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High Performance Liquid Chromatograph Mass Spectrometer

Application News

Analysis of Oligonucleotide Impurities Using Single Quadrupole Mass Spectrometer

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User Benefits

- Combination use of LCMS-2050 single quadrupole mass spectrometer and LabSolutions Insight™ Biologics provides comprehensive characterization of oligonucleotides and related impurities.
- Purity calculations for target components can be performed even on poorly separated peaks by utilizing MS spectra simultaneously acquired with UV chromatogram.

■ Introduction

In recent years, oligonucleotide therapeutics have been paid attention as a novel drug discovery modality because of their rapid progress of development. During the synthesis of oligonucleotide therapeutics, impurities that have differing base chain lengths are generated along with the target oligonucleotide. To ensure the safety and efficacy of the drug, comprehensive detection and identification of these impurities are required.

Reversed phase ion-pair chromatography is often employed as a method to separate the target compound from co-existing impurities. However, it is often difficult to establish analytical conditions that provide compete separation of all impurities that are formed in synthesis process due to their similar structures and properties to the principal compound of active ingredient. Against this drawback, liquid chromatography-mass spectrometry (LC-MS) is commonly used for impurity detection and identification. For analysis of oligonucleotide impurities, quadrupole time-of-flight mass spectrometers are mainly used because of their high mass resolution and ability to perform sequence and structure estimations via MS/MS analysis. However, in quality control process, the use of a single quadrupole mass spectrometer is expected to be advantageous because of providing mass information with easy operation when impurity information is already known.

This article presents a simulated impurity analysis of synthetic oligonucleotide using the LCMS-2050 high-performance liquid chromatography-mass spectrometer and LabSolutions Insight Biologics.

■ Sample

Oligonucleotide (FLP) modeled on Mipomersen's sequence as the target, along with n-1(5') molecule (missing one base from the 5' end) and n-3(5') molecule (missing three bases from the 5' end) as the related impurities were employed as a simulated sample for this study. Table 1 shows the sequences for FLP, n-1(5'), and n-3(5').

Table 1 Sequence information for respective components*

Abbreviations	Sequence(5′→3′)
FLP	MG-MC-MC-MU-MC-dA-dG-dT-dC-dT dG-dC-dT-dT-dC-MG-MC-MA-MC-MC
n-1(5′)	MC-MC-MU-MC-dA-dG-dT-dC-dT dG-dC-dT-dT-dC-MG-MC-MA-MC-MC
n-3(5′)	MU-MC-dA-dG-dT-dC-dT dG-dC-dT-dT-dC-MG-MC-MA-MC-MC

* Positions 5 of M: 2′-O-(2-methoxyethyl) nucleoside; d: 2′-deoxynucleoside; C: cytosine, and U: uracil are substituted by methyl groups. All phosphodiester bonds between nucleotides have been substituted by phosphorothioate bonds.

■ Analytical conditions

The employed instrument setup was Nexera™ XS inert and LCMS-2050. HPLC conditions are shown in Table 2, and MS conditions are shown in Table 3. The conditions were set where n-1(5') co-eluted with FLP, while n-3(5') was separated (Fig. 1) to demonstrate impurity analysis performance.

Table 2 HPLC analytical conditions

System	:	Nexera XS inert
Column	:	Shim-pack Scepter™ Claris C18-120 (100 mm × 2.1 mm I.D., 1.9 µm) *1
Mobile Phase A	:	100 mmol/L HFIP, 10 mmol/L TEA in water
Mobile Phase B	:	100 mmol/L HFIP, 10 mmol/L TEA in methanol
Time Program	:	15% B(0 min)→30% B(6 min) →45% B(6.1-8 min)→15% B(8.1-11 min)
Flow Rate	:	0.4 mL/min
Column Temp.	:	60 ℃
Injection Vol.	:	5 µL
UV Detection	:	190-800 nm (photodiode array detector)

*1 P/N : 227-31210-02

Table 3 MS detection conditions

Ionization	:	ESI/APCI (DUIS™), Negative mode
Mode	:	Scan (*m/z* 650-2000)
Nebulizing gas flow	:	3.0 L/min
Drying gas flow	:	5.0 L/min
Heating gas flow	:	7.0 L/min
Desolvation temp.	:	500 ℃
DL temp.	:	250 ℃
Interface voltage	:	-3.0 kV

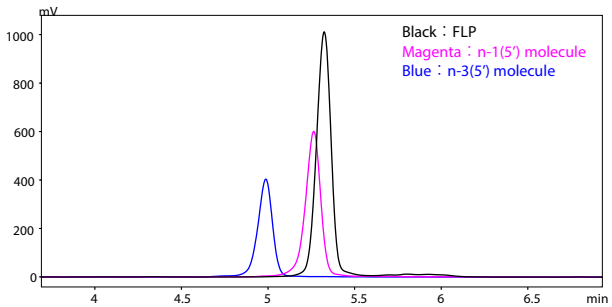

Fig. 1 Chromatograms of FLP, n-1(5′), and n-3(5′) (260 nm by PDA detector)

■ Data processing parameters

LabSolutions Insight Biologics is data processing software for oligonucleotides and related impurities. In the setting screen shown in Fig. 2, select nucleobases, linkers, ribose, nucleobase modifications, etc., to create the FLP sequence. These parameters can be freely edited. the molecular formula and mass information appear in the lower left, while the structural formula appears on the right after entering the sequence.

In LC-MS, even an isolated single component may be detected at different *m/z* due to differences in valence or isotopes. Insight Biologics can combine peaks originated from different valences or isotopes into a single component chromatogram to be displayed.

In this article, the peak areas of respective components are calculated using the following formula.

$$\text{Component peak area} = (\text{peak area in UV chromatogram}) \times \left(\frac{\text{MS peak area of target component involved in UV–corresponding peak}}{\text{Sum of MS peak areas of all components involved in UV–corresponding peak}} \right)$$

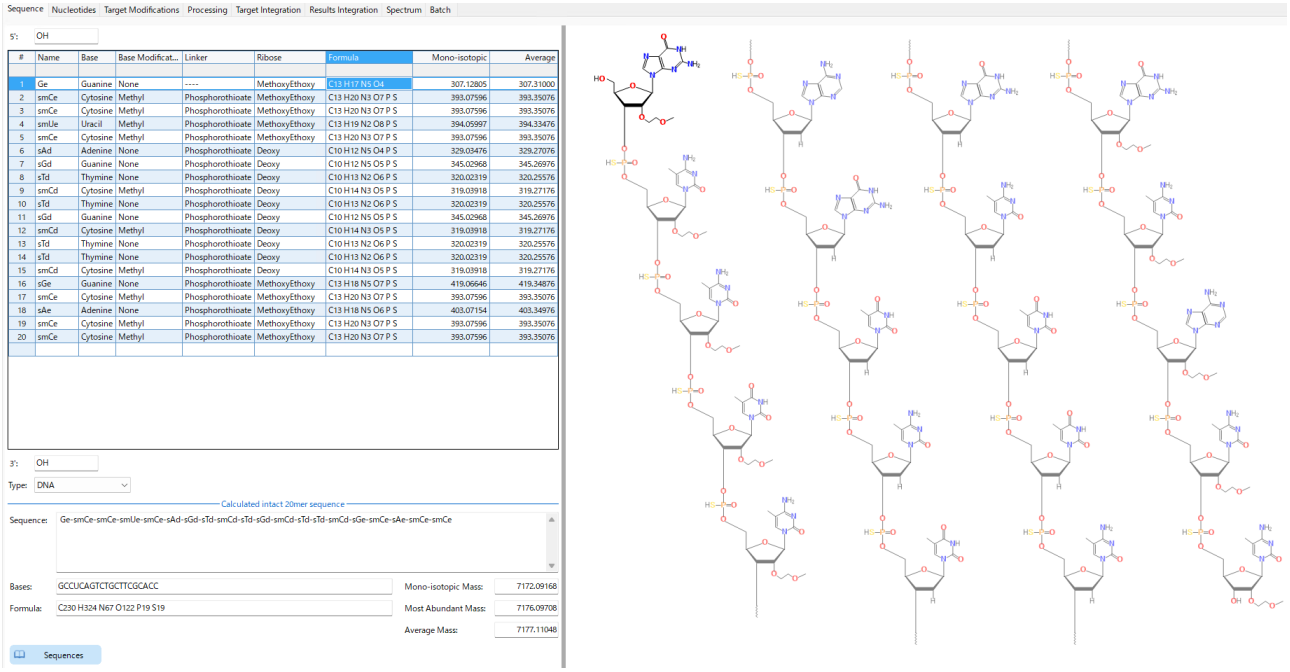

Fig.2 Setting screen of LabSolutions Insight Biologics (oligonucleotide sequence)

■ Quantitation od impurities in FLP

The linearity of the component peak area and concentration of FLP was confirmed using LabSolutions Insight Biologics. The results showed a coefficient of determination of 0.999 or higher over the range of 1, 5, 10, 25, and 50 µmol/L.

Next, samples were prepared and analyzed by adding each nucleotide-deficient to FLP at 20 µmol/L, which was within the linearity range. Both nucleotides-deficient of n-1(5') and n-3(5') were detected and identified when added at 0.25% or more relative to FLP (equivalent to an added concentration of 0.05 µmol/L). Fig. 3 shows the UV chromatogram and component chromatogram for n-1(5') added at 0.25% relative to FLP. Fig 4 and Fig. 5 show the results of multivalent ion data processing.

Fig. 6 shows the UV chromatogram and component chromatogram of adding 0.25% n-3(5') to FLP. Fig. 7 and Fig. 8 show the results of multivalent ion data processing.

The concentrations of each nucleotide-deficient contained in FLP and obtained component peak areas showed good linearity with a contribution rate of 0.999 or higher over the above addition concentration range (0.25, 0.5, 1, 2, 5%) as shown in Fig. 9.

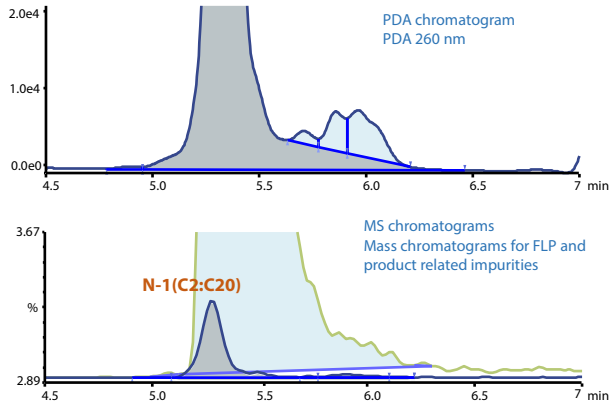

Fig. 3 Chromatograms of FLP containing 0.25% of n-1(5')

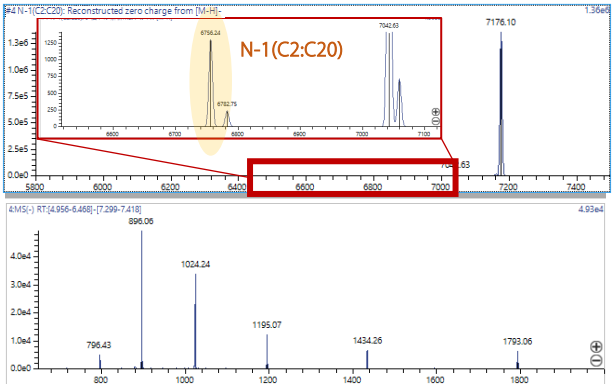

Fig. 4 Result of multivalent ion data processing for FLP containing 0.25% of n-1(5') (n-1(5') shown in Fig.3)
Upper : MS spectrum of multivalent ion data processing
Lower : MS spectrum

#	Peak	RT	Area	Mass	Mass Error (ppm)	Mass Error (mDa)	Multi-charge m/z(s)
2	N-1(G1:C19)	4.888	65	6782.43	-87.599	-594.14	846.96,967.75,1129.42
4	N-1(C2:C20)	5.338	16782	6756.24	-117.384	-793.07	749.80,843.57,964.16,1125.16,1349.81

Fig. 5 Result of multivalent ion data processing for FLP containing 0.25% of n-1(5')
Identification result

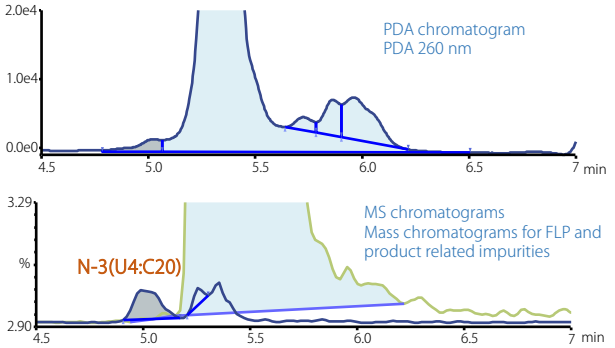

Fig. 6 Chromatograms of FLP containing 0.25% of n-3(5′)
Upper : MS spectrum of multivalent ion data processing
Lower : MS spectrum

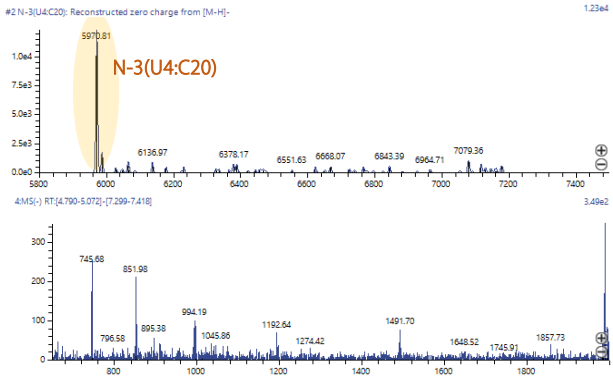

Fig. 7 Result of multivalent ion data processing for FLP containing 0.25% of n-3(5′)
(n-3(5′) shown in Fig. 6)
Upper : MS spectrum of multivalent ion data processing
Lower : MS spectrum

#	Peak	RT	Area	Mass	Mass Error (ppm)	Mass Error (mDa)	Multi-charge m/z(s)
1	N-3(G1:C17)	4.688	----	5985.24	-106.294	-636.20	746.76,854.23,996.65
2	N-3(U4:C20)	5.022	13506	5970.81	-42.054	-251.10	662.48,745.45,851.77,993.97,1193.28,1491.76
3	N-3(G1:C17)	5.022	107	5986.08	35.234	210.91	854.13,996.51,1196.62,1495.24
4	N-2(C3:C20)	5.022	99	6365.06	102.450	652.10	1059.94,1271.58,1590.41

Fig. 8 Result of multivalent ion data processing for FLP containing 0.25% of n-3(5′)
Identification result

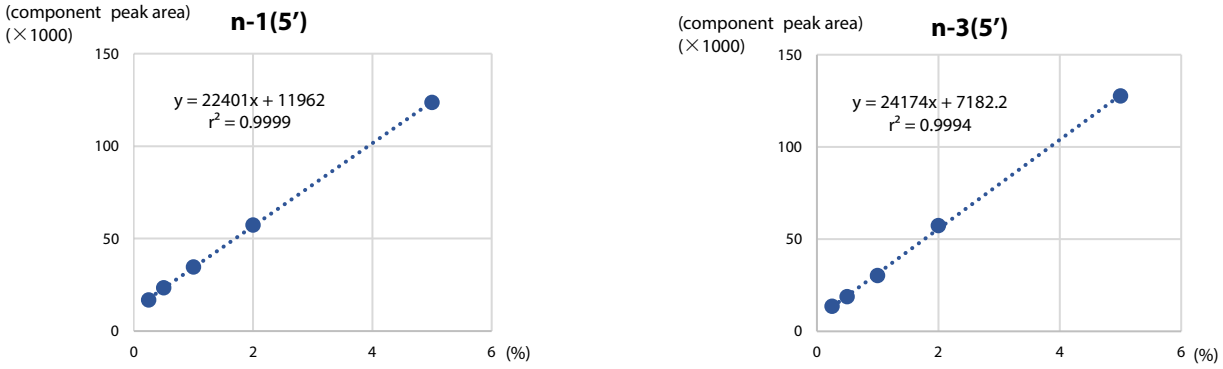

Fig. 9 Linearity evaluation for additional concentration

■ Conclusion

The simulated sample consisting of mainly FLP and small amounts of n-1(5')/n-3(5') was analyzed using the LCMS-2050 and LabSolutions Insight Biologics software and obtained data were processed appropriately. Detection, identification, and quantitation at low concentrations were accomplished not only for n-3(5') well-separated from FLP but also for n-1(5') co-eluted with FLP. In case that establishing analytical conditions is difficult, Using this instrument setup and software will provide efficient quality confirmation of oligonucleotide therapeutics.

<Acknowledgement>

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<Reference>

1. Ryutaro Takamine, Takao Yamaguchi, Challenges of Impurities Analysis in Manufacturing Therapeutic Oligonucleotides, J. Mass Spectrom. Soc. Jpn. 2023, vol.71, No.2, P51-54

<Related Application>

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.01-00656A-EN

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