Application Note

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Forced Degradation Studies of Synthetic Oligonucleotide

Using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Abstract

The rising interest in synthetic oligonucleotides as a new class of therapeutics requires the development of quality control methods. A forced degradation study is a critical tool for evaluating chemical stability and elucidating the degradation pathways of synthetic oligonucleotides. It is an essential component in the development of these therapeutic agents. In this application note, the antisense oligonucleotide Fomivirsen was evaluated under thermal and oxidative stress conditions using ion-pairing reversed-phase (IP-RP) LC coupled to LC/Q-TOF. Shortmers and linkage-oxidized degradation products were studied using dedicated target plus impurities (TPI) and sequence confirmation (SC) workflows.

Introduction

Synthetic oligonucleotides, as a novel class of biotherapeutics, hold great potential to treat a wide spectrum of diseases. As of 2024, there have been 22 FDA- and/or EMA-approved oligonucleotide drugs. Oligonucleotide therapeutics are produced through chemical synthesis and often modified to enhance their stability and potency.

Chemical stability and degradation pathway studies are regulatory requirements.[1] A forced degradation study is a critical tool for evaluating the chemical stability and elucidating the degradation pathways of synthetic oligonucleotides. Previous studies on oligonucleotide therapeutics have shown that the degradation products appear mostly from shortmers with losses of nucleotides from the 3'- and 5'-terminus, depurination, desulfurization, and oxidation.[2]

The complexity of the oligonucleotides and their degradation products demands more specific and sensitive analytical tools. Ion-pairing reversed-phase liquid chromatography tandem mass spectrometry (IP-RPLC-MS/MS) is an essential method to address the analytical requirements. The sensitivity and specificity of the method enables the identification and characterization of low-abundance degradation products.

In this application note, forced degradation studies were conducted under thermal and oxidative stress conditions to evaluate the stability of Fomivirsen, an antisense oligonucleotide. An Agilent 1290 Infinity II bio LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF was adopted as the analytical platform. Data analysis was conducted using Agilent MassHunter BioConfirm software, applying the target plus impurities (TPI) and sequence confirmation (SC) workflows.

Experiment

Sample and sample preparation

Fomivirsen was purchased from MedChemExpress (NJ, USA). It is an antisense 21-mer phosphorothioate oligonucleotide with the sequence of CGTTTGCTCTTCTTCTTGCG. The sample was dissolved in water at a concentration of 1 mg/mL and stored at –80 °C.

For the heat stress study, each aliquot of 40 µL sample was incubated at 80 °C in a Thermomixer (Eppendorf, USA). At 4, 8, and 24-hour intervals, three aliquots were collected and stored at 4 °C after cooling down before LC/MS analysis.

For the oxidative stress study, the sample was subjected to 0.30 and 3.00% H_2O_2 at a concentration of 1 mg/mL for 2 and 4 hours at room temperature.

Instrumentation

For separation, an Agilent 1290 Infinity II bio LC system was used, including:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent InfinityLab bio-inert Quick Connect heat exchanger, standard flow (option #065)#

Samples were analyzed on a 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Dual Jet Stream ESI source.

Software

The following software was used in this study:

- Agilent MassHunter acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for LC and MS. Table 3 displays the data analysis parameters in MassHunter BioConfirm software.

Table 1. LC parameters.

Agilent 1290 Infinity II Bio LC System	
Column	Agilent AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Thermostat	8 °C
Solvent A	15 mM TEA and 100 mM HFIP in water
Solvent B	Methanol
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 10 1.0 10 4.0 22 5.0 50
Post Time	6 min
Injection Volume	2 µL, with needle wash flush port 10 seconds of 50% methanol
Column Temperature	60 °C

Table 2. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
General Source Parameters Shared Across Two Workflows	
Source	Dual AJS
Polarity	Negative
Gas Temperature	275 °C
Gas Flow	12 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	65 V
Tune Mode	Extended Dynamic Range (2 GHz)
Reference Mass	*m/z* 1033.988109
Parameters for TPI Workflow	
Acquisition Mode	MS Scan
Mass Range	*m/z* 300 to 3,200
Acquisition Rate	3 spectra/s
Parameters for SC Workflow	
Acquisition Mode	Targeted MSMS
MS Scan Range	*m/z* 400 to 3,000
Acquisition Rate/Time	1 spectra/s
MS/MS Scan Range	*m/z* 100 to 3,000
Acquisition Rate/Time	2 spectra/s
Isolation Width	Medium (~ *m/z* 4)
Collision Energy (CE)	12, 14, 16, 18 V

Table 3. Data analysis parameters.

Agilent MassHunter BioConfirm Software	
Parameters for TPI Workflow	
Mods and Profiles	Oxidation (oxidative stress)
Matching Rules	5'-Truncation with linker maximum allowed 20 (thermal stress)
Matching Algorithm	Find by Formula
Match Tolerance	Mass ±10 ppm
Parameters for SC Workflow	
Extraction (MS/MS)	Group by collision energy Number of scans: 2
Match Tolerance	±15 ppm
Matching Criteria	Warn if score is < 90 Do not match if score is < 80

Results and discussion

Effect of heat at 80 °C

The Fomivirsen sample was heated at 80 °C for an extended period up to 24 hours. Significant degradation of the full-length product (FLP) was observed over the period. Figure 1 illustrates the total ion chromatograms (TICs) of the samples at 0, 4, 8, and 24 hours. The abundance of FLP gradually decreased and more degradation products became prominent over 24 hours. At 4, 8, and 24 hours, the abundance of FLP reduced by 15.17, 28.66, and 80.35%, compared to the zero-hour samples shown in Figure 2.

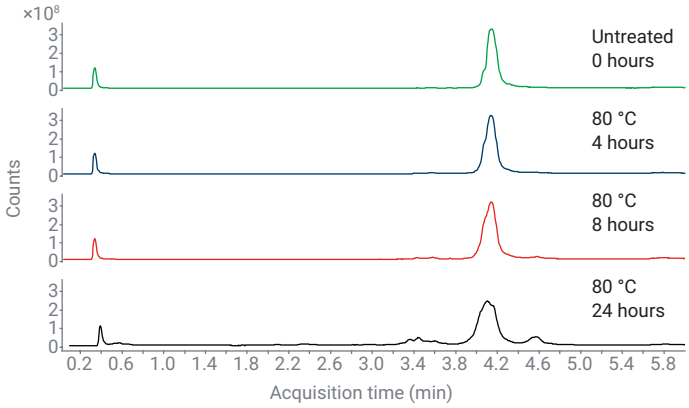

Figure 1. Total ion chromatograms of Fomivirsen samples heated at 80 °C for 0, 4, 8, and 24 hours.

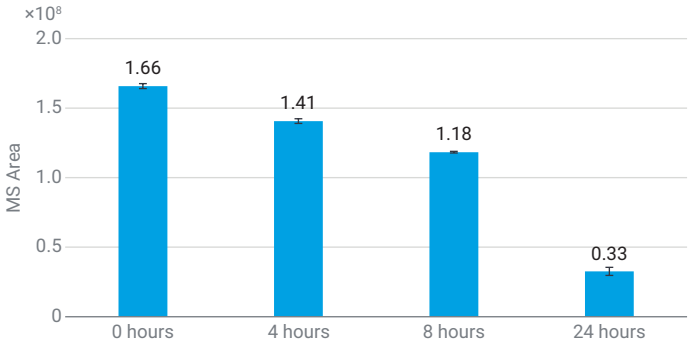

Figure 2. MS areas of full-length product in the heat-stressed samples at 0, 4, 8, and 24 hours (triplicates per condition).

The major group of degradation products identified through the TPI workflow was the 5'-end truncated shortmers with a phosphate group (linker) (Figure 3). To facilitate data analysis, the identified shortmers were classified into three categories: long (n-1 to n-5), medium (n-6 to n-10), and short (n-11 and above). In the zero-hour samples, there were mainly long shortmers (7.43%), low levels of medium (1.10%), and trace-level short-truncated sequences (0.05%). These are considered product impurities, formed during the chemical synthesis due to failed base coupling at the 5' end.

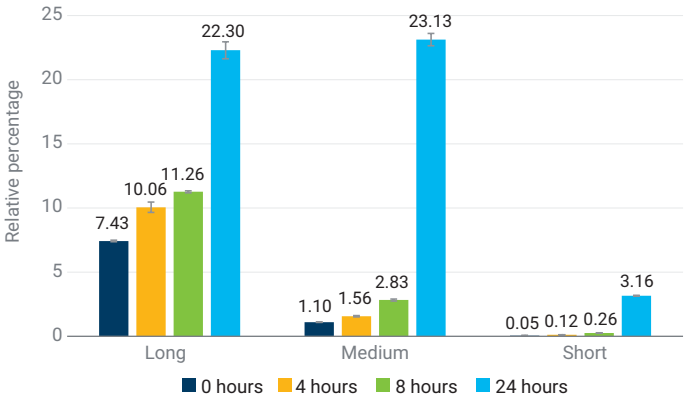

Figure 3. Relative percentage of long, medium, and short length 5' truncated shortmers with linker at 80 °C for 0, 4, 8, and 24 hours (n = 3).

At 4 and 8 hours, the degradation products consisted of predominantly long shortmers. However, gradual increase in relative percentage of medium and short shortmers was evident. By 8 hours, the medium and short shortmers increased by 2.57 and 5.20 times compared to zero hours. By 24 hours, the relative percentage of medium shortmers was almost the same as the long ones. In addition, a drastic increase of short shortmers was observed at 24 hours.

Effect of hydrogen peroxide

In the oxidative stress study, the samples were subjected to 0.30 and 3.00% H_2O_2 for 2 and 4 hours. The main degradation pathway was the oxidation of the phosphorothioate linkage to the corresponding phosphodiester analog (PO). Figure 4 illustrates the TICs of the stressed samples.

At the concentration of 0.30% H_2O_2, the FLP was degraded by 63.36 and 88.99% at 2 and 4 hours. Multiple oxidative degradants coeluted with the FLP, causing the wavy peak apex and fronting of the original FLP peak due to the less hydrophobic oxidated species. The relative percentage of oxidized oligo was 74.49 and 91.76% at 2 and 4 hours, respectively (Figure 5). The number of oxidized linkages ranged from one to six at 2 hours, and one to eight at 4 hours.

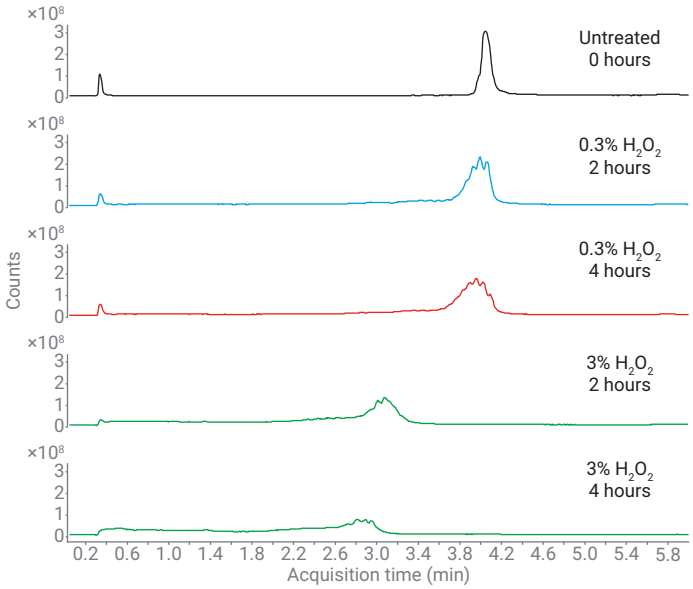

Figure 4. Total ion chromatograms of oxidative stressed samples.

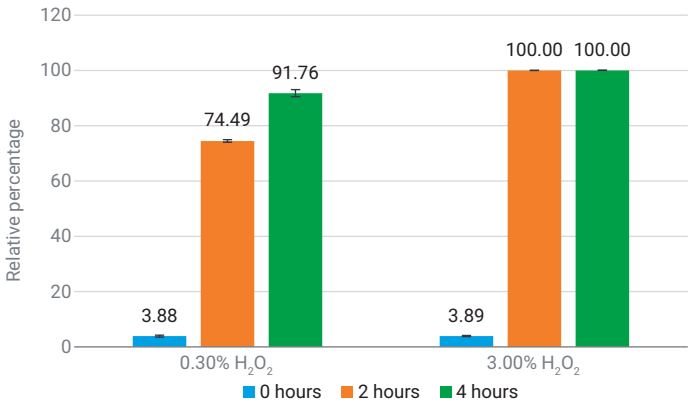

Figure 5. Relative percentage of total oxidized degradants in the stressed samples at 0, 2, and 4 hours (n = 3).

At the concentration of 3.00% H_2O_2, the FLP was completely oxidized at 2 hours; 15 to 19 PO linkages were generated at this time. By 4 hours, the number of PO linkages further increased to 19 and 20, which indicated that almost complete oxidation was achieved. Because Fomivirsen is the first generation of synthetic oligo[3] with only linkage modification, it is more susceptible to oxidative stress than 2' ribose-modified oligos.

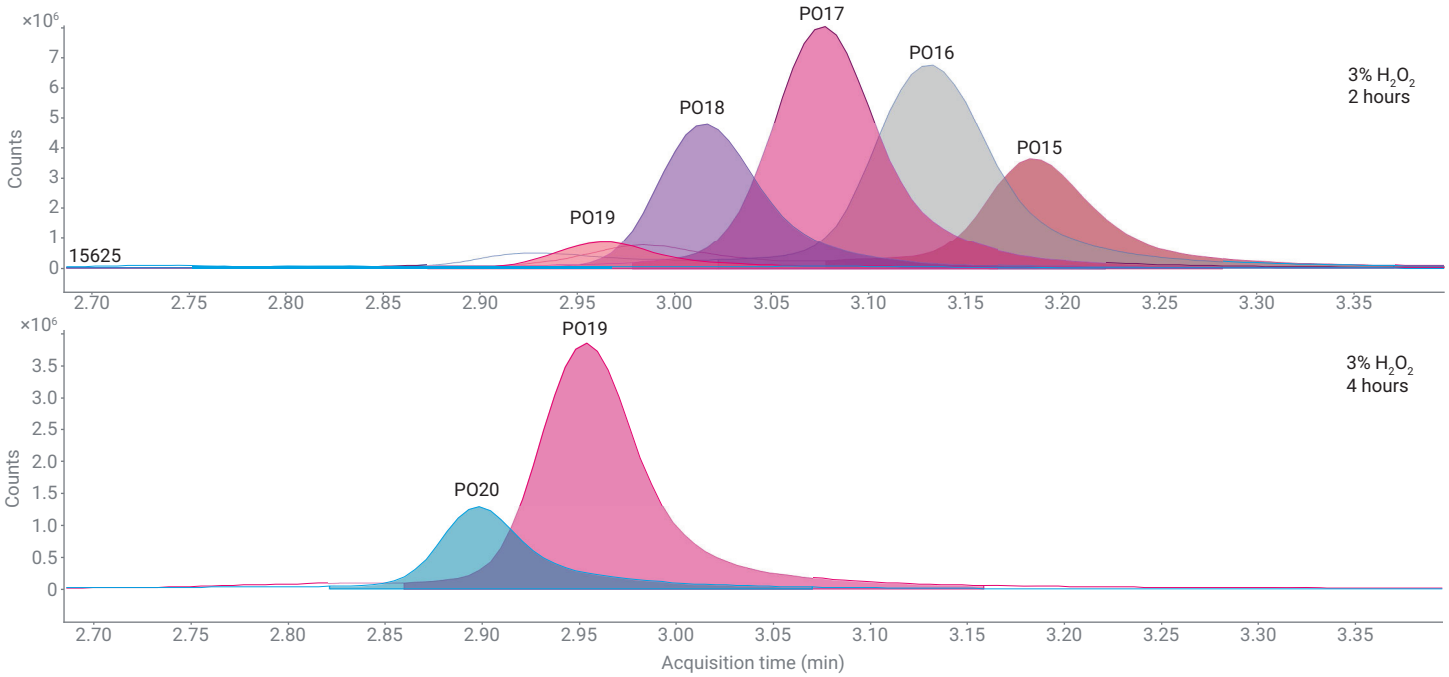

Figure 6. Overlaid biomolecule MS spectra of 3% H_2O_2-treated samples at 2 and 4 hours.

Sequence confirmation

In addition to degradation product investigation using the TPI workflow, the sequence confirmation workflow was employed to confirm the FLP and n-15 shortmer's sequences in the 24-hour heat-stressed sample. 100% coverage was achieved for both sequences, with adequate fragment ion types and numbers being identified as shown in the fragment confirmation ladder of each sequence in Figure 7. The fragment ions of FLP were all good quality with BioScore above 90. It is noteworthy that, although the relative abundance of the n-15 shortmer was as low as 0.07% and eluted as early as 0.48 minutes, its sequence was confidently confirmed by the SC workflow with BioScore all above 80.

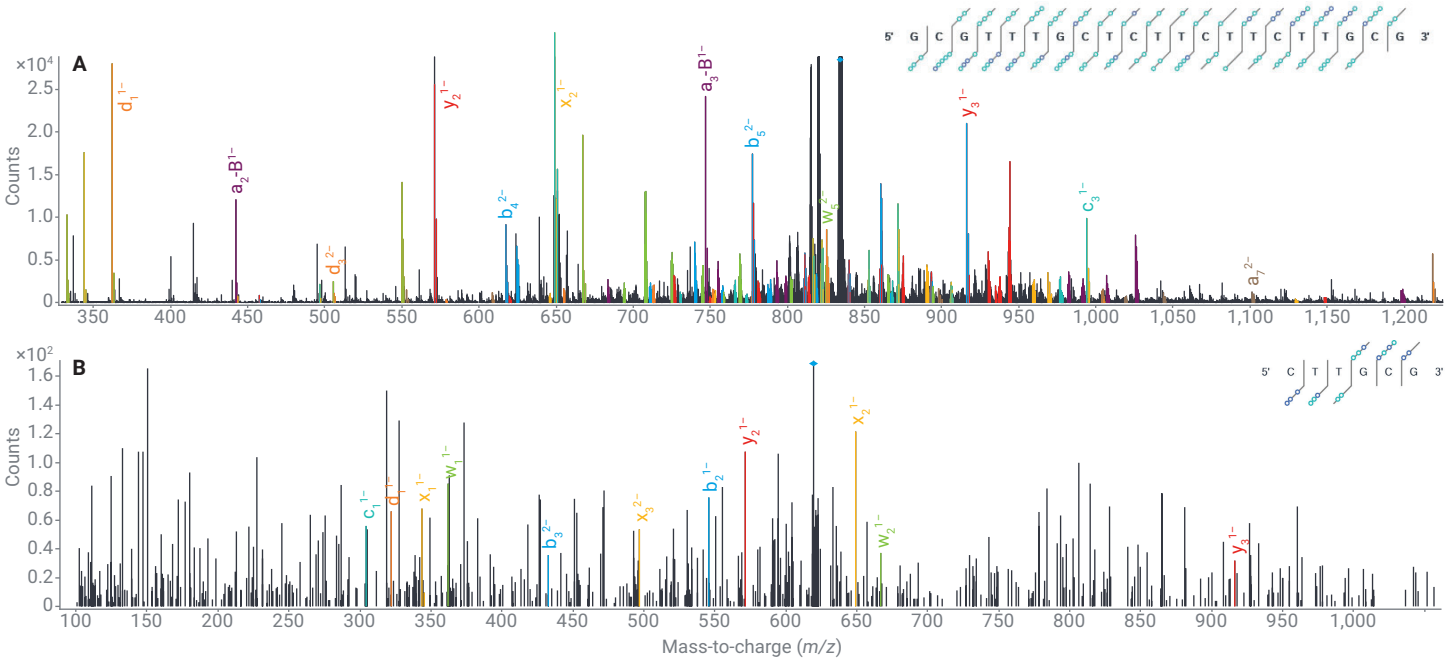

Figure 7. Sequence confirmation MS/MS spectra of (A) FLP and (B) n-15.

Conclusion

Forced degradation studies serve as a critical tool for evaluating chemical stability and elucidating the degradation pathways of synthetic oligonucleotides. They represent an essential component in the development and characterization of these therapeutic agents, ensuring safety as well as efficacy of the intended oligonucleotide therapeutic agent.

In this application note, the use of ion-pairing RP-HPLC on an Agilent 1290 Infinity II bio LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF was powerful in the identification and confirmation of various degradation products in the Fomivirsen sample. The results demonstrated high sensitivity and reproducibility of the method, suitable for conducting degradation studies.

References

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2. Pourshahian, S. Therapeutic Oligonucleotides, Impurities, Degradants, and Their Characterization by Mass Spectrometry. *Mass Spectrom. Rev.* **2021**, *40*, 75–109.
3. Elzahar, N. M.; Magdy, N.; El-Kosasy A. M.; Bartlett M. G. Degradation Product Characterization of Therapeutic Oligonucleotides Using Liquid Chromatography Mass Spectrometry. *Analytical and Bioanalytical Chemistry*. **2018**, *410*, 3375–3384.

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