SHIMADZU
Excellence in Science

Application Note

No. 110

Pharmaceutical

Analysis of Deaminated Oligonucleotides Using Supercritical Fluid Chromatography

Momoka Hayashida

Pharmaceutical

■ Abstract

Supercritical fluid chromatography (SFC) has been considered difficult to apply to oligonucleotides, which are highly polar compounds, due to the polarity of carbon dioxide used as the mobile phase. However, a previous study[1] found that 4-mer DNA was able to be applied to SFC. In this study, the applicability of SFC to oligonucleotide longer than 4-mer was investigated. In addition, to evaluate the separation behavior of SFC, we applied it to the separation of deaminated oligonucleotides, which are difficult to separate even by the existing ion-pair reversed-phase chromatography (IP-RPLC). Assuming that SFC would be applied to the purification of oligonucleotides after synthesis, sequences with and without dimethoxytrityl (DMTr) protecting group were employed as samples.

Using optimized modifier containing octylamine, 18-mer modified oligonucleotides were detected. As a result of optimization of analytical conditions for the separation of deaminated oligonucleotides, 10- and 18-mer target oligonucleotides and their deaminated products were able to be separated.

This article has been re-written based on the original paper[2] published in Journal of Chromatography A (Open Access version).

1. Introduction

Oligonucleotide therapeutics are new modalities of pharmaceuticals, whose active pharmaceutical ingredients are oligonucleotides. These oligonucleotides are mainly produced by phosphoramidite solid-phase synthesis (Fig. 1). In this synthesis, nucleotides are individually added to the growing chain mainly from the 3' to the 5' end through a coupling reaction with phosphoramidite (Fig. 1-1, 2). The 5' position of the sequence in coupling process is protected by a 4,4'-dimethoxytrityl (DMTr) group, but the 5' position of the sequence that has not undergone coupling is capped with acetyl group to terminate further reaction (Fig. 1-4). When coupling the next base, the DMTr group is deprotected (Fig. 1-5) and the next phosphoramidite is coupled (Fig. 1-2). By repeating this process, any sequence can be synthesized. After completing full-length elogation, the oligonucleotide is cleaved from the solid phase support and purified by one of the following two methods (Fig. 2): first, DMTr-protected (DMTr-on) oligonucleotide is cleaved from solid phase support and purified by liquid chromatography (LC); second, DMTr-protection is removed from oligonucleotide (DMTr-off) on the solid phase support then cleaved and subjected to LC purification.

In either purification method, it is difficult to completely remove many impurities produced in the synthesis process, and therefore, it is important to perform impurity analysis. For example, deaminated oligonucleotide is an impurity in which the amino group of the cytosine base is mainly replaced by a hydroxy group due to the basic conditions used in the cleavage and the deprotection. Deaminated cytosine is recognized as uracil and may cause off-target effects. IP-RPLC is often used as a separation method, but due to the similar polarity of the target and deaminated product, separation is difficult even with IP-RPLC depending on the sequence.

SFC is an analytical technique that employs pressurized carbon dioxide as the principal component of mobile phase, which shows less viscosity and more diffusivity than those of ordinary

liquid mobile phase, resulting in high separation performance. SFC is also used for preparative applications because it is easy to scale up to preparative use, and the collected fractions generally consist of almost organic solvent, providing easy post-processing.

SFC has been considered difficult to analyze highly polar compounds due to the low polarity of pressurized carbon dioxide, but optimizations of the composition of the co-solvent (modifier) and its mixing ratio with carbon dioxide have made it possible to be applied to highly polar compounds as well[3]. In our previous study[1], 4-mer oligonucleotides were analyzed using a modifier containing 2-aminoethanol. However, peak tailing was observed for highly polar sequences, indicating that further optimization of analytical conditions is essential to apply this method to analysis of highly polar sequences. Therefore, it was considered that the peak shape could be improved by adding octylamine, a highly hydrophobic alkylamine, to the modifier to form more hydrophobic ion pairs for improving the solubility of the oligonucleotide to the mobile phase.

In this study, the composition of the modifier was investigated to evaluate whether SFC could be applied to 5-, 10-, 15-, and 18-mer sequences. SFC was applied to the separation of deaminated sequences to evaluate its separation ability. Assuming that the more hydrophobic DMTr groups would be easier to be analyzed, DMTr-on and DMTr-off sequences were used for the evaluation, and the difference in retention behaviors of these sequences were also evaluated.

2. Experiments

Analytical conditions for SFC and IP-RPLC are shown in Table 2-6. The concentration of 2-aminoethanol was fixed at 50 mmol/L in the present study, since the addition of 40 mmol/L or more of 2-aminoethanol in the previous study showed good peak shape. The same concentration of acetic acid was also added to the modifier to neutralize the pH of the modifier. A Shim-pack Diol II column (hereafter referred to as Diol II column) was used as in the previous study.

MS conditions are shown in Table 1.

Table 1 MS conditions

System	: LCMS-9030
Polarity	: Negative
Interface temp	: 350 °C
Nebulizer gas	: 3.0 L/min
Heating gas	: 10.0 L/min
Drying gas	: 10.0 L/min
DL temp	: 250 °C
Heat block temp	: 400 °C
Interface Voltage	: -3.5 kV
TOF-MS	: *m/z* 150-2000

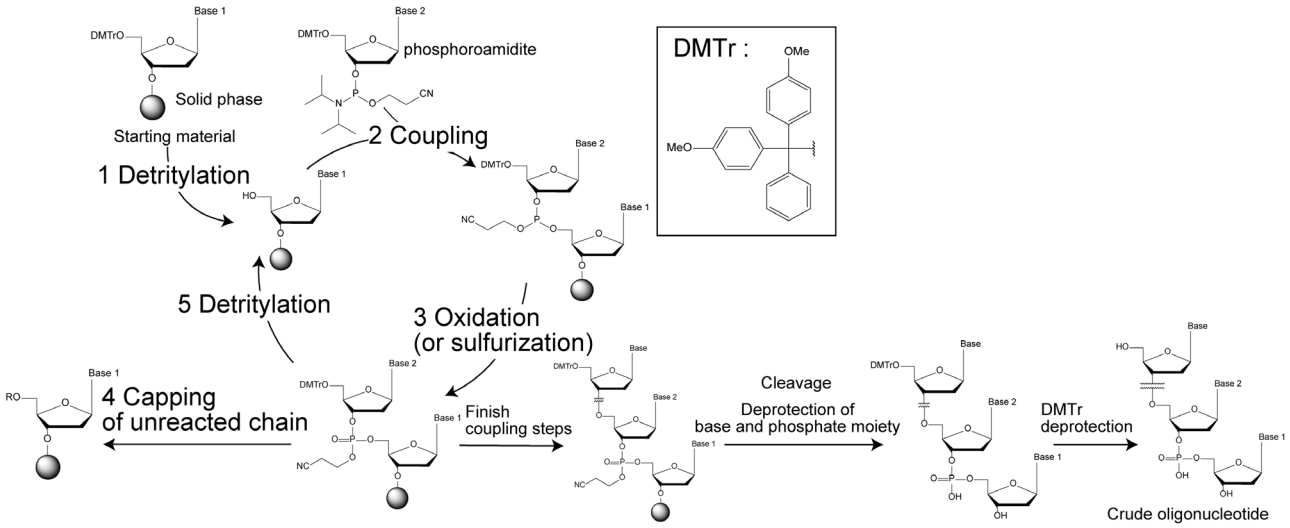


Fig. 1 phosphoramidite method

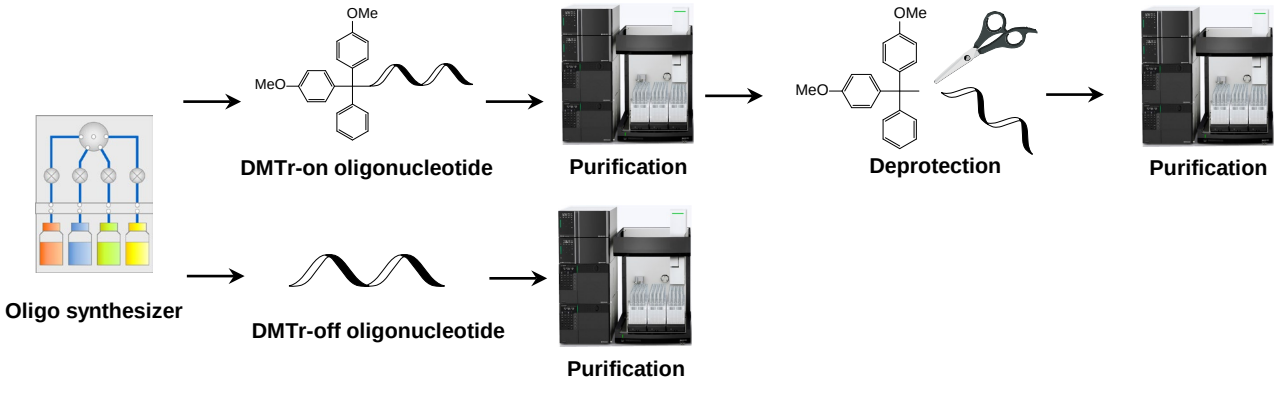


Fig. 2 Purification method for solid-phase synthesis

3. Results and Discussion

3-1. Evaluation of applicability of SFC to 10-, 15-, 18-mer oligonucleotides

First, the applicability of SFC to long oligonucleotides was evaluated by analyzing 5-, 10-, 15-, and 18-mer DNA (Fig. 3) using modifiers containing 2-aminoethanol, which was used in the previous study[1], or octylamine, which is highly hydrophobic (Fig. 4). In case of using 2-aminoethanol, the elution of 5-mer peak was confirmed, but 10-, 15-, and 18-mer peaks were not eluted. When octylamine was used, the 5- and 10-mer peaks were confirmed, but the 15- and 18-mer peaks were not. This suggests that octylamine can analyze longer oligonucleotides. Then sequences modified with 2′-*O*-methoxyethyl (2′-MOE) RNA (Fig. 3) and containing 5-methylcytosine instead of cytosine were subjected to SFC analyses to evaluate the effects of these modifications on retention behaviors and peak shapes. When octylamine was used, 5-, 10-, 15-, and 18-mer peaks were eluted. This suggests that SFC is more applicable to 2′-MOE-modified oligonucleotides than to DNA. In our previous study, it was shown that retention times for oligonucleotides depended on sequence polarity, which is derived from the type of base[1].

However, there was no significant difference in polarity between DNA and 2′-MOE-modified oligonucleotides, and analysis was also performed on modified sequences other than 2′-MOE, but their retention times did not match the polarity of the sequences. This indicates that the retention mechanism for the sugar moiety is different from that of the base moiety. Further studies are required to clarify which sequences can be analyzed with SFC. However, to our knowledge, this is the first time that longer oligonucleotides than 10-mer in length have been analyzed with SFC.

Table 2 Analytical conditions for Fig. 4

System	: Nexera™ UC
Column	: Shim-pack™ UC-Diol II (150 mm × 4.6 mm I.D., 3 µm*1)
Column oven temp.	: 35 °C
Injection vol.	: 1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2 B) 50 mmol/L 2-aminoethanol/octylamine and 50 mmol/L acetic acid in methanol and water (95:5, *v/v*)
B conc.	: 20% (0–2 min), 20–60% (2–20 min), 60% (20–24 min), 60–20% (24–25 min), 20% (25–30 min)
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: MS (Table 1)

*1 Custom-made

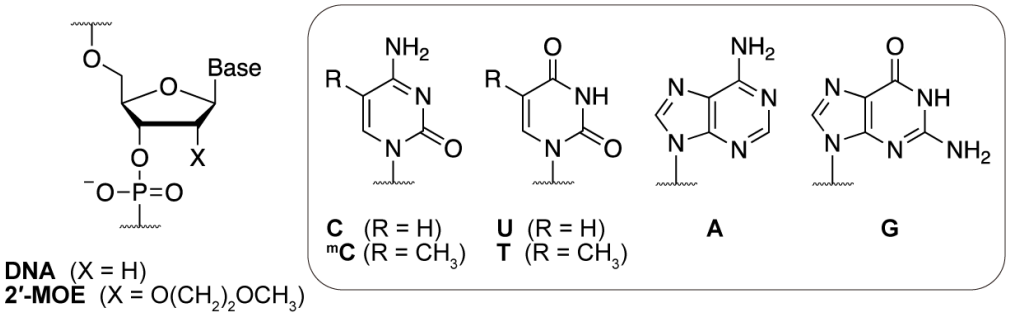
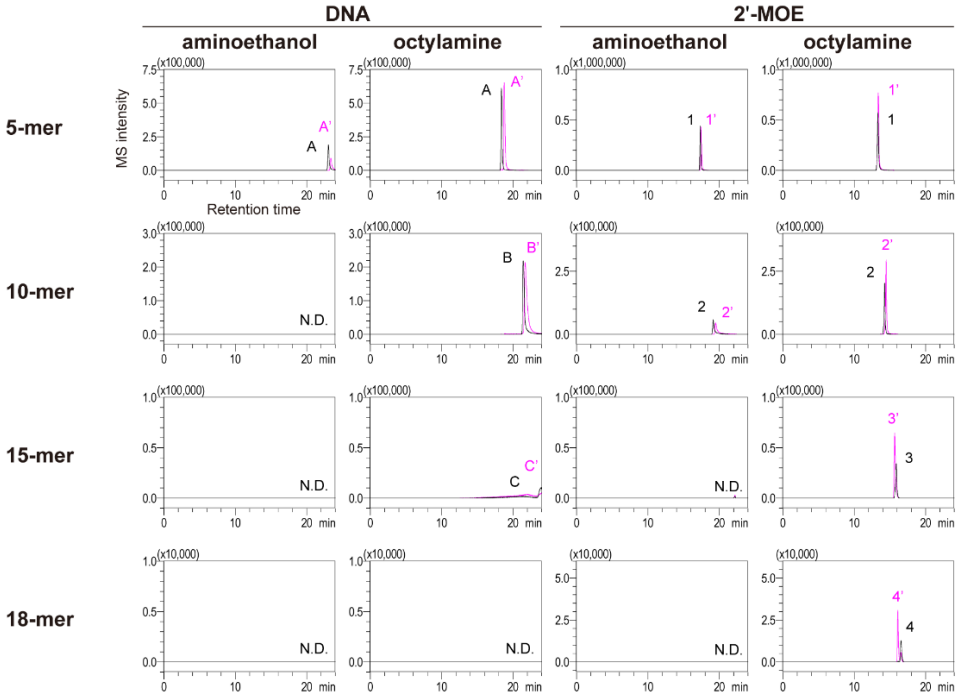


Fig. 3 Structures of DNA and 2′-MOE-modified oligonucleotides



Peak	Sequence (DNA, 5'-3')	*m/z*	Rt (min)
A	DMTr-TCACT	$[M-2H]^{2-}$, 868.70	18.4
A'	TCACT	$[M-2H]^{2-}$, 717.64	18.7
B	DMTr-TCACTTTCAT	$[M-2H]^{2-}$, 1626.32	21.4
B'	TCACTTTCAT	$[M-2H]^{2-}$, 1475.26	21.7
C	DMTr-TCACTTTCATAATGC	$[M-3H]^{3-}$, 1600.30	N.D.
C'	TCACTTTCATAATGC	$[M-3H]^{3-}$, 1499.59	N.D.
D	DMTr-TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1440.51	N.D.
D'	TCACTTTCATAATGCTGG	$[M-4H]^{4-}$, 1364.98	N.D.

Peak	Sequence (5'-3')	*m/z*	Rt (min)
1	DMTr-TCACT	$[M-2H]^{2-}$, 1068.31	13.3
1'	TCACT	$[M-2H]^{2-}$, 916.74	13.3
2	DMTr-TCACTTTCAT	$[M-3H]^{3-}$, 1344.69	14.3
2'	TCACTTTCAT	$[M-3H]^{3-}$, 1243.97	14.5
3	DMTr-TCACTTTCATAATGC	$[M-4H]^{4-}$, 1491.63	15.9
3'	TCACTTTCATAATGC	$[M-4H]^{4-}$, 1416.09	15.7
4	DMTr-TCACTTTCATAATGCTGG	$[M-5H]^{5-}$, 1430.15	16.5
4'	TCACTTTCATAATGCTGG	$[M-5H]^{5-}$, 1369.73	16.1

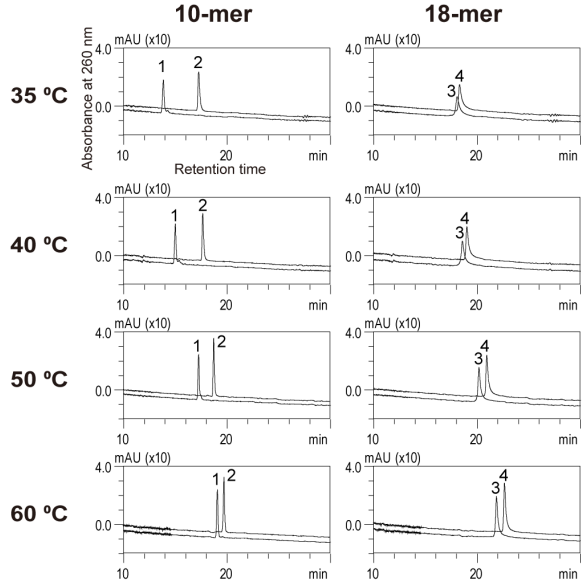
"C" indicates 5-methylcytosine, and the sequences are fully 2′-*O*-MOE modified.

Fig. 4 Extracted ion chromatograms (EICs) of 5-, 10-, 15-, 18-mer DNA and 2′-MOE-modified oligonucleotides

3-2. Optimization of analytical conditions for separating deaminated oligonucleotides from desired oligonucleotides

To evaluate the separation performance of deaminated oligonucleotides with SFC, 2′-MOE-modified oligonucleotides and their deaminated sequences were used to investigate the column oven temperature and the type of modifier additive. For the evaluation, 18-mer, which is the base length used in oligonucleotide therapeutics, and 10-mer, which is about half of the base length used in oligonucleotide therapeutics. Only DMTr-on sequences were used here because DMTr-on and -off sequences showed approximately the same retention times and peak shapes. Column oven temperature was investigated because it was an important factor for improving peak shape in IP-RPLC (Fig. 5). The 10-mer sequence showed good peak shape and complete separation from its deaminated sequence at 35 °C, while the 18-mer sequence showed a broadened peak and was co-eluted with its deaminated sequence. As the temperature increased, the width of the 18-mer peak was decreased, and complete separation from the deaminated sequence was achieved at temperatures above 50 °C. This indicates that the column oven temperature is an important factor in the separation of oligonucleotide and its deaminated sequence. Therefore, 50 °C was employed as the column oven temperature.

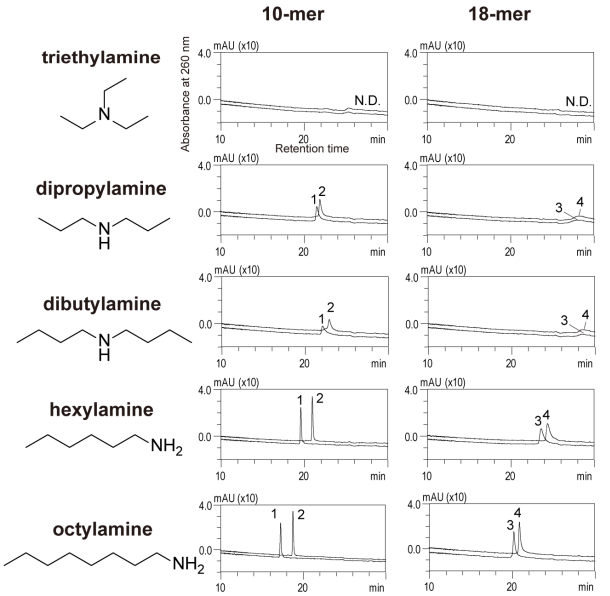
Then optimization of alkyl amines was executed. Since the choice of alkyl amine greatly affected the separation ability in IP-RPLC, same way of investigation was done on SFC (Fig. 6). First, primary, secondary, and tertiary alkylamines in same carbon number (hexylamine, dipropylamine, and triethylamine) were investigated. No peak was detected for triethylamine, a broad peak was detected for dipropylamine, and a sharp peak was detected for hexylamine. Since hexylamine is the most hydrophobic of the three alkyl amines, the hydrophobicity of the alkyl amine was thought to be involved in the peak shape. When dibutylamine, a secondary amine with greater hydrophobicity than hexylamine, was employed, a broad peak was detected. This suggests that long-chain, primary amines would be effective for obtaining good peak shapes. Finally, octylamine was continuously used because it provided the best peak shapes and the highest resolutions among the evaluated alkyl amines.



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2′-*O*-MOE modified.

Fig. 5 Chromatographic separations of target (peak 1 and 3) and deaminated (peak 2 and 4) sequences at column oven temperatures of 35, 40, 50, and 60 °C



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2′-*O*-MOE modified.

Fig. 6 Chromatographic separations of target (peak 1 and 3) and deaminated (peak 2 and 4) sequences using different alkyl amines in modifiers

Table 3 Analytical conditions for Fig. 5

System	: Nexera UC
Column	: Shim-pack UC-Diol II (150 mm × 4.6 mm I.D., 3 µm*1)
Column oven temp.	: 35, 40, 50, and 60 °C
Injection vol.	: 1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2 B) 50 mmol/L octylamine and 50 mmol/L acetic acid in methanol and water (95:5, *v/v*)
B conc.	: 30% (0–5 min), 30–60% (5–30 min), 60% (30–35 min), 30% (36–40 min)
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: PDA 260 nm

*1 Custom-made

Table 4 Analytical conditions for Fig. 6

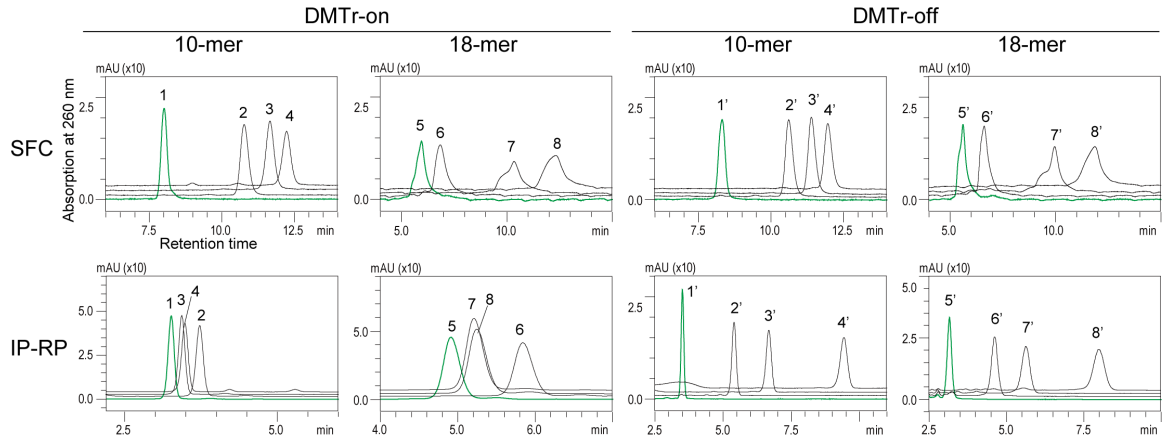
System	: Nexera UC
Column	: Shim-pack UC-Diol II (150 mm × 4.6 mm I.D., 3 µm*1)
Column oven temp.	: 50 °C
Injection vol.	: 1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2 B) 50 mmol/L alkylamine and 50 mmol/L acetic acid in methanol and water (95:5, *v/v*)
B conc.	: 30% (0–5 min), 30–60% (5–30 min), 60% (30–35 min), 30% (36–40 min)
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: PDA 260 nm

*1 Custom-made

3-3. Evaluation for separation behavior of deaminated oligonucleotides

Finally, the separation performances of SFC and IP-RPLC on target and their deaminated sequences were compared. Isocratic elution was employed for both SFC and IP-RPLC because gradient profile did not seem to affect the retentions in both separation modes equally. Propylamine, which has been reported to provide good separation of deaminated oligonucleotides in IP-RPLC, was used as the ion-pair reagent[4]. All sequences were analyzed with modifier ratios set to be eluted within 15 minutes. The SFC separations of sequences with different numbers of deamination from target oligonucleotides were successful for both DMT-on 10-mer and 18-mer sequences (Fig. 7). IP-RPLC also separated the samples in the same way, but SFC showed better separation than that of IR-RPLC.

The target oligonucleotides and their deaminated sequences of both DMTr-off 10- and 18-mer were able to be separated by SFC. It is suggested that the oligonucleotides interacted more strongly with the column than the DMTr group because DMTr-on and DMTr-off sequences were eluted at approximately the same retention time. (Fig. 8). IP-RPLC was also able to separate DMTr-off 10- and 18-mer oligonucleotides and their deaminated sequences and provided better separations than those of SFC. When analyzing DMTr-on sequences with IP-RPLC, the contribution to retention from the hydrophobic DMTr group seemed large, and the contribution to retention from the hydrophilic base seemed small. On the other hand, DMTr-off sequences do not have significantly more hydrophobic functional groups. Consequently, the separation of those sequences based on recognizing the difference of base could be done.



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TTATTTTCAT
4	DMTr-TTATTTTTAT
5	DMTr-TCACTTTCATAATGCTGG
6	DMTr-TTACTTTCATAATGCTGG
7	DMTr-TTATTTTCATAATGCTGG
8	DMTr-TTATTTTTATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Peak	Sequence (5'-3')
1'	TCACTTTCAT
2'	TTACTTTCAT
3'	TTATTTTCAT
4'	TTATTTTTAT
5'	TCACTTTCATAATGCTGG
6'	TTACTTTCATAATGCTGG
7'	TTATTTTCATAATGCTGG
8'	TTATTTTTATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Fig. 7 Chromatographic separations of target and deaminated sequences using SFC and IP-RPLC
Target (peak 1, 5, 1', and 5', green lines) and corresponding deaminated (peak 2-4, 6-8, 2'-4', and 6'-8', black lines) sequences

Table 5 Analytical conditions of SFC for Fig. 7 and 9

System	: Nexera UC
Column	: Shim-pack UC-Diol II (150 mm × 4.6 mm I.D., 3 µm*1)
Column oven temp.	: 50 °C
Injection vol.	: 1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) CO_2 B) 50 mmol/L octylamine and 50 mmol/L acetic acid in methanol and water (95:5, *v/v*)
B conc.	: 40% (DMTr-on and -off 10-mer), 45% (DMTr-on and -off 18-mer)
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: PDA 260 nm

*1 Custom-made

Table 6 Analytical conditions of IP-RPLC for Fig. 7 and 9

System	: Nexera XS
Column	: Shim-pack Scepter C18-120 (150 mm × 4.6 mm I.D., 3 µm*1)
Column oven temp.	: 50 °C
Injection vol.	: 1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, *v/v*)
Mobile phases	: A) 10 mmol/L propylamine (pH 9.6, adjusted with acetic acid) in water B) acetonitrile
B conc.	: 30% (0–5 min), 30–60% (5–30 min), 60% (30–35 min), 30% (36–40 min)
Flow rate	: 1.0 mL/min
Back pressure	: 10 MPa, 50 °C
Detection	: PDA 260 nm

*1 P/N 227-31016-05

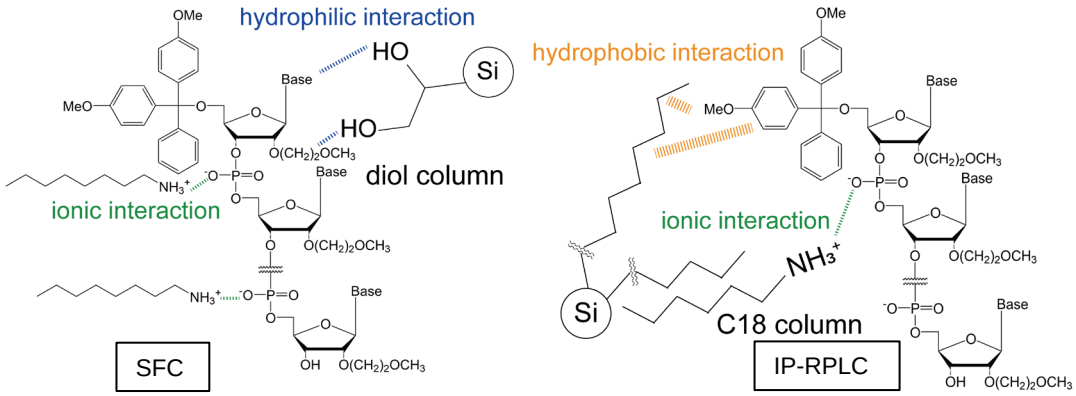


Fig. 8 Estimated retention mechanisms for SFC and IP-RPLC

Then, the separations of target oligonucleotides and sequences with different positions of deamination were investigated (Fig. 9). In the case of DMTr-on 10mer and -off 10-mer, the target sequence was able to be separated from deaminated sequences. In the case of IP-RPLC, some deaminated sequences were separated from DMTr-on 10-mer, but sequence d was co-eluted. IP-RPLC showed excellent separation of both DMTr-off 10-mer and 18-mer sequences. These results suggest that SFC and IP-RPLC showed different retention behaviors in oligonucleotide analysis.

4. Conclusions

In this study, the feasibility of analyzing of 5-, 10-, 15-, and 18-mer of DMTr-on and -off sequences by SFC was evaluated and it was found that 2'-MOE-modified sequences was able to be analyzed by using a modifier containing octylamine. Further studies are required to apply this method to all sequences used in oligonucleotide therapeutics. For example, it also confirmed that the phosphorothioate (PS)-modified sequences, a typical modification for oligonucleotide therapeutics, resulted in a heavy broadening of the peak shape due to incomplete separation of the diastereomers in SFC[5]. Since the desired separation may not be obtained due to the number of PS modifications in the sequence, optimization of the analytical conditions is essential.

Then, the feasibility of analyzing DMTr-on 10- and 18-mer, DMTr-off 10- and 18-mer, and their deaminated sequences by SFC was evaluated, and the results were compared to those from existing IP-RPLC analyses. The SFC provided almost same retention time for the target sequences regardless of the presence or absence of DMTr, and their deaminated sequences were separated from respective target sequences. In IP-RPLC, the target and the deaminated sequences were co-eluted in some DMTr-on sequences, while in DMTr-off sequences, the target and the deaminated sequences were well separated. These results suggest that the DMTr-on sequence may be applicable to the separation of impurity sequences with highly hydrophobic organ-targeting ligands as well as DMTr groups.

5. Acknowledgments

We would like to thank Prof. Satoshi Obika and Prof. Takao Yamaguchi (Graduate School of Pharmaceutical Sciences, Osaka University) for their guidance.

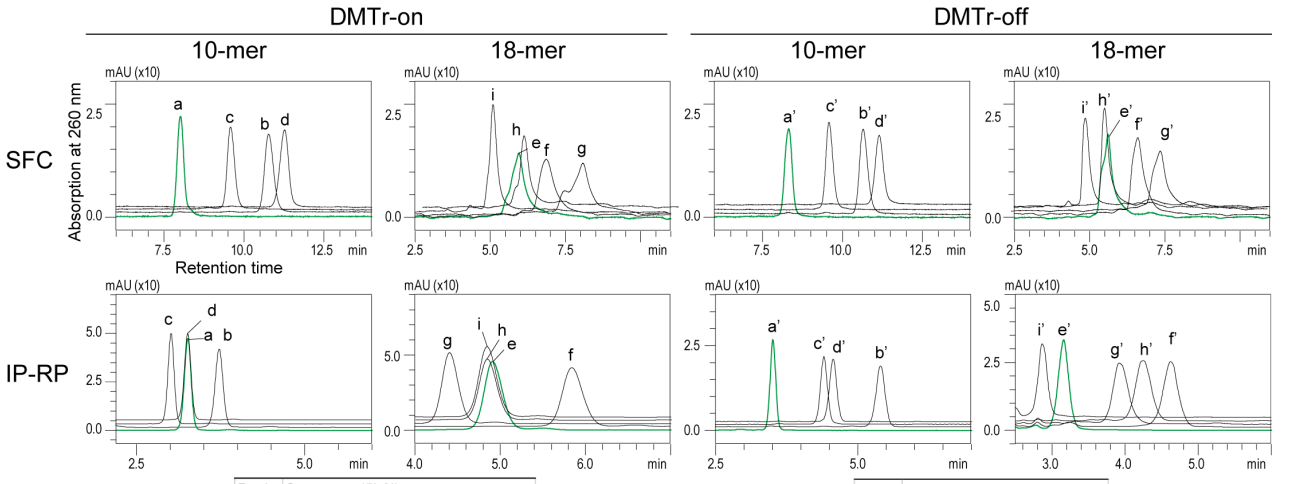
This research was partially supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

<References>

1) M. Hayashida *et al.*, *J. Chromatogr. A*, **2023**, 1708, 464333, DOI: 10.1016/j.chroma.2023.464333
2) M. Hayashida *et al.*, *J. Chromatogr. A*, **2025**, 1744, 465731, DIO: 10.1016/j.chroma.2025.465731
3) J. Molineau *et al.*, *J. Pharm. Biomed. Anal.*, **2021**, 193, 113736, DOI: 10.1016/j.jpba.2020.113736
4) S. G. Roussis *et al.*, *J. Chromatogr. A*, **2019**, 1594, 105-111 DOI: 10.1016/j.chroma.2019.02.026
5) M. Hayashida, **2024**, doctoral dissertation of Graduate School of Pharmaceutical Sciences, Osaka University, URL: https://hdl.handle.net/11094/96135

<Related Applications>

Short-Chain Oligonucleotide Analysis Using Supercritical Fluid Chromatography, Application Note No.100



Peak	Sequence (5'-3')
a	DMTr-TCACTTTCAT
b	DMTr-TTACTTTCAT
c	DMTr-TCATTTTCAT
d	DMTr-TCACTTTTAT
e	DMTr-TCACTTTCATAATGCTGG
f	DMTr-TTACTTTCATAATGCTGG
g	DMTr-TCATTTTCATAATGCTGG
h	DMTr-TCACTTTTATAATGCTGG
i	DMTr-TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Peak	Sequence (5'-3')
a'	TCACTTTCAT
b'	TTACTTTCAT
c'	TCATTTTCAT
d'	TCACTTTTAT
e'	TCACTTTCATAATGCTGG
f'	TTACTTTCATAATGCTGG
g'	TCATTTTCATAATGCTGG
h'	TCACTTTTATAATGCTGG
i	TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Fig. 9 Chromatographic separations of target and deaminated sequences using SFC and IP-RPLC
Target (peak a, e, a', and e', green lines) and corresponding deaminated (peak b-d, f-i, b'-d', and f'-i', black lines) sequences

First Edition: May 2025

Shimadzu Corporation
www.shimadzu.com/an/

Please fill out the survey

Related Products Some products may be updated to newer models.

Nexera UC

Supercritical Fluid Extraction / Supercritical Flu···

Related Solutions

Pharmaceutical and Biopharmaceutical

Oligonucleotide and mRNA Therapeutics

Price Inquiry

Product Inquiry

Technical Service / Support Inquiry

Other Inquiry