Optimization of Separation Conditions for the Analysis of Unmodified and Phosphorothioate-modified Oligonucleotides Using Various Ion-pairing Reagents

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1. Introduction

Ion-pair reversed-phase HPLC (IP-RP-HPLC) is widely used for the separation and analysis of therapeutic oligonucleotides. However, several analytical challenges remain, including the following:

- Adsorption of oligonucleotides to metal surfaces due to their phosphate backbone.
- MS signal suppression caused by ion-pairing reagents.
- Complex impurity profiles resulting from phosphorothioate (PS) modification.

In this study, an LC-MS/MS based analytical method was developed to evaluate the effects of various ion-pairing reagents and chromatographic stationary phases on the analysis of oligonucleotides. The retention behavior, peak shape, and MS detection sensitivity of both unmodified and phosphorothioate (PS)-modified oligonucleotides were systematically investigated. The target of this study was to establish optimized analytical conditions that minimize nonspecific adsorption to metal surfaces while maximizing MS sensitivity.

2. Material & Methods

2-1. Investigation of Hardware Adsorption Suppression

Oligonucleotides are prone to nonspecific adsorption to metal surfaces in LC systems due to their structural characteristics, resulting in reduced analytical reproducibility. Consecutive analyses were performed with and without metal-masking reagents to evaluate adsorption. The persistence of the masking effect was further investigated using acetylacetone (ACAC) and trifluoroacetylacetone (TFACAC) at concentrations of 0, 0.05, and 0.1%.

Analysis conditions

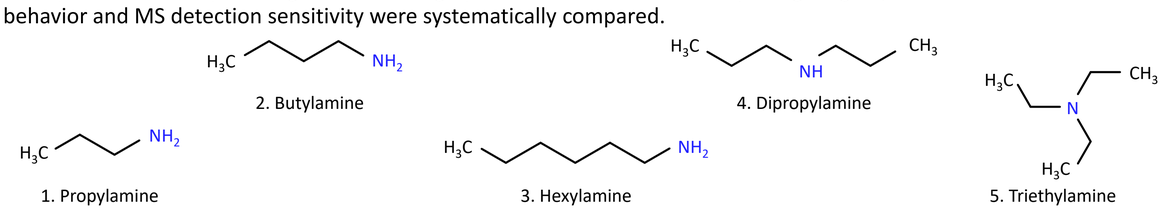
System： QTRAP 6500 + ExionLC System (SCIEX)
Column： Inertsil Hybrid-C18 (1.7 µm, 2.1 mm I.D. x 50 mm)
Mobile phase ： A) 100 mM HFIP + 5 mM TEA + 10% CH_3OH aq.
B) (0, 0.05, 0.1 %) acetylacetone in 90% CH_3OH aq.
A/B = 98/2 - 0.5 min - 98/2 - 5.5 min - 15/75 - 0.5 min - 98/2 - 1.5 min
Flow Rate ： 0.3 mL/min
Temp. ： 60 ℃

Oligonucleotides used (RNA or RNA All PS)

1. 5’-CATGACGTTCCTGATGCT-3’ (18-mer PO, m/z = 606.21/95.0)
2. 5’-CCATGACGTTCCTGATGCT-3’ (19-mer PO, m/z = 574.42/95.0)
3. 5’-TCCATGACGTTCCTGATGCT-3’ (20-mer PO, m/z = 604.81/95.0)
4. 5’-C^A^T^G^A^C^G^T^T^C^C^T^G^A^T^G^C^T-3’ (18-mer PS, m/z = 636.51/95.0)
5. 5’-C^C^A^T^G^A^C^G^T^T^C^C^T^G^A^T^G^C^T-3’ (19-mer PS, m/z = 670.46/95.0)
6. 5’-T^C^C^A^T^G^A^C^G^T^T^C^C^T^G^A^T^G^C^T-3’ (20-mer PS, m/z = 706.00/95.0) (^=Phosphorothioated)

2-2. Effect of Ion-Pairing Reagent (Alkylamine) Structure on Chromatographic Separation

Alkylamines are widely used as ion-pairing reagents for the reversed-phase LC analysis of oligonucleotides. In this study, five ion-pairing reagents with either linear or branched alkylamine structures were evaluated to investigate the effects of alkylamine structure and alkyl chain length on chromatographic separation and MS detection sensitivity. The linear alkylamines included propylamine, butylamine, and hexylamine, while the branched alkylamines included dipropylamine and triethylamine. After optimizing the chromatographic conditions for each ion-pairing reagent, their effects on retention behavior and MS detection sensitivity were systematically compared.



2-3. Evaluation of the Limit of Quantification (LOQ)

Based on the results of 2-1 and 2-2, an optimized LC–MS method was developed using the optimal metal-masking reagent and ion-pairing reagent. The limit of quantification (LOQ) was subsequently evaluated under the optimized conditions.

Analysis conditions

System： QTRAP 6500 + ExionLC System (SCIEX)
Column ： Inertsil Hybrid-C18 (1.7 µm, 2.1 mm I.D. × 50 mm)
Mobile phase： A) 100 mM HFIP + 5 mM TEA + 10% CH_3OH aq.
B) 0.05 % acetylacetone in 90% CH_3OH aq.
A/B = 98/2 - 0.5 min - 98/2 - 5.5 min - 15/75 - 0.5 min - 98/2 - 1.5 min
Flow Rate： 0.3 mL/min
Temp.： 60 ℃
Sample： All at 0.5 nM except 19-mer at 2 nM Oligo mix (in 5 mM Triethylamine/Methanol= 90/10)

3. Results & Discussion

3-1. Investigation of Hardware Adsorption Suppression

3-1-1. Behavior in the Absence of a Masking Agent

Eighty consecutive injections were performed without a metal-masking reagent, and the peak area was monitored over time (Figure 1). The chromatogram from the 10th injection is shown in Figure 2. In the absence of a metal-masking reagent, peak are as gradually increased with repeated injections due to progressive passivation of metal surfaces by adsorbed oligonucleotides. This resulted in poor quantitative stability and reduced analytical reproducibility.

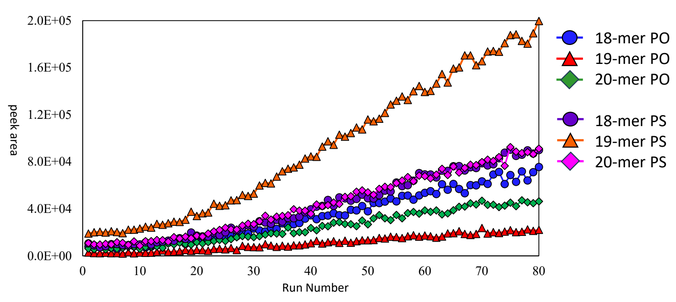


Fig. 1. Time course of peak area under metal-masking reagent-free conditions

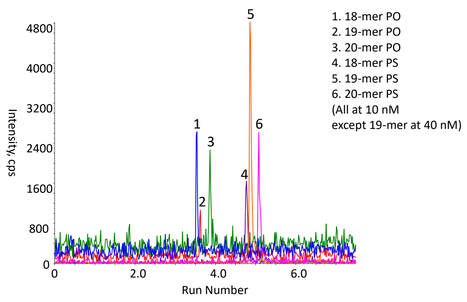


Fig. 2. Chromatogram obtained from the 10th injection under metal-masking reagent-free conditions.

3-1-2. Evaluation of ACAC Concentration and Persistence of Matal-masking Effect

ACAC was added to the mobile phase at 0.05 and 0.1%, and the stability of the peak area was evaluated over 60 consecutive injections (Figure 3).The addition of 0.05% ACAC markedly reduced peak area variability, demonstrating improved analytical stability.

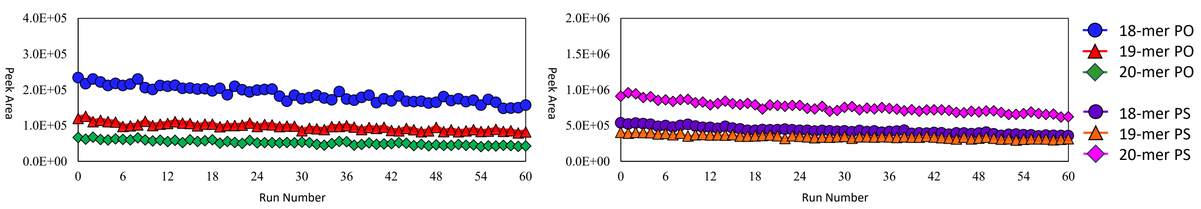


Fig. 3. Stability of peak area at 0.05% ACAC (left: PO form, right: PS form).

3-1-3. Comparison of Metal-masking Reagents

The performance of ACAC and TFACAC was compared using mobile phases containing 0.05% of each reagent (Figure 4). TFACAC perfromend significantly lower MS detection sensitivity than ACAC.

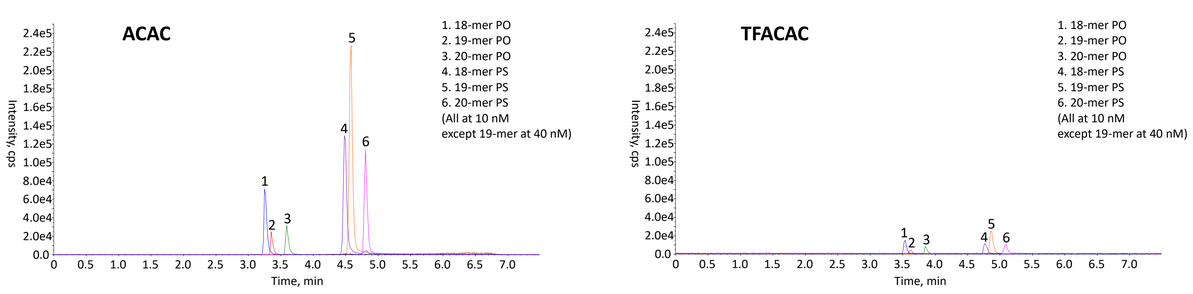


Fig. 4. Comparison of chromatograms under ACAC and TFACAC conditions

3-2. Investigation of the Effect of Ion-Pair Reagent (Alkylamine) Structure on Separation Behavior

3-2-1. Effect of Linear and Branched Alkylamines as Ion-Pairing Reagents on Separation

Different alkylamines were evaluated using the gradient conditions established for triethylamine, and the results are shown in Figure 5. Oligonucleotide retention increased with increasing alkylamine hydrophobicity (LogP), indicating that more hydrophobic alkylamines performed stronger retention.

Analysis conditions

Column ： Inertsil Hybrid-C18 (1.7 µm, 2.1 mm I.D. × 50 mm)
Mobile phase ： A) 100 mM HFIP + each 5 mM Alkylamine in 10% CH_3OH aq.
B) 0.05 % acetylacetone in 90% CH_3OH aq.
A/B = 98/2 - 0.5 min - 98/2 - 5.5 min - 15/75 - 0.5 min - 2/98 - 1.5 min
Flow Rate ： 0.3 mL/min
Temp. ： 60 ℃

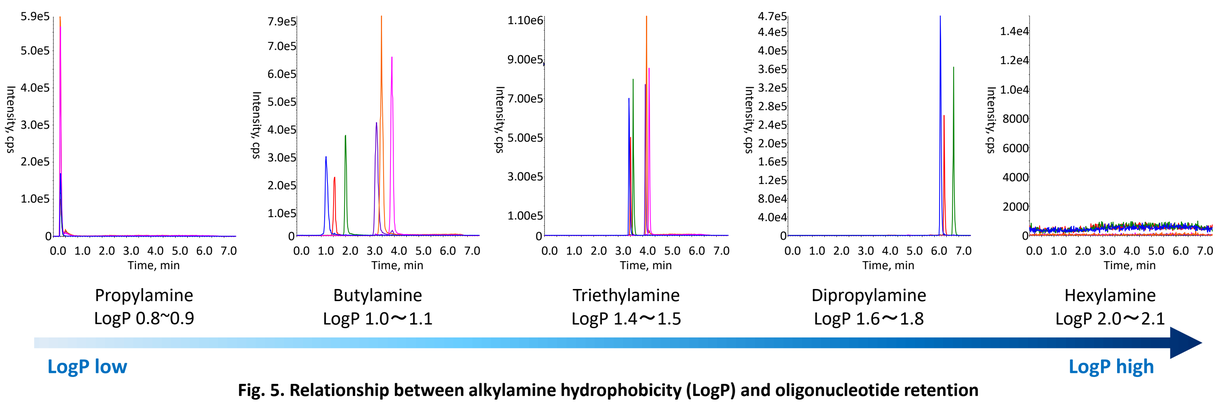


Fig. 5. Relationship between alkylamine hydrophobicity (LogP) and oligonucleotide retention

3-2-2. Evaluation of Detection Sensitivity Using Alkylamines with the Same Molecular Formula

To evaluate the effect of alkylamine structure on the MS detection sensitivity of oligonucleotides, alkylamines with the same molecular formula were compared under chromatographic conditions that ensured complete elution of all oligonucleotides (Figure 6). Among the phosphorothioate (PS)-modified oligonucleotides, the highest peak intensity was obtained with triethylamine, followed by dipropylamine and hexylamine.

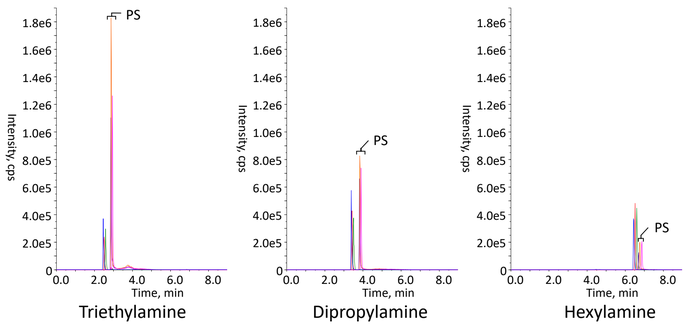


Fig. 6. Comparison of detection sensitivity for alkylamines with the same molecular formula

3-2-3. Evaluation of Chromatographic Separation and Detection Sensitivity Under Optimized Conditions Using Alkylamines with Same Molecular Formula

Alkylamines with the same molecular formula were evaluated under the conditions optimized to achieve the best separation for each reagent. The results are shown in Figure 7. Separation resolution of oligonucleotides tended to improve with increasing alkylamine hydrophobicity (LogP). However, triethylamine consistently provided the highest MS peak intensity, even under the optimized conditions. These results suggest that the structure of the alkylamine plays an important role in determining both separation performance and MS detection sensitivity.

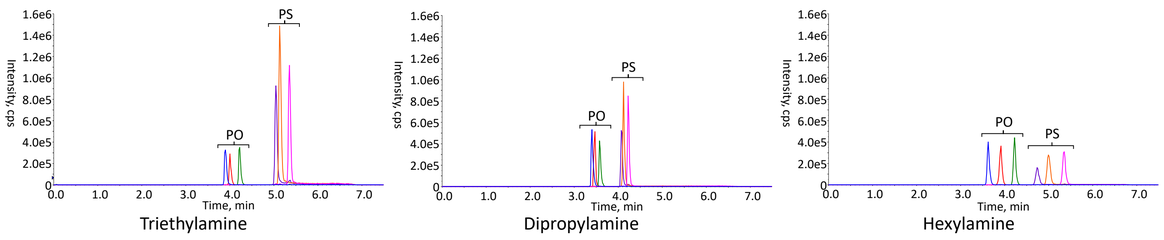


Fig. 7. Comparison of chromatograms obtained under the optimized conditions for each alkylamine.

Mobile phase :
A) 100 mM HFIP + each 5 mM alkylamine in 10% CH_3OH aq.
B) 0.05 % acetylacetone in 90% CH_3OH aq.

a) A/B = 98/2 - 0.5 min - 98/2 - 5.5 min - 15/75 - 0.5 min - 98/2 - 1.5 min – 98/2
b) A/B = 95/5 - 0.5 min - 95/5 - 5.0 min - 65/35 - 0.5 min - 95/5 - 1.5 min – 95/5
c) A/B = 65/35 - 0.5 min - 65/35 - 5.0 min - 50/50 - 0.5 min - 65/35 - 1.5 min – 65/35

3-3. Evaluation of the Limit of Quantification (LOQ)

Figure 8 shows the chromatograms of PO- and PS-modified oligonucleotides obtained under the optimized analytical conditions. All oligonucleotides were analyzed at 0.5 nM, except for the 19-mer PS oligonucleotide (2 nM). The LOQ was determined from seven replicate measurements according to USP <621> using the S/N ratio.

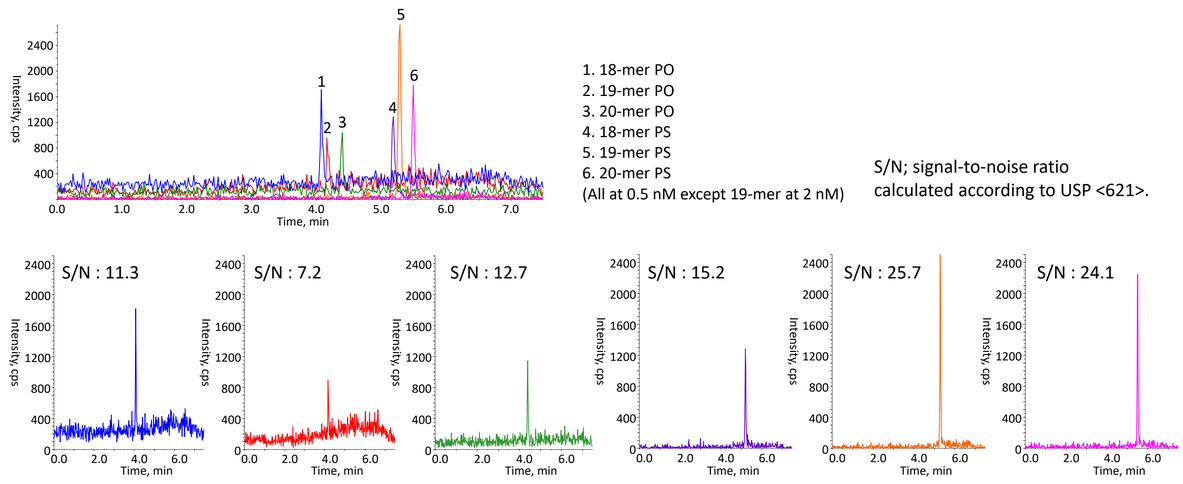


Fig. 8. LOQ evaluation of PO/PS oligonucleotides under optimized conditions
(top) Full chromatogram, (bottom) extracted ion chromatograms (EICs)

4. Conclusions

The effects of metal-masking reagents and ion-pairing reagents on oligonucleotide adsorption and MS detection sensitivity were investigated using a stainless (SS) column hardware.

The addition of 0.05% ACAC to the mobile phase provided stable peak areas, demonstrating effective suppression of nonspecific adsorption. However, TFACAC resulted in reduced MS detection sensitivity. This decrease was likely due to ionization suppression in ESI-MS caused by the increased fluorine-containing components derived from both TFACAC and hexafluoroisopropanol (HFIP) in the mobile phase.

Among the alkylamine ion-pairing reagents, oligonucleotide retention increased with increasing alkylamine hydrophobicity (LogP), indicating that hydrophobic interactions play a major role on retentivity. Furthermore, triethylamine provided the highest MS response for phosphorothioate (PS)-modified oligonucleotides among the alkylamines evaluated. This enhanced sensitivity is likely attributable to the higher volatility of triethylamine compared with the other alkylamines.

5. Future Work

- Evaluation of diethylmethylamine (DEMA-) and dimethylethylamine (DMEA-) based mobile phase conditions
- Extension to low-pM oligonucleotide analysis
- Development a HILIC-based analytical method
- Application to real samples