應用手冊

Analysis of siRNA Drugs at Denaturing UPLC Conditions Using MaxPeak Premier Column Technology

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Abstract

This application note demonstrates the utility of ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column for analysis of small interfering RNA (siRNA) therapeutic candidate compound. The siRNA duplex is analyzed at elevated temperature to ensure the duplex is denatured into two complementary single strand oligonucleotides. The aim of the application note is to quantify the siRNA oligonucleotides in the drug formulation. We selected the ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column because it showed a superior performance for analysis nucleic acids over the conventional stainless-steel column hardware. ACQUITY Premier Columns with MaxPeak High Performance Surfaces (HPS) Technology demonstrated a consistent quantitation with minimal fluctuation in siRNA recovery. Oligonucleotides signal repeatability and calibration linearity improved dramatically compared to conventional ACQUITY UPLC Peptide BEH C_{18}, 300 Å, Column.

Benefits

- An improved analysis repeatability was achieved with ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column compared to conventional version of the column
- ACQUITY Premier Columns eliminate an undesirable oligonucleotide adsorption on metal surfaces and column frits

- ACQUITY Premier Columns with MaxPeak HPS Technology removes the need for column conditioning
- Column conditioning is required for stainless-steel columns to stabilize their recovery of nucleic acid samples

Introduction

In the last decade, the oligonucleotides therapeutic compounds have gained an importance within the pharmaceutical industry. The raise on new modality of is related to their ability to treat a broad range of diseases and the technology advances in oligonucleotide manufacturing and drug delivery. Until 2016 three oligonucleotides were approved by pharmaceutical authorities, however, the number of oligonucleotide therapeutics studies since increased considerably.[1,2] The proliferation of the oligonucleotides in the clinical trials is accompanied with a demand for analytical methods suitable for quantitation and characterization of these compounds.

The methods for characterization of oligonucleotide therapeutics are required from early development stages, to quality control of an approved product. The analytics need to be robust, reliable, and easy enough to be performed in different environments and in many cases by non-expert analysists. This requirement conflicts with the considerable complexity of siRNA analysis.

siRNA drug is administered in duplex form, consisting of a pair of complementary chemically modified oligonucleotides. The siRNA is typically analyzed under denaturing LC conditions when the two oligonucleotides are denatured and analyzed as two separate entities known as single strands. Alternatively, the siRNA is analyzed as an intact duplex in non-denaturing conditions for verification that the duplex is not contaminated with an undesirable excess of single strand oligonucleotide species.

In this application note we describe the Ion-Pair Reversed-Phase Liquid Chromatography (IP-RP LC) used in denaturing conditions. The melting of siRNA duplex is accomplished by elevated column temperature to ensure complete dissociation of duplex siRNA structure into the corresponding single strand oligonucleotides. Denaturing IP-RP LC is the favored method for oligonucleotide LC-UV and LC-MS analysis in the industry. IP-RP LC method is suitable for sensitive analysis of antisense oligonucleotides (ASO) and siRNA therapeutic compounds due to its compatibility with MS analysis.

In the first step of method development we evaluated several BEH 18 columns lengths and sorbent pore size (130

or 300 Å). While adequate separation was achieved with several column configurations, the best separation selectivity and resolution for siRNA sample of interest was obtained with 2.1 mm x 150 mm ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column.

Experimental

Sample Preparation

A solution of 0.40 mg/mL was prepared by dissolving the duplex siRNA C in Milli-Q water. This concentration is labeled as 100% standard solution "Duplex C". For the construction of calibration curve 200% solution was prepared; the remaining concentrations (130%, 100%, 70%, 8%, 4%, 2%, and 0.2%) were prepared by dilution of this solution. One additional sample of different siRNA sequences was used for method development Solutions of 0.40 mg/mL was prepared by dissolving the correspondent mass of siRNA samples in Milli-Q water. The sample was labeled as 100% solution of "Duplex A" and "Duplex B".

Instrument

ACQUITY UPLC H-Class PLUS Bio System consisted of quaternary solvent manager QSM, column heater module CM-A, active column preheater APH, flow-through needle sample manager FTN SM, and photodiode array detector PDA equipped with titanium cell.

Data Management

Sample integration and quantitation was performed Empower 3.0 Software.

Method Conditions

Column 1:	ACQUITY UPLC Peptide BEH C_{18} Column, 300 Å, 1.7 µm, 2.1 mm x 150 mm (part no. 186003687)
Column 2:	ACQUITY Premier Oligonucleotide BEH C_{18} Column, 300 Å, 1.7 µm, 2.1 mm x 150 mm (part no.

	186010541)
Mobile phases:	Mobile phase 1: 0.07% (v/v) TEA and 0.60% v/v HFIP solution in Milli-Q water (5 mM TEA, 60 mM HFIP aqueous solution) Mobile phase 2: 70/30% v/v Methanol/acetonitrile Mobile phase 3: 85/15% (v/v) Mixture of mobile phase 1 and 2 Mobile phase 4: 30/70% (v/v) Mixture of mobile phase 1 and 2
Column temperature:	75 °C
Detection (UV):	260 nm PDA, titanium 5 mm detector cell
Injection volume:	3 µL

Gradient

Time (min)	Flow (mL/min)	A (% vol)	C (% vol)	D (% vol)	Curve
0	0.30	100	0	0	Initial
20	0.30	0	100	0	6
25	0.30	0	0	100	6
26	0.30	0	0	100	6
28	0.30	100	0	0	6
34	0.30	100	0	0	6

Data Treatment and Analysis

Peak area was calculated from UV 260 nm chromatograms by summing all dominant peaks areas (groups at ~15.9 min plus 17.5 min). The summed peak area is reported in Tables 1–4. For integration example see Figure 2.

Results and Discussion

Oligonucleotide Analysis on Conventional ACQUITY UPLC Peptide BEH C_{18}, 300 Å Column

In an initial experiment, ACQUITY UPLC Peptide BEH C_{18}, 300 Å Column is evaluated for analysis of siRNA. The samples were injected on a new column without previous conditioning. Figure 1 shows the results for initial five injections of Duplex C using denaturing IP-RP LC. Please note that signals close to 15.9 min and 17.5 min are single stranded oligonucleotides created by denaturation of siRNA duplex C. Because the oligonucleotide contains one or more phosphorothioate linkages, they are resolved into additional peaks is due to presence of the diastereomers.

Figure 1 shows that the initial injections have lower UV area than expected and the signal increases for the latter injection. This observation is consistent with a phenomenon described in literature (3–7), Briefly; the acidic oligonucleotides are adsorbed on the column metal surfaces (predominantly frits) by ionic interactions. This results in an incomplete sample recovery. Repeated injections saturate the available ionic adsorption sites and the sample recovery improves. This phenomenon is known as “sample conditioning”.

The area of first five injections of Duplex C is listed in the Table 1. While the conventional stainless-steel columns can be conditioned with repetitive sample injections of with other conditioning protocols to obtain consistent oligonucleotide recovery, the ionic adsorption of acidic samples on metallic column surfaces complicates the method development and sample quantitation. Therefore, we switched to ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column which has metal hardware modified with hybrid-silica layer. According to manufacturer, this modification eliminates the undesirable adsorption of oligonucleotides to column hardware.

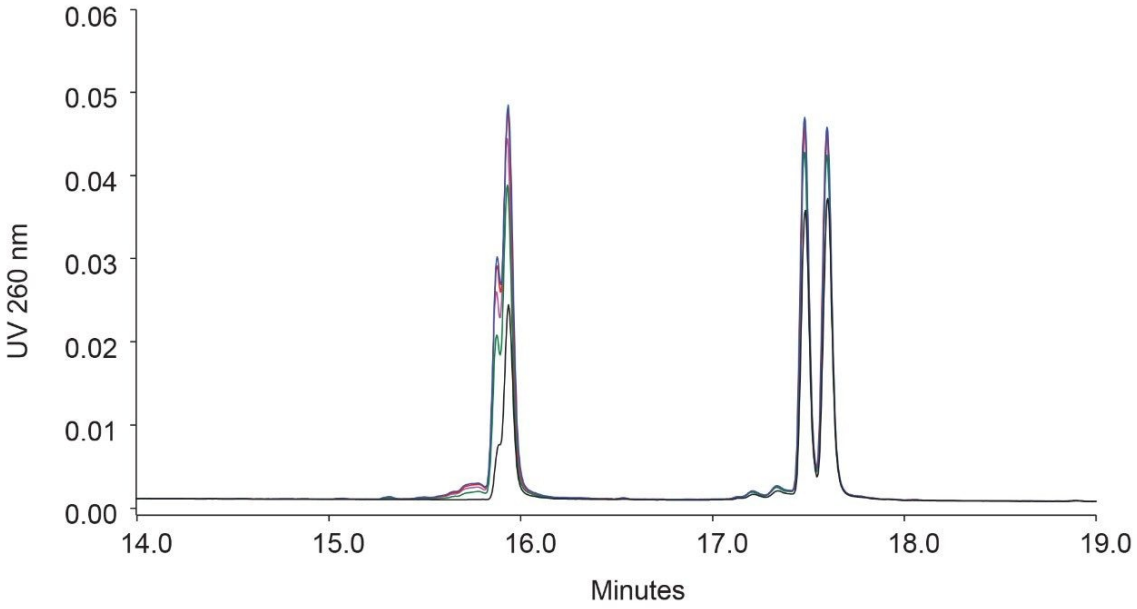

Figure 1. Five consecutive injections of 3 µL of Duplex C (0.40 mg/mL) using the ACQUITY UPLC Oligonucleotide BEH C_{18}, 300 Å Column. Injection 1 – black, injection 2 – green, injection 3 – pink, injection 4 – red, and injection 5 – blue line.

Replicate (n=5)	Peaks area (AU)
1	335658
2	474897
3	528923
4	559179
5	573862
RSD (%)	19.52

Table 1. Area results from chromatograms shown in Figure 1. The 'Peaks Area' corresponds to the sum of UV area of the main peaks in each replicate.

Oligonucleotide Analysis on ACQUITY Premier Oligonucleotide BEH C_{18} 300 Å Column

Figure 2 shows four injections of same sample (Duplex C) under the same LC conditions as described in previous section. The sample analysis was performed using ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column. Because the column is packed with the same sorbent as the conventional column used in Figure 1, the retention and separation of oligonucleotides is expected to be similar. As expected, the ACQUITY Premier Column shows consistent recovery for all four injections (peak areas are listed in Table 2). The variability in signal is so minimal that the four chromatograms in Figure 2 are practically indistinguishable. The results suggest that no detectable sample loss is observed on ACQUITY Premier Column; therefore we selected the ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column for further siRNA method development.

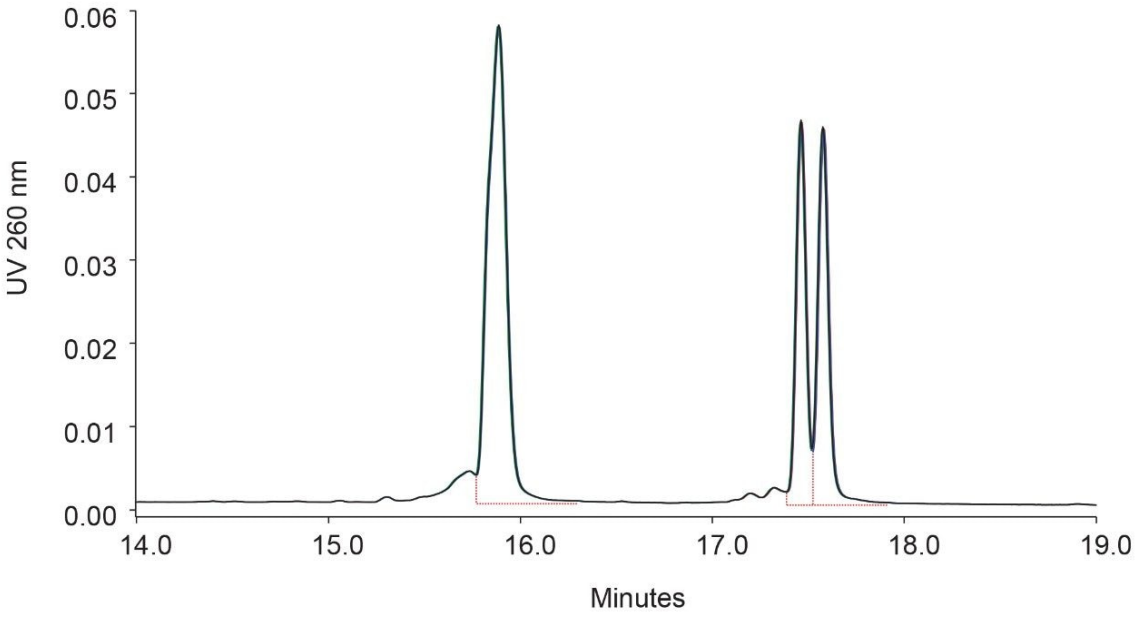

Figure 2. Four consecutive injections of 3 µL of Duplex C (0.40 mg/mL) using the ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column.

Replicate (n=4)	Peaks area (AU)
1	724961
2	726678
3	723674
4	724691
RSD (%)	0.17

Table 2. Area results from chromatograms shown in Figure 2. The 'Peaks Area' corresponds to the sum of UV area of the main peaks in each replicate.

Linearity, Recovery, Repeatability, and Accuracy of Oligonucleotide Duplexes on ACQUITY Premier Oligonucleotide BEH C_{18} 300Å Column

The analytical method developed with ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column was further qualified to evaluate whether it is fit for purpose for siRNA purity analysis. The parameters studied were calibration linearity, analyte recovery, and quantitation accuracy at concentration levels required for quality control application. Accuracy and recovery data at required concentration range for Duplex C are listed in Table 3. All the results are well within acceptance criteria.

Nominal concentration (%)	Measured recovery (%)
0.20	100.25
2.0	96.92
4.0	97.04
8.0	97.46
70.0	99.89
100.0	100.00*
130.0	100.02

Table 3. Individual recovery of Duplex C at different nominal concentrations considering 100.0% solution as normalization level.

** The value of 100% standard was used for data normalization.*

The Duplex C relative recovery for the lowest nominal concentration 0.20 was 99.88% for an initial injection without previous conditioning, and the recovery of the control reference solution at nominal concentration 100% was 100.07% for additional injection after all the nominal standards listed in Table 3 were injected. These numbers are exceptionally good for oligonucleotide compounds and indicate a minimal impact of sample carryover. The carryover was studied in more details in a separate study; the results are presented in the application note.[8]

% Relative Standard Deviation data of three replicates of 0.20% solution and five replicates of 100% solution for Duplex C is presented in Table 4. The results are well within the acceptance criteria (RSD ≤15.0% for three replicates at nominal concentration 0.20%; RSD ≤2.0% of five replicates at nominal concentration 100%).

Nominal concentration (%)	Peaks area (AU)	% RSD
Replicate 1 – 0.20%	5536	
Replicate 2 – 0.20%	5769	
Replicate 3 – 0.20%	5686	2.09 (n=3)
Replicate 1 – 100%	2618322	
Replicate 2 – 100%	2619747	
Replicate 3 – 100%	2619513	
Replicate 4 – 100%	2618797	
Replicate 5 – 100%	2611356	0.13 (n=5)

Table 4. %RSD at quantitation limit –0.20% and repeatability of 100% solution for Duplex C. The 'Peaks Area' corresponds to the sum of UV area of the main peaks in each replicate.

In the subsequent study we evaluated the linearity of quantitation for mixture of two siRNA molecules, Duplex A + Duplex B as test analytes. Linearity was evaluated using both ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å and ACQUITY Peptide BEH C_{18}, 300 Å Columns. (See Figure 3).

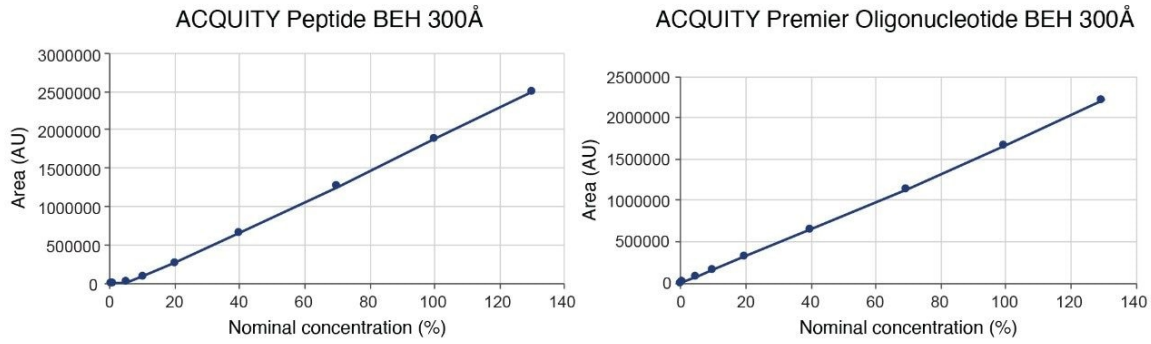

Figure 3. Calibration curves obtained with Duplex A + B mixture using conventional ACQUITY Peptide BEH C_{18}, 300 Å Column or ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column. Conventional column shows sample loss at low nominal concentration.

While the linear fit of the calibration curve has acceptable correlation coefficient for both columns (0.9977 and

0.9995, respectively), the signal at nominal concentrations 0.2% and 1.0% is equal to 0 for conventional stainless-steel column. Due to the nature of non-specific adsorption the sample loss is more pronounced at low concentrations, which leads to reduced dynamic range of calibration. In other words, it is not possible to reliably quantify the oligonucleotides on conventional columns at low nominal concentrations. Minor loss of linearity was observed for the ACQUITY Premier Column; the recovery values are shown in Table 5. The minor sample loss is related to metallic adsorption sites present in UPLC system (needle, tubing, detector cell).

Nominal concentration (%)	Conventional column sample recovery (%)	ACQUITY Premier Column sample recovery (%)
0.2	0	75.4
1.0	0	87.6
5.0	8.5	94.6
10.0	44.5	96.3
20.0	70.4	97.2
40.0	87.1	97.6
70.0	95.4	97.5
100.0	Normalization level	Normalization level
130.0	101.5	102.2

Table 5. Recovery values at different nominal concentrations injected in the linearity study of Duplex A + B sample. For the calibration curves see Figure 3.

Conclusion

Ion-pair reversed-phase denaturing LC method is suitable for analysis and quality control of siRNA. The denatured oligonucleotides were separated and quantified in the single strand form. ACQUITY Premier Columns with MaxPeak High Performance Surfaces Column hardware modified with hybrid-silica layer show superior recovery of the oligonucleotide sample compared to conventional stainless-steel columns packed with the same sorbent. We have shown that ACQUITY Premier Oligonucleotide BEH C_{18}, 300 Å Column is suitable for robust analysis of siRNA without a compromise or need for lengthy column conditioning. The ACQUITY Premier Column

afforded higher confidence in accuracy of quantitative results; its performance fulfils the quality requirements for the qualification parameters of recovery, repeatability, and linearity.

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ACQUITY UPLC H-Class PLUS Bio System <https://www.waters.com/10166246>

ACQUITY UPLC PDA Detector <https://www.waters.com/514225>

Empower Chromatography Data System <https://www.waters.com/10190669>

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