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应用纪要

Reproducible Hydrophilic Interaction Chromatography for Denaturing and Non-Denaturing Analyses of Oligonucleotides Using GTxResolve Premier BEH Amide Columns

Abraham S. Finny, Tatiana Johnston, Brent Sauner, Balasubrahmanyam Addepalli, Matthew A. Lauber

Waters Corporation

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Abstract

This application note discusses the benefits of GTxResolve Premier BEH Amide 300 Å 1.7 µm columns for performing Hydrophilic Interaction Chromatography (HILIC) of oligonucleotides up to 100 nucleotides in length. HILIC facilitates oligonucleotide analysis without the use of ion pairing reagents *(e.g.*, triethylamine (TEA), dibutylamine (DBA), *etc.*) to obtain optimal chromatographic performance. The GTxResolve Premier BEH Amide 300 Å 1.7 µm columns with larger pore size, greater column stability and no apparent silanol activity provides improved resolution of longer oligonucleotides in a multicomponent mixture. These oligonucleotides ranging from 20 to 100 mers exhibit excellent retention time reproducibility, greater peak symmetry, and size-dependent retention behavior. The identified mobile phase compositions and gradient conditions in this study enabled robust

chromatography, repeatable, and appropriate resolution of nucleic acid components. Further, GTxResolve Premier BEH Amide 300 Å 1.7 µm columns can help investigate the single stranded RNA impurities in siRNA under non-denaturing or denaturing conditions.

Benefits

- Optimized manufacturing procedures and oligonucleotide batch testing to create GTxResolve Premier BEH Amide 300 Å
- Low adsorption MaxPeak™ High Performance Surface hardware for reduced dependence on mobile phase ionic strength
- Excellent resolution for large biomolecules, including single and double stranded oligonucleotides up to 100 nucleotides in length
- Optimized separations at both low and high temperature for full characterization of product related impurities encountered while developing and release testing small interfering RNA (siRNA)

Introduction

HILIC presents a promising analytical approach for the characterization and quantification of oligonucleotide therapeutics. As the demand for these novel therapeutics grows, it is becoming increasingly important to develop LC-based approaches to confirm their identity and purity. HILIC offers several advantages in this context, including enhanced retention and separation of highly polar compounds, such as oligonucleotides.

Recent reports by Lardeux, Guillarme and Jora as well as Goyon, Wei and Zhang have highlighted the efficacy of HILIC in the analysis of oligonucleotides, noting its superior performance in terms of retention and resolution compared to traditional reversed-phase chromatography.[1–4] These studies have demonstrated the potential of HILIC to reduce the need for ion-pairing (IP) reagents, which can complicate the versatile use of both HPLC and MS instruments. Although IP agents are routinely used in reversed phase chromatography (IP-RP-LC), they can be difficult to flush from pumps and flow paths, and their residual concentrations can cause ion suppression when mass spectrometers are switched from negative ion mode detection back to positive ion mode polarity. The benefits of HILIC include

absence of IP agent-free mobile phases with complementary analytic behavior compared to IP-RP-LC.

The adsorption, retention, and elution of oligonucleotides onto and from a HILIC sorbent can be complicated. Retention in HILIC mode is driven by analyte partitioning into the aprotic mobile phase such as acetonitrile and a layer of temporarily immobilized water on stationary phase surface. Elevated salt in mobile phase promotes counterion hydration, and immobilized aqueous layer formation. Salt ions also help mitigate the electrostatic repulsion between negatively charged oligonucleotides and residual silanols. Apart from hydrophilic partitioning, adsorptive interactions such as hydrogen bonding and electrostatic interactions between the stationary phase ligands and ribose sugar, nucleobases also play a role in the HILIC retention process.[5] It is becoming increasingly appreciated that it is direct H-bond interactions that produces the strong retention of oligonucleotides on a HILIC stationary phase. Hydrophilicity of nucleobases (A<T<G<C<U) and their elution order is opposite to that of IP-RPLC separations.

As such, Waters™ has investigated the optimization of manufacturing procedures of HILIC stationary phases and begun to apply two rounds of application-specific batch testing to its amide bonded BEH sorbent. With GTxResolve Premier BEH Amide columns, analyst can have assured performance for both protein and oligonucleotide separations.

In this application note, we highlight the enhanced chromatography that can be obtained with the adoption of GTxResolve Premier BEH Amide 300 Å 1.7 µm columns. With robust stationary phase stability, excellent reproducibility and robust performance across multiple batches assure the analysts for smooth sailing of QC process in regulated environments. Another high light of these HILIC columns is their ability to perform impurity analysis in siRNA duplex samples and characterize the ssRNA components of the duplex with same mobile phase through adjustment of column temperature.

Experimental

Stock Ammonium Acetate Solution

5 M ammonium acetate solution (Invitrogen p/n: AM9071) or equivalent

ssDNA 20 to 100 Ladder

The ssDNA 20 to 100 Ladder (p/n: 186009448 <https://www.waters.com/nextgen/global/shop/standards--reagents/186009448-ssdna-20-to-100-ladder.html>) is a lyophilized, 10 µg quantity of a mixture of 20, 30, 40, 50, 60, 70, 80, 90, and 100-mer single stranded (ss) DNA. The components of this standard are chemically synthesized and confirmed by LC analysis to be present in reproducible quantities. Additional compositional information can be found in the table below.

Component	Elemental composition	Residue composition	Monoisotopic mass (Da)	Average mass (Da)
20mer	$C_{195}H_{246}N_{75}O_{118}P_{19}$	C:5 T:5 A:5 G:5	6114.05	6117.045
30mer	$C_{292}H_{368}N_{113}O_{177}P_{29}$	C:8 T:7 A:8 G:7	9186.555	9191.044
40mer	$C_{389}H_{489}N_{154}O_{234}P_{39}$	C:11 T:8 A:12 G:9	12268.073	12274.055
50mer	$C_{487}H_{612}N_{191}O_{295}P_{49}$	C:13 T:11 A:14 G:12	15371.573	15379.066
60mer	$C_{585}H_{734}N_{231}O_{354}P_{59}$	C:15 T:13 A:17 G:15	18484.085	18493.09
70mer	$C_{684}H_{857}N_{270}O_{414}P_{69}$	C:16 T:16 A:20 G:18	21611.596	21622.126
80mer	$C_{781}H_{979}N_{308}O_{474}P_{79}$	C:19 T:18 A:22 G:21	24700.097	24712.125
90mer	$C_{878}H_{1102}N_{343}O_{535}P_{89}$	C:22 T:21 A:24 G:23	27763.591	27777.111
100mer	$C_{976}H_{1224}N_{383}O_{594}P_{99}$	C:24 T:23 A:27 G:26	30876.103	30891.135

siRNA LC-MS Standard

The siRNA LC-MS standard (p/n: 186010598 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010598-sirna-lc-ms-standard.html>) is a lyophilized, 1 nmol quantity of a mixture of annealed 25-mer and 27-mer RNA strands. This siRNA molecule does not recognize any sequences within the human, mouse, or rat transcriptome. Additional compositional information can be found in the following table. The contents of one vial were reconstituted in 200 µL of 50:50 acetonitrile/50 mM ammonium acetate using gentle vortexing.

Component	Elemental composition	Monoisotopic mass (Da)	Average mass (Da)
Sense	5′ - rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArCrGrCrGrUdAdT - 3′	7922.0837	7925.8
Antisense	5′ - rArUrArCrGrCrGrUrArUrUrArUrArCrGrCrGrArUrUrArArCrGrArC - 3′	8597.1789	8601.2

Hydrophilic Interaction Chromatography

Column:	GTxResolve Premier BEH Amide 300 Å, 1.7 µm, 2.1 x 150 mm column (p/n: 186011251)
Mobile phase A:	ssDNA ladder: 50 mM ammonium acetate, 10% acetonitrile, 10% methanol (80% aqueous) siRNA: 50 mM ammonium acetate, 75% water, 25% acetonitrile
Mobile phase B:	ssDNA ladder: 50 mM ammonium acetate, 70% acetonitrile, 10% methanol (20% aqueous). siRNA: 50 mM ammonium acetate, 75:25 acetonitrile/18.2 MΩ water
LC system:	ACQUITY Premier System [Consisting of a Premier BSM with 50 µL Mixer (p/n: 700012635) Premier FTN-SM with 15µL MP35N Needle (p/n: 700012821) ACQUITY UPLC CH-A (p/n: 186015042) with an Active Preheater (p/n: 205002234) Post-column tubing to TUV: 0.005” ID x 22.5” LG MP35N Tubing p/n: 700008914
Detector:	ACQUITY Premier PDA or TUV detector with an analytical 500 nL flow cell
Wavelength:	260 nm
Flow rate:	0.4 mL/min for ssDNA (ssDNA ladder), 0.3 mL/min for siRNA
Injection:	1 µL of reconstituted 10 µg ssDNA ladder in 100

	µL, 1 µL of 5 nmol/mL siRNA LC-MS Standard solution (diluent: 50:50 acetonitrile/50 mM ammonium acetate)
Column temperature:	40 °C (ssDNA) 25 °C (siRNA, nondenaturing) 60 °C (denaturing)
Sample temperature:	5 °C
Injection vial:	QuanRecovery MaxPeak 12 x 32 mm Polypropylene 300 µL Screw Cap Vials 100 pack p/n: 186009186
Gradient:	Polyethylene Septum less Screw Cap for 12 x 32 mm Vials, 100/pk, p/n: 186004169

siRNA

Time	Flow rate (mL/min)	%A	%B	Curve
Initial	0.3	70	30	Initial
1.00	0.3	70	30	6
7.00	0.3	22	78	6
7.50	0.3	22	78	6
8.00	0.3	70	30	6
20.00	0.3	70	30	11

Results and Discussion

The novel GTxResolve Premier BEH (bridged ethylene hybrid) Amide 300 Å, 1.7 µm, columns utilize a chemically stable, trifunctionally bonded amide phase with stability to high pH and high temperature providing robust and versatile HILIC separations. Its wide pore format (300 Å) allows oligonucleotides (ONs) of various sizes to access pores to partition between organic and aqueous phase, engage with partially immobilized water layer through hydrogen bonding and electrostatic interactions for optimal retention and resolution of the resolution of longer ON versions (Figure 1 A). Packing of the larger pore size (300 Å) BEH Amide particles in columns featuring MaxPeak High Performance Surfaces prevents the non-specific adsorption of ON in the column flow path to provide out-of-box performance with no passivation requirements (Figure 1 B).

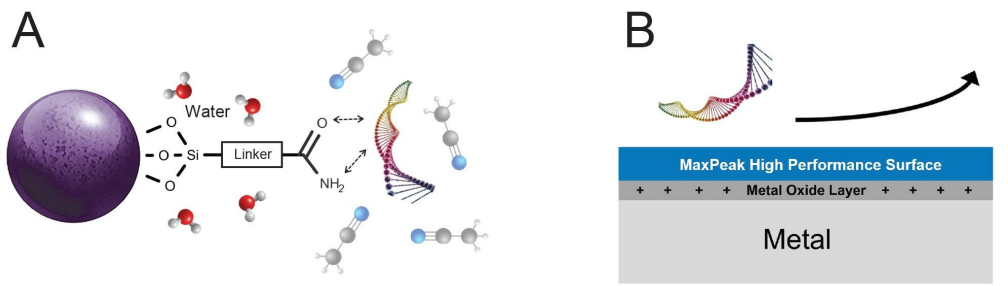

Figure 1. Schematic representation of the (A) GTxResolve Premier BEH Amide stationary phase and (B) MaxPeak High Performance Surfaces as used on column hardware to mitigate adsorptive interactions with oligonucleotides.

Presence of polar protic solvent such as methanol is known to weaken hydrogen bonding capacity and may destabilize the stagnant aqueous layer, but a small percentage addition (ACN and Methanol ratio of 1:1 for MPA and 7:1 for MPB) to the mobile phase can help improve the ON peak shape.[6] This mobile phase composition exhibited excellent peak shape and reproducible retention time upon screening of different batches of BEH Amide 300 Å materials using 20–100 mer ssDNA ladder (Figure 2). No or minimal tailing of chromatographic peaks across multiple batches indicate no silanol activity from stationary phase.

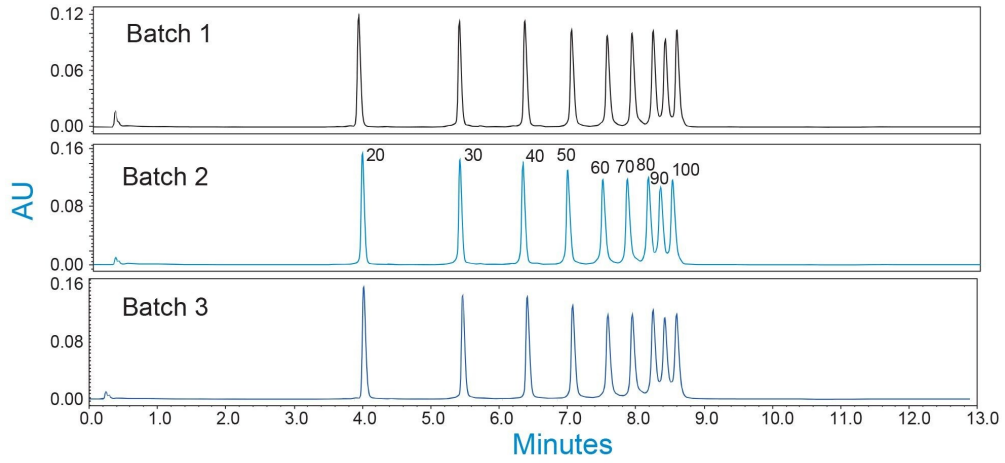

Figure 2. Reproducible retention behavior of 20–100 mer ssDNA LC-MS standard components using GTxResolve Premier BEH Amide 300 Å, 1.7 µm columns through HILIC analysis.

One of the major benefits HILIC chromatography is the ability to switch between nondenaturing and denaturing chromatography conditions while analyzing siRNA. Effective separation of siRNA duplexes can be achieved by simple manipulation of column temperature and/or mobile-phase ionic strength. Lower temperatures and higher ionic strengths helped stabilize the higher order structure of siRNA molecules. To demonstrate this point, we have used a GTxResolve Premier BEH Amide Column with an ambient column temperature to analyze the Waters siRNA LC-MS Standard. As shown in Figure 3.

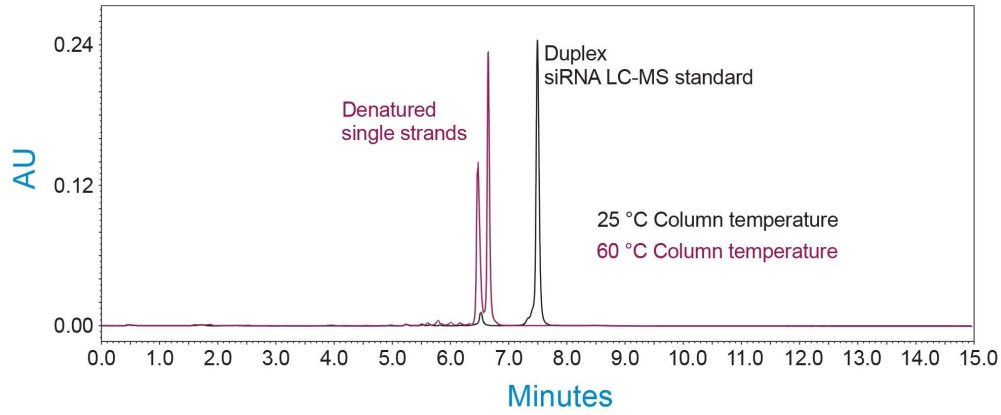

Figure 3. Hydrophilic interaction non-denaturing and denaturing A260 chromatogram of the siRNA LC-MS Standard as obtained with a GTxResolve Premier BEH 300 Å Amide 1.7 µm 2.1 x 150 Column using an ACQUITY Premier LC Ssystem, at 25 and 60 °C column temperature, 0.3 mL/min flow rate, and 1 µL injection volume.

Conclusion

The GTxResolve Premier BEH Amide 300 Å, 1.7 µm Column provides improved resolution for longer oligonucleotide analytes while maintaining excellent resolution of shorter oligonucleotides. MaxPeak Premier HPS hardware provides out of box performance without the need for passivation. Excellent batch-to-batch reproducibility is noticed when ssDNA components ranging from 20–100 mer oligonucleotides were analyzed under HILIC conditions. Identical retention times were observed across multiple batches of GTxResolve BEH Amide materials, which confirms their suitability for oligonucleotide therapeutic development and DMPK analysis. Such detection precision even by TUV response also enables smooth transfers of QC processes into regulated environments. The ability to analyze siRNA under nondenaturing and denaturing conditions through adjustment of column temperature enables both impurity analysis and characterization of ssRNA components with the same mobile phase. The combination of this high peak capacity column with a compliance ready LC system can ensure maximum productivity in regulated environments.

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