TN-1366

Impurity Profiling of GalNAc-conjugated Oligonucleotides by LC-MS-UV

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Introduction

Oligonucleotide (OGN) conjugates are successful drug therapies because of their efficiencies in delivering the oligonucleotide therapeutic to target cells and tissues. For example, Givorsiran is an approved OGN therapeutic conjugated to N-Acetylated Galactosamine (GalNAc), used to target liver cells and treat acute hepatic porphyria.

Characterization of OGN conjugates via ion-pairing reversed phase liquid chromatography (IP-RPLC) can be quite challenging because it requires a balance between the column's surface chemistry carbon load to mitigate general OGN recovery loss and the pore size to accommodate the size of the OGN conjugates. This technical application note describes the use of IP-RPLC coupled with MS and UV detection for the characterization of a siRNA duplex - GalNAc conjugated (**Figure 1**) using a Biozen™ C4 WidePore column under denaturing conditions.

LC Conditions

Columns: Biozen 2.6 µm Oligo (00D-4790-AN)
Biozen 2.6 µm WidePore C4 (00D-4786-AN)

Dimensions: 100 x 2.1 mm

Mobile Phase: A: Water with 5 mM Hexafluoroisopropanol + 3 mM N,N-Diisopropylethylamine
B: Acetonitrile / Water (65:35, v/v)

SecurityLink Connectors: Column to Tee: AJ1-2271
Tee to UV: AJ1-2411
Tee to MS: AJ1-2411
** Split flow after column was used to divert flow to MS and UV 1:1 at the same time*

Gradient:	Duplex Separation		Impurity Profiling	
	Time (min)	% B	Time (min)	% B
	0	6	0	5
	1	27	1	5
	8	67	1.1	10
	8.5	85	15	65
	9	85	16	65
	9.1	6	16.1	5
	14	6	20	5

Flow Rate: 400 µL /min | 0.300 µL /min

Injection Volume: 1 µL

Temperature: 60 °C to 80 °C in 5 °C increments, depending on experiment

LC System: Agilent® 1260 HPLC

Detection: MS and UV

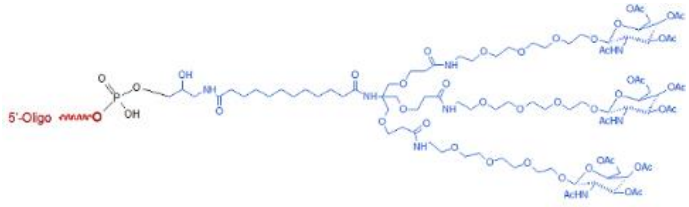
Detector: SCIEX® 7600 ZenoTOF® and UV @ 260 nm

Analytes:
1. *GalNAc RNA Sense Strand (8768.51 Da):*
5'-mC*mA*mG mAmAmA /i2FG/mA/i2FG/ mU/i2FG/mU /i2FC/mU/i2FC/ mAmUmC mUmUmA GalNAc trebler CPG -3'
2. *GalNAc RNA Antisense Strand (7547.80 Da):*
5'-mU*/i2FA/*/i2FA/ /i2FG/mA/i2FU/ mG/i2FA/mG /i2FA/mC/i2FA/ mC/i2FU/mC /i2FU/mU/i2FU/ mC/i2FU/mG* mG*mU-3'

MS Conditions

Scan Mode:	TOFMS	**Declustering Potential:**	-50
Polarity:	Negative	**CAD gas:**	7
Gas 1:	50 psi	**Start mass:**	500 m/z
Gas 2:	50 psi	**Stop mass:**	2000 m/z
Curtain Gas:	35 psi	**Q1 Resolution:**	Low
Source Temperature:	350 °C	**Accumulation time:**	0.25 min
Ion Spray Voltage:	-3500 V	**Collision energy:**	-10
Time bins to sum:	6	**Qjet RF amplitude:**	190

Figure 1. GalNAc TEG Trivalent Oligonucleotide at the 3' Sense Strand.



Results and Discussion

Initially, a Biozen™ Oligo C18 100 Å column was used to assess separation of a GalNAc-conjugated siRNA under denaturing conditions. **Figure 2** shows the extracted ion chromatograms (EICs) for the antisense strand eluting at RT 3.78 min while the duplex, in the form of dissociated strands due to high temperature at the source of the mass spectrometer, elutes at 4.78 min. **Figures 3** and **4** show the charged envelope under the same retention times. It is important to notice the overlapping envelopes in **Figure 4** that correspond to the sense and the antisense strands. **Figure 5** shows the deconvoluted spectra of the detected sense and antisense strands. Altogether, the Biozen Oligo C18 100 Å column did not resolve the individual strands for this GalNAc conjugated duplex potentially due to the column temperature being too low to fully dissociate the complex.

Double strands denature at elevated temperatures and the temperature required for dissociation depends on the OGN sequence and length. With the purpose of duplex dissociation, column temperatures ranging from 65 °C to 80 °C (in 5 °C increments) were analyzed. **Figure 6** shows the total ion chromatograms (TICs) at the various temperatures analyzed. The RNA duplex failed to completely dissociate into separate strands at all temperatures analyzed. Similarly, **Figure 7** shows the results under UV detection at 260 nm wavelength.

The Biozen Oligo column with organo-silica core-shell particle bonded to a C18 stationary phase offers high selectivity for even minute OGN differences alongside high and low pH robustness. Unfortunately, this column was not successful at resolving the GalNAc-conjugated duplex used in this study. Depending on the duplex-column chemistry interaction, solvent used, and column temperature conditions, the dissociated duplex can re-anneal on column. As an alternative, a Biozen WidePore C4 400 Å column was utilized. The Biozen WidePore C4 400 Å column provides the benefits of BioTi™ hardware and core-shell particle morphology identical to the Biozen Oligo column, a lower carbon load and a wider pore size (400 Å versus 100 Å).

Figure 8 shows the EIC of the antisense and sense strands on a Biozen WidePore C4 400 Å column. The charged envelope under the antisense strand peak and sense strand peak of **Figure 8** are shown in **Figures 9** and **10**, respectfully. The charge envelopes do not overlap in either peak, suggesting the Biozen WidePore C4 400 Å column was able to resolve both strands of the GalNAc-conjugated duplex. **Figure 11** shows the deconvoluted spectra for the detected sense and antisense strands. As **Figures 12** and **13** show, the Biozen WidePore C4 400 Å column successfully resolved sense strand from antisense strand at all temperatures, with the duplex completely dissociating at 80 °C.

Impurity profiling was also tested using the Biozen WidePore C4 400 Å column. As shown in **Figures 14** and **15**, this column is capable of baseline resolving the single strands from RNA duplex and from its respective impurities. Here, n-1 and n-2 species of the antisense strands were detected by HRMS while quantitation was done by UV detection (**Table 1**). Similarly, the loss of GalNAc on the sense strand was also detected.

Figure 2. EIC of Antisense and Sense Strands of GalNAc-Conjugated siRNA Duplex on a Biozen Oligo C18 100 Å column.

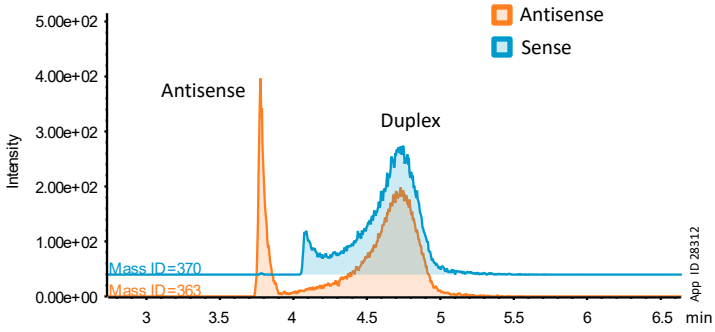


Figure 3. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Charge Envelope Under Antisense Peak.

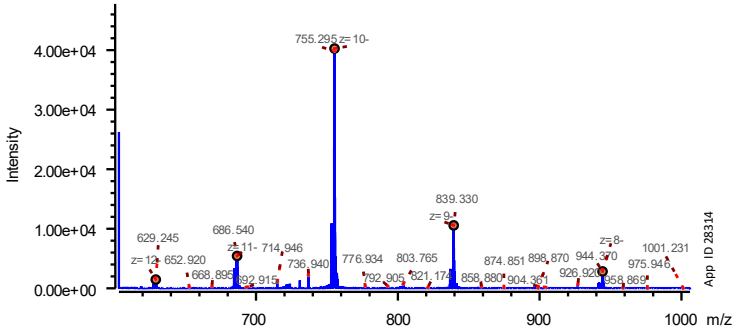


Figure 4. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Charge Envelope Under Antisense and Sense Strand Duplex Peak.

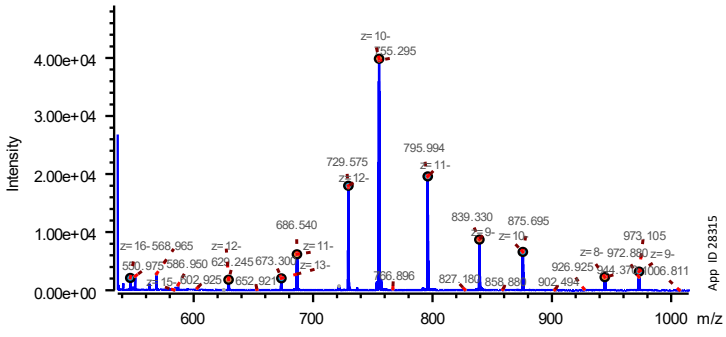


Figure 5. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Mass Reconstructed Spectra Showing Molecular Weights for Antisense Strand and Sense Strand.

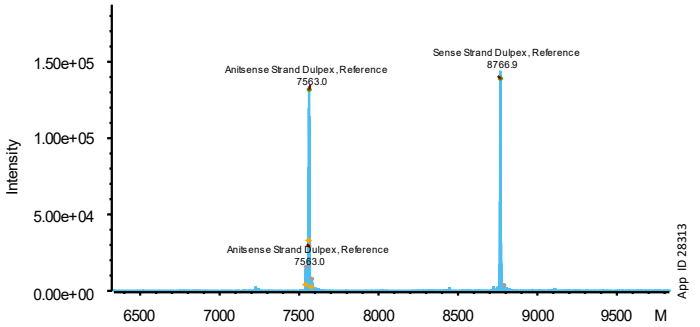


Figure 6. TIC of the Effect of Column Temperature on Separation of GalNAc-Conjugated siRNA Duplex Using a Biozen™ Oligo C18 100 Å Column.

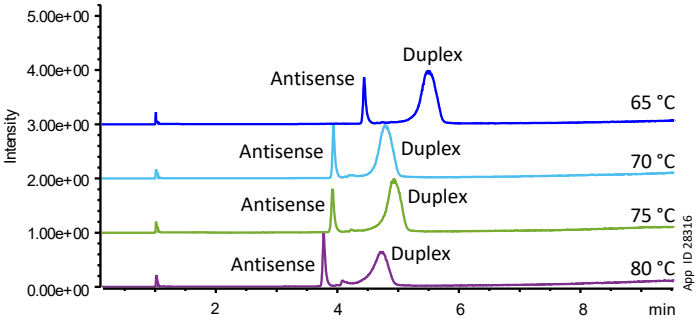


Figure 7. UV Chromatogram @ 260 nm of the Effect of Column Temperature on Separation of GalNAc-Conjugated siRNA Duplex Using a Biozen Oligo C18 100 Å Column.

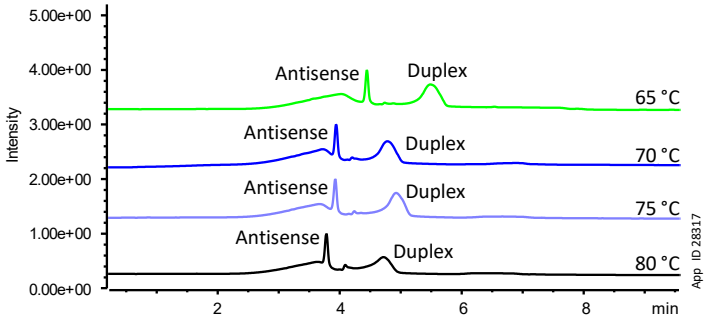


Figure 8. EIC of Antisense and Sense Strands of GalNAc-Conjugated siRNA Duplex on a Biozen WidePore C4 400 Å Column.

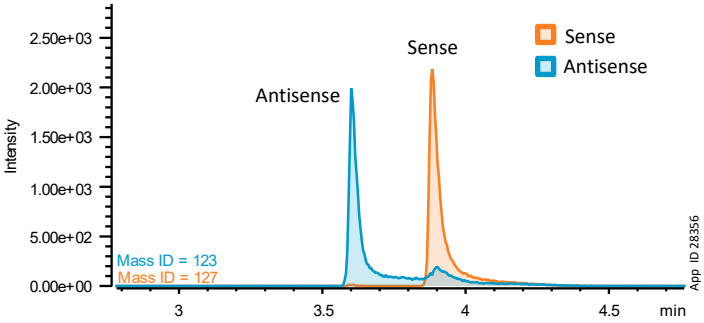


Figure 9. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Charge Envelope Under Antisense Peak Using a Biozen WidePore C4 400 Å Column.

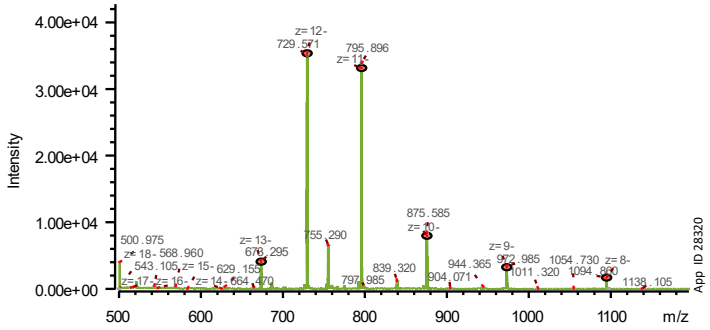


Figure 10. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Charge Envelope Under Sense Strand Peak Using a Biozen WidePore C4 400 Å Column.

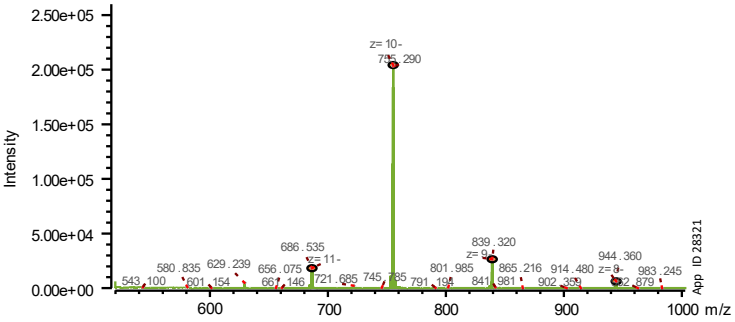


Figure 11. IP-RP-LC Analysis of GalNAc-Conjugated siRNA Duplex Mass Reconstructed Spectra Showing Molecular Weights for Antisense Strand and Sense Strand Using a Biozen WidePore C4 400 Å Column.

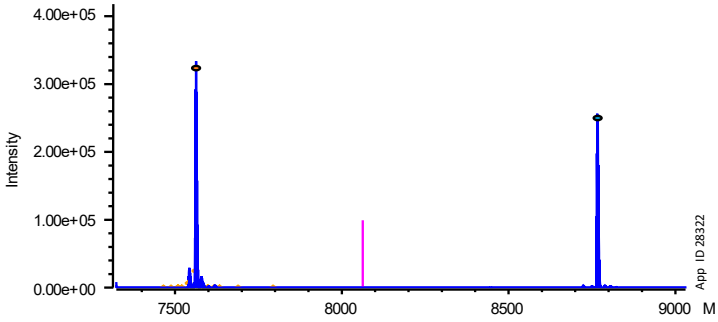


Figure 12. TIC of the Effect of Column Temperature on Separation of GalNAc-Conjugated siRNA Duplex Using a Biozen™ WidePore C4 400 Å Column.

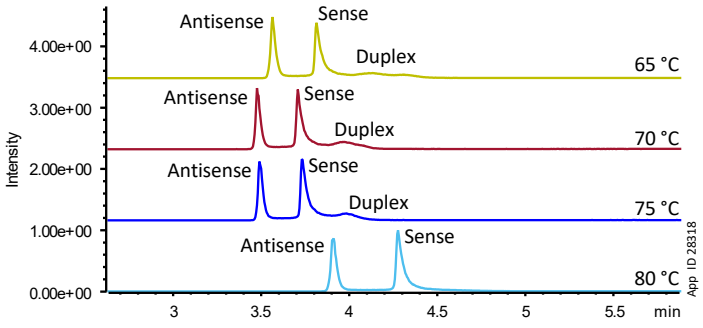


Figure 13. UV Chromatogram @ 260 nm of the Effect of Column Temperature on Separation of GalNAc-Conjugated siRNA Duplex Using a Biozen WidePore C4 400 Å Column.

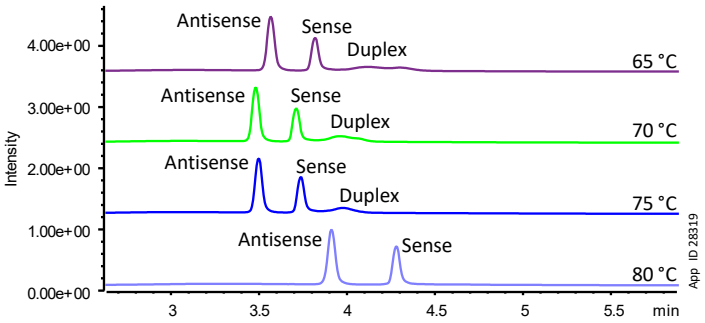


Figure 14. TIC of Impurity Profiling of GalNAc-Conjugated siRNA Using a Biozen WidePore C4 400 Å Column.

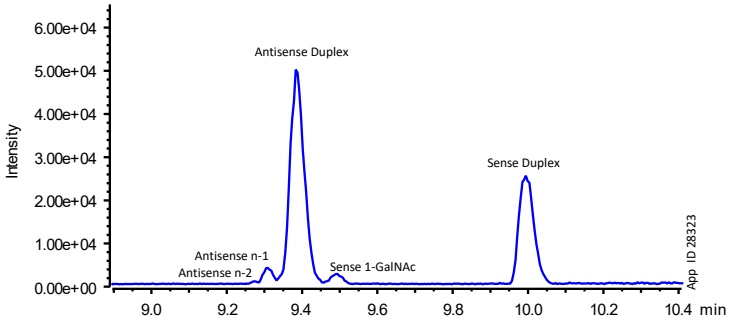


Figure 15. EICs of Intact Strands and Impurities of GalNAc-Conjugated siRNA Using a Biozen WidePore C4 400 Å Column.

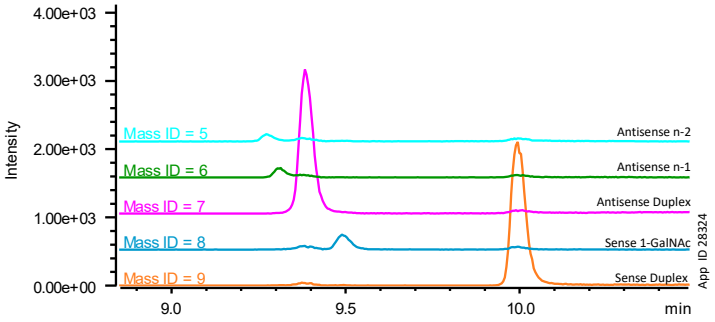


Table 1. Identified Oligonucleotide Species Using the Biozen WidePore C4 Column Demonstrating Corresponding Peak Areas, Retention Times, Ion Intensities, and PPM Errors Showcasing Both, Accurate Mass Assignments and Baseline Resolution of GalNAc-Conjugated Oligonucleotide and its Impurities.

Name	Retention Time (min)	Peak Width	Area	% Area	Intensity	Monoisotopic Mass	ppm Error
Antisense n-2	9.25	0.08	60.00	1.66	1.04E+04	6848.95	-3.82
Antisense n-1	9.30	0.04	1.30E+02	3.59	2.11E+04	7208.02	-3.08
Antisense Duplex	9.38	0.07	2.10E+03	58.01	2.73E+05	7560.01	-0.66
Sense 1-GalNAc	9.49	0.06	1.30E+02	3.59	3.20E+04	7569.33	-0.71
Sense Duplex	9.99	0.10	1.20E+03	33.15	2.29E+05	8764.00	-11.50

Conclusion

Impurity profiling and characterization of siRNA GalNAc-conjugated OGNs is a challenging undertaking because of the need to identify very small differences on both strands of the siRNA duplex. The large pore size of the Biozen WidePore C4 column in combination with its core-shell and bio-inert technology, provide the necessary resolving power for the triantennary structure of the GalNAc-conjugated OGN from its impurities and proves to be an excellent candidate for metabolite profiling and quantitation.

Ordering Information

Biozen™ Columns (mm)							Biocompatible Guard Cartridges		
	50 x 2.1	100 x 2.1	150 x 2.1	50 x 4.6	100 x 4.6	150 x 4.6	for 2.1 mm	for 4.6 mm	Holder
							/3pk	/3pk	ea
Biozen 1.7 µm Oligo	00B-4791-AN	00D-4791-AN	00F-4791-AN	—	—	—	AJ0-9820	AJ0-9822	AJ0-9000
Biozen 2.6 µm Oligo	00B-4790-AN	00D-4790-AN	00F-4790-AN	00B-4790-E0	00D-4790-E0	00F-4790-E0	AJ0-9820	AJ0-9822	AJ0-9000

Biozen Columns (mm)								Biocompatible Guard Cartridges		
	50 x 2.1	100 x 2.1	150 x 2.1	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	for 2.1 mm	for 4.6 mm	Holder
								/3pk	/3pk	ea
Biozen 2.6 µm WidePore C4	00B-4786-AN	00D-4786-AN	00F-4786-AN	00B-4786-E0	00D-4786-E0	00F-4786-E0	00G-4786-E0	AJ0-9816	AJ0-9818	AJ0-9000
Biozen 3.6 µm Intact XB-C8	00B-4766-AN	00D-4766-AN	00F-4766-AN	00B-4766-E0	—	00F-4766-E0	—	AJ0-9812	AJ0-9814	AJ0-9000

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