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Analytical Products

Application Note

Analysis of Oligonucleotide Standard 6 Mix by Liquid Chromatography-UV

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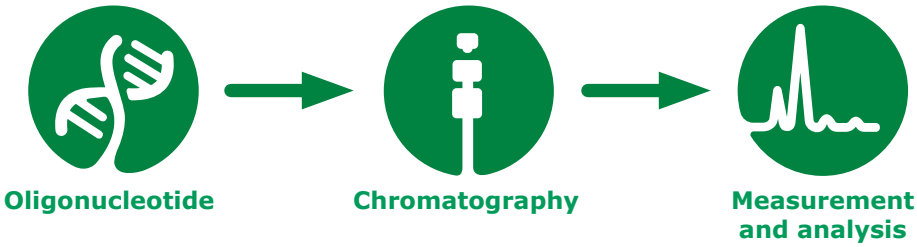

Introduction

With the COVID-19 pandemic, oligonucleotides (Oligos) have proven their importance in diagnostic and therapeutic applications. Currently, 11 oligonucleotide drugs crossing many disease areas have been approved by the FDA.[1, 2] Obstacles preventing quicker development of oligonucleotide therapeutics include the challenges of unfavorable absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies for many clinical trials.[2] Some strategies have been developed to tackle the challenges, such as chemical modification to improve drug delivery.

Synthetic oligonucleotides are typically small, single- or double-stranded modified nucleic acids.[2] There are many established techniques to analyze and characterize oligonucleotides, including capillary gel electrophoresis (CGE), ion exchange chromatography (IEX), and ion pair reversed phase liquid chromatography (IP-RPLC). Generally, liquid chromatography of Oligos is very challenging due to the similarity of oligonucleotide structures, very polar characteristics, presence of truncated and/or modified Oligos, ease of self-association into a variety of conformations, and affinity for metal surfaces.[1,2] This application describes the separation of an internally produced oligonucleotide standard (Oligo Standard 6) mix, which includes six oligonucleotides, on Supelco® Chromolith® RP-18e columns.

General Procedures

Oligo Standard 6 is an internal (in-house) system suitability mix for HPLC-UV evaluation of oligonucleotide separations. The standard contains six components with molecular weights of 3588.3 Da (Oligo 1), 4157.93 Da (Oligo 2), 7580.83 Da (Oligo 3), 10014.35 Da (Oligo 4), 6116.97 Da (Oligo 5), and 4395.8 Da (Oligo 6) following their elution order on Chromolith® RP-18e columns tested here.

Reagent Preparation

50 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 50 mM TEAA, 50 mL of TEAA (commercial 1 M solution) was added into 950 mL of HPLC grade water and mixed well.

20 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 20 mM TEAA, 20 mL of TEAA (commercial 1 M solution) was added into 980 mL of HPLC grade water and mixed well.

5 mM of Triethylammonium Acetate (TEAA)

To prepare 1 L of 5 mM TEAA, 5 mL of TEAA (commercial 1 M solution) was added into 995 mL of HPLC grade water and mixed well.

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Sample Preparation

5 µM of Oligo Standard 6 sample

1 mL of HPLC grade water was added into the sample vial which contains 5 nmol each of the six Oligo components and mixed well.

HPLC-UV System Setup and Data Analysis

Essential settings of the HPLC-UV chromatography system for analysis of Oligo Standard 6 are listed in Table 1 below.

Table 1. HPLC-UV general system settings.

Instrument Setup	
HPLC system	Agilent 1260 Infinity II
Software	Agilent ChemStation
Column	Chromolith® RP-18e, 100 x 4.6 mm; Chromolith® High Resolution RP-18e, 100 x 2.0 mm/50 x 2.0 mm
Column temp.	25 °C; 40 °C
Autosampler temp.	5 °C
Mobile phase A	5–50 mM TEAA
Mobile phase B	Acetonitrile
Flow	0.4–3 mL/min
Injection volume	5 µL
Run time	12 min
Detector	UV; 260 nm

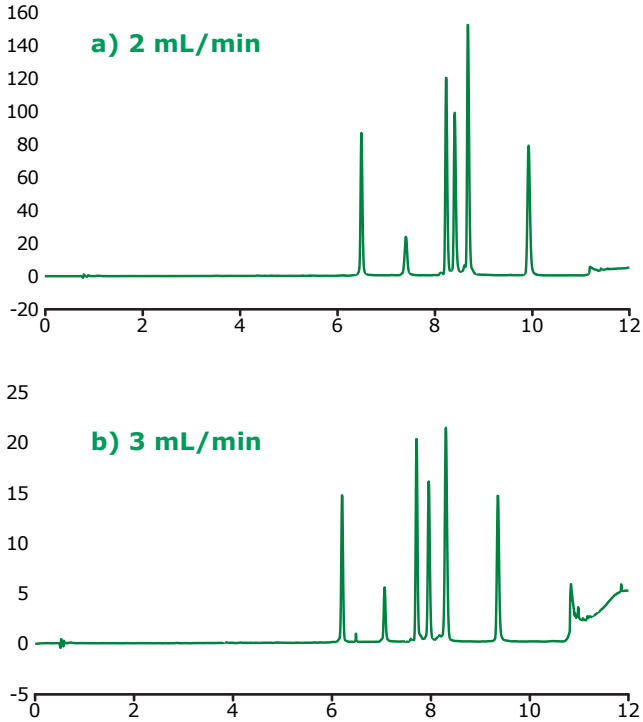


Figure 1. Oligo Standard 6 separation on Chromolith® RP-18e, 100 x 4.6 mm column at flow rates: a) 2 mL/min; b) 3 mL/min with a gradient of 5% B to 15 % B in 10 minutes. Mobile phase A: 50 mM TEAA in water; Mobile phase B: acetonitrile. Note: injection volume for 2 mL/min is 20 µL and 5 µL for 3 mL/min.

Results and Discussion

With the linkage of phosphate groups, oligonucleotides tend to stick to metal surfaces present in stainless steel column hardware and the LC system, resulting in reduced sensitivity and inaccurate quantitation. Researchers have made a variety of efforts to mitigate this adsorption inside instrumentation, such as treatment of the system with EDTA, high pH mobile phase, or utilizing bio-inert HPLC system components.[3] Conventional HPLC columns are typically packed in metal columns, exposing the metal surfaces with positive charge which can adsorb acidic molecules, such as oligonucleotides containing phosphate groups. Chromolith® HPLC columns are made of highly porous monolithic rods of silica, with an innovative bimodal pore structure and packed in metal-free PEEK (polyetheretherketone) columns, which make it a good candidate for oligonucleotide analysis.

Chromolith® RP-18e, 100 x 4.6 mm column

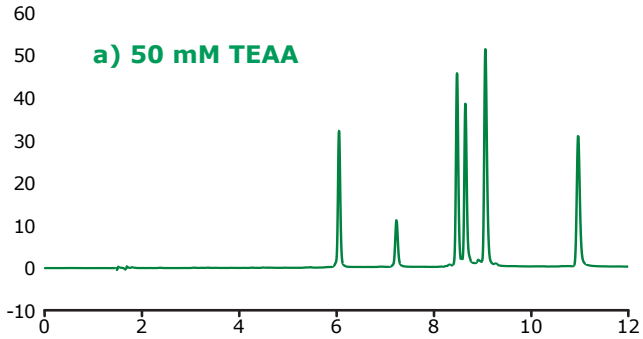
Flow Rate Test

To improve separation efficiencies, the particle size of packing material is usually reduced. Currently, conventional HPLC columns contain 5, 3, 2, and even sub 2 µm silica particles.[4] However, the smaller particle size will cause higher back pressure affecting the assay throughput, robustness, and column lifetime. The optimal solution is to use a column that offers faster throughput without the risk of high back pressure. Since Chromolith® is not packed with silica particles, but a single rod of high-purity, polymeric silica gel, the unique construction enables highly efficient separations at accelerated speeds, which is ideal for high throughput analysis.[4]

Figure 1 shows the separation of Oligo Standard 6 on a Chromolith® RP-18e column under flow rates of 2 mL/min and 3 mL/min with only 25 pmol on column injection for each oligonucleotide. 50 mM of TEAA was used as mobile phase A and acetonitrile as mobile phase B with a gradient of 5% B ramping to 15% B in 10 minutes. The typical back pressure at 2 mL/min and 3 mL/min is 30–50 bar which is beneficial for high throughput assays.

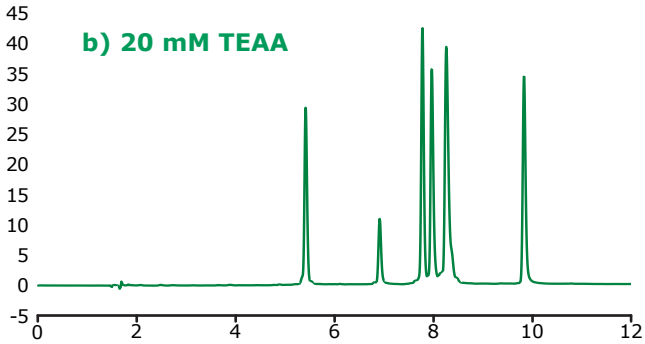
Ion-Pairing Additive Concentration Test

In the qualitative and quantitative analysis of oligonucleotide impurities, ion-pair reversed phase liquid chromatography has been the dominant technique. The ion-pairing reagents added in mobile phase are typically several alkylammonium salts which are adsorbed on the column sorbent with the positive charges exposed to interact with the negatively charged oligonucleotides. Triethylammonium acetate (TEAA) is one of the commonly used ion-paring reagents in LC-UV analysis of oligonucleotides. Optimizing ion-pairing additive concentration is important to achieve efficient separation while minimizing cost from additive consumption. In this work, optimization of TEAA concentration was conducted.



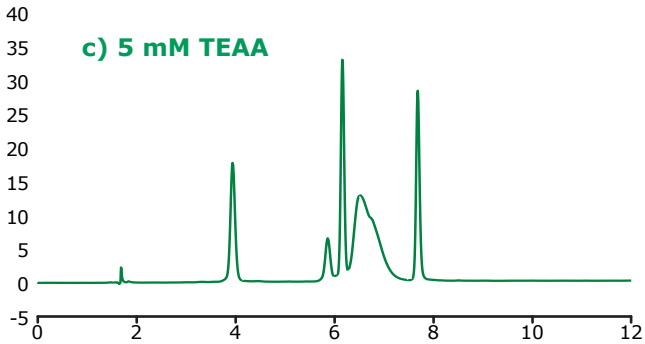
50 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.051	113.191	32.336	
Oligo 2	7.232	45.290	11.023	12.549
Oligo 3	8.476	153.993	45.496	13.167
Oligo 4	8.647	136.388	38.111	1.938
Oligo 5	9.058	205.826	50.765	4.293
Oligo 6	10.964	142.741	30.822	17.499



20 mM TEAA

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	5.418	110.992	29.261	
Oligo 2	6.916	44.280	10.811	15.225
Oligo 3	7.780	158.234	42.434	8.820
Oligo 4	7.969	145.570	35.631	1.917
Oligo 5	8.263	215.256	39.189	2.522
Oligo 6	9.835	144.133	34.367	13.334



5 mM TEAA

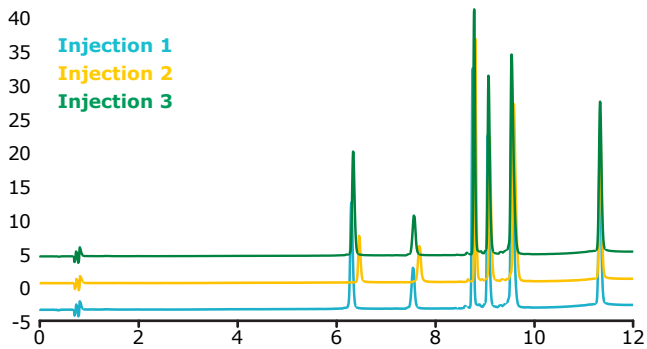
Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	3.942	121.392	17.759	
Oligo 2	5.865	40.805	5.964	10.811
Oligo 3	6.163	157.698	32.526	1.925
Oligo 4/5	6.517	368.327	12.332	0.749
Oligo 6	7.685	143.739	28.318	2.462

Figure 2. Oligo Standard 6 separation on Chromolith® RP-18e, 100 x 4.6 mm column with different TEAA concentration in mobile phase A: a) 50 mM TEAA; b) 20 mM TEAA; and c) 5 mM TEAA. Resolution is calculated between each two adjacent peaks.

Figure 2 shows the different concentrations of TEAA tested in mobile phase A with acetonitrile as mobile phase B in the separation. Five microliters of Oligo Standard 6 sample was injected on a Chromolith® RP-18e, 100 x 4.6 mm column at a flow rate of 1 mL/min with a gradient of 8% B to 15% B in 10 minutes for each test. With 50 mM of TEAA in mobile phase A, the oligonucleotides were well separated with the retention time as indicated in Figure 2. When the TEAA concentration was lowered to 20 mM, Oligo 1 to 6 eluted in the same order but with less retention on column. With the exception of Oligos 1 and 2, the resolution between each peak pair is seen to be lower as well. When TEAA concentration was further lowered to 5 mM, Oligos 4 and 5 were not separated which indicates the ion-pairing strength is not high enough to separate these two oligonucleotides. Comparing the peak heights of the six Oligos under the three different TEAA concentrations, 50 mM TEAA produced the highest peak height as shown in the table in Figure 2. Therefore, the ion-pairing additive concentration needs to be optimized based on the characteristics of the oligonucleotides.

Chromolith® High Resolution RP-18e Column

The Chromolith® High Resolution column possesses 1.15 µm macropores compared with 2 µm on the standard Chromolith® column. This modification results in higher separation efficiency and better peak shape. Although this creates higher back pressure, it is still less than half that of any particulate column of similar efficiency.[4]



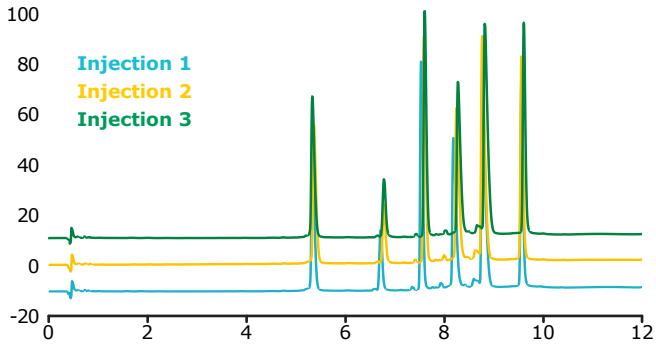
Chromolith® High Resolution RP-18e, 100 x 2.0 mm—3 µL injection

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	6.337	102.148	27.621	
Oligo 2	7.568	50.707	10.807	11.691
Oligo 3	8.781	167.619	65.201	13.279
Oligo 4	9.072	150.772	47.522	3.972
Oligo 5	9.539	228.983	53.013	4.936
Oligo 6	11.327	159.908	39.979	17.292

Figure 3. Oligo Standard 6 separation on Chromolith® High Resolution RP-18e, 100 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temperature: 40 °C.

Here, 3 µL of Oligo Standard 6 sample was injected onto the Chromolith® High Resolution RP-18e, 100 x 2.0 mm column at 0.4 mL/min with a gradient of 8% B to 15% B in 10 minutes. **Figure 3** is an overlay of three injections showing consistent retention and response. 50 mM TEAA concentration was used as mobile phase A and acetonitrile was mobile phase B. Resolution between Oligo 4 and 5 is 4.936. A shorter column of Chromolith® High Resolution RP-18e, 50 x 2 mm was compared with the same conditions with 5 µL of injection volume used in

Figure 3. As shown in **Figure 4**, on a 50 x 2 mm column, all six oligonucleotides were eluted within 10 minutes with the resolution between Oligo 4 and 5 of 3.921. Thus, Chromolith® HR RP-18e column is capable of oligonucleotide analysis using LC-MS compatible flow rates.



Chromolith® High Resolution RP-18e, 50 x 2.0 mm —5 µL injection

Peak ID	RT (min)	Peak Area	Peak Height	Resolution (USP)
Oligo 1	5.343	261.641	56.765	
Oligo 2	6.794	106.792	23.310	12.458
Oligo 3	7.618	357.882	90.331	7.641
Oligo 4	8.292	316.582	61.032	5.746
Oligo 5	8.836	479.91	84.329	3.921
Oligo 6	9.625	307.511	84.625	6.631

Figure 4. Oligo Standard 6 separation on Chromolith® High Resolution RP-18e, 50 x 2.0 mm column. Mobile phase A: 50 mM TEAA in water, Mobile phase B: acetonitrile; gradient: 8% B to 15% B in 10 minutes at flow rate of 0.4 mL/min, column temperature: 40 °C.

Conclusion

In this application note, the separation of Oligo Standard 6, an internally created HPLC-UV system suitability mix, was demonstrated on Chromolith® and Chromolith® High Resolution RP-18e columns. Flow rates up to 3 mL/min were evaluated on Chromolith® with excellent separation of the six Oligos indicating that it is ideal for high throughput assays. The results of the ion-pairing reagent optimization experiments indicate that 50 mM TEAA provides the best separation and sensitivity for Oligo Standard 6. Separation of Oligo Standard 6 on Chromolith® High Resolution column with flow rate of 0.4 mL/min produced better resolution of Oligo 4 and 5 compared to 3 mL/min method on Chromolith® column, with resolution (USP) of 3.9 vs 1.9. This result demonstrates that Chromolith® High Resolution column is suitable for oligonucleotide analysis by LC-MS with mass spectrometer favorable flow rates tested here. In addition, the polymeric column housing can be used as part of a metal free, or bio-inert HPLC system.

Acknowledgement

The authors would like to thank Pierre Potier for providing the Standard 6 oligonucleotide mix and for technical support.

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2. Hammond, S.M.; Aartsma-Rus, A.; Alves, S. et al. Delivery of oligonucleotide-based therapeutics: challenges and opportunities. EMBO Molecular Medicine 13: e13243 (2021).
3. Gilar M.; DeLano M.; Gritti F. Mitigation of analyte loss on metal surfaces in liquid chromatography. J. Chrom. A 1650 (2021) 462247
4. Chromolith® HPLC columns brochure-pb6401, Race through separations with revolutionary technology.

Product list

Description	Cat. No.
HPLC columns	
Chromolith® HPLC column RP-18e, L × I.D. 100 mm × 4.6 mm	1.02129.0001
Chromolith® HPLC column HR RP-18e, L × I.D. 100 mm × 2.0 mm	1.52322.0001
Chromolith® HPLC column HR RP-18e, L × I.D. 50 mm × 2.0 mm	1.52321.0001
Chemicals & reagents	
Triethylammonium Acetate, 1 M Solution	90358
Water, HPLC-Grade	270733
Acetonitrile, HPLC-Grade	900667
Instruments & consumables	
Eppendorf ThermoMixer® F1.5	EP5384000012
Vials, amber glass, volume 2 mL	27344
Pipette 0.5–10 µL	EP4924000223
Pipette 10–100 µL	EP4924000258
Pipette 100–1000 µL	EP4924000282
Pipette tips 0.1–20 µL box	Z640204
Pipette tips 2–200 µL box	Z640220
Pipette tips 50–1000 µL box	Z640247

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