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Application News

High Performance Liquid Chromatograph Mass Spectrometer

Analysis of Oligonucleotides Using a Single Quadrupole Mass Spectrometer with Hydrophilic Interaction Chromatography (HILIC)

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User Benefits

- Synthetic oligonucleotides and impurities can be analyzed using HILIC.
- Comprehensive characterization of synthetic oligonucleotides can be executed using LCMS-2050 single quadrupole mass spectrometer and LabSolutions Insight™ Biologics.
- Purity calculations for target components can be performed by utilizing MS spectra simultaneously acquired with UV chromatogram.

■ Introduction

In recent years, oligonucleotide therapeutics have been rapidly developed and have attracted attention as a new drug discovery modality. Comprehensive detection and identification of impurities generated during the synthesis process and having different base lengths from the target product are required[1].

Reverse-phase ion-pair chromatography is widely used for the separation of target compounds from impurities. Liquid chromatography-mass spectrometry (LC-MS) is commonly used as well since complete separation of the target product and all impurities seems almost impossible.

However, the development of analytical methods without using ion-pair reagents is expected because ion-pair reagents such as triethylamine have safety concerns and issues related to flow pass contamination of the instrument[2].

In this article, HILIC was focused on as the separation mode because it does not require ion-pair reagent and employs a mobile phase suitable for liquid chromatography-mass spectrometry then impurity analysis of synthetic oligonucleotides using HILIC with the LCMS-2050 high-performance liquid chromatography-mass spectrometry system and LabSolutions Insight Biologics was performed.

■ Sample

A mixture of the full-length product (FLP) modeled on Mipomersen sequence, the n-1(5′) variant, and the n-3(5′) variant was employed as a simulated sample containing the main product and two impurities. Table 1 shows the sequences of the FLP, the n-1(5′) variant, and the n-3(5′) variant.

Table 1 Sequence of Each Component

Sample	Sequence(5′→3′)	Chain length (mer)	Molecular weight
FLP	Ge-smCe-smCe-smUe-smCe-sAd-sGd-sTd-smCd-sTd-sGd-smCd-sTd-sTd-smCd-sGe-smCe-sAe-smCe-smCe	20	7177.11
n-1(5′)	mCe-smCe-smUe-smCe-sAd-sGd-sTd-smCd-sTd-sGd-smCd-sTd-sTd-smCd-sGe-smCe-sAe-smCe-smCe	19	6757.76
n-3(5′)	mUe-smCe-sAd-sGd-sTd-smCd-sTd-sGd-smCd-sTd-sTd-smCd-sGe-smCe-sAe-smCe-smCe	17	5971.06

* 【Nucleobase】
G : Guanine A : Adenine U : Uracil C : Cytosine T : Thymine
【Base modification】
m : methyl
【Linker】
s: phosphorothioate
【Ribose】
e : methoxy d : deoxy

■ Analytical Conditions

Nexera™ XS inert and LCMS-2050 were used for the analyses. HPLC conditions are shown in Table 2 and MS conditions in Table 3.

Table 2 HPLC Analytical Conditions

System	:	Nexera XS inert
Column	:	HILICpak VN-50 2D (Shodex)
Mobile Phase A	:	100 mmol/L CH_3COONH_4 /water =30:70
Mobile Phase B	:	100 mmol/L CH_3COONH_4 /acetonitrile =30:70
Time Program	:	92% B(0-1.5 min)→72% B(12.5 min)→50% B(12.51–14 min)→92% B(14.01–17 min)
Flow Rate	:	0.2 mL/min
Column Temp.	:	60 ˚C
Injection Vol.	:	15 µL
UV Detection	:	190–800 nm (photodiode array detector)

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	:	6.0 L/min
Desolvation Temp.	:	500 ˚C
DL Temp.	:	250 ˚C
Interface Voltage	:	-2.0 kV

■ Settings for Data Processing

LabSolutions Insight Biologics is software designed for the data processing of oligonucleotides and their impurities. In the setting screen, the FLP sequence is created by selecting oligonucleotide bases, linkers, ribose, and modifications, which can be freely added and edited. For further details, please refer to Application News 01-00974-en.

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 by 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.

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

■ Linearity Evaluation of Concentration and Peak Area Using Insight Biologics Processing

The linearity between FLP peak areas and concentrations was verified using LabSolutions Insight Biologics. A coefficient of determination of 0.999 or higher was observed at concentrations of 1, 2.5, 5.0, 10.0, and 20.0 µmol/L.

Then, detection and identification limits of additional concentration of the n-1(5′) and the n-3(5′) variants as impurities to the FLP concentration were evaluated.

Samples containing both nucleotide-deficient variants and 10 µmol/L of the FLP, which was within the linearity range, were prepared and subjected to analysis. For both the n-1(5′) and the n-3(5′) variants, when added at concentrations ranging from 2% (0.2 µmol/L) to 50% (5 µmol/L) relative to the FLP, both nucleotide-deficient variants were detected and identified.

Fig. 1 shows the UV chromatogram and the component chromatogram obtained when the n-1(5′) variant was added at 50% relative to the FLP.

In ESI, different m/z values are detected as multi-valent ions for high molecular weight compounds. Multi-valent ions ranging from trivalent to heptavalent were detected for the n-3(5′) variant, and from tetravalent to nonavalent for the n-1(5′) variant. The results of the multi-valent ions analysis for the n-3(5′) variant are shown in Fig. 2, and those for the n-1(5′) variant in Fig. 3. As is shown in Fig. 4, mass identification errors for the FLP and the variants were within 0.3 Da.

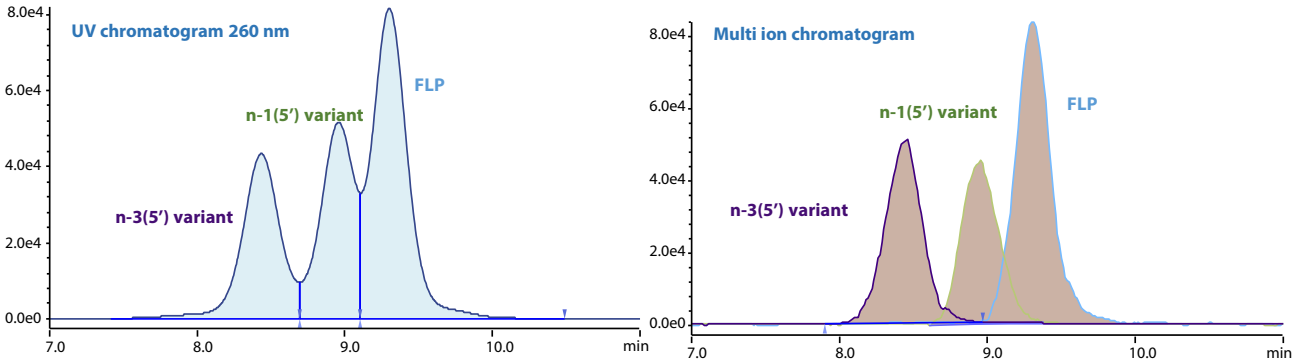

Fig.1 Chromatograms of FLP containing 50% of n-1(5) variant
Left: UV chromatogram Right: Component chromatogram

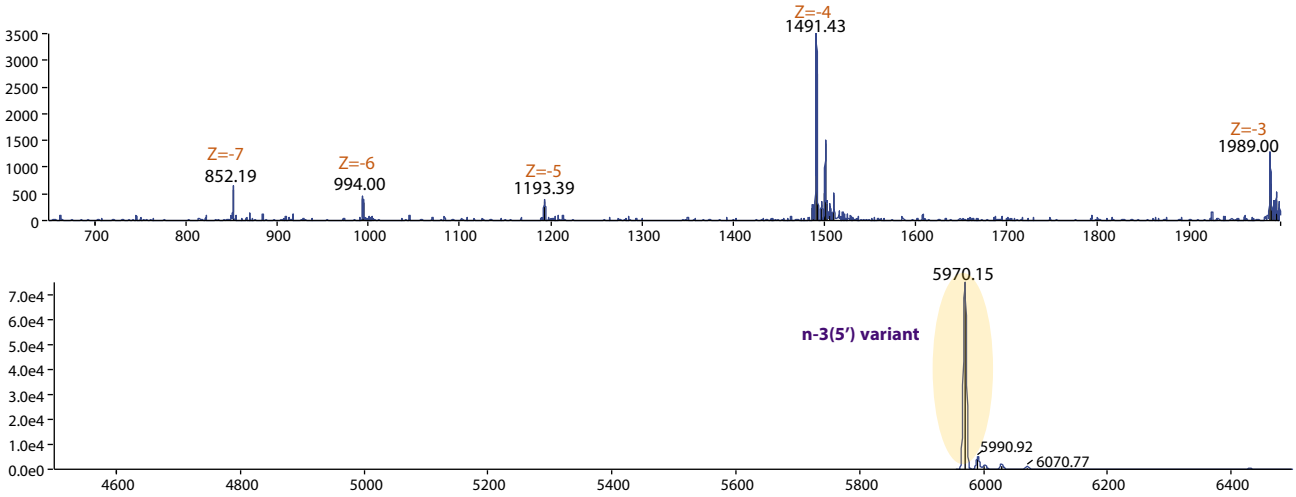

Fig. 2 Results of multi-valent ion processing when 50% of the n-1(5′) variant and n-3(5′) variant were added to FLP (Case of n-3(5′) variant shown in Fig. 1)
Upper: MS spectrum Lower: MS chromatogram from multi-valent ion processing

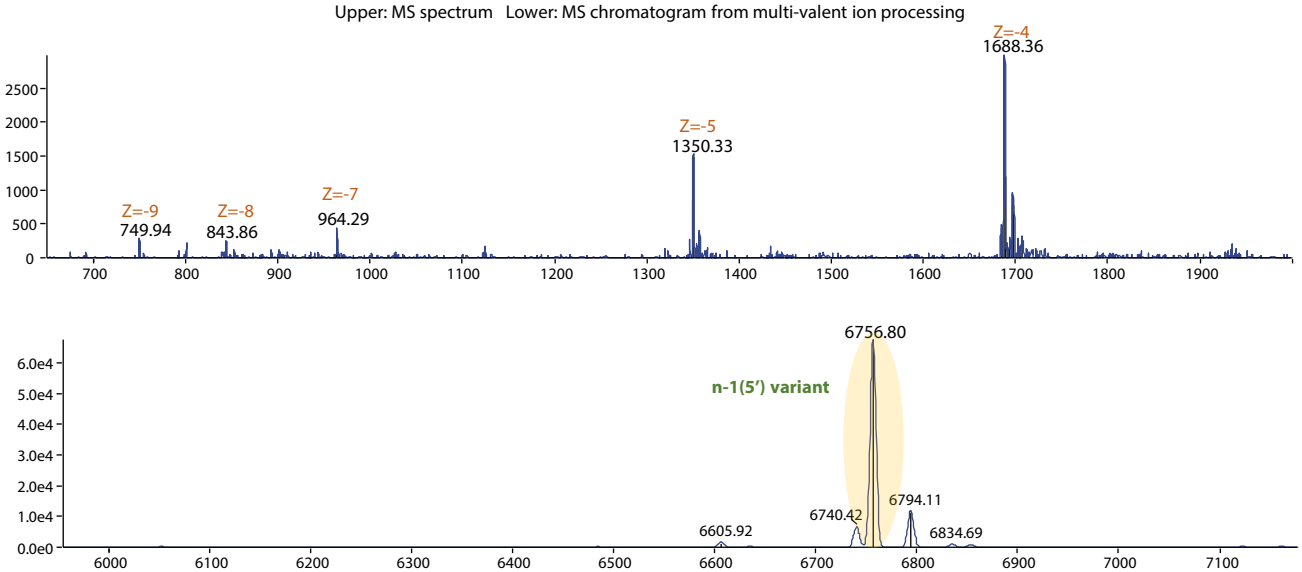

Fig. 3 Results of multi-valent ion processing when 50% of the n-1(5′) variant and n-3(5′) variant were added to FLP (Case of n-1(5′) variant shown in Fig. 1)
Upper: MS spectrum Lower: MS chromatogram from multi-valent ion processing

#	Peak	RT	Area	Mass	Mass Error (ppm)	Mass Error (mDa)	Multi-charge m/z(s)
1	N-3(U4:C20)	8.432	791081	5970.15	45.355	270.76	662.06,745.32,851.97,994.13,1193.09,1491.48,1988.98
2	N-1(C2:C20)	8.956	826030	6756.80	-34.210	-231.16	749.91,843.74,964.29,1125.22,1350.38,1688.14
3	FLP(G1:C20)	9.300	1397443	7176.08	-2.369	-17.00	796.47,896.03,1024.23,1195.15,1434.13,1792.99

Fig. 4 Identification results for FLP with 50% additions of n-1(5′) and n-3(5′) variants

The concentrations of each nucleotide deficient compound included in the FLP and obtained component peak areas exhibited good linearity with a coefficient of determination of 0.999 or more across the concentration range specified above (2, 5, 10, 20, 50%), as shown in Fig. 5. Good linearity was obtained by calculating the component peak areas based on the UV peak areas.

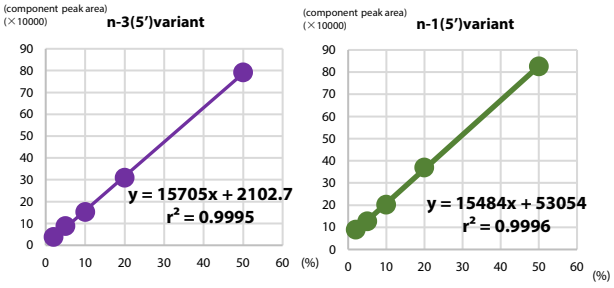

Fig. 5 Evaluation of Linearity between additional concentration (2–50%) and component peak area
Left: n-3(5′) variant Right n-1(5′) variant

■ Analysis using ammonium bicarbonate

Oligonucleotides are generally detected in negative ion mode by MS since phosphate is contained in their common structure. It is also known that detection sensitivity of oligonucleotides as negative ions tends to be improved owing to promoting deprotonation under high pH condition of mobile phase.

Therefore, a mobile phase containing ammonium bicarbonate (pH 8) was applied to the analysis and confirmed that detection and analysis were successfully performed using LabSolutions Insight Biologics. The HPLC conditions are shown in Table 4. The MS conditions are the same as those in Table 3. The UV chromatogram and component chromatogram of a mixture of the FLP, the n-1(5′) and the n-3(5′) variants (5 µmol/L each) are shown in Fig. 6.

When ammonium bicarbonate was used in the mobile phase, a gradual shortening of retention times was observed during consecutive analyses due to a change in the mobile phase composition caused by dissolved carbon dioxide from the air. Additionally, MS sensitivity was approximately half of that obtained under conditions using ammonium acetate in the mobile phase. Furthermore, no improvement in MS sensitivity was observed even when employing ammonia-added mobile phase of pH 10.

Based on the above-mentioned results, the conditions using ammonium acetate are suggested to be more suitable from the viewpoint of sensitivity and retention time stability although no significant difference in separation behavior was observed depending on the type of mobile phase under the conditions evaluated.

Table 4 HPLC Analytical Conditions

System	:	Nexera XS inert
Column	:	HILICpak VN-50 2D (Shodex)
Mobile Phase A	:	100 mmol/L NH_4HCO_3 (pH 8) /water =30:70
Mobile Phase B	:	100 mmol/L NH_4HCO_3 (pH 8) /acetonitrile =30:70
Time Program	:	99% B(0–1.5 min)→72% B(12.5 min)→50% B(12.51–14 min)→99% B(14.01–17 min)
Flow Rate	:	0.2 mL/min
Column Temp.	:	60 ˚C
Injection Vol.	:	15 µL
UV Detection	:	190-800 nm (photodiode array detector)

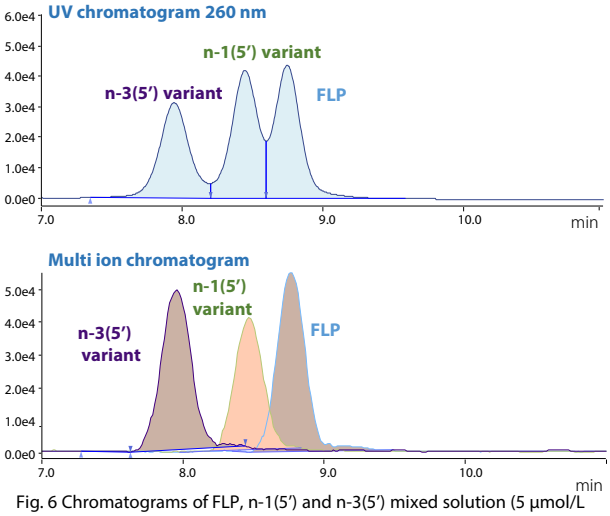

Fig. 6 Chromatograms of FLP, n-1(5′) and n-3(5′) mixed solution (5 µmol/L each)*
Upper: UV chromatogram Lower: Component chromatogram
*Obtained using ammonium bicarbonate (pH 8) in mobile phase

■ Conclusion

In this article, simulated samples of the FLP supplemented with the n-1(5′) and the n-3(5′) variants as impurities were analyzed using HILIC on a high-performance liquid chromatography-mass spectrometry LCMS-2050. Obtained data were processed with LabSolutions Insight Biologics, and each contained component was successfully identified. Furthermore, good linearities between concentrations and the component peak areas exhibited were observed across a relative concentration range of 2% to 50% to the FLP.

MS sensitivity was reduced by approximately one-fifth to one-tenth compared to reverse-phase ion-pair chromatography. There still remains a challenge of application to the low-concentration range.

However, it was demonstrated that combination use of this instrumentation and software makes it entirely possible to evaluate synthetic oligonucleotides. Furthermore, there is no need to dedicate the analytical system setup to this specific application as HILIC analysis does not require ion-pair reagents, resulting in the advantage of easily applying the instrument to other analytical uses.

References

1) Takamine et al., J. Mass Spectrom. Soc. Jpn. 2023, vol.71,No.2, P51-54
2) Guimaraes et al., Journal of the American Society for Mass Spectrometry, 2023, 34

Related Applications

1. Analysis of Oligonucleotide Impurities Using Single Quadrupole Mass Spectrometer, Application News No. 01-00974A-EN

01-01238-EN First Edition: Jul. 2026

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