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Application News

High Performance Liquid Chromatograph Mass Spectrometer

High-Throughput Analysis of Drug Metabolites Using a Triple Quadrupole LC-MS/MS System

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User Benefits

- The high-speed analysis time of 0.80 min contributes to higher throughput.
- Nexera™ X4's excellent high-speed gradient performance in combination with the LCMS™-8060RX CoreSpray technology enables consistent and reliable trace quantitative analysis.

■ Introduction

In pharmacokinetic studies, drug concentrations in biological samples are analyzed to clarify the absorption, distribution, metabolism, and excretion of drugs in vivo. Because rapid results are required and a large number of samples must be handled, faster analytical methods are increasingly important.

This report describes an example of high-throughput analysis of six drug metabolites using a system consisting of a Nexera X4 ultra high-performance liquid chromatograph and a LCMS-8060RX triple quadrupole mass spectrometer.

Fig. 1 Nexera™ X4 and LCMS-8060RX

■ Sample Preparation

Reaction solutions obtained after metabolic reactions by cytochrome P450 (CYP), with coumarin, diclofenac, and nifedipine added as substrates, were used as analytical samples. The sample preparation process is shown in Fig. 2.

Prepare 50 µL of microsome mixture
- 0.2 mg/mL Human liver microsome
- Substrates (coumarin and diclofenac 2 µmol/L, nifedipine 1 µmol/L)
- 100 mM Potassium phosphate buffer (pH 7.4)

↓

Preincubate for 5 min at 37 °C

↓

Add 50 µL of NADPH regeneration system
- 6.6 mM Magnesium chloride
- 6.6 mM Glucose-6-phosphate
- 2.6 mM NADP+
- 0.8 U/ml Glucose-6-phosphate dehydrogenase
- 100 mM Potassium phosphate buffer (pH 7.4)

↓

Incubate for 10 min at 37 °C

↓

Add 300 µL cold Acetonitrile (Stop the reaction)

↓

Centrifuge with 10,000 g for 10 min

↓

Dispense 150 µL of supernatant for measurement

Fig. 2 Pretreatment Procedure

■ Analysis Conditions

The LC-MS/MS analysis conditions are indicated in Table 1. The target compounds and MRM conditions are indicated in Table 2.

Table 1 Nexera X4 and LCMS-8060RX Analysis Conditions

HPLC Conditions	
System:	Nexera X4
Column:	Shim-pack Scepter C18-120 (20 mm × 2.1 mm I.D., 1.9 µm)*1
Temperature:	30 °C
Injection Volume:	1 µL
Mobile Phases:	A) 0.05 % Formic acid in water B) 0.05 % Formic acid in acetonitrile
Flowrate:	0.4 mL/min
Time Program:	B conc. 3 % (0.00 - 0.05 min) → 95 % (0.20 - 0.60 min) → 3 % (0.61-0.80 min)
Rinsing Type:	External rinse
Mixer:	Micro mixer
Sample Loop Volume:	15 µL
Vial:	Shim-vial H glass*2 (150 µL)
MS Conditions	
System:	LCMS-8060RX
Ionization:	ESI (Positive and negative mode)
Mode:	MRM
Nebulizing Gas Flow:	5 L/min
Heating Gas Flow:	15 L/min
Interface Temp.:	400 °C
DL Temp.:	300 °C
Block Heater Temp.:	400 °C
Drying Gas Flow:	5 L/min

*1 P/N: 227-31012-01 *2 P/N: 227-34500-11

Table 2 Target Compounds and MRM Conditions

Compound	Polarity	Target *m/z*	Reference *m/z*
Resorufin	+	214.10 > 186.10	214.10 > 103.10
4′-Hydroxydiclofenac	+	312.00 > 230.10	312.00 > 265.90
Oxidized Nifedipine	+	345.10 > 284.10	345.10 > 152.00
6-Hydroxychlorzoxazone	-	184.00 > 64.05	184.00 > 120.00
7-Hydroxycoumarin	-	161.00 > 133.10	161.00 > 105.10
Hydroxy Tolbutamide	-	285.10 > 186.00	285.10 > 104.10

Fig. 3 Shim-vial H (150 µL) Vial

A Shim-vial H (150 µL) was used as the vial (Fig. 3). This small-volume vials with their unique shape are made of the same materials and receive the same cleaning treatment as the 1.5 mL vials. They suppress adsorption more than other small-volume inserts by limiting the contact surface between the sample and the container. In addition, the bottom surface of the vial is hollowed out in order to increase thermal conductivity.
(Residual volume: Approx. 10 µL)

The LCMS-8060RX was used as the mass spectrometer. CoreSpray, adopted in the LCMS-8060RX, achieves stable ionization by further improving nebulizer flow uniformity and extending the upper flowrate limit to 7 L/min. Consistent ion injection reduces variation within the same sample and between instruments (Fig. 4).

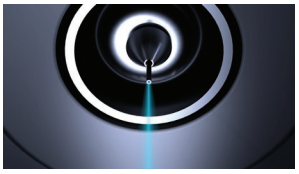

Fig. 4 CoreSpray Injection in the LCMS-8060RX

The Nexera X4 LC unit used, provides highly consistent solvent delivery during gradient analysis and demonstrates excellent retention time repeatability even in ultra-high-speed analysis. That significantly increases analysis speed while maintaining analytical quality equivalent to conventional HPLC.

The analysis time for this method, including column equilibration, is 0.80 min, enabling rapid processing of a huge number of samples. Furthermore, the Nexera X4 features tool-less fittings (Fig. 5) for column connections, allowing simple and reliable column replacement even for ultra-high pressure analysis.

Fig. 5 Tool-less Fittings in Nexera X4 System

■ Evaluation of Retention Time and Peak Area Repeatability

A 10 nmol/L standard sample was analyzed six times using the indicated method to evaluate the repeatability of retention times and peak areas.

The standard deviation of retention times was 0.010 to 0.013 sec, and the relative standard deviation of peak area was 3.0 % or less, confirming that sufficient analytical consistency was maintained even under high-speed gradient conditions.

Table 3 Evaluation of Retention Time and Peak Area Repeatability (n = 6)

Compound	Retention time (min)	Standard deviation of retention time (sec)	Area %RSD
Resorufin	0.399	0.010	2.54
4′-Hydroxydiclofenac	0.432	0.011	2.38
Oxidized Nifedipine	0.451	0.013	1.74
6-Hydroxychlorzoxazone	0.391	0.012	1.96
7-Hydroxycoumarin	0.399	0.010	1.65
Hydroxy Tolbutamide	0.400	0.012	2.28

■ Results for Standard Samples

Calibration standards were prepared over the range of 0.01 to 200 nmol/L, and linearity was confirmed from the results of six repeated analyses. The calibration range and coefficients of determination are summarized in Table 4, and representative chromatograms are shown in Fig. 6.

The results showed good linearity for all compounds, with R^2 > 0.995 within the specified concentration range. All calibration points satisfied the criteria of accuracy within 80–120 % and precision within 20 % RSD for concentration.

Table 4 Calibration Summary Table

Compound	Quantification Range (nmol/L)	Coefficient of Determination R^2
Resorufin	0.5 – 100	0.999
4′-Hydroxydiclofenac	0.1 – 200	0.996
Oxidized Nifedipine	0.01 – 50	0.999
6-Hydroxychlorzoxazone	0.5 – 200	0.995
7-Hydroxycoumarin	0.5 – 100	0.995
Hydroxy Tolbutamide	0.05 – 200	0.997

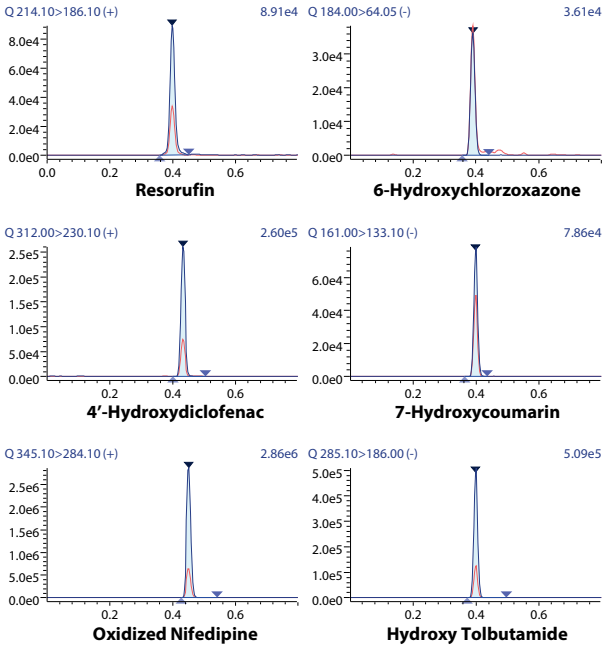

Fig. 6 Representative Chromatograms (10 nmol/L)

■ Results for CYP-Metabolized Samples

Using the same method, samples after CYP-mediated metabolic reactions were analyzed. As shown in the chromatograms in Fig. 7, no interfering peaks were observed in the unspiked sample solution, while the target metabolites (4′-hydroxydiclofenac, oxidized nifedipine, and 7-hydroxycoumarin) were detected with good peak shapes in the substrate-spiked sample solution.

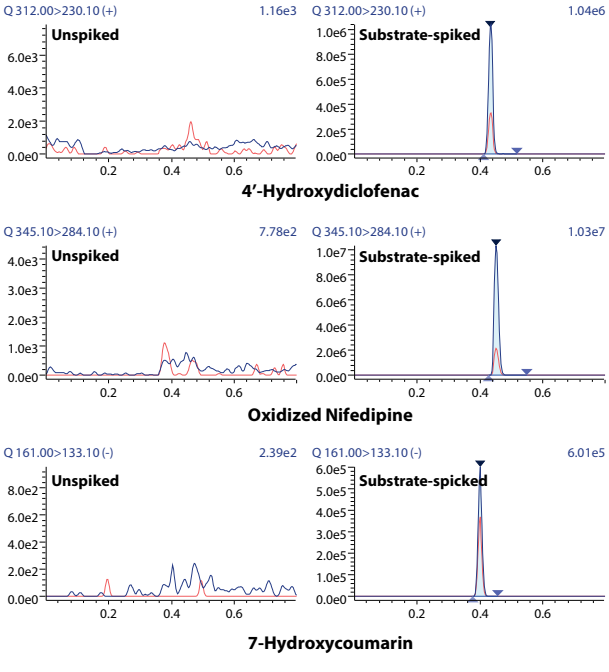

Fig. 7 Chromatograms of CYP-Metabolized Samples

■ Evaluation of Matrix Effects

The matrix factor (MF) was evaluated by adding the target metabolites to an unspiked CYP metabolic reaction sample solution. The results are indicated in Table 5. The MF ranged from 0.89 to 1.07, indicating no significant matrix effects and confirming that consistent analysis is possible with this method.

Table 5 Results of Matrix Effect Evaluation

Compound	Spiked conc.	MF (Average, n = 6)	Conc. %RSD (n = 6)
Resorufin	5 nmol/L	0.98	2.08
4'-Hydroxydiclofenac		0.90	2.98
Oxidized Nifedipine		0.89	2.98
6-Hydroxychlorzoxazone		1.07	4.03
7-Hydroxycoumarin		1.03	1.91
Hydroxy Tolbutamide		0.99	3.74

■ Conclusion

Using the Nexera X4 ultra high-performance liquid chromatograph and the LCMS-8060RX triple quadrupole mass spectrometer, six drug metabolites were analyzed. Despite ultra-fast 0.80 min analysis times, including column equilibration, retention time and peak area repeatability were good, confirming that consistent analysis is possible. This analytical system is expected to be useful for analyses that require high-speed performance, such as pharmacokinetic studies, where a large number of samples must be processed in a short time.

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