Ion-pair Reversed-phase Liquid Chromatographic Separation of Oligonucleotides

Takumi TAKANO,* Chiaki AOYAMA,* Yoshiro TERASAKI,* Kenichi SUZUKI,* Aki ANDO,* Yanting SONG,** and Makoto TSUNODA****

*GL Sciences Inc., 6-22-1 Nishi Shinjuku, Shinjuku, Tokyo 163-1130, Japan
**School of Pharmaceutical Sciences, Hainan University, Haikou 570228, China
***Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan

Therapeutic oligonucleotides have recently been approved in the United States, the EU, and Japan. Hence, the analysis of oligonucleotides is an important topic in drug development. Liquid chromatographic techniques are commonly used for purity verification and the determination of oligonucleotides. In ion-pair reversed-phase separation, several parameters, such as the pore size of the stationary phase, mobile phase additives, and column temperature, were investigated using three types of oligonucleotides (18, 19, and 20 mer). All of the investigated parameters could influence the separation, and they are expected to be useful for optimizing oligonucleotide separation.

Keywords Pore size, mobile phase additives, column temperature, DNA, HPLC

(Received July 22, 2021; Accepted August 24, 2021; Published December 10, 2021)
was 5 – 50%B over 60 min. The column temperature was TEAA (pH 6.5)/acetonitrile (80:20, v/v). The linear gradient acetate (TEAA; pH 6.5), and mobile phase B consisted of 5 mM adopted. Mobile phase A consisted of 5 mM triethylammonium was 0.6 mL/min. A binary gradient elution program was then C8 (3 used for LC. Separation was performed on four types of reversed-phase columns (GL Sciences), InertSustain C18 (3 μm, 3.0 × 100 mm), InertSustainSwift C18 (3 μm, 3.0 × 100 mm), Inertsil WP300 C18 (5 μm, 3.0 × 100 mm), and InertSustainSwift C8 (3 μm, 3.0 × 100 mm). The flow rate of the mobile phase was 0.6 mL/min. A binary gradient elution program was then adopted. Mobile phase A consisted of 5 mM triethylammonium acetate (TEAA; pH 6.5), and mobile phase B consisted of 5 mM TEAA (pH 6.5)/acetonitrile (80:20, v/v). The linear gradient was 5 - 50%B over 60 min. The column temperature was maintained at 40 or 60°C. The injection volume was 1 μL. UV detection was performed at a wavelength of 260 nm.

Results and Discussion

Most FDA-approved oligonucleotides are 18 – 30 mer; hence, 20 mer oligonucleotides (for chemical composition, see Materials and Methods section) were used as typical oligonucleotides. The 18 and 19 mer oligonucleotides were used as impurities. The effects of several parameters, such as the pore size of the stationary phase, mobile phase additives, column temperature, and type of stationary phase, were investigated for separating three types of oligonucleotides (18, 19, and 20 mer).

Effects of pore size of the stationary phase

First, the pore size of the reversed-phase column was investigated. In porous silica particles, most interactions of the analytes with stationary phases occur in the pores. For low-molecular-weight compounds (molecular weight <1000 Da), the average pore size used is 100 Å. Large-pore particles (~300 Å) are used for separating large molecules, such as proteins. Considering that the molecular weights of oligonucleotides used in this study were about 6000 Da, which is greater than that of low-molecular-weight compounds, but smaller than that of proteins, the pore size should influence the retention and separation. In this study, three types of reversed-phase columns with pore sizes of 100 Å (IntertSustain C18), 200 Å (IntertSustainSwift C18), and 300 Å (Inertsiil WP300 C18) were investigated. The separation results are shown in Fig. 1. The separation efficiency was poor for a pore size of 100 Å (Fig. 1(a)), probably because these pores were too small for the oligonucleotides investigated. Good separation was achieved with pore sizes of 200 and 300 Å, and a difference in retention was observed (Figs. 1(b) and 1(c)). The column with a pore size of 300 Å led to weaker retention than that with a pore size of 200 Å. This difference can be explained by the surface area of the particles. Columns with pore sizes of 200 and 300 Å had surface areas of 200 and 150 m²/g, respectively. Accordingly, a column with a pore size of 200 Å, which offered better separation, especially for 18 and 19 mer, was used in subsequent experiments.

Effects of hexafluoroisopropanol addition to mobile phases

Ion-pairing reagents are indispensable for the retention and separation of oligonucleotides in reversed-phase columns. However, in the case of detection, increasing the concentration of the ion-pairing reagent does not degrade the separation resolution, but decreases the sensitivity in mass spectrometry (MS) detection. Apffel et al. reported that the addition of hexafluoroisopropanol (HFIP) to the mobile phase can enhance the sensitivity of MS with electrospray ionization.13 Herein, we investigated the manner in which the addition of HFIP (0.1, 0.5, and 1.0%) to the mobile phases influenced the separation of the three oligonucleotides. The chromatograms are shown in Fig. 2. HFIP clearly affected the retention of the oligonucleotides, but an increase in the HFIP concentration did not improve the absolute resolution. We calculated the resolution of two pairs of oligonucleotides, 18 and 19 mer, and 19 and 20 mer. As shown in Fig. 3, the trends in the R, values of the two pairs were different. With an increase in the HFIP concentration, the R, values between 18 and 19 mer increased, while those between 19 and 20 mer decreased. This result indicated that HFIP can change the separation efficiency of oligonucleotides, and that the extent of change depends on the length of the oligonucleotides, and probably, on their sequence.

Effects of column temperature

In chromatographic separation, the separation selectivity and efficiency are generally influenced by temperature because the separation involves a series of equilibrium reactions where the analytes are either dissolved in the mobile phase or adsorbed
The higher is the temperature, the faster is the exchange of analytes between the mobile and stationary phases, and the shorter is the retention time of the analytes on the column. In the case of oligonucleotide separation, high temperatures exceeding 60°C are often employed to improve the peak shape and separation. Although such a high temperature is mandatory for the analysis of double-stranded oligos, such as siRNAs, it is unclear whether the same is also necessary for single-stranded oligonucleotides. Hence, the effect of temperature (40°C and 60°C) on the separation of the three oligonucleotides was evaluated using two different mobile phases (0.1% and 0.5% HFIP). As shown in Fig. 4, when the column temperature was increased to 60°C, shorter retention times, but similar resolutions, were observed with both mobile phases. This suggests that a high temperature is not necessary for separating single-stranded oligonucleotides. Separation at lower temperatures offers several advantages, such as improved column durability and easy scale-up to preparative HPLC.

**Effects of stationary phases**

Most studies on oligonucleotide separation have been performed using an ODS column. For the analysis of macromolecules, such as proteins, C8 columns are sometimes preferable. Considering that oligonucleotides are not small molecules, a C8 column (InertSustainSwift C8) was investigated instead of an ODS column, with mobile phases containing 0.5% HFIP at 40°C. The pore size of the C8 column is 200 Å. Figure 5 shows the chromatograms obtained with both the ODS and C8 columns. The retention and resolution of all
oligonucleotides were similar to those observed for the ODS column. This indicates that the C8 column provides a separation efficiency similar to that of the ODS column, thus being suitable for oligonucleotide separation.

Conclusions

With the increasing complexity of oligonucleotide therapeutics, the separation of oligonucleotides is gaining importance. As described in this study, several parameters can influence the separation efficiency of the oligonucleotides. Hence, with further investigation, it should be easier to optimize the separation of oligonucleotide drugs, even if their chemical compositions are different. Since most oligonucleotide-based drugs are chemically modified, e.g., by phosphorothioate modification, the separation of such oligonucleotides should also be examined.

References

1. N. Dias and C. A. Stein, *Mol. Cancer Ther.*, 2002, 1, 347.
2. B. D. Adams, C. Parsons, L. Walker, W. C. Zhang, and F. J. Slack, *J. Clin. Invest.*, 2017, 127, 761.
3. C. M. Perry and J. A. Balfour, *Drugs*, 1999, 57, 375.
4. O. A. Musaimi, D. A. Shaer, F. Albericio, and B. G. de la Torre, *Pharmaceuticals*, 2021, 14, 145.
5. K. Cook and J. Thayer, *Bioanalysis*, 2011, 3, 1109.
6. S. M. McCarthy, M. Gilar, and J. Gebler, *Anal. Biochem.*, 2009, 390, 181.
7. M. Gilar, K. J. Fountain, Y. Budman, U. D. Neue, K. R. Yardley, P. D. Rainville, R. J. Russell II, and J. C. Gebler, *J. Chromatogr. A*, 2002, 958, 167.
8. L. W. McLaughlin, *Chem. Rev.*, 1989, 89, 309.
9. A. J. Alpert, *J. Chromatogr. A*, 1990, 499, 177.
10. L. Gong and J. S. O. McCullagh, *J. Chromatogr. A*, 2011, 1218, 5480.
11. A. C. McGinnis, B. Chen, and M. G. Bartlett, *J. Chromatogr. B*, 2012, 883-884, 76.
12. A. P. McKeown, P. N. Shaw, and D. A. Barrett, *Chromatographia*, 2002, 55, 271.
13. A. Apffel, J. A. Chakel, S. Fischer, K. Lichtenwalter, and W. S. Hancock, *Anal. Chem.*, 1997, 69, 1320.
14. D. Guillarme, S. Heinisch, and J. L. Rocca, *J. Chromatogr. A*, 2004, 1052, 39.
15. M. Biba, C. J. Welch, J. P. Foley, B. Mao, E. Vazquez, and R. A. Arvary, *J. Pharm. Biomed. Anal.*, 2013, 72, 25.