

## Analysis of siRNA Duplexes at Non-Denaturing UPLC Conditions Using MaxPeak Premier Column Technology

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### Abstract

We explored the application of the Waters ACQUITY UPLC H-Class System with Oligonucleotide BEH C<sub>18</sub> 300 Å Column for the analysis of a mixture of two silencing RNA or small interfering RNA (siRNA) at non-denaturing conditions. A drug formulation consisting of two combined siRNA duplexes was analyzed with the goal to separate and quantify the intact duplexes, and their single stranded oligonucleotide contaminants. We developed the Ion-pair Reversed-Phase (IP-RP) UPLC method with UV detection for the therapeutic characterization. The column was operated at non-denaturing conditions (column temperature 20 °C) to maintain siRNA species in duplex form and achieve good chromatographic resolution from the single stranded oligonucleotides. We found that MaxPeak Premier Column Technology provides an excellent sample recovery, separation selectivity, quantitation repeatability and linearity for siRNA analysis.

### Benefits

- The Oligonucleotide BEH C<sub>18</sub>, 300 Å Column provides accurate and repeatable separation of siRNA duplexes

- ACQUITY Premier Columns eliminate an undesirable oligonucleotide adsorption on metal surfaces and column frits. On-column degradation and significant carry over were not observed with this column
- Chromatographic resolution of siRNA duplexes permits a confident identification and quantification of the individual species. Duplex molar ratio and accurate quantitation the duplex impurities/variants in drug product formulations were obtained
- Accurate determination of the excess of single strand oligonucleotides was achieved

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## Introduction

Oligonucleotides and siRNA duplexes belong to a growing class of nucleic acid based therapeutic compounds.<sup>1,2</sup> IP-RP LC method is suitable for sensitive analysis of these new modalities in both LC-UV and LC-MS setup. siRNA molecules are short (~20–25 base pairs), chemically modified RNA duplexes capable of *in-vivo* regulation of protein expression. siRNA can be analyzed in both at denaturing conditions when the duplex is transformed into two complementary oligonucleotides<sup>3</sup>, or in non-denaturing conditions as intact duplexes.<sup>4</sup> The latter method is used for example when it is desirable to estimate the excess of single stranded oligonucleotides in the siRNA duplex formulation. Non-denaturing IP-RP LC assay plays a pivotal role in the optimization of annealing procedure following the oligonucleotides synthesis. In this application note we describe analysis of formulation comprising of two siRNA duplexes. Quantitative measurement and characterization of the complex mixture is paramount for the safety and efficacy of the drug product formulations. IP-RP LC with MS detection is important for the interpretation of sample components in the complex analysis.

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## Experimental

### Sample Preparation

Reference Solutions of samples were prepared in demineralized water. Reference Solution of the two siRNA duplexes was prepared at concentration of 2.0 mg/mL; this represents 100% standard. Related reference solutions were prepared at 70, 100, and 130% of the Reference Solution concentration (high level) and 0.2, 2.0,

4.0, and 6.0% concentration (low level). The drug formulation was dissolved at a nominal concentration of 2.0 mg/mL in demineralized water. Individual single strand oligonucleotide standards and individual siRNA duplexes ("Duplex 1" and "Duplex 2") were prepared at concentrations of 0.10 mg/mL in demineralized water and used for separation selectivity study.

## 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 analytical flow cell. It is recommended to use THF/Hexane-compatible check valves (p/n: 700005273 <<https://www.waters.com/nextgen/us/en/shop/service-parts--kits/700005273-assembly-check-valve-dual-ball--seat.html>> ) in the QSM pump to ensure proper function with HFIP solvents. Preheater function in the Column Manager is enabled. Sample Manager (SM) Wash fluid was methanol, SM Purge and Seal wash fluids were water/acetonitrile (90/10, v/v).

## Data Management

Sample integration and quantitation was performed with Empower v3.0 Software.

## Method Conditions

Column: Oligonucleotide BEH C<sub>18</sub> Column, 300 Å, (p/n: 186010541). To ensure a reliable resolution of the duplexes with the column after the storage the column was rinsed with acetonitrile/water/formic acid (90/10, 0.1, v/v) for at least 2 hours at the flow rate of 0.30 mL/min and at column temperature of 50 °C.

Mobile phase A: 0.2% Hexylamine (HA) and 0.5% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution (v/v) in Milli-Q water (concentration correspond to 15 mM HA and 47.5 mM HFIP aqueous solution). To prepare the mobile phase A transfer 500 mL water into a 1000-mL volumetric flask and using a pipette add 5.0 mL of HFIP followed by 2.0 mL of HA. Mix well with magnetic stir bar until the amine dissolves. Add remaining 493 mL of water to total volume of 1000 mL and mix well.

Mobile phase B: 80% Methanol/20% acetonitrile (v/v). To prepare mobile phase B mix 800 mL of methanol with 200 mL of acetonitrile. Mix well before use.

Column temperature: 20 °C

Auto-Sampler temperature: 10 °C

Flow rate: 0.15 mL/min

Detection: UV 260 nm, sampling rate 5 points/s

Injection volume: 6 µL

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*A programmed linear gradient is listed in Table 1.*

## Gradient

Time (min)	Flow (mL/min)	A (% vol)	B (% vol)	Curve
0	0.15	50	50	Initial
30	0.15	20	80	6
31	0.15	50	50	6
40	0.15	50	50	6

## Data treatment and analysis

Example of peak area estimation is shown in Figure 1. Peak area was calculated from chromatogram(s) by summing peaks areas in Duplex 1 region (peaks eluting near 21.19 min) and Duplex 2 region (peaks eluting near 23.34 min). The summed peak area was used for method qualification (see Table 2).

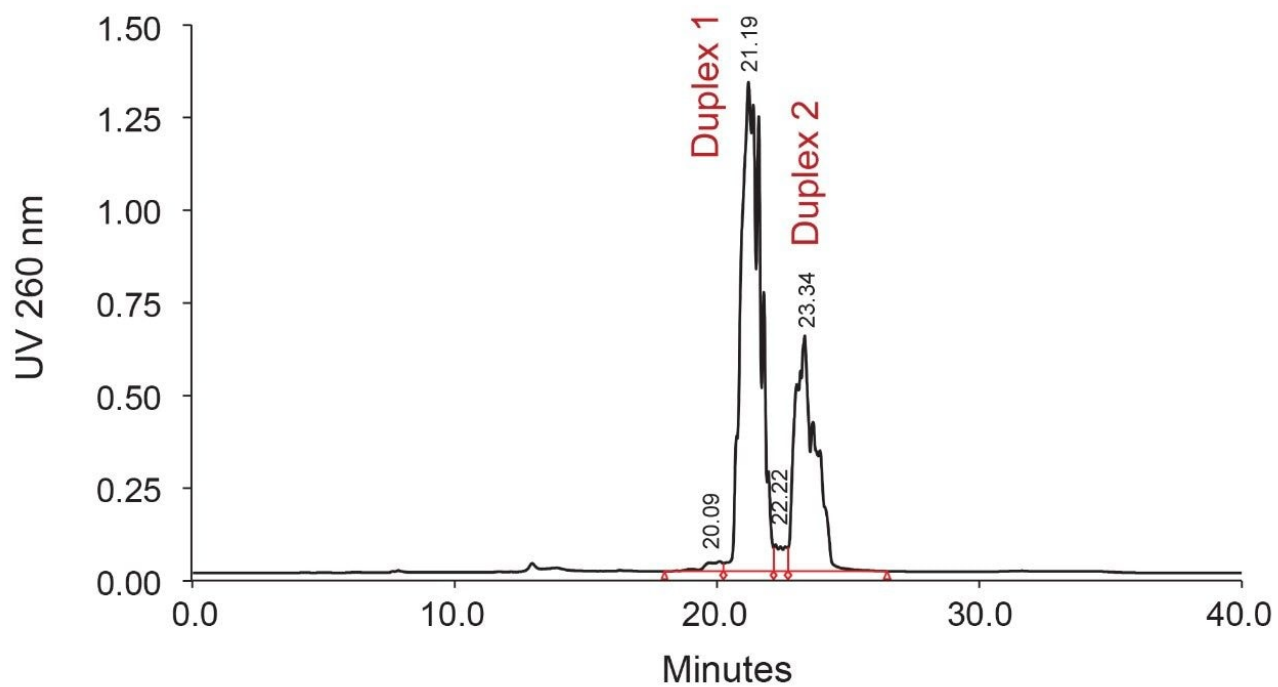


Figure 1. Analysis of reference solution, mixture of two siRNA duplexes by non-denaturing IP-RP LC. siRNA species elute in duplex form. The resolution of Duplex 1 and Duplex 2 into multiple peaks is due to presence of diastereomers in the parent oligonucleotides.

## Method qualification summary

Test/conc. level *	Acceptance range	Observed value	Result
0.2%	Recovery 50–150%	92.8%	pass
2%	Recovery 70–130%	97.3%	pass
4%	Recovery 80–120%	99.9%	pass
6%	Recovery 80–120%	101.1%	pass
70%	Recovery 95–105%	103.6%	pass
100%	Recovery 95–105%	102.5%	pass
130%	Recovery 95–105%	101.8%	pass
Precision 100%	Repeatability: $\leq 5.0\%$	0.1–0.2%	pass
Linearity 0.2–130%	$R \geq 0.99$ ; $RSD \leq 10.0\%$	1.00; 3.6%	pass
Detection limit 0.1%	Detectable	$S/N \geq 3$	pass

Table 2. IP-RP LC method siRNA duplex analysis results.

## Results and Discussion

### Coping with non-specific siRNA loss in LC

It has been reported that oligonucleotides and duplex nucleic acid suffer from high affinity towards positively charged metal surfaces.<sup>5–9</sup> Sample loss was observed for oligonucleotides in our lab using conventional ACQUITY UPLC Oligonucleotide BEH C<sub>18</sub>, 300 Å Column or, in general, columns constructed from stainless-steel materials.<sup>3,6,7</sup> Recent report illustrates that the sample loss occurs predominantly on stainless-steel (and on unmodified titanium) frits used in LC columns construction; this is due to high surface of frits available for the sample adsorption.<sup>6</sup> The sample loss is most apparent for acidic samples (such as nucleic acids) at low concentrations. New column technology based on MaxPeak Premier Column Technology was developed to mitigate the sample loss. The technology employs hybrid-silica to reduce the undesirable sample adsorption within the column hardware (or LC system).<sup>5,8,9</sup> After experiencing an improved sample recovery with ACQUITY Premier Oligonucleotide BEH C<sub>18</sub>, 300 Å Column compared to conventional columns from multiple vendors we selected this column for further method development.<sup>3</sup>

### Separation of siRNA duplexes from single stranded oligonucleotide impurities

Figure 2 shows the optimized method (see Experimental) developed for separation of single stranded oligonucleotides and two siRNA duplexes. In general, duplex species of DNA or RNA are more strongly retained in IP-RP LC compared to single stranded oligonucleotides.<sup>4</sup> Single stranded species elute early in the chromatogram and are well resolved from the duplexes. However, our separation task is complicated by the presence of two separate siRNA duplexes in the formulation. Separation of Duplex 1 from Duplex 2 proved to be a challenge. A long 150 x 2.1 mm ACQUITY Premier Oligonucleotide BEH C<sub>18</sub>, 300 Å Column packed with 1.7 µm sorbent provided the best separation from all tested columns. Please note that both Duplex 1 and 2 (see brown and dark blue chromatograms in Figure 2) are partially resolved into multiple peaks. All the peaks within the siRNA duplex elution region have their expected mass(es). Apparently, the separation is due to presence of optical isomers (diastereomers) present in the duplex species.<sup>10,11</sup> Parent single stranded oligonucleotides contain internucleotide phosphorothioate linkages generating optical isomers. The “peak broadening” due to the presence of diastereomers is apparent also for single stranded species, most notable for antisense peak of Duplex 2.

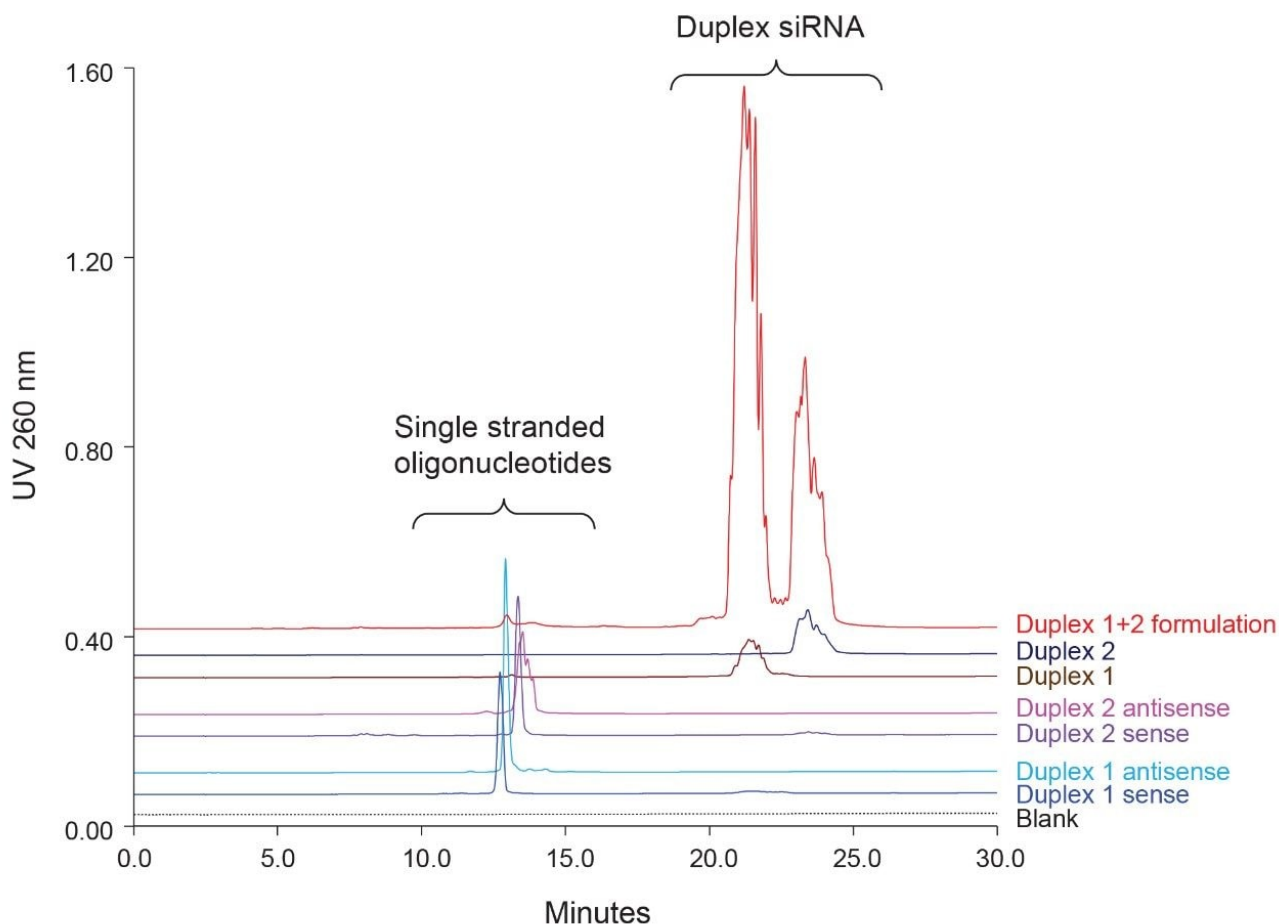


Figure 2. IP-RP LC retention study of single stranded and duplex siRNA constituents.

### siRNA carryover study

The adsorption of sample on metallic LC hardware and standard stainless-steel columns is a suspected contributor to an elevated sample carryover in nucleic acids LC analysis.<sup>6</sup> We evaluated the sample carryover for ACQUITY Premier Oligonucleotide BEH C<sub>18</sub>, 300 Å Column with our optimized method. Figure 3 shows that blank chromatogram does not contain any significant system peaks; we observe a modest baseline drift due to gradient program. Next, we injected 0.2% quantitation limit standard (LOQ sample level) for comparison; acceptable carryover should be significantly lower than the LOQ sample signal. Subsequently we injected 100 % sample standard (data not shown); the response for the main siRNA signals is 1.2 and 0.6 AU, respectively (see red chromatogram in Figure 2) followed by another blank injection (red chromatogram in Figure 3). Figure 3



illustrates that the carryover was below the noise level. This result confirms that carryover does not compromise the quantification results.

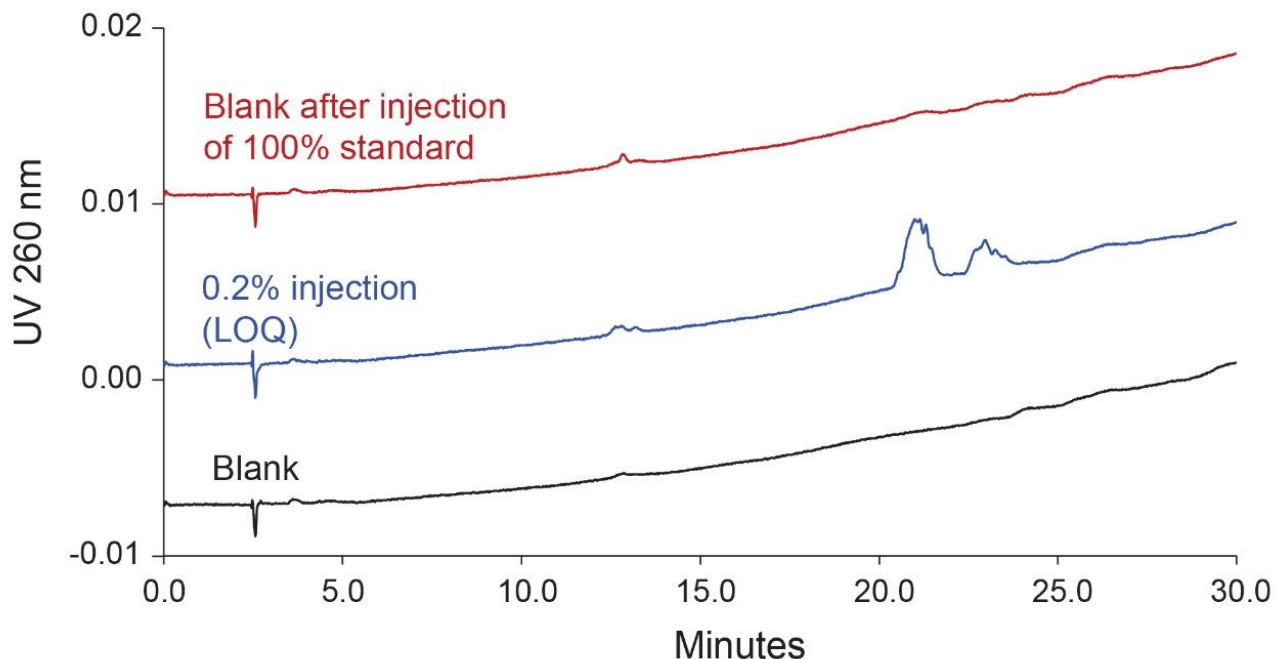


Figure 3. IP-RP LC carryover study.

## Conclusion

Ion-pair reversed-phase non-denaturing IP-RP LC method is suitable for separation of single stranded impurities from siRNA duplex. The optimized ion pairing system permitted separation and characterization of two siRNA duplexes present in the drug formulation. Mobile phase was compatible with MS detection which permitted the confirmation of eluting species according to their molecular weight. We demonstrate that ACQUITY Premier Oligonucleotide BEH C<sub>18</sub>, 300 Å Column is suitable for robust quantitation of siRNA duplexes without compromises or carryover. The ACQUITY Premier Column afforded higher confidence in accuracy of quantitation; its performance fulfils the quality requirements for the qualification parameters of recovery, repeatability, and linearity.

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## Acknowledgements

Pratheeba Yogendrarajah, Willy Verluyten, Jean-Paul Boon, Mario Hellings - Analytical Development, Janssen Pharmaceutical Companies of Johnson & Johnson

Evelien Dejaegere, Leslie Napoletano, Martin Gilar - Waters Corporation

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720007361, September 2021

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