Efficient Method Development of Small Interfering RNA by Reversed-Phase Ion-Pair Chromatography

○Risa Suzuki[1], Daiki Fujimura[1] , Yusuke Osaka[1] , [1]Shimadzu Corporation

1. Introduction

Due to the effort of the RNA interfering (RNAi) to prevent protein synthesis, short duplexes of RNA, such as small interfering RNA (siRNA) is being studied as a treatment for a variety of indications[1]. The methods for characterization of oligonucleotide therapeutics are required from development phase to quality control of a product. Ion-Pair Reversed-Phase Liquid Chromatography (IP-RP LC) under denaturing conditions is typically used for siRNA characterization. Separation from sense and antisense strands and contaminants is important for more accurate results but achieving an accurate analytical method requires consideration of complex parameters such as column chemistry, ion-pair reagent concentration, and column temperature. This study describes how to achieve the optimal separation on siRNA and related impurities efficiently by LabSolutions MD which is a dedicated software for supporting method development.

2. Experiment

2-1. System

Nexera™ XS inert (Method Scouting System : Fig.1) with Shim-pack Scepter™ Claris columns were used to offer complete inertness of the sample flow path to achieve optimal chromatographic separation of oligonucleotides (shown in Fig.2).

Fig.1 Method Scouting System

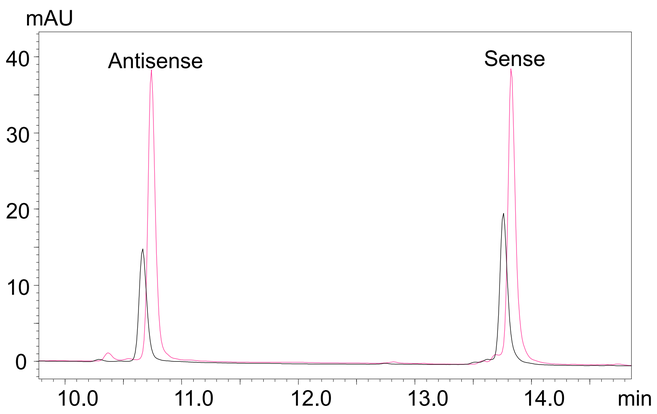

Fig.3 siRNA analysis using Scepter C18-300 (SUS, black line) and Scepter Claris C18-300 (inert column, pink line)

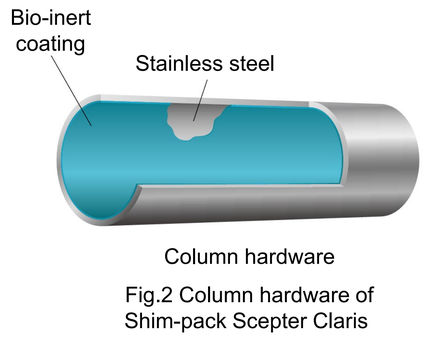

Fig.2 Column hardware of Shim-pack Scepter Claris

2-2. Sample and Analytical Conditions

A 21mer siRNA duplex standard was bought from NIPPON GENE Co.,LTD.. Stock solutions of siRNA was prepared in sterilized water at a concentration of 20 µmol/L form powder. Analytical sample was prepared by adding sterilized water to reach final concentration of 1.0 µmol/L. Analytical conditions are shown in Table 1.

Table 1 Analytical Conditions

System	: Nexera XS inert (Method Scouting System)	
Column	: Shim-pack Scepter Claris C18-120 (100 mm × 2.1 mm I.D., 3 µm, P/N：227-31210-05*)	
	Shim-pack Scepter Claris C18-300 (100 mm × 2.1 mm I.D., 3 µm, P/N：227-31209-05*)	
	Shim-pack Scepter Claris C4-300 (100 mm × 2.1 mm I.D., 3 µm, P/N：227-31208-05*)	
	*Shimadzu GLC product number	
Temperature	: 45, 55, 65 °C	
Injection Volume	: 2 µL	
Mobile Phases		
Pump A – Line A	: 100 mmol/L HFIP*1 and 20 mmol/L alkylamine in water	*1 1,1,1,3,3,3-hexafluoro-2-propanol
– Line B	: 100 mmol/L HFIP in water	
– Line C	: 200 mmol/L HFIP and 20 mmol/L alkylamine in water	
– Line D	: 200 mmol/L HFIP in water	
Pump B – Line A	: Methanol	
Flowrate	: 0.3 mL/min	
Time program (%B)	: 15% (0-1 min) →35% (1-21 min)→95% (21.01-26 min) →15% (26.01-33 min) (Final condition)	
Detection	: 260 nm (SPD-M40, UHPLC inert cell)	

3. Result

3-1. Comparison of separation patterns by ion-pair reagents

Chromatograms (siRNA, under denaturing condition) measured using different alkylamine (Triethylamine; TEA, N,N-diisopropylethylamine;DIPEA, and Dibutylamine;DBA) are shown in Fig.4. The result shows that the separation of sense strand, antisense strand and impurities are better when using alkylamines with longer carbon chains. The peak shape of the sense strand got worse when DBA was used. It is considered that impurities that could not be separated when using TEA or DIPEA is separated. However, this impurity peak was not be able to separate from sense strand perfectly. From this result, it was decided to use DIPEA for further studies.

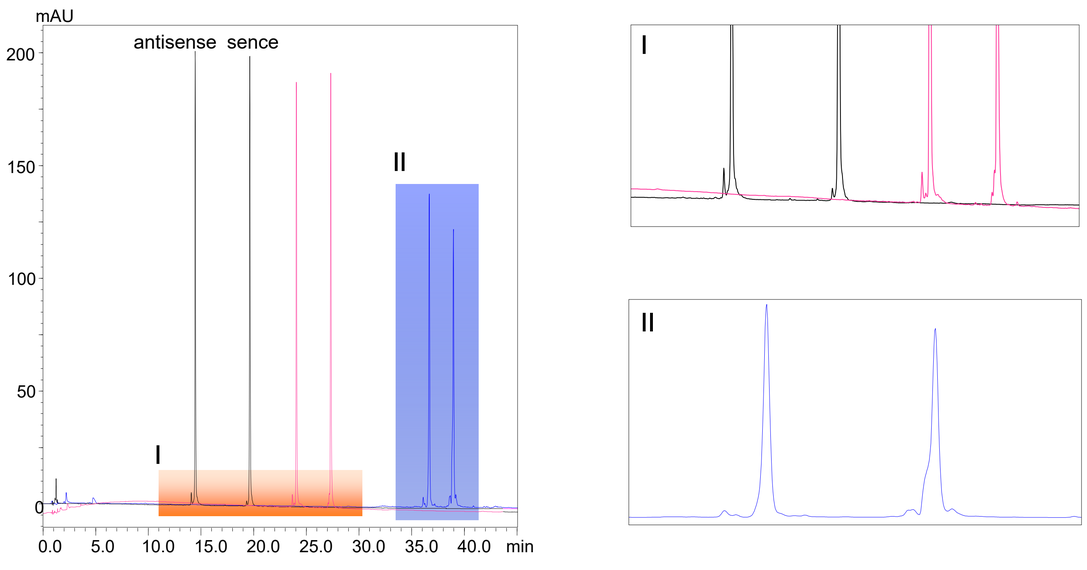

Fig.4 Chromatograms Obtained by mobile phase screening(under the gradient condition of increasing from a rate of 5%B to 1%/min for 45 minutes.)(10mmol/L of TEA: Brack line, DIPEA: Pink line, and DBA:Blue line)

Chromatograms measured under the concentration of HFIP (100 and 200 mmol/L) are shown in Fig.5. The result shows that the concentration of HFIP in the aqueous mobile phase have a slight effect on separation of sense strand, antisense strand and related impurities. It was decided the concentration of HFIP is 200 mmol/L for further studies.

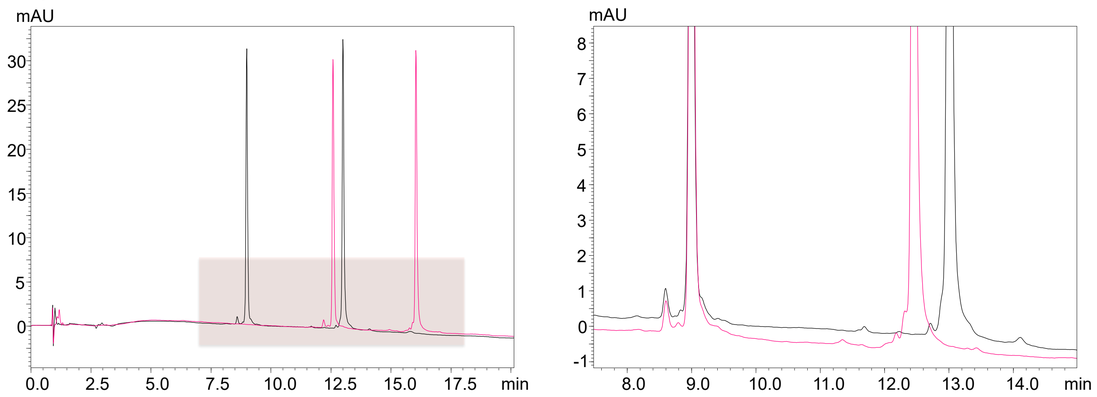

Fig.5 Chromatogram obtained by 100 mmol/L (Black line) and 200 mmol/L (pink line) of HFIP (Left)
Surrounded place is enlarged and chromatogram is shifted to overlap the peak position of antisense strand(Right)

3-2. Design Space Evaluation for Optimal Condition

Based on the optimal condition of mobile phase at initial screening phase (Fig.4 and 5), analytical conditions were further optimized for the separation of sense strand, antisense strand and related impurities. The parameters of concentration of DIPEA in the aqueous mobile phase (2, 5, 10, and 20%), column oven temperature (45, 55, and 65°C), and column stationary phase (C18 with 12 nm of pore size, C18 with 30 nm of pore size, and C4 with 30 nm pore size) were changed. The Resolution of antisense and related impurities were visualized by design spaces (Fig.9). The vertical line shows concentration of DIPEA and the horizontal line shows column oven temperature. The red region indicates higher resolution, and the blue region indicates lower resolution. By visualizing resolution, it became evident that the higher the column oven temperature and optimal concentration of DIPEA are the better the resolution of each peak. LabSolutions MD can automatically search for the optimal condition that meets several criteria by overlaying design spaces. Point A shown in the center of Fig.9 is the optimum analytical condition automatically calculated from the resolution of antisense strand and impurity by LabSolutions MD. This approach could maximize the resolution without depending on user's experience.

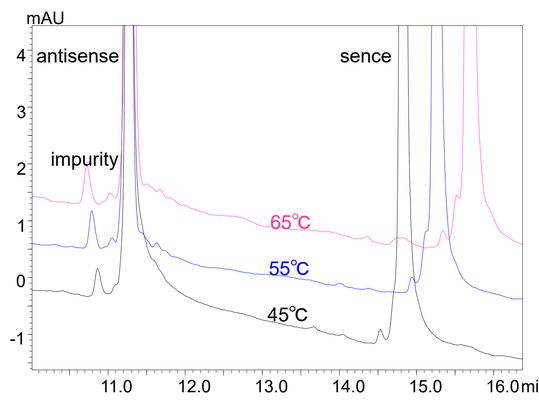

Fig.6 Chromatograms under various column temperature (Chromatograms were shifted to fix the peak position of antisense strand)

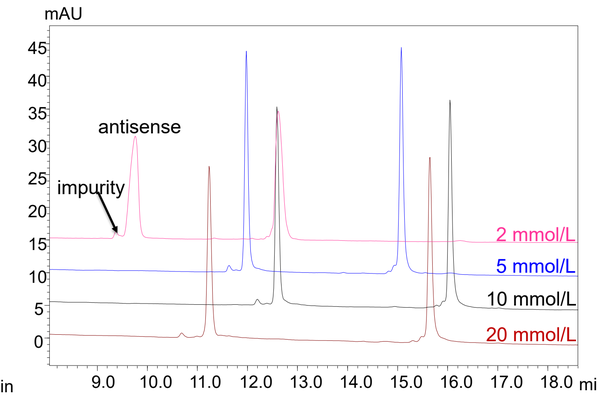

Fig.7 Chromatograms under various concentration of DIPEA

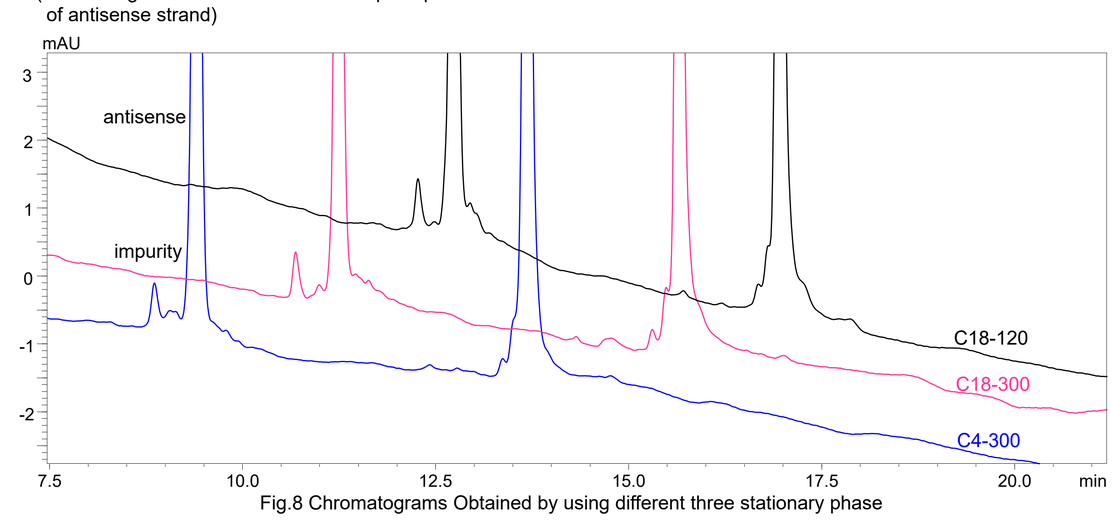

Fig.8 Chromatograms Obtained by using different three stationary phase

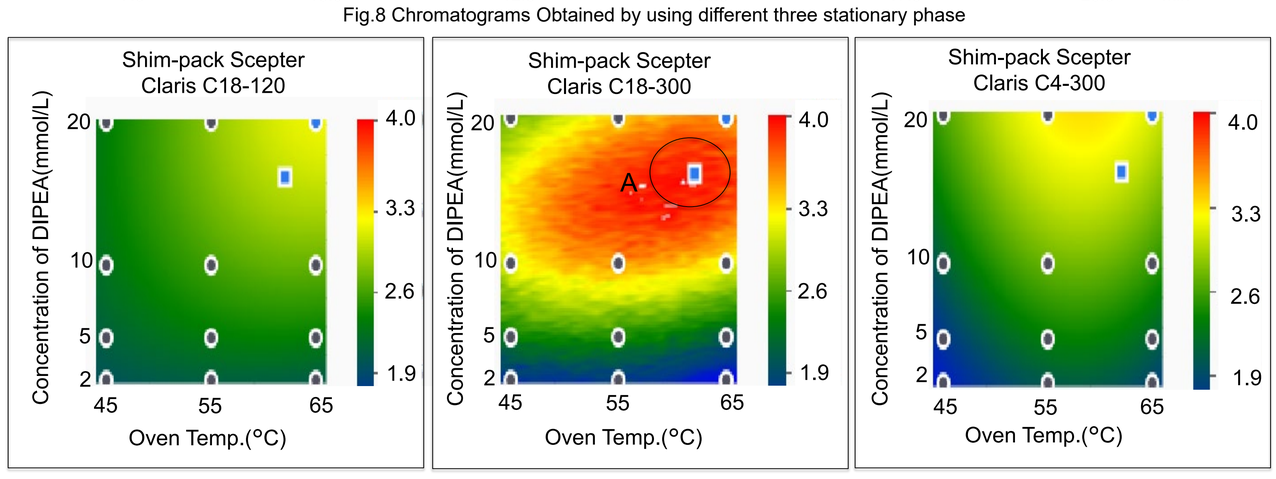

Fig.9 Design Spaces for Resolution of antisense strand and related Impurity

4. Conclusion

The separation pattern of siRNA can be different depending on the structure of sense, and antisense strand such as length, a type of impurities, and nucleobase. Therefore, it is required to optimize the separation for each sequence of strand individually. On the other hand, a number of evaluation and data processing are a time-consuming process. The latest software algorithm provides an effortless workflow to build optimal analytical conditions. Thanks to specific functionalities such as mobile phase blending function, column switching, and design space, the final output of the separation condition contributes to improving data quality, accelerating method development of siRNA.

5. Reference

Sean M. McCarthy, Martin Gilar, John Gebler; Analytical Biochemistry, Volume 390, Issue 2, 15 July 2009, Pages 181-188