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Application News

Liquid Chromatograph Mass Spectrometer

Rapid Analysis of Anticoagulants in Plasma Using LC-MS/MS

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User Benefits

- Nexera™ X4 significantly improves solvent delivery responsiveness, providing short analysis cycle time and rapid processing of a large number of samples.
- High-speed needle internal/external rinsing function completes needle cleaning within analysis time.
- Eight anticoagulants in plasma can be quantified with simple pretreatment of just deproteinization.

■ Introduction

In pharmacokinetic studies, blood drug level measurement is performed to clarify the dynamics of drug absorption, distribution, metabolism, and excretion within human body. High-speed analysis is paid important attention because of rapid requirement of analytical results and huge number of samples to treat.

This article presents ultra-high-speed analysis for eight anticoagulants involving five direct oral anticoagulants/new oral anticoagulants (DOACs/NOACs)*1 performed by combination use of Nexera X4 high-performance liquid chromatograph and triple quadrupole mass spectrometer (Fig. 1).

Nexera X4 achieves significantly improved solvent delivery responsiveness through four independently actuated plungers and pressure feedback mechanism. This provides reliable results in ultra-high-speed analyses as is described in this article.

*1 Direct/Novel Oral Anticoagulants, oral anticoagulants used for the treatment and prevention of thromboembolism as an alternative to vitamin K antagonists such as warfarin

Fig. 1 Nexera™ X4 and LCMS-8060RX

■ Sample pretreatment

Eight anticoagulants listed in Table 1 were added to human plasma to prepare calibration and QC samples. Calibration samples ranged from 10 to 500 µg/L (100 to 5000 µg/L for Acenocoumarol and Warfarin) were used with the stable isotope-labeled compound of each targeted compound as its internal standard (added at 100 µg/L in the solution). QC samples were prepared at four levels: 10 µg/L (LLOQ), 20 µg/L (Low), 200 µg/L (Middle), and 400 µg/L (High). (Acenocoumarol and Warfarin: 100 µg/L (LLOQ), 200 µg/L (Low), 2000 µg/L (Middle), 4000 µg/L (High))

Human plasma samples spiked with anticoagulants were deproteinized using the pretreatment shown in Fig. 2 to be subjected to LC/MS analyses.

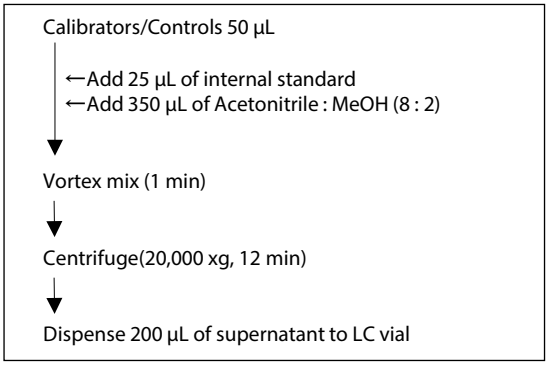

Fig. 2 Pretreatment procedure

Table 1 Targeted anticoagulants

Compound	Type	*m/z*	Reference *m/z*
Acenocoumarol	Target analyte	354.10>163.10	354.10>296.10
Apixaban		460.20>443.10	460.20>199.20
Argatroban		509.20>384.20	509.20>70.00
Betrixaban		452.10>324.10	452.10>279.10
Dabigatran		472.20>289.20	472.20>144.20
Edoxaban		548.20>366.20	548.20>152.10
Rivaroxaban		436.10>145.10	436.10>231.10
Warfarin		309.10>163.10	309.10>251.10
[$^{2}H_4$]-Acenocoumarol	Internal standard	358.10>167.10	358.10>300.10
[^{13}C, $^{2}H_8$]-Apixaban		469.20>452.20	469.20>199.10
[$^{13}C_6$]-Argatroban		515.20>390.20	515.20>70.00
[$^{13}C_6$]-Betrixaban		458.10>330.10	458.10>285.10
[$^{13}C_6$]-Dabigatran		478.20>295.20	478.20>144.20
[$^{2}H_6$]-Edoxaban		554.20>372.20	554.20>158.10
[$^{13}C_6$]-Rivaroxaban		442.10>145.10	442.10>237.10
[$^{2}H_6$]-Warfarin		315.10>163.10	315.10>257.10

■ Analytical conditions

Analysis was performed using ultra-high-speed liquid chromatograph Nexera X4 and triple quadrupole mass spectrometer LCMS-8060RX under the conditions shown in Table 1 and Table 2.

Nexera X4 provides high stability of solvent delivery during gradient analysis and demonstrates excellent retention time repeatability even in ultra-high-speed analysis. It significantly increases analysis speed while maintaining analytical quality equivalent to conventional HPLC. Solvent delivery unit of Nexera X4 employs four independently actuated plungers and pressure feedback mechanism. This provides individual plunger operation for optimized suction and discharge timing of mobile phase delivery, resulting in a significant reduction of pump induced pulsation. Consequently, the solvent delivery responsiveness is improved owning to the minimized internal volume required for the reduction of solvent delivery pulsation.

The analysis time for this method, including column equilibration, is 2.45 min, providing rapid processing of a huge number of samples. Furthermore, Nexera X4 employs tool-less fittings (Fig. 3) for column connection, allowing simple and reliable column replacement even for ultra-high pressure analysis.

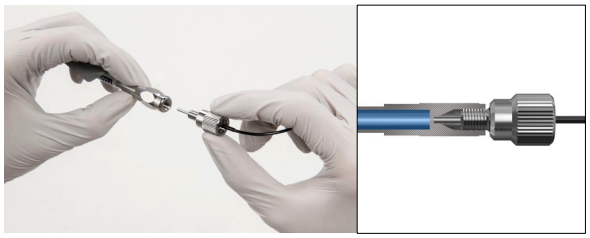

Fig. 3 Tool-less fittings equipped in Nexera X4

Table 2 Analytical conditions of Nexera X4 and LCMS-8060RX

HPLC conditions	
System	: Nexera X4
Column	: Shim-pack™ NovaCore C18-HB*2 (50 mm × 2.1 mm I.D., 1.7 µm)
Temperature	: 50 ˚C
Injection volume	: 1 µL
Mobile phases	: A) 5 mmol/L ammonium formate, 0.1 % formic acid in Water B) 5 mmol/L ammonium formate, 0.1 % formic acid in MeOH : Water (9 : 1)
Flow rate	: 0.5 mL/min (0.00-1.50 min)→0.8 mL/min (1.51-2.00 min)→0.5 mL/min(2.01-2.45 min)
Time program	: B conc. 5 % (0.00-0.20 min)→ 98 % (1.20-2.00 min)→5 % (2.01-2.45 min)
Rinsing Type	: Internal & External Rinse (Fast)
Sample loop volume	: 15 µL
MS conditions	
System	: LCMS-8060RX
Ionization	: ESI, Positive mode
Interface voltage	: 1.0 kV
Mode	: MRM
Nebulizing gas flow	: 3 L/min
Heating gas flow	: 15 L/min
Interface temp.	: 400 ˚C
DL temp.	: 200 ˚C
Block heater temp.	: 400 ˚C
Drying gas flow	: 5 L/min

*2 P/N : 227-32901-04

■ Analytical results

Typical chromatogram and gradient profile are shown in Fig. 4. Calibration curves and chromatograms at lowest calibration point of respective compounds are shown in Fig. 5. Table 3 summarizes the results of evaluating accuracy and precision (%CV) based on six repeated analyses of calibration and QC samples.

All compounds showed good linearities within the specified concentration ranges. The accuracies of the QC samples were within 100 ± 15%, and precisions were within 15%, indicating good repeatability[1].

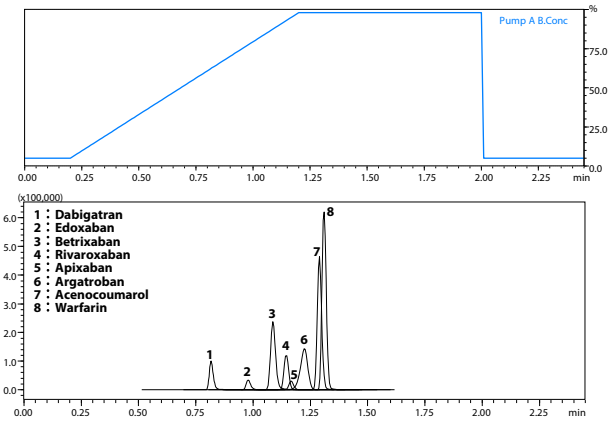

Fig. 4 Gradient profile and typical chromatogram

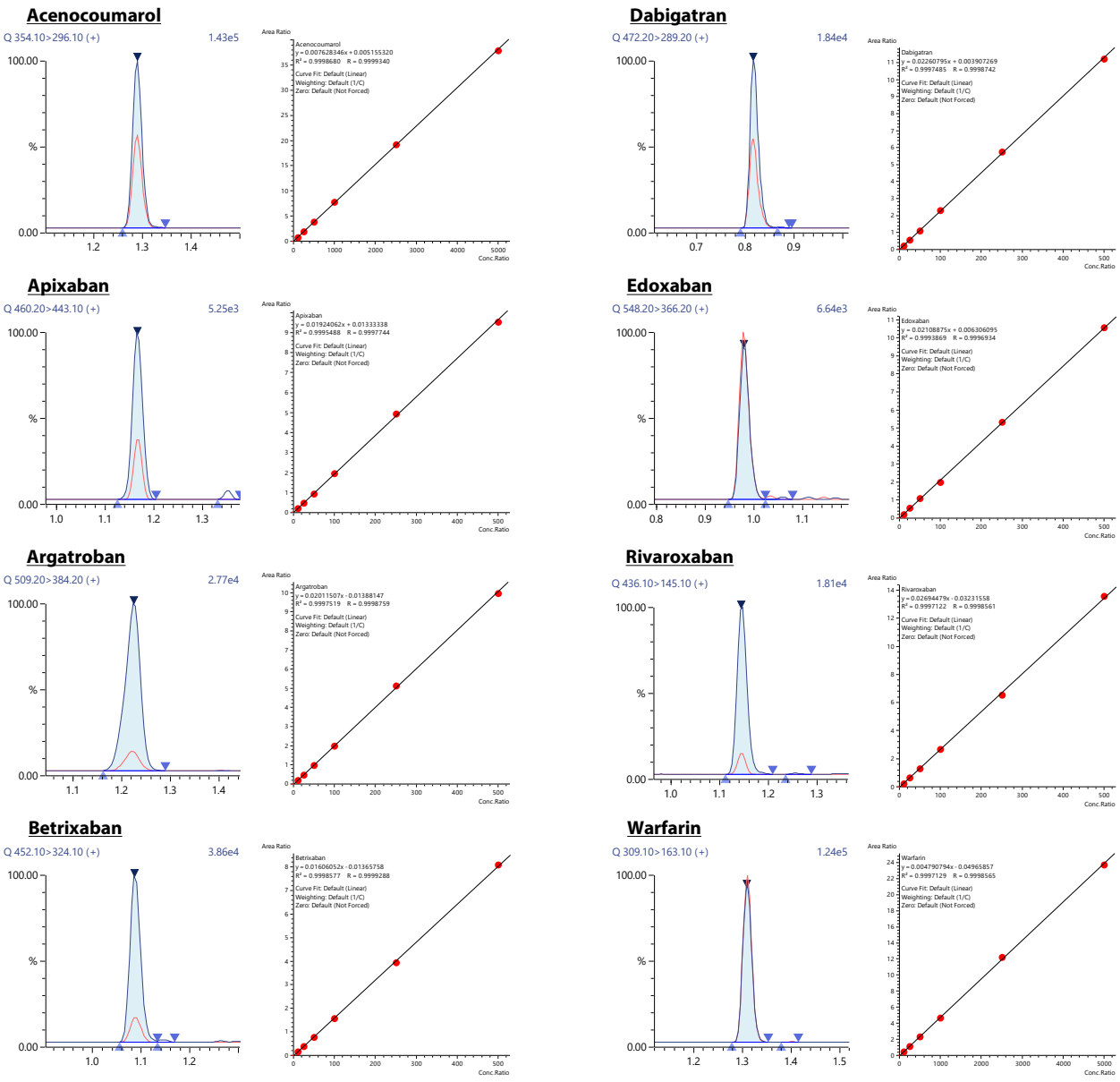

Fig. 5 Calibration curves and chromatograms (the lowest point of the curve)

Table 3 Analytical results of QC samples

Compound	Calibration range (µg/L)	Accuracy (%)				Precision (%CV), n=6			
		LLOQ (10 µg/L)	Low (20 µg/L)	Middle (200 µg/L)	High (400 µg/L)	LLOQ (10 µg/L)	Low (20 µg/L)	Middle (200 µg/L)	High (400 µg/L)
Acenocoumarol *3	100-5000	99.7	104.7	96.5	95.4	5.1	8.0	0.7	4.7
Apixaban	10-500	96.1	97.9	97.2	97.4	10.6	12.6	5.8	6.3
Argatroban	10-500	98.8	105.6	101.6	106.2	3.7	2.7	2.7	2.9
Betrixaban	10-500	104.0	108.3	104.0	104.7	3.8	7.4	3.7	2.5
Dabigatran	10-500	99.7	104.7	96.5	95.4	6.0	2.9	4.7	3.2
Edoxaban	10-500	96.1	97.9	97.2	97.4	13.7	3.6	6.4	5.6
Rivaroxaban	10-500	98.8	105.6	101.6	106.2	7.2	4.8	4.8	4.6
Warfarin *3	100-5000	104.0	108.3	104.0	104.7	4.0	6.5	3.7	6.6

*3 100 µg/L for LLOQ, 200 µg/L for Low, 2000 µg/L for Middle, 4000 µg/L for High

■ High-speed internal/external needle rinsing to suppress carryover

Evaluation of carryover effect is important as well in developing analytical methods. Observed carryover could be reduced or prevented in some cases using rinsing function equipped in autosampler, but it should be completed within an extremely short analysis time in case of ultra-high-speed analysis. SIL-40C X4 is a high-end autosampler equipped with a high-speed internal and external rinsing function. The analytical method described in this article completes both internal and external needle rinsing and the equilibration inside the needle within the analysis time as shown in Fig. 6. The high-speed internal/external needle rinsing function contributes to reduction of carryover while maintaining analysis cycle time.

SIL-40 X4 for Nexera X4 provides reliable HPLC analysis of a wide variety of samples from low concentration requiring high sensitivity to high concentration where carryover is a concern.

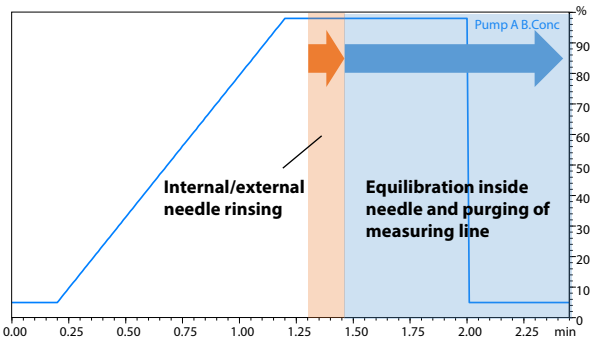

Fig. 6 Time sequence diagram for internal/external needle rinsing

■ Conclusion

Eight anticoagulants were analyzed using ultra-high-performance liquid chromatograph Nexera X4 and the triple quadrupole mass spectrometer LCMS-8060RX. With a simple pretreatment of just deproteination, favorable results were obtained in the evaluation of accuracy and precision of QC samples. The analysis time including column equilibration was 2.45 minutes, providing the rapid processing of a huge number of samples.

Nexera X4 HPLC, featuring industry-leading low-dispersion design and cutting-edge fluid control technology, demonstrates its high performance especially in analyses that require high-speed availability for rapid processing of a huge number of samples, such as pharmacokinetic studies.

<References>

1) Guideline on bioanalytical method validation and study sample analysis (December 2024, November, Ministry of Health, Labor and Welfare, Japan)

01-01078-EN First Edition: Mar. 2026

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