Improving Oligonucleotide Separation Efficiency in Ion Exchange and Reverse Phase Chromatography – Taking Advantage of UHPLC Instrumentation and New Column Technology

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Lisa Zang[1], H. Ji[2] [1]Agilent Technologies, Life Science and Chemical Analysis, 5301 Stevens Creek Blvd, Santa Clara, CA 95051; [2] Life Technologies, 6055 Sunol Blvd, Pleasanton, CA 94566

Introduction

Ion exchange and ion pairing reverse phase chromatographic techniques are the main HPLC methods used in both analyses and purifications of wide ranges of oligonucleotides today. Both techniques have shown advantages and disadvantages depending on the required resolution and the sample loading for preparative separations/purifications[1]. As the smaller particle size columns become widely available, the improvement in both resolution and mass transfer can be obtained with using new column technologies.

In addition, using UHPLC instrumentation is an important factor in delivering the high resolution separation with higher performance and higher power range (high pressure and flow) when running applications with these new columns.

In this presentation, we demonstrate the oligonucleotide quality analysis using ion-exchange chromatography on both conventional HPLC instrument and on UHPLC instrument. The analyses were also done using sub-2 um particle size reverse phase column with UHPLC instrument.

Experimental

Sample Preparation

A crude customer oligo sample was used in this work. The oligo of interest has a length of 34 base pairs. The sample was purified on Agilent 1200 semi-prep HPLC with an analytical scale fraction collector. The purification separation was done using Xterra C18 column, 4.6x50mm, 2.5um with a linear ACN and water gradient. TEA (triethylamine) is added to each mobile phase as the ion pairing reagent. The 100 ul fractions were collected continuously into a standard 96 wellplate by time mode across the region of interest (see Figure 1)

Experimental

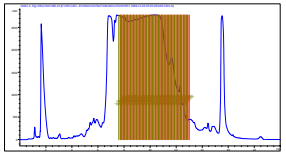

Fig. 1 Sample purification using ion pairing reverse phase chromatography. Fractions are collected from 7.5 min to 13 min by time mode into 96 well plate (100ul each).

HPLC Instrumentation, Column and Method

Agilent 1290 UHPLC system was used to perform quality analysis of the collected fractions using ion exchange chromatography. The 1290 pump is configured to use 35 ul Jet Weaver mixing. A TSK DNA-NPR 4.6x75 mm column was used. A 10 minutes ion exchange chromatography method with NaCl gradient in Tris buffer (pH 8.5) is used.

The flow rate is 1.25 ml/min.
Mobile phase A contains 0.1 M NaCl.
Mobile phase B contains 1M NaCl.
Separation graident: 50-56%B in 6 min.
Sample injection vol: 0.5 ul
Column temperature: 53 C.
DAD acquisition rate: 40 Hz

The same 96 wellplate sample analysis was run on a conventional HPLC (Agilent 1200 HPLC system) using the same column for comparison. The customer's method was used to run the samples with the identical mobile phases.

We also conducted the analysis on the fractions collected from the semi-preparative purification of another customer 24-bp oligo sample. The prep method is the same as shown in Figure 1. In this analysis, we ran reverse phase chromatography using Kinetex 2.1x150 mm, 1.7 um, 1000 bar column with Agilent 1290 system. The same ACN/H2O with TEA mobile phases used in semi-prep work were used with a 8.6 min gradient (25%-37% B) and flow of 0.65 ml/min. Injection volume is 0.3 ul.

Results and Discussion

The results obtained from Agilent 1290 UHPLC system running the ion exchange method are shown in Figure 2 along with the results obtained from customer's conventional HPLC (1200 LC). The 1290 system results show that a significant improvement in resolution is obtained comparing with the results obtained from conventional LC. Many impurity peaks from shorter oligomers are completely resolved. This demonstrates that the high precision of 1290 Infinity pump and the low system delay volume contribute to the huge improvement.

In Figure 4, we show the results of the fraction analysis obtained using a long sub-2 um particle size reverse phase column on the 1290 UHPLC system. The intent of the work is to investigate the possibility of using the same mobile phases and similar methods as those used in semi-preparative purification to run the quality control analysis.

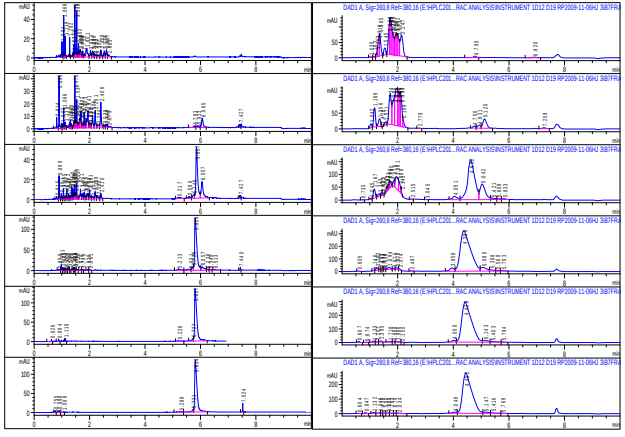

Figure 2. Ion Exchange Chromatography UHPLC Results vs. Conventional LC Results

The fractions 26 through 31 are plotted to show the time transition (increasing in target oligo purity). The left panel shows the results from 1290 Infinity system and right panel shows the results from regular HPLC. The same column and the same wellplate samples are used in the experiments. The injection volume for 1290 system is 0.5 ul and for regular LC is 1 ul. The method used for regular HPLC analysis is optimized and routinely used at the customer site.

Results and Discussion

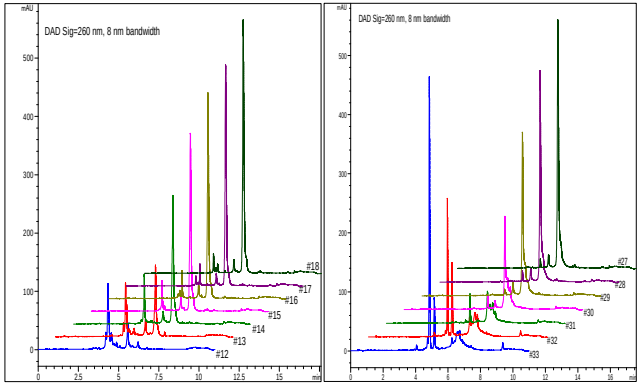

Figure 4. Reverse Phase Chromatography UHPLC results using a 2.1x150 mm sub-2 um particle size column. The fractions 12 to 18 are displayed on the left showing the increasing trend of the target oligomer purity. The fractions 21 to 33 are shown on the right side showing the decreasing trend of the target oligomer purity as the purification time slides passing the peak of collection.

With UHPLC instrument, it becomes possible to run the analysis using a longer sub-2 um particle size column (2.1x150mm) to maximize resolution without increasing run time.

In addition, this further opens the possibility of running both purification and quality analysis using the same UHPLC system to take advantage of the improved resolution demonstrated here.

Conclusions

We demonstrated the benefit of using UHPLC instrumentation in oligonucleotide applications. The Agilent 1290 UHPLC system not only provides the power to run long small particle size (150 mm) columns with higher flow rate to improve separation efficiency without increase in analysis time, it also delivers high quality performance for conventional ion exchange separation methods with significantly improved resolution.

[1] *L.L. Lloyd et al. / J. Chromatogr. A 1009 (2003) 223–230.*