LC/MS Analysis of Various Oligonucleic Acids with/without Modifications

Introduction

Oligonucleotide therapeutics, such as antisense nucleic acid drugs, are promising candidates for genetic and metabolic, and cancer treatments. Oligonucleic acids (Oligo-DNAs and Oligo-RNAs) of about 20mer sizes are generally used in approved nucleic acid drugs. Most oligonucleic acids are chemically modified. Among them, phosphorothioated oligonucleotides with which an oxygen atom is replaced by a sulfur atom at its phosphate linkage (Fig. 1) have higher stability and are less likely decomposed in vivo than non-modified oligonucleic acids. Apart from phosphorothioation, oligonucleic acids with chemically modified ribose sugars on the nucleic acid (Fig. 2) is another form of chemical modification. For example, nucleic acids having 5-bases at both ends of ribose modified with 2'-MOE (2'-Methoxyethyl) is already used in commercialized nucleic drugs.

The development and quality control of oligonucleotide therapeutics often require highly selective and sensitive analytical methods. The method utilizing an LC/MS system is one of them. The most often used method for the analysis of oligonucleotides 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 the eluent, which does not work well with MS detectors.

The Shodex HILICpak VN-50 columns used in this application are high performance HILIC columns. They are packed with multi-porous polyvinyl alcohol polymers modified with diol functional groups and designed to work well with LC/MS measurements. This technical article introduces methods developed for analyses of modified oligonucleotides. The analytes included were phosphorothioated oligonucleic acids and oligonucleic acids with chemically modified ribose sugars on nucleic acid. The method used a 2.0-mm internal diameter (I.D.) column (VN-50 2D) with a simple eluent containing acetonitrile with relatively low level of salt, i.e., without an ion-pair reagent. A 1.0 mm I.D. column (VN-50 1D, special order) was also tested to achieve higher sensitivity analysis.

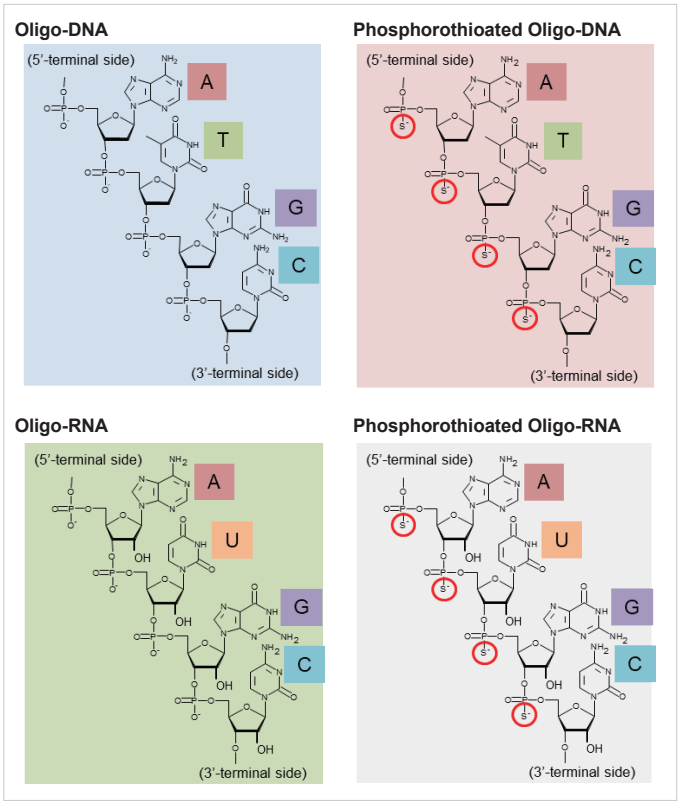


Fig. 1. Oligonucleic acids with/without phosphorothioation

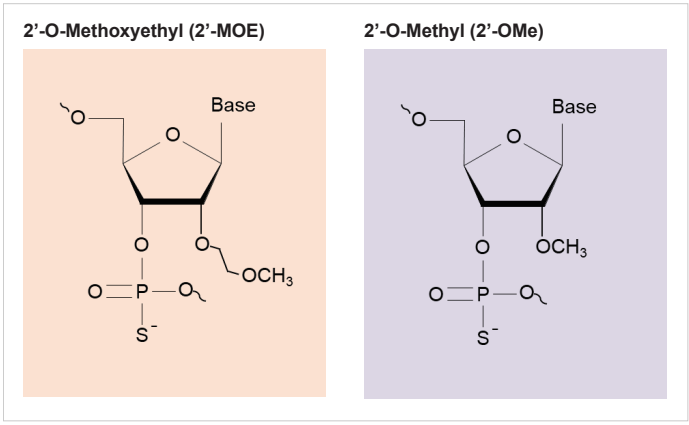


Fig. 2. Oligonucleic acids with chemically modified ribose sugars on nucleic acid

Experiments

The LC/MS system used was Shimadzu Nexera/LCMS-8030 Plus. The columns used were Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm; PEEK housing) and VN-50 1D (1.0 mm I.D. x 150 mm; PEEK lined SUS housing). Particle size of the packing material is 5 µm and its pore size is 100 Å.

High pressure linear gradient elution was used. The elution method was optimized during method development. Different ratios of Eluent A - ammonium formate aqueous solution and Eluent B - acetonitrile were used. The column temperature was set at 40 °C. An MS was coupled with a UV (260 nm) for detection. An ESI was used as a means of ionization and SIM (-) mode was used for the detection.

1. Analysis of various oligonucleic acids

The elution patterns of modified (phosphorothioated) and non-modified oligonucleic acids were studied. The analytes included were: Sample 1. Synthetic 20mer oligo-DNA, Sample 2. Synthetic phosphorothioated 20mer oligo-DNA, Sample 3. Synthetic 20mer oligo-RNA, and Sample 4. Synthetic phosphorothioated 20mer oligo-RNA. They were dissolved in ultra-pure water and0.1-mg/mL samples were injected for analysis.

2. Analysis of oligonucleic acids with chemically modified ribose sugars on nucleic acid

The elution patterns of oligo-RNA having ribose modified with 2'-MOE and non-modified oligo-RNA were studied as well. The analytes included were: Sample 5. Synthetic phosphorothioated 20mer oligo-RNA with all ribose modified with 2'-MOE, Sample 6. Synthetic phosphorothioated 20mer oligo-RNA with 5-bases at both ends of ribose modified with 2'-MOE, and Sample 7. Synthetic phosphorothioated 20mer oligo-RNA with all ribose modified with 2'-OMe (2'-O-Methyl). They were dissolved in ultra-pure water and 0.2-mg/mL samples were injected for analysis.

3. Comparisons of VN-50 1D and VN-50 2D columns on their sensitivities

A 1.0 mm I.D. column (VN-50 1D) was tested aiming to increase sensitivity of the LC/MS method. Its sensitivity was compared with that of a 2.0 mm I.D. column (VN-50 2D). Sample 6 was dissolved in ultra-pure water and 0.2-mg/mL samples were injected for the analysis.

4. Analysis of 10 to 50mer oligo-DNAs

Lastly, the separations of up to 50mer oligo-DNAs were optimized. The sizes of synthetic oligo-DNA samples included were: Sample 8. 10mer, Sample 9. 20mer, Sample 10. 30 mer, Sample 11. 40mer, and Sample 12. 50mer. They were dissolved in ultra-pure water and 0.02-mg/mL samples were injected for analysis.

Table 1 summarizes the oligonucleic acids analyzed in this application.

Table 1. Oligonucleic acids analyzed in this application

1. Oligo-DNA 20mer	ATACCGATTAAGCGAAGTTT
2. 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. Oligo-RNA 20mer	AUACCGAUUAAGCGAAGUUU
4. Phosphorothioated oligo-RNA 20mer	A*U*A*C*C*G*A*U*U*A*A*G*C*G*A*A*G*U*U*U
5. Phosphorothioated oligo-2'-MOE-RNA 20mer	[2MOeA]*[2MOeU]*[2MOeA]*[2MOeC]*[2MOeC]*[2MOeG]*[2MOeA]*[2MOeU]*[2MOeU]*[2MOeA]*[2MOeA]*[2MOeG]*[2MOeC]*[2MOeG]*[2MOeA]*[2MOeA]*[2MOeG]*[2MOeU]*[2MOeU]*[2MOeU]
6. Phosphorothioated oligo-2'-MOE(1-5/16-20)-RNA 20mer	[2MOeA]*[2MOeU]*[2MOeA]*[2MOeC]*[2MOeC]*[rG]*[rA]*[rU]*[rU]*[rA]*[rA]*[rG]*[rC]*[rG]*[rA]*[2MOeA]*[2MOeG]*[2MOeU]*[2MOeU]*[2MOeU]
7. Phosphorothioated oligo-2'-OMe-RNA 20mer	[2OMeA]*[2OMeU]*[2OMeA]*[2OMeC]*[2OMeC]*[2OMeG]*[2OMeA]*[2OMeU]*[2OMeU]*[2OMeA]*[2OMeA]*[2OMeG]*[2OMeC]*[2OMeG]*[2OMeA]*[2OMeA]*[2OMeG]*[2OMeU]*[2OMeU]*[2OMeU]
8. Oligo-DNA 10mer	TTCTTCGGAA
9. Oligo-DNA 20mer	CTTCTCATGGTTCTTCGGAA
10. Oligo-DNA 30mer	TGTTGTCATACTTCTCATGGTTCTTCGGAA
11. Oligo-DNA 40mer	CCACACCGGCTGTTGTCATACTTCTCATGGTTCTTCGGAA
12. Oligo-DNA 50mer	GACAACAGCCCCACACCGGCTGTTGTCATACTTCTCATGGTTCTTCGGAA

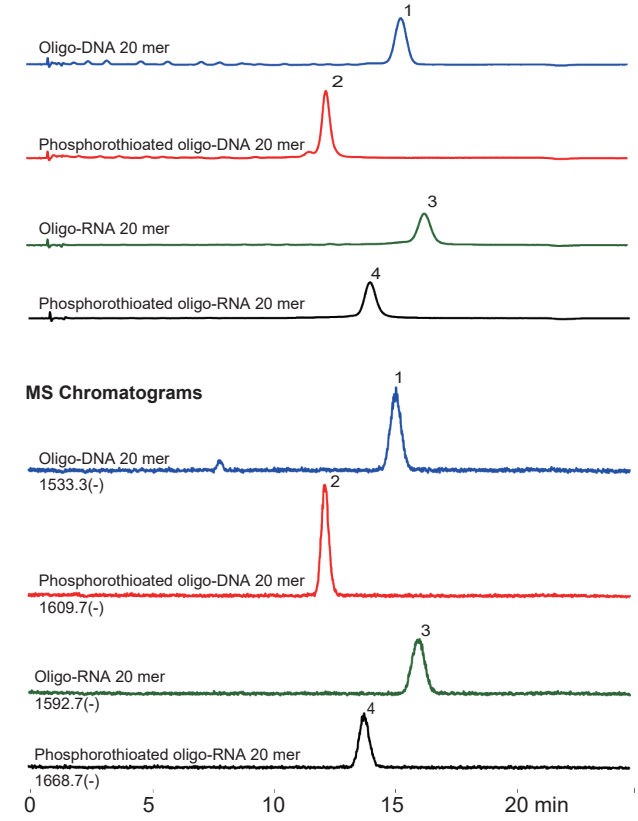
*phosphorothioated position

Results

1. Analysis of various oligonucleic acids

Figure 3 shows the LC/UV/MS chromatograms of various 20mer oligonucleic acids and their phosphorothioated analogs. The elution condition was a linear gradient from 64 to 56 % acetonitrile over 0 to 10 minutes, and then 56 % acetonitrile was held for 10 minutes.

Under HILIC mode, the higher the hydrophilicity, the stronger the retention becomes. Thus, since synthetic oligo-RNA has higher hydrophilicity than synthetic oligo-DNA, its retention becomes longer (compare peaks 1 and 3). Meanwhile, retention of a phosphorothioated oligonucleotide is weaker than its non-phosphorothioated analog (compare peaks 1 and 2 and peaks 3 and 4). This is because in a phosphorothioated oligonucleotide's phosphate linkage, an oxygen atom is replaced by a sulfur atom, which makes it more hydrophobic.



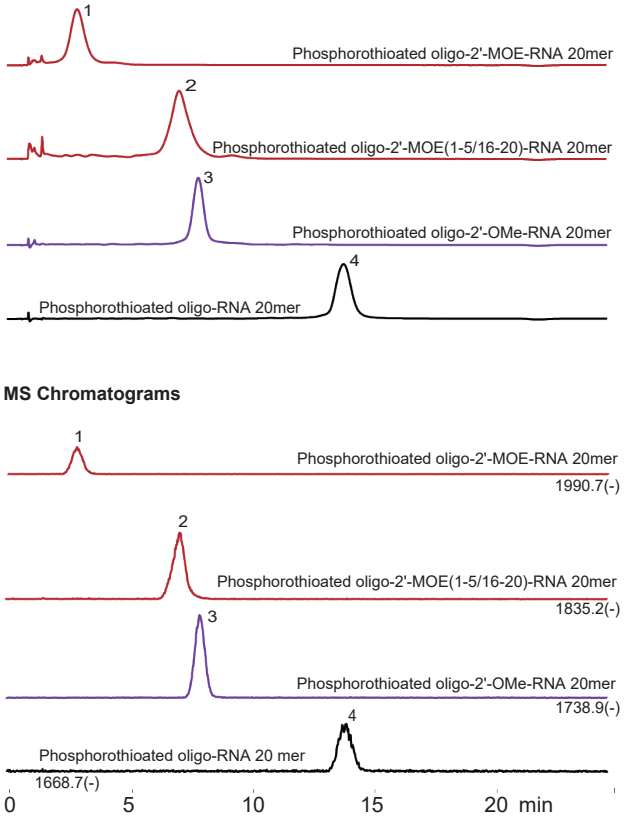
Sample : 0.1 mg/mL each (in H_2O), 1 µL
Column : Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm)
Eluent : (A) 50 mM $HCOONH_4$ aq.(pH 9.8) / (B) CH_3CN
High pressure Linear gradient
62 % B (0 min) → 56 % B (10 - 20 min) → 62 % B (20.01 - 25 min)
Flow rate : 0.3 mL/min
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Detector : UV (260 nm) + ESI-MS (SIM Negative)
Column temp.: 40 ºC

Fig. 3. UV/MS chromatograms of various olugonucleic acids

2. Analysis of oligonucleic acids with chemically modified ribose sugars on nucleic acid

Figure 4 shows the LC/UV/MS chromatograms of several modified and non-modified 20mer oligo-RNAs. The elution condition was a linear gradient from 64 to 57 % acetonitrile over 0 to 10 minutes, and then 57 % acetonitrile was held for 10 minutes.

Among synthetic phosphorothioated oligo-RNAs, one with more 2'-MOE modification has a lower hydrophilicity. Thus, 2'-MOE modified ones are less retained than their non-modified analogs (compare peaks 1, 2, and 4). On the other hand, 2'-OME has a higher hydrophilicity than 2'-MOE. Thus, even all ribose are modified with 2'-OME, its retention is not affected so much and elutes at about the same time as the one partially modified with 2'-MOE (compare peaks 2 and 3).



Sample : 0.2 mg/mL each (in H_2O), 1 µL
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
64 % B (0 min) → 57 % B (10 - 20 min) → 64 % B (20.01 - 25 min)
Flow rate : 0.3 mL/min
Instrument : Shimadzu Nexera / LCMS-8030 Plus
Detector : UV (260 nm) + ESI-MS (SIM Negative)
Column temp.: 40 ºC

Fig. 4. UV/MS chromatograms of oligonucleic acids with chemically modified ribose sugars on nucleic acid

3. Comparisons of VN-50 1D and VN-50 2D columns on their sensitivities

Figure 5 shows the MS chromatograms of Sample 6 analyzed by VN-50 1D (1.0 mm I.D.) and VN-50 2D (2.0 mm I.D.). The elution condition was a linear gradient from 64 to 57 % acetonitrile over 0 to 10 minutes, and then 57 % acetonitrile was held for 10 minutes. The required analysis times were about the same. However, 1.0 mm I.D. column with a use of lower flowrate led to increased LC/MS sensitivity. The study demonstrated a feasibility of achieving a higher sensitivity analysis.

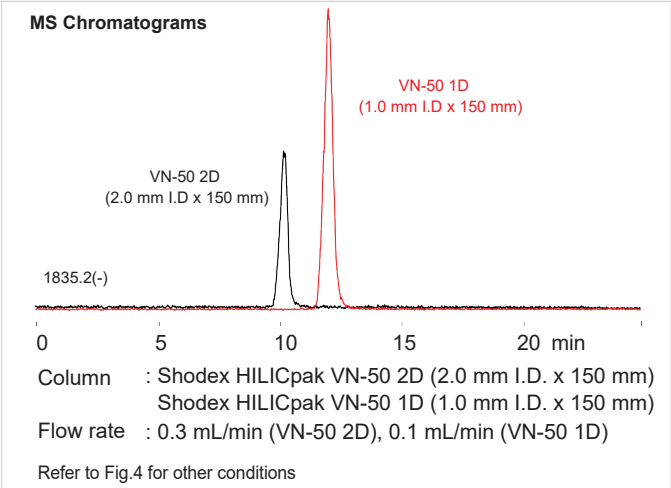


Fig. 5. Comparisons of 1.0 mm and 2.0 mm I.D. columns

4. Analysis of 10 to 50mer oligo-DNAs

Figure 6 shows the LC/UV/MS chromatograms of 10, 20, 30, 40, and 50mer oligo-DNAs. The optimized elution condition was a linear gradient from 65 to 45 % acetonitrile over 0 to 10 minutes, and then 45 % acetonitrile was held for 10 minutes. This gradient condition (higher ammonium formate aqueous solution ratio) provided the improved separations of longer chain oligo-DNAs.

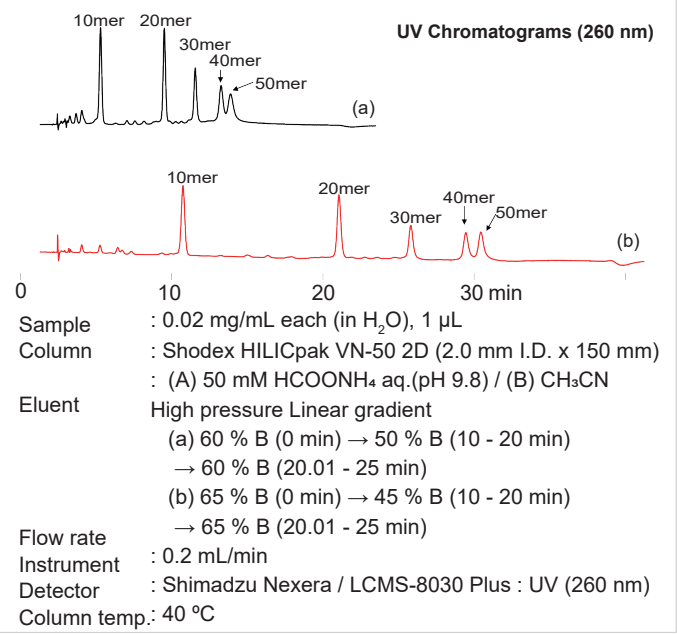


Fig. 6. UV chromatograms of 10 to 50mer oligo-DNAs

Conclusions

This application demonstrated the feasibility of Shodex HILICpak VN-50, a polymer-based diol HILIC column, coupled with an LC/UV/ESI-MS for the analysis of various oligonucleic acids with and without chemical modifications. The confirmed analytes include were phosphorothioated oligonucleic acids and oligonucleic acids with chemically modified ribose sugars on nucleic acid, which are used in nucleic acid drugs.

VN-50 works as a HILIC column with a use of 50-mM ammonium formate/acetonitrile eluent. This mild condition does not require ion-pair reagents required for reversed phase chromatography nor highly concentrated salts required for ion-exchange chromatography. Thus, it reduces the LC/MS system maintenance burden. Also, since ammonium formate/acetonitrile eluent mixture is volatile, it has an advantage in simplifying desalting process during purification.

Moreover, the polymer-based packing material 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.

Lastly, a special VN-50 1D column with 1.0 mm I.D. and reduced flow rate demonstrated a feasibility of providing increased LC/MS sensitivity analysis.

Therefore, the results presented the advantages of using VN-50 series columns in development, quality control, and purification of nucleic acid drugs.

Reference

Shodex Technical Article No.4: LC/MS Analysis of Oligonucleotides

https://www.shodexhplc.com/

Resonac America, Inc

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