for the high sensitivity trace level determination of nitrite and nitrate ions in pharmaceutical additives.

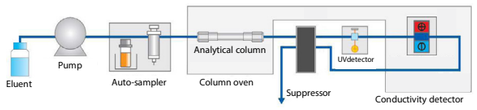

Fig.1 Flow Pass Diagram of Suppressor Type Ion Chromatography HIC-ESP

Table1 Analytical Conditions

System	: HIC-ESP＋SPD-40
Column	: Shim-pack IC-SA3*1 (250 mm×4.0 mm I.D., 5 µm)
	: Shim-pack IC-SA3(G)*2 (10 mm × 4.6 mm I.D., 5 µm)
Mobile Phase	: 3.6 mmol/L Sodium Carbonate
Flow Rate	: 0.8 mL/min
Column Temp.	: 45 ℃
Injection Vol.	: 50 µL
Detection	: UV 210 nm

*1 P/N : 228-41600-91, *2 P/N : 228-41600-92

■ Analysis of Standard Solutions

Fig. 2 shows a chromatogram at the lowest level of the calibration curve (0.002 mg/L each for nitrite and nitrate ions), and Fig. 3 shows a six-level linear calibration curve from 0.002 to 0.1 mg/L. The coefficients of determination were all larger than 0.9998, showing good linearities.

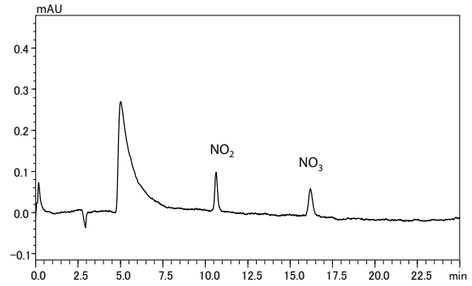

Fig.2 Chromatogram of Standard Nitrate and Nitrite Ions (Each 0.002 mg/L)

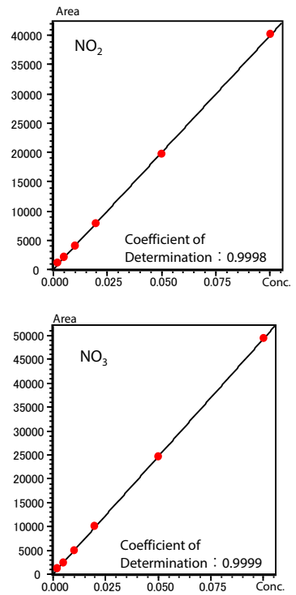

Fig.3 Calibration Curves for Nitrate and Nitrite Ions (Each 0.002-0.1 mg/L)

■ Applications

Polyvinyl Alcohol

10 mL of ultrapure water was added to 0.1 g of sample and heated to 60 ℃ to dissolve. Obtained chromatogram is shown in Fig. 4.

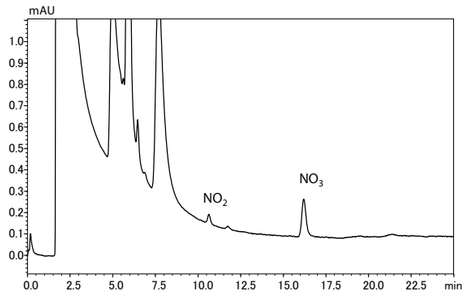

Fig.4 Chromatogram of Nitrate and Nitrite Ions in Polyvinyl alcohol

Silicic Acid Anhydride

5 mL of ultrapure water was added to 0.5 g of sample, and it was shaken with a shaker for 10 min, then centrifuged (2500 rpm, 10 min). obtained supernatant was subjected to analysis. Obtained chromatogram is shown in Fig. 5.

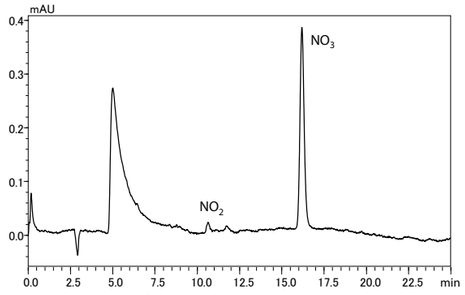

Fig.5 Chromatogram of Nitrate and Nitrite Ions in Silicic Acid Anhydride

Sodium Stearyl Fumarate

5 mL of ultrapure water was added to 0.2 g of sample, and it was shaken with a shaker for 10 min, then centrifuged (2500 rpm, 10 min). Obtained supernatant was filtered with a membrane filter (pore size 0.2 µm) for ion chromatography then subjected to analysis. Obtained chromatogram is shown in Fig. 6.

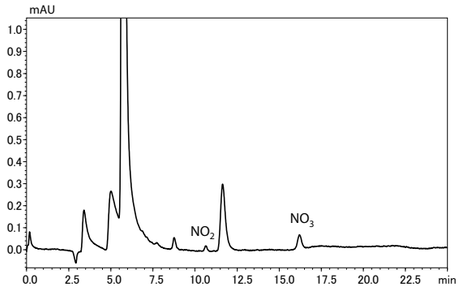

Fig.6 Chromatogram of Nitrate and Nitrite ions in Sodium Stearyl Fumarate

Determination results and recoveries

Resulting concentrations and recovery rates are shown in Table 2. Recovery rates were calculated by adding a standard nitrate and nitrite compounds to respective samples. and the results were good enough for ranging from 95% to 100% for both nitrite and nitrate ions.

■ Conclusion

A suppressor type ion chromatograph HIC-ESP was connected to a UV detector for high sensitivity analysis of nitrite and nitrate ions in pharmaceutical additives. Trace amounts of nitrite and nitrate ions were detected in all samples, and satisfactory recovery rates of more than 95% was obtained.

Many samples can be handled by dissolving or extracting them into water as a pretreatment for this method. A simple pretreatment enables high sensitivity analysis.

Table 2 Resulting concentrations and recovery rates

Sample	Sample amount g	Sample volume mL	Added standard mg/L		Resulting conc. mg/L		Recovery rate*3 %		Content rate µg/g	
			NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
Polyvinyl alcohol	0.100	10	–	–	0.001*4	0.006	100	95	0.1	0.6
	0.100	10	0.02	0.02	0.021	0.025				
Silicic acid anhydride	0.496	5	–	–	0.001*4	0.013	95	100	0.01	0.13
	0.499	5	0.02	0.02	0.020	0.033				
Sodium stearyl fumarate	0.200	5	–	–	0.001*4	0.002	95	100	0.03	0.05
	0.200	5	0.02	0.02	0.020	0.022				

*3 Recovery rate=(concentration after addition-concentration before addition)/additional amount x 100
*4 Obtained through extrapolation of calibration curve

＜References＞

1) FDA Control of Nitrosamine Impurities in Human Drugs
https://www.fda.gov/regulatory-information/search-fda-guidance-documents/control-nitrosamine-impurities-human-drugs
（Confirmed on Sep. 26th, 2023）

2) European Pharmaceutical Review
https://www.europeanpharmaceuticalreview.com/news/178501/ph-eur-adopts-revised-general-monographs-after-adding-para-on-n-nitrosamines/ （Confirmed on Sep. 26th, 2023）

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Application News

AIRsight™ Infrared/Raman Microscope

Contaminant Analysis of Pharmaceuticals (Tablets) Using AIRsight Infrared/Raman Microscope

Shoko Iwasaki

User Benefits

- Use of AIRsight makes it possible to acquire both infrared and Raman spectra at the same location without moving the measurement target sample.
- Highly accurate qualitative analysis is possible by measuring the infrared and Raman spectra at the same location.
- Even microscopic contaminants on pharmaceuticals (tablets) can be analyzed by simple operation.

■ Introduction

In recent years, consumers have shown heightened concern about contamination of products by foreign matter, and demand for analysis to respond to this problem has also increased. Although news reports that contaminants have been discovered in some foods and pharmaceuticals have appeared from time to time, it is difficult to eradicate this problem completely, as the causes of contamination are assumed to include various processes such as contamination of raw materials at the time of purchase, contamination of the product due to deterioration of component parts of the production line, and contamination of the product by the consumer. The types of foreign matter are also diverse, including not only organic materials such as human hair, plastics, and rubber, but also oxides, metal fragments, and other inorganic substances. For these reasons, higher accuracy is required in qualitative analysis in order to identify the cause of contamination.
The AIRsight Infrared/Raman microscope is a new microscope in which a Raman unit is incorporated in an infrared microscope, making it possible to carry out both Raman and infrared analysis with a single instrument, even though separate instruments had been required until now. Fig. 1 shows the appearance of the AIRsight. Since the infrared spectrum and Raman spectrum can be acquired at the same location, without moving the sample, the accuracy of qualitative analyses of micro regions is dramatically improved. Operation is also simple because both the infrared and Raman measurements can be controlled with one software program, AMsolution.
This article introduces an example of measurement of a contaminant adhering to the surface of a pharmaceutical (tablet) (hereinafter, "tablet") by micro-infrared spectroscopy and micro-Raman spectroscopy.

Fig. 1 Appearance of AIRsight™

■ Infrared Spectroscopy and Raman Spectroscopy

In infrared spectroscopy, infrared light is irradiated on the sample and the amount of light absorbed at each wavelength (wavenumber) is measured. In contrast, in Raman spectroscopy the light scattered by sample when it is irradiated with light of a specified wavelength is measured, and the difference between the incident light and the scattered light (i.e., the Raman shift) is then calculated. Like the infrared spectrum, the Raman spectrum is based on the vibrational spectrum of molecules. Both techniques are used for purposes such as identification of substances by comparison with known spectra and structural determination and quantitative analysis of molecules. However, the intensity and shape of the detected peaks differ in the two methods.

■ Measurement Samples and Conditions

Fig. 2 shows the appearance of a contaminant adhering to the surface of a tablet. The contaminant is reddish-brown and exists in a scattered form over a range of about 100 µm on the tablet surface. With conventional instruments, the single most time-consuming process is the work of setting the tablet on the microscope stage and adjusting the measurement position so that it is in the field of view (FOV) of the microscope. With AIRsight, this work can be completed easily because the wide-field observation camera, which is provided as standard equipment, makes it possible to observe a FOV at a size that is visible to the human eye (10 × 13 mm). The wide-field camera is also equipped with a digital zoom function with a maximum magnification of 5x (2.0 × 2.6 mm). The microscope camera, which is used in the actual measurement, is also equipped with a 10x zoom (0.03 × 0.04 mm) function, so even microscopic contaminants can be observed smoothly. Positional information is shared by the microscope camera and the wide-field camera, ensuring that the FOV does not shift due to switching between the two cameras.
Table 1 shows the measurement conditions.

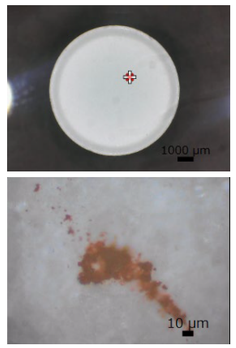

Fig. 2 Appearance of Contaminant on Tablet Surface
Top: Image of Entire Tablet Observed with Wide-Field Camera
Bottom: Image of Contaminant on Tablet Surface Observed with Microscope Camera

Table 1 Measurement Conditions

Instruments	:	IRTracer™-100, AIRsight
Infrared Spectrometry		
Resolution	:	8 cm^{-1}
Accumulation	:	100
Apodization Function	:	SqrTriangle
Detector	:	T2SL
Raman Spectrometry		
Accumulation	:	100
Exposure Time	:	1.0 sec
Objective Lens	:	50x
Excitation Wavelength	:	785 nm
Detector	:	CCD

■ Contaminant Analysis by Micro-Infrared Spectroscopy

First, the infrared spectra were acquired. An analysis of the normal area and an area with an adhering contaminant was conducted by micro-ATR measurement. Fig. 3 shows the acquired infrared spectra.

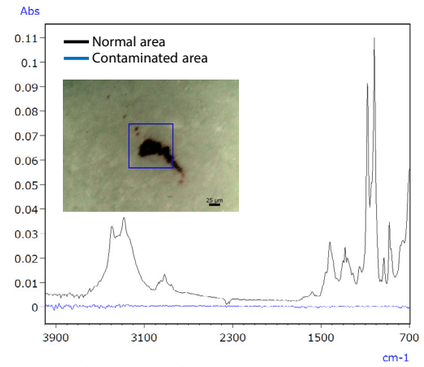

Fig. 3 Infrared Spectra of Normal and Contaminated Areas

The infrared spectrum of the normal area corresponded to the main component (mannitol) of a pharmaceutical product. However, it was not possible to identify the cause of contamination because no peaks were detected from the area with the adhering contaminant.

■ Contaminant Analysis by Micro-Raman Spectroscopy

Next, the Raman spectra were acquired and an analysis of the normal and contaminated areas was carried out by micro-Raman measurement. Fig. 4 shows the measurement results of the Raman spectra. The intensities of the spectra are also shown. The differences between the spectra of the normal area and the contaminated area were clearly evident.

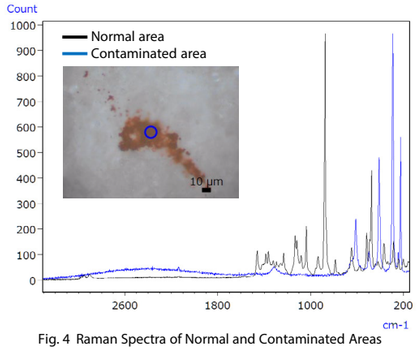

Fig. 4 Raman Spectra of Normal and Contaminated Areas

A Raman spectrum was acquired for iron oxide, which may possibly have adhered to the tablet surface, and the spectra of the contaminated part of the sample tablet and the iron oxide were overlaid, as shown in Fig. 5. Since the two spectra showed close agreement, the contaminant adhering to the tablet surface was inferred to be iron oxide.

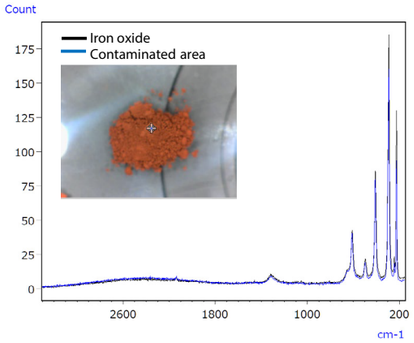

Fig. 5 Raman Spectra of Contaminated Area and Iron Oxide

■ Infrared Spectrum of Iron Oxide

As shown in Fig. 3, it was not possible to identify the cause of the contaminated area by micro-infrared spectroscopy. Fig. 6 shows the infrared spectrum (measurement method: single-reflection ATR) of iron oxide, which was estimated to be the contaminant by micro-Raman spectroscopy.

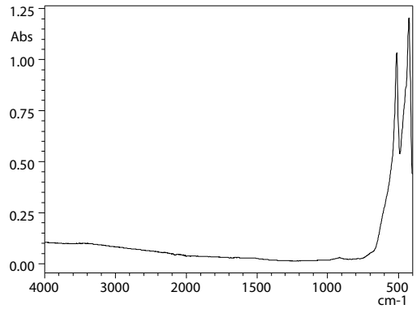

Fig. 6 Infrared Spectrum of Iron Oxide by Single-Reflection ATR Method

Because the characteristic peak in the infrared spectrum of iron oxide was located on the low wavenumber side from 510 cm^{-1}, it could not be detected by micro-infrared spectroscopy using an MCT detector. However, useful data could be obtained by Raman spectroscopy because this technique has higher qualitative capability for inorganic compounds than infrared spectroscopy.

■ Conclusion

As introduced in this article, a contaminant analysis of a pharmaceutical tablet was carried out by micro-infrared spectroscopy and micro-Raman spectroscopy. Although trace amounts of inorganic compounds are difficult to analyze qualitatively by infrared spectroscopy, identification of the inorganic contaminant in this experiment was possible by Raman spectroscopy.
Because the AIRsight Infrared/Raman microscope introduced in this article enables smooth infrared spectrometry and Raman spectrometry of the same location using only one instrument, it is extremely useful for qualitative analyses of unknown samples. In addition, the entire sequence of operations necessary in an analysis, that is, setting the observation and measurement position, measurement, and analysis, can be carried out automatically. We hope that analysts will use this new instrument when analyzing contaminants which high accuracy in qualitative analysis is required and identifying the causes of nonconforming products and other problems.

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Application News

LC/MS

No. C185

Structural Analysis of Impurities in Pharmaceuticals Using Trap-Free 2D HPLC and the LCMS™-9030

T. Iida, Y. Inohana

Management and confirmation of the type, quantity, and safety of trace impurities contained in products are important points for ensuring the quality of products in a wide variety of fields including pharmaceuticals (raw materials, final pharmaceutical agents, and generic drugs), foods (health foods and supplements), and fine chemical products (solvents, paints, surfactants, and various chemical products). In terms of impurities in products, known component checks are performed via the HPLC-UV method in quality control departments. Conversely, the measurement and analysis of unknown components using mass spectrometers, which provide better sensitivity and excellent specificity, based on the HPLC-UV method, are requirements in research departments.

However, it is not possible to apply the nonvolatile mobile phase conditions used with HPLC as is in LC/MS analysis. Accordingly, in research departments, these are replaced by volatile mobile phase conditions, which is a very labor intensive process. These condition changes carry significant risks regarding changes to the elution order, and the omission of impurities eluted in the vicinity of main components, so very careful investigations are required.

This report introduces an example of the structural analysis in which impurities detected under nonvolatile mobile phase conditions were converted into volatile mobile phase conditions on-line without any complicated examination using a trap free 2-dimensional(2D) HPLC, and then subjected to precision mass spectrometry using the LCMS-9030 quadrupole time-of-flight mass spectrometer.

HPLC Analysis via a Nonvolatile Mobile Phase (1st Dimension)

Here, a 1 mg/mL test solution was prepared using commercially available atorvastatin calcium as the test reagent. The measurements were performed based on the analysis conditions noted in the Japanese Pharmacopoeia, shown in Table 1. Because a nonvolatile citric acid buffer solution was used as the mobile phase, it could not be injected as is into the LC/MS.

Table 1 Analysis Conditions (1st Dimension)

Column	:	Shim-pack™ VP-ODS (250 mmL.×4.6 mmI.D., 5 µm)
Mobile Phase A	:	Citrate buffer pH 5.0 / Acetonitrile / Tetrahydrofuran (4/1/1 = v/v/v)
Mobile Phase B	:	Acetonitrile / Tetrahydrofuran (1/1 = v/v)
Time program	:	B.Conc 7 % (0-40 min) → 40 % (80 min) → 7 % (80.1-100 min)
Flow Rate	:	1.43 mL/min
Column Temp.	:	40 °C
Injection Volume	:	20 µL
Detection	:	UV 254 nm

The analysis was performed using a combination of the LCMS-9030 and a trap-free 2D HPLC, with the instrument configuration shown in Fig. 1. The flow of the mobile phases differs with the valve positions in each operation. The flow line for the nonvolatile mobile phase is shown in red. The flow line for the volatile mobile phase is shown in blue. The peak capture loop for the fractionated impurities is shown in green.

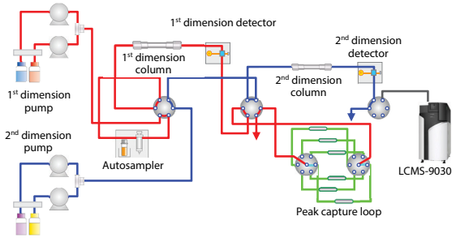


Fig. 1 Flow Line Diagram

The atorvastatin calcium solution was analyzed using this system, and the UV chromatogram obtained is shown in Fig. 2. Atorvastatin, the main component, was eluted with a retention time of approximately 16 minutes. Multiple impurity peaks can be confirmed in this vicinity. Of these, the 16 impurities (including the main component) shown in the figure were fractionated in the peak capture loop.

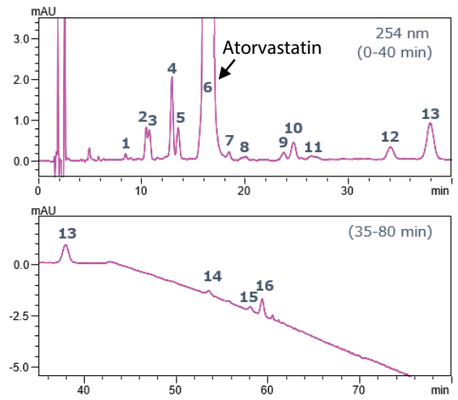


Fig. 2 UV Chromatogram for Atorvastatin Calcium (1st Dimension)

LC/MS Analysis via a Volatile Mobile Phase (2nd Dimension)

The main component and impurities (ID 1 to 16) fractionated in the peak capture loop were extruded from the loop using a volatile mobile phase via valve position switching and the 2nd dimensional solvent delivery unit, and were then injected into the LCMS-9030. The conditions used in the 2nd dimensional analysis are shown in Table 2.

Table 2 Analysis Conditions (2nd Dimension)

Column	:	Shim-pack XR-ODS (50 mmL.×2.0 mmI.D., 2.2 µm)
Mobile Phase A	:	10 mmol/L Ammonium Formate – water
Mobile Phase B	:	Acetonitrile
Time program	:	B.Conc 10 % (0 min) →100 % (6-6.5 min) → 10 % (6.51-10 min)
Flow Rate	:	0.3 mL/min.
Column Temp.	:	40 °C
Injection Volume	:	20 µL (Loop Volume)
Detection	:	UV 254 nm, MS, MS/MS scan (ESI Positive or Negative Mode)

Table 3 LCMS-9030 Measurement Results for the Impurities (Positive Mode)

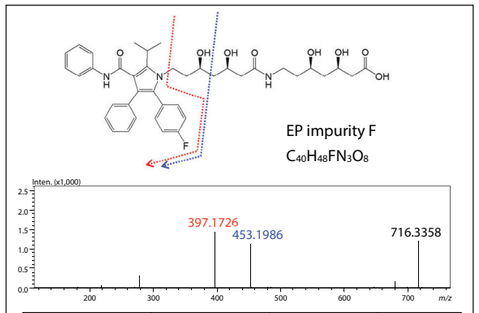
ID	RT (HPLC)	EP listed impurities	$[M+H]^+$ theoretical	$[M+H]^+$ observed	Error (PPM)	Content (%)
1	8.44			591.2501		0.01
2	10.44	Impurity F	718.3498	718.3497	-0.13	0.04
3	10.74			575.2551		0.04
4	12.92			575.256		0.12
5	13.53	Impurity A	541.2697	541.2695	-0.37	0.05
5	13.53			557.2445		0.05
6	16.30	Atorvastatin	559.2603	559.2601	-0.31	99.41
7	18.39			557.2446		0.01
8	20.10			557.2445		0.01
9	23.73			601.2709		0.01
10	24.68	Impurity G	573.2759	573.2762	0.48	0.03
11	26.43			591.25		0.01
12	34.07			573.2393		0.04
13	37.93	Impurity H	541.2497	541.2499	0.35	0.12
14	53.51			416.1655		0.02
15	58.02	Impurity D	432.1606	432.1607	0.32	0.02
15	58.02			362.1187		0.02
16	59.34	Impurity D	432.1606	432.1605	-0.15	0.07

From a comparison of the respective blanks and sample measurement, it is possible to reliably confirm the elution of the target components from the 2nd dimensional column. From the results of the scan analysis by the LCMS-9030, it is possible to estimate the molecular weights of the target impurities by investigating the ions observed specifically at the elution times for the applicable peaks. The measurement results in positive mode and negative mode are summarized in Tables 3 and 4. Additionally, atorvastatin, the main component, and the impurities noted in the European Pharmacopoeia (EU) were attributed with high mass accuracy by comparison to the theoretical mass values.

Table 4 Results of Measurements of the Impurities with the LCMS-9030 (Negative Mode)

ID	RT (HPLC)	EP listed Impurities	$[M-H]^-$ theoretical	$[M-H]^-$ observed	Error (PPM)
1	8.44			589.2354	
2	10.44	Impurity F	716.3353	716.3358	0.70
3	10.74			573.2406	
4	12.92			573.241	
5	13.53	Impurity A	539.2551	539.2552	0.10
5	13.53			555.2301	
6	16.30	Atorvastatin	557.2457	557.2457	0
7	18.39			555.2305	
8	20.10			N.D	
9	23.73			599.2565	
10	24.68	Impurity G	571.2614	N.D	
11	26.43			N.D	
12	34.07			N.D	
13	37.93	Impurity H	539.2352	N.D	
14	53.51			414.1506	
15	58.02	Impurity D	430.1460	N.D	
15	58.02			360.1035	
16	59.34	Impurity D	430.1460	430.1455	-1.19

Further, when an MS/MS scan was used, it was possible to obtain not only the molecular weight information for the impurities, but also a structural analysis from the product ion information. As an example, Impurity F noted in the EP was analyzed with high mass accuracy using MS/MS. As shown in the example in Fig. 3, the cleavage positions were automatically attributed using the MS Workbook Suite software from ACD/Labs.



Predicted formula	theoretical mass	observed mass	error (PPM)
C26H22FN2O (-)	397.1722	397.1726	1.01
C29H26FN2O2 (-)	453.1984	453.1986	0.44
C40H47FN3O8 (-)	716.3353	716.3358	0.70

Fig. 3 Results of an MS/MS Analysis of Impurity F noted in the EP (Negative Mode)

Summary

As shown here, it was possible to identify impurity peaks with high probability using nonvolatile mobile phase conditions as is, through a combination of the LCMS-9030, which is capable of accurate MS/MS analysis, and a trap-free 2D HPLC.

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Application News

Gas Chromatograph Mass Spectrometer GCMS-QP2020 NX

Screening of Ethylene Glycol and Diethylene Glycol in Medicinal Syrup by GCMS with FASST mode (Part 1 – as per Indonesian BPOM Method)

Chia Chee Geng, Cynthia Melanie Lahey, Chua Chun Kiang, and Jackie Jackie
Shimadzu (Asia Pacific) Pte Ltd

User Benefits

- GCMS-QP2020 NX delivers high scan speed capabilities for screening analysis
- Fast Automated Scan/SIM Type (FASST) mode enables consecutive collection of scan and single ion monitoring (SIM) data
- Accurately identify and quantify both EG and DEG in medicinal syrup with a single injection
- Superior reliability and reproducibility of the results obtained

■ Introduction

Chemical contamination in pharmaceutical products can lead to fatal consequences. In Indonesia, for example, at least 195 deaths among children have been reported relating to ethylene glycol (EG) and diethylene glycol (DEG) contamination in medicinal syrup [1]. In West Africa, Gambia, 70 child deaths were also suspected to be caused by contaminated medicinal syrup [2]. This has caught the attention of the World Health Organization (WHO), hence leading to the issuance of a global alert on this issue.

Propylene glycol, glycerol, and sorbitol are commonly used in medicinal syrup as excipients. Their presence helps improve the solubility of the active ingredients during formulation. They also function as thickeners and sweeteners impacting the taste of the medication. These raw materials are easily contaminated with toxic ethylene glycol (EG) and diethylene glycol (DEG). Over the past few decades, numerous contamination incidents of medicinal syrup with EG and DEG have been reported [3]. Accidental ingestion of EG and DEG may result in abdominal pain, vomiting, diarrhea, inability to pass urine, headache, altered mental state, and kidney injury which leads to death.

In this article, we will examine the usage of the Shimadzu GCMS-QP2020 NX to identify and quantify EG and DEG in medicinal syrup. The analysis will be demonstrated using the Fast Automated Scan/SIM Type (FASST) mode, which enables consecutive operation of scan mode and selected ion monitoring (SIM) mode for accurate qualification and quantitation within a single injection.

Figure 1. Shimadzu GCMS-QP2020 NX with AOC-20i+s Plus

■ Measurement Conditions

The analysis was performed using Shimadzu GCMS-QP2020 NX and AOC-20i+s Plus autosampler (**Figure 1**). Details of the analytical conditions were depicted in **Table 1**, in accordance with the method from the Indonesian national food and drug agency (BPOM) with slight modifications [4]. As the recommended method has minimum sample preparation, modifications were made to enhance the performance and robustness of the system. A capillary column with a 5 m integrated guard column was used to enhance the setup capability to handle a wide range of complex matrices without the risk of losing its performance. Due to the superior sensitivity of GCMS-QP2020 NX, a higher split ratio of 20:1 was used to minimize system contamination from the sample matrix. Event times for Scan and SIM were reduced to 0.2 and 0.1 sec, respectively, to increase the number of data points for better peak shape and integration.

Table 1. GCMS Parameters

Flow Control Mode	Constant Flow
Column Flow Rate	0.65 mL/min
Injection Mode	Split (Split Ratio = 20)
Injection Port Temp.	250 °C
Injection Volume	1 µL
Carrier Gas	Helium
Column	SH-PolarWax column with 5 m integrated guard column (30 m long, 0.25 mm I.D., 0.25 µm film thickness) [P/N: 227-36360-01]
Column Oven Temp. Program	Initial Temp 100 °C (hold for 1 min) - Increase to 130 °C with a rate of 10 °C/min (hold 7 min) - Increase to 240 °C with a rate of 20 °C/min (hold 3 min) - Increase to 250 °C with a rate of 20 °C/min (hold 3 min)
Ion Source Temp.	230 °C
Interface Temp.	240 °C
Acquisition Mode	FASST (Scan/SIM)
Event Time (sec)	Q3 Scan: 0.2 Q3 SIM: 0.1
Scan *m/z* Range	29 to 400 amu
SIM Ions	EG: 31 (target ion) 33 and 62 (reference ions) DEG: 45 (target ion) 75 and 31 (reference ions)

■ Sample Preparation

1000 ppm calibration stock solution preparation

EG and DEG were purchased from TCI, Japan. Standard solutions of EG and DEG in methanol were prepared by dissolving 100 mg of each in separate 100 mL volumetric flasks. To improve dissolution, sonicate EG and DEG with 50 mL methanol (MeOH) before topping up to the 100 mL mark. The standard solutions were subsequently used for the preparation of a series of various concentrations of calibration standard solutions in 5 mL volumetric flasks in accordance with **Table 2**.

Table 2. Preparation of EG and DEG calibration plots in 5 mL volumetric flasks

Level	Ethylene Glycol		Diethylene Glycol	
	Conc /ppm	Amount from 1000 ppm stock/ µL	Conc /ppm	Amount from 1000 ppm stock/ µL
1	6	30	12	60
2	8	40	16	80
3	10	50	20	100
4	12	60	24	120
5	14	70	28	140

Medicinal syrup sample preparation

A blank medicinal sample solution was prepared by transferring 10 mL of the medicinal syrup sample into a 100 mL volumetric flask. To improve dissolution, the medicinal syrup in 50 mL of methanol was sonicated for 5 minutes before topping it up to the mark. The diluted mixture was then filtered with a 0.45 µm PTFE membrane filter. A portion of the blank sample solution was subsequently spiked with 6 ppm of EG and 12 ppm of DEG for repeatability study.

Medicinal syrup samples (Sample A and Sample B) were purchased commercially and were prepared in a similar fashion. However, they were scaled down proportionally using 10 mL volumetric flasks instead. A portion of Sample A was spiked with both 4 ppm of EG and 20 ppm of DEG and similarly, a portion of Sample B was spiked with 20 ppm of DEG for recovery studies.

■ Results and Discussion

Setting up the FASST screening method

Calibration plots were obtained by spiking various concentrations of EG (6 to 14 ppm) and DEG (12 to 28 ppm) in methanol and analyzing them using FASST mode. **Figures 2a-d** demonstrated that the linearity plots obtained from two separate preparations have linear fits with R^2 of at least 0.9985 for both EG and DEG. The blank medicinal syrup and its spiked solutions (6 ppm EG and 12 ppm DEG) were analyzed subsequently. For the blank control medicinal syrup, DEG was not detected (**Figure 3b**), and only a negligible amount of EG (not quantifiable) was present (**Figure 3a**), thus the endogenous amount of EG was assumed to have an insignificant contribution to the results.

The results of the repeated injections (n=10) of the spiked blank medicinal syrup are summarized in **Table 3**. Concentration repeatability (%RSD) of 1% and 2% were obtained for 6 ppm EG and 12 ppm DEG, respectively, demonstrating the high degree of precision of the entire setup. Decent %Recovery values were also obtained for both EG (118% to 123%) and DEG (103% to 109%). The quantifications of 6 ppm EG (**Figure 3c**) and 12 ppm DEG (**Figure 3d**) were successfully demonstrated and established as the limits of quantification (LOQ), with a signal-to-noise (S/N) ratio above 200.

Figure 4a-b show the total ion chromatogram (TIC) scan profile and the SIM mass chromatogram (MC) profile for the target ions of EG (*m/z* 31) and DEG (*m/z* 45) of the level 5 calibration standard (**Table 2**). Based on the TIC profiles, two prominent peaks at the retention time of 8.817 mins (**Figure 4a**) and 15.374 mins (**Figure 4b**) were observed and that corresponded to EG and DEG, respectively.

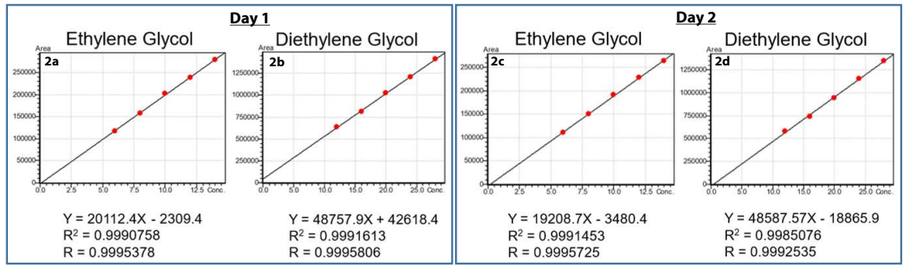

Figure 2a-d. Calibration plots of EG and DEG obtained on separate preparations

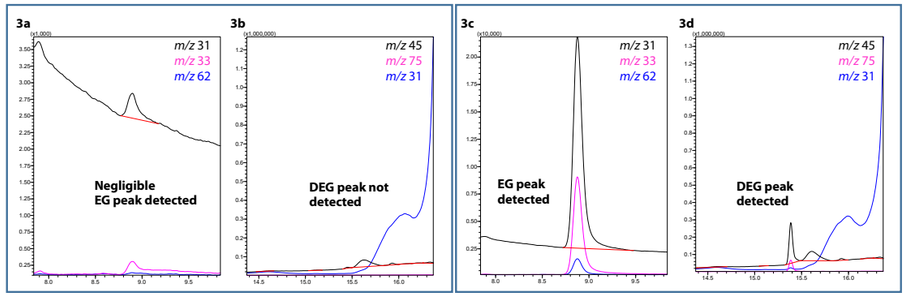

Figure 3a-b. SIM Mass Chromatogram (MC) of blank medicinal syrup

Figure 3c-d. SIM Mass Chromatogram (MC) of spiked (6 ppm EG and 12 ppm DEG) blank medicinal syrup

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Table 3. Repeatability (*n*=10) and recovery of the spiked blank medicinal syrup with 6 ppm EG and 12 ppm DEG

Injection	EG detected /ppm	DEG detected /ppm	%Recovery EG	%Recovery DEG
1	7.08709	13.07627	118%	109%
2	7.24933	12.75458	121%	106%
3	7.40988	12.76441	123%	106%
4	7.30253	12.45931	122%	104%
5	7.32590	12.47512	122%	104%
6	7.32913	12.57976	122%	105%
7	7.28901	12.60053	121%	105%
8	7.30924	12.54618	122%	105%
9	7.18782	12.31182	120%	103%
10	7.21388	12.38494	120%	103%
Std. Dev.	0.09001963	0.22249365		
Average	7.270381	12.595292		
%RSD	1.24%	1.77%		

As indicated by the BPOM method, the SIM profiles were used for quantitation. EG was quantitated with the target ion of *m/z* 31 and qualified with the reference ions of *m/z* 33 and 62. On the other hand, DEG was quantitated with the target ion of *m/z* 45 and qualified with reference ions of *m/z* 75 and 31. **Figures 5a-b** depict the mass spectrum profiles of identified EG and DEG, respectively, in spiked medicinal syrup, with high similarity indices of 90 and 93 when matched against the NIST 2020 mass spectral library.

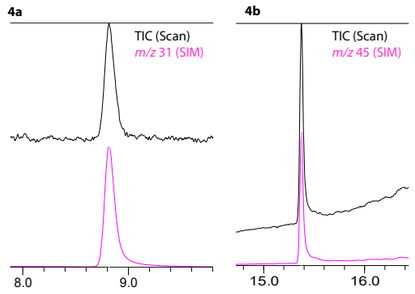

Figure 4a-b. Calibration standard level 5's Total Ion Chromatogram (TIC) scan profile and SIM target ion profiles of EG and DEG

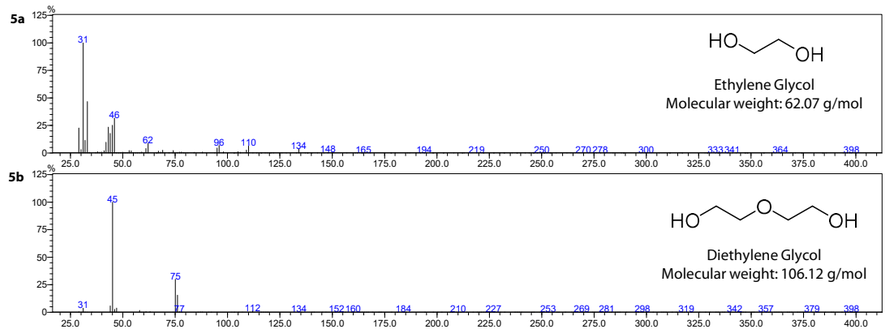

Figure 5a-b. Mass spectrum profiles of 6 ppm EG and 12 ppm DEG detected in spiked medicinal syrup

Screening of commercially purchased medicinal syrup

Commercially purchased Sample A and Sample B medicinal syrup underwent the same sample treatment as the blank medicinal syrup control. Briefly, 1 mL of the neat sample was transferred to a 10 mL volumetric flask containing 5 mL of MeOH. The diluted mixture was then sonicated for 5 minutes and topped up to the mark. The diluted mixture was then filtered through a 0.45 µm PTFE membrane filter. The filtered sample was then used for GCMS analysis directly.

Amount of EG and DEG detected in Sample A and Sample B

Table 4 and **Table 5** summarize the concentration results obtained. For Sample A, 7.18 ppm of EG (avg *n*=4) was detected. Thus, the sum of EG and DEG present in Sample A was equivalent to 0.07 mg/mL after considering the dilution factor. For Sample B, 10.20 ppm of EG (avg *n*=4) was detected. Meanwhile, a trace amount of DEG (below LOQ) was detected, which explained the poor inter-day concentration reproducibility for DEG in this sample. Thus, the sum of the concentration of EG and DEG present in Sample B after taking into consideration of the dilution factor was ~0.11 mg/mL.

This experiment demonstrated Shimadzu GCMS-QP2020 NX superior performance in terms of repeatability and sensitivity for detecting EG and DEG in contaminated medicinal syrup.

High repeatability observed

A high degree of reproducibility is observed when the concentration of the analyte of interest falls within the calibration plot range. Within the day, repeatability %RSD between 0.01% to 1.95% was observed. The high degree of precision in the results observed in **Table 4** and **Table 5** were in strong agreement with the data in **Table 3**. Good intermediate precision across different days was observed as well, ranging between 2.22% to 3.92%.

%Recovery of the experiment

Due to the lack of a blank control matrix for Sample A and Sample B, the %Recovery was determined by spiking into the Sample A and Sample B syrup directly. Sample A syrup was spiked with 4 ppm of EG and 20 ppm of DEG, while 20 ppm of DEG was spiked into Sample B syrup. To avoid exceeding the level in the calibration plot, Sample B was not spiked with EG.

The additional amount of spiked analyte detected in the individual sample was calculated by taking the difference between the result obtained for the spiked sample with respect to the average reading of the unspiked sample that day. As the level of endogenous DEG in Sample B was below LOQ, the basal level of DEG contribution will be assumed to be negligible. **Table 6** summarizes the result of the spiking experiment and its %Recovery.

Table 4. Summarized result of EG and DEG detected in Sample A syrup

Day	EG detected /ppm	Avg EG detected /ppm (n=2)	%RSD	Day	DEG detected /ppm	Avg DEG detected /ppm (n=2)	%RSD	(EG+DEG)/ (mg/mL)
1	7.17694	7.277225	1.95%	1	ND	ND	ND	0.07
1	7.37751			1	ND			0.07
2	6.98670	7.084415	1.95%	2	ND	ND	ND	0.07
2	7.18213			2	ND			0.07
Avg EG *n*=4	7.18082			**Avg DEG *n*=4**	ND		**Avg Sum *n*=4**	0.07
Intermediate Precision	2.22%			**Intermediate Precision**	ND			

EG detected
DEG not detected in Sample A

Abbreviation used: avg = average, ND = Not Detected

Table 5. Summarized result of EG and DEG detected in Sample B syrup

Day	EG detected /ppm	Avg EG detected /ppm (n=2)	%RSD	Day	DEG detected /ppm	Avg DEG detected /ppm (n=2)	%RSD	(EG+DEG)/ (mg/mL)
1	10.54466	10.54558	0.01%	1	0.51743	0.53152	3.75%	0.11
1	10.54650			1	0.54561			0.11
2	9.79652	9.857925	0.88%	2	1.48060	1.49213	1.09%	0.11
2	9.91933			2	1.50365			0.11
Avg EG *n*=4	10.20175			**Avg DEG *n*=4**	1.0118225		**Avg Sum *n*=4**	0.11
Intermediate Precision	3.92%			**Intermediate Precision**	54.83%			

EG detected
DEG detected, but below LOQ in Sample B

Abbreviations used: avg = average

Table 6. Summarized result of the spiked EG and DEG detected in Sample A and spiked DEG detected in Sample B

Sample A				Sample B	
Amount of spiked EG detected/ppm	**%Recovery**	**Amount of spiked DEG detected/ppm**	**%Recovery**	**Amount of spiked DEG detected/ppm**	**%Recovery**
4.405055	110%	23.93676	120%	23.24588	116%
4.705645	118%	24.05775	120%	23.23355	116%
3.144905	79%	24.74546	124%	24.36432	122%
3.225340	81%	25.07093	125%	22.95413	115%

In Sample A syrup, the %Recovery for EG was observed to be between 79% to 118% and 120% to 125% for DEG. For Sample B syrup, the %Recovery for DEG was observed to be between 115% to 122%. The slight deviation from the ideal accuracy was attributed to the observable matrix effect. This is within expectation, as with this approach, the sample undergoes minimum sample preparation before the injection into the GCMS system. A matrix-matched calibration curve is expected to improve the performance, and this will be explored in part 2 of this application news.

FASST advantage in complex matrices

Figure 6a-b and **Figure 7a-c** depict the TIC and MC of Sample A and Sample B samples, respectively, using FASST mode. As shown, EG was detected in Sample A, and both EG and DEG were detected in Sample B. In Sample A, it was observed that the retention time of the EG peak shifted from 8.760 min to 9.025 min (**Figure 6a-b**). This was attributed to the huge earlier eluting peak (**Figure 6a**) in the Sample A matrix, which overloaded the column. As the FASST analysis mode was used, EG was still successfully identified with high confidence even though its expected retention time has shifted. Using the scan mode data from FASST analysis mode, the huge interfering peak that affected the elution of EG was identified as propylene glycol, with a high similarity index of 97 when matched against the NIST 2020 library. The advantage of FASST mode analysis in identifying unknown interfering peaks while maintaining the sensitivity of target compounds was well-demonstrated in this experiment.

Calculation and tips

The concentration of the sample is inferred from the linear calibration plot equation obtained in the form of:

$$y = ax + c$$

where x = amount of EG or DEG in ppm
y = area obtained from GCMS
a = slope of the calibration plot
c = intercept

The sum amount of EG+DEG (mg/mL) for medicinal syrup is calculated using the following formula:

$$(EG+DEG) = x \cdot F/1000$$

where x = sum of EG and DEG in ppm
F = dilution factor

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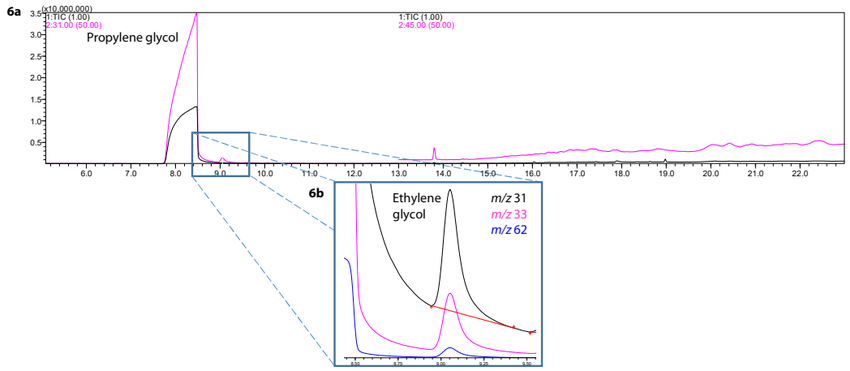

Figure 6a-b. TIC and MC (EG and DEG quantifier ion) profile of Sample A, and its zoom section (SIM profile) where EG was detected

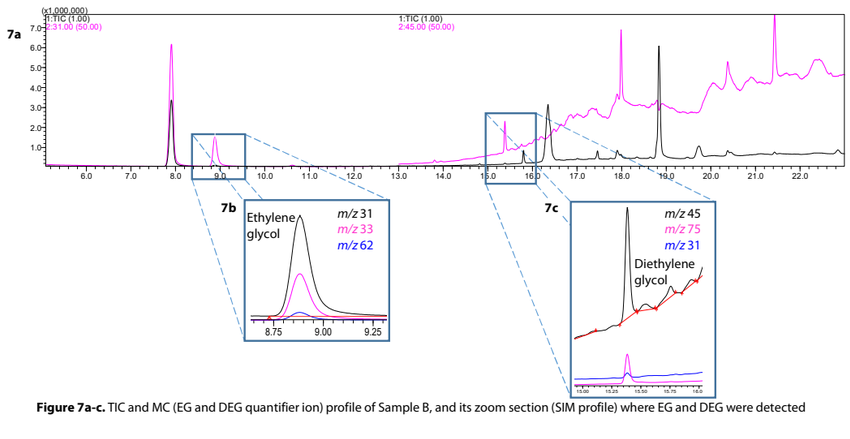

Figure 7a-c. TIC and MC (EG and DEG quantifier ion) profile of Sample B, and its zoom section (SIM profile) where EG and DEG were detected

For very viscous samples, it might not be practical to transfer the desired volume accurately. In such instance, the density of the syrup sample should be determined first, then the amount of sample transferred should be weighed precisely. The dilution factor of should be calculated as followed:

$$F = \rho \cdot Vt/Wt$$

where F = dilution factor
ρ = density of medicinal syrup (g/mL)
Vt = total volume during dilution (mL)
Wt = Weight of medicinal syrup (g)

If the sample viscosity did not pose difficulty in drawing the exact desired volume, the dilution factor should be calculated as below:

$$F = Vt/V1$$

where F = dilution factor
Vt = total volume during dilution (mL)
V1 = Volume of medicinal syrup (mL)

■ Conclusion

To prevent any future risk of mass poisoning tragedies due to the ingestion of contaminated medicinal syrup, stringent quality control should be advocated. In this application news, Shimadzu GCMS-QP2020 NX was demonstrated to provide sensitive, precise, and robust detection of EG and DEG in medicinal syrup in two different sample matrices with minimum sample preparation, as per the Indonesian BPOM method.

The limitation of the approach is that as minimal sample preparation is used, and due to matrix effect only decent %Recovery is obtained. While this method is appropriate for quick testing in labs for large variety of samples with different sample matrices, it might not meet the stricter accuracy requirement for quality control labs in pharmaceutical industry. A separate improvised method will be developed to address the said problem using matrix-matched calibration curve in Part 2 of this application news.

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4. Metode Analysis Uji Cemaran Etilen Glikol (EG) dan Dietilen Glikol, Badan Pengawas Obat dan Makanan, 24 Oct 2022.

04-AD-0283-EN First Edition: Dec. 2022

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Application News

Gas Chromatograph Mass Spectrometer GCMS-QP2020 NX

Screening of Ethylene Glycol and Diethylene Glycol in Medicinal Syrup by GCMS with FASST mode (Part 2 – Improved Method for QC Testing)

Chia Chee Geng, Cynthia Melanie Lahey, Chua Chun Kiang, and Jackie Jackie
Shimadzu (Asia Pacific) Pte Ltd

User Benefits

- GCMS-QP2020 NX delivers high scan speed capabilities for screening analysis
- Fast Automated Scan/SIM Type (FASST) mode enables consecutive collection of scan and single ion monitoring (SIM) data
- Accurately identify and quantify both EG and DEG in medicinal syrup with a single injection
- Superior reliability, reproducibility and selectivity of the results obtained
- Matrix-matched calibration greatly improves %Recovery

■ Introduction

In Part 1 of the application news, we have successfully demonstrated GCMS-QP2020 NX superior capability in screening of ethylene glycol (EG) and diethylene glycol (DEG) in medicinal syrup, using the recommended method by Indonesian BPOM [1]. The method requires minimum sample preparation, making it suitable for laboratories in relevant authorities to screen medicinal syrup from different brands easily, thus making it possible to make rapid assessment on the safety of the medicinal syrup on the market. However, notable matrix effect has been observed, thus affecting the %Recovery of the method. As a result, this approach might not be capable of meeting the stringent QC testing requirements in pharmaceutical industry. Part 2 of the application news intends to address this gap.

To improve the accuracy of the method, our team decided to modify the BPOM method slightly using matrix-matched calibration plot. This slight modification greatly enhances the method selectivity, enabling accurate quantitation of EG and DEG in the presence of complex sample matrices.

In this article, we will be using the exact setup as Part 1. We will examine the usage of the Shimadzu GCMS-QP2020 NX to identify and quantify EG and DEG in medicinal syrup. The analysis will be demonstrated using the Fast Automated Scan/SIM Type (FASST) mode, which enables consecutive operation of scan mode and selected ion monitoring (SIM) mode for accurate qualification and quantitation within a single injection.

Figure 1. Shimadzu GCMS-QP2020 NX with AOC-20i+s Plus

■ Measurement Conditions

The analysis was performed using Shimadzu GCMS-QP2020 NX and AOC-20i+s Plus autosampler (**Figure 1**). Details of the analytical conditions were depicted in **Table 1**, in accordance with the method from the Indonesian national food and drug agency (BPOM) with slight modifications [2]. As the recommended method has minimum sample preparation, modifications were made to enhance the performance and robustness of the system. A capillary column with a 5 m integrated guard column was used to enhance the setup capability to handle a wide range of complex matrices without the risk of losing its performance. Due to the superior sensitivity of GCMS-QP2020 NX, a higher split ratio of 20:1 was used to minimize system contamination from the sample matrix. Event times for Scan and SIM were reduced to 0.2 and 0.1 sec, respectively, to increase the number of data points for better peak shape and integration.

Table 1. GCMS Parameters

Flow Control Mode	Constant Flow
Flow Rate	0.65 mL/min
Injection Mode	Split (Split ratio = 20)
Injection Port Temp.	250 °C
Injection Volume	1 µL
Carrier Gas	Helium
Column	SH-PolarWax column with 5 m integrated guard column (30 m long, 0.25 mm I.D., 0.25 µm film thickness) [P/N: 227-36360-01]
Column Oven Temp. Program	Initial Temp 100 °C (hold for 1 min) - Increase to 130 °C with a rate of 10 °C/min (hold 7 min) - Increase to 240 °C with a rate of 20 °C/min (hold 3 min) - Increase to 250 °C with a rate of 20 °C/min (hold 3 min)
Ion Source Temp.	230 °C
Interface Temp.	240 °C
Acquisition Mode	FASST (Scan/SIM)
Event Time (sec)	Q3 Scan: 0.2 Q3 SIM: 0.1
Scan *m/z* Range	29 to 400 amu
SIM Ions	EG: 31 (target ion) 33 and 62 (reference ions) DEG: 45 (target ion) 75 and 31 (reference ions)

■ Sample Preparation

Medicinal syrup sample preparation

A medicinal sample solution was prepared by transferring 10 mL of the medicinal syrup sample into a 100 mL volumetric flask. To improve dissolution, the medicinal syrup in 50 mL of methanol was sonicated for 5 minutes before topping it up to the mark. The diluted mixture was then filtered with a 0.45 µm PTFE membrane filter. 1 µL filtered sample was then analyzed using GCMS, and only negligible amount of EG was detected, and DEG was not being detected [1]. Thus, this medicinal sample solution was used as a blank sample because endogenous level of EG will be assumed to have negligible contribution to the experimental results. Subsequently, this filtered blank medicinal syrup sample was used for the preparation of the matrix-matched calibration plot (**Table 2**), and in parallel, a separate preparation of spiked samples at corresponding to Level 1 (LOQ) and level 3 of the calibration plot was prepared.

Matrix-matched calibration plot preparation

EG and DEG were purchased from TCI, Japan. Standard solutions of EG and DEG in methanol were prepared by dissolving 100 mg of each in separate 100 mL volumetric flasks. To improve dissolution, sonicate EG and DEG with 50 mL methanol (MeOH) before topping up to the 100 mL mark (1000 ppm standard solution). The 1000 ppm standard solutions were subsequently used for the preparation of a series of various concentrations of calibration standard solutions in 5 mL volumetric flasks in accordance with **Table 2**, topped to the mark with the filtered blank medicinal syrup sample.

Table 2. Preparation of EG and DEG calibration plots in 5 mL volumetric flasks

Level	Ethylene Glycol		Diethylene Glycol	
	Conc /ppm	Amount from 1000 ppm stock/ µL	Conc /ppm	Amount from 1000 ppm stock/ µL
1	6	30	12	60
2	8	40	16	80
3	10	50	20	100
4	12	60	24	120
5	14	70	28	140

■ Results and Discussion

Matrix-matched calibration plot

Matrix-matched calibration plots were obtained by spiking various concentrations of EG (6 to 14 ppm) and DEG (12 to 28 ppm) in the blank medicinal syrup and analyzed them using FASST mode. **Figures 2a** to **2d** demonstrate the linearity of the calibration plot from two separate preparations, having linear fits with R^2 of at least 0.999, for both EG and DEG. The high degree of similarity between the equations of the linearity plot from the two different preparations reflects the robustness and reliability of the method used.

Like Part 1 of the application news, in accordance with the BPOM method, the SIM profiles were used for quantitation. EG was quantitated with the target ion of *m/z* 31 and qualified with the reference ions of *m/z* 33 and 62. On the other hand, DEG was quantitated with the target ion of *m/z* 45 and qualified with reference ions of *m/z* 75 and 31.

%Recovery at levels 1 and 3

Table 3 summarized the result for the %Recovery of the spiked samples using the conventional non-matrix-matched standards

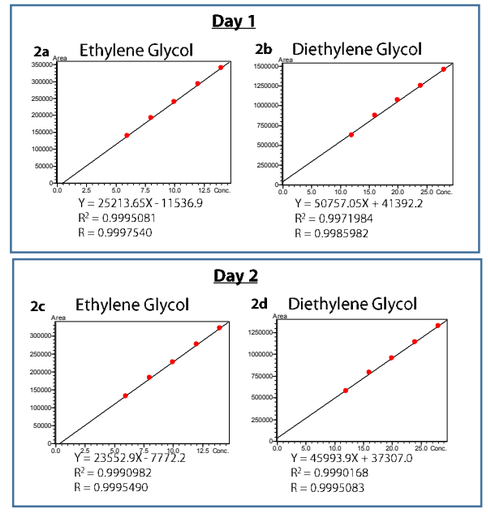

Figure 2a-d. Matrix-matched calibration plots of EG and DEG obtained on separate preparations

calibration plot approach vs matrix-matched calibration plot approach, at level 1 (LOQ) and level 3 concentration levels of EG (6 and 10ppm) and DEG (12 and 20 ppm). For the conventional non-matrix-matched calibration plot approach, %Recovery for EG ranges from 118% to 120%, and 102% to 110% for DEG. Significant positive bias is observed upon attempting to quantify EG and DEG in the spiked sample. This biasness was corrected when using the matrix-matched calibration approach. For the same set of data, using the matrix-matched calibration approach, the %Recovery for EG obtained ranges from 99% to 102%, and 98% to 105% for DEG. The results obtained were also in strong agreement with **Figure 2a** to **2d**, whereby the equations of the separate preparation of the matrix-matched calibration plots on different days shares high degree of similarity.

High degree of precision observed

The high degree of precision observed in **Table 3** is in strong agreement with the observation in Part 1 of the application news. The concentration %RSD (**Table 3**) of the results matrix-match calibration plots obtained were very similar to those of non-matrix-matched. Using the matrix-matched calibration plots, the concentration %RSD obtained ranged from 0.38% to 2.90%. This demonstrates that modifying the method has negligible effect on the precision of the data.

Improving selectivity and accuracy of measurement

As mentioned above, after the modification using the matrix-matched calibration plot to process the data, the %Recovery of the results approaches the ideal value of 100% (**Table 3**). Our team has thus successfully improved the selectivity of the setup that enables us to quantify EG and DEG with a better accuracy. This approach is suitable for QC lab in pharmaceutical industry with previously released batches of uncontaminated finished product that has negligible amount of EG and DEG presence (suitable for use as blank).

The limitation of this approach is the requirement of a corresponding blank matrix (with negligible amount of EG and DEG). In cases where a lab needs to test a diverse range of samples made up of various matrices, such as regulatory agencies or testing laboratories, the approach presented in Part 1 of the application note is more practical [1].

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Table 3. Comparison of results using conventional non-matrix-matched calibration plot (spiked in MeOH) with matrix-matched calibration plot approach.

		Non-Matrix-Matched Calibration plot				Matrix Matched Calibration plot			
EG spiked /ppm	DEG spiked /ppm	EG detected /ppm	DEG detected /ppm	%Recovery EG	%Recovery DEG	EG detected /ppm	DEG detected /ppm	%Recovery EG	%Recovery DEG
6	12	7.20433	12.9938	120%	108%	6.11272	12.50618	102%	104%
6	12	7.20901	13.15353	120%	110%	6.11644	12.65962	102%	105%
6	12	7.08610	12.80426	118%	107%	6.01840	12.32410	100%	103%
6	12	7.06675	12.29578	118%	102%	6.00297	11.83565	100%	99%
	%RSD	1.06%	2.91%		%RSD	1.00%	2.90%		
10	20	11.94484	20.35343	119%	102%	9.89412	19.57594	99%	98%
10	20	11.92798	20.42981	119%	102%	9.88068	19.64931	99%	98%
10	20	12.01733	21.17994	120%	106%	9.95195	20.36990	100%	102%
10	20	12.01584	21.47227	120%	107%	9.95076	20.65070	100%	103%
	%RSD	0.39%	2.65%		%RSD	0.38%	2.65%		

Calculation

In this application news, the %Recovery and precision calculations were based on the concentrations of the sample inferred from the linear calibration plot equation, and therefore the final concentrations of EG and DEG in the medicinal syrup were not shown.

Refer to Part 1 of the application news [1] for tips for performing concentration calculations.

■ Conclusion

As a follow-up to Part 1 of the application news, our team showed how we were able to improve the method's selectivity using the matrix-matched calibration plot approach. As a result, we were able to satisfy the demanding QC standards of the pharmaceutical industry for the release testing of finished goods. The highly precise results obtained in this Part 2 is in strong agreement with the observation made in Part 1 of this application news.

Both Parts 1 and 2 of the application news demonstrated that Shimadzu GCMS-QP2020 NX can provide accurate, sensitive, precise, and robust detection of EG and DEG in medicinal syrup with minimum sample preparation. The developed methods are suitable for meeting the stringent requirements of relevant authorities, testing labs, or pharmaceutical quality control.

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Application News

Gas Chromatograph Mass Spectrometer GCMS-TQ™8050 NX
High Performance Liquid Chromatograph Mass Spectrometer LCMS-8060NX

GC/MS and LC/MS Analysis of Metabolites in Hepatitis Model Mouse Serum

Yutaka Umakoshi

User Benefits

- Multi-omics Analysis Package can be used to analyze data obtained by GC/MS and LC/MS measurements.
- Multi-omics Analysis Package enables multivariate analysis, including principal component analysis.
- Acquired data can be easily visualized using a metabolic map template..

■ Introduction

Metabolomics is technology used to comprehensively measure all metabolites in living organisms. Metabolomics is used in various fields. In the medical field, it is used to search for biomarkers and determine pathogenesis. A variety of analytical instruments are used to measure metabolites. In particular, chromatographs and mass spectrometers are often used in combination because they can measure various metabolites with high sensitivity. Liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS) differ in detectable compounds, and both methods can be used to obtain complementary data.

This article describes using GC/MS and LC/MS data analysis methods with Multi-omics Analysis Package[1]. As samples, serum was measured from mice treated with a choline-deficient (CD) and a methionine- and choline-deficient (MCD) diet, known as nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH) models, respectively. By using Multi-omics Analysis Package, GC/MS and LC/MS data were integrated and visualized on a single metabolic map.

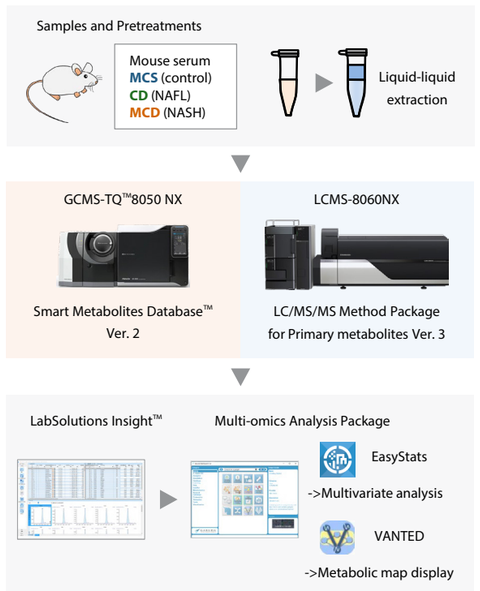

Fig. 1 Overall Workflow

■ Samples and Pretreatments

Serum samples were obtained from mice treated with a methionine- and choline-deficient (MCD, n = 6) diet, a choline-deficient (CD, n = 6) diet , and a methionine- and choline-supplemented (MCS, n = 5) diet. Mice treated with MCD diet are known as representative NASH models[2]. Serum samples were pretreated as shown in Fig. 2. 2-Isopropylmalic acid dissolved in ultrapure water was used as the internal standard. Derivatization for GC/MS was performed according to the Pretreatment Procedure Handbook for Metabolites Analysis[3].

• Serum	50 µL
• IS (2-Isopropylmalic acid, 0.5 mg/mL)	10 µL
• Methanol: Chloroform: Ultrapure water (10:5:3)	1000 µL

↓ Stir (5 min)
↓ Centrifuge (10 min, 15000 rpm, 4 °C)

• Supernatant	700 µL
• Ultrapure water	200 µL
• Chloroform	200 µL

↓ Stir (5min)
↓ Centrifuge (10 min, 15000 rpm, 4 °C)

500 µL of the upper layer was used for GC/MS analysis and 100 µL for LC/MS analysis.

GC/MS analysis
• Upper layer 500 µL
↓ Centrifugal concentration
• Methoxyamine-pyridine solution (20 mg/mL) 80 µL
↓ Shake (90 min, 1800 rpm, 30 °C)
• Add 40 µL of MSTFA
↓ Shake (30 min, 1200 rpm, 37 °C)
↓ Centrifuge (5 min, 15000 rpm, 4 °C)
Dispense into analytical vials

LC/MS analysis
• Upper layer 100 µL
↓ Centrifugal concentration
• Redissolve in 50 µLultrapure water
• Dispense into analytical vials

Fig. 2 Pretreatment Process Flows

■ Analytical Conditions

"Smart Metabolites Database Ver. 2" was used for GC/MS analysis. "LC/MS/MS Method Package for Primary Metabolites Ver. 3" was used for LC/MS analysis, with MRM event added for the 2-isopropylmalic acid added as an internal standard. The analysis conditions for GC/MS and LC/MS are shown in Table 1.

Table 1 Analytical Conditions

GC-MS:	**GCMS-TQ8050 NX**
Autoinjector:	**AOC20i Plus / 20s Plus**
GC	
Column:	BPX-5 (30 m, 0.25 mm I.D., 0.25 µm) P/N: 054101
Injection Temp.:	250 °C
Column Oven:	60 °C (2 min)→ 15 °C/min → 330 °C (3 min)
Injection Mode:	Split
Split Ratio:	30
Carrier Gas:	He
Carrier Gas Control:	Linear Velocity (39.0 cm/sec)
Injection Volume:	2 µL
MS	
Mode:	MRM
Ion Source Temp.:	200 °C
Interface Temp.:	280 °C
HPLC:	**Nexera™ X3**
Column:	Shim-pack™ GIST PFPP (2.1 mm I.D. x 150 mm, 3 µm) P/N: 227-30858-07
Column Oven:	40 °C
Solvent A:	0.1% Formic acid in water
Solvent B:	0.1% Formic acid in acetonitrile
Mode:	Gradient elution
Flowrate:	0.25 mL/min
Injection Volume:	1 µL
MS:	**LCMS-8060NX**
Ionization:	ESI positive/ negative (IonFocus™)
Mode:	MRM
Nebulizing Gas:	3.0 L/min
Drying Gas:	10.0 L/min
Heating Gas:	10.0 L/min
DL Temp.:	250 °C
Heat Block Temp.:	400 °C
Interface Temp.:	270 °C

■ Data Analysis

Data obtained by GC/MS and LC/MS were processed using LabSolutions Insight. LabSolutions Insight enables efficient waveform processing for GC/MS and LC/MS in a similar manner. GC/MS and LC/MS data were each corrected with internal standards and the resulting area ratios were used for subsequent analyses.

The corrected area values were further analyzed using the "Multi-omics Analysis Package." This software can analyze a large amount of data obtained by mass spectrometry using various methods, such as multivariate analysis, graphing volcano plots, and displaying metabolic maps. In addition, it includes visualization templates for various GC/MS and LC/MS method packages, allowing smooth visualization of the obtained data.

In this analysis, a metabolic map that integrates GC/MS and LC/MS was used, but metabolic maps were also provided for "Smart Metabolites Database Ver. 2" and "LC/MS/MS Method Package for Primary Metabolites Ver. 3" so that a metabolic map can be selected based on objectives (Fig. 3).

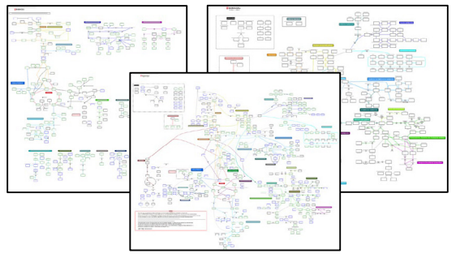

Fig. 3 Metabolic Map Templates
(From left to right: Metabolic maps for Smart Metabolites Database, integrated GC/MS and LC/MS analysis, and LC/MS/MS Method Package for Primary Metabolites)

■ Metabolites Detected

As shown in Fig. 4, a total of 122 compounds were detected using GC/MS and LC/MS. GC/MS detected 93 components, mainly amino acids, organic acids, and sugars. LC/MS detected 58 components, mainly amino acids, organic acids, and nucleobases. Twenty-nine compounds, including amino acids and organic acids, were detected by both methods. For metabolites detected by both GC/MS and LC/MS, the LC/MS data, which offered higher sensitivity, were used in subsequent data analysis.

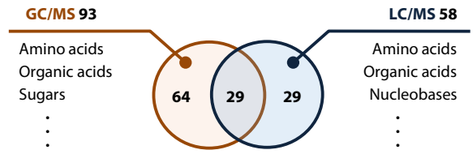

Fig. 4 Metabolites Detected by GC/MS and LC/MS

■ Principal Component Analysis Results

Principal component analysis was performed using GC/MS and LC/MS data (Fig. 5). Three groups were separated on the score plot. The MCD group was separated on the first principal component axis, whereas the CD group was separated on the second principal component axis.

Loading plots showed that small amino acids, such as glycine, alanine, serine and 2-aminobutyric acid, were more abundant in the MCD group. On the other hand, glucose and related metabolites were found to be lower in the MCD group. Sulfur-related metabolites, such as methionine and methionine sulfoxide, were also found to be more abundant in the CD group.

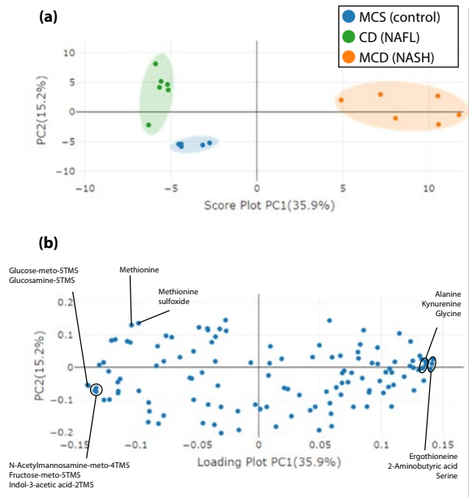

Fig. 5 Principal Component Analysis Using EasyStats
(a) Score plot, (b) Loading plot

■ Metabolic Map Display

The obtained data were visualized on a metabolic map using VANTED (Fig. 6). The names of metabolites identified by GC/MS, LC/MS, and both methods are indicated in blue, black, and green, respectively. This metabolic map includes key metabolic pathways, including glycolysis, amino acid metabolism, the TCA cycle, and the urea cycle.

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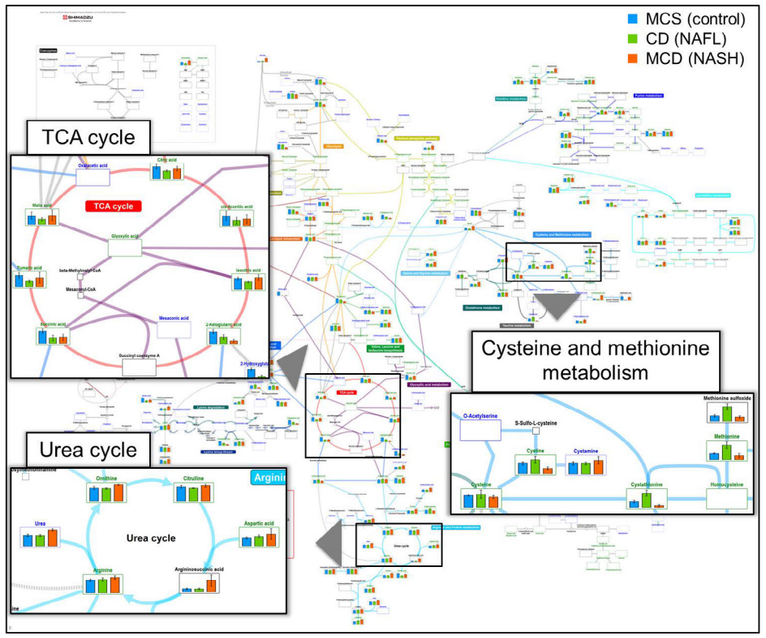

Fig. 6 Metabolic Map Display using VANTED (Overall)

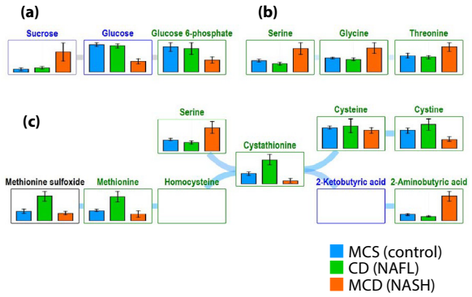

Fig. 7 Metabolic Map Display using VANTED (Excerpt)
(a) Sugar-related metabolites, (b) amino acids, and (c) sulfur-related metabolites

Some of the metabolites that changed among the three groups are shown in Fig. 7, which shows how metabolic maps can be edited to suit given objectives.

As in the previous study[4], glucose and other sugars decreased in the MCD group. Amino acids such as serine, glycine, and threonine increased in the MCD group. Sulfur-related metabolites also increased in the CD group.

■ Conclusion

Analysis using GC/MS and LC/MS detected 122 compounds in mouse serum. Area value data from both methods were integrated and visualized on a single metabolic map. The metabolic map display showed that sugars, amino acids, and sulfur-related metabolites were altered in the CD and MCD groups.

Using a metabolic map template makes it easy to visualize the obtained data. The Multi-omics Analysis Package provides strong support for interpreting metabolomic data results.

■ Acknowledgment

We appreciate Dr. Tsutomu Matsubara and Dr. Chiho Kadono of Osaka Metropolitan University for providing us with samples for this application.

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Application News

CLAM™-2030 Fully Automated Sample Preparation Module for LCMS
LCMS™-8060 Liquid Chromatograph Mass Spectrometer

Measuring Primary Metabolites in Human Plasma Using an LC/MS/MS System with Fully Automated Sample Preparation Module

E. Imoto and D. Kawakami

User Benefits

- Capable of comprehensive quantitative analysis of primary metabolites in plasma
- Performs time-consuming sample preparation automatically.
- Eliminates the need for manual sample preparation and reduces variability in quantitative results due to manual operations.

■ Introduction

Metabolomics analysis, a technique for comprehensive analysis of large numbers of metabolites, is used in a wide range of fields and applications, including functional analysis of food, improving fermentation productivity, and elucidating physiological and pathological mechanisms. Liquid chromatograph mass spectrometers and other mass spectrometer systems are used to perform metabolomics analysis. In recent years, metabolomics analysis is increasingly being used in clinical research, such as to search for disease markers and markers that predict the efficacy and toxicity of drugs. However, sample preparation for metabolomics analysis and the operation of mass spectrometers is much more complex than for general laboratory testing. As a result, there is a risk of procedure errors and variability due to operator differences. In addition, operator workload increases as the number of samples increases, and sample preparation can become a bottleneck in the analytical workflow when analyzing a large number of samples.

A normal sample preparation protocol for primary metabolite analysis performed on an LC/MS/MS system with fully automated sample preparation module involves steps such as adding organic solvent for deproteination, removing solid components by centrifugation, and recovering supernatant. This article introduces an example of using an LC/MS/MS system with fully automated sample preparation module, comprised of a CLAM-2030 fully automated sample preparation module and LCMS-8060 liquid chromatograph mass spectrometer (Fig. 1), to perform an analysis and resolve the issue of sample preparation encountered when metabolomics analysis is applied to clinical research.

Fig. 1 LC/MS/MS System with Fully Automated Sample Preparation Module (CLAM™-2030 + LCMS™-8060)

■ Workflow for Simultaneous Analysis of Primary Metabolites on an LC/MS/MS System with Fully Automated Sample Preparation Module

Blood collection tubes need only be set in the system since the sample preparation steps are performed automatically by the CLAM-2030. After sample preparation, samples are then automatically transferred to the auto-sampler for analysis by the LC/MS/MS system. Fig. 2 shows the sample pretreatment protocol and analysis workflow, including analysis by the LC/MS/MS system. LC conditions, MS conditions, and parameter settings for MRM transitions all follow LC/MS/MS Method Package for Primary Metabolites Ver. 2 (Table 1). Analytes were set by modifying part of this Method Package.

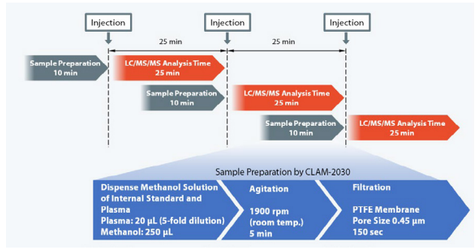

Fig. 2 Pretreatment of Plasma Samples by CLAM-2030

Table 1 LC and MS Analytical Conditions

Liquid Chromatograph	
System:	Nexera™ X2
Column:	Reversed-phase column
Mode:	Gradient elution
Injection Volume:	2 µL
Mobile Phase A:	0.1 % formic acid in Water
Mobile Phase B:	0.1 % formic acid in Acetonitrile
Flowrate:	0.25 mL/min
Mass Spectrometer	
System:	LCMS-8060
Ionization:	ESI (Positive/Negative)
Nebulizing Gas:	3 L/min
Drying Gas:	10 L/min
Heating Gas:	10 L/min
DL Temp.:	250 °C
Heat Block Temp.:	400 °C
Interface Temp.:	300 °C

■ Continuous Analysis of Clinical Samples and QC Samples Using an LC/MS/MS System with Fully Automated Sample Preparation Module

Ten clinical samples were analyzed each day for 22 days (220 samples in total) and commercial human plasma was used as a QC sample. The QC sample was analyzed first, followed by the 10 clinical samples. D3-creatine was used as an internal standard. Table 2 lists the primary metabolites detected in the QC sample using the LC/MS/MS system with fully automated sample preparation. A total of 55 components were detected, mainly amino acids, organic acids, and nucleosides included in LC/MS/MS Method Package for Primary Metabolites Ver. 2.

The peak area %RSD was calculated for each primary metabolite detected when 22 QC samples were measured. The relative peak area %RSD between each primary metabolite and D3-creatine was also calculated, and histograms were drawn showing the data range and frequency of the peak area %RSD and the relative peak area %RSD (Fig. 3). The peak area %RSD was 10 % or lower for 33 of 55 components (60 %), and 20 % or lower for 51 of 55 components (93 %). By contrast, when adjusted based on the D3-creatine internal standard, the relative peak area %RSD was 10 % or lower for 37 of 55 components (67 %), and 20 % or lower for 52 of 55 components (95 %).

Table 2 Primary Metabolites Detected in QC Sample

Compound Name	Compound Type	Compound Name	Compound Type
2-Aminobutyric acid	Amino acid	Valine	Amino acid
4-Aminobutyric acid	Amino acid	Carnitine	Amino acid derivative
4-Hydroxyproline	Amino acid	Creatinine	Amino acid derivative
Alanine	Amino acid	Kynurenine	Amino acid derivative
Arginine	Amino acid	S-Adenosylhomocysteine	Amino acid derivative
Asparagine	Amino acid	Adenine	Base
Aspartic acid	Amino acid	Choline	Choline
Asymmetric dimethylarginine	Amino acid	Acetylcarnitine	Lipid
Citrulline	Amino acid	Inosine	Nucleoside
Cystathionine	Amino acid	Uridine	Nucleoside
Cysteine	Amino acid	Adenosine 3′, 5′-cyclic monophosphate	Nucleoside
Glutamic acid	Amino acid	Adenosine monophosphate	Nucleoside
Glutamine	Amino acid	Niacinamide	Vitamin
Glycine	Amino acid	Allantoin	Purine
Histidine	Amino acid	Hypoxanthine	Purine
Isoleucine	Amino acid	Carnosine	Peptide
Leucine	Amino acid	2-Ketoglutaric acid	Organic acid
Lysine	Amino acid	Argininosuccinic acid	Organic acid
Methionine	Amino acid	Cholic acid	Organic acid
Methionine sulfoxide	Amino acid	Citric acid	Organic acid
Ornithine	Amino acid	Creatine	Organic acid
Phenylalanine	Amino acid	Guanidoacetic acid	Organic acid
Proline	Amino acid	Isocitric acid	Organic acid
Serine	Amino acid	Lactic acid	Organic acid
Symmetric dimethylarginine	Amino acid	Pantothenic acid	Organic acid
Threonine	Amino acid	Succinic acid	Organic acid
Tryptophan	Amino acid	Uric acid	Organic acid
Tyrosine	Amino acid		

Note: Partial change from compounds in Primary Metabolite LC/MS/MS Method Package Ver. 2.

These results show the LC/MS/MS system with a fully automated sample preparation module not only reduces sample preparation workload but also reduces measurement variability. Adding an internal standard also enables a more stable continuous analysis to be performed.

The peak area of D3-creatine measured while analyzing 10 clinical samples each day over 22 days (220 samples in total) is shown in Fig. 4. No instrument tuning was performed, nor was the analytical column changed during these 22 days. A peak area %RSD of 5.9 % was obtained for D3-creatine during continuous analysis. These results show that stable continuous analysis was achieved not just for QC samples but also for clinical samples.

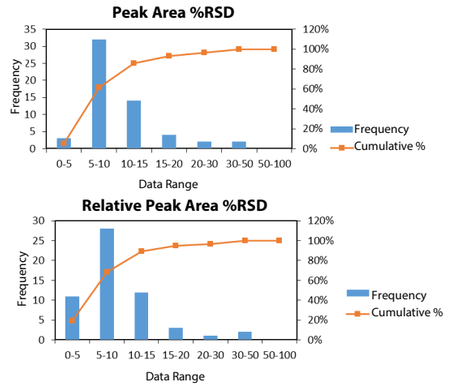

Fig. 3 Histograms Showing Data Range and %RSD and Relative Peak Area %RSD for D3-Creatine from 22 Analyses of QC Sample

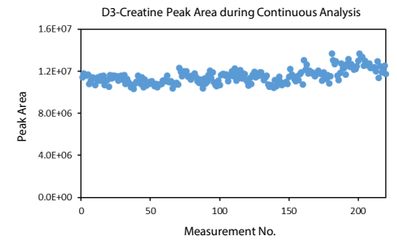

Fig. 4 Peak Area of D3-Creatine during Repeated Analysis of Clinical Samples Over 22 Days (220 Samples in Total)

■ Conclusion

Simultaneous analysis of primary metabolites was performed using an LC/MS/MS system with a fully automated sample preparation module comprised of the CLAM-2030 Fully Automated Sample Preparation Module and LCMS-8060 Liquid Chromatograph Mass Spectrometer. Stable continuous analysis was achieved for both clinical samples and QC samples. This system is expected to be developed in the future for use in the search and verification of marker candidates by metabolomics analysis.

■ Acknowledgments

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Application News

No. C183

Liquid Chromatography Mass Spectrometry

Simultaneous Analysis of Etizolam, Triazolam, and Their Metabolites in Biospecimens Using LCMS™-9030

■ Introduction

Etizolam and Triazolam are psychotropic drugs in the benzodiazepine class, which are used for a wide range of purposes due to their properties as a sedative, hypnotic drug, anxiolytic, anticonvulsant, and muscle relaxant. Benzodiazepines and their metabolites in the body are important analytes in the field of forensic toxicology and therefore more reliable toxicology test methods, such as liquid chromatography mass spectrometry (LC/MS), are needed.

Since Etizolam, Triazolam, and their metabolites (alpha-Hydroxyetizolam, alpha-Hydroxytriazolam, and 4-Hydroxytriazolam) have similar structures (Fig. 1) or almost the same molecular weight, it is difficult to separate them using high performance liquid chromatography (HPLC) or by the difference in *m/z* values that can be detected by a quadrupole mass analyzer with nominal mass resolving power. In this experiment, we simultaneously analyzed Etizolam, Triazolam, and their metabolites using the Nexera™ X2 ultra high performance liquid chromatograph and LCMS-9030 high-resolution mass spectrometer.

E. Imoto

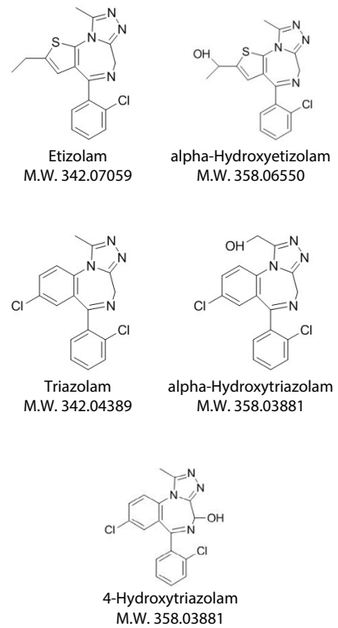

Fig. 1 Molecular Structures of Etizolam, Triazolam, and Their Metabolites

■ Instrument and Analysis Conditions

The analysis conditions of HPLC and the mass spectrometer are given in Table 1. As the HPLC conditions, a column packed with 1.9 µm particles was selected for the purpose of separating the metabolites. As for the mobile phases, 0.1 % formic acid solution and acetonitrile (containing 0.1 % formic acid) were used.

Table 1 Analysis Conditions

HPLC Conditions		
System	Nexera X2	
Column	YMC-Triart C18 (1.9 µm, 50 × 2 mm)	
Mobile phase A	0.1 % formic acid + water	
Mobile phase B	0.1 % formic acid + acetonitrile	
Flow rate	0.6 mL/min	
Time program	Time (min)	Concentration of B (%)
	0.00	20
	3.00	20
	11.50	30
	11.51	95
	13.00	95
	13.01	20
	15.00	STOP
Injection volume	2 µL	
Column temperature	40 °C	
MS Conditions		
System	LCMS-9030	
Ionization method	ESI (+)	
Nebulizer gas flow rate	3.0 L/min	
Heating gas flow rate	10.0 L/min	
Drying gas flow rate	5.0 L/min	
Interface temperature	300 °C	
DL temperature	250 °C	
Heat block temperature	400 °C	

■ Pretreatment of Spiked Blood and Plasma Samples

A volume of 100 mg of Q-sep™ QuEChERS extraction salt packet (Q150 packet, Restek) was placed in a 2.0 mL microtube as packing materials, to which three ϕ 3 mm stainless steel beads, 300 µL of acetonitrile, 200 µL of distilled water, standard substances of each compound, and 100 µL of human whole blood or blood plasma were added. Followed by centrifugal separation (10,000 rpm, 10 min), the supernatant was collected as a sample. The standards were added to matrices to achieve a concentration equivalent to 10 ng/mL and a spike and recovery test was performed. The pretreatment workflow is shown in Fig. 2.

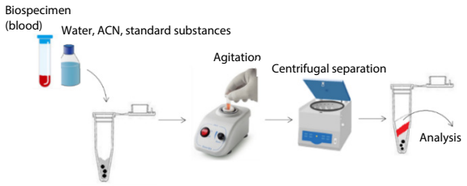

Fig. 2 Pretreatment Workflow Diagram

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Experiment Results

Fig. 3 shows an extracted-ion chromatogram (XIC) (extraction range: theoretical *m/z* value ± 2 mDa of each compound) obtained from the mixture of Etizolam, Triazolam, and their metabolites (50 ng/mL). Compounds with a difference of 26 mDa, such as Etizolam and Triazolam or alpha-Hydroxyetizolam and alpha-Hydroxytriazolam, were detected from peaks at different retention times. Etizolam and Triazolam were sufficiently separated from their retention times. Alpha-Hydroxytriazolam and alpha-Hydroxyetizolam, which cannot be sufficiently separated by HPLC, were measured individually to check the level of separation by accurate mass. The mass range was individually set for each XIC.

When alpha-Hydroxyetizolam was measured individually, a peak was detected only on the XIC with the following extraction range: the theoretical *m/z* value ± 2 mDa (*m/z*: 359.0708 to 359.0748) of the target compound. A peak was not detected on the XIC with an extraction range of *m/z* from 359.0441 to 359.0481 (figures on the left side of Fig. 4). In addition, as with the case of alpha-Hydroxyetizolam, a peak was detected only on the XIC with an extraction range of the theoretical *m/z* value ± 2 mDa (*m/z*: 359.0441 to 359.0481) when measured individually (figures on the right side of Fig. 4).

The peaks of alpha-Hydroxytriazolam and alpha-Hydroxyetizolam overlap each other because their retention times are nearly the same; however, the results indicate that they can be selectively quantified without interference from the other compound by using an XIC with the extraction range of the theoretical *m/z* value ± 2 mDa for the detection of each compound.

Fig. 5 shows the calibration curves created from the standard samples of Etizolam, Triazolam, and their metabolites. The results of spike and recovery tests performed by adding each standard to a whole blood or blood plasma sample at a concentration equivalent to 10 ng/mL are shown in Table 2. Each psychotropic drug and metabolite was successfully detected at a sufficient level of sensitivity at each concentration. Furthermore, the coefficient of determination of the calibration curve of all compounds reached 0.999, indicating an excellent linearity of the results. Also, good quantitative results were obtained from the matrices of the psychotropic drugs and their metabolites using these calibration curves: the range was 105 % to 115 % for whole blood samples and 117 % to 131 % for blood plasma samples.

The mass chromatograms of blank samples, whole blood and plasma samples to which the psychotropic drugs and their metabolites were spiked are shown in Fig. 6. As shown by the chromatograms, these compounds were detected only from the whole blood and plasma samples and were not detected from blank samples. In addition, alpha-Hydroxytriazolam and 4-Hydroxytriazolam in the matrix were separated by using liquid chromatography (LC).

Relative mass errors of standard solutions and matrices at each concentration are given in Table 3. The concentration hardly affected the relative mass errors and the results ranged from –0.229 mDa to 0.335 mDa, which were extremely good. The relative mass error range of whole blood and plasma samples was –0.270 mDa to 0.354 mDa, which confirmed that the range of relative mass errors of matrices is also stable.

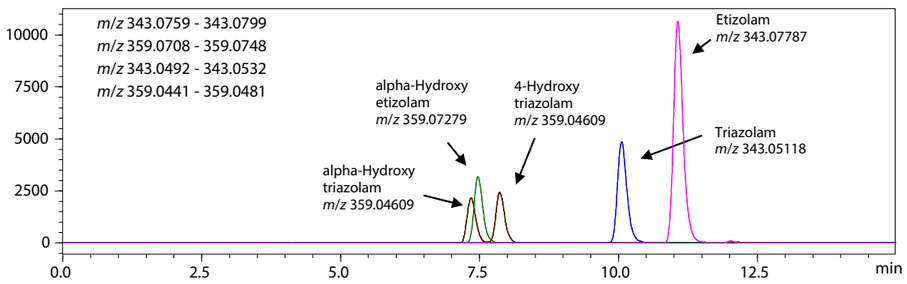

Fig. 3 Mass Chromatogram (Extraction Range: Theoretical Value ± 2 mDa) of Etizolam, Triazolam, and Their Metabolites (50 ng/mL)

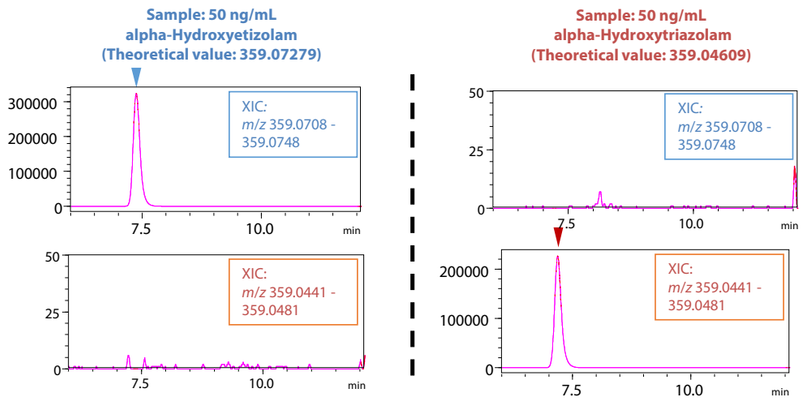

Fig. 4 Separation of alpha-Hydroxyetizolam and alpha-Hydroxytriazolam by Mass Resolution

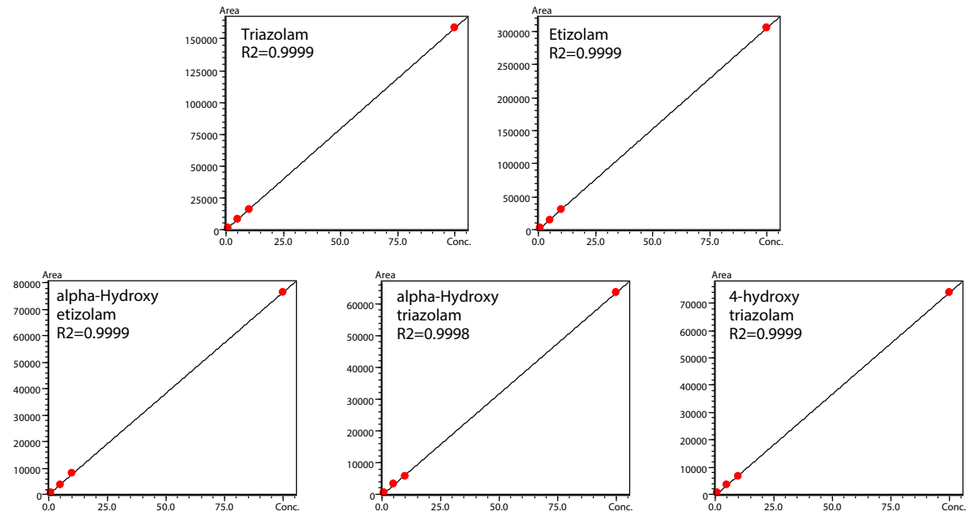

Fig. 5 Calibration Curves of Each Compound (1 to 100 ng/mL)

Table 2 Quantitative Results of Each Compound

Compound Name	Spike Concentration (ng/mL)					
	Standard Solution				Whole Blood	Blood Plasma
	1	5	10	100	10	10
	Accuracy (%)					
Etizolam	107	96	102	100	105	117
alpha-Hydroxy etizolam	92	97	103	100	105	120
Triazolam	103	99	100	100	109	122
alpha-Hydroxy triazolam	135	107	92	100	113	125
4-Hydroxy triazolam	141	100	96	100	115	131

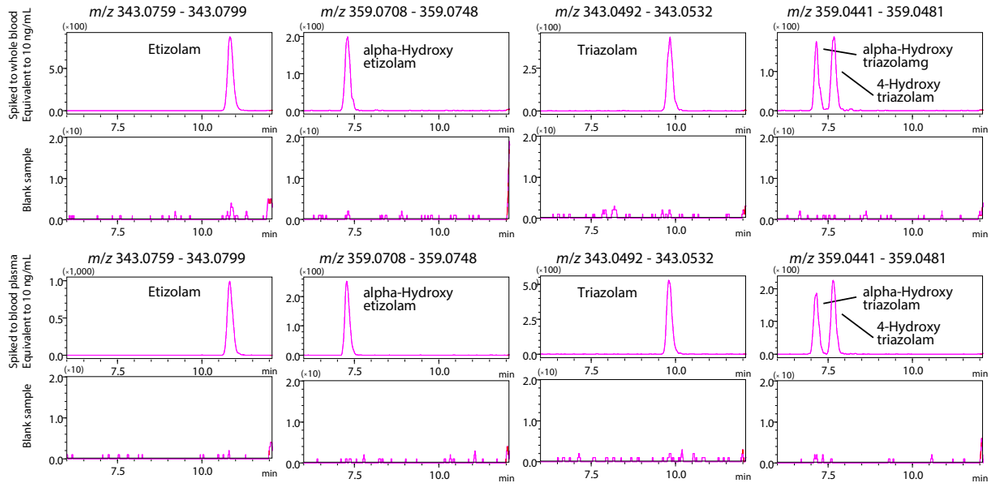

Fig. 6 Mass Chromatograms of Each Compound in Whole Blood and Blood Plasma

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Table 3 Mass Errors (mDa) of Matrices and the Standard Solution at Each Concentration

Compound Name	Spike Concentration (ng/mL)					
	Standard Solution				Whole Blood	Blood Plasma
	1	5	10	100	10	10
	Absolute errors (mDa)					
Etizolam	0.237	0.152	0.088	0.064	0.271	0.310
alpha-Hydroxy etizolam	0.101	0.335	–0.229	–0.091	0.354	–0.113
Triazolam	0.233	0.142	0.060	0.308	–0.270	0.002
alpha-Hydroxy triazolam	–0.037	–0.145	–0.102	–0.290	0.314	0.048
4-Hydroxy triazolam	–0.157	0.194	0.068	0.195	0.136	0.192

Conclusion

Etizolam, Triazolam, and their metabolites were simultaneously analyzed using the LCMS-9030 high-resolution quadrupole time-of-flight mass spectrometer (Q-TOF). While only some compounds were separated using LC alone, all compounds were selectively quantified by separating and detecting alpha-Hydroxyetizolam and alpha-Hydroxytriazolam, which have a mass difference of 26 mDa, by the high resolving power of the Q-TOF.

The linearity of the calibration curves created from standard samples prepared by serial dilutions was good and the quantitative results from the whole blood and plasma samples demonstrated high accuracy.

These results of mass errors indicate that the compounds can be measured stably with high mass accuracy without being affected by concentration or matrices.

<Acknowledgments>
We would like to express our sincere appreciation to Associate Professor Kei Zaitsu of the Department of Legal Medicine and Bioethics, Nagoya University Graduate School of Medicine for his contribution to the analyses described in this document.

- The products described in this document have not been approved or certified as a medical device under the Pharmaceutical and Medical Device Act of Japan.
- The analytical techniques described in this document cannot be used for the purpose of medical examinations.

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Application News

No. C230

LC-MS

Analysis of Favipiravir in Human Plasma Using Fully Automated Sample Preparation LC/MS/MS System

Introduction

Favipiravir (brand name: Avigan®), which was developed by FUJIFILM Toyama Chemical Co., Ltd, is one of the RNA polymerase inhibitors used for treating influenza. In Application News C229, we introduced a robust, highly-sensitive analysis using LC/MS/MS with manual pretreatment. However, manual pretreatment of plasma samples entails a certain level of workload. This report introduces a method of analyzing favipiravir using a fully automated sample preparation LC/MS/MS system that can reduce variation between procedures, sample mix-ups, and risk of exposure to the samples (Fig. 1).

E. Imoto, D. Kawakami

Fig. 1 Fully Automated Sample Preparation LC/MS/MS System (CLAM™+LC/MS/MS)

Fully Automated Sample Preparation of Favipiravir in Plasma

For analysis of low-molecular weight compounds in plasma using a LCMS™, it is common to use supernatant collected following deproteinization by adding an organic solvent. With the fully automated sample preparation LC/MS/MS system, these preparatory steps are done automatically just by placing a blood collection tube in the system after plasma separation (Fig. 2). Pretreatment of the next sample can also be performed in parallel with LC/MS/MS analysis, which can greatly reduce the time required to analyze each sample.

This analysis was performed in a per-sample cycle time of 6.5 minutes from plasma pretreatment to the analysis of favipiravir using LC/MS/MS.

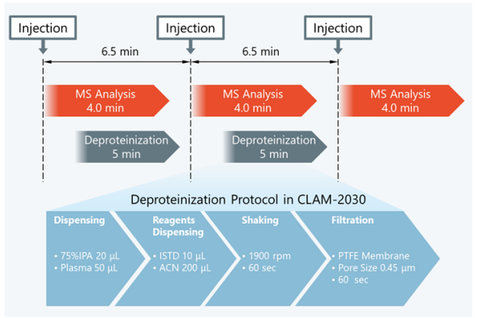

Fig. 2 Workflow of Fully Automated Sample Pretreatment

Analytical Conditions and Pretreatment of Samples

Favipiravir (PN: C8720*1), as the target compound, and [^{13}C,^{15}N]-favipiravir (PN: C8853*1), as its stable isotope, were purchased from Alsachim, one of the companies of the Shimadzu Group. [^{13}C,^{15}N]-favipiravir was used as the internal standard (ISTD). Favipiravir was spiked with commercially available human plasma treated with EDTA 2K to prepare calibration curves and QC samples. Analysis was performed using the LC and MS analytical conditions shown in Table 1 and the MRM transition in Table 2. Shim-pack Scepter™ C18-120 (50 mm×2.1 mm I.D., 1.9 µm, P/N: 227-31012-03) was used as the analytical column. Fig. 3 shows the MS chromatograms and structural formulas of the compounds.

A calibration curve was prepared using calibration points at plasma concentrations of 1, 2, 5, 10, 20, 50 and 100 µg/mL for favipiravir (n = 5 for each calibration point). [^{13}C,^{15}N]- favipiravir (20 µg/mL) solution was prepared using acetonitrile and used as ISTD. The pretreatment steps for the plasma sample spiked with favipiravir are shown in Fig. 2. Samples were automatically prepared through the following series of steps: mixing 20 µL of 75% isopropyl alcohol (IPA), 50 µL of plasma, 10 µL of ISTD and 200 µL of acetonitrile, shaking the mixture, and then filtration of the mixture using a PTFE membrane filter. Finally, the prepared sample was used for analysis.

*1 Shimadzu GLC and Alsachim Product numbers

Table 1 LC and MS Analytical Conditions

<LC analytical conditions>		<MS analytical conditions>	
UHPLC	Nexera™ X2	LC/MS/MS system	LCMS-8060
Analysis column	Shim-pack Scepter C18-120 (50 mm×2.1 mm I.D., 1.9 µm)	Interface	Heated ESI
Mobile phase	A: 0.05 % Formic acid – water B: 0.05 % Formic acid – acetonitrile	MS analysis mode	MRM (+)
Gradient program (%B)	5 % (0 – 0.30 min) ➡ 30 % (0.35 min) ➡ 90 % (1.50 – 2.50 min) ➡ 5 % (2.60 – 4.00 min)	Heat block temperature	400 °C
		DL temperature	250 °C
Flow rate	0.4 mL/min	Interface temperature	300 °C
Column oven temperature	40 °C	Nebulizing gas flow rate	3 L/min
Injection volume	1.0 µL	Drying gas flow rate	10 L/min
Rinse solution (for external rinse only)	MeOH	Heating gas flow rate	10 L/min

Table 2 MRM Transitions of Favipiravir and [^{13}C,^{15}N]-Favipiravir

Compound	Ion	Precursor ion (*m/z*)	Product ion (*m/z*)
Favipiravir	Quantifier ion	157.70	85.10
	Qualifier ion	157.70	113.20
[^{13}C,^{15}N]-Favipiravir	Quantifier ion	159.70	85.10
	Qualifier ion	159.70	113.20

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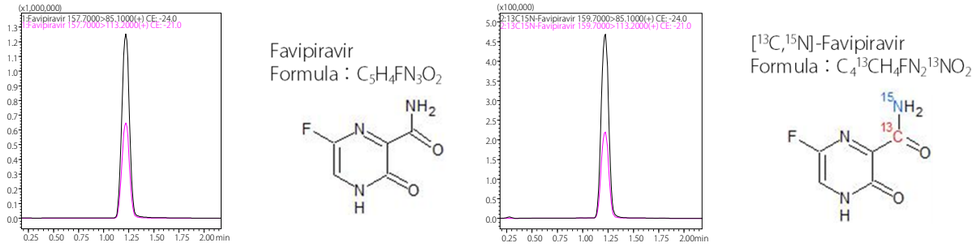


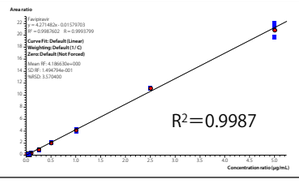
Fig. 3 MS Chromatograms and Structural Formulas of Favipiravir (Left) and [^{13}C,^{15}N]-Favipiravir (Right)

Preparation of Calibration Curve

The calibration curve prepared using the fully automated sample preparation LC/MS/MS are shown in Table 3. Good linearity with R^2 of 0.9987 was obtained in the set calibration range. The precision of favipiravir (%RSD) was 1.0 % – 5.6 % over the entire concentration range, including the quantitative lower limit. The accuracy of favipiravir ranged between 95 % – 105 %, within acceptance limits of 100 ± 15 %.

Table 3 Linearity, Precision and Accuracy of Favipiravir in Plasma Using Fully Automated LC/MS/MS Obtained from Analysis

Favipiravir					
ID	Spiked Conc. (µg/mL)	Average Conc. (µg/mL)	Precision %RSD	Accuracy %	Calibration Curve
Blank	---	---	---	---	
Level 1	1	1.04	4.3	104	
Level 2	2	2.00	1.9	100	
Level 3	5	4.77	2.4	95	
Level 4	10	9.87	1.9	99	
Level 5	20	19.7	3.5	99	
Level 6	50	52.7	1.0	105	
Level 7	100	97.9	5.6	98	



Validation of Analytical System Using QC Samples

Favipiravir was prepared at the following plasma concentrations as QC samples: 3, 50, 90 µg/mL to evaluate its repeatability (Table 4) and between-days reproducibility comparing results obtained over three days (Table 5). Based on the repeatability test result, the precision of favipiravir (%RSD) was 1.6 % – 3.0 %. The accuracy ranged between 94 % – 97 % with acceptance limit of 100 ± 15 %. Based on the test results for between-days reproducibility, the precision of favipiravir (%RSD) was 0.2 % – 7.6 %. The accuracy ranged between 88 % - 99 % within acceptance limit of 100 ± 15 % during QC sample analyses on each of the three days.

Table 4 Repeatability of Favipiravir in Plasma

Compound	QC Sample	Spiked Conc. (µg/mL)	Intra-Assay (*n*=6)		
			Average Conc. (µg/mL)	Precision %RSD	Accuracy %
Favipiravir	Low	3	2.90	2.2	97
	Medium	50	48.3	3.0	97
	High	90	84.8	1.6	94

Table 5 Between-Days Reproducibility of Favipiravir in Plasma

Compound	QC Sample	Spiked Conc. (µg/mL)	Day 1st (*n*=3)			Day 2nd (*n*=3)			Day 3rd (*n*=3)		
			Average Conc. (µg/mL)	Precision %RSD	Accuracy %	Average Conc. (µg/mL)	Precision %RSD	Accuracy %	Average Conc. (µg/mL)	Precision %RSD	Accuracy %
Favipiravir	Low	3	2.95	0.2	98	2.94	7.6	98	2.64	3.7	88
	Medium	50	49.3	1.0	99	47.1	2.8	94	46.0	3.6	92
	High	90	85.8	1.3	95	81.3	1.0	90	79.4	1.7	88

Conclusion

Using favipiravir spiked with plasma, a fully automated sample preparation LC/MS/MS analytical system has been developed. The prepared calibration curve showed good linearity. The repeatability and between-days reproducibility of favipiravir were evaluated using QC samples. Good accuracy and reproducibility were obtained.

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Application News

No. C228

LC-MS

High Sensitivity Analysis of Aldosterone in Human Serum Using LCMS™-8060NX

N. Kato, Y. Inohana

Introduction

Aldosterone (Fig. 1), a mineralocorticoid produced in the cortex of the adrenal gland, plays key roles in the homeostasis of electrolytes, circulating blood volume and blood pressure regulation. The aldosterone levels in blood are used for diagnosis of hypertensive diseases, such as primary hyperaldosteronism, diabetic nephropathy or nephritis interstitial, etc.

For the measurement of aldosterone levels, radioimmunoassay (RIA) or enzyme immunoassay (EIA) has been used, but the aldosterone concentration levels measured using the immune responses may be greater due to the cross reaction with steroid hormones having similar structures in the samples. The use of LC/MS/MS with high selectivity prevents the problems caused by the conventional method and enables more accurate quantitative analysis.

This report introduces an example of high sensitivity quantitative analysis of aldosterone in serum using LCMS-8060NX.

This report introduces an example of high sensitivity analysis of aldosterone in serum using LCMS-8060NX, a triple quadrupole mass spectrometer. Calibration curves prepared using standard samples showed linearity in the concentration range of 0.5 - 10000 pg/mL (R^2 =0.9998), with %RSD of concentration of 3.96% at the lower limit of quantification and accuracy at each calibration point ranged from 95.58 - 109.84%. Furthermore, the accuracy of serum samples spiked with aldosterone (1 pg/mL) was 106%, indicating that the method used in the analysis is suitable for the actual samples.

Sample Preparation

Standard samples were serially diluted with 50% methanol, prepared at concentrations of 0.5 - 10000 pg/mL and used for preparation of the calibration curve.

For the samples spiked with serum, commercially available steroid-free human serum was spiked with aldosterone to make a serum concentration of 1 pg/mL. The standard sample and internal standard were spiked with 100 µL of serum, and then shaken well with a mixture of hexane and ethyl acetate (9 : 1). The solution obtained was centrifuged for 10 minutes, followed by evaporating the organic solvent layer to dryness, and then re-dissolved with 50% methanol.

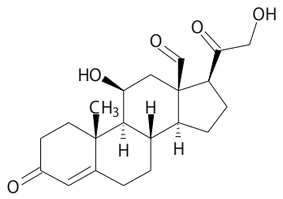

Fig. 1 Structural Formula of Aldosterone

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Analytical Conditions

The analytical conditions for HPLC and MS are shown in Table 1. The MRM transitions are shown in Table 2.

Table 1 Analytical Conditions

[HPLC conditions] (Nexera™ X3)	
Column	: Shim-pack Scepter™ C18-120, 50 mm × 2.1 mml.D., 1.9 µm*1
Mobile phases	: A) 0.05 mM NH_4F in water B) methanol
Mode	: Gradient elution
Flow rate	: 0.3 mL/min
Injection volume	: 25 µL
[MS conditions] (LCMS-8060NX)	
Ionization	: ESI (Positive mode)
Mode	: MRM
Interface voltage	: 1 kV
IonFocus voltage	: 3.5 kV
Nebulizing gas flow	: 3.0 L/min
Drying gas flow	: 10.0 L/min
Heating gas flow	: 5.0 L/min
DL temp.	: 250 °C
Block heater temp.	: 300 °C
Interface temp.	: 400 °C
Probe position	: +1.5

*1 : P/N 227-31012-03

Table 2 MRM Transitions

Compound	Precursor *m/z*	Product *m/z*
Aldosterone	361.20	343.30
Aldosterone-[D4]	365.25	346.25

Calibration Curve

The calibration curve prepared using the standard sample (internal standard method, n=3 for each concentration) showed good linearity in a wide dynamic range from 0.5 - 10000 pg/mL with a coefficient of determination (R^2) of 0.9998. The %RSD of concentration at the lower limit of quantification was 3.96% with the accuracy at each calibration point ranging from 95.58 - 109.84%. Fig. 2 shows the calibration curve, and Fig. 3 shows the MRM chromatogram of the 0.5 pg/mL standard solution.

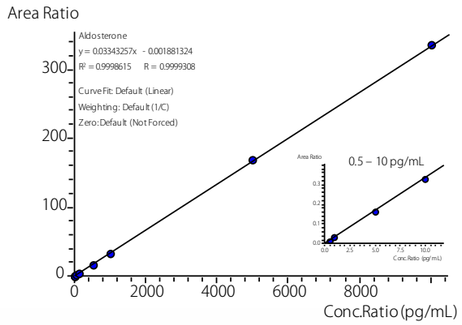

Fig. 2 Calibration Curve (0.5 - 10000 pg/mL)

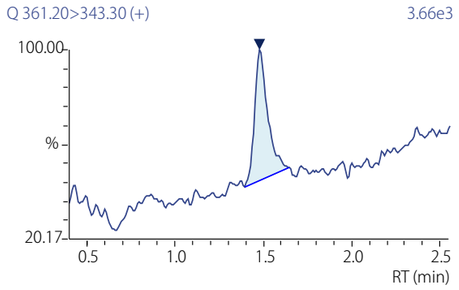

Fig. 3 MRM Chromatogram (neat STD: 0.5 pg/mL)

Sample Spiked with Human Serum

Based on the area obtained by the measurement of the pretreated sample, the aldosterone level was calculated using the calibration curve shown in Fig. 2. Good accuracy was obtained and the mean level of aldosterone spiked at 1 pg/mL with serum was 1.06 pg/mL (n=3). Fig. 4 shows the MRM chromatogram of the sample spiked with human serum.

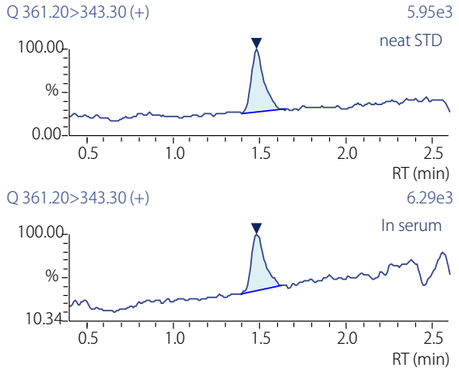

Fig. 4 MRM Chromatogram at 1 pg/mL (Upper: neat STD, Lower: Sample Spiked with Human Serum)

Conclusion

Quantitative analysis of aldosterone using LCMS-8060NX demonstrated that the aldosterone level can be determined across a wide range of 0.5 - 10000 pg/mL, and that good accuracy is achieved with samples spiked with human serum.

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Application News

Liquid Chromatography Mass Spectrometry

No. C213

High-Sensitivity Analysis of Drugs in Ultra-Small Volume Plasma Samples Using Microflow LC/MS/MS

It is known that drugs and other xenobiotics are generally subject to metabolism in the body, which facilitates their detoxification and elimination from the body. Therefore, pharmacokinetic (PK) studies of the metabolic fate of drugs in the body are conducted by preclinical and clinical tests as part of the drug development process [(1)].

In preclinical PK studies, the concentration of drugs and their metabolites in biological samples obtained from animal experiments is analyzed by LC/MS/MS.

However, since the size of the animal model generally limits the amount of sample volume that can be taken safely, a large number of animals and a significant volume of drug may be necessary for the evaluation, and this becomes an issue from the ethical and economic point of view.

An effective approach to overcome those issues, together with the use of micro sampling technology, is the development of high sensitivity LC MS/MS methods that allows drug detection from the small amount of sample.

This article introduces an example of a high-sensitivity microflow LC/MS/MS method, for the analysis of drugs in ultra-small volume plasma samples.

D. Vecchietti, K. Matsumoto

Nexera Mikros™ Microflow Liquid Chromatography Mass Spectrometry System

This study was conducted using a Shimadzu Nexera Mikros (Fig. 1), which can be used effectively in a wide range of flow rates from the microflow region to the semi-microflow region (1 to 500 µL/min).

Fig. 1 Nexera Mikros™ Microflow LC-MS System

The Nexera Mikros can detect target components with higher sensitivity than the semi-microflow LC/MS systems in general use, which operates in the flow rate range of approximately 100 to 500 µL/min, and also offers excellent robustness and shorter analysis times in comparison with nano LC/MS system, which operates in a flow rate range of several 100 nL/min to 1 µL/min.

Fig. 2 shows the flow diagrams during trapping and during elution. Because acetonitrile is commonly used as a sample solvent for deproteinization, the peak shape in direct injection is broadened due to the elution strength of acetonitrile, and this limits the injection volume. In order to overcome this broadening of the peak by the sample solvent, the trap & elute injection method is used in the Nexera Mikros system. After the target compound is retained in the trap column (trap), the column is backflushed with the analytical mobile phase by switching a valve, and the target compound is transported to the analytical column (elute) for separation and detection.

Sample Preparation

Plasma samples (2 µL) were prepared by spiking with Verapamil and Nor-Verapamil, which are shown in Fig. 3. The spiked samples were diluted by adding 58 µL of a precipitant solution (containing acetonitrile + formic acid 0.1 % and a labelled internal standard, Verapamil-D6). After allowing the samples to stand quietly for 20 min (incubation) at a room temperature, the samples were centrifuged and the supernatant was transferred to a vial, and 5 µL of the samples was injected for analysis.

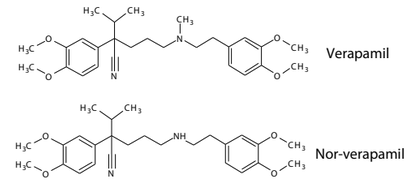

Fig. 3 Chemical Formulas of Verapamil and its Metabolite Nor-Verapamil

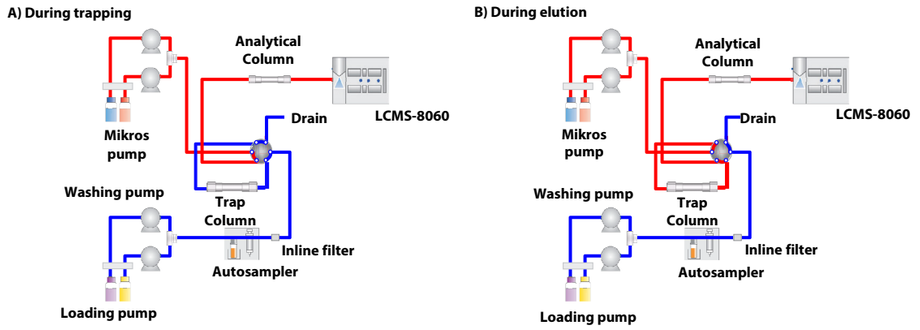

Fig. 2 Flow Diagrams of Trap and Elute System

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Analytical Conditions

Table 1 and Table 2 show the conditions of HPLC using the Nexera Mikros and MS, respectively. Sample loading in the trap column was carried out using water/acetonitrile = 98/2 containing 0.1 % formic acid, and the analysis was conducted by gradient separation of the water and acetonitrile containing 0.1 % formic acid. During the analysis, 100 % acetonitrile was delivered to the trap column to wash. A comparison of microflow analysis and semi-microflow analysis was also carried out using the same device configuration.

Table 1 LC Conditions (Microflow)

Analytical column	: Shim-pack™ MC PLONAS Biphenyl (2.7 µm, 100 mm × 0.2 mm I.D.)
Trap column	: Shim-pack™ MCT LC8 (5 µm, 5 mm × 0.3 mm I.D.)
Temp.	: 40 °C
Mobile phase A	: Water + Formic acid 0.1 %
Mobile phase B	: Acetonitrile + Formic acid 0.1 %
Flow rate	: 250 µL/min (Load), 4 µL/min (Elution)
Gradient	: B Conc. 26 % (0-1 min)-95 % (4-5 min)
Injection volume	: 5 µL

Table 2 MS Conditions

Ionization	: Micro ESIMicro-ESI 8060
Probe voltage	: +2.6 kV (positive ionization)
Temp.	: Interface: no heating Desolvation line: 250 °C Heater block: 400 °C
Gas flow	: Nebulizing Gas: 1 L/min Heating gas: -- Drying gas: --
MRM	: (Quant / Qual) Verapamil (455.0 > 150.25 / 455.0 > 303.3 (165.2)) Nor verapamil (440.95 > 165 / 440.95 > 150) Verapamil D6 (461.3 > 309.3 / 461.3 > 165.25 (150.25))

Evaluation of Signal Intensity in Microflow Analysis

The effect of microflow analysis on the signal intensity of the target compound was evaluated using the Verapamil and Nor-Verapamil spiked plasma (lowest concentration calibration point, 0.5 µg/L). The sample injection volume and the linear velocity in the columns were adjusted to be the same, and the signal intensities with the microflow and semi-microflow were compared. A Micro-ESI 8060 microflow ionization unit was used as the ionization unit under the microflow condition, and a standard ESI ionization unit was used with the semi-microflow. Table 3 shows analysis conditions.

Table 3 Analysis Conditions with Microflow and Semi-Microflow LC/MS/MS

Parameter	Micro LC/MS/MS method	Semi-Micro LC/MS/MS method
Injection mode	Trap & Elute	Direct
Injection volume (µL)	5	5
Flow rate (µL/min)	4	441
Analytical column	0.2×100 mm, 2.7 µm	2.1×100 mm, 2.7 µm
Linear velocity (cm/s)	4.145	4.145
Sample concentration (µg/L)	0.5	0.5

In comparison with the semi-microflow analysis, the signal intensity for Verapamil increased by 4.5 times or more and that of Nor-Verapamil increased by 3.5 times or more when the microflow was used (Fig. 4), confirming that sensitivity can be enhanced with the same sample volume by using the microflow method.

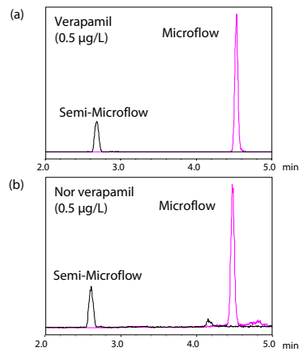

Fig. 4 Comparison of Signal Intensities of (a) Verapamil and (b) Nor-Verapamil in Microflow and Semi-Microflow LC/MS/MS (Spiked Plasma Sample at 0.5 µg/L)

Linearity of Calibration Curve

Fig. 5 shows the calibration curve (linear regression model with 1/X weighting) by the internal standard method. Calibration curves with linearity of $R^2 = 0.998$ or higher and good accuracy were obtained for both Verapamil and Nor-Verapamil (Fig. 5, Table 4).

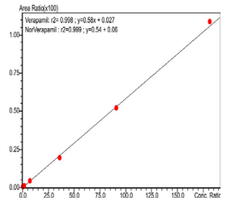

Fig.5 Calibration Curve of Verapamil

Table 4 Accuracy of Calibration Points of Microflow and Semi-Microflow LC/MS/MS

Conc. (µg/L)	Accuracy % (Verapamil)	Accuracy % (Nor verapamil)
0.5	101.4	94.2
0.9	110.7	108.4
1.8	93.9	91.2
7.3	100.5	108.5
36.3	93.5	98.1
90.8	97.3	98.9
181.6	102.6	100.7

Conclusion

Quantitation of Verapamil and Nor-Verapamil was conducted with a Nexera Mikros microflow liquid chromatography mass spectrometry system, utilizing the trap & elute system to overcome broadening of the peaks by the sample solvent.

For drug quantitation in biosamples, the use of microflow LC MS/MS method showed increased sensitivity compared to a semi micro flow method. Based on this result a reduction of initial plasma sample was possible without affecting the overall analytical performances of the method.

The use of microflow LC MS/MS technology can therefore finds a successful application in discovery-stage PK studies.

<References>
(1) Rapid Commun. Mass Spectrom. **2014**, 28, 1293–1302.

First Edition: Aug. 2020

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Application News

No. C227

LC-MS

High Sensitivity Analysis of Testosterone in Human Serum Using LCMS™-8060NX

This report introduces an example of high sensitivity analysis of testosterone in serum using LCMS-8060NX, a triple quadrupole mass spectrometer. Calibration curves prepared using standard samples showed linearity in the concentration range of 0.05 - 1000 pg/mL (R^2 =0.9996), with %RSD of concentration of 2.47% at the lower limit of quantification and accuracy at each calibration point ranged from 94.64 - 111.04%. Furthermore, the accuracy of serum samples spiked with testosterone (1 pg/mL) was 105%, indicating that the method used in the analysis is suitable for the actual samples.

N. Kato, Y. Inohana

Introduction

Testosterone (Fig. 1), an androgen produced in the testes and the adrenal glands, is a steroid hormone which contributes to the growth enhancement of bones and muscles and hematopoiesis. The free-testosterone level in blood plays a key clinical role as a biomarker for a variety of diseases, such as Cushing's syndrome and tumors. Testosterone levels in blood have usually been measured using immunoassay, but these levels may be affected by the cross reactivity caused by steroid hormones having similar structures. LC/MS/MS with high selectivity prevents the problems caused by the conventional method and enables more accurate quantitative analysis, and it is expected that it will come into clinical use.

This report introduces an example of high sensitivity quantitative analysis of testosterone in serum using LCMS-8060NX.

Sample Preparation

Standard samples were serially diluted with 50% methanol, prepared at concentrations of 0.05 - 1000 pg/mL and used for preparation of the calibration curve.

For the samples spiked with serum, commercially available steroid-free human serum was spiked with testosterone to make a serum concentration of 1 pg/mL. The standard sample and internal standard were spiked with 100 µL of serum, and then shaken well with a mixture of hexane and ethyl acetate (9 : 1). The solution obtained was centrifuged for 10 minutes, followed by evaporating the organic solvent layer to dryness, and then re-dissolved with 50% methanol.

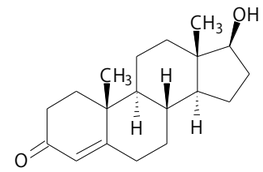

Fig. 1 Structural Formula of Testosterone

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Analytical Conditions

The analytical conditions for HPLC and MS are shown in Table 1. The MRM transitions are shown in Table 2.

Table 1 Analytical Conditions

[HPLC conditions] (Nexera™ X3)	
Column	: Shim-pack Scepter™ C18-120, 50 mm × 2.1 mm I.D., 1.9 µm*1
Mobile phases	: A) 0.05 mM NH_4F in water B) methanol
Mode	: Gradient elution
Flow rate	: 0.3 mL/min
Injection volume	: 25 µL
[MS conditions] (LCMS-8060NX)	
Ionization	: ESI (Positive mode)
Mode	: MRM
Interface voltage	: 1 kV
IonFocus voltage	: 3 kV
Nebulizing gas flow	: 3.0 L/min
Drying gas flow	: 15.0 L/min
Heating gas flow	: 15.0 L/min
DL temp.	: 250 °C
Block heater temp.	: 500 °C
Interface temp.	: 350 °C
Probe position	: +1.5

*1 : P/N 227-31012-03

Table 2 MRM Transition

Compound	Precursor *m/z*	Product *m/z*
Testosterone	289.25	97.15
Testosterone-[13C3]	292.25	100.20

Calibration Curve

The calibration curve prepared using the standard sample (internal standard method, n=3 for each concentration) showed good linearity in a wide dynamic range from 0.05 - 1000 pg/mL with a coefficient of determination (R^2) of 0.9996. The %RSD of concentration at the lower limit of quantification (0.05 pg/mL) was 2.47% with the accuracy at each calibration point ranging from 94.64 - 111.04%. Fig. 2 shows the calibration curve, and Fig. 3 shows the MRM chromatogram of the 0.05 pg/mL standard solution.

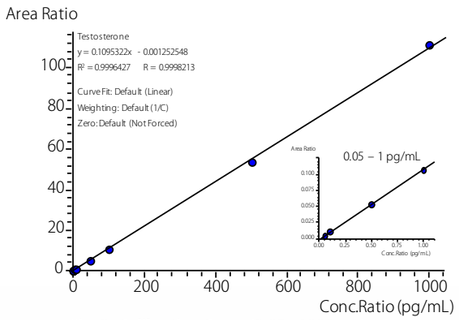

Fig. 2 Calibration Curve (0.05 - 1000 pg/mL)

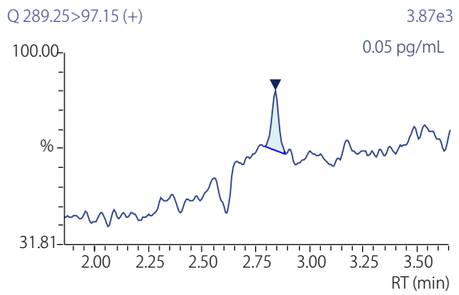

Fig. 3 MRM Chromatogram (neat STD: 0.05 pg/mL)

Sample Spiked with Human Serum

Based on the area obtained by the measurement of the pretreated sample, the testosterone level was calculated using the calibration curve shown in Fig. 2. Good accuracy was obtained and the mean level of testosterone spiked at 1 pg/mL with serum was 1.05 pg/mL (n=3). Fig. 4 shows the MRM chromatogram of the sample spiked with human serum.

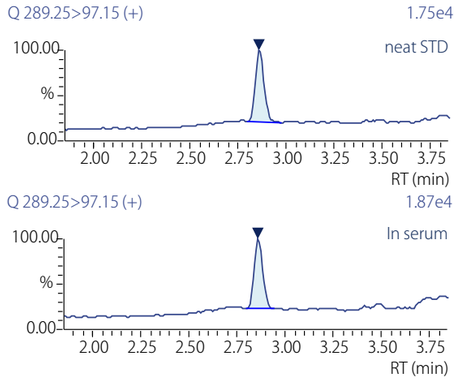

Fig. 4 MRM Chromatogram at 1 pg/mL (Upper: neat STD, Lower: Sample Spiked with Human Serum)

Conclusion

Quantitative analysis of testosterone using LCMS-8060NX demonstrated that the testosterone level can be determined across a wide range of 0.05 - 1000 pg/mL, and that good accuracy is achieved with samples spiked with human serum.

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Application News

LC-MS

No. C217

Simultaneous Analysis of Remdesivir and Metabolites in Human Plasma Using Fully Automated Sample Preparation LC/MS/MS System

Introduction

Remdesivir (brand name: Veklury®), which was developed by Gilead Sciences (U.S.) for treatment of Ebola virus disease, is a prodrug having antiviral activity against single-strand RNA viruses. It is known to be partly metabolized to activated GS-441524, the main metabolite of remdesivir, in vivo[1]. In Application News C218, we introduced an robust, highly-sensitive simultaneous measurement method using LC/MS/MS with manual pretreatment. Meanwhile, manual pretreatment of plasma samples requires a certain level of workload. This report introduces a method of simultaneously analyzing remdesivir and its metabolite using the automated sample preparation LC/MS/MS system that can reduce variation between procedures, mix-ups of the samples, and risk of exposure to the samples (Fig. 1).

E. Imoto, D. Kawakami

Fig. 1 Fully Automated Sample Preparation LC/MS/MS System (CLAM™+LC/MS/MS)

Analysis of Remdesivir in plasma with Fully Automated Pretreatment

For analysis of low-molecular compounds in plasma using LCMS™, it is common to use supernatant collected following deproteinization by adding an organic solvent. With the fully automated sample preparation LC/MS/MS system, these preparatory steps are done automatically just by placing a blood collection tube in the system after plasma separation (Fig. 2). Pretreatment of the next sample can also be performed in parallel with LC/MS/MS analysis, which can greatly reduce the time required to analyze each sample.

This analysis was performed in a per-sample cycle time of seven minutes from plasma pretreatment to the simultaneous analysis of remdesivir and its metabolite using LC/MS/MS.

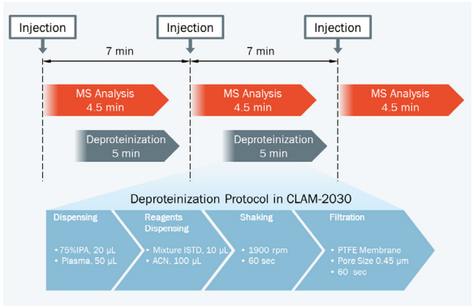

Fig. 2 Workflow of Fully Automated Sample Pretreatment

Analysis Conditions and Pretreatment of Samples

Remdesivir (P/N: C8799*) and GS-441524 (P/N: C8847*), as the target compounds, and [U-Ring-$^{13}C_6$]-remdesivir (P/N: C8845*) and [$^{13}C_5$]-GS-441524 (P/N: C8855*), as their stable isotopes, were purchased from Alsachim, one of the companies of the Shimadzu Group. [U-Ring-$^{13}C_6$]-remdesivir and [$^{13}C_5$]-GS-441524 were used as materials of the internal standard. To commercially available human plasma treated with EDTA 2K, remdesivir and GS-441524 were added. Following this, the calibration curves and QC samples were prepared. Analysis was performed using the LC and MS analysis conditions shown in Table 1 and the multiple reaction monitoring (MRM) data acquisition parameters shown in Table 2. Shim-pack Scepter™ C18-120 (50 mm×2.1 mm I.D., 1.9 µm) was used as the analytical column. Fig. 3 shows the MS chromatograms.

Calibration was performed using 5 calibration points at concentrations of 100, 500, 1000, 2500 and 5000 ng/mL for remdesivir and 5 calibration points at concentrations of 5, 25, 50, 250 and 500 ng/mL for GS-441524 (n = 5 for each calibration point). [U-Ring-$^{13}C_6$]-remdesivir (2.5 µg/mL) and [$^{13}C_5$]-GS-441524 (0.25 µg/mL) were mixed with methanol to be used as the internal standard (ISTD). Samples are automatically prepared through a series of steps. These comprise mixing 20 µL of 75%IPA, 50 µL of plasma, 10 µL of ISTD and 100 µL of acetonitrile, shaking the mixture, and then filtration of the mixture using a PTFE membrane filter, as shown in Fig. 2. Finally, the prepared sample is used for LC/MS/MS analysis.

*Alsachim's product numbers

Table 1 LC and MS Analytical Conditions

<LC Analysis Conditions>		<MS Analysis Conditions>	
UHPLC	Nexera™ X2	LC/MS/MS system	LCMS-8060
Analysis column	Shim-pack Scepter C18-120 (50 mm × 2.1 mm I.D., 1.9 µm)	Interface	Heated ESI
Mobile phase	A: 0.05 % Formic acid–water B: 0.05% Formic acid–acetonitrile	MS analysis mode	MRM (+)
Gradient program (%B)	5 % (0 – 0.30 min) ➡ 30 % (0.35 min) ➡ 70 % (1.50 min) ➡ 90 % (1.80 – 2.80 min) ➡ 5 % (2.90 – 4.50 min)	Heat block temperature	400 °C
Flow rate	0.4 mL/min	DL temperature	200 °C
Column oven temperature	40 °C	Interface temperature	300 °C
Injection volume	2.0 µL (co-injected with 20 µL of water)	Nebulizing gas flow rate	3 L/min
Rinse solution (for external rinse only)	MeOH: IPA = 1:1 (v/v)	Drying gas flow rate	10 L/min
		Heating gas flow rate	10 L/min

Table 2 MRM Transitions of Remdesivir and GS-441524

Compounds	Ion	Precursor ion (m/z)	Product ion (m/z)
Remdesivir [$C_{27}H_{35}N_6O_8P$]	Quantitation ion	603.05	272.10
	Qualification ion	603.05	229.00
[$^{13}C_6$]-Remdesivir [$C_{21}{}^{13}C_6H_{35}N_6O_8P$]	Quantitation ion	609.05	278.20
	Qualification ion	609.05	229.15
GS-441524 [$C_{12}H_{13}N_5O_4$]	Quantitation ion	291.90	163.05
	Qualification ion	291.90	173.05
[$^{13}C_5$]-GS-441524 [$C_7{}^{13}C_5H_{13}N_5O_4$]	Quantitation ion	296.90	164.10
	Qualification ion	296.90	174.10

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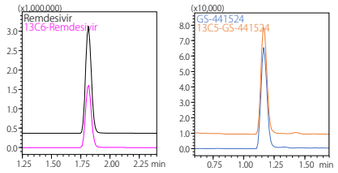
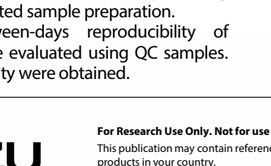
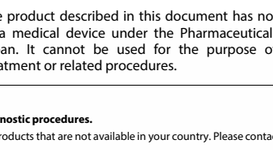


Fig. 3 MS Chromatograms of Remdesivir, [U-Ring-$^{13}C_6$]-Remdesivir (Left) and GS-441524, [$^{13}C_5$]-GS-441524 (Right)

Preparation of Calibration Curves

Calibration curves prepared using the fully automated sample preparation LC/MS/MS are shown in Table 3. Good linearity was obtained in the set calibration range. The precision (reproducibility) of remdesivir and GS-441524 in the entire concentration range, including the quantitative lower limit, was %RSD 0.5 %—2.9 % and %RSD 2.4 %—4.9 %, respectively. Similarly, the accuracy of remdesivir and GS-441524 was 87.8 %—108 % and 94.5 %—105 %, respectively, indicating that the accuracy of both was within 100 ± 15 %.

Table 3 Linearity, Accuracy and Precision of Remdesivir and GS-441524 in plasma Obtained from Analysis Using Fully Automated LC/MS/MS

Compound	Remdesivir				GS-441524			
ID	Spiked Conc. (ng/mL)	Measured Conc. (ng/mL)	Precision %RSD	Accuracy %	Spiked Conc. (ng/mL)	Measured Conc. (ng/mL)	Precision %RSD	Accuracy %
Blank	---	---	---	---	---	---	---	---
Level 1	100	87.8	0.5	88	5	4.72	2.4	95
Level 2	500	539	0.8	108	25	26.2	4.8	105
Level 3	1000	1052	1.2	105	50	50.8	4.4	102
Level 4	2500	2536	2.9	101	125	125	4.9	100
Level 5	5000	4885	2.5	98	250	248	4.1	99
Calibration Curve								

Validation Test of the Analytical System Using QC Samples

Remdesivir and GS-441524 were prepared at the following concentrations as QC samples: for remdesivir, 100, 750, 1000 and 3750 ng/mL; for GS-441524, 5, 37.5, 50 and 187.5 ng/mL to evaluate their repeatability (Table 4) and between-days reproducibility comparing results of three days (Table 5). Based on the repeatability test result, the precision of remdesivir was %RSD 0.9 %—2.0 %, while that of GS-441524 was %RSD 2.3 %—3.6 %. The accuracy of remdesivir was 90.5 %—106 %, while that of GS-441524 was 88.5 %—91.6 %, indicating that their reproducibility was within 100 ± 15 %. Based on the test results for between-days reproducibility, the precision of remdesivir was %RSD 0.1 %—7.2 %, while that of GS-441524 was %RSD 0.4 %—7.8 %. Additionally, the accuracy of remdesivir was 82.2 %—107 %, while that of GS-441524 was 86.7 %—92.8 %, indicating that their accuracy was within 100 ± 20 % at the LLOQ and within 100 ± 15 % in other concentration ranges.

Table 4 Repeatability of Remdesivir and GS-441524 in plasma

Compounds	QC Sample	Spiked Conc. (ng/mL)	Intra-Assay (*n*=6)		
			Average Conc. (ng/mL)	Precision %RSD	Accuracy %
Remdesivir	LLOQ	100	90.5	2.0	91
	Low	750	797	1.7	106
	Medium	1000	1045	0.9	105
	High	3750	3393	2.0	91
GS-441524	LLOQ	5	4.51	3.1	90
	Low	37.5	33.2	2.5	89
	Medium	50	45.2	2.3	90
	High	187.5	171.7	3.6	92

Table 5 Between-Days Reproducibility of Remdesivir and GS-441524 in plasma

Compounds	QC Sample	Spiked Conc. (ng/mL)	Day 1st (*n*=3)			Day 2nd (*n*=3)			Day 3rd (*n*=3)		
			Average Conc. (ng/mL)	Precision %RSD	Accuracy %	Average Conc. (ng/mL)	Precision %RSD	Accuracy %	Average Conc. (ng/mL)	Precision %RSD	Accuracy %
Remdesivir	LLOQ	100	91.6	1.1	92	82.2	4.9	82	85.1	1.9	85
	Low	750	788	1.8	105	734	1.4	98	770	0.1	103
	Medium	1000	1037	0.7	104	999	0.7	100	1018	0.6	102
	High	3750	3765	1.3	100	3441	1.3	92	3994	7.2	107
GS-441524	LLOQ	5	4.54	4.3	91	4.54	7.7	91	4.50	7.8	90
	Low	37.5	33.1	1.8	88	34.1	2.9	91	32.5	3.2	87
	Medium	50	44.8	3.2	90	44.5	2.7	89	43.8	0.4	88
	High	187.5	174.0	3.7	93	172.6	3.0	92	167.5	0.7	89

Conclusion

A system for analyzing remdesivir and GS-441524, its metabolite, by adding them to plasma was developed using the LC/MS/MS with fully automated sample preparation.
The repeatability and between-days reproducibility of remdesivir and GS-441524 were evaluated using QC samples. Good accuracy and reproducibility were obtained.

<References>
1) Richard T et.al., "Remdesivir: A Review of Its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19", ACS Cent. Sci.

First Edition: Jul. 2020

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Liquid Chromatograph Mass Spectrometer LCMS-8060NX

Application News

Highly Sensitive and Selective Method for Estimation of Formoterol at Sub-pg/mL in Human Plasma Using Shimadzu LCMS-8060NX

Avinash Gaikwad[1], Chaitanya krishna Atmakuri[1], Yogesh Arote[1], Jitendra Kelkar[2], Pratap Rasam[2]
[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., [2] Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Simple, selective and highly sensitive method with lower limit of quantification of 0.2 pg/mL
- Linear dynamic range suitable for pharmacokinetic studies, spanning from 0.2 pg/mL to 100 pg/mL
- Single-step sample extraction method that increases sample productivity

1. Introduction

Formoterol is an inhaled long-acting beta2-adrenergic receptor agonist used as a bronchodilator in the management of asthma and COPD (refer Fig.1 for structure of formoterol). It acts on bronchial smooth muscle to dilate and relax airways and is administered as a racemic mixture of its active (R;R)- and inactive (S;S)-enantiomers. A major clinical advantage of formoterol over other inhaled beta-agonists is its rapid onset of action (2-3 minutes), which is at least as fast as salbutamol, combined with a long duration of action (12 hours) . Following single/multiple dose administration of formoterol, the drug exhibits very low bioavailability and requires a highly sensitive and selective quantification of formoterol from plasma. In this work, a highly sensitive, and selective method was developed for the accurate quantification of formoterol from plasma.

We therefore developed a new LCMS method to solve problems like low bioavailability and low extraction recovery. The developed method has a high level of accuracy and can detect very low concentration of formoterol in human plasma. We focused on making the extraction process easier, improving the chromatography, and increasing sensitivity. This helped us to develop the most sensitive bioanalytical LCMS method for formoterol in human plasma.

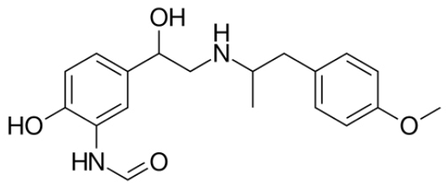

Fig. 1 Structure of Formoterol [1)]

2. Salient Features

- Quantitative method for estimation of Formoterol in human plasma was developed and partially validated as per US major Guidelines (results are presented in Table 1)
- Highly sensitive method to detect and quantify the analyte of interest at very low concentrations.
- Rapid turnaround time for sample processing and data generation.
- Exhibits a linear relationship between the analyte concentration and the analytical response.
- Straightforward sample preparation with minimal steps and handling.
- Partially validated method for quick implementation of assay

Table 1 Method Validation Summary

Calibration curve range		0.20 pg/mL to 100 pg/mL
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	113.60
	Precision (% RSD)	4.8
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	95.89 to 108.82
	Precision (% RSD)	2.10 to 4.70
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	105.43
	Precision (% RSD)	11.55
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	98.82 to 101.43
	Precision (% RSD)	5.99 to 9.82
Global % recovery	Recovery (%)	62.09
	Precision (% RSD)	5.33
Matrix effect	Mean Matrix Factor	1.01

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC- Middle Quality Control and HQC- Higher Quality Control

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3. Experimental

3.1. Sample preparation and analytical conditions

- Two hundred microliters of extraction buffer was added to plasma samples and vortexed to mix for 30 seconds.
- After vortexing the samples were processed by using solid phase extraction technique. The sample extraction protocol is mentioned below:

Extraction protocol

- Conditioning and equilibration- 1mL methanol followed by 1 mL water
- Sample loading
- Wash 1 - 1.000 mL 0.05 % ammonia in water
- Wash 2 - 1.000 mL milli Q water
- Dry the cartridges for few seconds.
- Elution - 0.200 mL of 10% acetonitrile in water
- Transfer the solution into the prelabelled HPLC vials for analysis.

3.2. Instrument parameters on LCMS-8060NX

Refer to Table 2 for analytical conditions and instrument parameters. Refer to Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack Velox™ C18 100 × 2.1 mm, 2.7 µm (P/N: 227-32009-03)
Mobile Phase	Gradient – Acetonitrile (B): 0.1% formic acid in 5 mM Ammonium Acetate (A)
Flow Rate	0.3 mL/min
Oven Temp	50 ℃
Injection	20 µL
Parameter	**MS**
Interface	ESI
Interface voltage and temp	1 kV and 300 ℃
MS Mode	MRM, Positive
Heat Block Temp	100 ℃
DL Temp	200 ℃
CID Gas	270 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of Formoterol on LCMS

Compound	MRM (*m/z*)	CE (V)
Formoterol	345.35-149.10	-15.0

Fig. 2 Nexera™ X2 with LCMS-8060NX system

4. Result and Discussion

4.1. Method Development

Mass Spectrometry Optimization Conditions:
The analysis was conducted in positive electrospray ionization (ESI) mode to achieve efficient ionization of formoterol. Various mobile phase compositions, including different concentrations of formic acid and ammonium formate, were evaluated to optimize formoterol ionization. Source parameters, such as temperature, capillary voltage, and desolvation gas flow, were optimized to maximize signal intensity and reproducibility. The protonated molecular ion [M+H]+ at m/z 345.35 was selected as the precursor for formoterol, as it provided the highest response. The most abundant and specific product ion was identified at m/z 149.1 for quantification purpose, and for formoterol D6 the MRM was set at m/z 351 > 152. The collision energy was optimized to obtain the highest signal for the selected product ions, ensuring robust and reliable quantification.

Chromatographic Conditions:
Reversed-phase high-performance liquid chromatography (RP-HPLC) was selected as the chromatographic technique, employing a Shimadzu Shim-pack Velox C18, 2.1 x 100 mm, 2.7 µm particle size for excellent peak shape and resolution. The mobile phase consisted of 0.1% formic acid in 5 mM ammonium acetate (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with a gradient elution program starting at 10% B and increasing to 90% B over 5 minutes, followed by a 2-minute hold. The flow rate was set at 0.3 mL/min, and the column temperature was maintained at 50°C to improve peak symmetry and reproducibility. The injection volume was optimized at 20 µL.

Sample preparation:
The samples were prepared using a slightly changed version of an already published method. Because we used plasma as a matrix and formoterol binds strongly to plasma proteins, we had to break this binding before extraction to get the best results. Usually, pure methanol is used for this, but because the sample sticks to the sorbent through hydrophobic interaction, using too much methanol can cause problems. So, we treated the plasma samples with 20% methanol to make the proteins settle. After spinning the samples in a centrifuge, we put the clear liquid onto a cartridge for cleaning. We found that up to 50% methanol could be used on the cartridge without losing the compounds. So, we used 50% methanol to wash away unwanted substances. Finally, we used pure acetonitrile to get the compounds of interest. This method allowed us to recover over 60% of the compounds and removed most of the unwanted substances.

4.2. Method Validation Summary

The bioanalytical method demonstrated acceptable selectivity, with no interfering peaks observed at the retention time of the analyte or the internal standard. The method was linear over the concentration range of 0.2 to 100 pg/mL, with a correlation coefficient (r2) of 0.9967. The accuracy and precision of the method were within the acceptance criteria, with intra-day accuracy ranging from 95.89% to 108.82% and intra-day precision (% CV) less than 5.0%. The global accuracy and precision were also within the acceptable limits, with values ranging from 98.82% to 101.43% and % CV less than 10.0%, respectively. The recovery of the analyte from the matrix was consistent, with an average recovery of 62.09%. The matrix effect assessment showed that the matrix did not significantly influence the analyte response, with a matrix factor of 1.04 ± 0.08. Overall, the partially validated bioanalytical method was demonstrated to be selective, linear, accurate, precise, and is suitable for the quantification of the analyte in the matrix samples

Table 4 Selectivity

Plasma lot no.	Formoterol		
	Blank Plasma	LLOQ area	% Interference
V1102	0	19,359	0.00
V8245	0	17,936	0.00
V6132	0	17,594	0.00
V11886	0	18,480	0.00
V11782	0	23,070	0.00
V11911	0	19,115	0.00

Table 5 Intra-day precision and accuracy

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (0.20 pg/mL)	0.23	113.60	4.80
LQC (1.57 pg/mL)	1.71	108.82	4.70
MQC (10.00 pg/mL)	9.59	95.89	4.20
HQC (50.00 pg/mL)	49.95	99.90	2.10

Table 6 Global precision and accuracy

Inter-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (0.20 pg/mL)	0.21	105.43	11.55
LQC (1.57 pg/mL)	1.55	98.82	9.82
MQC (10.00 pg/mL)	10.14	101.43	9.05
HQC (50.00 pg/mL)	50.62	101.24	5.99

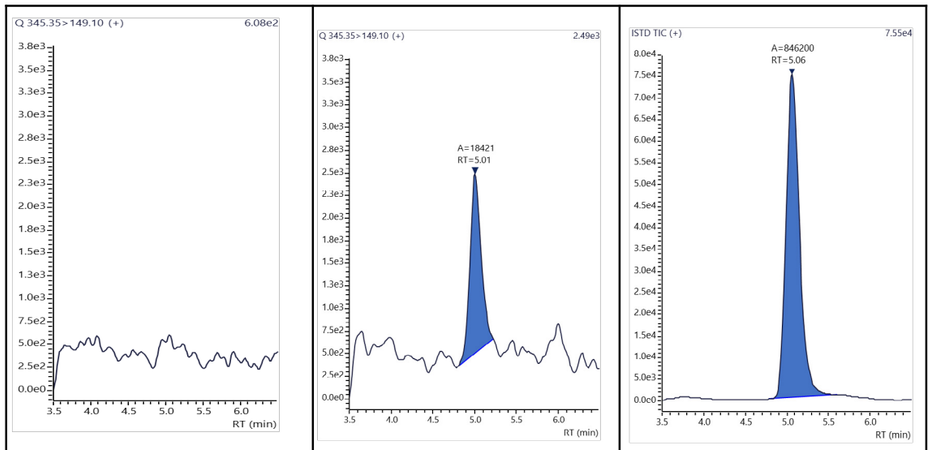

Fig. 3 Chromatograms of Formoterol (Extracted Blank, extracted LLOQ and extracted IS)

Table 7 Recovery

Sr.No.	Ext- Sample	PE-Sample	Ext- Sample	PE-Sample	Ext- Sample	PE-Sample
	LQC		MQC		HQC	
1	41,604	64,591	3,43,507	5,37,660	18,07,964	28,65,374
2	46,653	72,793	3,21,080	5,44,004	16,34,704	28,97,701
3	43,708	73,741	3,43,610	5,29,191	16,58,957	27,33,750
4	40,121	68,189	3,32,688	5,06,571	15,86,493	28,23,271
5	48,724	67,748	3,36,506	5,23,299	15,64,788	27,71,630
6	45,982	64,346	3,18,991	5,28,119	16,74,796	29,03,347
AVERAGE	44,465	68,568	3,32,730	5,28,141	16,54,617	28,32,512
STD DEV	3,251.35	3,976.64	10,707.57	12,900.07	86,079.02	69,127.51
% RSD	7.31	5.80	3.22	2.44	5.20	2.44
% Recovery	64.85		63.00		58.42	
Note: Read Ext-Sample as extracted sample and PE-Sample as post extracted sample						

Table 8 Global Recovery

QC level	Recovery
LQC (n=6)	64.85
MQC (n=6)	63.00
HQC (n=6)	58.42
Mean	**62.09**
SD	**3.31**
% RSD	**5.33**

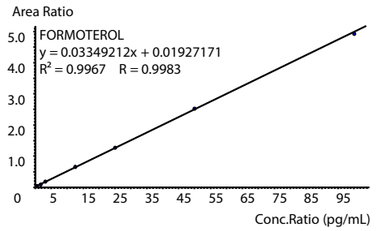

Fig. 4 Calibration curve of formoterol

Table 9 Matrix effect

Formoterol	Aqueous sample	Post extracted sample	Matrix factor	Formoterol-D6	Aqueous sample	Post extracted sample	Matrix factor	IS Normalized Matrix Effect
LQC	59,541	67,353	0.88	LQC	11,63,053	13,67,542	0.85	1.04
	52,732	73,516	0.72		11,51,676	15,03,566	0.77	0.94
	53,781	72,457	0.74		11,34,897	14,93,306	0.76	0.98
	55,868	74,502	0.75		11,24,465	15,10,743	0.74	1.01
	58,883	73,438	0.80		11,23,858	15,03,789	0.75	1.07
	60,559	68,817	0.88		11,20,498	14,70,161	0.76	1.15
Mean								**1.03**
SD								**0.08**
% RSD								**7.45**

Formoterol	Aqueous sample	Post extracted sample	Matrix factor	Formoterol-D6	Aqueous sample	Post extracted sample	Matrix factor	IS Normalized Matrix Effect
HQC	30,43,248	28,33,021	1.07	HQC	12,26,890	11,81,358	1.04	1.03
	30,52,981	29,09,235	1.05		12,33,019	12,14,213	1.02	1.03
	29,95,738	28,31,373	1.06		12,43,741	11,76,539	1.06	1.00
	30,00,218	28,51,836	1.05		12,48,614	11,61,478	1.08	0.98
	30,30,254	28,46,925	1.06		12,31,934	11,38,770	1.08	0.98
	26,20,942	30,16,549	0.87		9,95,497	10,55,653	0.94	0.92
Mean								**0.99**
SD								**0.04**
% RSD								**4.23**

5. Conclusion

In this study, a sensitive, rapid, and less plasma volume LC-MS method was developed and validated for the quantification of the formoterol in human plasma. The method utilized a simple sample preparation and provided a short chromatographic runtime, enabling efficient high-throughput analysis. The assay demonstrated excellent analytical performance characteristics, including good linearity (0.2 pg/ml to 100 pg/ml, precision, accuracy, and selectivity. The method's low LLOQ of 0.2 pg/ml and minimal matrix effects make it a valuable tool for supporting PK studies.

6. References

1) https://www.chemspider.com/Chemical-Structure.2340731.html (Accessed Feb 13,2024)

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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Selective and Sensitive Method for Estimation of Liraglutide in Human Plasma using Shimadzu LCMS-8060

Avinash Gaikwad [1], Chaitanya Krishna Atmakuri [1], Yogesh Arote [1], Pratap Rasam [2], Jitendra Kelkar [2]
[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., [2] Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Simple, novel and most sensitive method with LLOQ - 0.5 ng/mL
- Low plasma volumes in sample extraction extends the life of mass spectrometer
- Quick sample extraction method increased sample productivity

1. Introduction

Liraglutide is a glucagon-like peptide-1 receptor agonist (GLP-1 receptor agonist) also known as incretin mimetics. It works by increasing insulin release from the pancreas and decreases excessive glucagon release [(1)]. Liraglutide is a medication used for treatment of type 2 diabetes or obesity. The prolonged action of liraglutide is achieved by attaching a fatty acid molecule at one position of the GLP-1-(7-37) molecule, enabling it to both self-associate and bind to albumin with-in the subcutaneous tissue and blood stream. The active GLP-1 is then released from albumin at a slow, consistent rate. Albumin binding also results in slower degradation and reduced renal elimination compared to GLP-1-(7-37).
Following subcutaneous administration, a mean C max of 35 ng/mL was achieved after 8-12 hours of dosing with an absolute bioavailability of 55 %. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of 0.50 ng/mL.
Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over and low extraction recovery.
We have therefore developed a method with high chromatographic resolution, good sensitivity with lowest limit of quantification (LLOQ) of 0.50 ng/mL for liraglutide in human plasma using LCMS-8060. Method was developed keeping some key criteria in focus namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of liraglutide for the pharmacokinetic investigation.

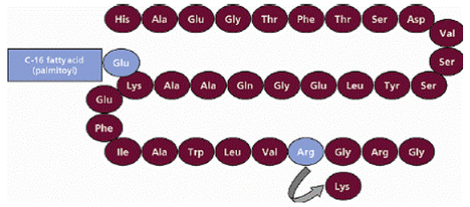

Fig. 1 Structure of Liraglutide [(2)]

2. Salient Features

- A rapid, sensitive, and high throughput method for quantification of liraglutide in human plasma
- Ready to use validated method easy to transfer to customers laboratories
- Single step SPE method increased sample throughput
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of liraglutide at 0.50 ng/mL.
- Less plasma volume (200 µL) avoided unnecessary wastage of plasma samples and at the same time increase the instrument life.

3. Method Validation

Liraglutide LCMS method was validated as per US major guidelines. All method validation parameters evaluated met the acceptance criteria. Summary of liraglutide method validation results are given in Table 1.

Table 1 Method Validation Summary

Calibration curve range		0.50 ng/mL to 202.70 ng/mL
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	102.63
	Precision (% CV)	14.52
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	96.10 to 101.01
	Precision (% CV)	2.85 to 12.79
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	109.98
	Precision (% CV)	18.52
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	102.40 to 106.78
	Precision (% CV)	7.45 to 13.09
Global % recovery	Recovery (%)	50.92
	Precision (% CV)	13.06

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC_ Middle Quality Control and HQC- Higher Quality Control

Link to Content Page →

4. Experimental

4.1. Sample preparation and analytical conditions

- Samples were processed using positive pressure solid phase extraction. 200 µL of analyte spiked in plasma samples were pre-treated with 600 µL buffer and vortexed to mix. Samples were loaded on pre-conditioned SPE cartridges, washed with organo-aqueous solution and eluted in a solution of methanol-acetonitrile. This eluted solution was injected directly on a Shimadzu LCMS-8060 system.

4.2 Instrument parameters on LCMS-8060

Refer to the Table 2 for analytical conditions and instrumental parameters. MRM transitions are described in the Table 3.

4.2 Instrument parameters on LCMS-8060

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ Velox Biphenyl 2.7 µm, 2.1 × 100 mm column (P/N: 227-32015-03)
Mobile Phase	A: 0.1% formic acid in water B: 0.1% formic acid in Acetonitrile
Flow Rate	0.25 mL/min
Oven Temp	40 ˚C
Injection volume	25 µL
Parameter	**MS**
Interface	ESI
Interface Voltage and temp	5 kV and 400 ˚C
MS Mode	MRM, Positive
Heat Block Temp	400 ˚C
DL Temp	300 ˚C
CID Gas	270 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 3 MRM transition and parameters of Liraglutide on LCMS

Compound	MRM (m/z)	CE (V)
Liraglutide	938.5-1128.4	-20.0

Fig. 2 Nexera™ X2 with LCMS-8060 system

5. Result and Discussion

5.1. Method Development

Liraglutide was dissolved in solvent consisting of 50/50 Acetonitrile/water. However poor linearity was observed in the range of 0.5 to 200 ng/mL when analyzing the working solution and we suspect that this was due to strong peptide adsorption. After enhancing the solvent strength, we got good linearity of the aqueous working solutions.

In this study, Shimadzu LCMS-8060 was selected to quantify liraglutide in human plasma. The m/z 938.5 was selected as a precursor with 4+ charge state. Many ion fragments with similar intensities were produced under a certain collision energy. Initially several possible ion fragments (m/z 1064, m/z 1129, m/z 1185 and m/z 523 were selected for investigation. .After careful MRM optimization and pre-analysis of biological samples, the transition m/z 938.5 → 1128.4 which showed high intensity and minimum interference was finally selected to quantify liraglutide in human plasma. Refer Table 3 for optimized MRM.

Optimization of chromatographic conditions was simple and focused on evaluating the composition of the mobile phase, buffer and type of analytical column.

Based on previous research experience, we found acetonitrile was more suitable than methanol as the organic phase and the presence of formic acid was beneficial for the sensitivity of liraglutide. Final mobile phase comprised of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Several HPLC columns were evaluated for better peak shape. However, Shimadzu's Shim-pack Velox Biphenyl column (100 x 2.1 mm, 2.7um pore diameter) provided minimum matrix interference and a good chromatographic resolution and sharp peak shape. The analysis time was set to 10.0 mins. Refer Fig. 3 for liraglutide extracted blank and extracted LLOQ chromatograms. Signal to noise of liraglutide at LLOQ level was found S/N > 20.

In terms of sample preparation, SPE was the only means tested. In our experience with quantitative bioanalysis of peptides in particular, the use of SPE represents the opportunity for the most selective extraction technique, the most tunable and most amenable to truly rugged methodology.

In any case, considering the emphatically polar nature of Liraglutide, it was easily predictable that any format of liquid–liquid extraction would afford negligible recovery. Protein precipitation with organic solvent may afford decent recovery, but this technique is highly unselective, only eliminating the largest proteins from the sample and leaving a myriad of potential interferences that will experience an equal concentration effect to what the analyte and internal standard experience during sample preparation

In a method where it is known that an extensive concentration of test article in a sample extract will be required in order to attain sufficient sensitivity for a given purpose, protein precipitation becomes all the less feasible in the context of matrix effect. Besides, our established LC limitation of phosphatidylcholines non-elution within a single gradient cycle meant that it would be required for the sample extraction to provide this selectivity. Selective over-retention of these interferences is readily achievable on SPE sorbents that involve reversed-phase retention and where optimization is performed fully and adequately.

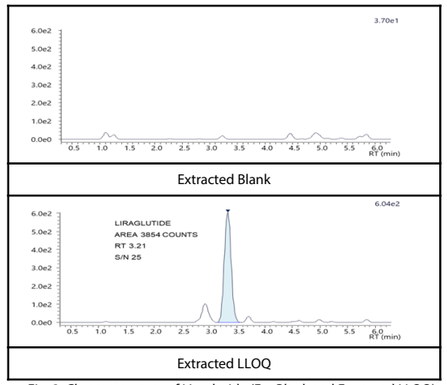

Fig. 3 Chromatograms of Liraglutide (Ext Blank and Extracted LLOQ)

5.2. Method Validation

- **Selectivity**

  Selectivity of the method was evaluated by extracting and analyzing 6 different lots of blank human plasma. Blank matrices from six different lots showed no significant interference at the retention time and MRM transition of liraglutide. Results are presented in Table 4.

Table 4 Selectivity

Plasma lot no.	Liraglutide		
	Area in blank matrix	LLOQ area	% Interference
V2533	335	1,699	19.72
V2528	169	3,597	4.70
P8072	54	2,880	1.88
V2534	510	2,842	17.95
V2537	87	2,049	4.25
P8200	189	3,961	4.77

- **Linearity**

  A linear equation was judged to produce the best fit for the concentration vs area response relationship. The regression type was 1/ Concentration^2 and peak area ration for an 8-points calibration curve was found linear from 0.5 to 202.70 ng/mL for liraglutide. The goodness of fit (r^2) was consistently greater than 0.99 during course of validation. Refer to Fig. 4 for a calibration curve.

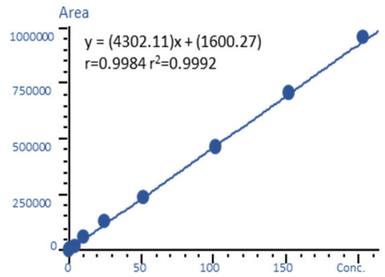

Fig. 4 Calibration curve

- **Intra-day accuracy and precision**

  Intraday accuracy and precision of liraglutide was determined for lowest limit of quantification (LLOQ QC), low (LQC), medium (MQC) and high (HQC) concentration of quality control samples in the biological matrix based on the expected range. Accuracy for intra-day was within 85-115% of the nominal value for all quality control samples except for LLOQ QC which was within 80-120%. For precision, the %CV was ≤ 15% for all quality control samples, except LLOQ QC which was ≤ 20%. Quantitative data is summarized in Table 5

Table 5 Intra-day accuracy and precision

Intra-day (n=6)			
Nominal Conc (ng/mL)	Observed Conc (ng/mL)	Accuracy (%)	Precision (%CV)
LLOQ QC (0.50 ng/mL)	0.51	102.63	14.52
LQC (1.52 ng/mL)	1.54	101.01	12.79
MQC (25.39 ng/mL)	24.40	96.10	2.85
HQC (152.03 ng/mL)	149.40	98.27	4.92

- **Global precision and accuracy**

  Inter-day accuracy and precision of liraglutide was evaluated on 3 PA batches. Accuracy for inter-day was within 85-115% of the nominal value for all quality control samples except for LLOQ QC which was within 80-120%. For precision, the %CV was ≤ 15% for all quality control samples, except LLOQ QC which was ≤ 20%. The results are presented in Table 6 for 18 QCs at each level, analyzed over 3 batches.

Table 6 Global precision and accuracy

Inter-day (n=18)			
Nominal Conc (ng/mL)	Observed Conc (ng/mL)	Accuracy (%)	Precision (%CV)
LLOQ QC (0.50 ng/mL)	0.55	109.98	18.52
LQC (1.52 ng/mL)	1.62	106.78	13.09
MQC (25.39 ng/mL)	26.00	102.40	7.45
HQC (152.03 ng/mL)	158.18	104.04	12.87

- **Recovery**

  Recovery experiments was evaluated by comparing peak area response of extracted QC samples with post extracted QC samples at three concentrations (LQC, MQC and HQC). Post extracted QC samples represent 100% recovery. The global recovery of liraglutide was 50.92%. Global recovery results are presented in Table 7. Recovery for liraglutide was found precise, consistent and reproducible at all levels.

Table 7 Recovery

QC level	Recovery
LQC (n=6)	43.24
MQC (n=6)	54.54
HQC (n=6)	54.97
Mean	**50.92**
SD	**6.65**
% CV	**13.06**

- **Matrix effect**

  Matrix factor was evaluated by comparing peak area ratio in presence of matrix ions with mean peak area ratio In absence of matrix ions.

  Un-extracted blank quality control samples were prepared from six different human blank plasma batches and processed, followed by reconstitution with aqueous LQC and HQC samples. Single injection was given from each lot. Peak area ratio in presence of matrix ions was obtained from these un-extracted LQC and HQC samples.

  The mean peak area ratio in absence of matrix ions was calculated from the results obtained from total recovery.

  Precision of LQC and HQC samples was 3.69% and 3.02% respectively, which was within the acceptance criteria of ≤15% .Representative data of matrix factor is shown in Table 8. The results confirm the suitability of the method for quantitative estimation of liraglutide in human plasma.

Link to Content Page →

Table 8 Matrix effect

Liraglutide	Aqueous sample	Post extracted sample	Matrix factor
LQC	6,217	5,983	0.96
	5,883	5,777	0.98
	5,746	5,372	0.93
	6,024	5,723	0.95
	6,343	6,232	0.98
	5,718	5,936	1.04
Mean			**0.97**
SD			**0.04**
%CV			**3.69**

Liraglutide	Aqueous sample	Post extracted sample	Matrix factor
HQC	5,43,668	5,22,379	0.96
	5,81,895	5,76,332	0.99
	6,36,429	6,06,953	0.95
	6,19,884	5,61,864	0.91
	6,03,813	5,89,440	0.98
	5,81,429	5,50,638	0.95
Mean			**0.96**
SD			**0.03**
%CV			**3.02**

- **Carry-over effect**

  Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was observed at the retention time and MRM transition of liraglutide in the extracted blank sample following the highest standard calibrator.

6. Conclusion

A Bioanalytical LCMS method has been successfully developed and validated for quantification of liraglutide in human plasma as per US major guidelines. This method is applicable for the determination of liraglutide in human plasma over the range of 0.50 to 202.70 ng/mL with a validated lower limit of quantification of 0.50ng/mL. LCMS-8060, along with special sample preparation and optimized chromatography provides a very selective and sensitive method for bioanalysis of liraglutide study samples in human plasma. Ultra-high speed and high-separation analysis was achieved on Nexera™ X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.250 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

7. References

1. https://en.wikipedia.org/wiki/Liraglutide (accessed Jan 02, 2020)
2. https://www.rxlist.com/victoza-drug.htm (accessed Jan 02, 2020)

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Liquid Chromatograph Mass Spectrometer LCMS-8045

Application News

A Fast, Simple and Sensitive Method for Estimation of Leuprolide in Human Plasma using Shimadzu LCMS-8045

Avinash Gaikwad[1], Chaitanya krishna Atmakuri[1], Yogesh Arote[1], Jitendra Kelkar[2], Pratap Rasam[2]
[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., [2] Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Rapid, simple and sensitive method with LLOQ of 25 pg/mL
- Low plasma volume helps to reduce matrix effect and increase lifespan of column and LCMS
- Single step SPE method helps boost overall productivity, saving time and reducing errors.

1. Introduction

Leuprolide acetate is a synthetic nonapeptide analog of naturally occurring gonadotropin releasing hormone (GnRH or LH-RH). The analog possesses greater potency than the natural hormone. The chemical name is 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide acetate (salt) and the structure is provided in Fig.1 [1)].

Leuprolide also known as Leuprorelin acetate, is orally inactive and generally given subcutaneously or intramuscularly. Leuprorelin may be used in the treatment of hormone-responsive cancers such as prostate cancer and breast cancer. It may also be used for estrogen-dependent conditions such as endometriosis or uterine fibroids. It may be used for precocious puberty in both males and females, and to prevent premature ovulation in cycles of controlled ovarian stimulation for in vitro fertilization. It may be used to reduce the risk of premature ovarian failure in women receiving cyclophosphamide for chemotherapy [2)].

The peptide drug is released from the depot formulations at a functionally constant daily rate for 1, 3 or 4 months, depending on the polymer type [polylactic/glycolic acid (PLGA) for a 1-month depot and polylactic acid (PLA) for depot of >2 months], with doses ranging between 3.75 and 30mg.

Mean peak plasma leuprorelin concentrations of 13.1, 20.8 to 21.8, 47.4, 54.5 and 53 µg/L occur within 1 to 3 hours of depot subcutaneous administration of 3.75, 7.5, 11.25, 15 and 30 mg, respectively, compared with 32 to 35 µg/L at 36 to 60 min after a subcutaneous injection of 1mg of a non-depot formulation. Sustained drug release from the PLGA microspheres maintains plasma concentrations between 0.4 and 1.4 µg/L over 28 days after single 3.75, 7.5 or 15mg depot injections [3)]. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of sub-picogram level as low as 400 pg/mL.

Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over, and low extraction recovery.

We have therefore developed a method with high chromatographic resolution and ample sensitivity giving lowest limit of quantification (LLOQ) of 25 pg/mL for leuprolide in human plasma using LCMS-8045 (refer Fig.2). Method was developed keeping some key criteria in focus- namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of leuprolide for the pharmacokinetic investigation

2. Salient Features

- Quantitative method for estimation of leuprolide in human plasma was developed. Method was partially validated as per US major guidelines; results are presented in table 1.
- Effective throughput for quantitative assessment is increased by use of a quick simple extraction procedure.
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of leuprolide at 25 pg/mL.
- Low plasma volume helps to reduce matrix effect and increase lifespan of column and LCMS
- Customized gradient method enhances the chromatographic resolution of leuprolide with consistent and reproducible peak area and retention time.
- Method was partially validated as per US major guidelines for
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Matrix effect

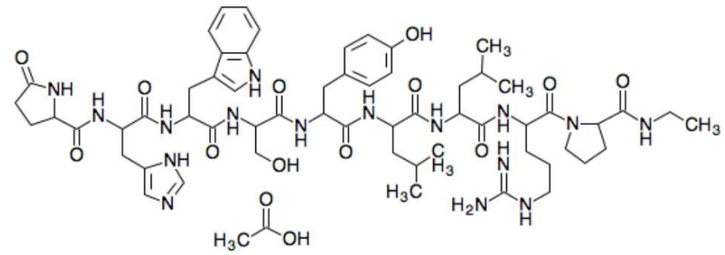

Fig.1 Structure of Leuprolide [1)]

Table 1 Partial Method Validation Summary

Calibration curve range	25.00 pg/mL to 30000 pg/mL	
Intraday precision and accuracy (For LLOQ QC)	Accuracy (%Nominal)	117.33
	Precision (%RSD)	15.50
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (%Nominal)	104.64 to 107.45
	Precision (%RSD)	1.97 to 6.08
Global precision and accuracy (For LLOQ QC)	Accuracy (%Nominal)	111.11
	Precision (% RSD)	16.13
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (%Nominal)	102.17 to 105.27
	Precision (% RSD)	3.00 to 6.37
Global % recovery	Recovery (%)	73.50
	Precision (% RSD)	5.08
Matrix effect	LQC	0.98
	HQC	0.87

Note: LLOQ QC- Lower Limit of Quantification Quality Control, LQC- Lower Quality Control, MQC- Middle Quality Control and HQC- Higher Quality Control

3. Experimental

3.1. Sample preparation and analytical conditions

- Fifty microliters of internal standard (50 ng/mL of Leuprolide-d10) was added to 200 µL of the pre-spiked calibration curve standards and quality control samples.
- To the blank and non-zero standard, 50 µL diluent was added to compensate the volume of internal standard.
- Samples were vortexed to mix and further precipitated with precipitating agent.
- Leuprolide was extracted by vortexing the samples for 5 minutes at 2000 rpm followed by centrifugation for 10 minutes at 5 °C.
- 200 µl of supernatant was transferred into autosampler vials and injected 25 µl on LCMS-8045 system

3.2. Instrument parameters on LCMS-8045

Refer to the Table 2 for analytical conditions and instrument parameters and Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ GIST C18, 3um, 2.1 × 50 mm (P/N: 227-30008-03)
Mobile Phase	Pump A-0.1% formic acid in Water Pump B-0.1 % formic acid in Acetonitrile
Flow Rate	0.6 mL
Oven Temp	40 °C
Injection volume	25 µL
Parameter	MS
Interface	ESI
Interface Voltage and temp	3 kV and 400 °C
MS Mode	MRM, Positive
Heat Block Temp	500 °C
DL Temp	350 °C
CID Gas	230 kPa
Nebulizing Gas	3 L/min
Drying Gas	10 L/min

Table 3 MRM transition and parameters of Leuprolide on LCMS

Compound	MRM (m/z)	CE (V)
Leuprolide	605.5-249.1	-35.0
Leuprolide	605.6-159.1	-35.0
Leuprolide-D10	610.6-221.1	-40.0
Leuprolide-D10	610.6-249.1	-40.0
Leuprolide-D10	610.6-159.2	-40.0
Leuprolide-D10	610.6-110.1	-40.0

Fig.2 Nexera™ X2 with LCMS-8045 system

4. Result and Discussion

4.1. Method Development

Optimization of the mass spectrometric condition

Leuprolide is an oligopeptide containing a histidine (His) and an arginine (Arg) in its structure. The presence of these two basic amino acids resulted in favorable sensitivity for leuprolide in the positive ESI ionization mode. In the positive ESI interface, both leuprolide and its IS leuprolide D10 formed predominantly doubly charged protonated molecules $[M^+2H]2^+$ at *m/z* 605.5 and m/z 610.6 in Q1 while the [M+H]+ ions at m/z 1209.9 and m/z1168.6 were less than 5% relative abundance of $[M^+2H]2^+$.The corresponding product ions was selected as the precursor ion. Leuprolide and IS both have fragment ions at *m/z* 159.1 and *m/z* 249.1. The optimal collision energy for leuprolide and IS were both set at 35 eV and 40 eV respectively.

Optimization of the sample preparation and chromatographic condition

Different extraction techniques such like SPE, LLE and PPT were tried to extract leuprolide and leuprolide D10 from human plasma. However, protein precipitation extraction technique was found to be more economic and time saving without compromising on sensitivity and recovery of leuprolide and leuprolide-d10. The extraction process includes addition of two hundred microliter of precipitating reagent to 200ul of the plasma samples, followed by vortex and centrifugation. Two hundred microliter of the supernatant was aliquoted in the autosampler vials and 25 ul of samples was injected on LCMS-8045 system.

LC conditions were optimized to maximize sensitivity, speed, and peak shape. Mobile phase composition was optimized through flow injection analysis, utilizing varying percentages of organic solvents. Comparison between acetonitrile and methanol revealed that both solvents yielded similar sensitivity, yet acetonitrile proved more effective for chromatographic separation of leuprolide and Leuprolide-d10. Additionally, the addition of acidic modifiers, such as formic acid, to the mobile phase enhanced sensitivity by facilitating analyte ionization. Consequently, Shim-pack™ C18 column was employed for the analysis. The use of Shimadzu Shim-pack C18 column achieved excellent separation for leuprolide and leuprolide-d10, allowing for a total analysis run time of 4.0 minutes under the specified chromatographic conditions on Shim-pack C18 Column.

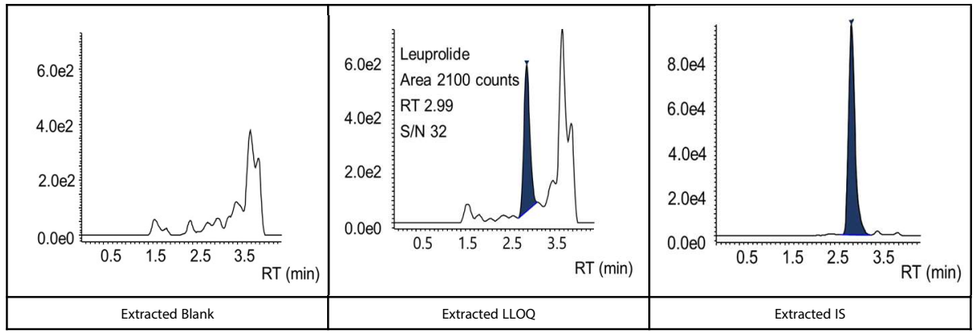

Fig.3 Extracted Chromatograms of Leuprolide Blank, LLOQ level and IS

4.2. Partial Method Validation

Assay Selectivity

Selectivity of the method was evaluated by analyzing 6 different lots of blank human plasma and blank plasma spiked with leuprolide and Leuprolide-d10. No significant interference was observed at the retention time and MRM of analyte(s) and IS (refer to the Table 4 and Fig.3).

Table 4 Selectivity

Plasma lot no.	Leuprolide		
	Blank Plasma	LLOQ area	% Interference
V3071	159	1,362	11.67
V1889	267	1,416	18.86
V1789	208	1,912	10.88
V1166	0	1,371	0.00
V3074	374	2,051	18.24
V3077	168	1,267	13.26

Plasma lot no.	Leuprolide-d10		
	Blank Plasma	LLOQ area	% Interference
V3071	205	93,692	0.22
V1889	243	86,611	0.28
V1789	638	89,217	0.72
V1166	690	89,838	0.77
V3074	574	91,397	0.63
V3077	864	89,865	0.96

Linearity of calibration curve and lower limit of quantification

The linear regression of the peak-area ratios vs. concentrations were fitted over the concentration range of 25 pg/ml to 30,000 pg/ml for leuprolide in human plasma. A typical equation of the calibration curve on a validation run was as follows: y = 0.0618078) X + (0.000289935) (r^2 = 0.9971) where y represents the peak-area ratio of analyte to IS and x represents the plasma concentration of leuprolide (refer Fig.4). Good linearity was obtained in this concentration range. The lower limit of quantification was established as 25 pg/ml for leuprolide. The precision and accuracy values corresponding to LLOQ are shown in Table 1.

Intra-day precision and accuracy

Intraday precision and accuracy were conducted using 6 replicates of LLOQ-QC, LQC, MQC and HQC over one P&A batch. Quantitative data is summarized in Table 5.

Table 5 Intra-day precision and accuracy

Intra-day (n=6)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (25.00 pg/mL)	29.33	117.33	15.5
LQC (340.00 pg/mL)	365.33	107.45	6.08
MQC (18900.00 pg/mL)	19776.33	104.64	1.97
HQC (25050.00 pg/mL)	26892.67	107.36	2.27

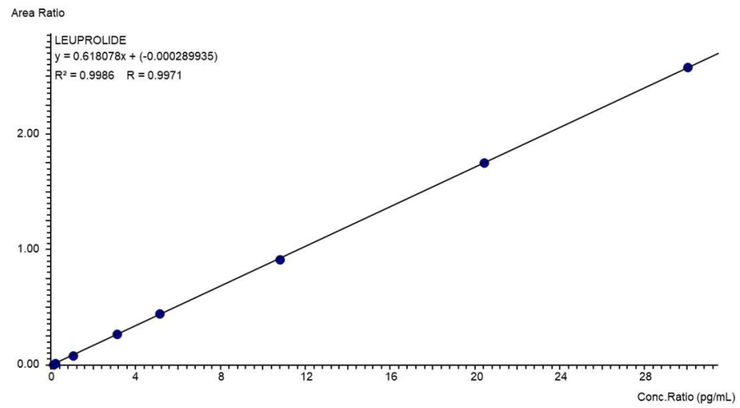

Fig.4 Calibration curve

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Global precision and accuracy

Global precision and accuracy were evaluated in 3 batches. Excellent accuracy and repeatability were observed with % RSD less than 6.37 % and percent accuracy between 102.17 % to 105.27 % for LQC, MQC and HQC level. At LLOQ QC level, the %RSD was found to be 16.13 % and percent accuracy as 111.11 %. The results are presented in Table 6. These values were within the acceptable range, and the method was thus judged to be suitably accurate and precise.

Table 6 Global precision and accuracy

Intra-day (n=18)			
Nominal Conc (pg/mL)	Observed Conc (pg/mL)	Accuracy (%)	Precision (% RSD)
LLOQ QC (25.00 pg/mL)	27.78	16.13	111.11
LQC (340.00 pg/mL)	351.61	6.37	103.42
MQC (18900.00 pg/mL)	19310.22	4.24	102.17
HQC (25050.00 pg/mL)	26370.33	3.00	105.27

Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

Extraction recovery

Recovery of leuprolide extracted from plasma were 76.97 %, 73.98 % and 69.55 % at concentrations of LQC, MQC and HQC levels (n=6) as shown in Table 7. Refer to the Table 8 for Global recovery.

Table 7 Statistics of Recovery

	LQC		MQC		HQC	
	Ext-Sample	PE-Sample	Ext-Sample	PE-Sample	Ext-Sample	PE-Sample
Recovery	6,133	7,137	2,51,587	3,42,538	4,28,310	6,10,029
	5,946	7,365	2,39,034	3,45,354	4,18,507	6,19,069
	5,785	7,679	2,61,793	3,46,481	4,42,164	6,02,507
	5,723	7,679	2,64,605	3,47,864	4,17,093	6,18,910
	5,875	8,413	2,54,268	3,45,665	4,14,249	6,23,025
	6,588	8,564	2,56,364	3,37,096	4,44,670	6,14,441
Mean	6008.3	7806.2	254608.5	344166.3	427498.8	614663.5
SD	317.7	568.7	9016	3881.4	13230.8	7438.6
% CV	5.29	7.29	3.54	1.13	3.09	1.21
% Recovery	76.97		73.98		69.55	

Note: Read Ext-Sample as extracted sample and PE-Sample as post extracted sample

Table 8 Global Recovery

QC level	Recovery
LQC (n=6)	76.97
MQC (n=6)	73.98
HQC (n=6)	69.55
Mean	**73.50**
SD	**3.73**
% RSD	**5.08**

Matrix effect

Matrix effect was studied for both leuprolide and Leuprolide-D10 internal standard using LQC and HQC samples. Mean matrix factor was found to be 0.98 and 0.87 at LQC and HQC respectively. Representative data of matrix effect is shown in Table 9. The results confirm the suitability of method for quantitative estimation of leuprolide in human plasma.

Table 9 Matrix factor

Leuprolide	Response ratio of Aqueous standard	Response ratio of Post extracted sample	Matrix factor
LQC	0.019	0.020	1.01
	0.020	0.018	0.90
	0.017	0.017	0.96
	0.019	0.018	0.95
	0.018	0.018	1.04
	0.020	0.019	0.93
Mean			**0.98**
SD			**0.05**
% RSD			**5.48**

Leuprolide	Response ratio of Aqueous standard	Response ratio of Post extracted sample	Matrix factor
HQC	1.294	1.167	0.9
	1.305	1.158	0.9
	1.364	1.156	0.8
	1.364	1.152	0.8
	1.335	1.149	0.9
	1.36	1.165	0.9
Mean			**0.87**
SD			**0.02**
% RSD			**2.66**

5. Conclusion

By optimizing the chromatographic conditions, a sensitive and rapid LC–MS/MS method for the quantification of leuprolide in human plasma was developed and partially validated. This method was sensitive enough to monitor low-dosage pharmacokinetic or depot formulation studies of leuprolide in human plasma. Compared with previously reported analytical methods, this method showed high throughput (4.0 min each sample) and greater sensitivity, with an LLOQ of 25 pg/mL. It could be applied to characterize the pharmacokinetics of leuprolide in healthy volunteers.

6. References

1) https://www.kegg.jp/entry/D00989 (accessed Jun 14, 2024).
2) https://en.wikipedia.org/wiki/Leuprorelin (accessed Jul 08, 2019).
3) Periti P, Mazzei T, Mini E. Clinical pharmacokinetics of depot leuprorelin. Clin Pharmacokinetic. 2002; 41(7):485-504.

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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Simple, Sensitive and Rapid Quantification of Teriparatide in Human Plasma by LCMS-8060

Avinash Gaikwad[1], Chaitanya krishna Atmakuri[1], Yogesh Arote[1], Jitendra Kelkar[2], Pratap Rasam[2]
[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., [2] Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Highly sensitive, validated and ready to use teriparatide LC/MS method
- Simple single step SPE method
- Linear dynamic range suitable for pK studies which ranged between 5 pg/mL to 300 pg/mL
- Consistent, reproducible, and precise recovery

1. Introduction

Teriparatide, a mid size peptide shown in Fig. 1, is a recombinant parathyroid hormone used for the treatment of osteoporosis. Daily injections of teriparatide stimulates new bone formation leading to increased bone mineral density [(1)]. The subcutaneous dose of teriparatide results in very low plasma levels characterized by rapid absorption and elimination, thus requires a highly sensitive method for estimation of analyte in human plasma. In addition, the critical challenges in method development are poor ionization, non-specific adsorption, low recovery and predominantly carryover issues.

This motivated us to develop a highly sensitive quantification method for determination of teriparatide in human plasma using Shimadzu LCMS-8060 triple quadrupole mass spectrometry coupled with Nexera™ X2 UHPLC.

Shimadzu Application Development Centre (ADC-SAIP), Mumbai has developed and validated a rapid, simple, sensitive and novel method with the lowest limit of quantification (LLOQ) of 5 pg/mL. Precision and accuracy (PA) of the analyte were evaluated at lowest limit of quantification quality control (LLOQ QC), low quality control (LQC), middle quality control (MQC) and high quality control (HQC) samples.

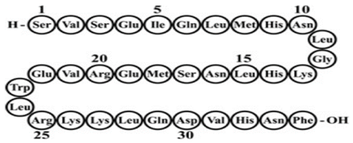

Fig.1 Structure of Teriparatide [(2)]

2. Salient Features

- A rapid, simple and sensitive method was developed for estimation of teriparatide in human plasma.
- Simple extraction procedure enhanced the selectivity of the method.
- Single step SPE method increased sample throughput.
- Heated ESI along with new UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitivity and selectivity of analyte.
- Customized gradient method satisfied the peak shape, retention time and background noise.
- Ready to use validated teriparatide method as per the US major guidelines.

Table 1 Method Validation Summary

Calibration curve range	5.00 to 300.00 pg/mL	
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (%Nominal)	105.49
	Precision (%RSD)	8.22
Intraday precision and accuracy (For LQC, MQC, HQC)	Accuracy (%Nominal)	98.33 to 111.99
	Precision (%RSD)	5.22 to 7.23
Global precision and accuracy (For LLOQ-QC)	Accuracy (%Nominal)	102.29
	Precision (%RSD)	15.12
Global precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)	93.13 to 102.17
	Precision (%RSD)	11.37 to 13.68
Global % recovery	Recovery (%)	80.37
	Precision (%RSD)	7.67
Matrix effect	Mean matrix Factor	1.03

3. Experimental

3.1. Sample preparation and analytical conditions

Teriparatide was extracted from plasma samples under basic conditions using Solid-Phase extraction technique using the protocol mentioned below:

- Conditioning and equilibration (1 mL methanol followed by 1 mL water)
- Sample loading - Wash 1 (1 mL wash solution 1 x 2 times)
- Wash 2 (1 mL wash solution 2 x 1 time)
- Elution (1 mL of elution solution)
- SPE eluent was blown under nitrogen gas and was reconstituted in 0.1 mL reconstitution solution before analysis on LC-MS/MS system

3.2. Instrument parameters on LCMS-8060

Refer to Table 2 for analytical conditions and instrument parameters and Table 3 for MRM transition.

Table 2 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack™ Velox C18 column 100 x 2.1 mm, 2.7 µm (P/N: 227-32015-03)
Mobile Phase	A: 0.1% formic acid in water B: Acetonitrile
Flow Rate	0.3 mL
Oven Temp	40 °C
Injection	20 µL

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Table 2 Analytical conditions and instrument parameters (continued)

Parameter	MS
Interface	ESI
Interface temp and Voltage	3 KV and 300 ˚C
MS Mode	MRM, Positive
Heat Block Temp	300 ˚C
DL Temp	250 ˚C
CID Gas	230
Nebulizing Gas	2
Drying Gas	10
Heating Gas	10

Table 3 MRM transition and parameters of Teriparatide on LC/MS

Compound	MRM (m/z)	CE (V)
Teriparatide	687.25-787.45	-20.2

4. Result and Discussion

4.1. Method Development

The initial LC/MS method development for teriparatide showed that the peptide obtained 5+, 6+, and 7+ charge state in MRM mode. However, 6+ charge state gave slightly higher signal in MRM mode compared to other 2 charges. The LC method development for teriparatide showed that analyte eluted off the C18 column under gradient conditions with 22 and 25% organic content. The gradient run was established with total run time of 10 min. The analyte peak eluted from the LC column at the retention time of 5.74 min. A representative chromatogram of extracted blank and LLOQ sample are shown in Fig. 2. Liquid-liquid extraction and solid phase extraction (SPE) were tried initially for extraction of teriparatide from blank plasma. However, SPE extraction method showed consistent, reproducible and precise results without any matrix effect and hence was selected as the final sample extraction method.

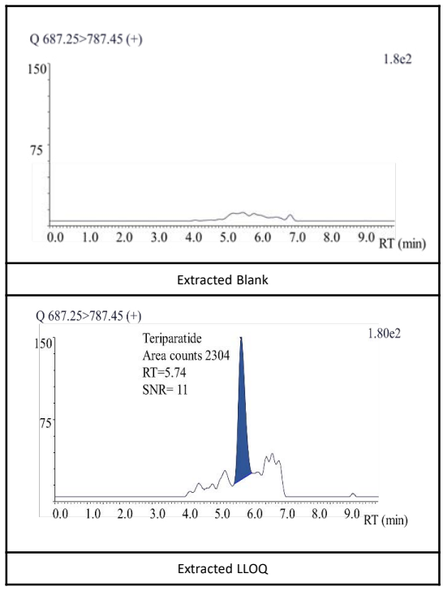

Fig. 2 Chromatograms of Teriparatide

4.2. Method Validation

Selectivity

Six blank human plasma lots were evaluated for their selectivity. No significant interference was observed at the retention time and MRM transition of analyte in any of the human blank plasma lots, refer Table 4 below.

Table 4 Selectivity

Plasma lot no.	Area in blank matrix	LLOQ area	% Interference
V 1403	80	2318	3.45
V 1744	63	3060	2.06
V 2117	79	2204	3.58
V 3509	13	1652	0.79
V 1493	66	2076	3.18
V 8076	93	1601	5.81

Linearity

Calibration curve was found linear from 5.00-300.00 pg/mL. The goodness of fit was consistently greater than 0.980 during the course of validation. Signal to noise ratio (s/n) at LLOQ level was found greater than 10:1, across 6 PA batches. Representative calibration curve is shown in Fig. 3.

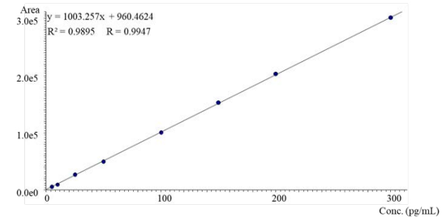

Fig. 3 Calibration curve

Intra-day and Inter-day accuracy and precision

Accuracy and precision of teriparatide was determined for LLOQ QC, LQC, MQC & HQC in biological matrix based on the expected range. Accuracy and precision batches were evaluated on intra-day and inter-day basis. Results of are shown in Table 5. Accuracy and precision results were measured at six determination per concentration at each level with four different concentration levels (LLOQ, LQC, MQC and HQC) and were found within acceptance criteria.

Table 5 Intra-day and Inter-day accuracy and precision

Intra-day (n=6)			
Nominal Conc (pg/mL)	**Observed Conc (pg/mL)**	**Accuracy (%)**	**Precision (%RSD)**
LLOQ (5.0 pg/mL)	5.27	105.49	8.22
LQC (10.0 pg/mL)	9.83	98.33	7.23
MQC (150.0pg/mL)	148.57	99.05	5.22
HQC (250.0 pg/mL)	279.98	111.99	6.28
Inter-day (n=18)			
Nominal Conc (pg/mL)	**Observed Conc (pg/mL)**	**Accuracy (%)**	**Precision (%RSD)**
LLOQ (5.0 pg/mL)	5.11	102.29	15.12
LQC (10.0 pg/mL)	9.84	98.4	13.68
MQC (150.0pg/mL)	139.69	93.13	11.37
HQC (250.0 pg/mL)	255.43	102.17	12.94

Recovery

Recovery experiment was conducted to evaluate precision, reproducibility and consistency of the analyte during extraction at LQC, MQC and HQC level. Recovery of Teriparatide was found precise, consistent, and reproducible at all levels. Global recovery was found 80.37 % refer Table 6.

Table 6 Recovery

QC level	Recovery
LQC (n=6)	85.85
MQC (n=6)	73.70
HQC (n=6)	81.55
Mean	**80.37**
SD	**6.16**
%RSD	**7.67**

Matrix effect

Matrix effect was studied at LQC and HQC levels. Mean matrix factor was found to be 1.03 at both LQC and HQC levels. Representative data of matrix effect is shown in Table 7. The results confirm the suitability of the method for quantitative estimation of Teriparatide in human plasma.

Table 7 Matrix effect

Teriparatide	Aqueous sample	Post extracted sample	Matrix factor
LQC	7,100	6,216	1.14
	5,500	5,240	1.05
	6,500	6,676	0.97
	6,418	6,435	1.00
	6,719	6,426	1.05
	6,700	6,830	0.98
Mean			1.03
SD			0.06
%CV			6.11
Teriparatide	**Aqueous sample**	**Post extracted sample**	**Matrix factor**
HQC	2,16,207	1,95,725	1.10
	1,96,279	1,86,088	1.05
	1,92,251	1,96,876	0.98
	2,14,920	1,98,680	1.08
	2,08,751	1,92,847	1.08
	1,30,259	1,50,699	0.86
Mean			1.03
SD			0.09
%CV			8.91

Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

5. Conclusion

LCMS-8060, along with special sample preparation method, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of Teriparatide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a gradient flow rate of 0.3 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

1. https://go.drugbank.com/drugs/DB06285 (accessed March 08,2022)
2. https://www.rxlist.com/forteo-drug.htm (accessed March 08, 2022)

06-SAIP-LC-056-EN First Edition: May. 2023

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Application News

High Performance Liquid Chromatograph i-Series LC-2050C

Quantification of Six Porphyrin Biomarkers in Urine Using LC-2050C with Fluorescence Detection

Devika Tupe, Bhaumik Trivedi, Purushottam Sutar, Pratap Rasam, Jitendra Kelkar
Shimadzu Analytical (India) Pvt. Ltd.

User Benefits

- A simple and robust HPLC method for the determination of porphyrins in urine
- No sample pre-treatment is required.

■ Introduction

Porphyria are a group of seven metabolic disorders of haem biosynthesis. These include aminolevulinic acid dehydratase porphyria (ADP), acute intermittent porphyria (AIP), variegate porphyria (VP), hereditary coproporphyria (HCP), porphyria cutanea tarda (PCT), erythropoietic protoporphyria (EPP) and congenital erythropoietic porphyria (CEP). People with certain types of porphyria can have a sudden life-threatening crisis. These attacks are rare and difficult to diagnose because they are non-specific. In many cases, the disease is misdiagnosed because of the rarity of some types of porphyria. For early diagnosis and identification of the type of porphyria, accurate quantification of porphyrins in urine and faeces is necessary.

This application note describes an HPLC method with fluorescence detection for the quantification of six porphyrin biomarkers; namely, uroporphyrin I, heptacarboxyporphyrin I, hexacarboxyporphyrin I, pentacarboxyporphyrin I, copro-porphyrin I and coproporphyrin III in urine.

■ Experimental

A commercial lyophilized porphyrin standards kit was purchased from RECIPE Chemicals+Instruments GmBH (Munich, Germany). The kit contains a urine calibrator mixture of uroporphyrin I, heptacarboxyporphyrin I, hexa-carboxyporphyrin I, pentacarboxyporphyrin I, copro-porphyrin I and coproporphyrin III of known concentration; urine control level I and level II. The calibrator and controls were reconstituted in 5 mL of Milli Q water.

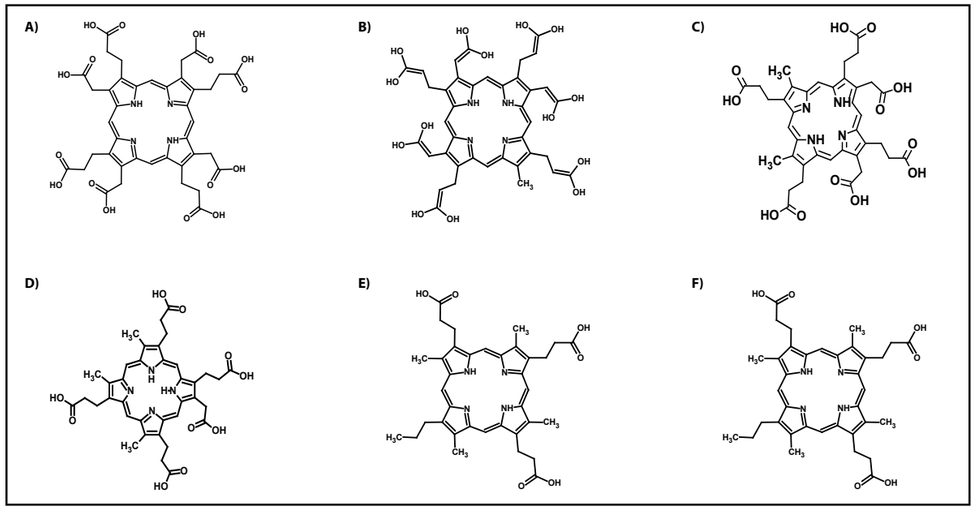

Figure 1 Structure of porphyrins - A) Uroporphyrin I, B) Heptacarboxyporphyrin I, C) Hexacarboxyporphyrin I, D) Pentacarboxyporphyrin I, E) Coproporphyrin I, and F) Coproporphyrin III.

Structurally, porphyrin consists of four pyrrole rings (five-membered closed structures containing one nitrogen and four carbon atoms) linked to each other by methine groups (—CH=). Figure 1 shows the structures of porphyrins. All porphyrin compounds absorb light intensely at or close to 410 nm. Although various methods have been developed for the analysis of urine porphyrins, the property of porphyrins to exhibit florescence at a specific wavelength make reverse-phase high-pressure liquid chromatography (HPLC) coupled with fluorescence detection the gold standard method for this purpose.

Further dilutions for the calibrator were made using mobile phase as diluent under dark conditions in amber vials, while the controls were injected without dilution. The samples were analyzed using Shimadzu i-series LC-2050C HPLC system coupled with RF-20 AXS fluorescence detector shown in Figure 2 using the analytical conditions given in Table 1.

■ Linearity of the Porphyrins

Six-point calibration curve for the porphyrin calibrator was prepared by serially diluting the calibrator mixture 5 times in diluent.

Figure 2 LC-2050C coupled with RF-20Axs detector

Table 1 Analytical conditions

HPLC system	: LC-2050 C
Column	: Shim-pack Scepter™ C18-120 (250 mm x 4.6 mm, 5 µ) (P/N :227-31020-06)
Column oven temp.	: 25 ˚C
Autosampler temp.	: 4 ˚C
Mobile phases	: A- 10 mM Ammonium acetate in water (pH: 5.6) B- Acetonitrile
Flow rate	: 1.0 mL/min
Gradient program (B%)	: 0 - 17 min → 0 - 45 (%) 17 - 17.1 min → 45 - 10 (%) 25 min → STOP
Detector	: RF-20 Axs (fluorescence detector)
Wavelength	: Excitation - 395 nm Emission - 630 nm
Injection volume	: 20 µl
Diluent	: Mobile phases A / B = 90 / 10 (v/v)

A representative chromatogram of porphyrin calibrator is shown in Figure 3. The Figures 4-A to 4-F depict the calibration curve and level 1 (L1) calibrator chromatograms for uroporphyrin I, heptacarboxyporphyrin I, hexacarboxy-porphyrin I, pentacarboxyporphyrin I, coproporphyrin I and coproporphyrin III, respectively.

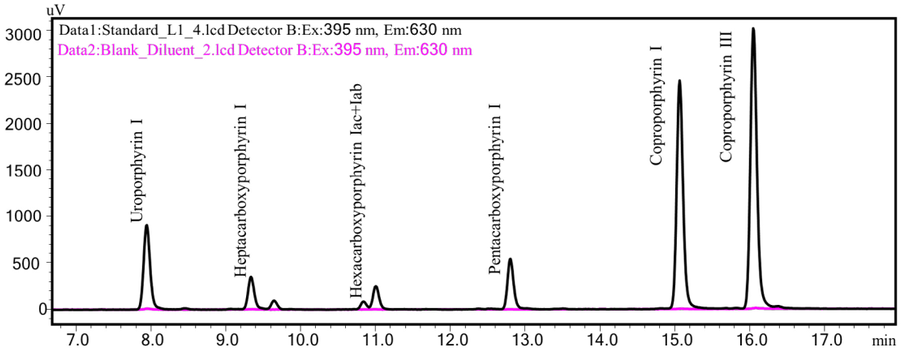

Figure 3 Representative chromatogram of Porphyrin Calibrator

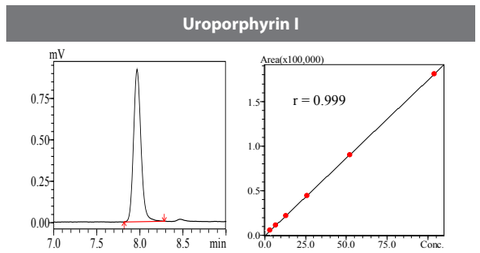

Figure 4-A L1 calibrator chromatogram and calibration curve for uroporphyrin

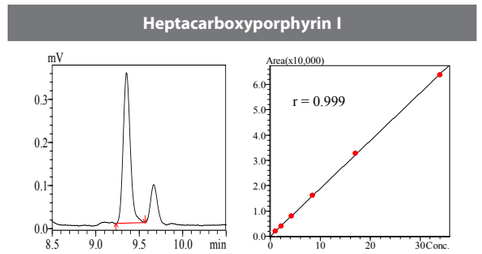

Figure 4-B L 1 calibrator chromatogram and calibration curve for heptacarboxyporphyrin I

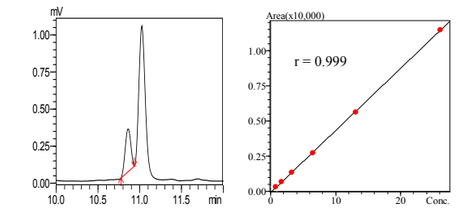

Figure 4-C L1 calibrator chromatogram and calibration curve for hexacarboxyporphyrin I

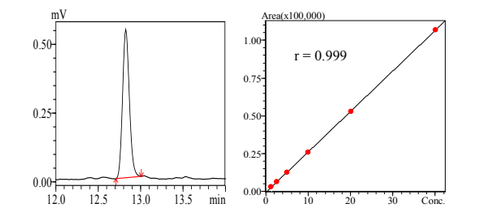

Figure 4-D L1 calibrator chromatogram and calibration curve for pentacarboxyporphyrin I

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Coproporphyrin I

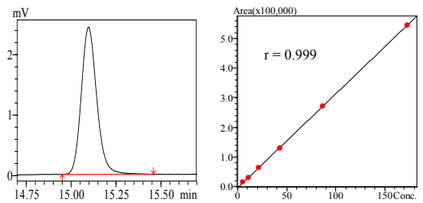

Figure 4-E L1 calibrator chromatogram and calibration curve for coproporphyrin I

Coproporphyrin III

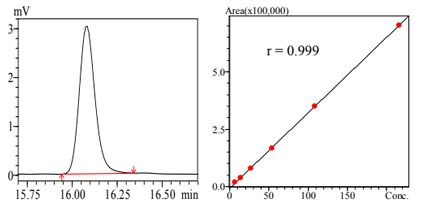

Figure 4-F L1 calibrator chromatogram and calibration curve for coproporphyrin III

Table 2 Concentration and accuracy results of porphyrins (n=3)

	Uroporphyrin I			Heptacarboxyporphyrin I			Hexacarboxyporphyrin I		
Calibration Level	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy [%]
L1	3.25	3.50	108	1.0625	1.00	94	0.82	0.95	117
L2	6.5	6.69	103	2.125	2.07	98	1.63	1.71	105
L3	13	12.95	100	4.25	4.19	99	3.26	3.26	100
L4	26	25.59	98	8.5	8.46	100	6.53	6.35	97
L5	52	51.84	100	17	17.38	102	13.05	12.92	99
L6	104	104.17	100	34	33.83	100	26.10	26.20	100
	Pentacarboxyporphyrin I			**Coproporphyrin I**			**Coproporphyrin III**		
Calibration Level	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy	Expected conc. (µg/L)	Observed conc. (µg/L)	Accuracy [%]
L1	1.25	1.39	111	5.41	6.07	112	6.75	7.82	116
L2	2.51	2.58	103	10.81	11.08	102	13.50	13.91	103
L3	5.01	4.97	99	21.63	21.19	98	27.00	26.41	98
L4	10.03	9.85	98	43.25	42.31	98	54.00	52.56	97
L5	20.05	19.98	100	86.50	86.88	100	108.00	108.31	100
L6	40.10	40.17	100	173.00	173.06	100	216.00	216.22	100

Table 3 Summary result of urine control I (C1) and urine control II (C2) analysis (n=6)

Compound	Observed Conc. (µg/L)		Expected Conc. (µg/L)		Control range (µg/L)		Accuracy[%]		% RSD of Retention Time		% RSD of Conc.	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
Uroporphyrin I	16.03	190.38	16.70	194.00	12.5 to 20.9	155 to 233	96	98	0.00	0.05	0.11	0.12
Heptacarboxyporphyrin I	5.98	56.44	6.36	56.10	4.46 to 8.27	44.9 to 67.3	94	101	0.04	0.07	0.17	0.09
Hexacarboxyporphyrin I	5.03	44.52	4.67	45.10	3.27 to 6.07	36.0 to 54.1	108	99	0.06	0.04	0.62	0.52
Pentacarboxyporphyrin I	7.41	59.13	6.94	58.90	4.86 to 9.03	47.1 to 70.6	107	100	0.04	0.06	0.09	0.05
Coproporphyrin I	35.86	295.80	34.70	295.00	27.7 to 41.6	236 to 354	103	100	0.03	0.03	0.84	0.06
Coproporphyrin III	75.41	358.78	71.90	356.00	57.5 to 86.3	285 to 427	105	101	0.04	0.03	0.08	0.08

■ Results

Linearity:
The summary results for concentration and accuracy of porphyrins in urine calibrator are given in Table 2. Good linear correlations along with correlation coefficients greater than 0.999 were acquired for all porphyrins as shown in Figures 4-A to 4-F.

Obtained Controls:
Urine control level I (C1) and level II (C2) were analyzed. The summary of results of urine controls are given in Table 3. The retention time %RSD for the six porphyrins was between 0.00 to 0.07 and the concentration %RSD was between 0.05 to 0.84. The measured concentrations of both control samples were well within the control range specified.

■ Conclusion

- A home brew, simple and low-cost method for analysing urinary six porphyrins was developed on Shimadzu i-series LC-2050C HPLC system coupled with RF-20 AXS fluorescence detector which needs no sample preparation.
- Ease of operation, no sample preparation, rugged technology, reduced cost of analysis, unambiguous outcome and overall performance, will, definitely, appeal to industries for reduction of time and cost merit.
- This method may also assist in the differential analysis of various porphyria.

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Revision A: Jul. 2024

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High Performance Liquid Chromatography Nexera™FV

Application News

On-line Dissolution Test of Loxoprofen Sodium Tablets

Ayano Tanabe, Kana Matsuoka

User Benefits

- On-line dissolution test can significantly reduce the man-hours required from test solution sampling to HPLC analysis.
- Fraction analysis mode provides sampling of test solution at minimum 5-minute intervals and is expected to save labor and prevent human error.
- Direct injection analysis mode doesn't require fractionation vials, resulting in cost reduction.

■ Introduction

Disintegration tests and dissolution tests are specified by the Japanese Pharmacopoeia (JP) and the U.S. Pharmacopeia (USP). Nexera FV is an on-line HPLC system for automated dissolution testing of drug products. By connecting to a dissolution tester, the system can provide tablet loading, analysis of the test solution at each sampling time, calculation of dissolution rate, and preparation of report output automatically. This system eliminates human error and realizes labor-saving and efficient dissolution testing.

This article reports the results of an on-line dissolution test of loxoprofen sodium tablets using Nexera FV.

■ On-line dissolution testing with Nexera FV

A sampling device for dissolution tester and a filtration device are required to build up an on-line dissolution testing system. Test solution sampled from the dissolution tester is filtered by the filtration device and introduced directly into a flow vial (see Fig. 2) contained in the autosampler of Nexera FV. Analysis is started by injecting the test solution into the HPLC flow path, following the aspiration of it in the flow vial.

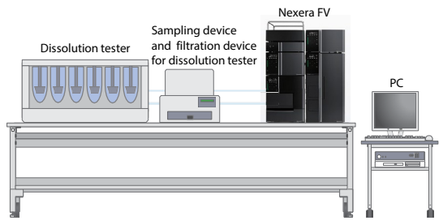


Fig.1 On-line dissolution test system setup using Nexera FV

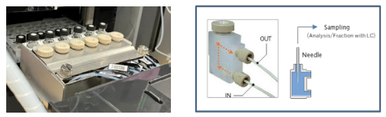


Fig.2 Flow vial

Nexera FV offers two different analysis modes: fraction analysis mode and direct injection analysis mode.

In fraction analysis mode, the test solution from the dissolution tester is once fractionated into vials or microtiter plate, and sampling intervals as short as 5 minutes can be supported. LC analyses can be performed collectively after the dissolution testing is completed or inserted between sampling intervals. An automatic dilution and an automatic addition of internal standard can be supported in this mode as well.

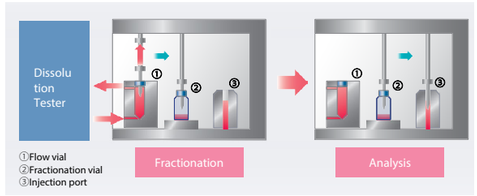


Fig.3 Fraction analysis mode

In direct injection analysis mode, the test solution from the dissolution tester is injected directly from a flow vial into the HPLC. After the test solution in first flow vial has been analyzed, the test solution in the second flow vial is subjected to HPLC analysis. This mode is useful when the sampling interval is large enough.

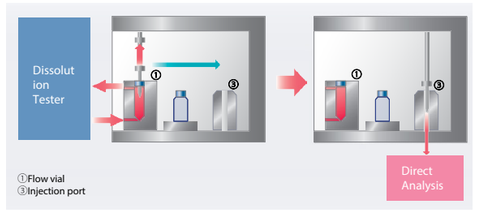


Fig.4 Direct injection analysis mode

■ On-line dissolution test of loxoprofen sodium tablets

On-line dissolution test of loxoprofen sodium tablets was performed. The HPLC conditions were optimized based on those described in the Japanese Pharmacopoeia 18th Edition as determination method for "Loxoprofen Sodium Tablets" to allow ultra high-speed analysis, which provides reductions of the retention time to 1 minute and the analysis time to 1.5 minutes. Table 1 shows the dissolution test conditions and Table 2 shows the HPLC analytical conditions.

Table 1 Dissolution test conditions

System	:	NTR-6600AST (TOYAMA SANGYO CO., LTD.)
Dissolution method	:	Paddle
Dissolution media		Water
Media volume	:	900 mL
Rotation speed	:	50 rpm
Bath temperature	:	37 ℃
Total time	:	30 min
Sampling time	:	5、10、15、20、25 and 30 min

Table 2 HPLC analytical conditions

Column	:	Shim-pack™ XR-ODS Ⅱ *1 (75 mm×3.0 mm I.D., 2.2 μm)
Mobile phase	:	Methanol/Water/Acetic Acid/Triethylamine =600 : 400 : 1 : 1
Flow rate	:	0.8 mL/min
Column temp.	:	40 ℃
Injection vol.	:	2 μL
Detection	:	UV 222 nm

*1 P/N：228-41624-91

●Analysis of standard solution

Using a 1.5 mL ordinary vial, six repeated analyses of a standard solution of loxoprofen sodium (60 mg/L) were performed. Fig. 5 shows the chromatograms and Table 3 shows the results. Good results of around 0.05% RSD for both retention time and peak area values were obtained.

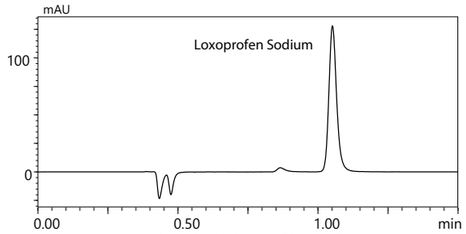


Fig.5 Chromatogram of standard solution of loxoprofen sodium (60 mg/L)

Table 3 Retention Time repeatability and peak area repeatability for a standard solution (60 mg/L、n=6)

	Retention time (min)	Area
1st	1.051	248,209
2nd	1.052	248,265
3rd	1.053	248,129
4th	1.052	248,232
5th	1.052	248,380
6th	1.051	248,038
Averages	1.052	248,209
%RSD	0.07	0.05

●Fraction analysis mode

The test solutions were sampled six times at 5, 10, 15, 20, 25, and 30 minutes. Fig. 6 shows the chromatogram of the test solution loxoprofen sodium tablet (68.1 mg per tablet indicated, dissolution time; 30 minutes).

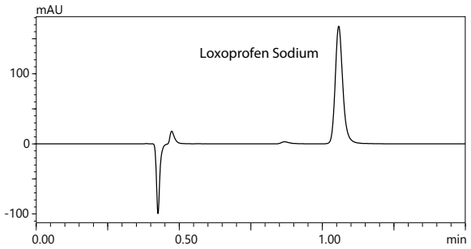


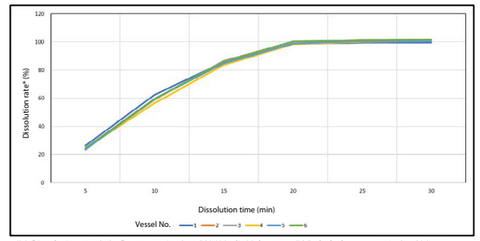
Fig.6 Chromatogram of test solution of loxoprofen sodium tablet (dissolution time; 30 min)

Table 4 shows the time-dependent variation of the dissolution rate in each vessel, and Fig. 7 shows the dissolution curve of loxoprofen sodium tablets.

The Japanese Pharmacopoeia, as ascribed, specifies that the dissolution rate of loxoprofen sodium tablets must be at least 85% in 30 minutes, and the results obtained from this study met this criterion.

Table 4 Time-dependent variation of dissolution rate in each vessel (%)

Time (min) / Vessel No.	5	10	15	20	25	30
1	26.28	62.34	85.67	98.45	99.53	99.49
2	23.88	59.71	84.98	99.07	101.08	100.89
3	23.90	58.78	86.68	99.80	101.27	101.18
4	24.33	56.44	83.55	98.18	100.25	100.64
5	23.54	59.62	84.81	98.77	100.54	100.61
6	24.77	59.54	85.97	100.41	101.15	101.59



*：Dissolution rate(%)=Concentration(mg/L)×Media Volume 0.9(L)/Labeled amount 68.1(mg)×100

Fig.7 Dissolution curves

●Direct injection analysis mode

Then, on-line dissolution test in direct injection analysis mode was performed. The test solutions from six vessels were sampled at 30 minutes only. Table 5 shows the dissolution rate in each vessel. In direct injection analysis mode, each dissolution rate 30 minutes was over 85%, which met the criterion as well.

Table 5 Dissolution rate (%) in each vessel

Time(min) / Vessel No.	30
1	103.49
2	101.67
3	100.40
4	100.29
5	102.42
6	102.32

■ Conclusion

In this article, an on-line dissolution test of loxoprofen sodium tablets using Nexera FV has been reported. The fraction analysis mode allows 5-minute sampling interval. The direct injection analysis mode doesn't require preparing vials for fractionation resulting in cost reduction. Thus, Nexera FV is an on-line HPLC system setup that can provide automated and efficient dissolution testing for drug products.

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Application News

Ultra High Performance Liquid Chromatograph Nexera™ FV

USP-Compliant Online Dissolution Testing of Antipyretic Analgesic: Automatic Addition of Internal Standard

K. Matsuoka, A. Tanabe

User Benefits

- Dissolution testing can be automated.
- The automatic sampling function enables testing with good reproducibility.
- The automatic dilution and the automatic addition of internal standard improve work efficiency.

■ Introduction

Dissolution testing is conducted for formulation development, quality control, bioequivalence tests of generic drugs, etc.. In dissolution testing, the dissolution properties of a drug product are checked under specific conditions for certain periods. Dissolution testing takes a lot of labor and time, since dissolution media must be sampled from multiple vessels every sampling time and analyzed.

Nexera FV is an HPLC system for online dissolution testing. It can automate the processes from sampling dissolution media, HPLC analysis, up to report output. By automating tasks that used to be conducted manually by operators, the system realizes labor saving and throughput improvement. In addition, automating tasks prevent human errors from whole processes.

This article introduces an example of automatic addition of the internal standard (ISTD) to the dissolution medium and a USP-compliant online dissolution testing of an antipyretic analgesic tablet using the Nexera FV. For additional information on online dissolution testing, please refer to Application News 01-00029 and 01-00031.

■ Online Dissolution Testing with Nexera FV

Fig. 1 shows a comparative example of the workflow of conventional method and online dissolution testing with Nexera FV. The Nexera FV system can automate sampling of dissolution medium at designated times, filtration, dilution, addition of internal standards, HPLC analysis, and report generation processes which had been conducted manually.

Furthermore, the HPLC analytical conditions can be set easily by the dedicated software DT-Solution (Fig. 2), and a report summarizing multiple data such as the dissolution rate is prepared simultaneously with completion of the analysis (Fig. 3).

Nexera FV has two modes of analysis: direct injection mode to inject the dissolution media delivered from the dissolution tester directly to the HPLC, and fraction analysis mode to fractionate the dissolution media into vials and analyze them. The former is effective when an analysis is enough short to complete by the next sampling time. The latter is used in tests with short sampling intervals, and in cases where dilution or addition of internal standards is required.

As described in this article, use of the Nexera FV fraction analysis mode to automatically add the ISTD made it possible to achieve high efficiency in work that had been done manually in the conventional process.

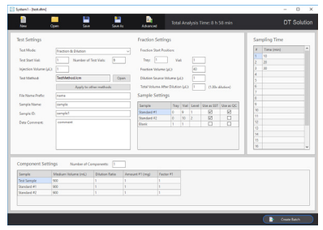

Fig. 2 DT-Solution Setting Window

Fig. 3 Multi-Data Report*1 Windows

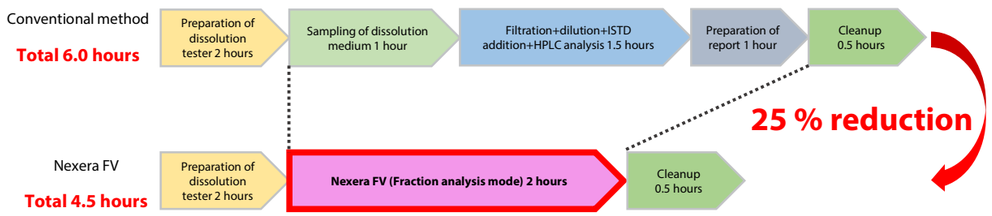

Fig. 1 Comparison of Workflow of Online Dissolution Testing*2

*1 Multi-Data Report is an optional function of LabSolutions™DB/CS to generate reports automatically.
*2 The time above is an example of an antipyretic analgesic with a dissolution test of 1 hour and an HPLC analysis of 0.5 hours (6 analyses of 6 minutes each).

■ Addition of ISTD Using Sample Preparation Function

In this article, the acetaminophen, aspirin, and caffeine in an antipyretic analgesic tablet were determined using the internal standard method. The sample preparation function of a Shimadzu SIL-30ACFV autosampler was used to add the ISTD to the standard sample and the fractions of dissolution medium. Fig. 5 shows the flow of ISTD addition using the autosampler, and Table 1 shows the sample preparation program. Fig. 4 shows an example of the vial arrangement in the autosampler when the sample preparation program in Table 1 is carried out. The ISTD vial (❶) is set in the control vial rack, and the flow vials which receive the dissolution media from the dissolution tester are set in the flow vial rack before starting the test. As dissolution medium fractionation vials (❷) and dilution/ISTD addition vials (❸), empty vials are set at the positions shown in Fig. 4. When the test is started and the specified time is reached, (1) the dissolution media from the dissolution tester are pumped to the flow vials, and (2) the dissolution media are fractionated to the dissolution medium fractionation vials (❷) by the autosampler. Following this, (3) the specified amounts of the ISTD in the ISTD vial (❶) and the fractionated dissolution medium in the fractionation vials (❷) are suctioned with a needle, and are then discharged together with the diluent into the dilution/ISTD addition vials (❸) and mixed. Finally, (4) 10 µL of the dissolution medium with addition of the prepared diluted ISTD by (3) is injected from the dilution/ISTD addition vials (❸) into the injection port (❹). These procedures of (❸),(❹) are carried out for all fractions. The ISTD used here was a methanol solution (before addition 3600 mg/L) of benzoic acid, and the diluent was methanol/glacial acetic acid = 95 : 5.

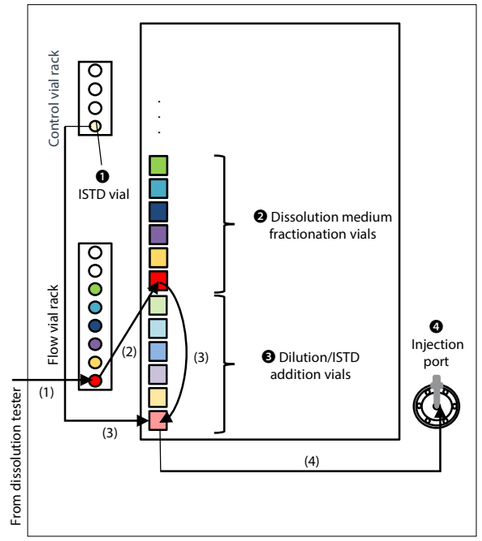

Fig. 4 Example of Vial Arrangements in Autosampler

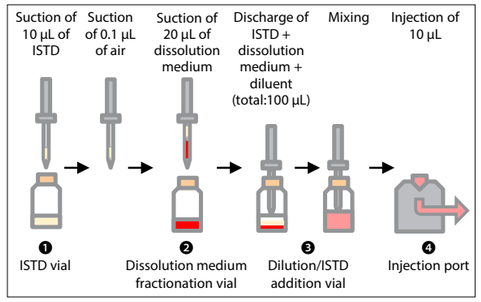

Fig. 5 Flow of Internal Standard (ISTD) Addition by Autosampler

Table 1 Sample Preparation Program

1	n.drain	13	n.strk ns
2	disp 600,rs	14	disp 100,ss
3	vial.n 0,9	15	mix 3,5,45,2,10
4	n.strk 52	16	n.drain
5	aspir 10,ss	17	disp 600,rs
6	air.a 0.1,ss	18	d.rinse
7	d.rinse	19	inj.p
8	a0=sn+6	20	v.inj
9	vial.n 1,a0	21	wait 2.0
10	n.strk ns	22	goto f0
11	aspir 20,ss	23	end
12	vial.n rn,sn		

■ System Suitability Test

Table 2 shows the analytical conditions, and Fig. 6 shows the chromatogram of the mixed standard sample of acetaminophen, acetylsalicylic acid, and caffeine. It is thought that the sample also contained a small amount of salicylic acid which was formed by hydrolysis of the acetylsalicylic acid, and this appeared as a peak.

Table 3 shows the result of the system suitability test. The test conditions are described in the United States Pharmacopeia USP43-NF38 Acetaminophen, Aspirin, and Caffeine Tablets DISSOLUTION[1]. Table 4 shows the requirements of the system suitability test in USP43-NF38. System suitability was confirmed by the repeated analyses (n=6) of a 100 mg/L solution under the conditions shown in Table 2. Both the performance and the reproducibility of the system satisfied the requirements of USP43-NF38.

Table 2 HPLC Conditions

Column	Shim-pack™ Scepter C18-120*1 (100 mm × 4.6 mm I.D., 5 µm)
Mobile phase	Methanol / Glacial Acetic Acid / Water = 28 : 3 : 69
Flow rate	2 mL/min
Column temp.	45 °C
Injection vol.	10 µL
Vial	Shimadzu Vial, LC, 1.1 mL, Glass*2 Shimadzu Vial, LC *3
Detection	UV 275 nm

*1 P/N : 227-31020-04 *2 P/N : 228-21283-91
*3 P/N : 228-31600-91

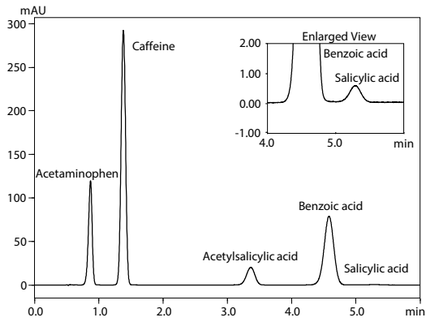

Fig. 6 Chromatograms of Mixed Standard (each 100 mg/L) of Acetaminophen, Acetylsalicylic Acid and Caffeine

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Table 3 Results of System Suitability Test (each 100 mg/L)

Suitability requirements		Target Compound			Internal Standard	Judgement
		Acetaminophen	Caffeine	Acetylsalicylic acid	Benzoic Acid	
Resolution (n=6)	≥1.4	19.32	15.73	4.66		PASSED
Tailing factor (n=6)	≤1.2	0.87	0.98	0.94	0.97	PASSED
Retention Time (%RSD)	≤2.0	0.08	0.07	0.28	0.17	PASSED
Area (%RSD)	≤2.0	0.36	0.38	0.35	0.44	PASSED

Table 4 Requirements of System Suitability Test (USP43-NF38)

Resolution	≥1.4 between any of the analyte and ISTD
Tailing factor	≤1.2 for each analyte peak
Relative standard deviation	≤2.0 %

■ Dissolution Test

Fig. 7 shows the chromatogram of the dissolution medium from the commercially-available antipyretic analgesic tablet. Table 5 shows the dissolution conditions. The HPLC analytical conditions are the same as that shown in Table 2. The dissolution media were sampled automatically and filtered at the specified time, the ISTD was added automatically by using the Nexera FV, and then analyzed.

Table 6 shows the dissolution rates of each component at a dissolution time of 60 min. USP43-NF38 Acetaminophen, Aspirin, and Caffeine Tablets DISSOLUTION requires dissolution of 75 % of the label contents of each of the three components acetaminophen, acetylsalicylic acid, and caffeine within the dissolution time of 60 min. In this test, the results were within the tolerance required in the USP, as dissolution rates of 75 % or more were achieved in the dissolution time of 60 min with all components and all vessels.

Table 5 Dissolution Conditions

System	NTR-6600AST (TOYAMA SANGYO CO., LTD.)
Dissolution method	Paddle
Dissolution media	Pure water
Media volume	900 mL
Rotation speed	100 rpm
Bath temperature	37 ˚C
Total time	60 min
Sampling time	60 min

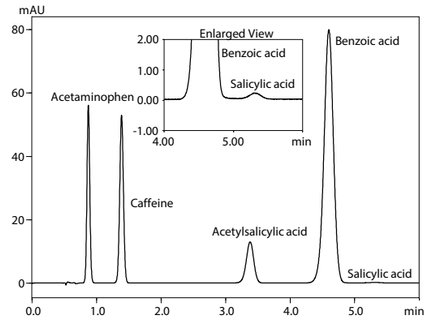

Fig. 7 Chromatograms of Antipyretic Analgesic (Dissolution Time : 60 min)

Table 6 Dissolution Rates* in Dissolution Test of Antipyretic Analgesic Tablets (Dissolution Time : 60 min, %)

Compound / Vessel No.	Acetaminophen	Caffeine	Acetylsalicylic acid
1	109.3	123.1	104.6
2	106.4	121.5	109.7
3	107.7	122.9	105.6
4	108.9	119.3	103.7
5	113.2	131.8	102.8
6	110.7	121.2	103.2
Judgement	PASSED	PASSED	PASSED

* Dissolution rate (%) = Concentration (mg/L) × Media volume 0.9 (L) / Labeled amount (mg) × 100

■ Conclusion

A USP-compliant online dissolution test was carried out using a commercially-available antipyretic analgesic tablet containing acetaminophen, acetylsalicylic acid, and caffeine. In comparison with the conventional method, a large reduction in work time and increase in work efficiency were achieved by automatic dilution and addition of the internal standard using the Nexera FV. As a result, it was found that the time required for the total process can be reduced by approximately 25 %.

<Reference>
(1) USP43-NF38-49 “Acetaminophen, Aspirin, and Caffeine Tablets DISSOLUTION”

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Application News

Gas Chromatograph HS-20 NX USTL/Brevis™ GC-2050

Efficient Analysis of Residual Solvents in Pharmaceuticals Using the Compact Model, Brevis GC-2050 (1)
—JP18 and USP467, Water-Soluble Samples—

Yui Higashi, Eisuke Kobayashi

User Benefits

- The slim and compact design of the Brevis GC-2050 enables the expansion of the number of operational units in the laboratory, allowing for efficient high-throughput analysis.
- Brevis GC-2050 can perform analysis using alternative carrier gases.
- Analysis can be performed with *tert*-butyl alcohol and cyclopentyl methyl ether, which have been newly added as Class 2 solvents in ICH Q3C (R8).

■ Introduction

Residual solvents in pharmaceuticals are defined as organic volatile chemical substances that are used or produced in the manufacture of drug substances or additives or in the preparation of drug products. In the Japanese Pharmacopoeia 18th Edition (JP18) or the United States Pharmacopeia (USP) General Chapters <467> Residual Solvents, residual solvents are classified as Class 1 to 3, according to their risk to human health, and the headspace GC method is mainly used to analyze them. The carrier gas that is normally used is He. However, He supply shortages have become an issue recently, so there is a demand to perform analysis using alternative carrier gases, such as H_2 or N_2.

This article introduces the results of analysis of Class 1 and 2 water-soluble samples using the compact design Brevis GC-2050. For the Procedure A, H_2 and N_2 in addition to He were used. When using an alternative carrier gas, it is necessary to first verify the operation based on USP General Chapter <1467>.

Fig. 1 HS-20 NX USTL (Ultra Short Transfer Line) + Brevis™ GC-2050

■ Instrument Configuration and Analytical Conditions

Table 1 Analytical Conditions of Water-Soluble Samples

GC Analytical Conditions (Procedure A and B)		
Model	:	Brevis GC-2050
Detector	:	FID (Flame Ionization Detector)
Column	:	A) SH-I-624Sil MS (0.32 mm I.D. × 30 m, d.f. = 1.8 µm) B) SH-PolarWax (0.32 mm I.D. × 30 m, d.f. = 0.25 µm)
Column Temp.	:	A) 40 °C (20 min) – 10 °C/min – 240 °C (20 min) Total 60 mins B) 50 °C (20 min) – 6 °C/min – 165 °C (20 min) Total 59.17 mins
Injection Mode	:	A) Split 1:5 B) Split 1:10
Carrier Gas Controller	:	Linear velocity (He, N_2, H_2)
Linear Velocity	:	35 cm/sec
Detector Temp.	:	250 °C
FID H_2 Flowrate	:	32 mL/min
FID Makeup Flowrate	:	24 mL/min (N_2)
FID Air Flowrate	:	200 mL/min
Injection Volume	:	1 mL

HS-20 NX Analytical Conditions (Same for Procedure A and B)		
Model	:	HS-20 NX USTL (Ultra Short Transfer Line)
Oven Temp.	:	80 °C
Sample Line Temp.	:	110 °C
Transfer Line Temp.	:	120 °C
Vial Shaking Level	:	Off
Vial Volume	:	20 mL
Vial Equilibrating Time	:	60 min
Vial Pressurizing Time	:	1 min
Vial Pressure	:	75 kPa
Loading Time	:	0.5 min
Load Equilib. Time	:	0 min
Needle Flush Time	:	5 min

■ Class 1 Standard Solution Analysis (Water-Soluble Samples)

Figs. 2 and 3 show the analysis results for the Class 1 standard solution.

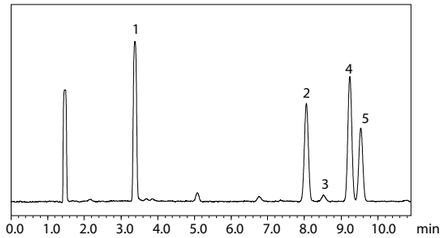

Fig. 2 Class 1 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A
1. 1,1-Dichloroethane, 2. 1,1,1-Trichloroethane,
3. Carbon tetrachloride, 4. Benzene, 5. 1,2-Dichloroethane

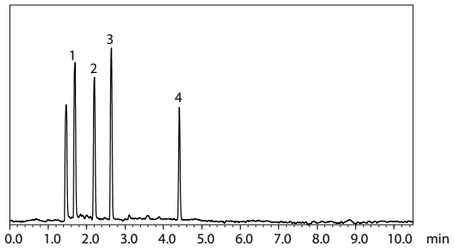

Fig. 3 Class 1 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure B
1. 1,1-Dichloroethane, 2. 1,1,1-Trichloroethane+ Carbon tetrachloride,
3. Benzene, 4. 1,2-Dichloroethane

Link to Content Page →

■ Class 2 Standard Solution Analysis (Water-Soluble Samples)

Fig. 4 shows the analysis results for Procedure A, and Fig. 5 shows the analysis results for Procedure B (Class 2A: black, Class 2B: pink, TBA, CPME, MiBK: blue). For system suitability, JP18 specifies that "the resolution between acetonitrile and methylene chloride in the Class 2 mixture A standard solution is not less than 1.0" when using Procedure A, and "the resolution between acetonitrile and cis-1,2-dichloroethene in the Class 2 mixture A standard solution is not less than 1.0" when using Procedure B. Satisfactory results were obtained with both procedures.

Note: The resolutions shown in the Figs. 4 and 5 are reference values and not guaranteed.

Note: A mixture of standard samples of *tert*-butyl alcohol (TBA), cyclopentyl methyl ether (CPME), and methyl isobutyl ketone (MiBK) was separately prepared to the prescribed concentration.

■ Analysis of Class 1 and 2 Standard Samples (Water-Soluble Samples) by Procedure A Using H_2 or N_2 Carrier Gas

Figs. 6 to 9 show the separation patterns for Procedure A using the alternative gases H_2 and N_2. When using an alternative carrier gas, the system suitability should be checked before performing the operation.

■ Conclusion

Even though the Brevis GC-2050 is small and compact, it is capable of analyzing residual solvents in pharmaceuticals in accordance with the JP18 and USP General Chapters <467> Residual Solvents. That compactness means the number of units installed in a laboratory can be increased compared with high-end models, so residual solvents in pharmaceuticals can be efficiently analyzed.

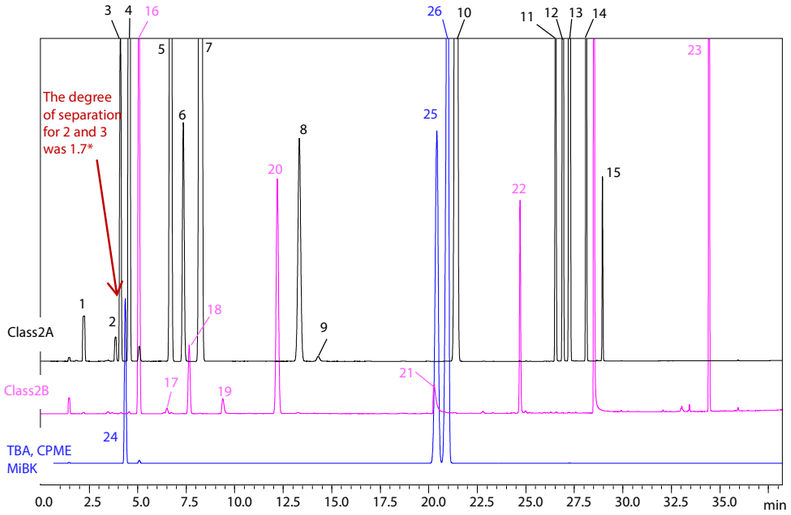

Fig. 4 Class 2 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A

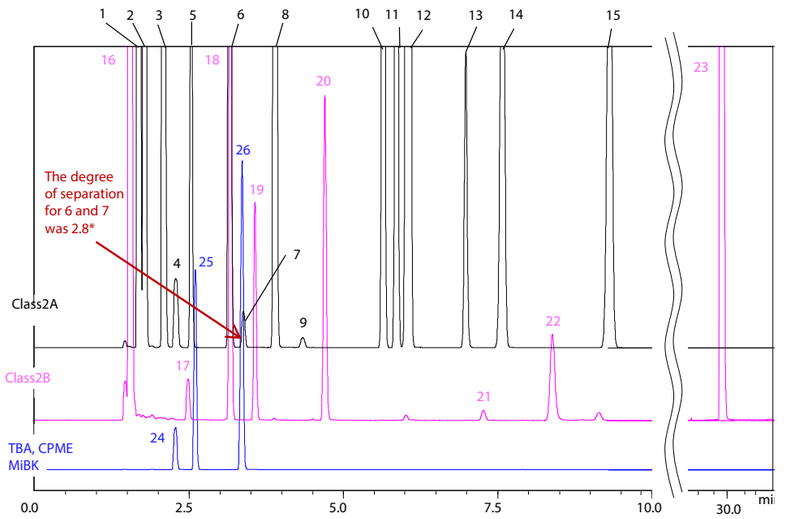

Fig. 5 Class 2 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure B

■ Examples of Analysis of Class 1 and 2 Standard Samples (Water-Soluble Samples) Using an Alternative Carrier Gas

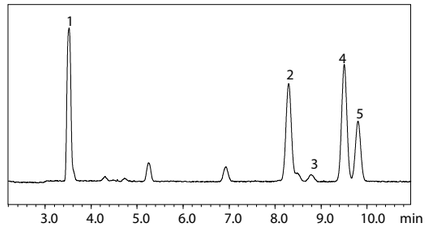

Fig. 6 Class 1 Standard Solution Chromatogram (Water-Soluble Sample) by Procedure A Using N_2 Carrier Gas
1,1-Dichloroethane, 2. 1,1,1-Trichloroethane,
3. Carbon tetrachloride, 4. Benzene, 5. 1,2-Dichloroethane

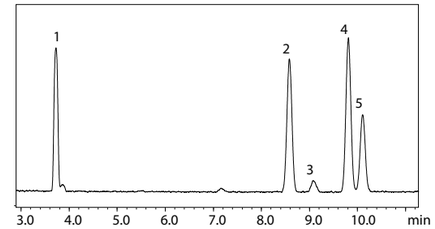

Fig. 7 Class 1 Standard Solution Chromatogram (Water-Soluble Sample) by Procedure A Using H_2 Carrier Gas
1. 1,1-Dichloroethane, 2. 1,1,1-Trichloroethane,
3. Carbon tetrachloride, 4. Benzene, 5. 1,2-Dichloroethane

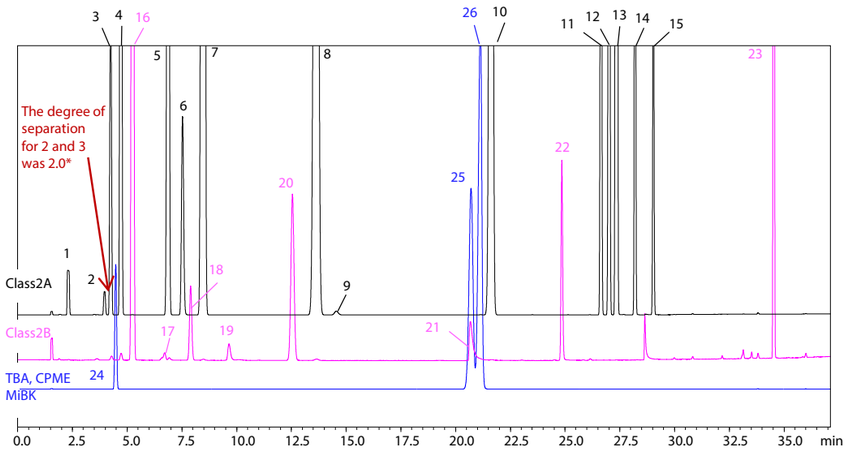

Fig. 8 Class 2 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A with N_2 Carrier Gas

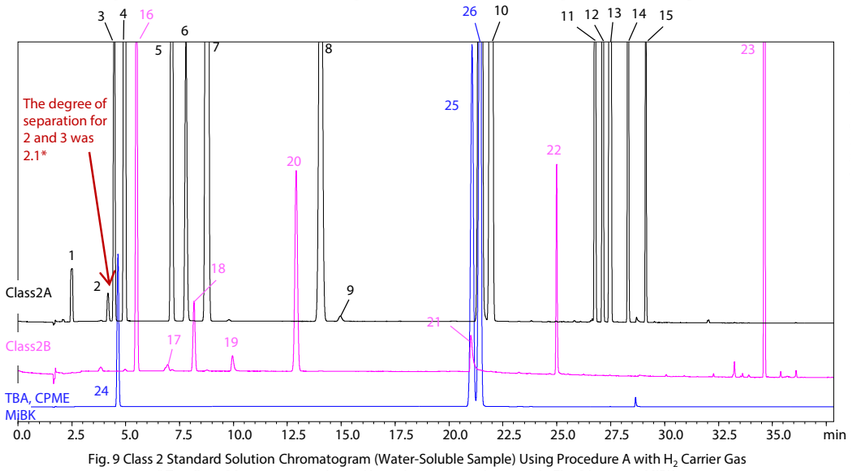

Fig. 9 Class 2 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A with H_2 Carrier Gas
* The Class 2A reagent used in the H_2 carrier gas analysis shown in Fig. 9 included MiBK.

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Application News

Gas Chromatograph HS-20 NX USTL/Brevis™ GC-2050

Efficient Analysis of Residual Solvents in Pharmaceuticals Using the Compact Model, Brevis GC-2050 (2)
—JP18 and USP467, Water Insoluble Samples—

Yui Higashi, Eisuke Kobayashi

User Benefits
- The slim and compact design of the Brevis GC-2050 enables the expansion of the number of operational units in the laboratory, allowing for efficient high-throughput analysis.
- Brevis GC-2050 can comply with the analysis methods of the pharmacopoeias.
- Analysis can be performed on *tert*-butyl alcohol and cyclopentyl methyl ether, which have been newly added as Class 2 solvents in ICH Q3C (R8).

■ Introduction

The methods of testing for residual solvents in pharmaceuticals are strictly prescribed in the Japanese Pharmacopoeia 18th Edition (JP18) and the United States Pharmacopeia (USP) General Chapters <467> Residual Solvents. In order to efficiently perform tests for residual solvents in pharmaceuticals under prescribed test methods, it is important to increase the number of instruments installed in laboratories, which often have limited space. The Brevis GC-2050 (Fig. 1) has a compact design, and compared with existing GC, the width of the system can be reduced by about 35 %. In addition, headspace samplers with ultra-short transfer lines can be used to further increase the number of units in the lab and enhance analysis efficiency. This article introduces the results of analysis of Class 1 and 2 water-insoluble samples using the compact Brevis GC-2050, in accordance with Supplement 2 of the JP18. DMF was used as the solvent.

Fig. 1 HS-20 NX USTL (Ultra Short Transfer Line) + Brevis™ GC-2050

■ Analytical Conditions

Table 1 Analysis Conditions of Water-Insoluble Sample

GC Analytical Conditions (Procedure A and B)		
Model	:	Brevis GC-2050
Detector	:	FID (Flame Ionization Detector)
Column	:	A) SH-I-624Sil MS (0.53 mm I.D. × 30m, d.f. = 3.0 µm) B) SH-PolarWax (0.32 mm I.D. × 30m, d.f. = 0.25 µm)
Column Temp.	:	A) 40 °C (20 min) – 10 °C/min – 240 °C (20 min) Total 60 mins B) 50 °C (20 min) – 6 °C/min – 165 °C (20 min) Total 59.17 mins
Injection Mode	:	A) Split 1:5 B) Split 1:10
Carrier Gas Controller	:	Linear velocity (He, N_2, H_2)
Linear Velocity	:	35 cm/sec
Detector Temp.	:	250 °C
FID H_2 Flowrate	:	32 mL/min
FID Makeup Flowrate	:	24 mL/min (N_2)
FID Air Flowrate	:	200 mL/min
Injection Volume	:	1 mL
HS-20 NX GC Analytical Conditions (Same for Procedure A and B)		
Model	:	HS-20 NX USTL (Ultra Short Transfer Line)
Oven Temp.	:	80 °C
Sample Line Temp.	:	90 °C
Transfer Line Temp.	:	105 °C
Vial Shaking Level	:	Off
Vial Volume	:	20 mL
Vial Equilibrating Time	:	45 min
Vial Pressurizing Time	:	1 min
Vial Pressure	:	68.9 kPa
Loading Time	:	0.5 min
Load Equilib. Time	:	0 min
Needle Flush Time	:	5 min

■ Class 1 Standard Solution Analysis (Water-Insoluble Samples)

Figs. 2 and 3 show the analysis results for the Class 1 standard solution.

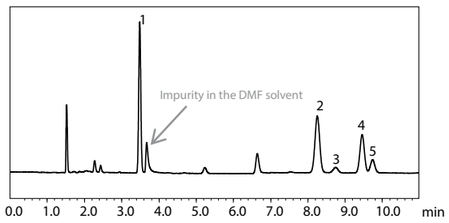


Fig. 2 Class 1 Standard Solution Chromatogram (Water-Insoluble Sample) Using Procedure A
1. 1,1-Dichloroethane, 2. 1,1,1-Trichloroethane,
3. Carbon tetrachloride, 4. Benzene, 5. 1,2-Dichloroethane

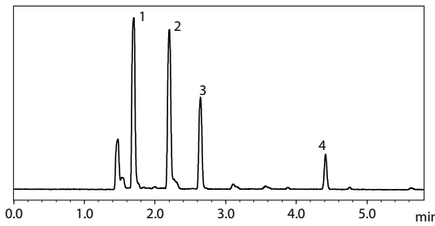


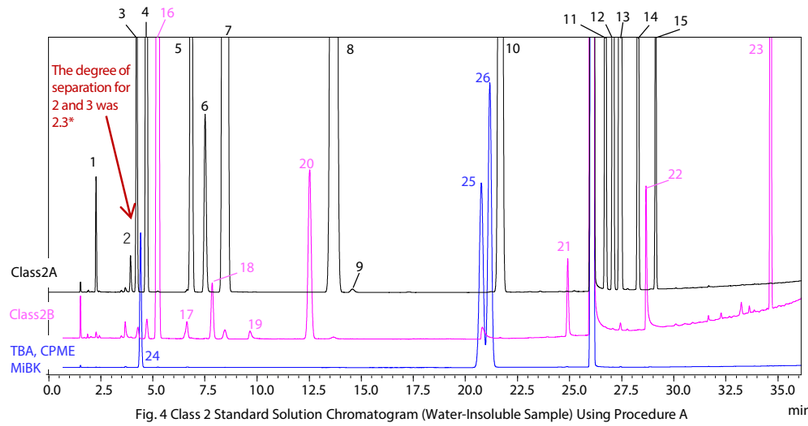
Fig. 3 Class 1 Standard Solution Chromatogram (Water-Insoluble Sample) Using Procedure B
1. 1,1-Dichloroethane, 2. 1,1,1-Trichloroethane+ Carbon tetrachloride,
3. Benzene, 4. 1,2-Dichloroethane

■ Class 2 Standard Solution Analysis (Water-Insoluble Sample)

Fig. 4 shows the analysis results for Procedure A, and Fig. 5 shows the analysis results for Procedure B (Class 2A: black, Class 2B: pink, TBA, CPME, MiBK: blue). For system suitability, JP18 specifies that "the resolution between acetonitrile and methylene chloride in the Class 2 mixture A standard solution is not less than 1.0" when using Procedure A, and "the resolution between acetonitrile and cis-1,2-dichloroethene in the Class 2 mixture A standard solution is not less than 1.0" when using Procedure B. Satisfactory results were obtained with both procedures.

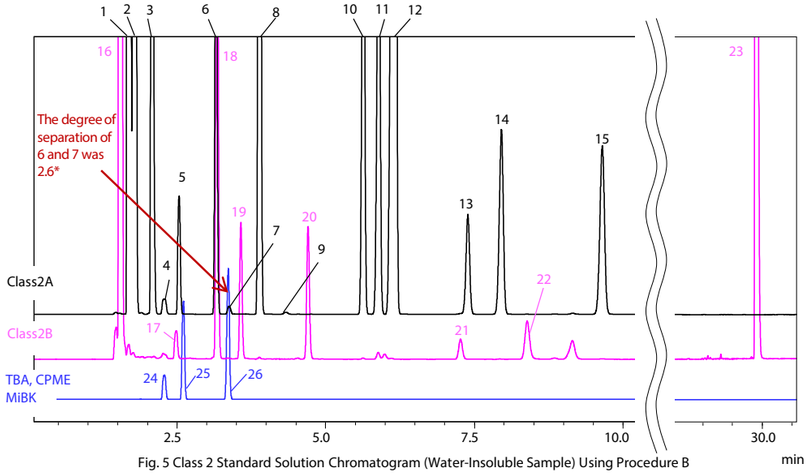
Note: The resolutions shown in the Figs. 4 and 5 are reference values and not guaranteed.

Note: A mixture of standard samples of *tert*-butyl alcohol (TBA), cyclopentyl methyl ether (CPME), and methyl isobutyl ketone (MiBK) was separately prepared to the prescribed concentration.



1: Methanol
2: Acetonitrile
3: Methylene chloride (DCM)
4: *trans*-1,2-Dichloroethylene
5: *cis*-1,2- Dichloroethylene
6: Tetrahydrofuran
7: Cyclohexane
8: Methyl cyclohexane
9: 1,4-Dioxane
10: Toluene
11: Chlorobenzene
12: Ethylbenzene
13: *m,p*-Xylene
14: *o*-Xylene
15: Cumene
16: Hexane
17: Nitromethane
18: Chloroform
19: 1,2-Dimethoxyethane
20: Trichloroethene
21: Pyridine
22: Methyl butyl ketone
23: Tetraline
24: *tert*-Butyl alcohol (TBA)
25: Cyclopentyl methyl ether (CPME)
26: Methyl isobutyl ketone (MiBK)

Fig. 4 Class 2 Standard Solution Chromatogram (Water-Insoluble Sample) Using Procedure A



1: Cyclohexane
2: Methyl cyclohexane
3: *trans*-1,2-Dichloroethene + Tetrahydrofuran
4: Methanol
5: Methylene chloride
6: *cis*-1,2-Dichloroethene
7: Acetonitrile
8: Toluene
9: 1,4-Dioxane
10: Ethylbenzene
11: *p*-Xylene
12: *m*-Xylene
13: Cumene
14: *o*-Xylene
15: Chlorobenzene
16: Hexane
17: 1,2-Dimethoxyethane
18: Trichloroethene
19: Chloroform
20: Methyl butyl ketone
21: Nitromethane
22: Pyridine
23: Tetraline
24: *tert*-Butyl alcohol (TBA)
25: Cyclopentyl methyl ether (CPME)
26: Methyl isobutyl ketone (MiBK)

Fig. 5 Class 2 Standard Solution Chromatogram (Water-Insoluble Sample) Using Procedure B

■ Conclusion

Even though the Brevis GC-2050 is small and compact it is capable of analyzing residual solvents in pharmaceuticals in accordance with the JP18 and USP General Chapters <467> Residual Solvents. That compactness means the number of units installed in a laboratory can be increased compared with high-end models, so residual solvents in pharmaceuticals can be efficiently analyzed.

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Application News

Fourier Transform Infrared Spectrophotometer IRSpirit™-X

Identification Test of the Ibuprofen Active Ingredient Compliant with European Pharmacopoeia and United States Pharmacopeia and Analysis of Final Product (Commercial Pharmaceutical) by ATR Method

Shoko Iwasaki, Yasushi Suzuki, and Takahide Hiramatsu

User Benefits

- A variety of infrared spectroscopy techniques can be used to easily perform identification tests for not only ibuprofen drug substances but also final products (commercial drugs).
- Identification tests compliant with European Pharmacopoeia and United States Pharmacopeia requirements can be easily performed using an IRSpirit-ZX spectrophotometer.
- The IRSpirit-ZX offers high humidity resistance for worry-free operations even in high-temperature and high-humidity environments.

■ Introduction

Infrared spectrophotometers are commonly used in combination with ultraviolet-visible spectrophotometers to perform identification tests of pharmaceuticals. The infrared spectroscopy method measures the degree of light absorption at each wavenumber as infrared light passes through the sample. The infrared spectrum shows the wavenumbers where light is absorbed and the intensity of that absorption, which are determined by the chemical structure and concentration of the target substances. Consequently, it can be used to identify or quantitate substances.

The pharmacopoeia of a country usually specifies the same measurement techniques for the same samples, but in a few rare cases, they specify different measurement techniques. This article describes differences in results obtained using the different identification test techniques specified for ibuprofen active ingredients in the European Pharmacopoeia (EP)[1] and the United States Pharmacopeia-National Formulary (USP-NF).[2] The article also describes using an ATR attachment to analyze not only the active ingredient but also the final drug product, which is the commercially marketed pharmaceutical.

■ Ibuprofen

Ibuprofen, which was used in this example is a type of non-steroidal anti-inflammatory pain reliever used to reduce symptoms of joint inflammation or fever and pain from inflamed areas. The market for ibuprofen and other nonsteroidal anti-inflammatory drugs (NSAID) is expected to expand due to continued global aging and increases in chronic pain.[3]

The chemical structure of ibuprofen is shown in Fig. 1. Due to its widespread use, the corresponding requirements are specified in the EP, USP-NF, and Japanese Pharmacopoeia (JP). They all specify using infrared spectroscopy for identification testing, but they recommend different measurement methods. The EP permits a variety of ATR and other techniques for analyzing potassium bromide (KBr) pellets, whereas the USP-NF specifies the paste (Nujol) method and the JP specifies analyzing KBr pellets.

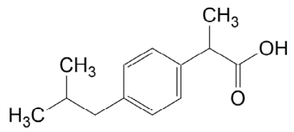

Fig. 1 Chemical Structure of Ibuprofen ($C_{13}H_{18}O_2$)

■ Instruments Used and Measurement Conditions

This article describes measuring the ibuprofen active ingredient using the KBr pellet method, the single reflection ATR method, and the paste method.

Samples were measured using an IRSpirit-ZX Fourier transform infrared spectrophotometer. The system is shown in Fig. 2, and the measurement parameters are listed in Table 1.

Fig. 2 IRSpirit™-X Series Fourier Transform Infrared Spectrophotometer

Table 1 Measurement Conditions

Instrument:	IRSpirit™-ZX spectrophotometer and QATR™-S (diamond) attachment
Resolution:	2 cm^{-1}
Number of scans:	45
Apodization function:	SqrTriangle
Detector:	DLATGS
Measurement wavenumber range:	4,000 to 550 cm^{-1}

Despite the compact case size (W390 × D250 × H210 mm) of the IRSpirit-X series systems, the TX model offers the highest sensitivity in its class. The ZX model features a ZnSe beam splitter that ensures worry-free operability even in high-temperature and high-humidity environments. However, due to the transmittance characteristics of its ZnSe beam splitter, the ZX model has a narrower measurable wavenumber range on the low-wavenumber end than the LX/TX models. Nevertheless, the ZX model is capable of measurements in the 4,000 to 650 cm^{-1} range, as specified in the EP, and the 3,800 to 650 cm^{-1} range, as specified in the USP-NF, for compliant measurements. But the ZX model is not capable of measuring the 4,000 to 400 cm^{-1} range, which is specified in the JP. To ensure identification tests are compliant with the JP, the LX or TX model should be used.

Due to the wide variety of instruments required for analyzing pharmaceuticals, laboratories tend to become cramped, so the extremely compact design of the IRSpirit-X series FTIR spectrophotometers makes them especially suitable for installation in sites with limited space.

■ EP-Compliant Identification Test (KBr Pellet Method)

Monographs 0721 in EP11.2 (in effect since July 1, 2023) specifies using the infrared spectroscopy method to compare the results of tested samples with ibuprofen CRS (CAS registration number (CAS RN®) 15687-27-1) in ibuprofen identification tests. Therefore, in this example, the infrared spectra from the tested ibuprofen reagent and the ibuprofen CRS reference sample were compared.

First, they were measured using the KBr pellet method, which is commonly used for identification testing. In the KBr pellet method, the sample is dispersed in KBr powder and formed into pellets with a pellet press. The pellet press used in this case was a Pixie mini-hydraulic press, which formed 7 mm diameter pellets. It is shown in Fig. 3.

The infrared spectra obtained are shown in Fig. 4. The test sample results closely match the standard reference sample results.

Fig. 3 Pixie Hydraulic Pellet Press

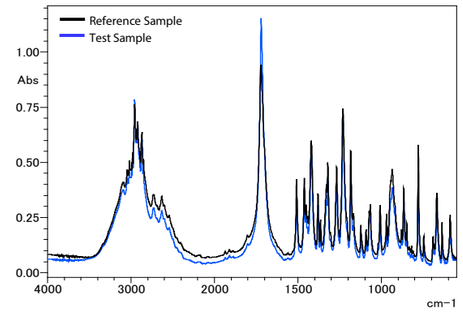

Fig. 4 Infrared Spectra of Ibuprofen (KBr Pellet Method)

■ EP-Compliant Identification Test (Single-Reflection ATR Method)

Aside from the KBr tablet method, a variety of other methods are also permitted for ibuprofen identification testing. Therefore, in this example, a diamond crystal was used to measure attenuated total reflectance (ATR). Ibuprofen reagent test samples were prepared in the same manner as in the KBr pellet method, and the results were compared with the results for ibuprofen CRS, the EP reference sample. The infrared spectra obtained are shown in Fig. 5.

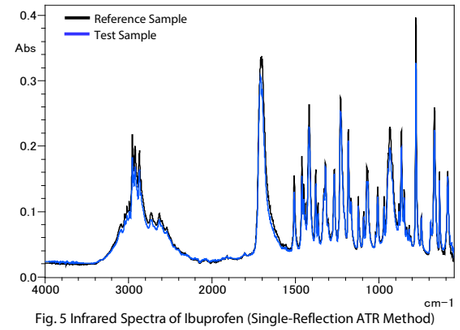

Fig. 5 Infrared Spectra of Ibuprofen (Single-Reflection ATR Method)

Just like the KBr pellet method results, the test sample results using the ATR method closely match the reference sample results.

Note that the ATR method offers the major advantage of not requiring pretreatment because the method involves positioning samples close to the crystal. However, the peak intensity obtained by the ATR method depends on the wavenumber, with higher peak intensities having lower wavenumbers.

Next, test sample spectra obtained from the KBr pellet and ATR methods were overlaid to identify the differences, as shown in Fig. 6. An enlarged view near 1,700 cm^{-1} is shown in Fig. 7. The intensity of peaks with the highest intensity is closely matched in both figures. A comparison of the spectra obtained by KBr pellet and ATR methods shows that the large absorption peak near 1,700 cm^{-1} shifted toward the low-wavenumber end in the ATR spectrum. Peak wavenumber positions are known to shift, particularly at peak positions with high absorption due to higher sample refractive index values.[4)-6)]

Thus, using different measurement techniques can result in different spectral intensity levels and peak positions, so it is essential to measure both the test sample and reference sample using the same technique.

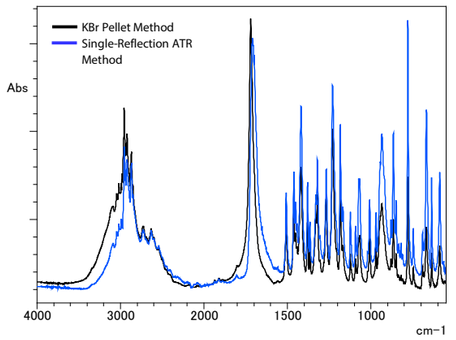

Fig. 6 Infrared Spectra of Ibuprofen (KBr Pellet and Single-Reflection ATR Methods)

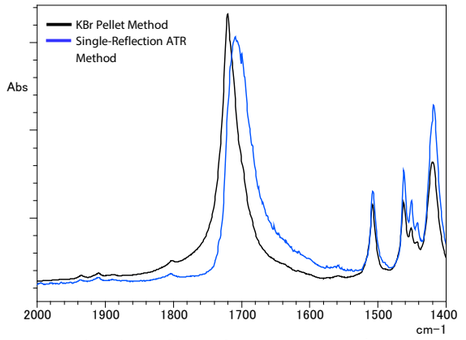

Fig. 7 Enlarged View of Spectra from 2,000 to 1,400 cm^{-1} in Fig. 6

■ USP-NF-Compliant Identification Test (Paste Method)

Monographs 206.28 in USP-NF 2023 Issue 2 (in effect since August 1, 2023) specifies the paste method for infrared spectroscopy used to compare results from test samples to ibuprofen CRS (CAS registration number (CAS RN) 15687-27-1) in ibuprofen identification tests.

Therefore, in this example, the paste method was used to obtain infrared spectra from the ibuprofen reagent test sample and the ibuprofen CRS reference sample and then the spectra were compared. The infrared spectra obtained are shown in Fig. 8. They show that the spectrum from the test sample closely matches the spectrum from the reference sample.

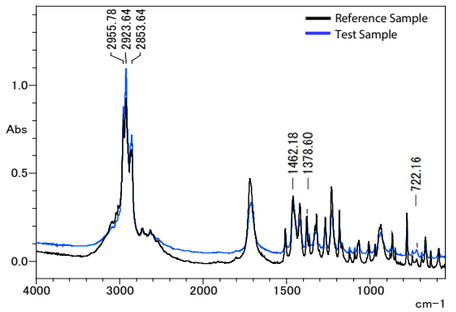

Fig. 8 Infrared Spectra of Ibuprofen (Paste Method)

Note that the paste method involves thoroughly mixing a small quantity of ground sample powder with a liquid paraffin (mineral oil) measuring spectra from that mixture sandwiched between two KBr window plates. Because the liquid paraffin absorbs light near 3,000 to 2,800 cm^{-1}, 1,500 to 1,350 cm^{-1}, and 720 cm^{-1} positions, the spectra obtained are for a mixture of ibuprofen and the liquid paraffin, as shown in Fig. 8.

Link to Content Page →

■ Analysis of Final Product (Commercial Pharmaceutical) by ATR Method

The following describes measuring ingredients contained in commercial drug tablets as an example of identification testing of a final product. The surfaces of most commercial drugs are coated with a substance to protect them from humidity, etc., or to make them easier to swallow by masking their odor or taste. For this example, the results from directly analyzing the coating layer on the surface of a tablet and the results from measuring a powder obtained by grinding the tablet are presented below.

The results from directly measuring the tablet coating layer by the single-reflection ATR method are shown in Fig. 9. When using the ATR method, light only penetrates samples to the shallow depth of a few micrometers, which only enables measuring the coating layer on tablet surfaces. A library search detected cellulose and talc components.

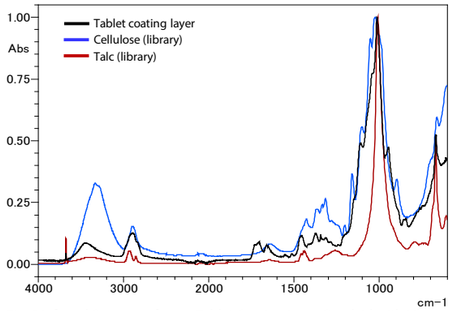

Fig. 9 Infrared Spectrum from a Tablet Coating Layer Overlaid with Library Search Results (Single-Reflection ATR Method)

Next, the tablet was ground to a powder, and the powder was measured by the single-reflection ATR method. Infrared spectra obtained from the powdered tablet and the ibuprofen CRS EP reference sample are shown overlaid in Fig. 10.

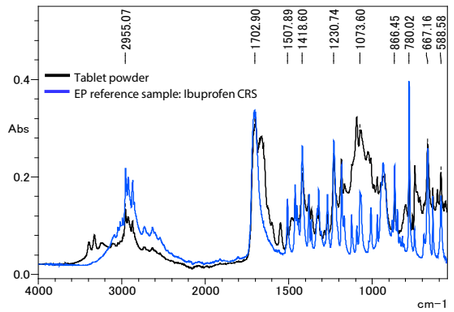

Fig. 10 Infrared Spectra of Powdered Tablet and Ibuprofen Reference Sample (Single-Reflection ATR Method)

The infrared spectrum from the tablet powder includes peaks that typically appear (at wavenumbers indicated at the top of Fig. 10) for ibuprofen, but also includes other peaks. To qualitatively analyze such non-ibuprofen ingredients, the infrared spectrum from the ibuprofen CRS was subtracted from the infrared spectrum from the tablet powder. The resulting difference spectrum is shown overlaid with library search results in Fig. 11. Based on that overlay, it can be inferred that the tablet analyzed in this case contains caffeine and silicate in addition to ibuprofen, cellulose, and talc.

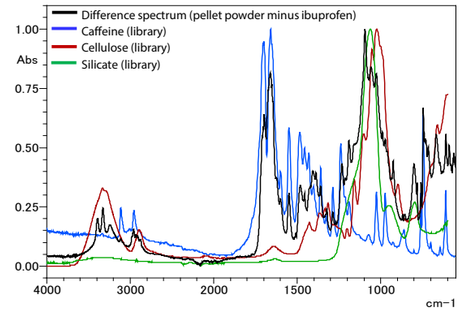

Fig. 11 Overlay of Ibuprofen Difference Spectrum with Library Search Results (Single-Reflection ATR Method)

■ Conclusion

Using the widely used drug ibuprofen, as a test sample, an IRSpirit-ZX FTIR spectrophotometer was used to perform EP and USP-NF-compliant identification tests based on the KBr pellet, ATR, and paste methods. For the EP-compliant identification test, results obtained by the KBr pellet and ATR methods were also compared. Since different measuring techniques will result in infrared spectra that do not match, it is essential to measure both test samples and reference samples using the same measuring techniques.

The system can also be used to qualitatively analyze surface coating layers or ingredients other than the main ingredient, ibuprofen, by using the ATR method to measure the commercial pharmaceutical, rather than only the active ingredient ibuprofen.

The IRSpirit-ZX model offers high humidity resistance for worry-free operations even in high-temperature and high-humidity environments. The LabSolutions™ IR software for data analysis and controlling IRSpirit-X series systems includes a program (IR Pilot™) designed specifically for easy identification testing, which has standard Spectral Advisor functionality that can suggest improvement measures that are based on comparing acquired data to measurement examples. With the IR Pilot program, procedural steps can be performed automatically, from performing operations by following instructions displayed in the window to correctly measuring samples, analyzing data, and printing results. It is highly recommended for users who intend to perform identification tests.

Reference Documents

1) EUROPEAN PHARMACOPOEIA SUPPLEMENT 11.2 (viewed August 2023)
2) The United States Pharmacopeia-National Formulary 2023 Issue2 (viewed August 2023)
3) A. Rastogi et al. A review on environmental occurrence, toxicity and microbial degradation of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). *J. Environ. Manag.* 2021, 300, 113694.
4) FTIR TALK LETTER Vo.1
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5) FTIR TALK LETTER Vo.2
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01-00645-EN First Edition: Dec. 2023

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Application News

Software for Efficient Method Development "LabSolutions™ MD"

USP-Compliant Analysis and Robustness Evaluation of Pramipexole by i-Series

Shinichi Fujisaki

User Benefits

- LC-2050C, with delay volume compatibility kit, is compatible with the delay volume of LC-2010, facilitating smooth method transfer.
- Design space generated by LabSolutions MD allows robustness evaluation of a test method without relying on the user's experience in chromatography.

■ Introduction

Pramipexole is a drug used to treat Parkinson's disease and other disorders. The United States Pharmacopeia (USP) specifies analytical conditions for this drug. "i-Series LC-2050C" integrated high-performance liquid chromatograph is equipped with delay volume compatibility with LC-2010, our former integrated HPLC, by using the optional delay volume compatibility kit. This compatibility ensures a smooth method transfer from LC-2010. In this paper, a case study is presented, analyzing Pramipexole hydrochloride, a compound listed in the USP, utilizing LC-2050C with the delay volume compatibility kit. The scientific basis (specificity and robustness) of the test method for the instrument change from LC-2010 to LC-2050C was evaluated using LabSolutions MD which is a dedicated software for supporting method development. Specifically, the effect on system suitability test, such as resolution and symmetry factor, was assessed by intentionally varying the flow rate and column oven temperature specified by the USP within a small range.

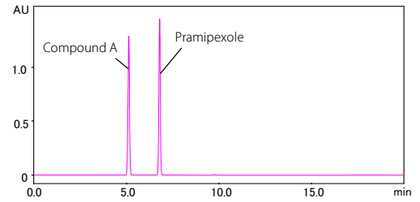

Fig. 1 Chromatogram by LC-2050C (with delay volume compatibility kit)

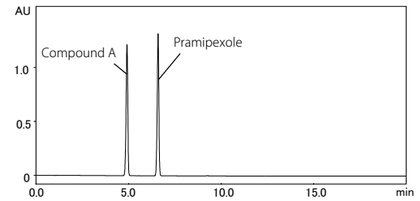

Fig. 2 Chromatogram by LC-2010

■ Analytical Conditions

The analytical conditions (system suitability test) for Pramipexole hydrochloride are presented in Table 1. Additionally, the analysis result using LC-2050C with the delay volume compatibility kit is shown in Fig. 1, while the analysis result using LC-2010 is illustrated in Fig. 2. These results indicate that LC-2050C and LC-2010 are delay volume compatible. Table 2 summarizes the results of the system suitability test for Pramipexole hydrochloride. Consequently, it was confirmed that both LC-2050C and LC-2010 systems met the system suitability criteria for the resolution and symmetry factor.

Table 1 Analytical Conditions (System suitability test)

System	: LC-2050C with compatibility kit
Sample	: Pramipexole Dihydrochloride (1.5 mg/mL) and Compound A (0.8 mg/mL)
Mobile phase	: A) 67 mmol/L (potassium) phosphate buffer containing 21 mmol/L 1-octanesulfonic acid sodium salt (pH 3.0) : B) Mobile Phase A/Acetonitrile = 50 : 50
Column	: Shim-pack Scepter™ C18-120 (150 mm × 4.6 mm I.D., 5 µm)*1
Injection Vol.	: 5 µL
Time program	: B Conc. 40%(0 min)→80%(15min) →40%(15.1-20min)
Column Temp.	: 40 ℃
Flow rate	: 1.5 mL/min
Detection (PDA)	: 264 nm

*1 P/N: 227-31020-05

Table 2 Results of System Suitability Test

System suitability item	Criteria	Result (LC-2050C)	Result (LC-2010)
Resolution (Pramipexole and compound A)	≧6.0	10.7	9.9
Symmetry factor (Pramipexole)	≦2.0	0.94	0.93

■ Robustness Evaluation by LabSolutions MD

Robustness evaluations are crucial for understanding the effects of parameter changes on measurement results and confirming the reliability of the test method. LabSolutions MD supports robust method development based on Analytical Quality by Design (AQbD) approach. The robustness of Pramipexole hydrochloride under USP specifications was assessed by varying the flow rate by ±0.1 mL/min (at three levels : 1.4, 1.5, and 1.6 mL/min) and the column oven temperature by ±1 ˚C (at three levels : 39, 40, and 41 ˚C), resulting in nine different combinations (refer to Fig. 3). Plots illustrating the resolution between Pramipexole and compound A as well as the symmetry factor of Pramipexole at each flow rate and oven temperature level are provided in Fig. 4 and Fig. 6, respectively. In addition, design spaces of resolution and symmetry factor across the entire range of variation, with flow rate on the vertical axis and oven temperature on the horizontal axis, are displayed in Fig. 5 and Fig. 7.

Link to Content Page →

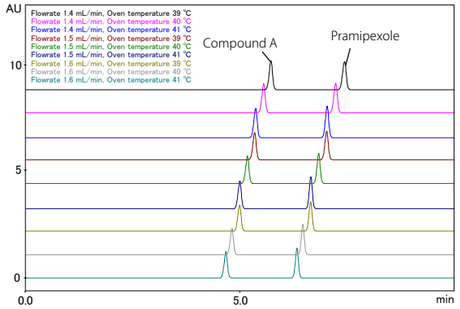

Fig. 3 Chromatograms at Each Level of Flow Rate and Oven Temperature

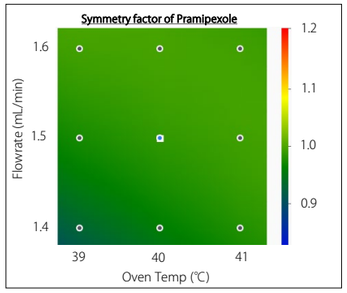

Fig. 7 Design Space of Symmetry Factor

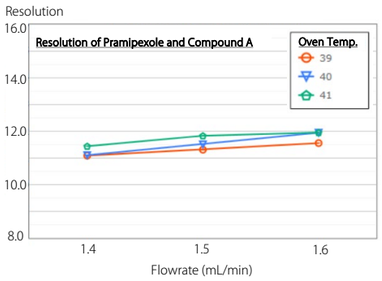

Fig. 4 Plots of Resolution

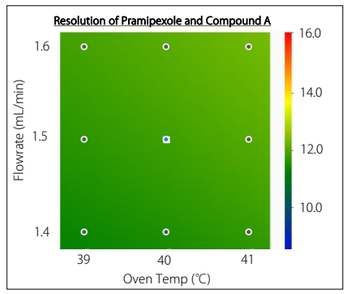

Fig. 5 Design Space of Resolution

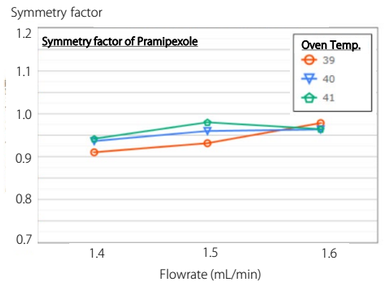

Fig. 6 Plots of Symmetry Factor

At their respective levels of flow rate and column oven temperature, the resolutions between Pramipexole and compound A varied between 11 and 12 (refer to Fig. 4). This outcome is represented as a design space (refer to Fig. 5). In Fig. 5, the green area (resolution : 11-12) covers the entire variation range, indicating minimal variation in resolution and meeting the criterion (≥6.0) for resolution in the system suitability test. The symmetry factor of Pramipexole varied between 0.9 and 1.0 at their respective levels of flow rate and column oven temperature (refer to Fig. 6). In the design space of the symmetry factor (Fig. 7), the green area (symmetry factor : 0.9-1.0) extends across the entire region, indicating minimal variation in the symmetry factor and meeting the criterion (≤2.0) for the symmetry factor in the system suitability test. Therefore, utilizing the design space can verify the robustness when parameters are varied, without relying on user's experience on chromatography.

■ Conclusion

Through USP-compliant analysis of Pramipexole, it has been demonstrated that LC-2050C (utilizing delay volume compatibility kit) and LC-2010 are delay volume compatible, thereby facilitating smooth method transfer. Moreover, method validation is commonly necessary during test method alterations. The design space function of LabSolutions MD effectively assesses analytical performance parameters such as specificity (resolution) and robustness, enabling reliable method management without depending on user experience.

＜References＞
1) US Pharmacopeia 43-NF38, 2022
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01-00698-EN First Edition: Feb. 2024

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Application News

Gas Chromatograph Nexis™ GC-2030, AOC™-30i+20s U

Determination of Organic Impurities from Valproic Acid as per proposed USP monograph GC method

Nitish Suryawanshi, Sanket Chiplunkar, Dheeraj Handique, Prashant Hase, Durvesh Sawant, Aseem Wagle, Rahul Dwivedi, Jitendra Kelkar and Pratap Rasam
Shimadzu Analytical (India) Pvt. Ltd.

User Benefits

- Shimadzu Nexis GC-2030 can be effectively used for organic impurities test of valproic acid drug substance as per the proposed USP monograph GC method.
- The Nexis GC-2030 easily meets the acceptance criteria as per the proposed USP monograph for valproic acid

■ Introduction

Valproic acid, or valproate, is a fatty acid derivative and anticonvulsant originally used as a popular organic solvent in industry and pharmaceutical manufacturing for nearly a century, Currently it is a compound of interest in the field of oncology for its anti-proliferative effects and is the subject of many clinical trials in a variety of cancer types. It is on the World Health Organization's List of Essential Medicines and is available as a generic medication. The impurities such as valproic acid impurity B & valproic acid impurity K (Figure 1) originate through the manufacturing process of valproic acid.

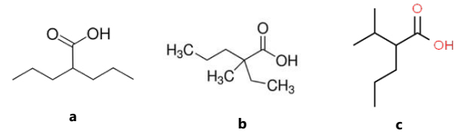


Figure 1: Structure of a) Valproic acid ; b) Valproic acid compound K; c) Valproic acid compound B

Their presence should be controlled in finished valproic acid drug substance, this led United States Pharmacopeia (USP) to incorporate a gas chromatography procedure named "Organic Impurities" in the proposed new monograph for valproic acid below are the changes proposed in the upcoming monograph.

1. Revision of organic impurities test to add valproic acid related compound K with an acceptance criteria of NMT 0.15%, using USP G35 stationary phase.
2. Addition of USP valproic acid related compound K RS
3. Deletion of USP valproic acid related compound A RS

This application note demonstrates the determination of organic impurities from valproic acid as per proposed USP monograph GC method using Shimadzu's Nexis GC-2030 system. (Figure 2)

Figure 2: Nexis™ GC-2030 system

Nexis GC-2030, Key features

- ✓ Tool-free Column Installation
- ✓ One-Touch Inlet Maintenance
- ✓ Remote Operations and Monitoring
- ✓ Achieves Exceptional Reproducibility (AFC with CPU)
- ✓ Best-in-class sensitivity for most of the detectors

■ Experimental

Chromatographic conditions (Table 1), system suitability, standard and sample preparations were done in accordance with the proposed USP monograph for valproic acid. System suitability parameters were also checked as per the requirements of USP monograph. (Table 2, 3, 4 & 5)

Table 1: Instrument configuration and analytical conditions

GC System	**: Nexis GC-2030 with AOC-30i+20s U**		
Column	: SH-PolarD Cap. Column, 60 m, 0.32 mm, 0.50 µm (P/N: 227-36276-01)		
Injection Mode	: Split (5:1)		
Flow Control Mode	: Column flow		
Injector Port Temp.	: 220 °C		
Carrier Gas	: Helium		
Flow Rate	: 2.0 mL/min		
Injection Volume	: 1.0 µL		
Temp. Program	Ramp Rate (°C/min)	Temp. (°C)	Hold Time (min)
	-	100	5
	4	200	15
Detector	: Flame Ionization Detector (FID)		
Detector Temp.	: 220 °C		
Detector Gases	: Hydrogen, Air for flame & Helium for make up		
Air	: 200 mL/min		
Hydrogen	: 32 mL/min		
Helium (Make up)	: 24 mL/min		

System suitability, standard and sample preparations:

System suitability solution: 0.5 mg/mL of USP valproic acid RS, 0.05 mg/mL of USP valproic acid related compound B RS, and 0.05 mg/mL of USP valproic acid related compound K RS in n-heptane.

Standard solution: 0.005 mg/mL of USP valproic Acid RS in n-heptane.

Sensitivity solution: 0.0025 mg/mL of USP valproic Acid RS from standard solution in n-heptane.
(Further, 5-time diluted solution (0.0005mg/mL) was prepared & injected to showcase the sensitivity at even lower levels using Shimadzu GC-2030)

Sample solution: 5 mg/mL of valproic acid in n-heptane.

Results:

System suitability (SST) requirements

Relative Retention Time (RRT): The relative retention times for valproic acid related compound B, valproic acid related compound K & valproic acid are about 0.97, 0.98 & 1.0, respectively.

Table 2: RRTs of valproic acid impurities

Compound	RRT	
	Expected	Found
Valproic acid related compound B	0.97	0.97
Valproic acid related compound K	0.98	0.98

Resolution: Not less than (NLT) 1.5 between valproic acid related compound B and valproic acid related compound K, System suitability solution

Table 3: Resolution between valproic acid impurities

Compound	Resolution	
	Limit	Found
Valproic acid related compound K	NLT 1.5	2.3

Tailing factor: Not more than (NMT) 1.5 for valproic acid, Standard solution

Table 4: Tailing factor for valproic acid

Compound	Tailing Factor	
	Limit	Found
Valproic acid	NMT 1.5	1.0

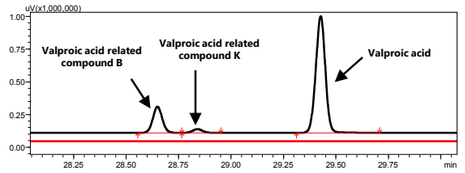
Signal-to-noise ratio (S/N) : NLT 10 for valproic acid, Standard solution

Table 5: S/N for valproic acid

Valproic acid-sensitivity solution	Signal-to-noise ratio	
	Expected	Found
0.5 ppm (Shimadzu)	NLT 10	19
2.5 ppm (USP)	NLT 10	181

The system suitability for organic impurities test passed as per criteria mentioned in proposed USP monograph.

Chromatograph of diluent blank & SST solution (Figure 3)



Black: SST solution ; Red: Diluent blank

Figure 3 Representative chromatograph for blank and SST solution

Sample results

Calculate the percentage of each impurity in the portion of valproic acid taken: Result = $(r_U / r_S) \times (C_S / C_U) \times (1/F)\ 100$

r_U = peak response of each impurity from the Sample
r_S = peak response of valproic acid from the Standard
C_S = concentration of USP valproic acid RS in the Standard solution (mg/mL)
C_U = concentration of valproic acid in the sample solution (mg/mL)
F= relative response factor

Table 6: Summary of content (%) of individual impurities in valproic acid sample replicates

Injection #	Unk.	Unk.	Unk.	B	K	VA
Injection-1	0.04	0.03	0.02	0.03	0.04	NA
Injection-2	0.04	0.03	0.02	0.02	0.04	NA
Injection-3	0.04	0.03	0.02	0.02	0.04	NA
Injection-4	0.04	0.03	0.02	0.03	0.04	NA
Injection-5	0.04	0.03	0.02	0.03	0.04	NA
Injection-6	0.04	0.03	0.02	0.02	0.04	NA

Unk.: Unknown ; B: Valproic acid related compound B; K: Valproic acid related compound K ; VA: Valproic acid

■ Conclusion

- This study successfully demonstrated the performance of Shimadzu Nexis GC-2030 system to determine the content of organic impurities in valproic acid sample as per the proposed USP monograph.
- The parameters for SST such as RRT, resolution, tailing factor and S/N meets the expected criteria.

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Application News

Liquid Chromatograph Nexera™ XS

Determination of Dexamethasone and its organic impurities content as per USP monograph UHPLC method

Purushottam Sutar[1], Nirmal Thakker[2]
1 Shimadzu Analytical (India) Pvt. Ltd., 2 Spinco Biotech Pvt. Ltd.

User Benefits

- Shimadzu Nexera XS can be effectively used for assay and organic impurities test of Dexamethasone as per the USP monograph UHPLC method
- The Nexera XS easily meets with all the acceptance criteria as per the USP monograph for Dexamethasone

■ Introduction

Dexamethasone (see Fig. 1) is a glucocorticoid medication used to treat rheumatic problems, a number of skin diseases, severe allergies, asthma, chronic obstructive lung disease, croup, brain swelling, eye pain following eye surgery, and along with antibiotics in tuberculosis. It may be given by mouth, as an injection into muscle and vein, as a topical cream or ointment for the skin or as a topical ophthalmic solution to the eye. The long-term use of dexamethasone may result in thrush, bone loss, cataracts, easy bruising, or muscle weakness. Dexamethasone also has anti-inflammatory and immunosuppressant effects.

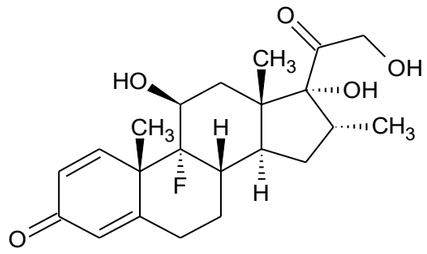

Fig. 1 Structure of Dexamethasone

The United States Pharmacopeia (USP) monograph defines an UHPLC (Ultra High-Performance Liquid Chromatography) method for high throughput fast analysis demanding a competent UHPLC system.

Here, we demonstrate the analysis for Dexamethasone as per the official monograph in USP using Shimadzu Nexera XS (see Fig. 2) fast LC system in compliance with system suitability requirements of the monograph.

Nexera series

Key features- Analytical Intelligence

- Automated support functions utilizing digital technology, such as machine-to-machine communication (M2M), Internet of things (IoT), and Artificial Intelligence (AI), that enable higher productivity and maximum reliability.

- Allows a system to monitor and diagnose itself, handle any issues during data acquisition without user input, and automatically behave as if it were operated by an expert.

- Supports the acquisition of high quality, reproducible data regardless of an operator's skill level for both routine and demanding applications.

Fig. 2 Nexera™ XS system

■ Experimental

Chromatographic conditions, mobile phase preparations, standard and sample preparations were done in accordance with the USP monograph for dexamethasone (see Table 1 for assay and Table 2 for organic impurities). System suitability parameters were also checked as per the requirements of USP monograph.

Assay

Solution A: 3.4 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.0.

Solution B: Acetonitrile.

Diluent: Acetonitrile/Water = 56:44

System suitability solution: 0.3 mg/mL of USP dexamethasone RS and 20 µg/mL of USP betamethasone RS in diluent. Sonicate to dissolve as needed.

Standard solution: 0.3 mg/mL of USP dexamethasone RS in diluent. Sonicate to dissolve as needed.

Sample solution: 0.3 mg/mL of dexamethasone in diluent. Sonicate to dissolve as needed.

Table 1 LC acquisition parameters for Assay

Column	: 100mm x 2.1 mm I.D., 1.7 µm USP packing L1
Oven temperature	: 35°C
Mobile Phase A	: Solution A
Mobile phase B	: Solution B
Gradient program (B %)	: 0.0-10.0 min → 24.0 (%); 10.0-15.0 min → 24.0-55.0 (%); 15.0-16.0 min → 55.0-90.0 (%); 16.0-16.1 min → 90.0-24.0 (%); 16.1-20.0 min → 24.0 (%).
Flow Rate	: 0.4 mL/min
Total Run Time	: 20.0 min
Injection Volume	: 2.0 µL
Autosampler Temperature	: 10°C
Detector wavelength	: 240 nm

Organic impurities

Solution A: 3.4 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.0.

Solution B: Acetonitrile.

Diluent: Acetonitrile/water = 56:44

System suitability solution: 0.3 mg/mL of USP dexamethasone RS and 20 µg/mL of USP betamethasone RS in diluent. Sonicate to dissolve as needed.

Standard solution: 4.0 µg/mL of USP dexamethasone RS, 6.0 µg/mL each of USP betamethasone RS and USP desoximetasone RS, and 12.0 µg/mL of USP dexamethasone acetate RS in diluent.

Sample solution: 4.0 mg/mL of dexamethasone in diluent. Sonicate to dissolve as needed.

Table 2 LC acquisition parameters for Organic impurities

Column	: 100 mm x 2.1 mm I.D.,; 1.7 µm USP packing L1
Oven temperature	: 35°C
Mobile Phase A	: Solution A
Mobile Phase B	: Solution B
Gradient program (B %)	: 0.0-10.0 min → 24.0 (%); 10.0-15.0 min → 24.0-55.0 (%); 15.0-16.0 min → 55.0-90.0 (%); 16.0-16.1 min → 90.0-24.0 (%); 16.1-20.0 min → 24.0 (%).
Flow Rate	: 0.4 mL/min
Total Run Time	: 20.0 min
Injection Volume	: 2.0 µL
Autosampler Temperature	: 10°C
Detector wavelength	: 240 nm

■ Results for Assay

The retention time of dexamethasone in standard and sample solutions is found to be about 8.961 minutes (see Fig. 3). The resolution between betamethasone and dexamethasone in system suitability solution is found to be 1.65 (see Fig. 4). The tailing factor for peak due to dexamethasone in the standard solution is well within the system suitability criteria of NMT 2.0 (see Table 3). The % relative standard deviation (%RSD) for retention time and peak area for six replicates of standards solution complies with the acceptable criteria of RSD NMT 0.73% (see Table 3) Overlay of five replicates of standards is shown in Fig. 5.

Table 3 Dexamethasone Assay standard

Parameter	Observed	USP Criteria
%RSD Retention Time (n=6)	0.056	NMT 0.73
%RSD Area (n=5)	0.161	NMT 0.73
Tailing factor	1.795	NMT 2.0

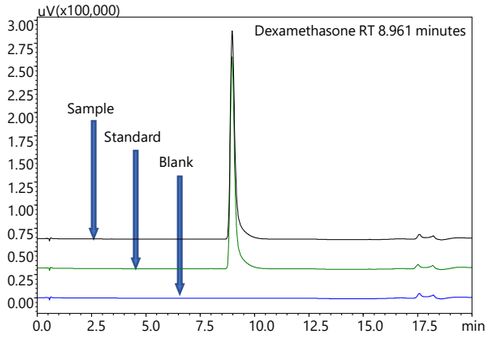

Fig. 3 Overlay of Assay sample, standard and blank

Sample analysis for Assay

The results obtained for analysis of sample using this method is found to be within the acceptance criteria of 97.0-102.0% (see Table 4).

Table 4 Content of Dexamethasone

Sample	Content (%)	USP Criteria (%)
Preparation-1	101.3	97.0-102.0
Preparation-2	100.7	

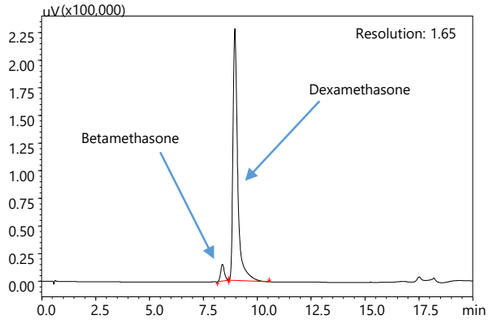

Fig. 4 System suitability solution

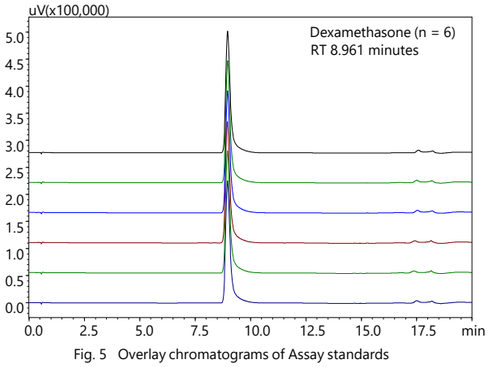

Fig. 5 Overlay chromatograms of Assay standards

■ Results for Organic impurities

The resolution between the peaks due to betamethasone and dexamethasone in the system suitability solution is found to be 1.65 which within the acceptance criteria of NLT 1.5 (see Fig. 4). The retention time of dexamethasone in standard and sample solutions (see Fig. 6 and Fig. 7, respectively) is found to be about 8.9 minutes. The observed %RSD for area and retention time of betamethasone, dexamethasone, desoximetasone and dexamethasone acetate for replicate injections in standard solution is within the criteria of NMT 5.0 (see Table 5). The observed relative retention time for betamethasone, desoximetasone and dexamethasone acetate w.r.t. dexamethasone is found to be 0.934, 1.632 and 1.790 respectively.

Table 5 Dexamethasone organic impurities standard

Analyte	Parameter	%RSD (n=6)	USP Criteria
Dexamethasone	Retention time	0.090	NMT 5.0
	Area	0.888	NMT 5.0
Betamethasone	Retention time	0.086	NMT 5.0
	Area	0.278	NMT 5.0
Desoximetasone	Retention time	0.030	NMT 5.0
	Area	0.086	NMT 5.0
Dexamethasone acetate	Retention time	0.022	NMT 5.0
	Area	0.143	NMT 5.0

Sample analysis for Organic impurities

The percentage of betamethasone, desoximetasone, and dexamethasone acetate in the portion of dexamethasone taken is calculated using below equation.

$$\text{Result} = (r_u / r_s) \times (C_u / C_s) \times 100$$

where,

r_u = peak response of betamethasone, desoximetasone, or dexamethasone acetate from the sample solution.

r_s = peak response of the corresponding USP reference standard from the standard solution.

C_s = concentration of the corresponding USP reference Standard in the Standard solution (mg/mL).

C_u = concentration of dexamethasone in the sample solution (mg/mL).

The percentage of 16α-methylprednisone, dexamethasone 7,9-diene, and any individual unspecified impurity in the portion of dexamethasone taken is calculated using:

$$\text{Result} = (r_u / r_s) \times (C_s / C_u) \times (1/F) \times 100$$

where,

r_u = peak response of 16α-methylprednisone, dexamethasone 7,9-diene, or any individual unspecified impurity from the sample solution

r_s = peak response of dexamethasone from the standard solution

C_s = concentration of USP dexamethasone RS in the standard solution (mg/mL)

C_u = concentration of dexamethasone in the sample solution (mg/mL)

F = relative response factor for respective impurities.

The content of individual known impurity, any unspecified unknown impurity and total impurities were found to be within the acceptance criteria (see Table 6, Table 7 and Table 8). The typical chromatogram of sample showing overlay with blank is shown in Fig. 7.

Table 6 Dexamethasone Organic impurities sample

Label claim	Main peak area %	Total impurities %area
Sample-1	99.691	0.309
Sample-2	99.687	0.313
USP criteria	-	NMT 0.5

Table 7 Dexamethasone organic impurities USP acceptance criteria

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
16α-Methylprednisone	0.86	1.0	0.15
Betamethasone	0.94	-	0.15
Dexamethasone	1.00	-	-
Dexamethasone 7,9-diene	1.40	1.7	0.10
Desoximetasone	1.58	-	0.15
Dexamethasone acetate	1.74	-	0.30
Any individual unspecified impurity	-	1.0	0.10
Total impurities	-	-	0.5

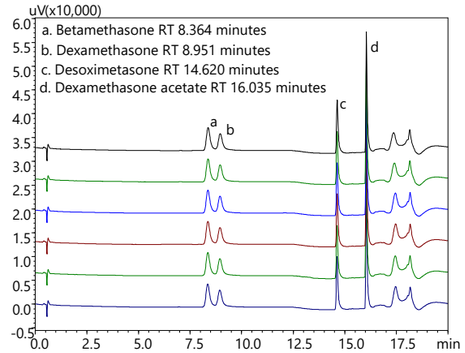

Fig. 6 Overlay chromatograms of Organic impurities standards

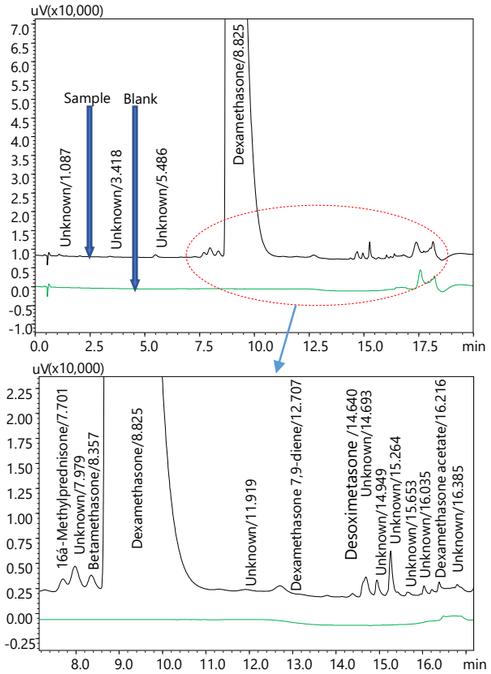

Fig. 7 Overlay of Organic impurities sample and blank

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Table 8 Percent area for impurity peaks in samples

Peak#	Name	RRT	Sample-1	Sample-2
1	Unknown	0.122	0.005	0.005
2	Unknown	0.382	0.004	0.004
3	Unknown	0.614	0.018	0.017
4	16á-Methylprednisone	0.862	0.024	0.024
5	Unknown	0.893	0.054	0.054
6	Betamethasone	0.935	0.025	0.025
7	Dexamethasone	0.987	99.691	99.687
8	Unknown	1.333	0.005	0.006
9	Dexamethasone 7,9-diene	1.422	0.024	0.030
10	Dosimethasone	1.638	0.011	0.010
11	Unknown	1.644	0.025	0.025
12	Unknown	1.672	0.017	0.017
13	Unknown	1.708	0.048	0.048
14	Unknown	1.751	0.005	0.005
15	Unknown	1.794	0.008	0.008
16	Dexamethasone acetate	1.814	0.003	0.003
17	Unknown	1.833	0.007	0.007

■ Conclusion

- This study successfully demonstrated the performance of Shimadzu Nexera series UHPLC system to determine the content of dexamethasone and its organic impurities in conformity with the USP monograph acceptance criteria and system suitability requirements.
- The reproducibility i.e., the relative standard deviation of retention time and area for standard are well within the acceptance criteria of NMT 0.73% for assay test and NMT 5.0% for the organic impurities test.
- The resolution between the peaks due to betamethasone and dexamethasone is found to be well within the acceptance.
- The relative retention times for all known impurities complies with the acceptance value in the USP monograph.
- The sample shows content of dexamethasone and its organic impurities within the permissible limits of USP.

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Application News

Liquid Chromatograph Mass Spectrometer LCMS-8060

Highly Sensitive Single LC-MS/MS Method for Cleaning Validation of Synthetic Peptides Using Shimadzu LCMS-8060

Avinash Gaikwad[1], Chaitanya krishna Atmakuri[1], Yogesh Arote[1], Jitendra Kelkar[2], Pratap Rasam[2]
[1] ADC - Shimadzu Analytical (India) Pvt. Ltd., [2] Shimadzu Analytical (India) Pvt. Ltd.,

User Benefits

- Six synthetic peptides quantified using a single LC-MS/MS method with LLOQ of 0.5 ng/mL
- Customized analytical method for peptides of different therapeutic categories
- Non-specific binding and low sensitivity issues addressed to make the method compatible for biological sample analysis

1. Introduction

As per supplementary guidelines on good manufacturing practices by WHO - "Cleaning validation is required as documented evidence to establish that cleaning procedures are removing residues to predetermined levels of acceptability, taking into consideration factors such as batch size, dosing, toxicology and equipment size". The objectives of good manufacturing practices (GMP) include the prevention of possible contamination and cross-contamination of pharmaceutical starting materials and products. Pharmaceutical products can be contaminated by a variety of substances such as contaminants associated with microbes, previous products (both active pharmaceutical ingredients (API) and excipient residues), residues of cleaning agents, airborne materials, such as dust and particulate matter, lubricants and ancillary material, such as disinfectants, and decomposition residues from:

A rapid, simple, sensitive, single method for,

- Product residue breakdown occasioned by, e.g. the use of strong acids and alkalis during the cleaning process; and
- Break down products of the detergents, acids and alkalis that may be used as part of the cleaning process.

Adequate cleaning procedures play an important role in preventing contamination and cross-contamination. Validation of cleaning methods provides documented evidence that an approved cleaning procedure will provide clean equipment, suitable for its intended use [(1)].
The analytical methods required for cleaning validation of active pharmaceutical ingredients require a different approach for development of different drug substances. In case of critical pharmaceutical products like peptides, these methods has to address the challenges posed by peptides viz poor ionisation, non-specific adsorption and carry-over. Shimadzu Application Development Centre (ADC) has developed a highly sensitive LC-MS/MS method for quantitation of six synthetic peptides i.e., abaloparatide, glucagon, cosyntropin, semaglutide, ganirelix acetate and liraglutide. The developed method is applicable to detect an LLOQ of 0.50 ng/mL for all 6 analytes and can be utilized for conducting the cleaning validation studies.

2. SALIENT FEATURES

- A rapid, simple, sensitive, single method for estimation of six synthetic peptides is developed to support the cleaning validation studies.
- Heated ESI along with New UF-Qarray™ ion guide technology contributes by increasing ion production and enhancing transmission, respectively. This ensures sensitive and selective quantification at 0.50 ng/mL
- Customized flow with gradient method satisfied the peak shape, retention time and background noise

3. Experimental

3.1 Sample preparation and analytical conditions

A primary stock solution of 1 mg/mL of each individual peptide was prepared by dissolving the 10 mg of working standard in a 10 mL of mixture containing DMSO: Methanol (10 : 90, v/v) in a clean and dry volumetric flask. Subsequent intermediate stock solution of 1 µg/mL was prepared in diluent containing Methanol : Water - (50 : 50, v/v).

Calibration curve standards were prepared in acetonitrile: water (50 : 50, v/v) containing 1.0% formic acid at nominal concentrations ranging from 0.50 - 250.00 ng/mL for abaloparatide & cosyntropin and 0.50-500.00 ng/mL for ganirelix, glucagon, liraglutide & semaglutide respectively.

An analytical investigation was performed using a Shimadzu LCMS-8060 mass spectrometer system (Fig.1). Chromatographic separation was carried out using a Shim-pack Velox™ column with a set column temperature of 60 ℃. The mobile phases consisted of 1.0% formic acid in water (phase A) and 1.0% formic acid in acetonitrile (phase B). A gradient elution method was employed, starting at 0% B and reaching 100% B after 10 minutes, with a flow rate of 0.20 ml/min. Subsequently, the gradient was reset to 0% B within the following 0.50 minutes, and followed by a 1.00 minute equilibration step at 0% B.

The ionization of the molecule was carried out using electrospray ionization (ESI) in positive-ion mode, with an interface temperature and voltage set at 300 ℃ and 4 kV respectively. Mass spectrometry parameters were fine-tuned by introducing a solution of the analytes at a concentration of 1 ng/ml through the LC column into the ion source, and subsequently optimizing the MS parameters manually using LabSolutions™ software. The mass spectra for the synthetic peptides were recorded within the mass range of *m/z* 100 to 1200. The compounds were differentiated based on their characteristic product ions. The acquired data was processed using LabSolutions software, and calibration curves were generated by utilizing the analyte peak area and fitting them with a linear regression model.

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3.2. Instrument parameters on LCMS-8060

Refer to Table 1 for analytical conditions and instrument parameters and Table 2 for MRM transition.

Table 1 Analytical conditions and instrument parameters

Parameter	HPLC
Column	Shim-pack Velox™ C18 column 100 x 2.1 mm, 2.7 µm, (P/N: 227-32009-03)
Mobile Phase	A: 1.0 % formic acid in water. B: 1.0 % formic acid in Acetonitrile
Flow Rate	0.20 mL/min
Oven Temp	60 ℃
Injection volume	40 µL
Parameter	**MS**
Interface	ESI
Interface temp and Voltage	300 ℃ and 4 kV
MS Mode	MRM, Positive
Heat Block Temp	400 ℃
DL Temp	250 ℃
Nebulizing Gas	3 L/min
Drying Gas	10 L/min
Heating Gas	10 L/min

Table 2 MRM transition and parameters of synthetic peptides on LCMS

Compound	MRM (*m/z*)	CE (V)
Abaloparatide	661.00-309.15	-37.0
Cosyntropin	587.20-223.10	-23.0
Ganirelix	524.20-170.05	-37.0
Glucagon	871.15-225.10	-37.0
Liraglutide	938.8-1128.60	-35.0
Semaglutide	1029.15-136.20	-41.0

Fig. 1 Nexera™ X2 with LCMS-8060 system

4. Result and Discussion

4.1. Optimization of ESI-MS/MS parameters

Regarding the mass spectrometry optimization, six synthetic peptides were subject to tuning using the LCMS-8060 system in positive ion mode. The fragmentation pattern and mass dependent parameters for each of the six synthetic peptides is presented in Table 2. The protonated precursor ion (Q1) of the synthetic peptides exhibited multiple charged states with corresponding *m/z* values of 661.00 (abaloparatide), 587.20 (cosyntropin), 524.20 (ganirelix), 871.15 (glucagon), 938.8 (liraglutide), and 1029.15 (semaglutide). Amongst the full scan of protonated precursors, the most dominant charge state Q1 masses were +6 (abaloparatide), +5 (cosyntropin), +4 (ganirelix), +3 (glucagon), +4 (liraglutide), and +4 (semaglutide). The product ion scan displayed predominant fragment ions at respective *m/z* values of 309.15 (abaloparatide), 223.10 (cosyntropin), 170.05 (ganirelix), 225.10 (glucagon), 1128.60 (liraglutide), and 136.20 (semaglutide). Detailed information on the multiple reaction monitoring (MRM) transitions utilized for the analysis can be found in Table 2.

4.2 Optimization of LC parameters

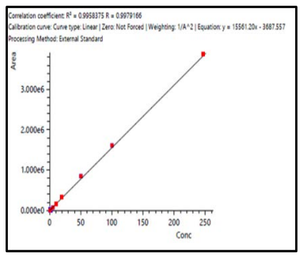
To optimize the chromatographic conditions, including the selection of column type, mobile phase composition, and nature, several trials were conducted to enhance retention and signal of the six synthetic peptides. The best chromatographic separation for simultaneous quantification of the six synthetic peptides in a single run was successfully achieved using customized gradient flow conditions on a Shim-pack Velox C18 column. To obtain well-defined peaks, the injection volume was optimized at 40 µL and the column oven temperature was set at 60 ℃.

- **Linearity**

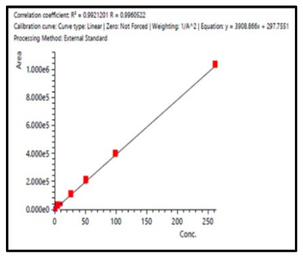
  Linearity experiments was evaluated by analysing calibration curves of abaloparatide, cosyntropin, ganirelix, glucagon, liraglutide and semaglutide. The standard curves were calculated by a weighted ($1/x^2$) least squares linear regression method through the measurement of the peak-area. The correlation coefficient (r^2) for all the six peptides was found more than 0.98 as shown in Figure 2. Calibration curve was found linear in the range of 0.50 - 250.00 ng/mL for abaloparatide and cosyntropin and 0.50 - 500.00 ng/mL for ganirelix, glucagon, liraglutide and semaglutide, respectively.

Table 3 Calibration curve data

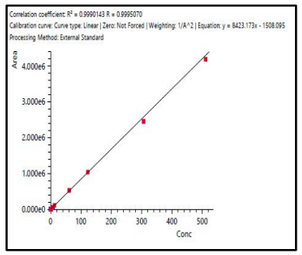
Analyte	Calibration curve range (ng/mL)	Weighing factor	Correlation coefficient (r^2)	Accuracy (%)	SNR of LLOQ (0.5 ng/mL)
Abaloparatide	0.50-250.00	$1/x^2$	0.9958	98 to 101	25
Cosyntropin	0.50-250.00	$1/x^2$	0.9921	99 to 105	22
Ganirelix	0.50-500.00	$1/x^2$	0.9990	95 to 99	15
Glucagon	0.50-500.00	$1/x^2$	0.9890	96 to 103	18
Liraglutide	0.50-500.00	$1/x^2$	0.9979	98 to 106	35
Semaglutide	0.50-500.00	$1/x^2$	0.9989	97 to 102	19



(a) Abaloparatide

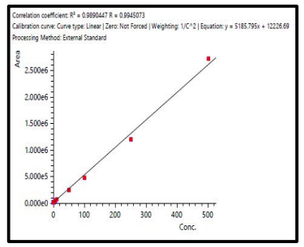


(b) Cosyntropin

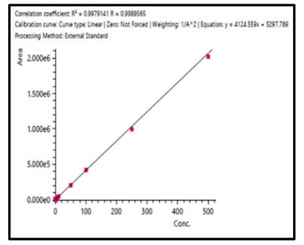


(c) Ganirellix

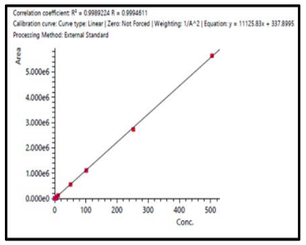
Fig. 2 Representative Calibration Curve



(d) Glucagon



(e) Liraglutide



(f) Semaglutide

Fig. 2 Representative Calibration Curve (continued….)

5. Conclusion

A highly selective and sensitive single LCMS method has been developed with the LLOQ of 0.50 ng/mL, which is more than an order of magnitude (refer to Fig.3). The method uses simple and customized gradient program. The developed method is sensitive enough to support the cleaning validation of regulatory studies. Shimadzu LCMS-8060, along with optimized chromatography provides a very selective and sensitive method for simultaneous quantification of abaloparatide, cosyntropin, ganirelix, glucagon, liraglutide and semaglutide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a gradient flow rate of 0.200 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavors.

6. References

1. https://www.who.int/medicines/areas/quality_safety/quality_assurance/SupplementaryGMPValidationTRS937Annex4.pdf?ua=1

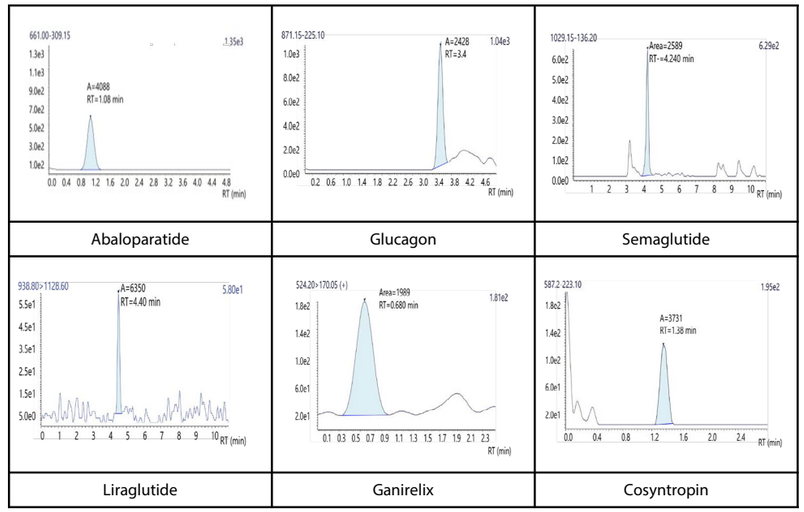


Fig. 3 Chromatograms

06-SAIP-LC-064-EN First Edition: Dec., 2023

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Application News

Fourier Transform Infrared Spectrophotometer IRXross™/IRSpirit™ Series

Identification Testing of *Myo*-Inositol Conforming to the European Pharmacopoeia (EP)

H. Iwamae

User Benefits

- Smooth identification testing is possible with the IRXross, which offers both high sensitivity and excellent operability, and the IRSpirit, a compact FTIR, by using the IR Pilot™ dedicated program included as a standard.
- IR Pilot is beneficial in routine analysis work including identification testing because the optimum measurement conditions can be set and accessed easily.
- The "peak designation judgment" function, which was newly added to IR Pilot, enables automatic pass/fail judgments based on wavenumbers specified by the user.

■ Introduction

The instrument control/data analysis software LabSolutions™ IR of the new IRXross and the IRSpirit compact FTIR (Fig. 1) includes the dedicated program IR Pilot as a standard feature, enabling simple measurement/analysis of four types: identification testing, contaminant analysis, quantitative analysis, and film thickness measurement. With this dedicated program, all steps from the actual measurement to analysis and printout can be performed automatically by the correct procedure merely by following the guidance on the screen. IR Pilot offers a total of 23 dedicated application programs as standard. It is also possible to register the entire analysis flow after a measurement and start the analysis from the main menu with one click from the next time.

This article introduces an example of identification testing of *myo*-inositol, which is an additive used in foods and pharmaceutical products, in accordance with the European Pharmacopoeia (EP).

Fig. 1 Appearance of IRXross (Left) and IRSpirit (Right)

■ *Myo*-Inositol

Myo-inositol is one type of cyclitol, which is listed as 1,2,3,5/4,6-inositol or (1R,2R,3S,4S,5R,6S)-cyclohexane-1,2,3,4,5,6-hexanol in the IUPAC nomenclature of chemistry (IUPAC: International Union of Pure and Applied Chemistry), and is a compound that exists universally in many animals and plants. Fig. 2 shows its chemical structural formula. *Myo*-inositol is the most generally distributed of the 9 isomers of inositol and is known as a water-soluble vitamin-like active substance. In addition to using an ingredient in infant formula and dietary supplements, it is also used as a liver hydrolysate preparation. *Myo*-inositol is included in various standards, including the EP and the United States Pharmacopeia (USP), and in Japanese Pharmaceutical Excipients 2018 and Japan's 9th Edition of Specifications and Standards for Food Additives (JSFA-IX), which specify identification tests utilizing the infrared spectrum.

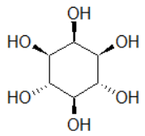


Fig. 2 Chemical Structural Formula of *Myo*-Inositol $C_6H_{12}O_6$

■ Identification Testing of *Myo*-Inositol

Monographs 1805 of the European Pharmacopoeia EP10.6 describes an identification test method for *myo*-inositol using infrared spectroscopy, in which the sample is compared with *myo*-inositol CRS (CAS Registry Number (CAS RN®): 87-89-8). The identification test in this article was conducted on the assumption that the infrared spectrum registered in the search software KnowItAll Spectroscopy Edition (Wiley Science Solutions) is the spectrum of the standard sample. Since EP Monographs 1805 does not mention the peak wavenumber to be confirmed or the allowable range of deviation (allowable error), 12 main peak positions were selected for this experiment. The allowable error between the sample and *myo*-inositol CRS was set at within 1.0 cm^{-1} from the measured resolution of 2 cm^{-1}.

A potassium bromide (KBr) pellet (φ 4 mm) was prepared as the test sample, and its infrared spectrum was measured. Table 1 shows the measurement conditions, and Fig. 3 shows the acquired infrared spectrum.

Table 1 Measurement Conditions

Instrument	:	IRXross Fourier transform infrared spectrophotometer (KBr window)
Resolution	:	2 cm^{-1}
Accumulation	:	45 times
Apodization function	:	SqrTriangle
Detector	:	DLATGS

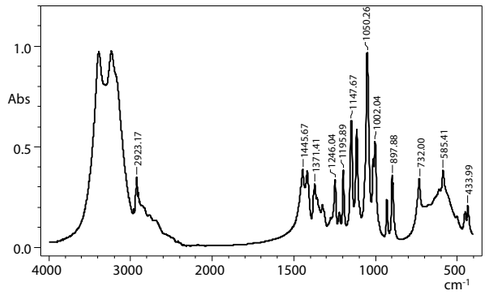
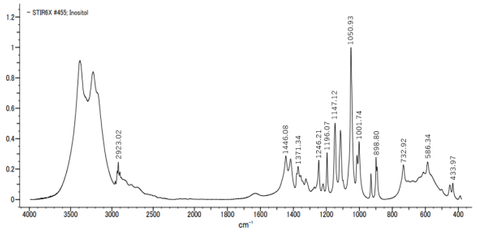


Fig. 3 Infrared Spectrum of Test Sample

Fig. 4 shows the infrared spectrum assumed here as the standard sample. Table 2 summarizes the peak wavenumber positions and the errors of the wavenumbers for the 12 main peak positions used in this experiment.



Name	Inositol
CAS Registry Number	87-89-8
Catalog Number	109102
Formula	C6H12O6
InChI	InChI=1S/C6H12O6/c7-1-2(8)4(10)6(12)5(11)3(1)9/h1-12H/t1-2-,3-,4+,5-,6-
InChI Key	CDAISMWEOUEBRE-GPIVLXJGSA-N
Instrument Name	Bruker Tensor 27 FT-IR
Melting Point	222 - 227 °C
Mol Weight	180.156 g/mol
Purity	>98%
Source of Sample	Spectrochem Pvt. Ltd.
Source of Spectrum	Bio-Rad Laboratories, Inc.
Technique	KBr-T

Fig. 4 Infrared Spectrum of *Myo*-Inositol (CAS Registry No.: 87-89-8)
(Source: Search Software KnowItAll Spectroscopy Edition)

Table 2 Main Peak Wavenumbers and Errors

Peak wavenumber in Fig. 3 (cm^{-1})	Peak wavenumber in Fig. 4 (cm^{-1})	Error of wavenumber (cm^{-1})
2923.17	2923.02	0.15
1445.67	1446.08	0.41
1371.41	1371.34	0.07
1246.04	1246.21	0.17
1195.89	1196.07	0.18
1147.67	1147.12	0.55
1050.26	1050.93	0.67
1002.04	1001.74	0.30
897.88	898.80	0.92
732.00	732.92	0.92
585.41	586.34	0.93
433.99	433.97	0.02

According to Table 2, peaks could be confirmed at the same wavenumbers and the largest error of the wavenumbers was 0.93 cm^{-1}. Therefore, it can be surmised that the measured sample is *myo*-inositol.

■ Higher Efficiency in Identification Testing by Using IR Pilot

As described above, an identification test was carried out by comparing the peak positions in the infrared spectra of a standard sample and the test sample. The following introduces an example of labor-saving in this operation.

Sample measurement and peak detection can be automated by using the IR Pilot dedicated program, which is provided with the IRXross and the IRSpirit as a standard feature.

IR Pilot is a convenient program that can simplify the entire workflow in identification test, from measurement and analysis of the target sample to printout, merely by selecting the four items ① Analysis purpose, ② Measurement technique, ③ Accessory to be used, and ④ Necessary data process in accordance with the guidance shown on the screen.

The measurement under the optimum conditions, as shown in Table 1, is possible merely by selecting "Identification test corresponding to pharmacopoeia," "Transmission spectroscopy," and "KBr pellet." By using "Peak pick" and "Peak designation judgment" for the analysis after the measurement, it is possible to prepare a report that includes detection of the main peaks and the detection results, compare the infrared spectra of the standard sample and test sample at the specified peak wavenumbers, and make a pass/fail judgment by a simple procedure.

The following explains this procedure using the condition setting screen of "Peak designation judgment" as an example (see Fig. 5). Here, the major peak positions were registered referring to the infrared spectrum of *myo*-inositol (CAS Registry No.: 87-89-8) registered in the search software KnowItAll Spectroscopy Edition. When performing an identification test, the user should purchase a standard sample and acquire the infrared spectrum. A maximum of 10 peak wavenumbers can be used in the comparison, and a maximum of 4 peak intensity ratios can be set. The test sample is judged as Pass if the error of the wavenumbers is within the allowable range set by the user. If the user registers the program after executing the analysis, only the background (BKG) measurement and the sample measurement operations are necessary when conducting identification tests in the future. In addition to the national pharmacopoeias, this system can also be used in identification tests by other official analysis methods.

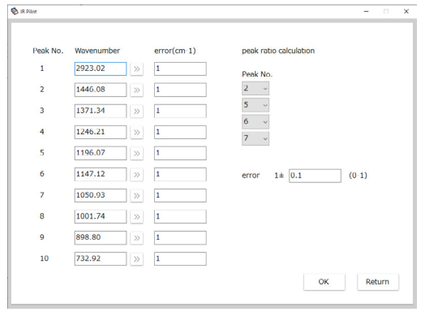


Fig. 5 Condition Setting Screen for Peak Designation Judgment

■ Conclusion

An identification test was conducted for *myo*-inositol, which is a type of additive used in food and pharmaceutical products. Measurement and peak detection conforming to the European Pharmacopoeia (EP) were carried out, and agreement of the absorbed wavenumbers of the test sample with the spectrum of an assumed standard sample was confirmed. If a standard sample is prepared, the entire process, including the pass/fail judgment, can be carried out automatically, allowing further labor-saving in identification tests. In identification tests based on official analysis methods, IR Pilot saves the user the time and trouble required to set analysis conditions and prepare reports, resulting in more efficient testing.

<Reference>
European Pharmacopoeia (EP 10.6), Monographs, *myo*-inositol

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07

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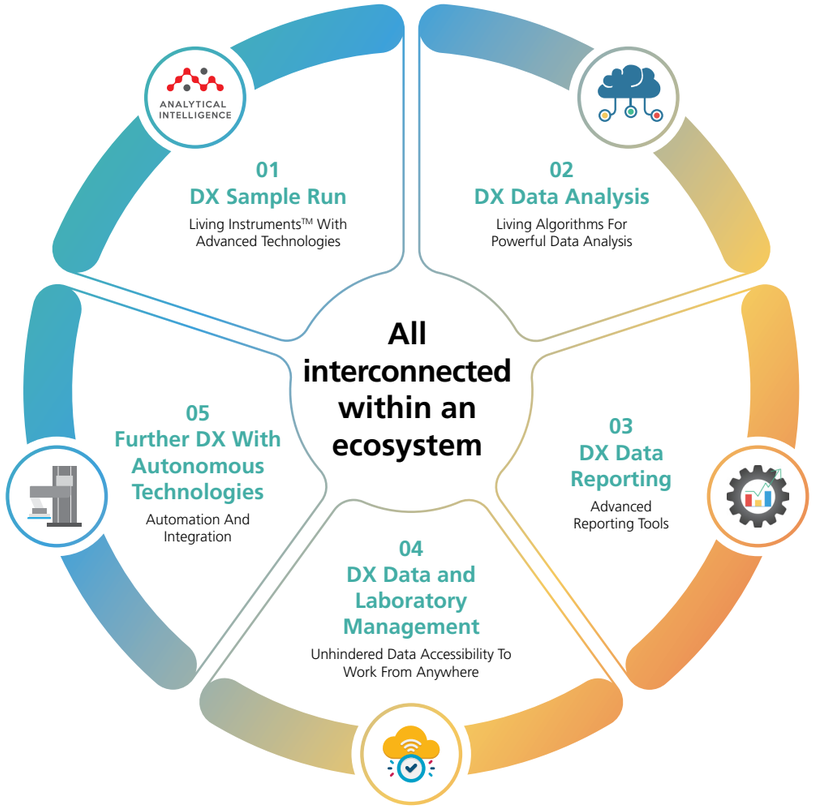

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