Analysis of nusinersen using a wide-pore reversed-phase YMC Accura Triart Bio C18 column

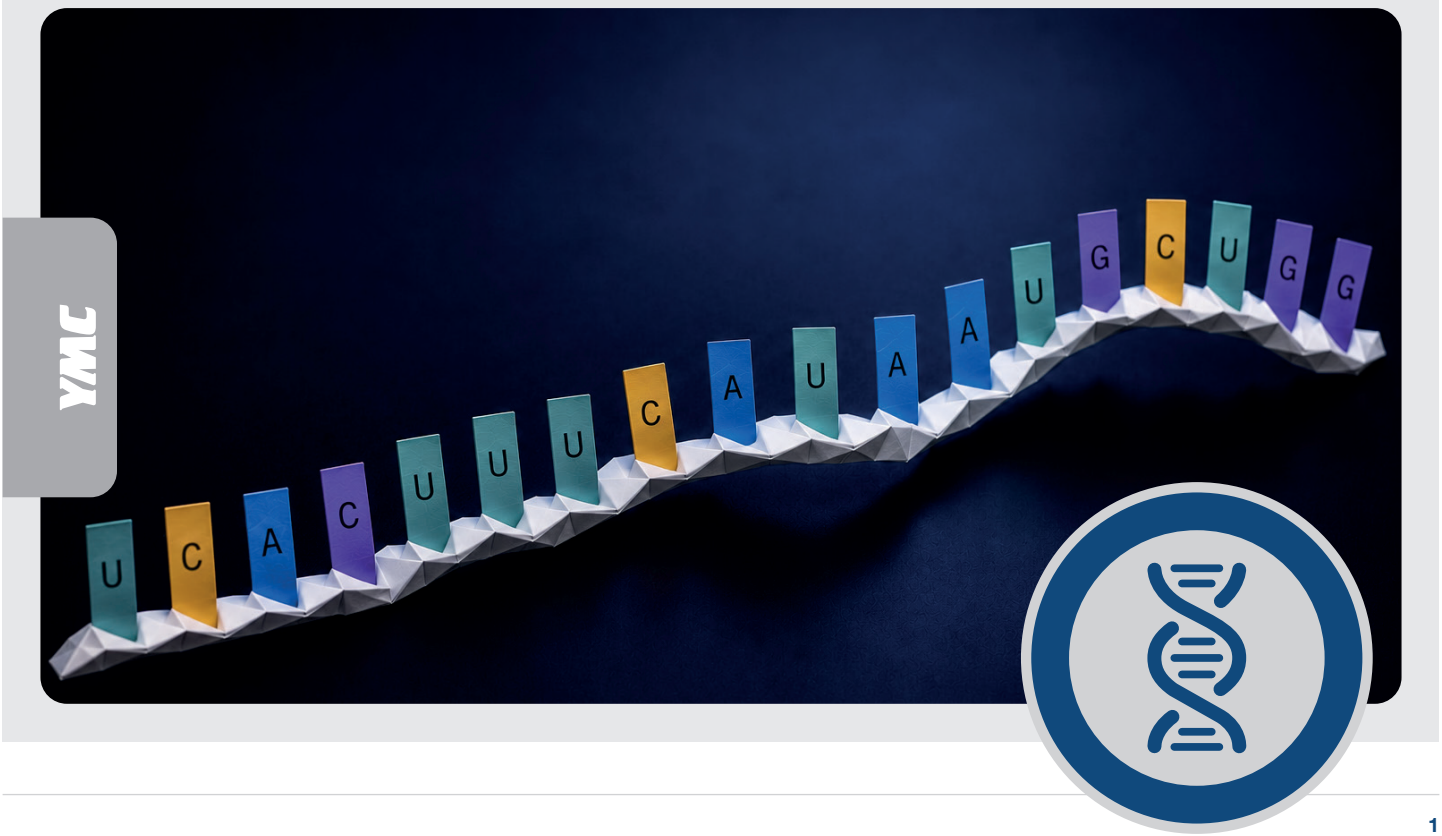
Oligonucleotide therapeutics have emerged as a major class of drugs, offering broad applications across various disease areas.

Antisense oligonucleotides (ASOs) constitute a well-established therapeutic modality, with several drugs approved by the U.S. Food and Drug Administration (FDA). Among them, nusinersen, an 18mer ASO incorporating phosphorothioate (PS) backbone linkages and 2'-O-methoxyethyl (2'-O-MOE) sugar modifications, is a prominent example, providing effective treatment for spinal muscular atrophy (SMA).

For synthetic oligonucleotides, solid-phase synthesis and subsequent chemical modifications inherently generate a variety of closely related impurities, including truncated species, sequence variants, and linkage-specific by-products. Comprehensive analytical characterisation is therefore essential to ensure product quality and support regulatory compliance.

Ion-pair reversed-phase liquid chromatography (IP-RP LC) is a reliable method for the analysis of therapeutic oligonucleotides, with its ability to resolve structurally similar species. When coupled with mass spectrometry (MS) detection, this approach enables an accurate characterisation of the complete impurity profile of oligonucleotides.

In this Application Note, the effects of temperature, organic solvent composition, and ion-pair reagent types on nusinersen retention and selectivity using an YMC Accura Triart Bio C18 column are investigated. The optimised analytical conditions are subsequently applied to LC-MS to further identify impurities



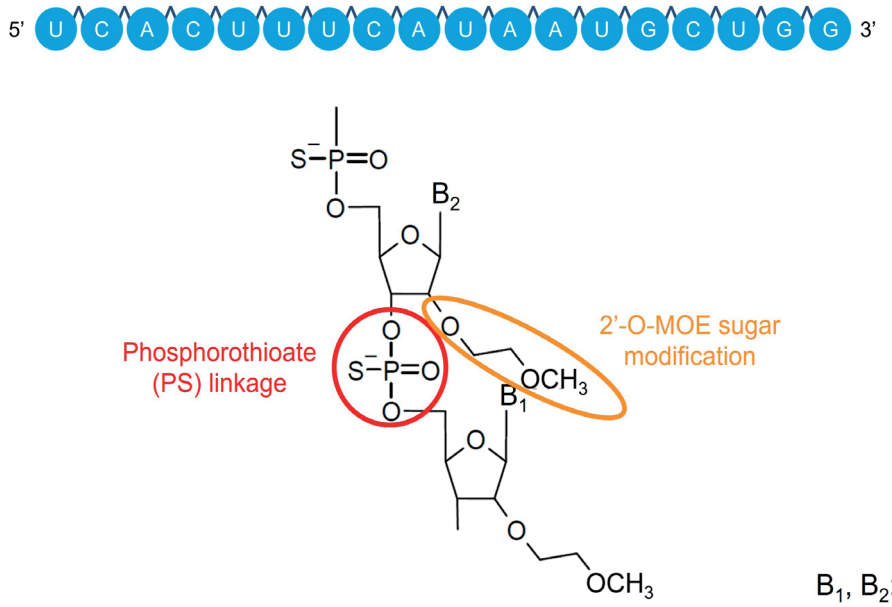


Figure 1: Structure of nusinersen showing the PS linkage and sugar modifications.

Effect of temperature

Temperature significantly influences retention and separation (Figure 2). A temperature range of 30 to 85°C is investigated. As the temperature increases, the retention time of nusinersen decreases, and the peaks become sharper. At 30°C, nusinersen is not eluted during the gradient elution step but instead during the column washing step. At 50°C, separation of early-eluting impurities improves.

Further increasing the temperature to 70°C enables the separation of both early- and late-eluting impurities associated with nusinersen. No significant difference in peak separation is observed between 70 and 85°C. Therefore, 70°C is selected for its effective separation of impurities, sharp peak shapes, and sufficient retention time.

Table 1: Overall chromatographic conditions unless otherwise stated.

Column:	YMC Accura Triart Bio C18 (1.9 µm, 30 nm) 100 x 2.1 mm ID
Eluents:	A) 5 mM HA–100 mM HFIP* B) acetonitrile/methanol (25/75)
Gradient:	0.86%B/min (1-30 min)
Initial %B:	27%B
Flow rate:	0.2 mL/min
Temperature:	70°C
Injection:	4 µL (200 ng/µL)
Detection:	UV at 260 nm
Sample:	nusinersen

* HA: hexylamine; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol

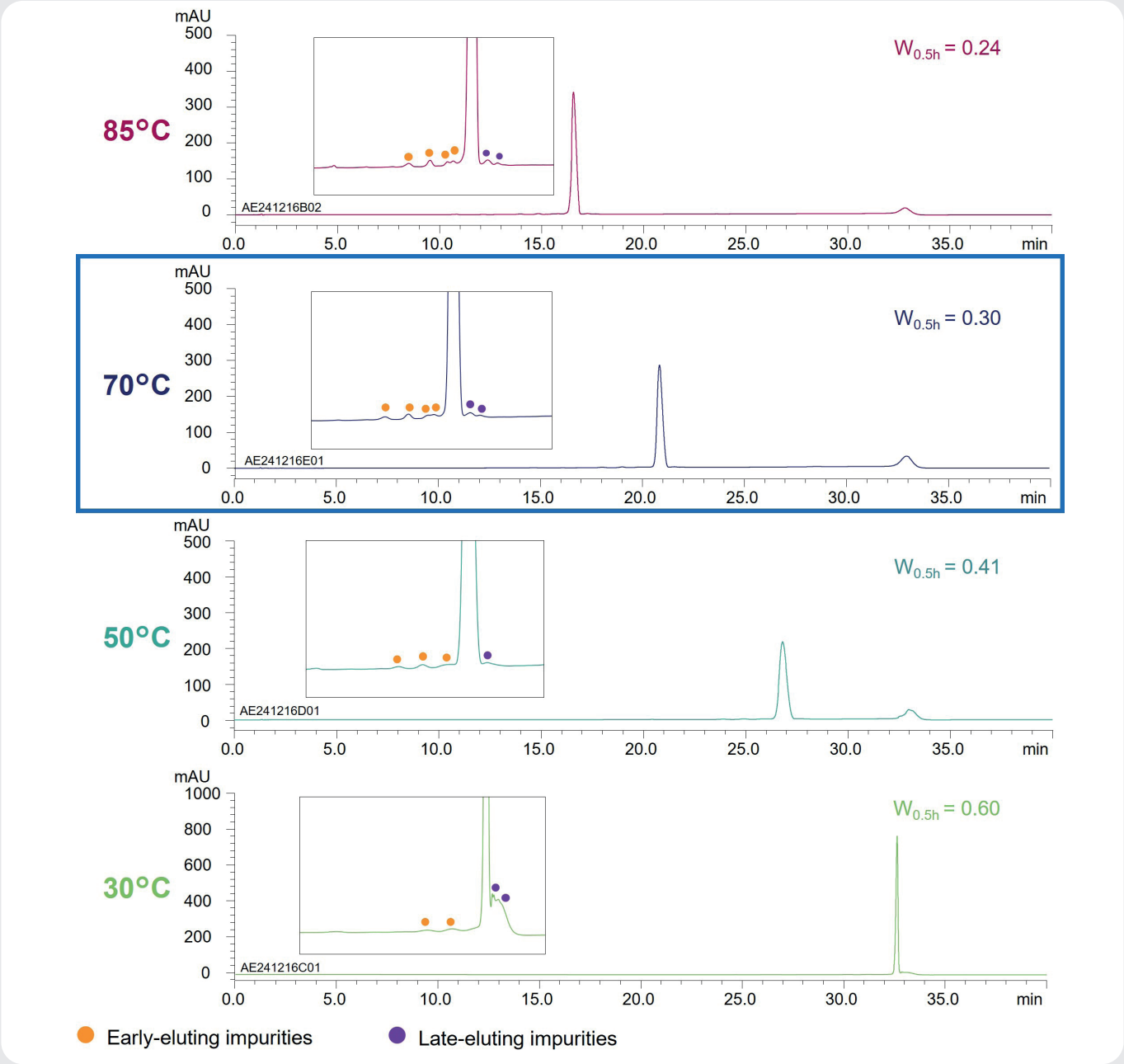


Figure 2: Effect of temperature on the separation of nusinersen and its associated impurities.

Effect of organic solvent composition

The composition of the organic solvent in eluent B affects separation and selectivity (Figure 3). Various ratios (v/v) of acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and 2-propanol (IPA) are investigated. While using 100% ACN allows separation of early-eluting impurities, using ACN/MeOH (25/75) yields an additional early-eluting impurity but results in broader peaks. Increasing the solvent strength of the mobile phase with ACN/EtOH (25/75) results in sharper peaks while maintaining separation of early-eluting impurities. Further increasing the strength with 100% IPA or ACN/IPA (25/75) enhances peak sharpness. Therefore, 100% IPA is selected for its ability to yield sharper peaks and efficient separation of early-eluting impurities.

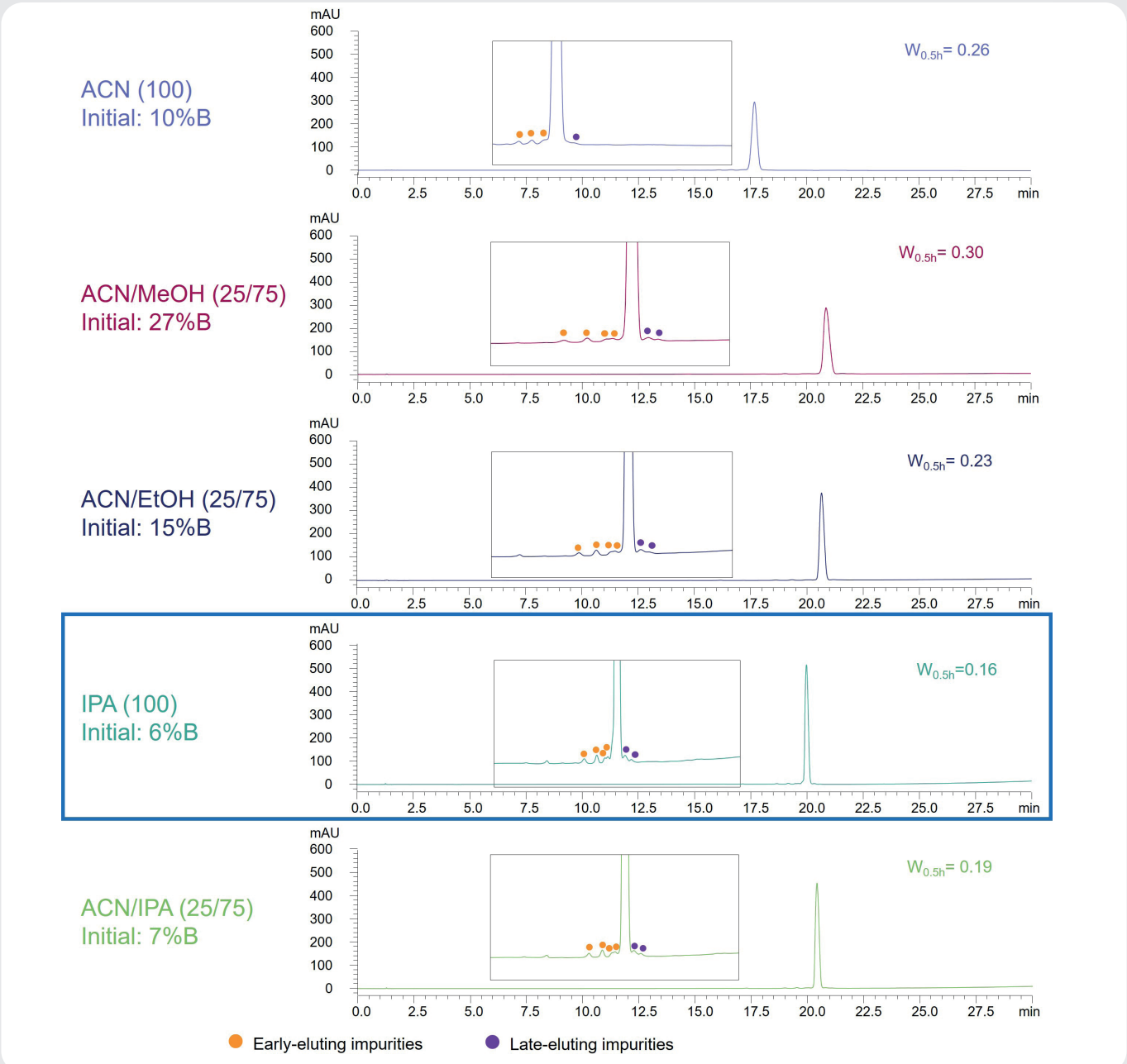


Figure 3: Effect of organic solvent composition on the separation of nusinersen and its associated impurities.

Effect of ion-pair reagents

The type of ion-pair reagent influences selectivity (Figure 4). Four ion-pair reagents with increasing hydrophobicity are investigated, including triethylamine (TEA), N,N-diisopropylethylamine (DIPEA), dibutylamine (DBA), and hexylamine (HA), listed in ascending order of hydrophobicity. No significant differences in peak shapes are observed across the four ion-pair reagents. Because TEA is the least hydrophobic, nusinersen is expected to elute earlier with TEA than with the other reagents; therefore, a lower initial concentration of eluent B (0.5%B) is used. Under these conditions, TEA still exhibits poor resolution of the early-eluting impurities. In contrast, HA provides the strongest retention of nusinersen and the best resolution of the early-eluting impurities. These results indicate that the hydrophobicity of the ion-pair reagent affects the retention and separation of nusinersen and its associated impurities.

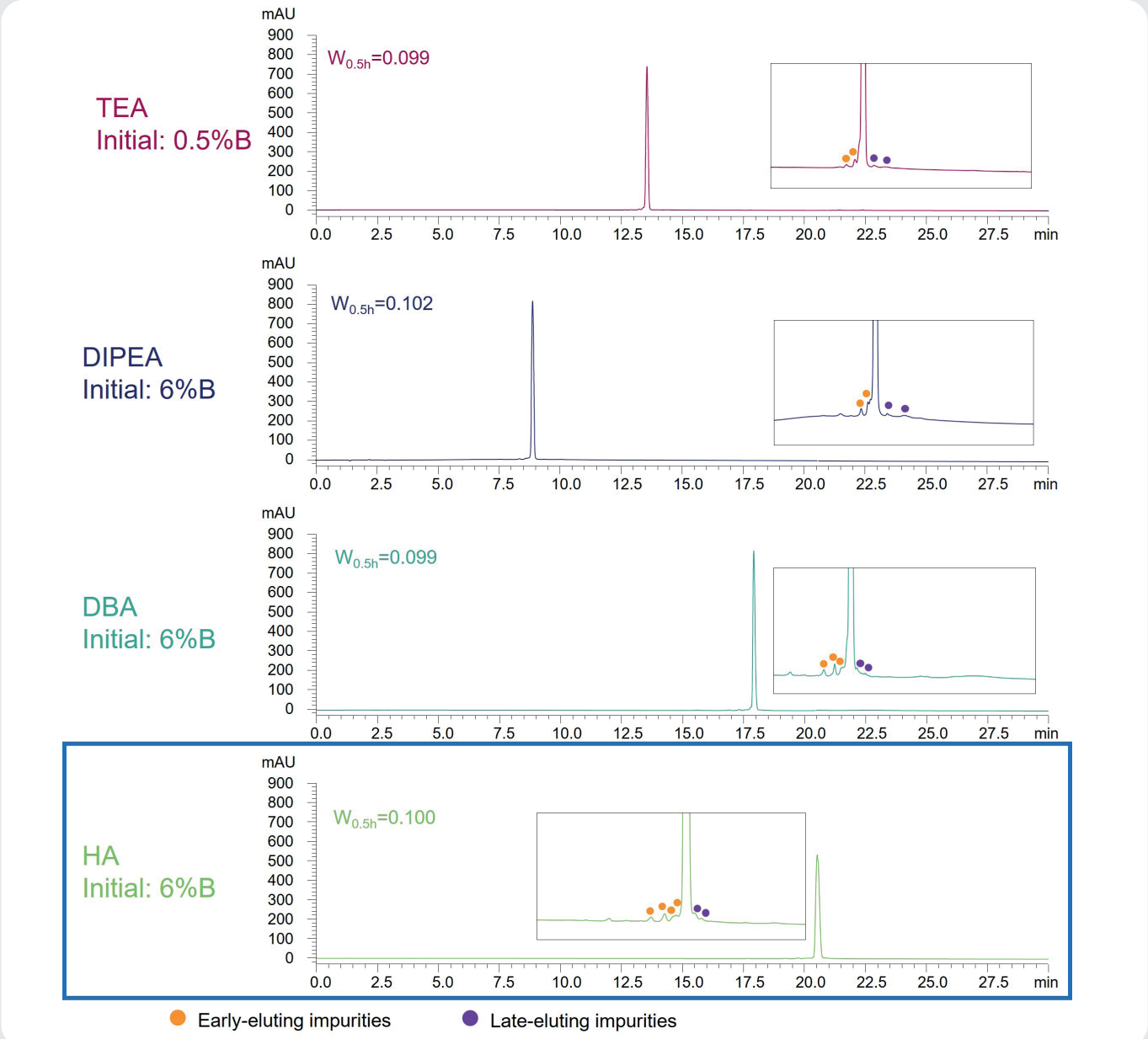


Figure 4: Effect of ion-pair reagents on the separation of nusinersen and its associated impurities.

LC-MS analysis of nusinersen

Liquid chromatography-mass spectrometry (LC-MS) enables the identification of early-eluting impurities associated with nusinersen **(Figure 5)**. The mass-extracted ion chromatogram (MIC) reveals three distinct early-eluting peaks corresponding to the N-3, N-2, and N-1 truncated species, eluting in this order. Although an additional minor peak corresponding to the N-4 truncated species is seen on the total ion chromatogram (TIC), this peak is not detected in the MIC profile. Examination of the main peak shows that a single-linkage PO variant co-eluted with nusinersen (all-PS), reflecting a known challenge in the analysis of PS-modified oligonucleotides.

Despite this limitation, the optimised IP-RP LC-MS method achieves comprehensive resolution and confident identification of all truncated impurities associated with nusinersen. These results demonstrate the strong capability of the YMC Accura Triart Bio C18 column for resolving closely related oligonucleotide species within complex impurity profiles. Although nusinersen serves as an example oligonucleotide, the analytical approach and chromatographic platform described here are applicable to a wide range of oligonucleotide therapeutics.

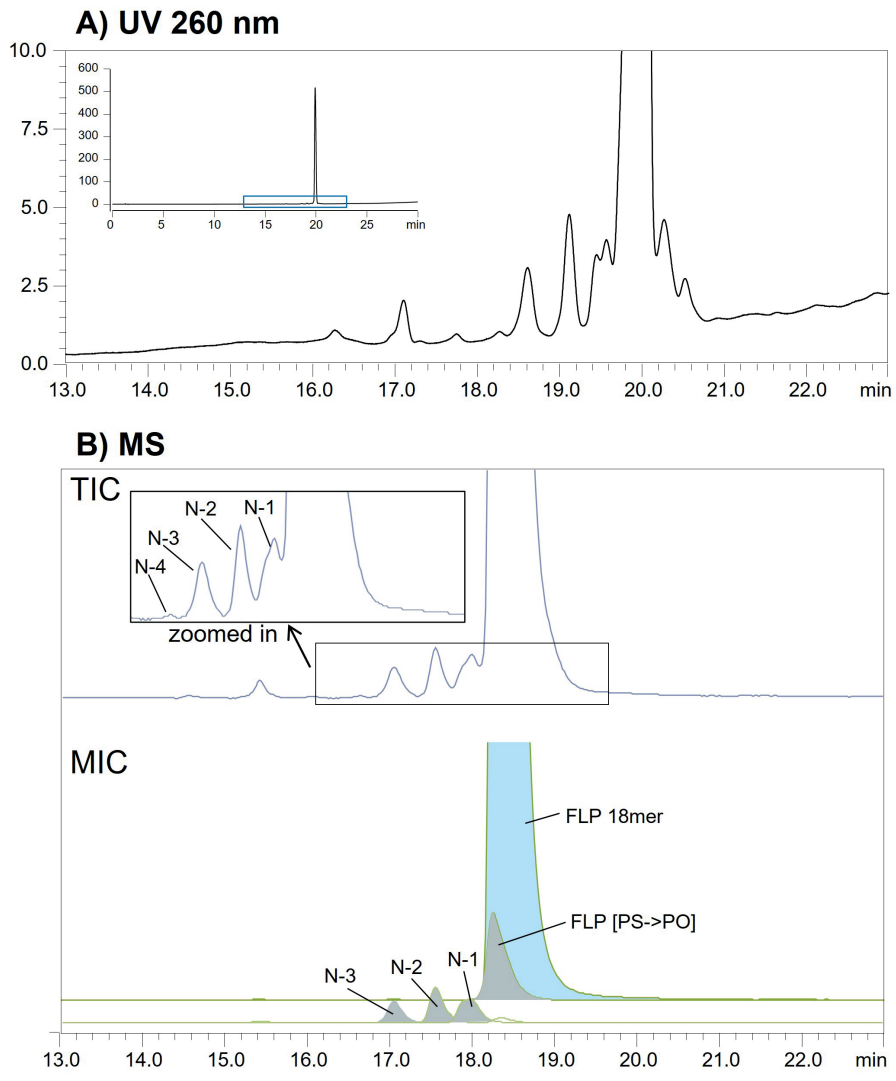


Figure 5. Detection of nusinersen and its associated impurities by UV 260 nm (A) and mass spectrometry (B).

Table 2: Optimised analytical conditions.

Column:	YMC Accura Triart Bio C18 (1.9 µm, 30 nm) 100 x 2.1 mm ID
Eluents:	A) 5 mM HA–100 mM HFIP*
	B) 2-propanol
Gradient:	0.86%B/min
Initial %B:	6%B
Flow rate:	0.2 mL/min
Temperature:	70°C
Injection:	4 µL (200 ng/µL)
System:	Shimadzu Nexera X2/SPD-M30A with UV-Vis detector
	Shimadzu Nexera XS/LCMS-9030 with Q-TOF LC-MS
Sample:	nusinersen

* HA: hexylamine; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol

Acknowledgement

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Application data by YMC CO., LTD.