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# LC/MS Method Development for Various Oligonucleotides Using Polymer-Based HILIC Columns Having Diol Group

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## Introduction

Development and quality control of oligonucleotide therapeutics often require highly selective and sensitive analytical methods. An LC/MS method is one of them. The most often used LC method for the oligonucleotide analyses have been reversed phase with a use of ion-pair reagents. However, the ion-pair reagents have a tendency to remain on the LC system and cause problems.

In this work, we used Shodex™ HILICpak™ VN-50 series columns, polymer-based HILIC columns modified with diol functional groups. The developed method enabled the LC/MS analysis of oligonucleotides without a need of an ion-pair reagent. Gradient condition was optimized with a mixture of acetonitrile and relatively low-concentration volatile basic solvent.

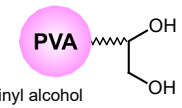
## Shodex™ HILICpak™ VN-50 Series Columns

| Product name | Column size (mm) I.D. x Length |
|---|---|
| HILICpak VN-50 4D | 4.6 x 150 |
| HILICpak VN-50G 4A | 4.6 x 10 |
| HILICpak VN-50 2D | 2.0 x 150 |
| HILICpak VN-50G 2A | 2.0 x 10 |
| HILICpak VN-50 1D | 1.0 x 150 |

※VN-50 1D will be available soon

**< Features >**

- Suitable for oligosaccharides and oligonucleotides analysis using HILIC mode
- Retains hydrophilic compounds without derivatization nor ion-pair reagent




**< Specifications >**

- Base Material: Spherical porous particles of polyvinyl alcohol modified with diol functional group
- Particle Size: 5 µm
- Pore Size: 100 Å
- Column Housing: PEEK
- Usable pH: 2 - 13 *Workable in alkaline condition*
- Usable temperature: 4 – 60 °C
- Applicable solvents: Mixture of water and acetonitrile or water and methanol of any ratio. Up to 100 % water, acetonitrile, or methanol.

## Method

**< Analytical Condition>**

Instrument : Shimadzu Nexera / LCMS-8030 Plus
Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Shodex HILICpak VN-50 1D (1.0 mm I.D. x 150 mm)
Eluent : (A) 50mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
Linear gradient (High pressure)
*No Ion-paring reagent nor HFIP used*
Flow rate : 0.3 mL/min (VN-50 2D), 0.1 mL/min (VN-50 1D)
Detector : PDA(190 - 350 nm) & ESI-MS SIM(-)
Column temp.: 40 °C
Injection vol. : 1 µL

**< Sample >**

Synthetic phosphorothioated oligo-RNA (Cartridge purified)
1. 19 mer U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
2. 20 mer A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
3. 21 mer G*A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U

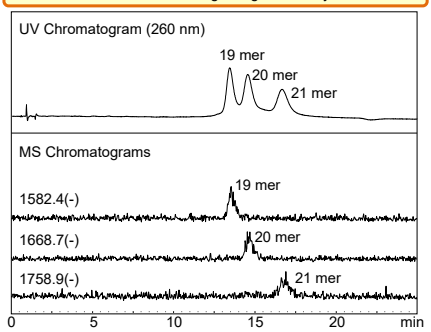
Synthetic phosphorothioated oligo-DNA (salt-free)
4. 20 mer A*T*A*C*C*G*A*T*T*A*A*G*C*G*A*A*G*T*T*T

Synthetic oligo-DNA (salt-free)
5. 20 mer ATACCGATTAAGCGAAGTTT

## Results and Discussion

### < Phosphorothioated Oligo-RNA Analysis >

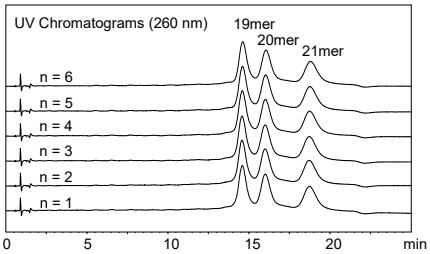
Elute in order of smaller to larger oligo-RNAs by HILIC mode




Sample : Synthesized phosphorothioated oligo-RNAs, 0.1 mg/mL each (in $H_2O$), 1 µL
1. 19mer U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
2. 20mer A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
3. 21mer G*A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.3 mL/min
Detector : PDA (190-350 nm) + MS SIM(-)
Column temp: 40 °C

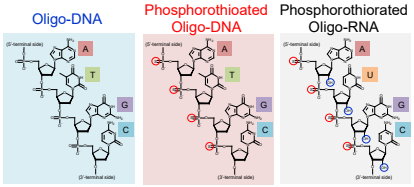
### < Repeatability >

Retention time deviations for all peaks: ≤ 0.5% RSD (n = 6)

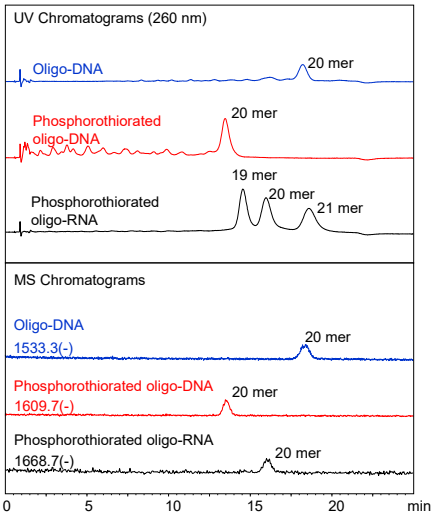



Sample : Synthesized phosphorothioated oligo-RNAs, 0.1 mg/mL each (in $H_2O$), 1 µL
1. 19mer U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
2. 20mer A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
3. 21mer G*A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.3 mL/min
Detector : PDA (190-350 nm)
Column temp: 40 °C

### < Comparisons of Oligonucleotides >



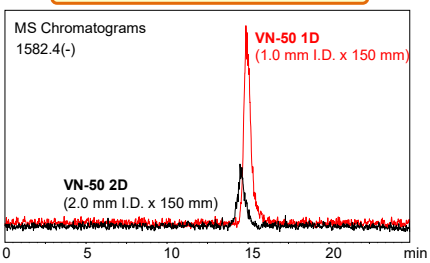

- Less retention for phosphorothiorated oligonucleotides (higher hydrophobicity)
- Stronger retention for phosphorothiorated oligo RNAs than phosphorothiorated oligo-DNA (RNA with OH group at the ribose 2'position makes it more hydrophilic than DNA)




Sample : 0.1 mg/mL each (in $H_2O$), 1 µL, * means phosphorothioated position
1. Synthesized oligo-DNA, 20mer ATACCGATTAAGCGAAGTTT
2. Synthesized phosphorothioated oligo-DNA, 20mer A*T*A*C*C*G*A*T*T*A*A*G*C*G*A*A*G*T*T*T
3. Synthesized phosphorothioated oligo-RNA, 20mer A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.3 mL/min
Detector : PDA (190-350 nm) + MS SIM(-)
Column temp: 40 °C

### < Comparisons of Column I.D. Size>

Superior ESI-MS sensitivity with VN-50 1D



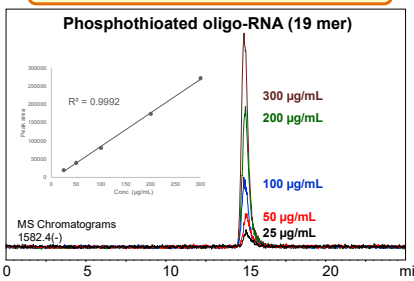

Sample : Synthesized phosphorothioated oligo-RNA, 0.1 mg/mL (in $H_2O$), 1 µL
1. 19mer U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Detector : MS SIM(-)
Column temp: 40 °C

Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.3 mL/min

Column : Shodex HILICpak VN-50 1D (1.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.1 mL/min

### < Calibration Curves >

- Good overall linearity
- Need further work in the ng/mL level quantification




Sample : Synthesized phosphorothioated oligo-RNA, 1 µL
1. 19mer U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Column : Shodex HILICpak VN-50 1D (1.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) $CH_3CN$
High pressure linear gradient ; B %=64 % (0 min)→57 % (10-20 min)→64 % (20.01-25 min)
Flow rate : 0.1 mL/min
Detector : MS SIM(-)
Column temp: 40 °C

## Conclusions

The Shodex™ HILICpak™ VN-50 series, polymer-based diol type HILIC columns, demonstrated highly selective LC/UV/ESI-MS measurements. The oligonucleotides were separated and eluted in the order of smaller to higher degree of polymerization. The gradient setting with a mixture of 50-mM aqueous ammonium formate solution and acetonitrile enabled the analysis without a need of ion-pair reagents for reversed phase mode or highly concentrated salt for ion exchange mode generally used. The developed method will also be beneficial for preparative purposes, as it does not require de-salting process. Furthermore, 1.0 mm I.D. column showed an improved sensitivity for the synthetic phosphorothioated oligonucleotides MS measurements. Because of their stabilities compared to regular oligonucleotides, phosphorothioated oligonucleotides are more often chosen for oligonucleotide therapeutics. The results presented in this work proved that the VN-50 series columns are expected to be effective tools for the development and quality control of oligonucleotide therapeutics.