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Application News

High Performance Liquid Chromatography / Nexera™ XS inert

Exploration and Optimization of Efficient Separation Conditions for Oligonucleotides by Reversed-Phase Ion-Pair Chromatography

Chihiro Hosoi, Emiko Ando

User Benefits

- LabSolutions MD significantly reduces the manual effort associated with HPLC method development, including mobile phase screening and the investigation of various chromatographic parameters.
- The integrated AI algorithm facilitates the optimization of gradient profiles, independent of the user's level of expertise.
- LabSolutions MD is particularly effective in refining separation conditions for oligonucleotide samples containing impurities, such as species with varying chain lengths.

■ Introduction

Nucleic acid drugs such as antisense oligonucleotides are mainly manufactured by chemical synthesis. Reversed-phase ion-pair chromatography (RP-IP) is used to separate and purify these compounds with HPLC. However, the separation pattern varies depending on the type and concentration of the organic solvent and ion-pair reagent used in the mobile phase, and the behavior of the variation depends on the base composition and chain length in the sequence, as well as the presence or absence of modified nucleic acids.

In this article, the separation conditions in PR-IP for a simulated sample containing different oligonucleotides assumed as a target sequence and related impurities of different chain length were optimized. Efficient method screening was performed in the three steps of "initial screening of mobile phase," "optimization of pH and oven temperature of mobile phase," and "gradient optimization" using LabSolutions MD, an analytical method development supporting software.

■ Samples to be analyzed

Oligonucleotides with contiguous thymine bases (dT(x), where x is the number of bases) were used for analysis, and eleven sequences with different chain lengths (dT(6), (10), (15)-(20), (25), (30), (40)) were prepared. All are unmodified single-stranded DNA and were chemically synthesized (HPLC purified). Oligonucleotide mixture samples were prepared by dissolving each oligonucleotide in water to make 5 µmol/L.

■ Initial Mobile Phase Screening

Ion-pair reagents added to the mobile phase, their concentrations, and organic solvents used for the mobile phase were investigated, followed by optimization of the separation conditions. Triethylamine (TEA), dibutylamine (DBA), and hexylamine (HA) were used as ion-pair reagents in acetic acid aqueous solutions for pH adjustment. Acetonitrile and methanol were used as organic solvents. Since high concentrations of DBA and HA were hardly dissolve in water at room temperature, they were added to water, allowed to stand overnight with continuous stirring, and then dissolved with adding acetic acid aqueous solution.

The simulated sample was a mixture of seven sequences (dT(6), (10), (15), (20), (25), (30), and (40)), and a total of eighteen combinations of ion-pair reagents, their concentrations, and organic solvents were investigated under the analytical conditions shown in Table 1. When TEA was used as the ion-pair reagent, multiple peaks overlapped in all combinations with organic solvents (Fig. 1). On the other hand, when DBA and HA were used, the separations were improved, although both separations tended to be improved with the increases of their concentrations. In preparation process, HA seemed difficult to be dissolve at higher concentrations.

Therefore, from here, 50 mmol/L of DBA and HA as ion-pair reagents and both acetonitrile and methanol as organic solvents were employed for further investigation.

Table 1 Analytical conditions

System	:	Nexera XS inert
Column	:	Shim-pack Scepter™ Claris C18-120 (100 mm × 2.1 mm I.D., 3 µm) *1
Mobile phase A	:	(1)20/50/100 mmol/L TEA acetic acid aqueous solution pH6.5 (2)5/20/50 mmol/L DBA acetic acid aqueous solution pH6.5 (3)20/50/100 mmol/L HA acetic acid aqueous solution pH6.5
Mobile phase B	:	Acetonitrile or Methanol
Flow rate	:	1.0 mL/min
B Conc.	:	(1)TEA : 10-50%(0-8 min)→100%(8.01-11 min) →10%(11.01-15 min) (2)DBA : 20-70%(0-8 min)→100%(8.01-11 min) →20%(11.01-15 min) (3)HA : 20-80%(0-8 min)→100%(8.01-11 min) →20%(11.01-15 min)
Column temp.	:	40 °C
Flow rate	:	0.35 mL/min
Injection volume	:	5 µL
Detection	:	UV 260 nm (SPD-M40, UHPLC inert cell)
Vial	:	Shim-vial H glass*2

*1 P/N : 227-31210-05, *2 P/N : 227-34500-02(Shimadzu GLC)

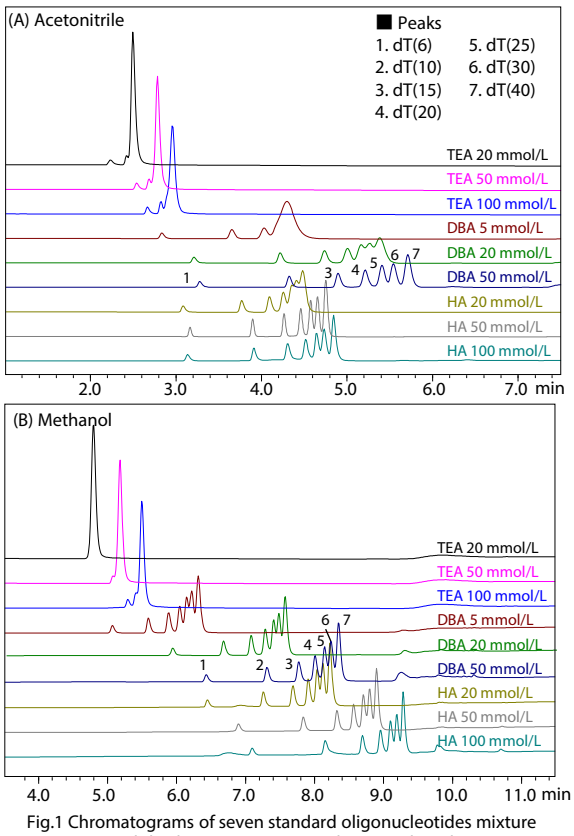

Fig.1 Chromatograms of seven standard oligonucleotides mixture
Mobile Phase B : (A)Acetonitrile, (B)Methanol

■ Optimization of oven temperature and mobile phase pH

Further investigation for analytical conditions were continued using 50 mmol/L DBA and 50 mmol/L HA as mobile phase A and acetonitrile and methanol as mobile phase B. The gradient profile was fixed to the conditions in (2) of Table 1, the column oven temperature was varied to 40, 50, 60, 70, and 80 ℃, and the pH of mobile phase A was varied to 6.0, 6.5, 7.0, and 7.5. Analytical batch was created and analyzed to cover each combination. Since the retention time of each sequence varied greatly due to changes in these parameters and differences in organic solvents, each peak was identified using the peak tracking function of LabSolutions MD. As UV spectra are not suitable for identification because of too small spectrometric differences in nucleic acid sequences, we used the elution order as the information for identification.

A design space was created with the pH of mobile phase A on the vertical axis and the column oven temperature on the horizontal axis to visualize the regions where the minimum resolution was 1.5 or higher (1.2 or higher only for the combination of HA and methanol) (Fig. 2). The black dots in Fig. 2 indicate actual measurement points, the red and blue regions indicate areas of high and low resolution, respectively. It was suggested that the optimal pH of the mobile phase varied depending on the type of organic solvent. As for the column oven temperature, the higher temperature provided the higher resolution, regardless of the combination of ion-pair reagent and organic solvent.

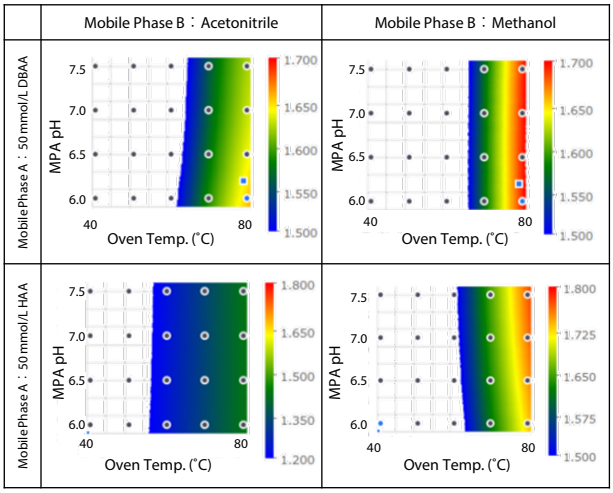

Fig.2 Design space for minimum resolution of seven standard oligonucleotides mixture

■ Automatic optimization of gradient profile

The workflow for automatic optimization of gradient profile in LabSolutions MD is shown in Fig. 3. LabSolutions MD is equipped with a unique AI algorithm that automatically searches for conditions that meet separation requirements by repeating "gradient profile improvement by AI (condition search)" and "analysis under improved conditions (correction analysis).

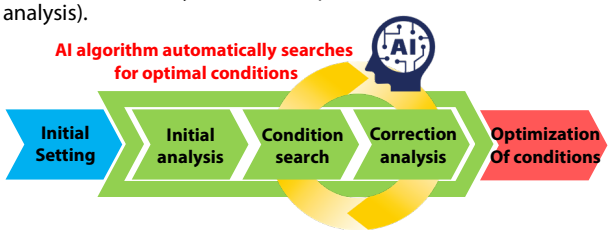

Fig.3 Workflow for automatic optimization of gradient profile using LabSolutions MD

Optimal gradient profile for a mixture of eleven nucleotide sequences (dT(6), (10) - (15), (20), (25), (30), (40)) containing a single base difference in chain length was automatically searched for under the conditions of minimum separation of 1.5. Mobile phase B was methanol, and the column oven temperature was fixed at 80 ℃. The AI algorithm repeated the correction analyses using two mobile phase A conditions (50 mmol/L DBA pH 7.5 and 50 mmol/L HA pH 7.5), and finally gradient profiles that met the minimum resolution of 1.5 was automatically searched for in each mobile phase condition. Chromatograms under the optimized conditions for respective ion-pair reagent are shown in Fig. 4.

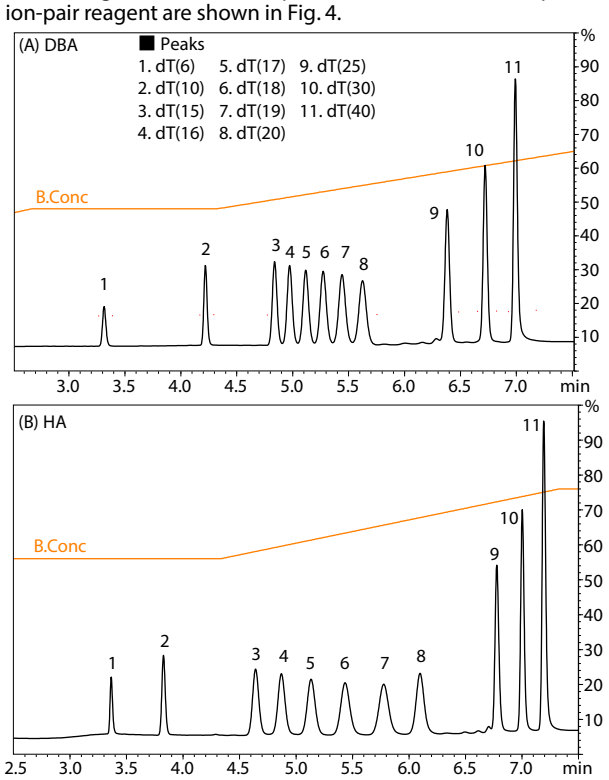

Fig.4 Chromatograms of eleven oligonucleotides mixture under auto-searched conditions
Mobile Phase A : (A)50 mmol/L DBA pH7.5, (B)50 mmol/L HA pH7.5

■ Conclusion

Using a standardized mixture comprising eleven oligonucleotide sequences of varying chain lengths as a model sample, separation conditions were systematically investigated via reversed-phase ion-pair chromatography. LabSolutions MD facilitated a comprehensive evaluation of key chromatographic parameters, including ion-pair reagent selection, mobile phase pH, and column oven temperature. The platform enabled efficient visualization of experimental outcomes, thereby streamlining the exploration of optimal conditions.

Furthermore, LabSolutions MD automatically optimized gradient profiles to achieve a minimum resolution of 1.5 within a few hours, resulting in substantial reductions in manual workload.

<Related application>

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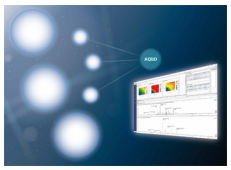

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