LC/MS Analysis of Oligonucleotides

Introduction

Oligonucleotide therapeutics have high expectations in the areas of genetic and metabolic treatments, but also for anti-cancer drugs and vaccines of various diseases. The wide range of applications is due to the oligonucleotide unique mechanisms of action. They have abilities to target specifically bind with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They also control specific gene expressions that are responsible for certain diseases. The development and quality control of the oligonucleotide therapeutics often require highly selective and sensitive analytical methods. The method with an LC/MS system is one of them. The most often used method for the oligonucleotide analyses have been reversed phase chromatography with the use of an ion-pair reagent. However, the ion-pair reagents have a tendency to remain on the LC system and cause several problems. Ion-exchange chromatography is another method commonly used, but it generally requires addition of highly concentrated salt in its eluent, which does not work well with MS detector.

The Shodex HILICpak VN-50 2D column used in this application is a high performance HILIC column. It is packed with multi-porous polyvinyl alcohol polymers modified with diol functional groups. The 2.0 mm internal diameter column is designed to work well with LC/MS measurements. We have successfully measured various measuring various oligosaccharides using the VN-50 series columns. The column is also found to be effective for the analysis of oligonucleotides. This application introduces the methods for analyzing oligonucleotides using the VN-50 2D column with a gradient elution employing a volatile basic solvent and acetonitrile. Although there was no need for an ion-pair reagent, the salt level was relatively low.

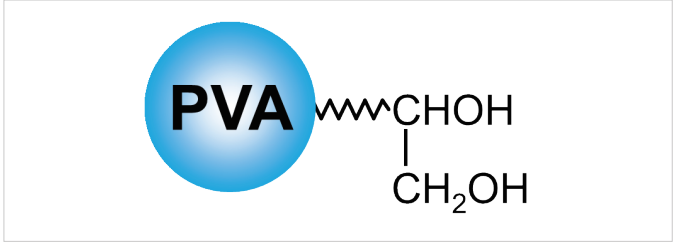


Fig. 1. Schematic diagram of VN-50 packing material

Experimental

There are two types of oligonucleotide therapeutics; DNA and RNA types. We studied three synthesized DNA oligos (unpurified, salt-free grade) dissolved in ultra-pure water as test samples. An DNA oligo has a structure having a side chain composed from four nucleobases (adenine (A), thymine (T), guanine (G), and cytosine (C)) in different combinations. Each test sample used was commercially synthesized, supposed to have base sequences as shown below. However, it is likely that all sample contain some fragmented components from the extension interruptions, deletion of bases, and such.

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

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

Test Sample 3 (62 mer)
5'-CATGAGAAGTATGACAACAGC
CCCACACCGGCTGTTGTCATA
CTTCTCATGGTTCTTCGGAA-3'

The LC/MS system used was Shimadzu Nexera/ LCMS-8030 Plus. The column used was Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm; particle size 5 µm; pore size 100 Å). The methods used were either (a) high pressure isocratic method or (b) linear gradient method with Eluent A: ammonium formate aqueous solution and Eluent B: acetonitrile. The injection volume used was 1 µL.

Results and Discussion

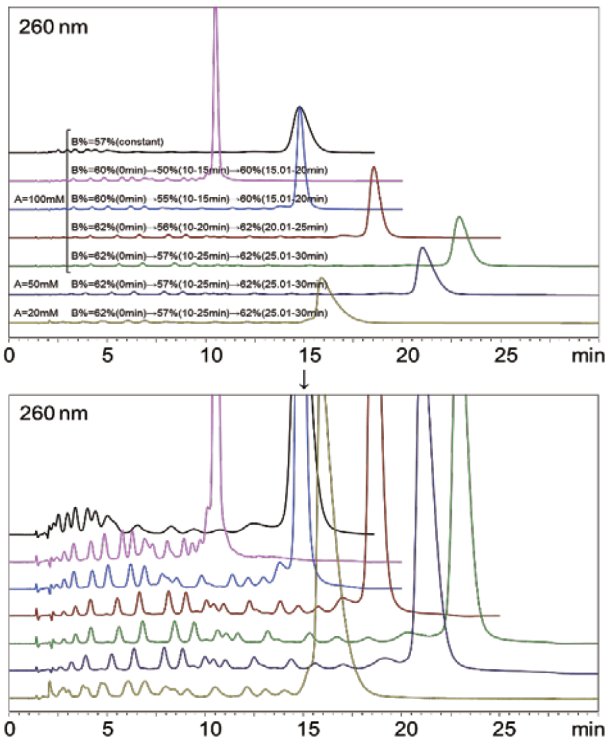
1. Analysis of a 20-mer DNA oligo (Test Sample 1)

1.1 Optimization of elution conditions

We tested several eluents, including ammonium formate, ultra-pure water, aqueous ammonium hydrogen carbonate, and ammonium water. DNA oligos were retained only by ammonium formate, and thus we decided to use it as Eluent A.

Next, we optimized the eluent conditions. Figure 2 shows the UV chromatograms of Test Sample 1 using different concentrations of the 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 at around 15 minutes.

From the MS chromatograms, the DNA oligos smaller than 19 mer were detected before the 20-mer DNA oligo's peak, i.e., DNA oligos were eluted in the order of smaller to the larger degree of polymerization. Since DNA oligos' hydrohilicities increase as their degree of polymerization increase, this elution trend indicates the main separation mode worked in this analysis was HILIC mode.



Column : Shodex HILICpak VN-50 2D
Eluent : (A) $HCOONH_4$ aq./(B) CH_3CN
Linear gradient
Flow rate : 0.2 mL/min
Detector : PAD (260 nm)
Column temp.: 40 ºC

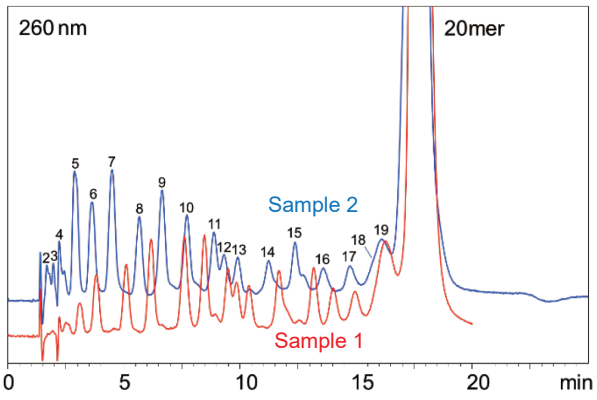
Fig. 2. Comparison of various eluent conditions for the analysis of Test Sample 1

Sufficient separation of smaller DNA oligos was not achieved by the method. To improve their separations, we introduced gradient conditions as such: The initial concentration of Eluent B (62 %) was linearly decreased to 56 % over a 10 minute, and this concentration was held for the next 10 minutes. This optimized condition provided the most well balanced resolutions and the analysis time.

An eluent with low salt concentration is desired to increase the MS detection sensitivity. Thus, we tested 20- and 50-mM ammonium formate for the Eluent A. 50-mM ammonium formate did not influence the separation nor peak shapes significantly, however, disturbances in the peak shapes were observed when 20-mM ammonium formate was used. Therefore, we decided to use 50-mM ammonium formate as Eluent A. After each run, it required a 5-minute equilibration to bring the column back to the initial condition.

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

Figure 3 shows the UV chromatograms of Test Samples 1 and 2. Test Sample 1, which contains nucleobase G, seemed to be retained longer than the Test Sample 2, which does not contain nucleobase G. It is known that when DNA forms double strands, G and C form stronger base pair than A and T pair does. This indicates that hydrogen bonding between G and the packing material has an influence on its retention.



Column : Shodex HILICpak VN-50 2D
Eluent : (A) 50-mM $HCOONH_4$ aq./(B) CH_3CN
Linear gradient
62 % B (0 min) → 56 % B (10 - 20 min)
→ 62 % B (20.01 - 25 min)
Flow rate : 0.2 mL/min
Detector : UV (260 nm)
Column temp.: 40 ºC

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

Figure 4 shows a mass spectrum (-) of 20-mer DNA oligo in 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. Refer to figure 3 for the analytical condition used.

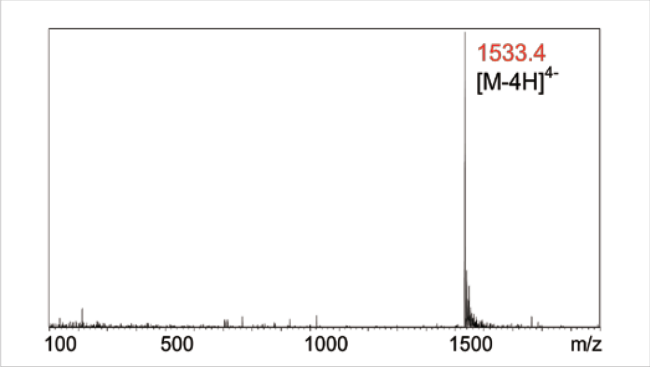


Fig. 4. Mass spectrum (-) of Test Sample 1

Figures 5 and 6 show MS chromatograms of Test Samples 1 and 2, respectively. In both samples, the DNA oligos of various chain lengths were detected in the order of lower to the higher degree of polymerization. Between some peaks, the resolutions of the UV chromatogram was not sufficient to identify each peak (e.g., between 11 and 12 mer or 18 and 19 mer). However, they can be identified by using an MS. Refer to figure 3 for the analytical condition used.

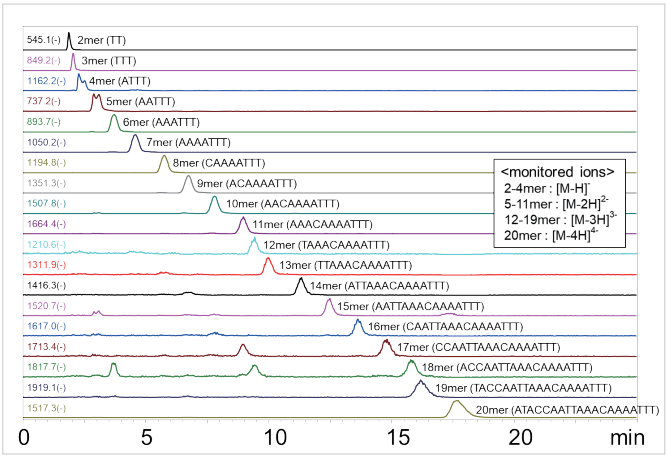


Fig. 5. MS chromatograms of Test Sample 1

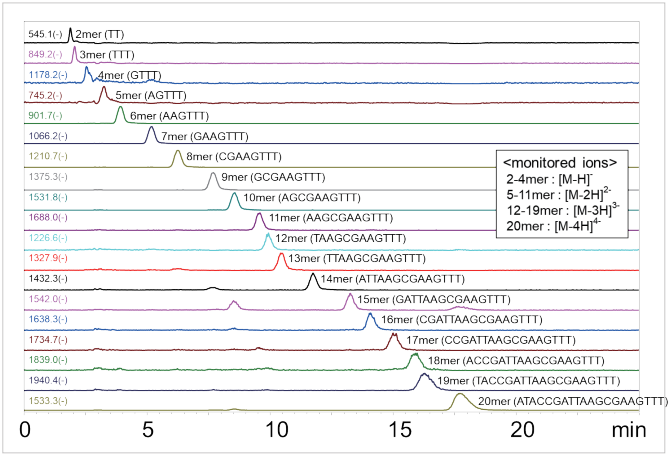
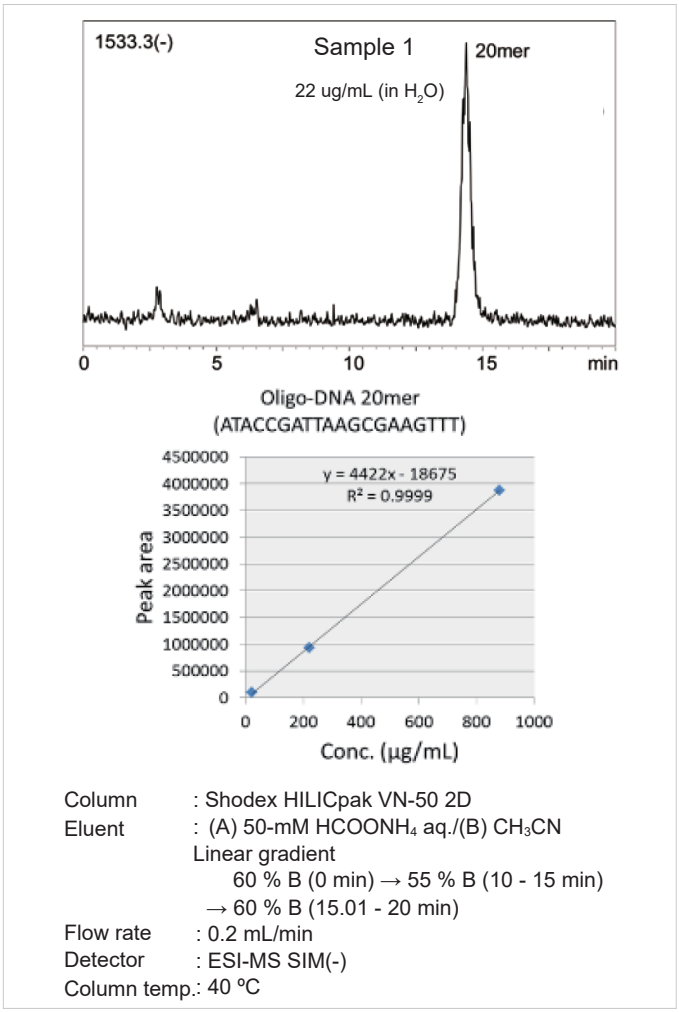


Fig. 6. MS chromatograms of Test Sample 2

Figure 7 shows the MS chromatogram and the calibration curve of the 20-mer DNA oligo in Test Sample 1. To shorten the analysis time, the gradient condition was modified. Linearity of the calibration curve obtained was well. Using the SIM mode, 20-mer DNA oligo can be selectively quantified in 10 µg/mL level. The analysis time can be shortened even more by decreasing the concentration of acetonitrile.



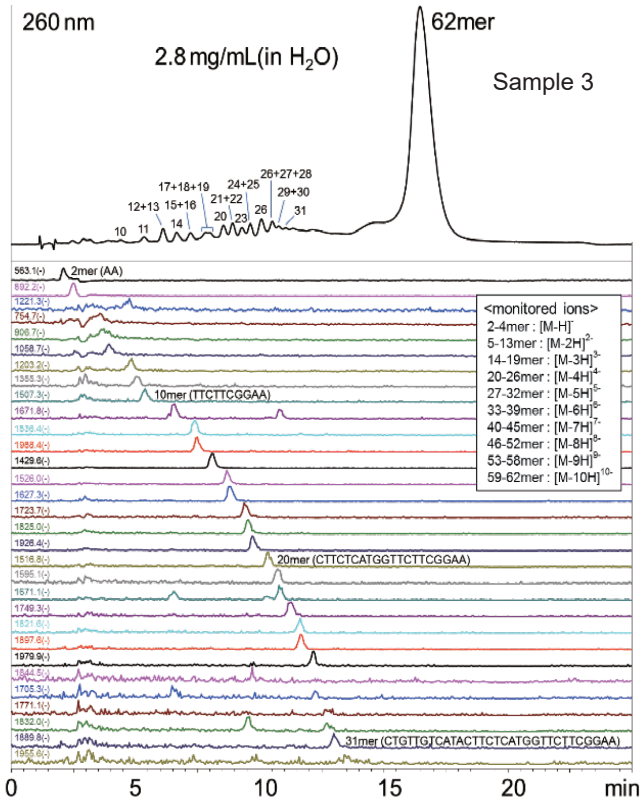
Column : Shodex HILICpak VN-50 2D
Eluent : (A) 50-mM $HCOONH_4$ aq./(B) CH_3CN
Linear gradient
60 % B (0 min) → 55 % B (10 - 15 min)
→ 60 % B (15.01 - 20 min)
Flow rate : 0.2 mL/min
Detector : ESI-MS SIM(-)
Column temp.: 40 ºC

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

2. Analysis of 62-mer DNA oligo

Figure 8 shows the UV and the MS chromatograms of Test Sample 3. It required to decrease percentage of the 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 caused by the multi valent ions that have formed during the ionization, and as their m/z value were over the MS's scanning upper limit (m/z 2000).



Column : Shodex HILICpak VN-50 2D
Eluent : (A) 50-mM $HCOONH_4$ aq./(B) CH_3CN
Linear gradient
60 % B (0 min) → 50 % B (10 - 20 min)
→ 60 % B (20.01 - 25 min)
Flow rate : 0.2 mL/min
Detector : UV (260 nm)
Column temp.: 40 °C

Fig. 8. UV and MS chromatograms of Test Sample 3

Conclusions

This application demonstrated the feasibility of Shodex HILICpak VN-50 2D, a polymer-based diol HILIC column, coupled with an LC/UV/ESI-MS for the analysis of up to about 30-mer oligonucleotides. The method is very selective by separating and eluting the oligonucleotides in the order of lower to higher degree of polymerization. The gradient elution of 50-mM ammonium formate/acetonitrile used in this method was very simple. Especially when compared to reversed phase chromatography methods that require ion-pair reagent or ion-exchange chromatography methods that require highly concentrated salts in their eluents. Moreover, since ammonium formate/acetonitrile eluent mixture is volatile, it has an advantage in simplifying 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 analyses requiring high precision and accuracy or in preparative scale measurements. In this application, we demonstrated the methods using the VN-50 series columns to be useful in the development, quality control, and the purification of oligonucleotide therapeutics.

https://www.shodexhplc.com/

Resonac America, Inc

TA. NO. 004. (2) 20. D. APR. P