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Liquid Chromatograph Mass Spectrometer

Application News

Ion-Pair Reversed-Phase LC/MS Analysis of GalNAc-siRNA Conjugates under Denaturing and Non-Denaturing Conditions

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User Benefits

- The LCMS-2050 single quadrupole mass spectrometer and the LabSolutions Insight™ Biologics analysis software can be used to confirm the molecular weight of GalNAc-siRNA conjugates.
- LabSolutions Insight Biologics enables analysis of multiple oligonucleotide sequences at the same time. Nucleobases, linkers, riboses, and base modifications can be added and removed as required.

■ Introduction

Among oligonucleotide therapeutics, antisense and siRNA therapeutics are actively studied as new modalities for treating genetic and intractable diseases. Oligonucleotide therapeutics modified with N-acetylgalactosamine (GalNAc) have attracted attention as drug delivery systems (DDS) designed to enhance the uptake of these therapeutics into the liver. GalNAc binds to the asialoglycoprotein receptor (ASGPR), which is highly expressed in the liver, and is subsequently internalized into hepatocytes. By exploiting this mechanism, GalNAc-modified oligonucleotide therapeutics can be efficiently delivered into hepatocytes. In many cases, a tri-antennary GalNAc (Tri-GalNAc) structure containing three GalNAc residues is used as a highly efficient ligand for ASGPR.

This application describes an example of ion-pair reversed-phase LC/MS analysis of GalNAc-siRNA conjugates using a single quadrupole mass spectrometer under denaturing and non-denaturing conditions.

■ Samples

Double-stranded siRNA based on the sequence of Givosiran was used.

Sense:
*C-s*A-s*G-*A-*A-*A-fG-*A-fG-*U-fG-*U-fC-*U-fC-*A-*U-*C-*U-*U-*A-R

Antisense:
*U-sfA-sfA-fG-*A-fU-*G-fA-*G-fA-*C-fA-*C-fU-*C-fU-*U-fU-*C-fU-*G-s*G-s*U

(*: 2′-methoxy, f: 2′-deoxy-2′-fluoro, s: phosphorothioate, R: A modification group containing Tri-GalNAc)

■ Analytical Conditions

Analysis was performed with Nexera™ XS inert UHPLC and LCMS-2050 single quadrupole spectrometer systems. The analytical conditions are shown in Table 1. The LCMS-2050 is equipped with a heated DUIS™ ion source for ionization, which offers the advantages of both ESI and APCI methods.

Table 1 Analytical Conditions

UHPLC (Nexera XS inert)	
Column:	Shim-pack Scepter Claris C18-300*1 (100 mm x 2.1 mm I.D., 1.9 µm)
Mobile Phase A:	100 mM HFIP, 10 mM TEA - water
Mobile Phase B:	Methanol
Gradient Program:	B Conc. 10 % (0 min) – 50 % (10 min) – 90 % (10.1-12 min) – 10 % (12.1-20 min)
Flowrate:	0.3 mL/min
Column Temp.:	25 or 60 °C
Injection Volume:	1 µL

*1: P/N 227-31209-02

MS (LCMS-2050)	
Ionization:	ESI/APCI (DUIS) negative
Mode:	MS *m/z* 550-2000
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	7.0 L/min
Desolvation Temp.:	450 °C
DL Temp.:	200 °C

■ Configuring the Data Analysis Parameters

First, the user configures an oligonucleotide sequence in the parameter configuration window using the nucleobases, linkers, ribose and modifications provided by the software (Fig. 1). Nucleobases, linkers, ribose, and base modifications can be added and removed on each tab page as required. In this example, the modification containing Tri-GalNAc was entered as a 3′-terminal modification of the sense strand (red box in Fig. 1). Once an oligonucleotide sequence is entered, the software displays the molecular formula, monoisotopic mass (to the left side), and structural formula (to the right side) of that oligonucleotide.

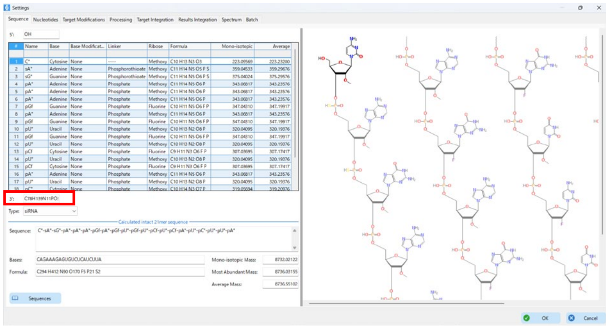

Fig. 1 Parameter Configuration Window

Insight Biologics can analyze multiple sequences. As an example, in Fig. 2, the sequences of sense and antisense strands were set as analysis targets. The specified sequences and optionally added information about nucleobases, linkers, riboses, and base modifications can be saved as an analysis settings file.

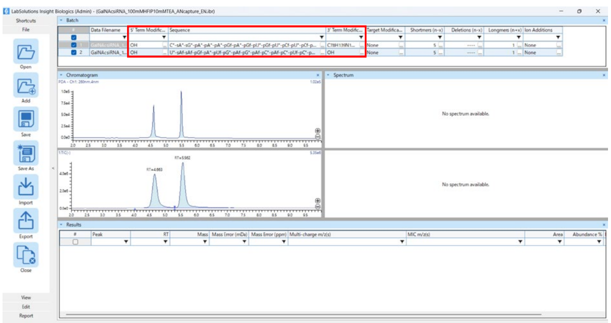

Fig. 2 Example of Multiple Sequence Settings

■ Separation of the GalNAc-siRNA Conjugates under Denaturing and Non-Denaturing Conditions

The UV chromatograms of the GalNAc-siRNA conjugate are shown in Fig. 3. At a 25 °C column oven temperature, it was eluted as one main peak. On the other hand, at a 60 °C column oven temperature, two peaks were detected. The GalNAc-siRNA conjugates were detected as denatured and dissociated into sense and antisense strands.

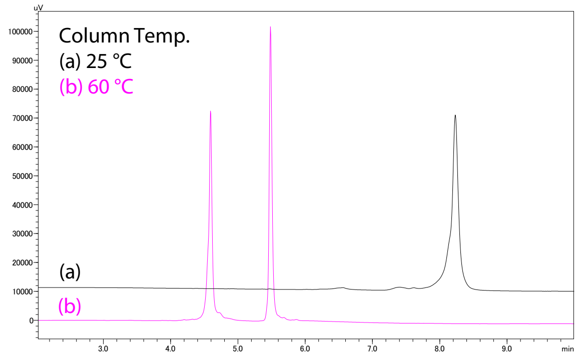

Fig. 3 UV Chromatogram (260 nm)

■ Results of Identification by LC/MS

Insight Biologics displays , identified oligonucleotide sequences as component chromatograms based on the MS1 spectrum, with all valence differences and isotopes added. Fig. 4 and 5 show the component chromatograms obtained by LC/MS analysis of GalNAc-siRNA conjugates at 25 and 60 °C column oven temperatures, respectively. At the 25 °C column oven temperature, the sense strand and antisense strand sequences were identified at the same retention time. On the other hand, at a 60 °C column oven temperature, the antisense and sense strand sequences were respectively identified in the order they were eluted. That confirmed that the two peaks detected in the UV chromatogram (Fig. 3) were eluted in the single-stranded (denatured) state.

The sense and antisense strand sequences were identified with a mass error of 1 Da from their theoretical molecular weights at both25 and 60 °C column temperatures. As an example, Fig. 6 shows the identification results for the sense strand at a column temperature of 60 °C.

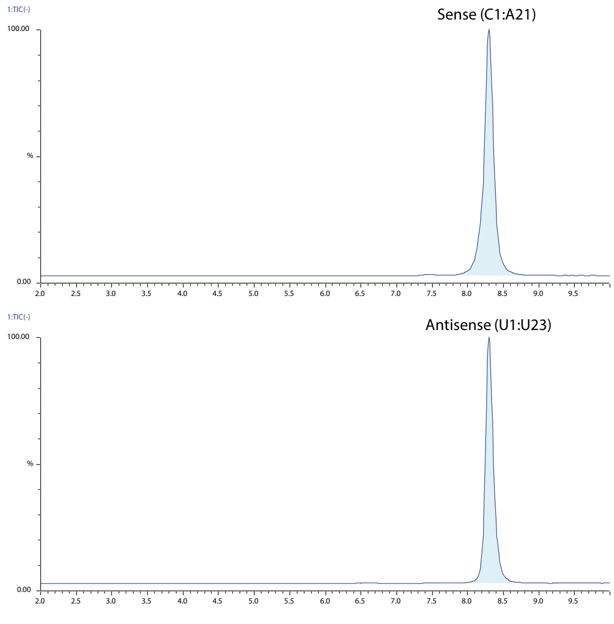

Fig. 4 Component Chromatograms with a Column Temperature of 25 °C

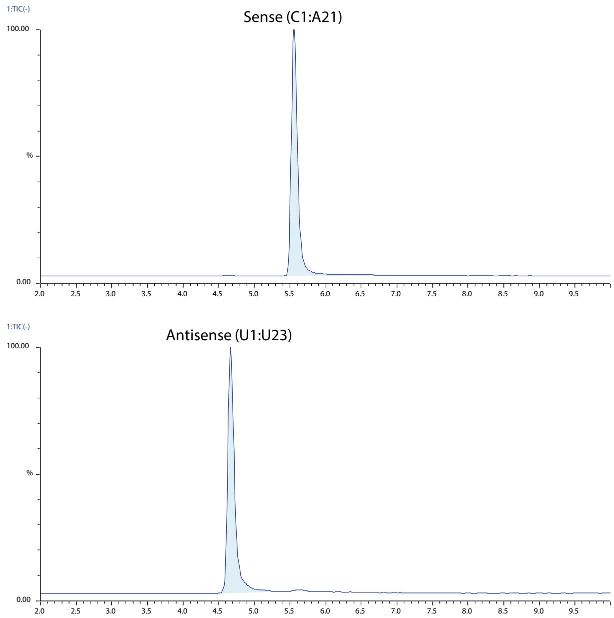

Fig. 5 Component Chromatograms with a Column Temperature of 60 °C

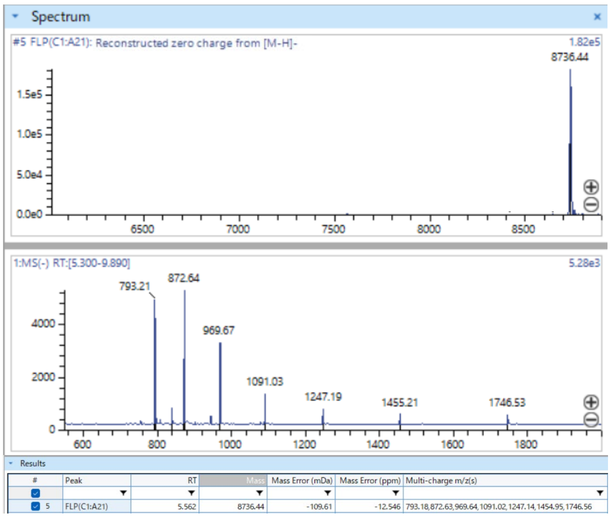

Fig. 6 Result of Identification for the Sense Strand with a Column Temperature of 60 °C
(A) Deconvoluted Spectrum; (B) Mass Spectrum

■ Conclusion

LC/MS analysis of GalNAc-siRNA conjugates under denaturing and non-denaturing elution conditions was performed using an LCMS-2050 single quadrupole mass spectrometer. Under denaturing conditions with a column temperature of 60 °C, the GalNAc-siRNA conjugate was eluted in a dissociated single-stranded state, but with a column temperature of 25 °C, it was eluted in a double-stranded state. Both sense and antisense strands were detected with a mass error of less than 1 Da from the theoretical molecular weight value.

Single quadrupole mass spectrometers cannot be used for MS/MS analysis, but they are easy to use and can be operated similarly to an LC system, so they are increasingly popular for confirming molecular weights, such as for quality control. Quadrupole time-of flight mass spectrometers are useful for sequence analysis by MS/MS, as described in Application News No. 01-01176.

■ Acknowledgments

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Related Applications

1. Characterization of GalNAc-siRNA Conjugates Using a Quadrupole Time-of-Flight Mass Spectrometer Application News No. 01-01176-EN
2. Reversed-Phase Ion-Pair LC/MS Analysis of siRNA under Denaturing and Non-Denaturing Conditions Application News No. 01-00915-EN
3. Efficient Method Development for Separation of Capped mRNA Fragments Application News No. 01-00898-EN

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