LC/MS Analysis of Oligonucleotides Using a Polymer-Based Diol Column - Shodex™ HILICpak™ VN-50 2D

Introduction

Oligonucleotide therapeutics have high expectations for treating genetic and metabolic disorders, anti-cancer drugs, and vaccines. They have the ability to specifically bind with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), allowing the control over specific gene expression linked to genetic diseases. The development and quality control of oligonucleotide therapeutics require highly selective and sensitive analytical methods, including LC/MS measurements. The method most often used for the analysis of oligonucleotides has been reversed phase chromatography with the use of ion-pair reagents. However, the ion-pair reagents tend to remain on the LC system and cause several other problems. Ion-exchange chromatography is another method commonly used, but it requires using highly concentrated salt in the eluent which is not ideal for MS detection.

The Shodex™ HILICpak™ VN-50 2D column used in this application is a high performance HILIC column, packed with multi-porous polyvinyl alcohol polymers modified with diol functional groups. The 2.0 mm internal diameter of the column is designed to work well for LC/MS measurements. The VN-50 series has successfully measured various oligosaccharides. It was shown to be effective for the analysis of oligonucleotides. This application introduces methods for the analysis of oligonucleotides using the Shodex HILICpak VN-50 2D column with a gradient elution of a volatile basic solvent and acetonitrile.

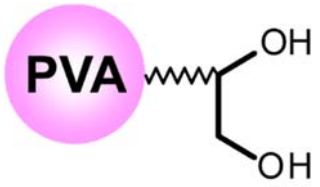

Fig.1 VN-50 packing material

Experimental

The two types of oligonucleotide therapeutics are categorized under DNA and RNA. In this application, three synthesized DNA oligos (unpurified, salt-free grade) were dissolved in ultra-pure water as the test samples were analyzed. DNA oligo's structure has a side chain composed from a combination of four nucleobases; adenine (A), thymine (T), guanine (G), and cytosine (C). Each test sample used was commercially synthesized with the base sequences shown below. However, it is likely that all samples contain some fragmented components from the extension interruptions, deletion of bases, etc.

Test Sample 1 (20mer): 5'-ATACCGATTAAGCGAAGTTT-3'

Test Sample 2 (20mer): 5'-ATACCAATTAAACAAAATTT-3'

Test Sample 3 (62mer): 5'-ATGAGAAGTATGACAACAGCCCCACACCGGCTGTTGTCATACTTCTCATGG TTCTTCGGAA-3'

Shimadzu Nexera / LCMS-8030 Plus was used with Shodex™ HILICpak™ VN-50 2D (2.0 mm I.D. x 150 mm; particle size 5 µm; pore size 100 Å). High pressure isocratic or linear gradient was used with Eluent A - ammonium formate aqueous solution and Eluent B - acetonitrile were used. The flow rate was set at 0.2 mL/min and the column temperature was set at 40 °C. The MS detector was coupled with a PAD detector (190-350 nm). An ESI was used as a means of ionization and Scan (-) or SIM (-) mode was used for the detection. The injection volume used was 1 µL.

Results and Discussion

1. Analysis of a 20 mer DNA oligo (Test Sample 1)

1-1. Optimization of the elution conditions

Several solvents were evaluated before choosing a suitable Eluent A including ammonium formate, ultra-pure water, aqueous ammonium hydrogen carbonate, and ammonium water. The DNA oligo was only retained by ammonium formate, leading to its use as Eluent A.

Fig.2 shows the UV chromatograms obtained for analyzing the Test Sample 1 under different concentrations of Eluent A and different ratios of Eluent A/B for the gradient elution. When an isocratic elution of 100 mM ammonium formate with 57% acetonitrile was used, the main component of the Test Sample 1, 20 mer DNA oligo, was detected around 15 minutes. MS data revealed the DNA oligos smaller than 19 mer were detected before the 20 mer DNA. The smaller mers eluted in the order of smallest to the largest degree of polymerization. As the degree of polymerization increased the hydrophilicity increased thus, the main separation mode worked in this analysis was assumed to be HILIC.

The separation of smaller DNA oligos were not sufficiently separated in this method. Gradient conditions were modified to improve separation. The initial concentration of the Eluent B (62%) was linearly decreased to 56% over 10 min with the concentration held for the next 10 minutes.

Eluents with a low to no salt concentrations increase the MS's detection sensitivity. 20 and 50 mM ammonium formate for the Eluent A. Using 50 mM ammonium formate did not cause any significant influence on the separation nor peak shapes, however, disturbances in the peak shapes were observed when 20 mM ammonium formate was used. Therefore, 50 mM ammonium formate as Eluent A was more preferable. After the elution of the target DNA oligo, it took about 5 minutes to equilibrate the column back to the initial conditions.

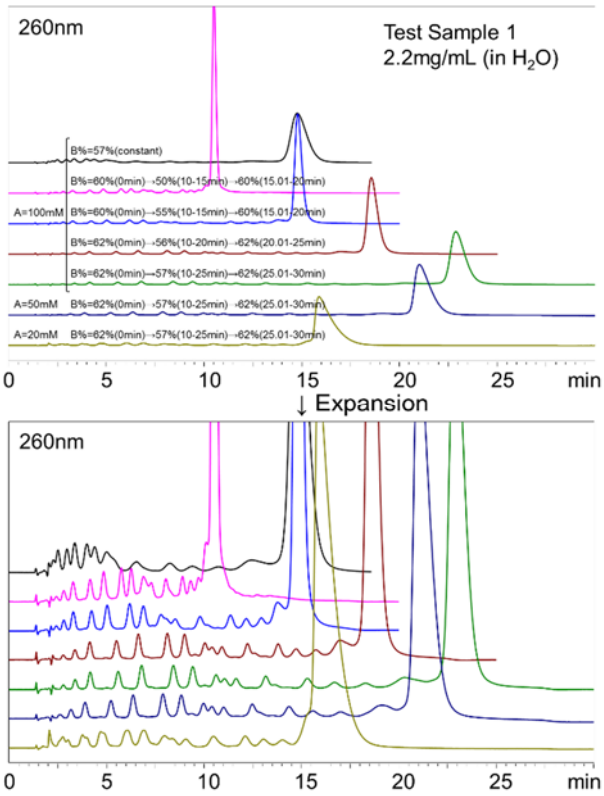

Fig.2 Comparison of various eluent conditions for the analysis of the Test Sample 1.

1-2. LC/UV/ESI-MS analysis of the Test Samples 1 and 2

Fig.3 shows the comparison of UV chromatograms (260 nm) for the Test Samples 1 and 2. The initial concentration of the Eluent B (62%) was linearly decreased to 56% over 10 minutes, and the concentration was held for the next 10 minutes, then returned to 62% to equilibrate the column. The Test Sample 1 containing the nucleobase G seemed retain longer than the Test Sample 2 which does not contain the nucleobase G. When DNA forms double strands, G and C form stronger base pairs than A and T, indicating hydrogen bonding between G and the packing material has an influence on its retention.

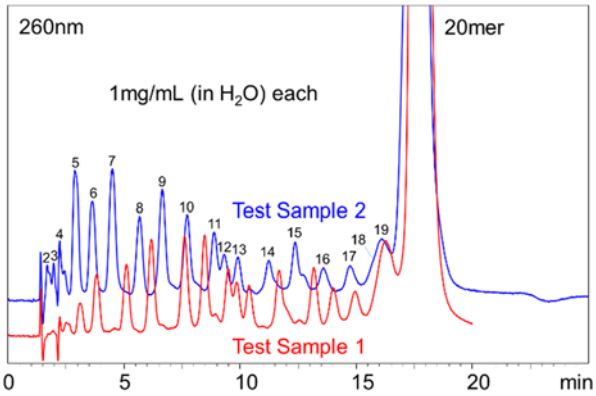

Fig.3 UV Chromatograms of the Test Sample 1 and 2

Fig. 4 shows the MS (-) analysis of the 20 mer DNA oligo in the Test Sample 1. It was confirmed that the DNA oligos were detected as multi-valent negative ions formed by losing multiple protons. The multi-valent negative ions on each DNA oligo chains were monitored using the SIM mode. Fig. 5 and 6 show the MS chromatograms of the Test Samples 1 and 2 respectively. In both samples, the DNA oligos of various chain lengths eluted in the order of degree of polymerization. Between some peaks, the resolution of chromatogram was not sufficient to identify each peak (e.g., between 11 and 12 mer or 18 and 19 mer). However, they can be sufficiently detected by using the MS detection.

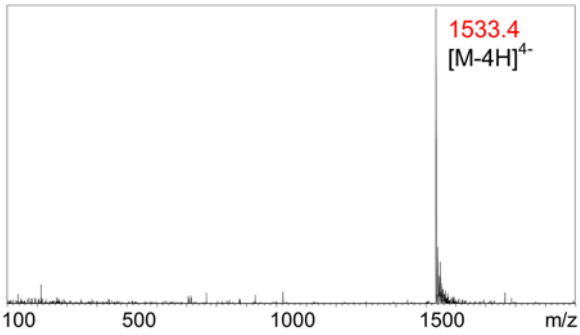

Fig.4 Mass spectrum (-) of the Test Sample 1 (20 mer)

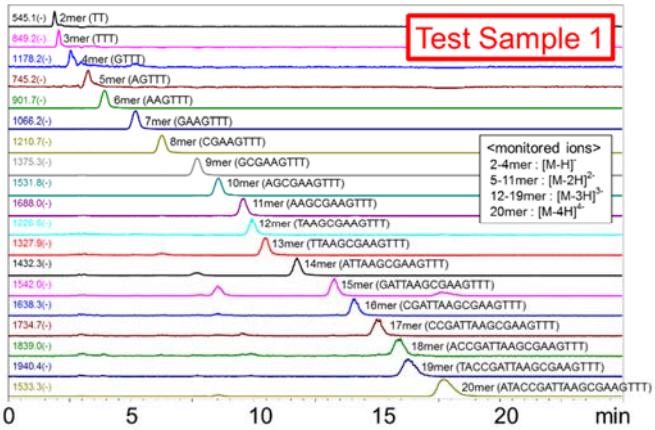

Fig.5 Mass chromatograms of the Test Sample 1

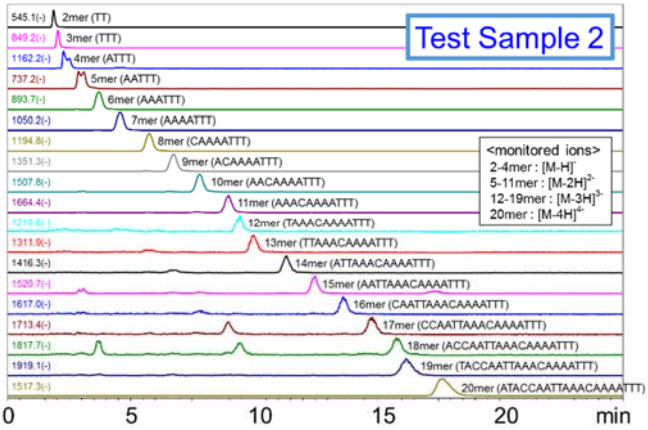

Fig.6 Mass chromatograms of the Test Sample 2

Fig 7 shows the MS chromatogram and the calibration curve of the 20 mer DNA oligo in the Test Sample 1. To shorten the analysis time, the gradient condition was modified. The initial concentration of the Eluent B (60%) was linearly decreased to 55% over a 10 minute, and the concentration was held for the next 5 minutes. The linearity of the calibration curve obtained was good as R^2=0.9999. Using the SIM mode, 20 mer DNA oligo can be selectively quantified in the 10 µg/mL level. The analysis time can be further shortened by decreasing the concentration of acetonitrile.

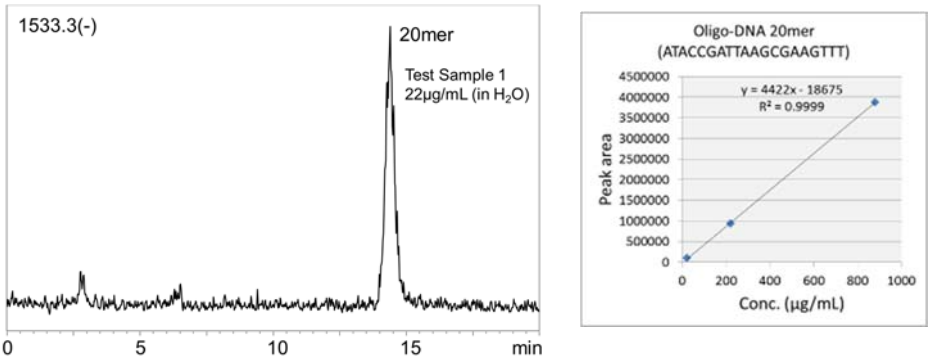

Fig. 7 MS chromatogram and the calibration curve of the Test Sample 1

2. Analysis of 62 mer DNA oligo

Fig. 8 shows the UV and the MS chromatograms of Test Sample 3. The initial concentration of the Eluent B (60%) was linearly decreased to 50% over 10 minutes, and the concentration was held for the next 10 minutes. Decreasing Eluent B to 50% to let the 62 mer DNA oligo to elute. Up to 31 mer DNA oligos were detected using the MS detector. However, the DNA oligos larger than 32 mer were not detected by the MS. This might be due to the multi valent ions formed during ionization, and as their m/z value were over the MS's scanning upper limit (m/z 2000).

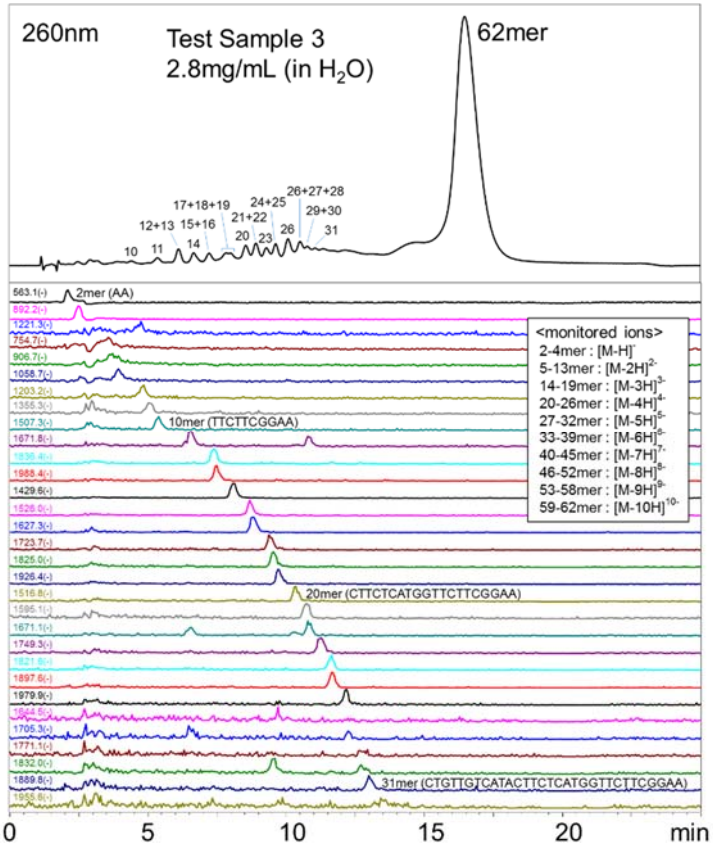

Fig.8 UV and Mass chromatograms of the Test Sample 3

Conclusions

This application demonstrated the feasibility of analyzing up to about 30 mer oligonucleotides using the polymer-based diol HILIC column, Shodex™ HILICpak™ VN-50 2D, coupled with an LC/UV/ESI-MS. The method is very selective by separating and eluting the oligonucleotides by degree of polymerization. The gradient elution of 50 mM ammonium formate / acetonitrile used in this method was very simple, especially when compared to the commonly used reversed phase chromatography method that requires an addition of ion-pair reagent or to the ion-exchange chromatography method that requires an addition of highly concentrated salts. Moreover, as the ammonium formate / acetonitrile eluent used here is volatile, it has an advantage in simplifying the desalting process during purification. The polymer-based packing material also allows the use of alkaline washing solutions which helps preventing the non-specific adsorption and carry-over related problems that are concerned in the analysis requiring high precision and accuracy or in the preparative scale measurements. Therefore, the methods using the VN-50 series columns introduced in this application have high potentials in the development, quality control, and the purification of oligonucleotide therapeutics.

TA. NO. 004. ①18. D. JUL. P