SHIMADZU
Excellence in Science

Application News

Liquid Chromatograph Mass Spectrometer

Characterization of GalNAc-siRNA Conjugates Using a Quadrupole Time-of-Flight Mass Spectrometer

Junna Nakazono

User Benefits

- The LCMS-9050 quadrupole time-of-flight mass spectrometer and the LabSolutions Insight™ Biologics analysis software can be used to characterize GalNAc-siRNA conjugates, such as with molecular weight confirmation and sequence analysis.
- 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 article describes an example of ion-pair reversed-phase LC/MS analysis for characterization of GalNAc-siRNA conjugates using a quadrupole time-of-flight mass spectrometer.

■ 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-9050 quadrupole time-of-flight mass spectrometer systems. The analytical conditions are indicated in Table 1.

Table 1 Analytical Conditions

UHPLC (Nexera XS inert)	
Column:	Shim-pack Scepter™ Claris C18-300*1 (100 mm × 2.1 mm I.D., 1.9 µm)
Mobile Phase A:	50 mM HFIP, 5 mM TEA – water
Mobile Phase B:	Methanol
Gradient Program:	B Conc. 10 % (0 min) – 50 % (10 min) – 90 % (10.01 - 12 min) – 10 % (12.1 - 20 min)
Flowrate:	0.3 mL/min
Column Temp.:	60 °C
Injection Volume:	2 µL
	*1: P/N 227-31209-02
MS (LCMS-9050)	
Ionization:	ESI negative
Mode:	MS *m/z* 550 - 2500, DDA (MS/MS) *m/z* 100 - 2500
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
Heating Gas Flow:	10.0 L/min
Interface Temp.:	350 °C
DL Temp.:	250 °C
Block Heater Temp.:	400 °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. It is also possible to display fragment positions within the structural formula.

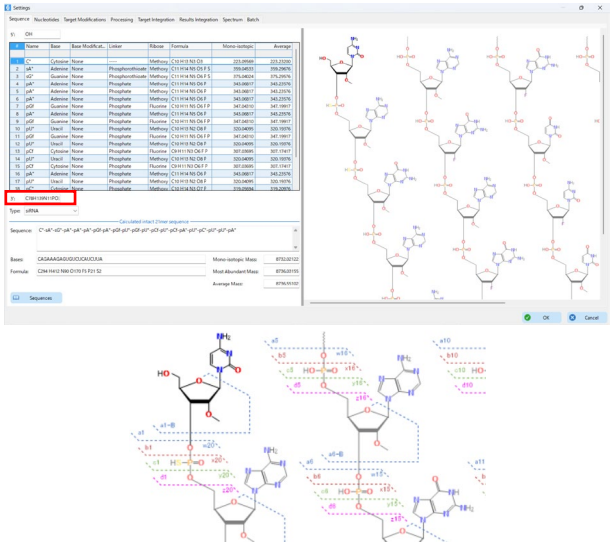

Fig. 1 Parameter Configuration Window

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.

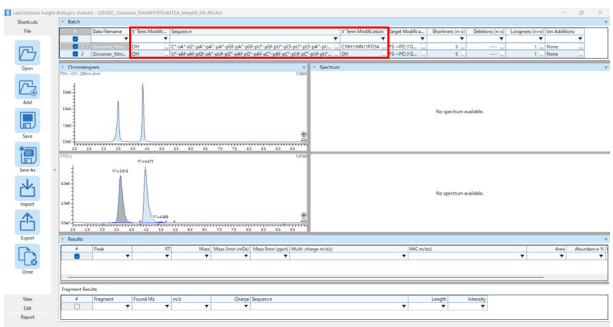

Fig. 2 Example of Multiple Sequence Settings

■ Molecular Weight Confirmation by MS

The UV chromatogram peaks of GalNAc-siRNA conjugates are shown in Fig. 3. At a 60 °C column oven temperature, double-stranded siRNA was detected as denatured and dissociated into sense and antisense strands.

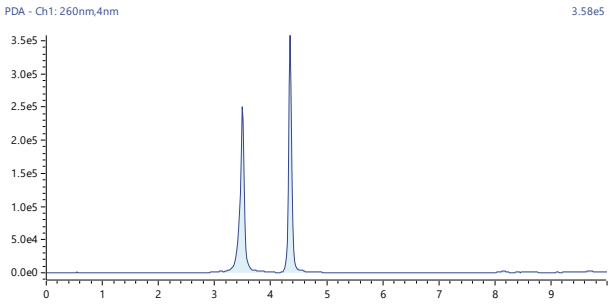

Fig. 3 UV Chromatogram (260 nm)

Fig. 4 shows the component chromatograms obtained by LC/MS analysis. In Insight Biologics, the identified oligonucleotide sequence is displayed as a component chromatogram based on the MS1 spectrum and summed with different valences and isotopes. As an example, the mass spectrum and the deconvoluted spectrum of multiply-charged ions of the sense strand peak are shown in Fig. 5. The sequences of both the sense strand and the antisense strand were identified.

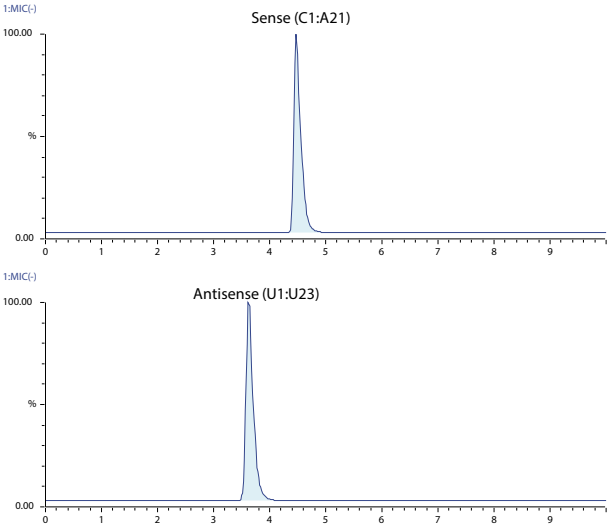

Fig. 4 Component Chromatograms

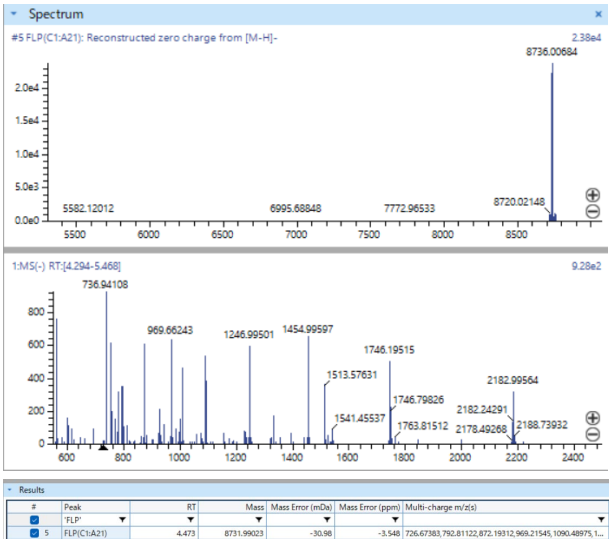

Fig. 5 Result of Identification for the Sense Strand
Top: Deconvoluted Spectrum; Bottom: Mass Spectrum

■ Sequence Analysis by MS/MS

Sequence can be confirmed using MS/MS fragment ion information. In addition to the identification results obtained from MS1 analysis, fragment ion information is also listed. As an example, the fragment ion information for the sense strand is listed in Fig. 6. Based on the fragment ion information, the results of sequence coverage visualization for the sense strand and the antisense strand are shown in Fig. 7 and 8, respectively. Sequence coverage can be displayed by switching between the branch mode (Fig. 7), which allows confirmation of the types of fragment series, and the fill mode (Fig. 8), which enables confirmation of the intensity information and overall coverage of the assigned ions. For both sequences, fragment ions resulting from cleavage between each nucleotide were detected, and 100 % sequence coverage was obtained. Sequence confirmation was also possible for sequences containing GalNAc modifications.

Fragment Results

#	Fragment	Found Mz	m/z	Charge	Sequence	Length	Intensity
4	b2	615.13793	615.13922	-1	C*-sA*	2	42113
1	c1	334.02611	334.02682	-1	C*-s	1	28557
5	c2	693.06943	693.07214	-1	C*-sA*-s	2	25070
14	c3	525.56028	525.56398	-2	C*-sA*-sG*-p	3	2520
2	d1	352.03723	352.03738	-1	C*-s	1	2237
80	a16	1060.16505	1060.16232	-5	C*-sA*-sG*-pA*-pA*-pA*-pGf-pA*-pGf-p...	16	1474
17	c4	697.10259	697.09806	-2	C*-sA*-sG*-pA*-p	4	1413
95	b19	697.10259	697.10356	-9	C*-sA*-sG*-pA*-pA*-pA*-pGf-pA*-pGf-p...	19	1413
16	b4	666.11829	666.12018	-2	C*-sA*-sG*-pA*	4	1312
70	y8	719.05809	719.05334	-6	U*-pCf-pA*-pU*-pC*-pU*-pU*-pA*	8	1091

Fig. 6 List of the Fragment ions for the Sense Strand
(Top 10 Peaks by Intensity)

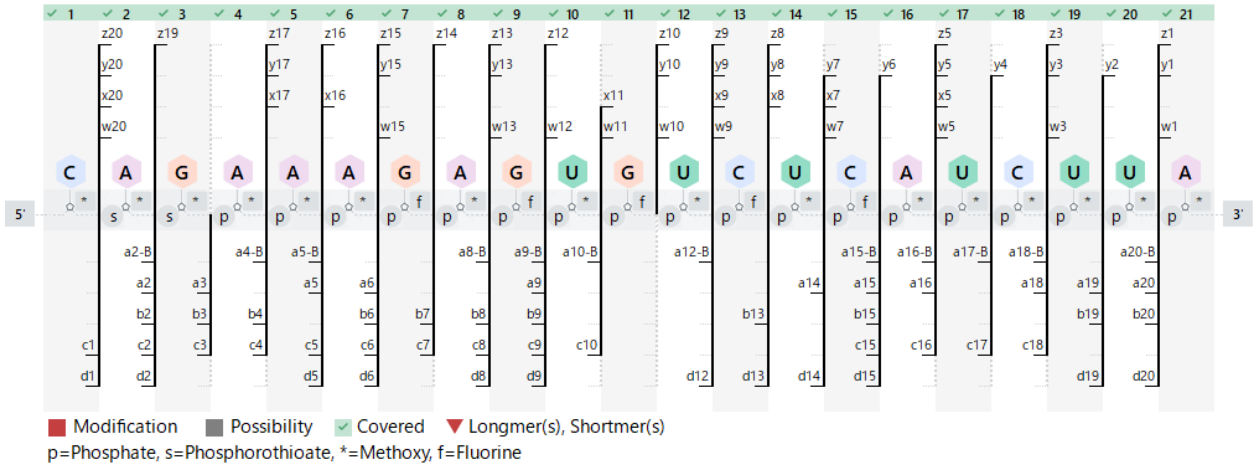

Fig. 7 Sequence Coverage of the Sense Strand Shown in Branch Mode

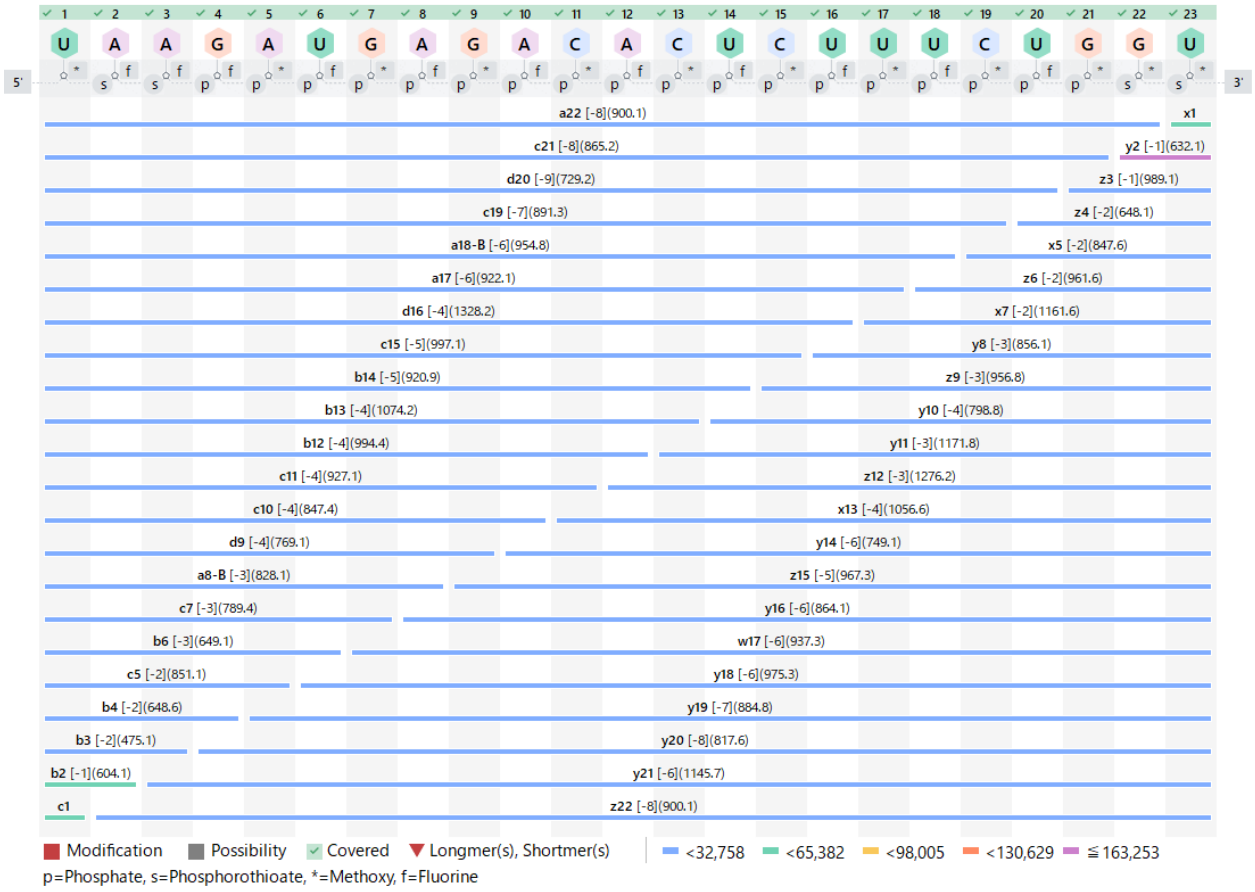

Fig. 8 Sequence Coverage of the Sense Strand Shown in Fill Mode

■ Conclusion

Ion-pair reversed-phase LC/MS analysis of GalNAc-siRNA conjugates was performed using an LCMS-9050 quadrupole time-of-flight mass spectrometer. The LCMS-9050 enables molecular weight confirmation by MS1 and sequence analysis by MS/MS.

By using the LabSolutions Insight Biologics oligonucleotide analysis software, multiple nucleic acid sequences can be analyzed at the same time. Furthermore, sequence coverage for each sequence can be visually confirmed in an easy-to-understand manner.

■ Acknowledgments

This research was supported by AMED under grant numbers JP21ae0121022, JP21ae0121023, and JP21ae0121024 (Project leader: Satoshi Obika).

Related Applications

1. Ion-Pair Reversed-Phase LC/MS Analysis of GalNAc-siRNA Conjugates under Denaturing and Non-Denaturing Conditions
   Application News No. 01-01177-EN
2. Ion-Pair Reversed-Phase 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

01-01176-EN First Edition: Apr. 2026

SHIMADZU
Shimadzu Corporation
www.shimadzu.com/an/

› Please fill out the survey

Related Products Some products may be updated to newer models.

› LCMS-9050

Quadrupole Time-of-Flight Liquid Chromatograph Mas···

› Nexera XS inert

Ultra High Performance Liquid Chromatograph

› LabSolutions Insight Biologics

Software for Oligonucleotide Sequence Characteriza···

Related Solutions

› Pharmaceutical and Biopharmaceutical

› Oligonucleotide and mRNA Therapeutics

› Price Inquiry

› Product Inquiry

› Technical Service / Support Inquiry

› Other Inquiry