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Application Note

Deploying the Alliance™ iS Bio HPLC System as a Modern HPLC for Biopharmaceutical Analysis in QC Environments

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Abstract

The Alliance iS Bio HPLC System with MaxPeak™ High Performance Surfaces (HPS) Technology is a bio-inert LC designed to reduce surface/analyte interaction for biopharmaceutical manufacturing environments. As a modern HPLC instrument, the Alliance iS Bio HPLC System is engineered with features including Waters innovative MaxPeak HPS Technology, intuitive touchscreen operation, automated pre-run checks to minimize errors, and Intelligent Method Translator App (iMTA). Together, these features enable manufacturing environments to modernize methods, save resources, and increase throughput for biopharmaceuticals. In this study, we evaluate the Alliance iS Bio HPLC System in a routine QC testing environment for biopharmaceuticals. Oligonucleotides were chosen as a test case given their relevance as an emerging modality and potential to benefit from the MaxPeak HPS Technology featured in the Alliance iS Bio HPLC System, given their susceptibility to analyte/surface interactions. In this study, up to a 30% increase in peak height and 15% increase in peak area was observed in the separation of oligonucleotides when compared to a legacy system. Furthermore, analysis run time, solvent, and sample consumption were reduced up to threefold when scaling to modern column chemistries. These results demonstrate the Alliance iS Bio HPLC System is well suited for QC environments as a next generation HPLC platform for analysis and routine testing of biopharmaceuticals.

Benefits

- The Alliance iS Bio HPLC System delivers consistent performance for QC environments.
- MaxPeak High Performance Surfaces increase recovery of metal-sensitive analytes.
- The Intelligent Method Translator App reduces errors and saves time in method transfer.
- The Alliance iS Bio HPLC System reduces operating costs and waste in scaled methods.

Introduction

Biopharmaceuticals have seen a steady increase over the past decade in their application as therapeutics to treat cancer and rare diseases. Their success is partly attributed to their ability to be tailored toward diseases while minimizing adverse side effects, making them superior in specificity and safety over traditional treatment methods. Oligonucleotide based therapies represent some of the most recent advancements in biologics in their treatment of diseases including muscular dystrophy, spinal muscular atrophy, and more recently as vaccines for controlling the spread of COVID-19.[1] These types of cell and gene therapies are based on nucleic acid interaction, which are formed through the coupling of base nucleosides to form a sequence designed to elicit a specific expression in the cell. While the synthesis procedure is well understood and controlled, it is crucial to monitor and control the impurities that form during the synthesis process.

To ensure drug products are safe, supporting labs in manufacturing environments are required to monitor known impurities or critical quality attributes (CQAs) associated with the drug product prior to release. Ion-pairing reversed phase chromatography (IP-RPLC) is a widely used technique to separate oligonucleotides.[2] As a technique, IP-RPLC effectively separates the oligonucleotides by size based on the ion pairing between the oligonucleotide's phosphate backbone and positively charged amine from the ion-pairing agent that adsorbs to the stationary phase. However, phosphate is a known chelator and can form complexes with metal. This can be problematic for oligonucleotides as the negatively charged phosphate groups of the backbone are prone to interact strongly with metal surfaces. Such interactions could lead to chromatographic artifacts including reduced recovery, increased variability, and peak tailing. This contrasts with QC labs which desire methods that are robust and can deliver consistent results in a timely manner. Using LC platforms that are designed for the analysis of biopharmaceuticals to help minimize these interactions while delivering robust performance is ideal for manufacturing environments.

Based on the innovative design of the Alliance iS HPLC System, the Alliance iS Bio HPLC System is the Waters™ answer to these challenging modalities. The Alliance iS Bio HPLC System is a bio-inert HPLC system specifically engineered for biopharmaceutical manufacturing environments. As part of its design, this innovative HPLC platform features a bio-inert flow path with biocompatible components throughout the system, and the MaxPeak HPS Technology, making it ideal for quality control labs working on biopharmaceutical applications. The system, which supports MaxPeak Premier Columns, enables increased resolution, increased sensitivity, and reduced peak tailing in the analysis and routine testing of biopharmaceutical samples.[3] These combined features allow for efficient method development while delivering consistent and reliable results.

Here in, the scope of this study is to evaluate the potential benefits of the Alliance iS Bio HPLC System by analyzing two samples: an oligonucleotide standard (OST) and a fully thiolated phosphorothioate (PS) oligonucleotide sample, GEM91.[4] Comparative analysis was performed between a legacy platform, and the new Alliance iS Bio HPLC System as a modern HPLC platform.

Experimental

Alliance iS Bio HPLC System performance was compared to a Legacy System. The MassPREP™ Oligonucleotide Standard (OST) contains 1 nmol of 15, 20, 25, 30, and 35 mer oligodeoxythymidines (p/n: 186004135 < https://www.waters.com/nextgen/global/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html>). The vial contents were dissolved in 250 µL of deionized water, giving a final concentration of 4 pmol/µL. 10 µL of this sample was injected (40 pmol) on column. GEM91: A 25 mer fully thiolated phosphorothioate (PS) oligonucleotide CTC TCG CAC CCA TCT CTC TCC TTC T, MW 7776 Da. GEM91 was injected at a concentration of 0.5 mg/mL.

Legacy System Conditions

LC system:	Legacy system
Detection:	UV/Vis 2489, λ=260 nm
Column:	XBridge™ BEH™ C_{18}, 5 µm, 130Å, 4.6 mm X 100 mm,

	(p/n: 186003115); XBridge BEH C_{18}, 2.5 µm, 130Å, 4.6 mm X 100 mm, (p/n: 186006039)
Column temperature:	60 °C
Sample temperature:	5 °C
Injection volume:	10 µL
Flow rate:	0.65 mL/min for OST, 0.50 mL/min for GEM91
Mobile phase:	A: 25 mM HAA in water, pH=7.0 B: 25 mM HAA in water/ACN=40/60, pH=7.0 C: Water D: Acetonitrile
Chromatography software:	Empower™ 3, FR4

Alliance iS Bio HPLC System Conditions

LC system:	Alliance iS Bio HPLC System
Detection:	TUV, λ=260 nm
Column:	XBridge Premier Oligonucleotide BEH C_{18}, 2.5 µm, 130Å, 4.6×50 mm (p/n: 186009901; +eConnect p/n: 186009901RF); 2.5 µm, 130Å, 4.6×100 mm (p/n: 186009902; +eConnect p/n: 186009902RF); 2.5 µm, 130Å, 4.6×150 mm (p/n: 186009903; +eConnect p/n: 186009903RF)

	Note: Full benefits of column tracking are achieved on Empower 3.8.0
Column temperature:	60 °C
Sample temperature:	5 °C
Injection volume:	10 µL for OST; as of GEM91, 10 µL for 50mm column, 20 µL for 100 mm column and 30 µL for 150 mm column.
Flow rate:	0.65 mL/min for OST; for GEM91: 0.50 mL/min for 5 µm column, 1.00 mL/min for 2.5 µm column
Mobile phase:	A: 25 mM HAA in water, pH=7.0 B: 25 mM HAA in water/ACN=40/60, pH=7.0 C: Water D: Acetonitrile
Chromatography software:	Empower 3.8.0

Gradient Table for Waters MassPrep OST analysis

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
initial	0.650	46.0	54.0	0.0	0.0	initial
1.00	0.650	46.0	54.0	0.0	0.0	6
8.50	0.650	33.0	67.0	0.0	0.0	6
11.50	0.650	33.0	67.0	0.0	0.0	6
12.50	0.650	25.0	0.0	0.0	75.0	6
14.50	0.650	25.0	0.0	0.0	75.0	6
15.50	0.650	46.0	54.0	0.0	0.0	6
20.00	0.650	46.0	54.0	0.0	0.0	6

Gradient Table for GEM91 analysis

Time (min)	Flow (mL/min)	%A	%B	%C	%D	Curve
initial	0.500	56.0	44.0	0.0	0.0	initial
4.00	0.500	56.0	44.0	0.0	0.0	6
48.00	0.500	34.0	66.0	0.0	0.0	6
60.00	0.500	34.0	66.0	0.0	0.0	6
62.00	0.500	25.0	0.0	0.0	75.0	6
74.00	0.500	25.0	0.0	0.0	75.0	6
84.00	0.500	56.0	44.0	0.0	0.0	6
100.00	0.500	56.0	44.0	0.0	0.0	6

Results and Discussion

Oligonucleotide MaxPeak HPS Technology Benefits

Oligonucleotides are notorious for low recovery in LC separations due to the chelating nature of the phosphate backbone. This can result in lengthy passivation procedures of surfaces to increase analyte recovery for analysis. As part of its bio-inert design, the Alliance iS Bio HPLC System is engineered with MaxPeak HPS Technology to

reduce analyte/surface interactions including those encountered in the analysis of oligonucleotides. To evaluate the Alliance iS Bio HPLC System in this respect, the recovery of an oligonucleotide standard was compared on two systems: A legacy system with a 2.5 µm, 130Å, 4.6 × 100 mm, XBridge BEH C_{18} Column and the Alliance iS Bio HPLC System (modern HPLC) with a 2.5 µm, 130Å, 4.6 × 100 mm, XBridge Premier Oligonucleotide BEH C_{18} Column. Each system was subjected to 20 injections of the MassPrep OST standard. Hexylammonium acetate (HAA) was utilized as the ion-pairing reagent. The mobile phase was prepared using pH 7 aqueous 25 mM HAA in water (MP A) and a mixture of water and acetonitrile (MP B). As depicted in Figure 1, the Alliance iS Bio HPLC System with a MaxPeak Premier Oligonucleotide Column demonstrated a significant improvement in the analysis of oligonucleotides. Specifically, using the 20 nt peak as a representative fully resolved peak, an average increase of up to 30% in peak height (Fig. 1A) and a 14% increase (Fig. 1B) in peak area was observed indicating improved sensitivity and recovery. Moreover, the Alliance iS Bio HPLC System achieved maximum recovery in a shorter time span, thereby eliminating the need for lengthy passivation procedures. This efficiency is attributed to the bio-inert aspect of the Alliance iS Bio HPLC System and column, wherein the MaxPeak HPS Technology effectively reduces the non-absorptive chelation between the phosphate backbone of the oligonucleotide and the metal surface of the system and column hardware, which in turn enhances analyte recovery.

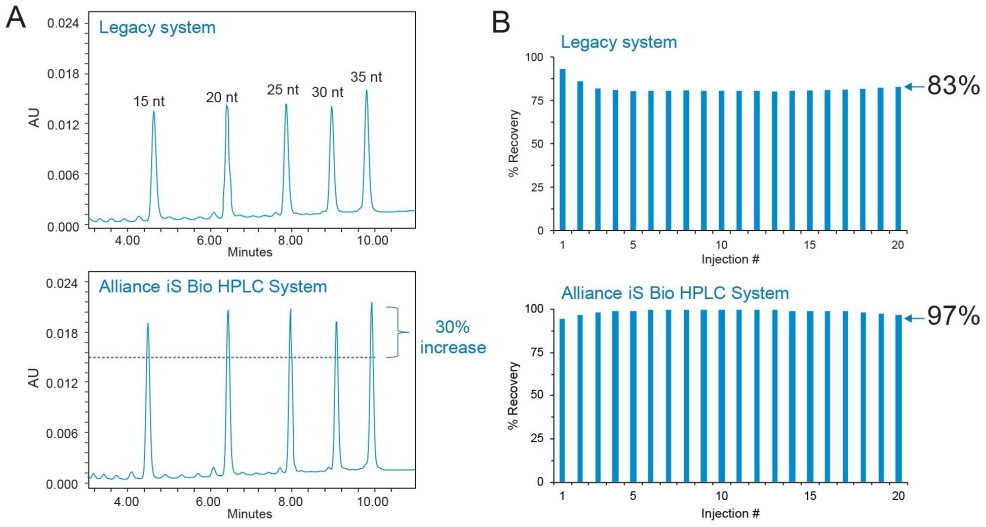


Figure 1. A) Separation of Waters MassPREP OST on an Alliance HPLC System with an XBridge BEH C_{18} Column (top); and on a Alliance iS Bio HPLC System with an XBridge Premier Oligonucleotide BEH C_{18} Column (bottom). B) Comparison of the % recovery versus the number of injections on both systems.

Method migration from a Legacy System to Alliance iS Bio HPLC System

With the benefit of the bio-inert surfaces confirmed, a fully thioated oligonucleotide therapeutic candidate, GEM91, was analyzed to further investigate the performance of the Alliance iS Bio HPLC System using a sample more representative of industry. Despite its refinement as a drug substance, GEM91 still contains trace impurities that necessitate monitoring. To establish comparability between instruments the same method and column were used on both systems. To assist in transferring methods, the Alliance iS Bio HPLC System offers users access to the Intelligent Method Translator App (iMTA) as a means to expedite the transfer process and reduce transcription errors. In this study, the iMTA (Fig 2A, 2B) was used to export the method conditions from the legacy system and re-create the method within the Alliance iS Bio HPLC System. As shown in the data, using a chemistry format representative of a traditional HPLC method (5 µm, 130Å, 4.6 × 100 mm), the legacy method chromatogram (Fig 2C) was reproduced on the Alliance iS Bio HPLC System (Fig 2D) without issue. Upon closer examination of the impurity profile (identified as peaks 1-3), we observed that the integrated MaxPeak HPS of the Alliance iS Bio HPLC System resulted in an increase of up to 40% in the signal-to-noise ratio for the trace impurities. This improvement translates to enhanced recovery and increased accuracy when analyzing critical species for advanced therapies such as oligonucleotides.

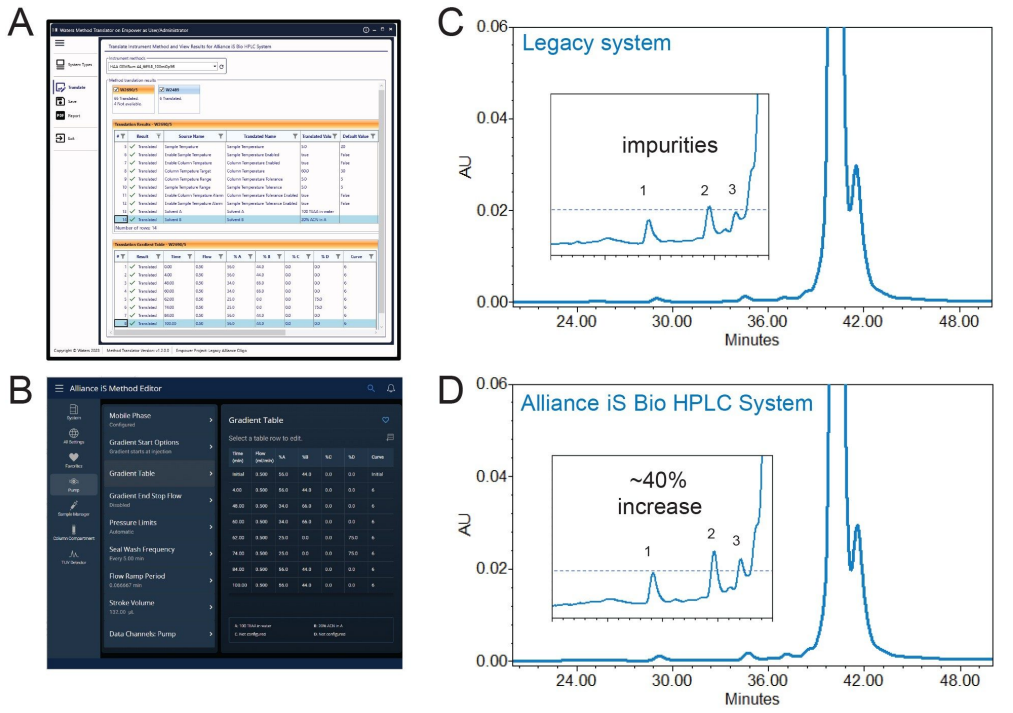


Figure 2. The intelligent Method Translator App (iMTA) was used to transfer a legacy method from the A) Alliance HPLC System to the B) Alliance iS Bio HPLC System. Comparison of GEM91 analysis on the C) Alliance HPLC System and D) Alliance iS Bio HPLC System with an XBridge BEH C_{18} Column.

Method modernization

As a modern HPLC instrument, the Alliance iS Bio HPLC System is capable of supporting pressures up to 10,000 psi enabling users to leverage the advantages of modern column technology to save resources and increase throughput. This technology is characterized by smaller particle sizes and column diameters, which are designed to increase separation efficiency. To demonstrate this, the Waters Columns Calculator was used to scale the method from a traditional 4.6 mm i.d. column format with 5 µm particle size to a more contemporary 2.5 µm particle stationary phase. Column lengths of 50 mm, 100 mm, and 150 mm were tested to evaluate scaling benefits. As depicted in Figure 3, the peak selectivity for GEM91 and its associated impurities was preserved across the smaller particle columns. This was determined based on the relative retention time listed for the four impurity peaks. The modernization of the method not only maintains selectivity in support of validated methods, but also introduces new benefits. By reducing the column length from 150 mm to 50 mm, a threefold reduction in

terms of time, sample, and solvent consumption was observed, demonstrating the cost-effectiveness of the Alliance iS Bio HPLC System.

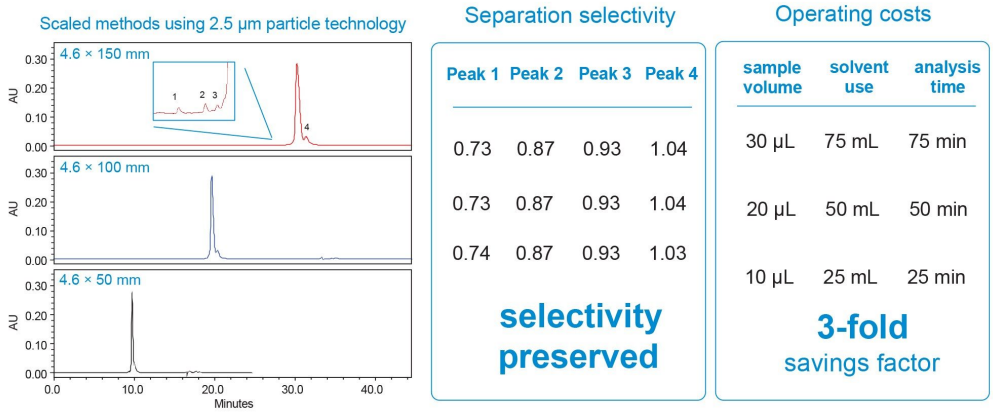


Figure 3. Impact of column lengths on the separation of GEM91 using 2.5 µm XBridge Premier Oligonucleotide BEH C_{18} Columns. Separation selectivity and operating cost are shown in the table.

Method Modernization

The value of the Alliance iS Bio HPLC System extends beyond its ability to support modern column chemistries. As part of its design, the Alliance iS Bio HPLC System is engineered with a quaternary solvent manager providing QC labs the ability to optimize methods more efficiently. Qualification and validation of methods often involve a degree of development and optimization. This process may include multiple preparations of samples and mobile phases where conditions are tested in a step-wise fashion. As an iterative process, this can lead to increased operating costs, loss time, and solvent wastage. The Alliance iS Bio HPLC System, with its four mobile phase reservoirs, offers quaternary solvent blending enabling increased flexibility and efficiency in method optimization. To demonstrate this, a concentrated 200 mM stock solution of HAA was prepared as mobile phase A. Water and acetonitrile were configured in mobile phases C and D to control HAA concentration and deliver the gradient. By adjusting mobile phase A from 50% to 12.5%, HAA concentrations ranging from 25 mM - 100 mM can be effectively evaluated using a single mobile phase preparation. An example of this is shown in Figure 4 where the acetonitrile was increased from 5% (MP D) in all runs to scout optimal HAA concentration to elute the GEM91 oligonucleotide (indicated by the arrow) within the gradient. In this example, 100 mM HAA was observed to be too high in concentration with the oligonucleotide eluting at the tail end of the gradient. In

addition, the final acetonitrile concentration was determined not to be high enough to elute some mobile phase impurities following the oligonucleotide. In this instance, the ion-pairing concentration was reduced to conserve reagent use and gain more control in the gradient development space. As a result, 25 mM HAA as an ion-pairing concentration was determined to be sufficient to elute the oligonucleotide within the gradient space, while still allowing for further method optimization.

Using the 25 mM conditions (MP A=12.5%), peak selectivity was further optimized using the same mobile phase preparation. This approach not only saved time at the bench but also reduced the experiment's environmental footprint through decreased solvent usage and waste generation. Mobile phase D was employed to optimize the gradient slope to increase the resolution between the impurities and the main peak. As shown in Figure 5D, the 11-minute gradient was optimized from 5% D/min to 1.36% D/min allowing for increased resolution between impurities (peaks 1-4) while keeping the total run time relatively short at 25 minutes.

Method reproducibility was evaluated for five replicate injections of GEM91 using the optimized conditions using the 2.5 µm, 130Å, 4.6 × 50 mm, XBridge Premier Oligonucleotide BEH C_{18} Column. As shown in Figure 6A, the Alliance iS Bio HPLC System can deliver rapid and consistent results as demonstrated in the overlay of five replicate injections. The impurity peaks (1-4) exhibit less than 0.05% RSD for retention time and less than 3% RSD in percentage peak area, as depicted in Figure 6B. Overall this approach enables users to lower overall operating costs and develop environmentally friendly methods for biopharmaceuticals that yield consistent and robust results with less solvent and reagent usage.

100 mM HAA gradient table

Time (min)	Flow (ml/min)	%A	%B	%C	%D	Curve
Initial	1.000	50.0	0.0	45.0	5.0	Initial
1.00	1.000	50.0	0.0	45.0	5.0	6
12.00	1.000	50.0	0.0	0.0	50.0	6
15.00	1.000	50.0	0.0	0.0	50.0	6
15.50	1.000	12.5	0.0	12.5	75.0	6
18.50	1.000	12.5	0.0	12.5	75.0	6
21.00	1.000	50.0	0.0	45.0	5.0	6
25.00	1.000	50.0	0.0	45.0	5.0	6

25 mM HAA gradient table

Time (min)	Flow (ml/min)	%A	%B	%C	%D	Curve
Initial	1.000	12.5	0.0	82.5	5.0	Initial
1.00	1.000	12.5	0.0	82.5	5.0	6
12.00	1.000	12.5	0.0	0.0	87.5	6
15.00	1.000	12.5	0.0	0.0	87.5	6
15.50	1.000	12.5	0.0	12.5	75.0	6
18.50	1.000	12.5	0.0	12.5	75.0	6
21.00	1.000	12.5	0.0	82.5	5.0	6
25.00	1.000	12.5	0.0	82.5	5.0	6

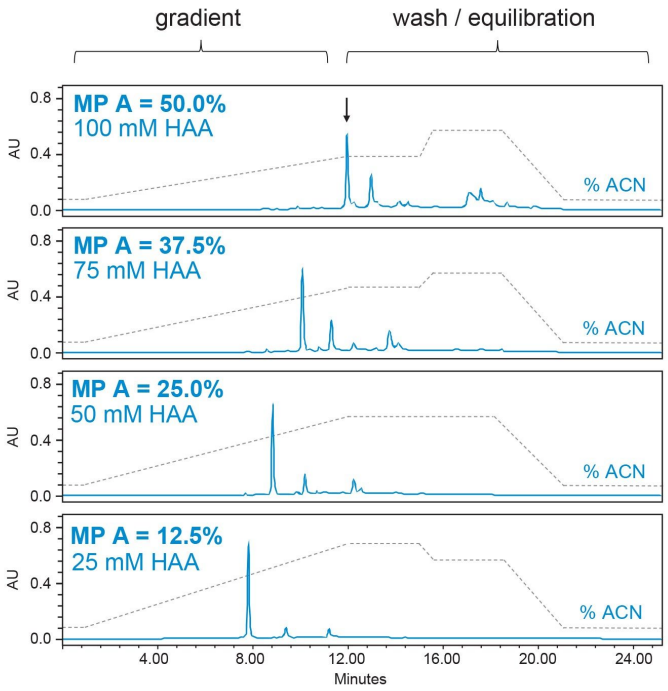


Figure 4. Separation of GEM91 using different concentrations of HAA. The gradient tables of 100 and 25 mM HAA are shown as examples. MP A:200 mM HAA, pH=7, MP C:Water, MP D:Acetonitrile.

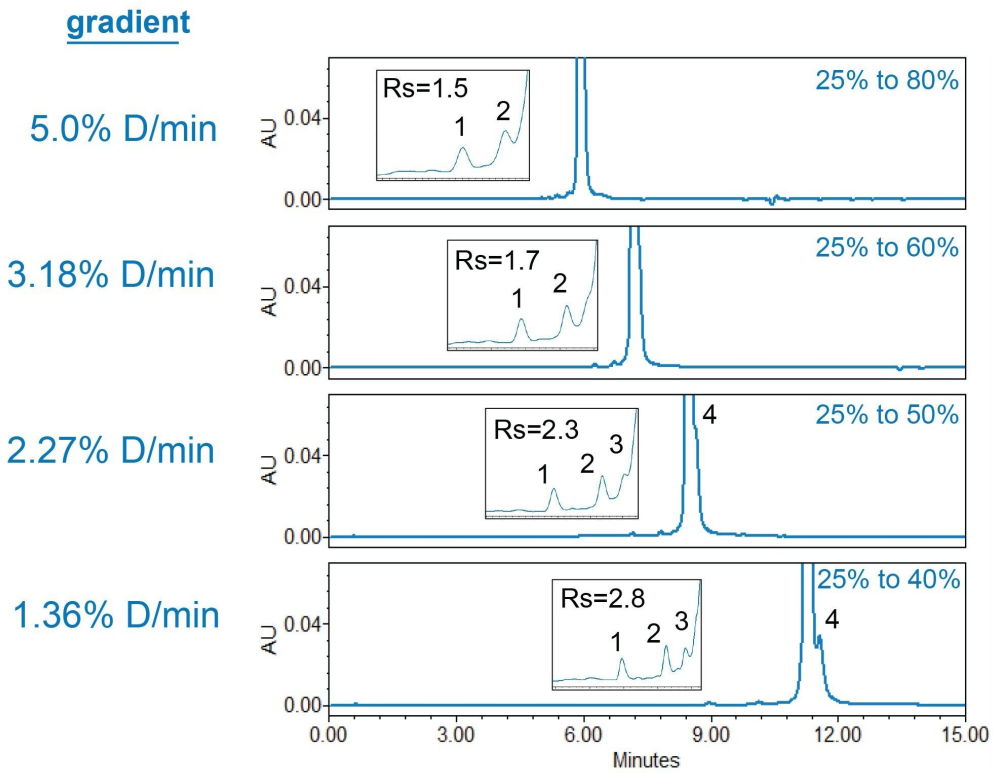
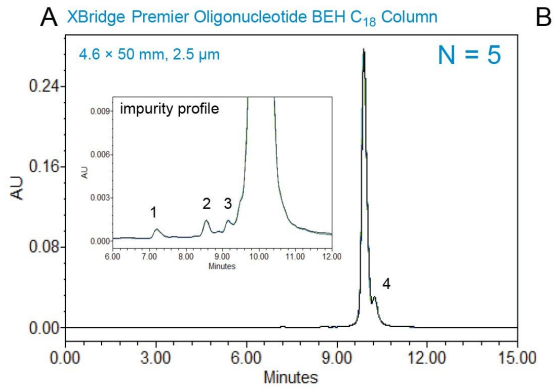


Figure 5. Separation of GEM91 with different percentage change of acetonitrile per minute (% D/min). Resolution between peak 1 and 2 noted as Rs in inset.

A



B

	Retention time		% Area	
	Average	RSD	Average	RSD
Peak 1	7.69	0.04%	0.24	2.28%
Peak 2	9.07	0.03%	0.45	1.35%
Peak 3	9.67	0.04%	0.46	2.03%
Main	10.41	0.03%	87.32	0.14%
Peak 4	10.77	0.03%	11.53	1.03%

Figure 6. A) Five replicate injections of GEM91 on Alliance iS Bio System with an XBridge Premier Oligonucleotide BEH C_{18} Column, inset shows the zoom in view of the impurities. B) Data table of mean RSD % for retention time and % peak area.

Conclusion

The observations found in this study suggest that the Alliance iS Bio HPLC System is well suited for the analysis of biopharmaceuticals such as oligonucleotides and is well positioned to handle current and future needs of supporting labs in manufacturing environments. The utilization of the Alliance iS Bio HPLC System and XBridge Premier Oligonucleotide Columns resulted in a 15% improvement in peak recovery of an oligonucleotide polyT standard and a 40% increase in the signal-to-noise ratio of impurities in a representative oligonucleotide sample (GEM91). The performance of the system was demonstrated with a relative standard deviation (RSD) of less than 3% in the area percentage of GEM91 sample's impurities across five replicate injections. The quaternary solvent manager of the Alliance iS Bio HPLC System was able to scout method conditions rapidly and efficiently demonstrating the value the Alliance iS Bio HPLC System offers in reducing costs and saving time in the analysis of biopharmaceuticals.

References

1. Ziyi Chen, Zhiliang Liu, Yali Feng, Aochen Shi, Liqing Wu, Yi Sang, Chenxi Li, Global research on RNA vaccines for COVID-19 from 2019 to 2023: a bibliometric analysis. Sec. *Vaccines and Molecular Therapeutics*, Volume 15–2024.
2. Koshel BM, Birdsall RE, Yu YQ. Improving Recovery and Quantitation of Oligonucleotide Impurities Using ACQUITY Premier with MaxPeak HPS Technology. Waters Application Note 720007238. April 2021.
3. Martin Gilar, Zhimin Li, Jason MacLean. Improved Chromatographic Analysis of Oligonucleotides with ACQUITY Premier Oligonucleotide BEH C18 Columns. Waters Application Note 720007012. August 2023.
4. Kathryn Brennan, Mary Trudeau, Michael Donegan, Paul D. Rainville. Improved Oligonucleotide SPE-LC-MS Analysis Using MaxPeak High Performance Technology. 720007019, September 2020.

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