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Application News

Software for Efficient Method Development
Ultra High Performance Liquid Chromatograph

Improvement of Oligonucleotide Peak Shape Using Automatic Pretreatment Function (Co-injection)

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User Benefits

- Sample dilution can be automated using the automatic pretreatment function "co-injection" for purified fractions of oligonucleotides containing high concentrations of salts.
- The automatic pretreatment function "co-injection" enables the simultaneous injection of a sample and a selected co-injection solvent. The co-injection solvent and its injection volume can be easily set via LabSolutions™ MD interface.

■ Introduction

In HPLC analysis, the composition of the sample solvent is critical for achieving proper peak shape. If the sample solvent is a stronger eluting solvent than the mobile phase, sample band condensation at the column inlet may be insufficient, leading to sample band broadening. For instance, increasing the organic solvent ratio in the sample solvent to dissolve low-polar compounds may deteriorate the peak shapes of early-eluting compounds in reversed-phase chromatography. Oligonucleotides are synthesized using the phosphoramidite method and subsequently purified by anion exchange chromatography or reversed-phase ion-pair chromatography (RP-IP). Purified fractions from anion exchange chromatography often contain high concentrations of salts, such as sodium chloride (NaCl) and sodium bromide (NaBr). When these purified fractions are analyzed using RP-IP, the high salt concentration in the sample solvent may interfere with the ion-pair formation capacity of the oligonucleotides. This interference can result in improper peak retention and adversely affect the chromatogram. This article introduces a case study demonstrating an improvement in oligonucleotide peak shape by suppressing the sample solvent effect through simultaneous injection with a desired solvent. This automatic pretreatment function "co-injection" is a standard feature included in the autosamplers of Nexera™ series. Furthermore, by utilizing LabSolutions MD, a dedicated software for supporting method development, comprehensive evaluation of co-injection solvent types and co-injection volumes was efficiently performed.

■ "Co-injection" Function

The co-injection function, equipped in autosamplers, provides automatic pretreatment. A specified amount of reagent or solvent from any vial can be mixed with the sample solution in the needle and injected (Fig. 1).

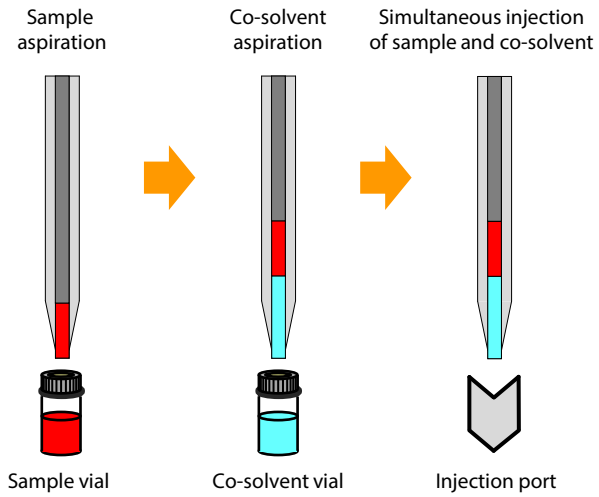

Fig. 1 Flow Diagram of Co-injection Sequence

■ Analytical Conditions

The analytical conditions are shown in Table 1. Two types of salts, 3 mol/L NaCl and 3 mol/L NaBr, were added to the sample solvent to simulate purified fractions containing high concentrations of salts. To assess the effects of co-solvents and co-injection volumes on peak shape, four different co-solvents were used : water and three solutions containing HA (hexylamine) at concentrations of 10, 30, and 50 mmol/L, each prepared in 100 mmol/L HFIP (1,1,1,3,3,3-hexafluoro-2-propanol). Additionally, three injection volumes (1, 3, and 5 µL) were evaluated. By simply entering the vial numbers and injection volumes of the co-injection solvents via the LabSolutions MD (red frame in Fig. 2), the autosampler automatically adjusts the co-injection solvent types and volumes during analysis. This enables automated comprehensive evaluation of conditions, greatly reducing manual labor. The target sample was one of the models of oligonucleotide therapeutics synthesized in the AMED project*1.

*1 Grant Number : JP21ae0121022, JP21ae0121023, JP21ae0121024

Table 1 Analytical Conditions

System	: Nexera XS inert (Method Scouting System)
Column	: Accura Triart Bio C18*2 (100 mm × 2.1 mm I.D., 1.9 µm)
Sample solvent 1	: 20 mM Tris-HCl (pH 8.5) containing 3 mol/L NaCl
Sample solvent 2	: 20 mM Tris-HCl (pH 8.5) containing 3 mol/L NaBr
Co-solvent 1	: Water
Co-solvent 2	: 100 mmol/L HFIP*3 and 10 mmol/L HA*4 in water
Co-solvent 3	: 100 mmol/L HFIP and 30 mmol/L HA in water
Co-solvent 4	: 100 mmol/L HFIP and 50 mmol/L HA in water
Co-injection volumes	: 1, 3, 5 µL
Mobile phases	
Pump A	: 100 mmol/L HFIP and 10 mmol/L HA in water
Pump B	: 100 mmol/L HFIP and 10 mmol/L HA in Methanol
Temperature	: 60 °C
Injection volume	: 1 µL
Flow rate	: 0.2 mL/min
Time program (%B)	: 25 % (0-2 min) →67 % (26 min)→ 100 % (26-27 min) →25 % (27-35 min)
Detection	: 260 nm (SPD-M40, UHPLC inert cell)

*2 YMC.CO., LTD.
*3 1,1,1,3,3,3-hexafluoro-2-propanol
*4 Hexylamine

			Pretreatment Program	
Use	Sample Name	Vial	co-solvents vial	co-injection volume(µL)
☑	Oligonucleotide 1	1	2	1
☑	Oligonucleotide 2	1	2	3
☑	Oligonucleotide 3	1	2	5
☑	Oligonucleotide 4	1	3	1
☑	Oligonucleotide 5	1	3	3
☑	Oligonucleotide 6	1	3	5
☑	Oligonucleotide 7	1	4	1
☑	Oligonucleotide 8	1	4	3
☑	Oligonucleotide 9	1	4	5
☑	Oligonucleotide 10	1	5	1
☑	Oligonucleotide 11	1	5	3
☑	Oligonucleotide 12	1	5	5

Fig. 2 Setting Screen of co-injection (LabSolutions MD)
Vial 2 : Co-solvent 1、 Vial 3 : Co-solvent 2 、 Vial 4 : Co-solvent 3 、 Vial 5 : Co-solvent 4

■ Improved Peak Shape Using Co-injection Function

Chromatograms obtained without the co-injection function are shown in Fig. 3(2) (sample solvent : NaCl) and Fig. 5(2) (sample solvent : NaBr), respectively. The ion-pair formation ability was suppressed due to the high concentration of salts in the sample solvents. Consequently, the oligonucleotide was not properly retained, and part of the peak was eluted as a split peak. On the other hand, simultaneous co-injection of pure water resulted in increased peak areas for the full-length product (FLP) (Fig. 3 (3)–(5) and Fig. 5 (3)–(5)), confirming that it effectively provided appropriate peak retention. When the co-injection volume was 3 µL or more (Fig. 3(4), (5) and Fig. 5(4), (5)), split peaks were no longer observed. The relationship between the co-solvents, co-injection volumes, and the normalized peak areas (%) of FLP is shown in Fig. 4 (sample solvent : NaCl) and Fig. 6 (sample solvent : NaBr), respectively. Regardless of the co-injected solvent type, the normalized peak areas of FLP remained nearly constant when the co-injection volume was 3 µL or more. This suggests that co-injection is effective in suppressing the peak splitting phenomenon caused by the sample solvent effect.

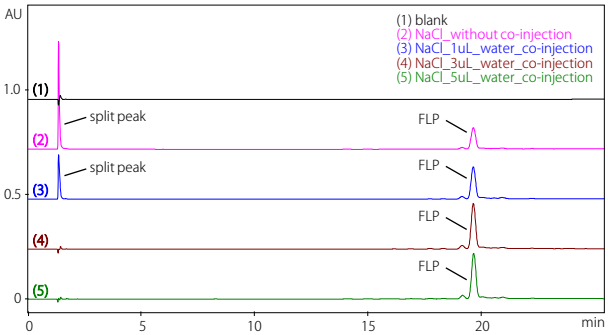

Fig. 3 Comparison of Chromatograms with/without Co-injection (sample solvent : NaCl)

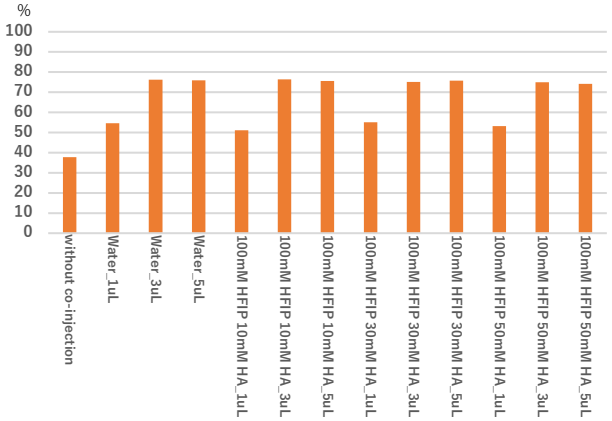

Fig. 4 Normalized FLP Peak Areas (sample solvent : NaCl)
* normalization based on sum of all peaks including FLP related impurities

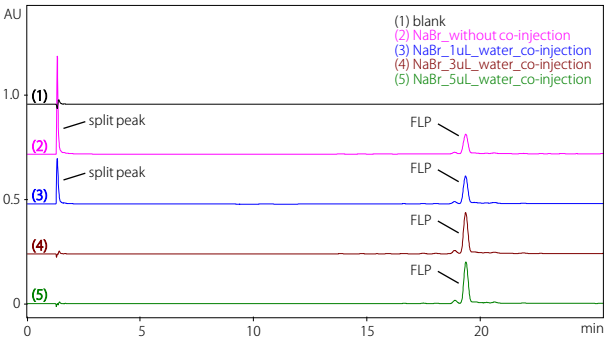

Fig. 5 Comparison of Chromatograms with/without Co-injection (sample solvent : NaBr)

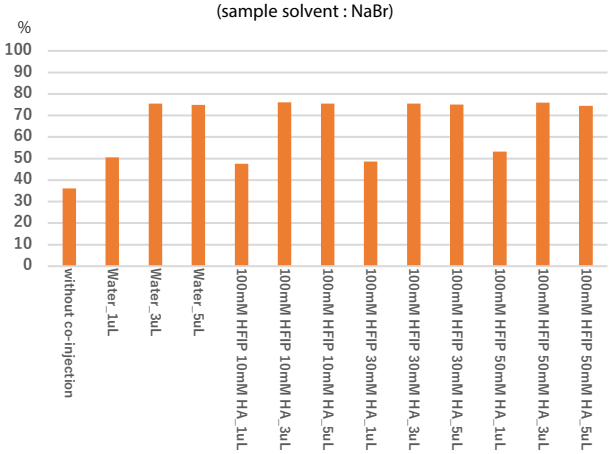

Fig. 6 Normalized FLP Peak Areas (sample solvent : NaBr)
* normalization based on sum of all peaks including FLP related impurities

■ Conclusion

When purified fractions of oligonucleotides containing high concentrations of salt are analyzed by RP-IP, the ion-pair formation capacity is suppressed, and peaks may not be properly retained, resulting in partial early elution as a split peak. Pretreatment, such as desalination or dilution of samples, is usually required before analysis, but it is time-consuming. The co-injection function, equipped in Nexera series autosamplers, allows for the simultaneous injection of a desired solvent with purified fractions, providing automatic pretreatment that enables the direct analysis of oligonucleotides in purified fractions with high salt concentrations. In particular, when many purified fractions need to be analyzed, significant labor savings can be achieved because the necessary pretreatment procedures are automated for all fractions. The optimal co-injection solvent and volume depend on the analytical conditions, such as oligonucleotide sequence and mobile phase composition. Therefore, although optimization of conditions is required each time, the use of LabSolutions MD enables efficient comprehensive evaluation of conditions.

■ Acknowledgments

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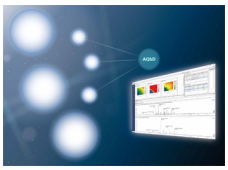

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