SHIMADZU

Efficient Method Development of Oligonucleotides by Reversed-Phase Ion-Pair Chromatography

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1. Introduction

Nucleic acid drugs are produced through chemical synthesis, but their synthesis process often introduces impurities such as shorter and longer lengths of products and protection groups. Therefore, proper chromatographic separation of the target oligonucleotide and impurities is critical for drug quality control and safety investigation. Liquid chromatography (LC) is one of the most popular techniques, combined with reversed-phase ion-pair (RP-IP) chromatography. The separation with RP-IP chromatography is affected by various parameters, including the column chemistry, the concentration of the ion-pair reagent in the mobile phase, organic solvent composition, gradient slope, etc. In addition, the separation performance also varies based on the molecule's chemical properties, such as sequence, length, molecule modifications, etc. Method development of LC separation is obviously challenging when considering the complexity of separation science and molecule chemistry facets. On the other hand, establishing a concrete analytical method in the early phase is vital for lengthy following research work.

To aim to develop a robust LC method for oligonucleotides in a short time, we studied the most efficient method development procedure, from a condition screening through the evaluation of method robustness. In a poster presentation, we will introduce a case of method development with LabSolutions™ MD, designed to support the entire method development procedure based on AQbD concept.

2. Experiment

2-1. System

Nexera™ XS inert (Method Scouting System, Fig. 1) with Shim-pack Scepter™ Claris (inert-coating metal-free column) was used to offer complete inertness of the sample flow path to achieve optimal chromatographic separation of oligonucleotides. In addition, a single quadrupole mass spectrometer LCMS-2050 was used for accurate peak tracking of oligonucleotides and related impurities.

Fig. 1 Nexera Method Scouting System

2-2. Sample and Analytical Conditions

A target oligonucleotide and five related impurities that have different sequences are used as a model sample of synthetic antisense oligonucleotide (Table 1). Full-length product (FLP), deletion sequences of n-1(3'), n-1(5'), and n-3 as shortmers, addition sequence of n+1 as longmer, and PO (modified from phosphorothioate to phosphate diester at 5') are prepared as a sample mixture. Analytical conditions are shown in Table 2.

Table 1 Sequences of Oligonucleotide and Related Impurities

Name	Sequence (5'→3') Note : * = 2'-O-methoxyethyl, m = 5-methyl, d = 2'-deoxy, PS (full)	Length
FLP	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*	20 mer
n-1(3')	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*	19 mer
n-1(5')	mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	19 mer
n-3	T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	17 mer
n+1	T*-T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	21 mer
PO	FLP (modified from phosphorothioate to phosphate diester at 5')	20 mer

Table 2 Analytical Conditions

System	: Nexera XS inert (Method Scouting System)
Column	: Shim-pack Scepter Claris (100 mm × 2.1 mm I.D., 3 μm)
Temperature	: 60 ℃
Injection Volume	: 2 μL
Mobile Phases	
Pump A – Line A	: 100 mmol/L HFIP[*1] and 20 mmol/L TEA[*2] in water *1 1,1,1,3,3,3-hexafluoro-2-propanol
– Line B	: 100 mmol/L HFIP in water *2 Triethylamine
– Line C	: 200 mmol/L HFIP and 20 mmol/L TEA in water
– Line D	: 200 mmol/L HFIP in water
Pump B – Line A	: Acetonitrile
– Line B	: Methanol
Flowrate	: 0.4 mL/min
Time program (%B)	: 6 % (0 min)→24 % (36 min)→50 % (36-37 min)→6 % (37-46 min)
Detection	: 260 nm (Photodiode array detector with UHPLC inert cell)
System	: LCMS-2050 (single quadrupole mass spectrometer)
Ionization	: ESI/APCI (DUIS™), negative mode
Mode	: SCAN (m/z 500-2000)
Nebulizing Gas	: 2.0 L/min (N₂)
Drying Gas	: 5.0 L/min (N₂)
Heating Gas	: 7.0 L/min (N₂)
DL Temp.	: 200 ℃
Desolvation Temp.	: 450 ℃
Interface Voltage	: -2.0 kV

3. Result

3-1. Initial Screening of Mobile Phase

Chromatograms (FLP and each impurity) measured under different conditions of the concentration of HFIP (100 and 200 mmol/L) and TEA (5, 10, 15, and 20 mmol/L) as well as different mixture ratio of acetonitrile and methanol (0, 50, and 100 %) are shown in Fig. 2. Mobile phase blending function automatically prepares these different conditions of the mobile phases. The result shows that the concentration of HFIP and TEA in the aqueous mobile phase, and the mixture ratio of acetonitrile and methanol have a large effect on separation of FLP and related impurities.
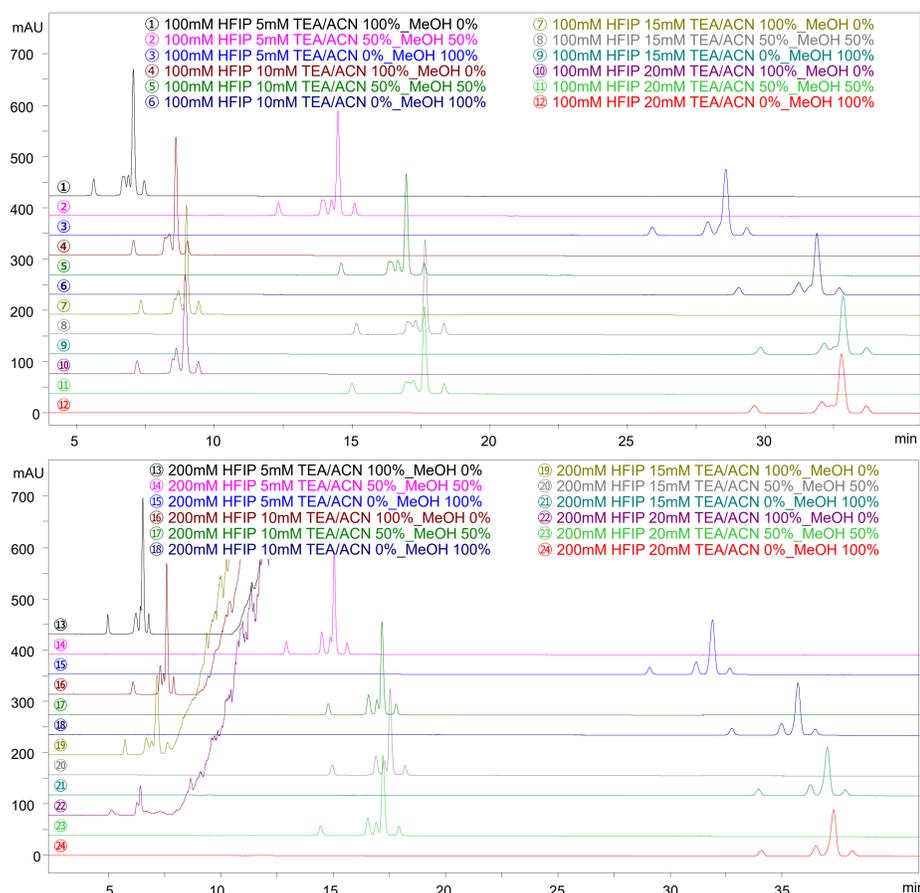

Fig. 2 Chromatograms in Mobile Phase Screening Phase (100 mmol/L HFIP (Upper) and 200 mmol/L HFIP (Lower))

Because screening generates as many chromatograms as the number of analysis schedules, they must be evaluated to determine which one is optimal. A manual procedure to check all chromatograms is troublesome and time-consuming. LabSolutions MD can quickly and easily find optimal conditions using the equation (Eq. 1) below to quantitatively evaluate the chromatographic separation.

$$(Evaluation\ Value) = P \times (Rs_1 + Rs_2 + \ldots + Rs_{P-1}) \cdot\cdot\cdot (Eq.\ 1)$$

The Evaluation Value is calculated as the number of peaks detected (P) multiplied by the sum of the resolution factor (Rs) for all peaks. Fig. 3 shows Evaluation Value obtained through mobile phase screening and listed in order from the highest to the lowest. It indicates that 100 mmol/L HFIP, 10 mmol/L TEA, and 50% acetonitrile ratio provided the best value as an optimal condition.

MPA Nick Name	MPB Nick Name	Evaluation Value
100mM HFIP 10mM TEA	ACN 50%_MeOH 50%	54.074
100mM HFIP 15mM TEA	ACN 50%_MeOH 50%	53.77
100mM HFIP 5mM TEA	ACN 50%_MeOH 50%	52.477
100mM HFIP 20mM TEA	ACN 50%_MeOH 50%	51.919
200mM HFIP 20mM TEA	ACN 50%_MeOH 50%	47.016
200mM HFIP 15mM TEA	ACN 50%_MeOH 50%	46.926
200mM HFIP 10mM TEA	ACN 50%_MeOH 50%	46.836
200mM HFIP 5mM TEA	ACN 50%_MeOH 50%	45.719
100mM HFIP 10mM TEA	ACN 100%_MeOH 0%	38.822
200mM HFIP 10mM TEA	ACN 100%_MeOH 0%	37.732

Fig. 3 Ranking of Each Condition by Evaluation Value

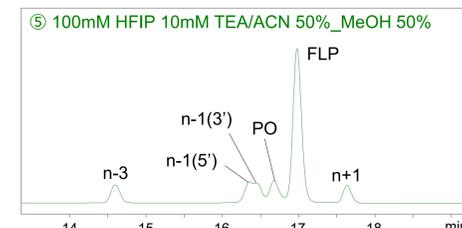⑤ 100mM HFIP 10mM TEA/ACN 50%_MeOH 50%
Fig. 4 Chromatogram of Highest Evaluation Value (Enlarged Chromatogram of Fig. 2 ⑤)

3-2. Design Space Evaluation for Optimal Conditions

Based on the optimal condition of the mobile phase at the initial screening phase (Fig. 4), the analytical conditions were further optimized for the separation of FLP and related impurities. The parameters of acetonitrile ratio in the organic solvent (40, 50, and 60 %), column oven temperature (55, 60, and 65 ℃), and initial concentration of gradient program (6, 7, and 8 %) were changed, and each peak was accurately tracked through all the data by LCMS-2050 to visualize resolution of FLP and related impurities by design spaces (Fig. 5). The vertical line shows acetonitrile ratio and the horizontal line shows column oven temperature. The red and blue areas indicate the better and the poor peak resolution area, respectively. By visualizing peak resolution, it became evident that the higher the column oven temperature is, the better the resolution of each peak gets, while an optimal ratio of acetonitrile is compound-related.
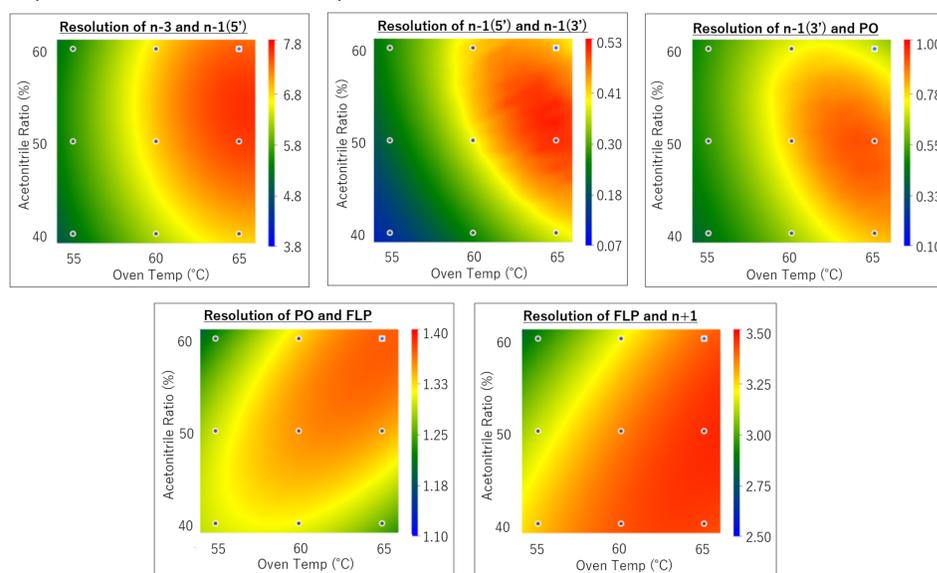

Fig. 5 Design Spaces for Resolution of FLP and Related Impurities (Initial concentration 8 %)

To achieve the most robust analysis, we searched for the optimal conditions that fulfill multiple criteria by overlaying design spaces. Fig. 6 illustrates that the final output met the following all conditions; (a) peak resolution of n-1(5')/n-1(3') is maximized, (b) peak resolution of n-1(3')/PO > 0.7, and (c) retention time (tR) of the final peak (n+1) < 16 min. The areas enclosed by the green and orange lines are where n-1(3')/PO < 0.7 and tR of the final peak (n+1) > 16 min, respectively. Point A (a red circle), in the area meeting the condition (b) and (c) (with the black hatching), indicates the analytical conditions providing the maximum peak resolution of n-1(5')/n-1(3'), meaning to fulfill all three demands. To proof it, the chromatogram obtained in the conditions of point A is shown in Fig. 7. As predicted in Fig. 6, the peak resolution of n-1(3')/PO was larger than 0.7, and tR of the final peak (n+1) was around 15 min, fulfilling all criteria for optimization. Although it was unfortunately impossible to obtain the perfect separation between n-1(5') and n-1(3') due to their very similar molecular structure, this approach could maximize the resolution automatically.
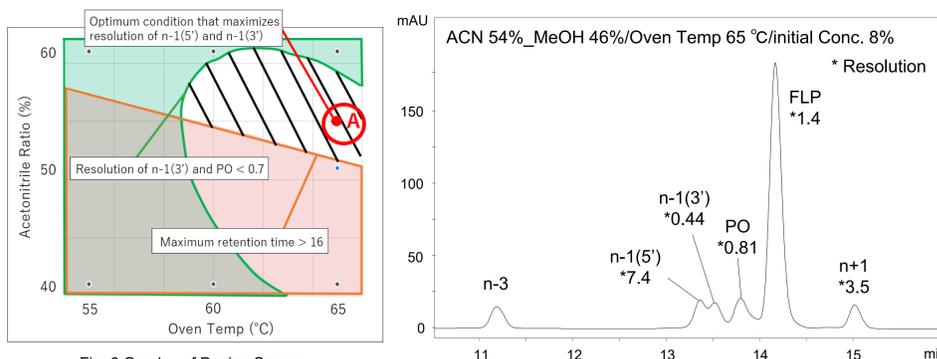

Fig. 6 Overlay of Design Spaces

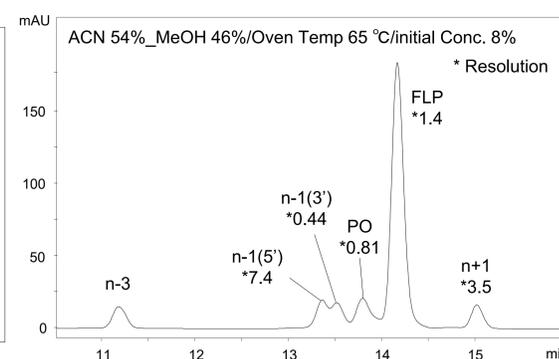ACN 54%_MeOH 46%/Oven Temp 65 ℃/initial Conc. 8%
Fig. 7 Chromatogram in Optimal Conditions (100 mmol/L HFIP and 10 mmol/L TEA)

4. Conclusion

The LC separation for oligonucleotides is affected by various parameters, not only the molecular structure and chemical property but also the separation conditions including column chemistry, mobile phase composition, etc. Instead of manually exploring the appropriate separation conditions of the target oligonucleotide and its impurities, the latest software algorithm provides an effortless workflow to reach robust analytical conditions. Thanks to the unique equation with the automated peak tracking function with LC/MS and design space concept, the final output of the separation conditions contributes to improving data quality, accelerating nucleic drug development.

5. Acknowledgment