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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

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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

Multivalent ion analysis was performed using LabSolutions Insight™ Biologics. In the parameter settings, pre-registered nucleic acid bases, linkers, riboses, and modifications were selected to create a sequence of target sample. Shortmers (n-x) was selected as the target modification (expected impurity). Sequence components and target modifications can be freely added.

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 10 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), 5988 for n-3 (3') (theoretical MW: 5987), 6775 for n-1 (3') (theoretical MW: 6776), and 7168 for the FLP (theoretical MW: 7169). These results showed small mass errors from the theoretical values, indicating high accuracy of the LCMS-2050 system.

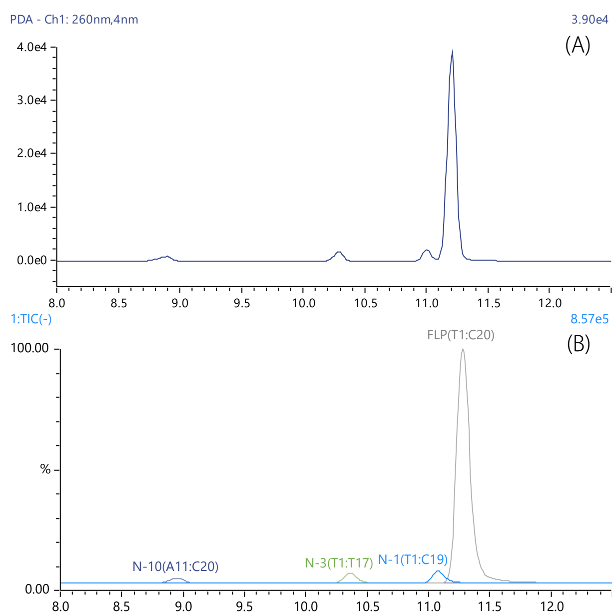

Fig. 2 Chromatograms of Model Oligonucleotide
(A) UV Chromatogram, (B) TIC Chromatogram

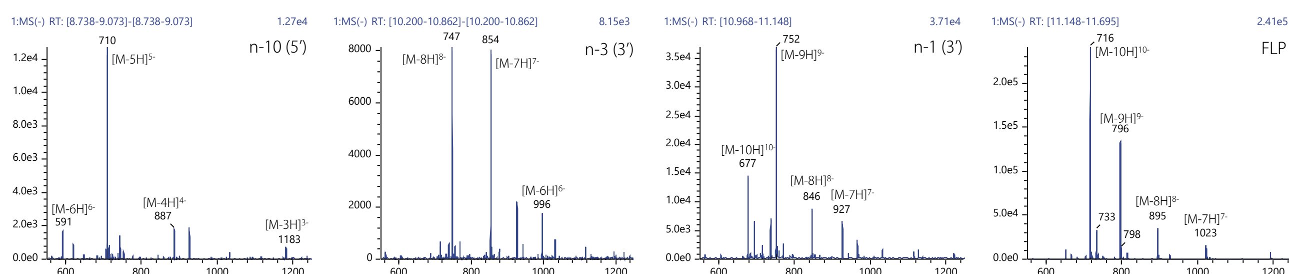

Fig. 3 Mass Spectra of Impurities and FLP

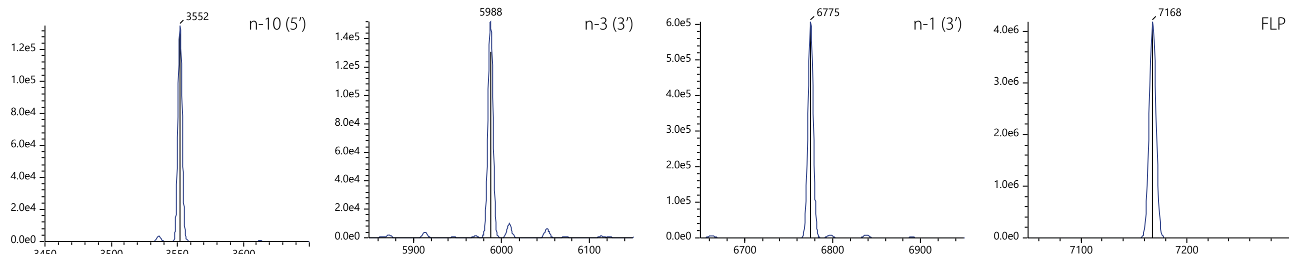

Fig. 4 Deconvolved Mass Spectra

■ 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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