Development of LC-MS method for Oligonucleotide Analysis with RP-HPLC Column Designed for Separation of Highly Polar Compounds

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1. Introduction

Reversed-phase HPLC has been widely applied to analysis and purification of synthetic oligonucleotides, such as primers of DNA sequencing or PCR, anion-exchange columns, and antiviral drugs. Because it is difficult to elute and separate highly polar compounds like short oligonucleotides on ordinary reversed-phase columns, an ion-pairing buffer containing triethylammonium acetate (TEAA) at a high concentration, e.g., 100-200 mM, has been commonly used to improve retention and resolution. However, a buffer containing TEAA at a concentration higher than 50 mM causes signal intensity decreases in electrospray ionization mass spectrometry (ESI-MS), which is one of the most important analytical methodology for oligonucleotides. Though it has been reported that the mobile phase of 11.5:8.5 hexane-2-propanol (HFP):TEAA buffer/methanol gives an advantage in MS sensitivity, it is necessary to add HFP, which is not so commonly used for RP-HPLC, with a relatively high concentration such as 400 mM to achieve good LC-MS analysis.

2. Experimental

HPLC conditions for separation of d(pT)20 in Fig. 2

- Column: Hydrosphere C18, 50 x 4.6 mm i.d., 3 μm
- Flow rate: 1.6 ml/min
- Temperature: 35°C
- Detection: UV at 269 nm
- Injection: 5 μl (25 pmol/component)
- Mobile phase: Four kinds of gradient systems are used

- Scan ES-TIC
- Total ion chromatograms (TIC)
- raw mass spectra

3. Results and Discussion

Figure 2 compares separation of poly(deoxythymidylic acid), d(pT)20, among four different gradient systems of TEAA buffer/acetonitrile (a), DBAA buffer/acetonitrile (b, c), TEAA buffer/methanol (d), and DBAA buffer/methanol (e, f), with the gradient slope which has been optimized at 10 mM buffer concentration. In case of the DBAA buffer, d(pT)20 can be separated favorably with higher initial concentration and higher gradient slope of organic solvent with both acetate and methanol than in case of the TEAA buffer. This result indicates that the ion-pairing interaction between di-n-butylamine and oligonucleotides is stronger than that between triethylamine and oligonucleotides. Even at a lower concentration of DBAA, such as 5 mM, the acceptable separation and good peak shape was maintained under the same gradient condition as 10 mM DBAA buffer, as shown in Chromatograms 2a and 2d.

4. Conclusions

- Oligonucleotide separation was compared under mobile phase conditions containing two different ion-pairing buffers, TEAA and DBAA, using Hydrosphere C18. It is possible to reduce the salt concentration and increase the organic solvent concentration with the DBAA buffer.
- Hydrosphere C18 showed strong retention and good resolution of short oligonucleotides at a relatively low concentration of DBAA, e.g., 5-10 mM, compared to the ordinary C18 phase.
- There was sufficient intensity of ESI-MS when 5-10 mM DBAA buffer was used to analyze oligonucleotides with both acetate and methanol. The intensity obtained with 5 mM DBAA buffer was equal or greater than that obtained with 400 mM HFP-TEA-buffer.

This simple method using Hydrosphere C18 with a low concentration of DBAA buffer provides a great potential for various chromatography of oligonucleotides, such as analytical and preparative HPLC or HPLC-MS.