Effect of Alkylamine Based Ion-pairing Reagents in Analysis of siRNA

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Overview

Silencing RNA, or siRNA, are short, double-stranded RNA molecules which are not only an important research tool in molecular biology, but also an emerging therapeutic modality. Numerous chromatographic methods have been developed for analysis of these double stranded RNA molecules, including LC-ESI-MS and LC-UV methods.

Initial oligonucleotide LC-MS methods used high mounts of TEA and HFIP (16 and 400 mM, respectively) to facilitate the retention of these negatively charged, polar compounds[1]. However, with modern HPLC media, this mobile phase composition may not be optimal for both chromatographic performance and electrospray ionization. Although many alkylamine ion-pair have been investigated for MS optimization[2], in this application note, we show that chromatographic separation of siRNA sense and anti-sense strands may be more optimal with TEA.

Figure 1 shows a 21mer double-stranded RNA using TEA as the ion-pairing reagent. Even with 12.5 mM HFIP, the relatively low concentration 4 mM TEA provides sufficient retention of the siRNA, with good resolution of sense and anti-sense strand; although not baseline, this would be acceptable to obtain high quality spectral data if characterizing each strand separately by high resolution MS.

N,N-diisopropylethylamine (DIEA) is another commonly used alkylamine, which often provides superior chromatographic separation and improved electrospray desorption for oligos. However, as seen in **Figure 2** for the same 21mer double-stranded RNA, we see some aberrant chromatography. Although there are seemingly more earlier eluting impurities, loss of resolution sense and anti-sense is observed. Further MS characterization would need to be performed to determine the actual impact on analysis. Similar chromatographic behavior is observed with more hydrophobic alkylamine, including N,N-dimethylcyclohexylamine (DMCHA) and hexylamine (HA). After optimizing ion pair concentration and gradient profile, separation using DMCHA is not favorable (**Figure 3**), and this odd chromatographic behavior is even more pronounced with HA (**Figure 4**).

It is important to note that if the intent is MS sensitivity, TEA may not perform as well as other alkylamine, as reported in the literature. Further, the more hydrophobic alkylamine show more impurities, though without the MS characterization it is unclear if these are indeed impurities/failure sequences or if these impurity peaks are a result of chromatographic aberration. Replicate injections showed consistent chromatography, though day to day data showed different chromatography (data not shown).

In summary, if the separation of sense and anti-sense strand when characterizing double-stranded oligonucleotides is prioritized, TEA will likely yield more favorable chromatography and more reproducible results. As such, other factors impacting MS performance, including modulation of HFIP concentration, might need to be implemented.

LC-UV Conditions

Column:	bioZen™ 2.6 µm Oligo
Dimension:	100 x 2.1 mm
Part No.:	00D-4790-AN
Mobile Phase:	A: 12.5 mM HFIP, IP Reagent as mentioned in Water B: 12.5 mM HFIP, IP Reagent as mentioned in MeOH
Gradient:	As mentioned
Flow Rate:	0.3 mL/min
Injection:	2 µL (1 µg/mL)
Temperature:	55 °C
Detection:	UV-Vis @ 260 nm
Sample:	siRNA

[1]Gilar M, Fountain KJ, Budman Y, et al. Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides: retention prediction. *J Chromatogr A*. 2002;958(1-2):167-182
[2]Basiri, Babak et al. "Assessing the Interplay between the Physicochemical Parameters of Ion-Pairing Reagents and the Analyte Sequence on the Electrospray Desorption Process for Oligonucleotides." *Journal of the American Society for Mass Spectrometry* vol. 28,8 (2017): 1647-1656.

Figure 1. Separation of 21mer double-stranded RNA, 4 mM TEA

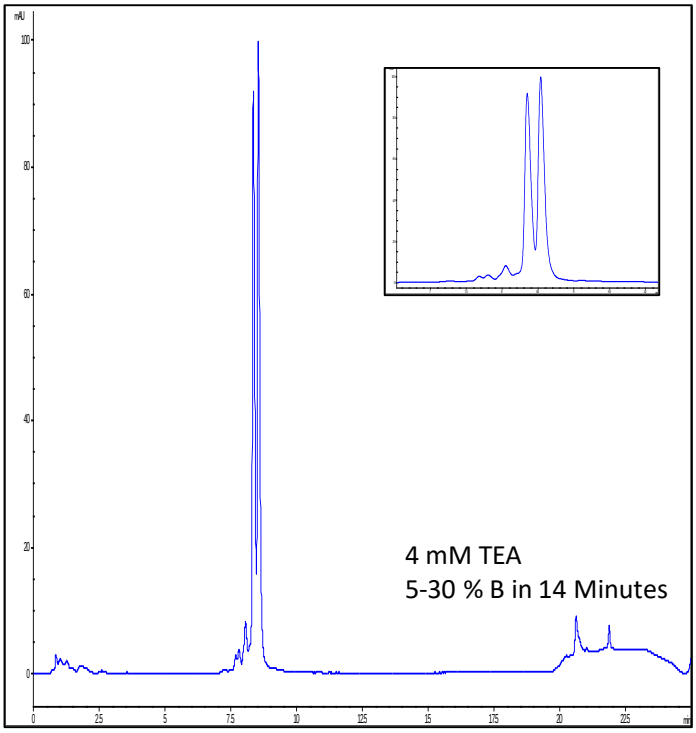


Figure 2. 21mer double-stranded RNA, 2 mM DIEA

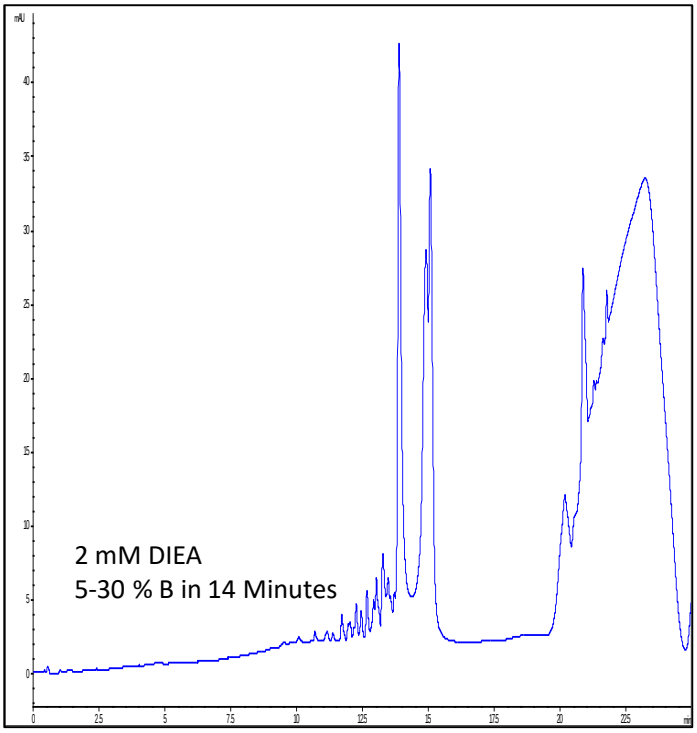


Figure 3. 21mer double-stranded RNA, 0.5 mM DMCHA

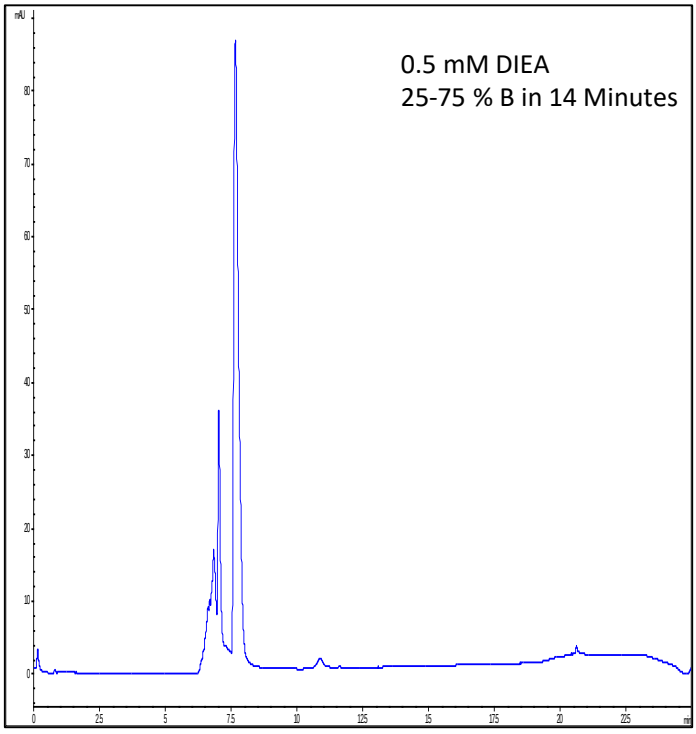
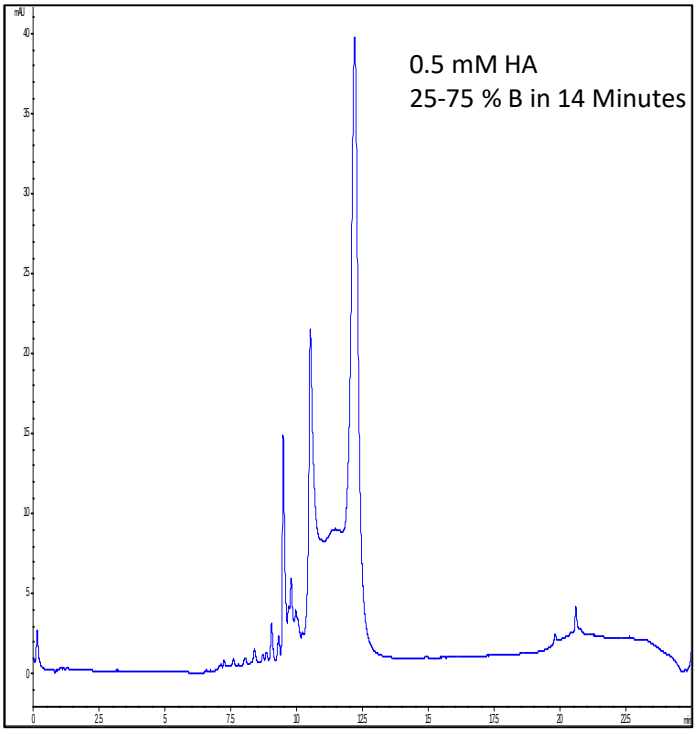


Figure 4. 21mer double-stranded RNA, 0.5 mM HA



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