Application Note

Biopharmaceuticals

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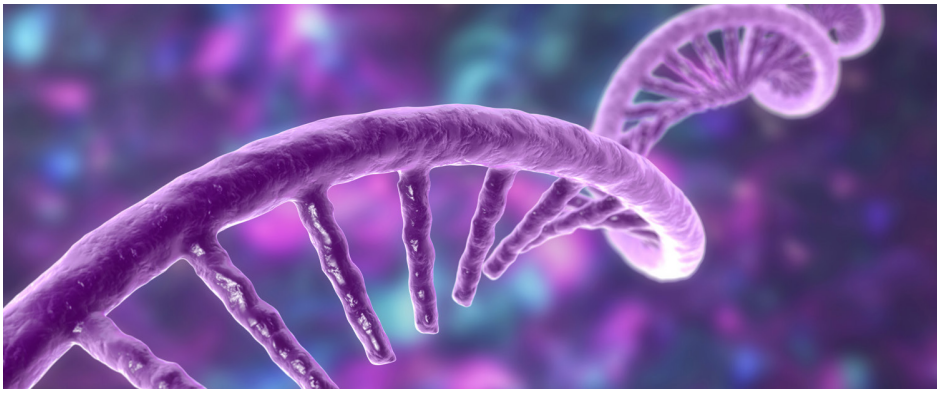
Analysis of Oligonucleotides Using an Ion-Pairing-Free Reversed-Phase Method with TOF LC/MS



Authors

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Abstract

This application note details a reversed-phase separation methodology for oligonucleotides without the use ion pairing reagents. The method was developed on an Agilent time-of-flight liquid chromatography/mass spectrometer with Agilent MassHunter BioConfirm biopharmaceutical software to analyze the data. A wide range of oligonucleotide samples were studied to show the flexibility and adaptability of the methodology. This application note describes the development of an alternative method for the analysis and separation of a wide array of oligonucleotides.

Introduction

The most common method for the analysis of synthetic oligonucleotides is ion-pairing reversed-phase (IPRP) however, it is costly, environmentally harmful, and requires dedicated instrumentation. This application note presents an alternative method using time-of-flight liquid chromatography/mass spectrometry (TOF LC/MS) and a reversed-phase (RP) chromatographic method that enables accurate and repeatable analysis of diverse oligonucleotides without the use of ion-pairing reagents. The analysis of 13 unique oligonucleotides with unmodified and heavily modified components demonstrates the versatility of this LC/MS method.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

- Agilent 6230B TOF LC/MS system
- Agilent MassHunter Acquisition software for LC/TOF, version 11
- Agilent MassHunter BioConfirm biopharmaceutical software, version 12.1
- Agilent 1290 Infinity II Bio Binary Pump (G7120A)
- Agilent 1290 Infinity II Bio Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1260 Infinity II Diode Array Detector HS (G7117C)

Sample preparation

All samples were resuspended in deionized water to a concentration of 1 or 10 µM, depending on the specific oligonucleotide, and stored at −80 °C. Samples were transferred to polypropylene vials and stored in the temperature-controlled autosampler for up to 2 days prior to analysis.

Table 1. Oligonucleotide sample sequences.

Oligonucleotide Name	Length	Sequence
ASO-1	18	dU/MOErC//MOErA//MOErC/dUdUdU/MOErC//MOErA/dU/MOErA//MOErA/dU/MOErG/CdU/MOErG/G
ASO-2	20	dU/MOErC/dUdU/MOErG/TT/MOErA//MOErC//MOErA//MOErT//MOErG//MOErA//MOErA//MOErA/dU/MOErC//MOErC//MOErC/C
Fomivirsen	21	GCGTTTGCTCTTCTTCTTGCG
Givosiran	22 S	mC*mA*mGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA/L96/
	23 AS	mU*mG*mGfUmCfUmUfUfCmUfCfAmCfAmGfAmGfUmAmGfA*fA*mU
sgRNA	103	Proprietary
20-mer DNA	20	TGCATGCATGCATGCATGCA
30-mer DNA	30	CATGCATGCATGCATGCATGCATGCATGCA
40-mer DNA	40	TGCATGCATGCATGCATGCATGCATGCATGCATGCATGCA
60-mer DNA	60	TGCATGCATGCATGAATGCATGCATACAAGCATGCATACAAGAATGAATACATGCAAGCA
14-mer RNA	14	rCrArCrUrGrArArUrArCrCrArArU
17-mer RNA	17	rUrCrArCrArCrUrGrArArUrArCrCrArArU
20-mer RNA	20	rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
21-mer RNA	21	rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mC	2'-O-methyl C
mG	2'-O-methyl G
mU	2'-O-methyl U
rA	Ribose adenine
rC	Ribose cytosine
rG	Ribose guanine

Code	Description
rU	Ribose uracil
Code	Description
/MOErA/	Methoxyethoxy A
/MOErC/	Methoxyethoxy C
/MOErT/	Methoxyethoxy T
/MOErG/	Methoxyethoxy G
dU	Deoxyuridine
fA	2-fluoroadenosine
fC	2-fluorocytidine
fG	2-fluoroguanadine
fU	2-fluorouridine

LC/MS analysis

Table 2. Source parameters for the Agilent 6230B TOF LC/MS.

Parameter	Value
Ion Source	Dual Agilent Jet Stream (AJS) electrospray (ESI) source
Polarity	Positive
Gas Temperature	300 °C
Drying Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,000 V
Nozzle Voltage	1,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF	750 V
Mass Range	750 to 3,000 *m/z*
Acquisition Rate	1.25 spectra/sec

Table 3. HPLC parameters.

Parameter	Value
Column	Agilent AdvanceBio Oligonucleotide column, 2.1 × 50 mm, 2.7 µm, (p/n 659750-702)
Multisampler Temperature	8 °C
Mobile Phase A	20 mM Ammonium bicarbonate in water
Mobile Phase B	Methanol
Flow Rate	0.8 mL/min
Injection Volume	2 µL
Multi Wash	20:80 Water:methanol; flush port; 5 seconds 90:10 Water:methanol; flush port; 3 seconds
Column Temperature	75 °C
Post Time	1.0 min
Gradient Program	Time (min) %B 0 10 0.5 10 5.0 50 5.1 90 6.0 90 6.1 10

Results and discussion

Three mobile phase modifiers were evaluated to determine the impact on oligonucleotide LC/MS separation and sensitivity (Figure 1). The mobile phase buffers evaluated in this study included ammonium bicarbonate, ammonium acetate, and ammonium formate, all at 20 mM concentration in mobile phase A. The ammonium acetate and ammonium formate buffers were adjusted to pH 8.5 with ammonium hydroxide, whereas the ammonium bicarbonate buffer was not pH-adjusted. The LC/UV results showed that the 14-, 17-, 20-, and 21-mer RNA samples separated similarly on the RP column with an average resolution of R = 1.47 between the 20- and 21-mer RNA. This indicated that further gradient optimization could achieve baseline separation (R = 1.5) for n–1 impurities, which are often monitored for biopharmaceuticals.

Notably, the LC/MS results showed significant sensitivity differences between the mobile phase buffers even though the same chromatography method was used. The results showed that the ammonium bicarbonate buffer yielded the best ionization efficiency for oligonucleotides relative to the other tested mobile phase buffers. We also confirmed the loss of oligonucleotide retention in the absence of the mobile phase buffers by replacing mobile phase A with unbuffered deionized water (Figure 1).

To determine the applicability of the analytical method for therapeutic-relevant molecules, the non-IPRP method was applied to modified antisense oligonucleotides (ASO), siRNA, DNA, and sgRNA molecules. With minor adjustments in gradient, the desired chromatographic outcomes were achieved on a diverse range of analytes.

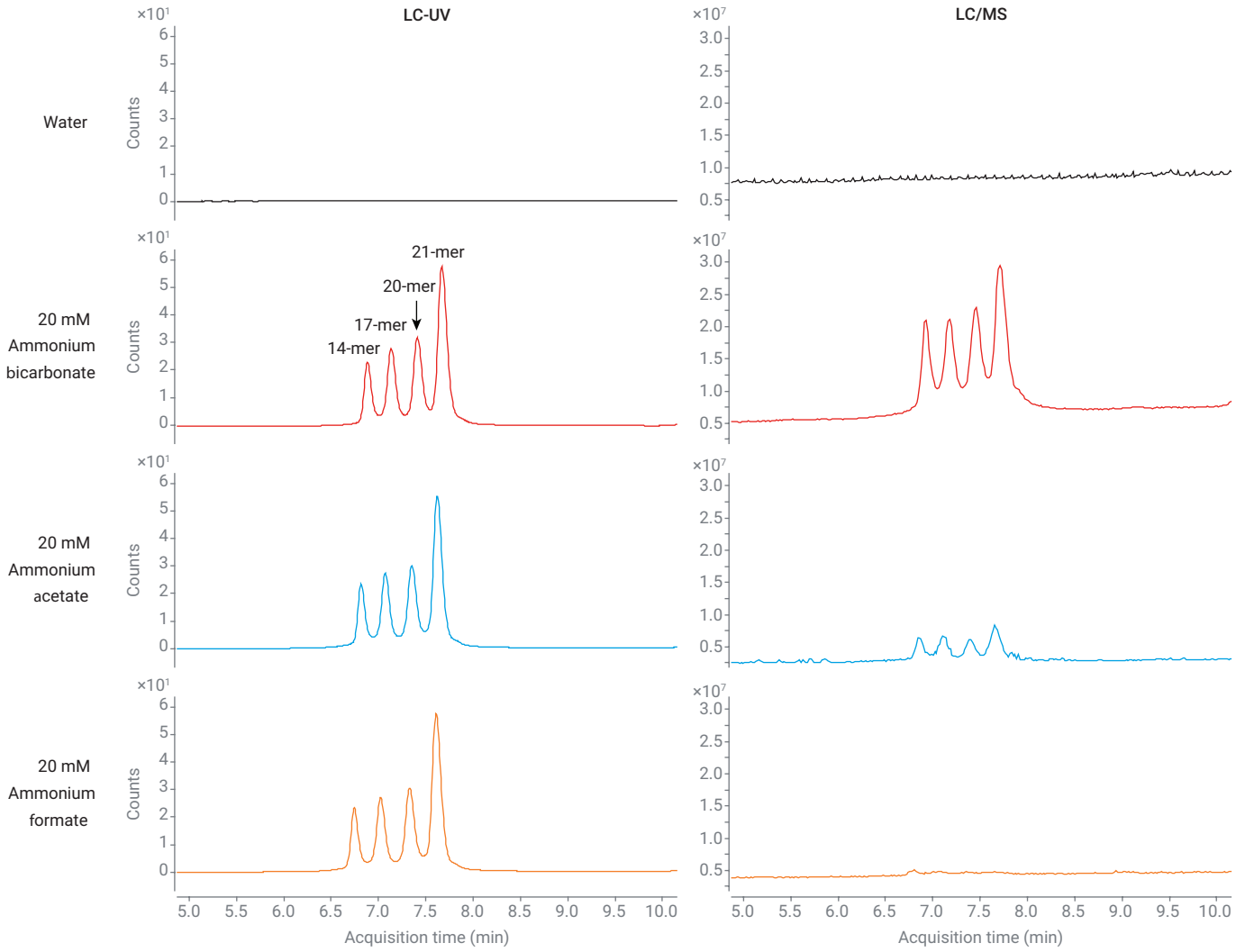

Figure 1. Separation of 14-, 17-, 20-, and 21-mer RNA using different mobile phase buffers at pH 8.5 with UV and MS detectors.

Figure 2 shows the chromatographic separation of sense and antisense strands of givosiran achieved with this method. All samples were analyzed using BioConfirm 12.1. The results showed that across all oligonucleotides evaluated, the average measured mass was within 12.5 ppm of the expected mass, as shown in Table 4.

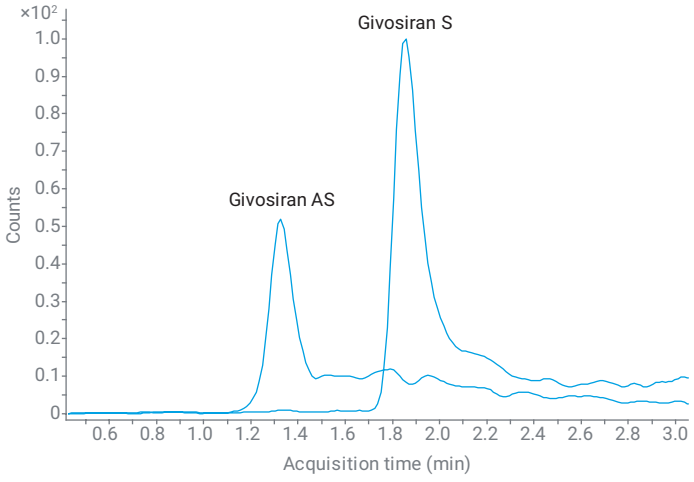

Figure 2. EIC of sense- and antisense strands of givosiran

Table 4. Deconvoluted results for each target analyte (ASO, siRNA, DNA, and RNA).

Oligonucleotide Name	Expected Mass	Measured Mass	Difference (ppm)
ASO-1	6,348.2792	6,348.3583	12.45
ASO-2	7,309.2227	7,309.1506	9.86
Fomivirsen	6,682.404	6,682.3895	2.16
Givosiran	8,736.5007	8,736.6134	12.91
	7,563.8424	7,563.8719	3.9
sgRNA (103-mer)	32,394.3	32,395.0582	23.4
20-mer DNA	6,116.9809	6,116.8979	13.56
30-mer DNA	9,190.9483	9,190.895	5.8
40-mer DNA	12,295.9265	12,295.9407	1.16
60-mer DNA	18,525.9881	18,525.9695	1.0

All MS data from the experiment were processed in BioConfirm 12.1 using the oligonucleotide target plus impurities data analysis workflow as shown in Figure 3. Using the targeted Find-by-Formula approach it was shown that across all oligonucleotides evaluated, the average measured mass was within 8.6 ppm of the expected mass, as shown in Table 4.

To ensure the repeatability of the method and stability of the stationary phase at higher temperatures, 55 replicate injections of ASO-2 were run, and the retention time and TIC area response were monitored (Figure 4). ASO-2 was chosen for this test because of the difficulty presented by the peak shape associated with the partial separation of R/S diastereomers. Over the course of the 55 injections, the method achieved an RT %RSD of 0.72 and an area response %RSD of 1.74, thus demonstrating excellent peak RT and response stability.

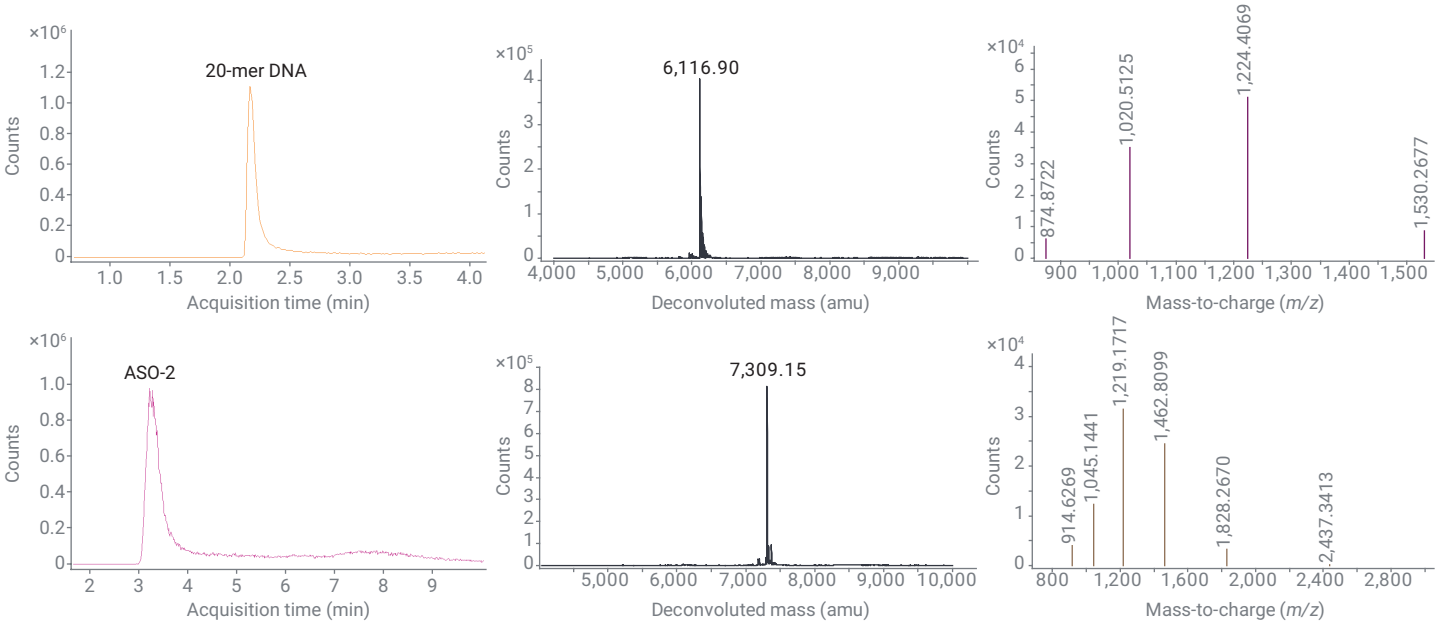

Figure 3. Agilent BioConfirm analysis results for varying oligonucleotides (ASO and DNA) showing extracted ion chromatograms, deconvoluted MS spectra, and raw MS spectra.

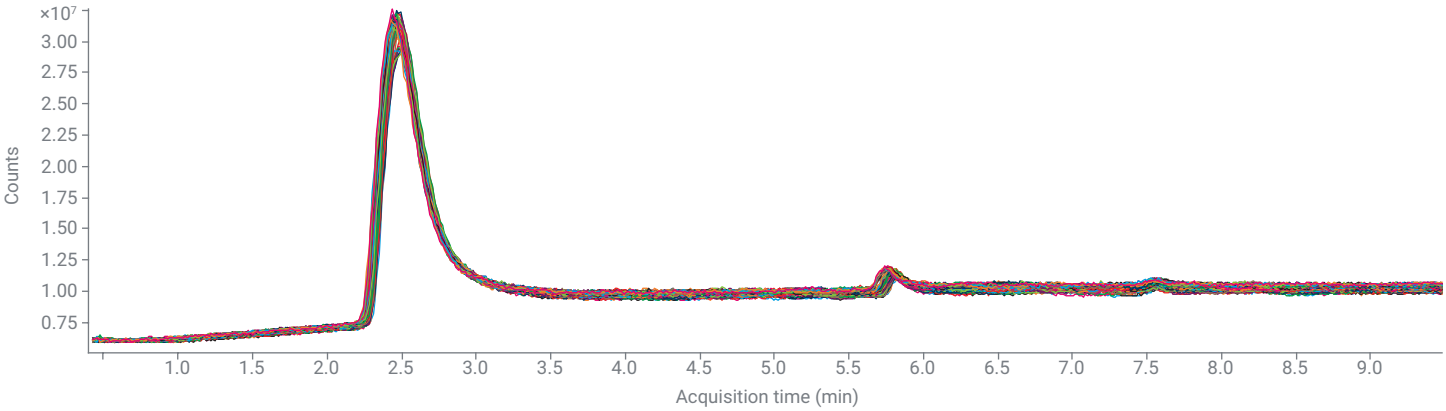

Figure 4. Overlay of 55 injections of ASO-2 showing the repeatability of the retention times.

Conclusion

The described method allows accurate and repeatable analysis of diverse oligonucleotides using non-ion-pairing reversed-phase chromatography combined with TOF LC/MS detection. The results illustrate suitable performance on highly modified ASOs, DNA, sgRNA, and siRNA ranging from 14- to 103-mer oligonucleotides. This methodology provides an improved solution for measuring the masses of pharmaceutically relevant oligonucleotides. This analysis using TOF LC/MS provides an attractive alternative to traditional RPLC methods, which often require dedicated systems. Additionally, this non-ion-paring method is more practical and sustainable when the cost of ion-pairing solvents is high and frequent flushing of the ion-pairing LC is required to maintain system cleanliness.

Reference

1. Hayashi, Y.; Sun, Y. Overcoming Challenges in Oligonucleotide Therapeutics Analysis: A Novel Nonion Pair Approach. *J. Am. Soc. Mass Spec.* **2024**, *35(9)*. DOI: 10.1021/jasms.4c00270

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DE-002889

Printed in the USA, December 20, 2024
5994-8013EN

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