Ultra-sensitive quantification of GalNAc- and lipid-conjugated siRNA using trap-and-elute microflow LC

Increasing sensitivity for oligonucleotide quantification using the SCIEX Triple Quad 7500 system, powered by SCIEX OS software

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This technical note describes how to achieve ultra-low limits of quantification for small interfering RNAs (siRNAs) conjugated with lipid and N-Acetylgalactosamine (GalNAc) (Figure 1) in rat plasma by using a microflow trap-and-elute method and a highly sensitive triple quadrupole mass spectrometer.

Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as their potency improves and delivery challenges are addressed. Modalities such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) are becoming more critical due to their high specificity and ability to reach formerly untreatable targets. Sensitive and robust methods for quantitative analysis of oligonucleotides are needed to support the development of these therapeutics. Hybridization methods can achieve sensitive detection limits, however, this type of assay is unable to differentiate oligonucleotides from impurities and metabolites. LC-MS/MS can provide excellent selectivity, high sensitivity and the ability to analyze multiple oligonucleotides in a single assay. However, sensitivity can still be insufficient for studies in which the sample is limited, such as preclinical pharmacokinetic or pharmacodynamic studies in rats or mice.

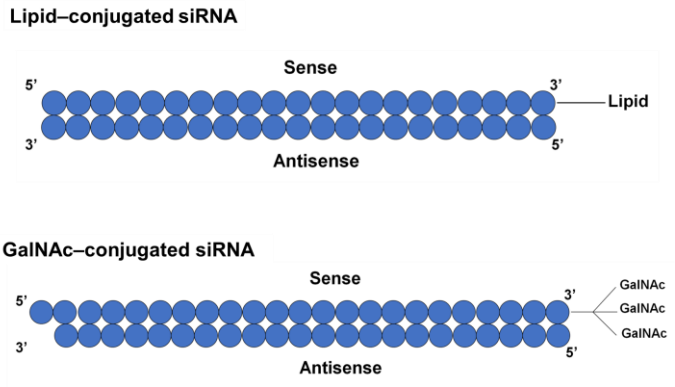

Figure 1. Structural representation of lipid-conjugated and GalNAc-conjugated siRNA. Modifications were linked to the 3' end of the strand.

Published data show that microflow LC lowered the LLOQ by an order of magnitude for several ASOs in an LC-MS/MS assay using the QTRAP 6500+ system.[1] The use of a trap-and-elute microflow approach allowed injection of large volumes of extracted plasma samples, up to 30 µL, without significantly increasing run times. This technical note describes using the same microflow setup to achieve ultra-low LLOQs for lipid- and GalNAC-conjugated siRNAs.

Key features of the microflow workflow using the SCIEX 7500 system for oligonucleotide quantification

- Achieve low-pg/mL LLOQs for lipid- and GalNAc-conjugated siRNAs in rat plasma for pharmacokinetic studies faced with sample limitations that require improved specificity compared to traditional hybridization assays
- Attain high sample throughput without increasing method run time by performing large sample volume analysis
- Save time, using the OptiFlow Pro ion source with E Lens probe, by easily switching between analytical and microflow setup with no need for electrode position optimization
- Increase robustness and decrease costs with reduced consumption of LC-MS grade ion-pairing reagents
- Leverage the greater ion generation and ion transmission on the SCIEX 7500 system that enable significant gains in sensitivity and result in lower LLOQs, compared to the previous generation QTRAP 6500+ system
- Increase productivity with the user-friendly interface and integrated platform for data acquisition, processing and management in the SCIEX OS software

Methods

Samples and reagents: siRNAs conjugated with 21-mer lipid or 22-mer GalNAc were obtained from a collaborator. Internal standards for both siRNAs with similar structures to the target compounds were also obtained from a collaborator. The ion-pairing reagents 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, ≥ 99.8%) and diisopropylethylamine (DIEA, ≥ 99.5%) were purchased from Sigma Aldrich.

Sample preparation: Calibration curves were prepared separately for both siRNAs by spiking analyte and internal standard (IS) in extracted rat plasma (Sprague Dawley, K2 EDTA, BioIVT). The IS for the lipid-conjugated siRNA was spiked in at a concentration of 2 ng/mL and the IS for the GalNAc-conjugated siRNA was spiked in at 5 ng/mL. An aliquot of 1 mL plasma samples were extracted using Clarity OTX solid phase extraction (SPE) cartridges (Phenomenex) following the manufacturer's protocol for extracting oligonucleotide therapeutics from biological samples. After drying with nitrogen gas at 40°C, the plasma extract was reconstituted in 1 mL mobile phase A.

Chromatography: A M5 MicroLC system was used in trap-and-elute mode. A separate sleeve-type column oven was used to control the trap column temperature. Gradient and other chromatographic conditions for both assays, and columns used, are summarized in Tables 1 and 2. A volume of 30 µL of reconstituted sample was loaded onto the trap column.

Table 1. Chromatographic conditions.

Parameter	Setting
Mobile phase A	*water + 15 mM N,N-diisopropylethylamine + 35 mM hexafluoroisopropanol*
Mobile phase B	*methanol*
Trap column	*0.3 x 5 mm, 3 µm, 120 Å, YMC Triart C18*
Trap column temperature	*70°C*
Analytical column	*0.3 x 50 mm, 1.7 µm, 130 Å, Waters BEH C18*
Analytical column temperature	*70°C*
Injection volume	*30 µL*

Loading flow rate was 35 µL/min for 2 min, after which the trap valve was switched and the sample eluted onto the analytical column. The analytical gradient was ramped from 5% to 90% mobile phase B in 3 minutes for the lipid-conjugated siRNA and from 10% to 30% B in 3 minutes for the GalNAc-conjugated siRNA. Flow rate was 5 µL/min. The trap valve was switched back to the load position 3.5 minutes after the start of the analysis for washing the trap at the loading flow rate.

Table 2. LC method for lipid-conjugated siRNA and GalNAc-conjugated siRNA. The % B values in parentheses are for the GalNAc-conjugated method.

Time (min)	Loading pump %B	Analytical pump %B	Trap valve position
0.0	*0*	*5 (10)*	*LOAD*
2.0	*0*	*5 (10)*	*INJECT*
3.0	*60*		
5.0		*90 (30)*	
5.5		*90 (80)*	*LOAD*
7.0	*60*		
7.2	*90*		
8.0	*90*		
8.2	*5*		
9.0		*90 (80)*	
9.2		*5 (10)*	
11.0	*5*	*5 (10)*	

Mass spectrometry: A SCIEX 7500 system with an OptiFlow Pro ion source with E Lens probe and a microflow probe and electrode was used in MRM mode. All source and MS parameters were optimized and are reported in Tables 3, 4a and 4b. The MS methods included multiple MRM transitions for both the sense and antisense strand of each analyte and internal standard (IS).

Table 3. Source conditions.

Parameter	Setting
Polarity	*Negative*
Curtain gas	*32 psi*
Gas 1	*20 psi*
Gas 2	*40 psi*
CAD gas	*12*
Ion spray voltage	*-3000 V*
Source temperature	*100°C*

Data processing: MRM data were processed with SCIEX OS software 2.0 using the MQ4 integration algorithm. A $1/x^2$ weighting was used for quantification.

Table 4a. MRM transitions and MS parameters for the lipid-conjugated siRNA method.

ID	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Dwell time (ms)	Q0D (V)	CE (V)	CXP (V)	EP (V)
*lipid-siRNA-AS 697.40 -134.1**	*697.4*	*134.1*	*25*	*-60*	*-34*	*-23*	*-10*
lipid-siRNA-AS 697.40 -335	*697.4*	*335*	*25*	*-60*	*-34*	*-23*	*-10*
lipid-siRNA-AS 697.40 -593.30	*697.4*	*593.3*	*25*	*-60*	*-30*	*-23*	*-10*
*lipid-siRNA-SS 759.80-134.1**	*759.8*	*25*	*25*	*-60*	*-38*	*-23*	*-10*
lipid-siRNA-SS 690.8-134.1	*690.8*	*134.1*	*25*	*-60*	*-60*	*-23*	*-10*
*IS-AS 685.5-358**	*685.5*	*358*	*25*	*-60*	*-30*	*-23*	*-10*
IS-AS 754.2-358	*754.2*	*358*	*25*	*-60*	*-40*	*-23*	*-10*
IS-SS 711.7-632.1	*711.7*	*632.1*	*25*	*-60*	*-30*	*-23*	*-10*
*IS-SS 711.7-358**	*711.7*	*358*	*25*	*-60*	*-30*	*-23*	*-10*

**Used for quantification.*

Table 4b. MRM transitions and MS parameters for the GalNAc-conjugated siRNA method.

ID	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Dwell time (ms)	Q0D (V)	CE (V)	CXP (V)	EP (V)
*GalNAc-siRNA-AS 620.4-318.9**	*620.4*	*318.9*	*30*	*-60*	*-29*	*-23*	*-10*
GalNAc-siRNA -AS 620.4-410.2	*620.4*	*410.2*	*30*	*-60*	*-41*	*-23*	*-10*
GalNAc-siRNA -SS 676.3-334.3	*676.3*	*334.3*	*30*	*-60*	*-35*	*-23*	*-10*
*GalNAc-siRNA -SS 676.3-319**	*676.3*	*319*	*30*	*-60*	*-41*	*-23*	*-10*
*IS-AS 786.3-604.2**	*786.3*	*604.2*	*30*	*-60*	*-33*	*-23*	*-10*
IS -AS 786.3-410	*786.3*	*410*	*30*	*-60*	*-53*	*-23*	*-10*
*IS -SS 771.3-399**	*771.3*	*399*	*30*	*-60*	*-53*	*-23*	*-10*

**Used for quantification.*

Results and discussion

Figure 2 shows the chromatography for the GalNAc-conjugated and lipid-conjugated siRNAs and their respective internal standards. Antisense and sense strands were chromatographically separated using high column temperatures. No separation was observed between the analyte and internal standard for either siRNAs. Interference between the MRM transitions for the lipid-conjugated siRNA strands and the internal standard strands was observed. In order to keep this interference below the noise level for the analyte, the IS concentration could not exceed 2 ng/mL.

Calibration curves were measured in triplicate by spiking the analytes and their respective internal standards into plasma extract in concentrations ranging from 0.05 to 30 ng/mL. Saturation was observed at concentrations higher than 30 ng/mL. Good accuracy and precision were achieved for both siRNAs (Table 5). LLOQs and linear calibration curve ranges were determined based on the requirements that the %CV of the calculated mean of the concentration is below 20% at the LLOQ and below 15% for higher concentrations, while the accuracy of the calculated mean is between 80% and 120% at the LLOQ and between 85% and 115% for higher concentrations.

The LLOQ for the sense and antisense strands of the lipid- and GalNAc-conjugated siRNAs was 0.05 ng/mL. Considering the higher molecular weights of the (duplex) siRNAs, these LLOQs are comparable to the LLOQs found earlier for ASOs in rat matrix using a similar method on the SCIEX 7500 system.[1] The calibration curves demonstrated good linearity from 0.05 to 30 ng/mL (Figures 3 and 4).

Table 5. Calculated concentration, precision and accuracy for antisense and sense strands of the conjugated siRNAs. All concentrations are based on the antisense/sense duplex.

	Lipid-conjugated siRNA						GalNAc-conjugated siRNA					
	Antisense strand			Sense strand			Antisense strand			Sense strand		
Actual (ng/mL)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)
0.05	0.0508	9.55	101.5	0.0498	13.1	99.52	0.0478	2.07	95.56	0.0512	8.17	102.4
0.1	0.0957	9.15	95.74	0.104	14.0	104.1	0.106	6.36	106.26	0.0916	6.38	91.59
0.3	0.306	12.5	102.1	0.262	14.9	87.28	0.318	5.55	106.01	0.327	7.06	109.0
1	1.04	6.56	104.5	1.12	13.4	112.1	1.08	2.39	107.94	1.07	1.71	107.0
3	3.07	2.77	102.5	2.88	9.37	95.92	2.98	1.46	99.22	3.06	2.95	102.1
10	9.36	5.48	93.64	10.1	3.76	100.6	8.50	1.58	85.01	8.80	6.26	87.99
30	NA	NA	NA	30.1	2.09	100.5	NA	NA	NA	NA	NA	NA

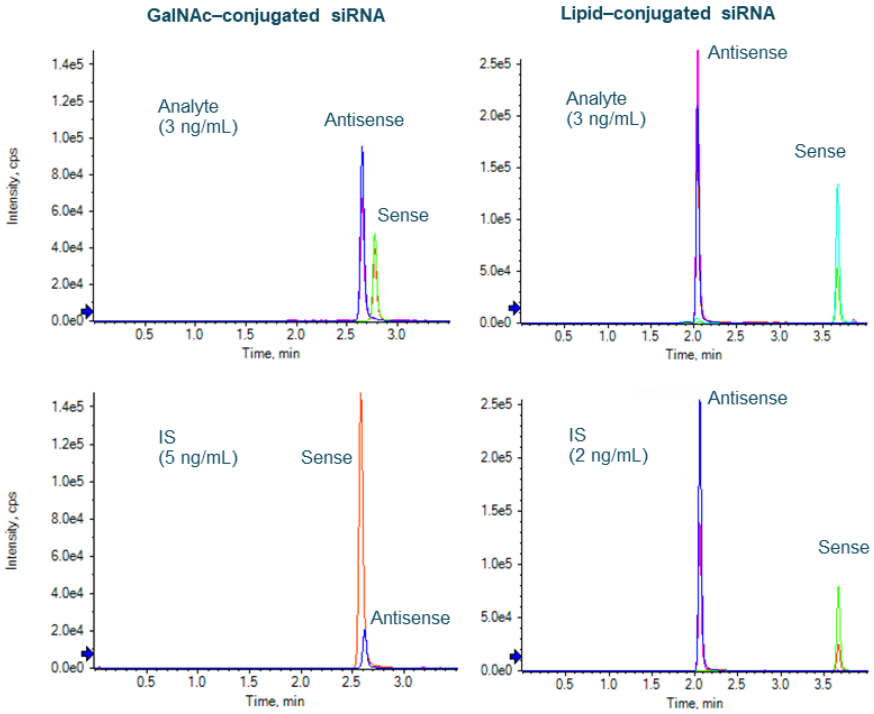

Figure 2. Chromatography for the GalNAc-conjugated (left) and lipid-conjugated siRNA (right) and their respective internal standards. Antisense and sense strands are separated, but no separation was observed between analyte and internal standard for both siRNAs.

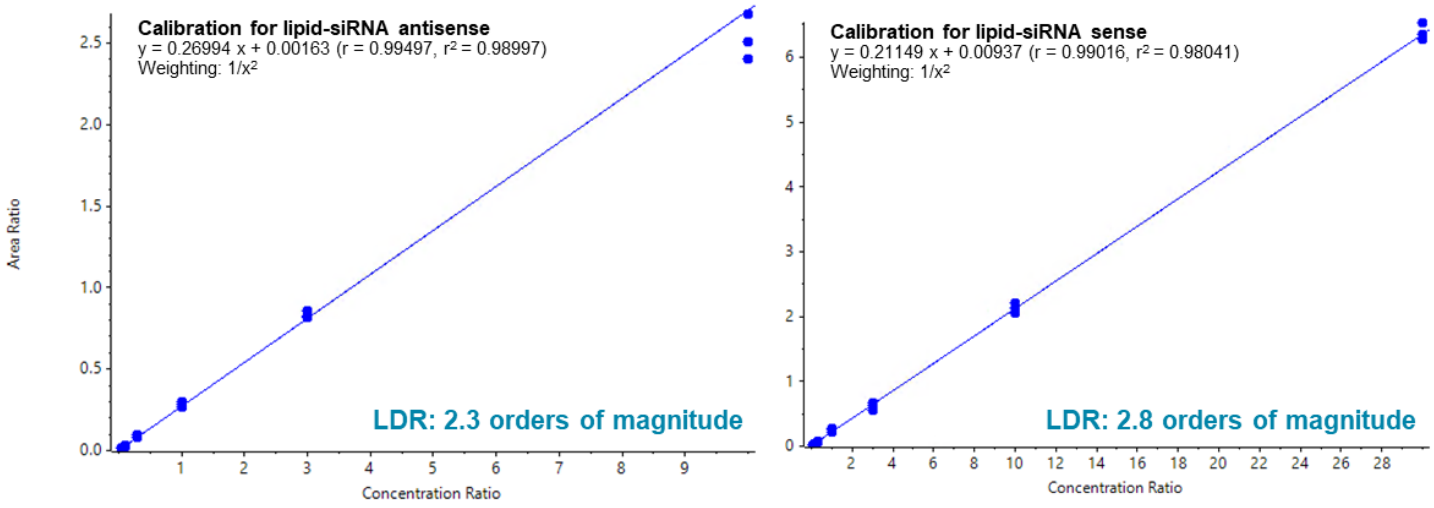

Figure 3. Calibration curves based on IS-adjusted XIC areas for the antisense and sense strands of the lipid-conjugated siRNA. Good linearity was achieved from 0.05 ng/mL to 10 ng/mL for the antisense strand and from 0.05 ng/mL to 30 ng/mL for the sense strand.

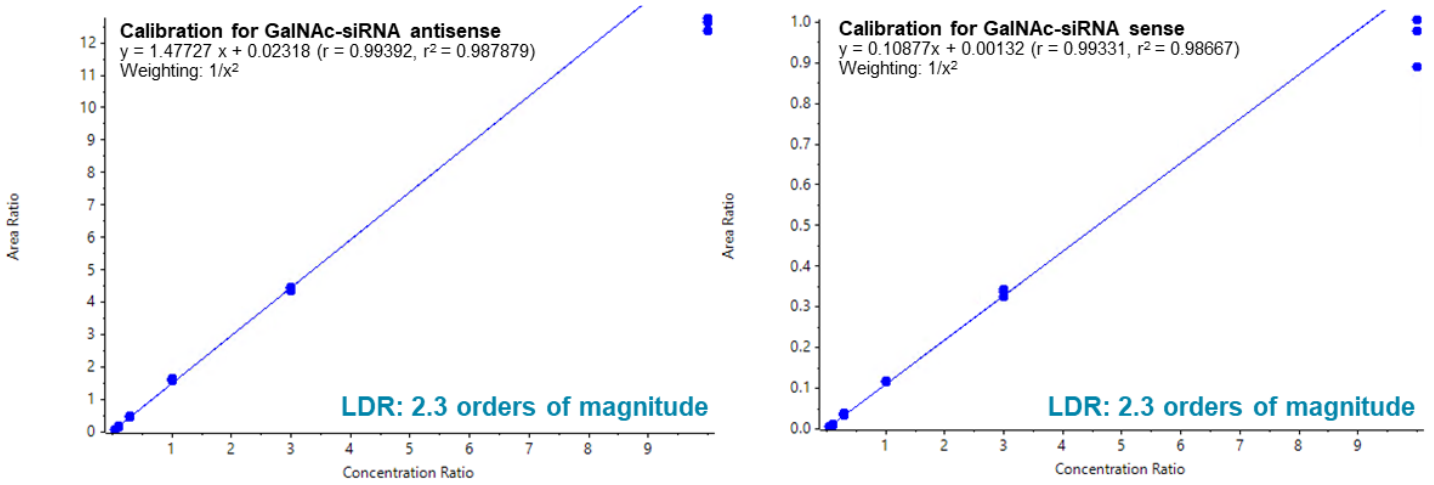

Figure 4. Calibration curves based on IS-adjusted XIC areas for the antisense and sense strands of the GalNAc-conjugated siRNA. Good linearity was achieved from 0.05 ng/mL to 10 ng/mL for both the antisense and sense strands.

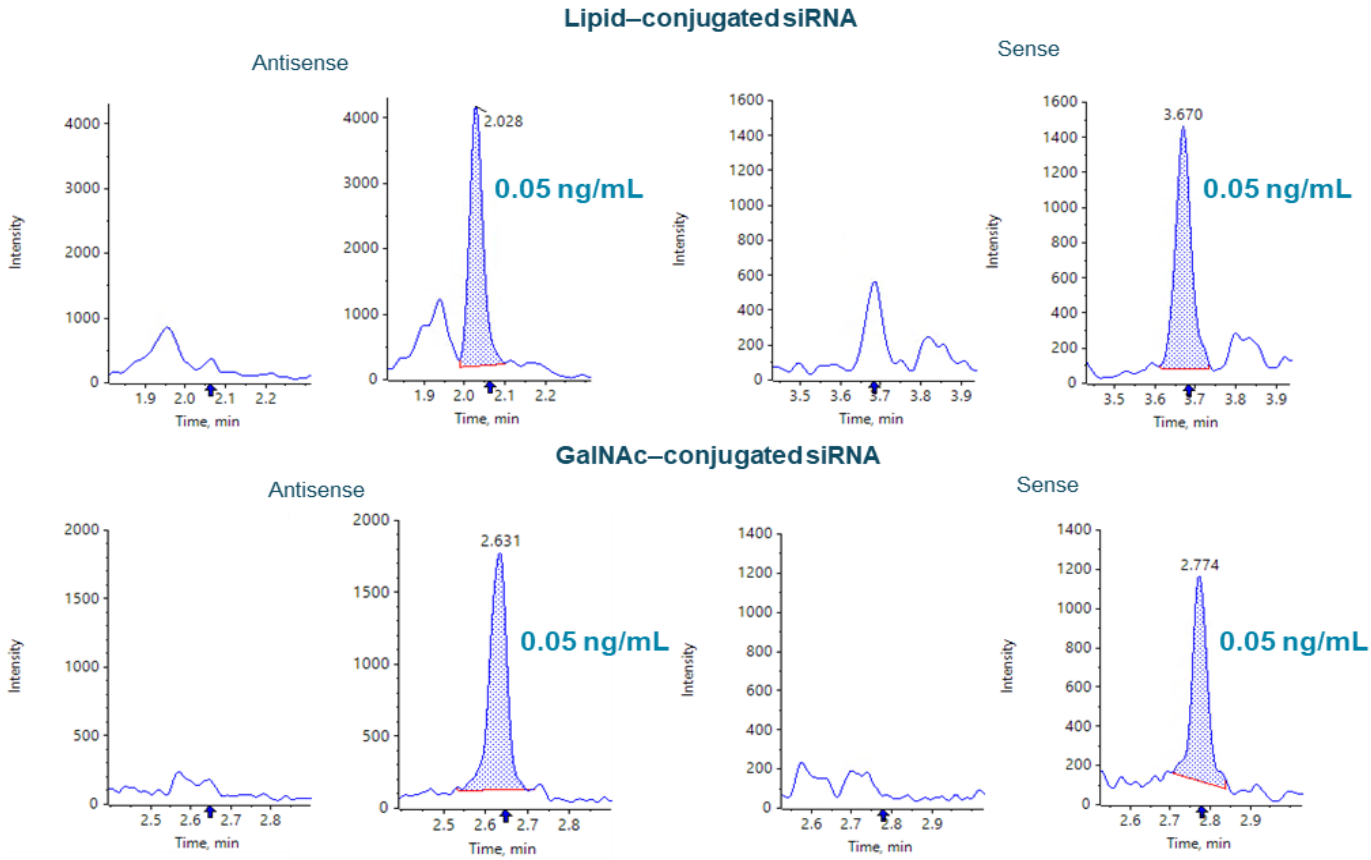

Figure 5. XICs for the transitions of each analyte. Blanks from extracted plasma (left) and ASOs at their LLOQs (right). No noticeable interference was observed in the matrix blank.

Figure 5 shows the extracted ion chromatograms (XICs) of the antisense and sense strands for the lipid- and GalNAc-conjugated siRNAs. For each condition, a blank (left) and spiked plasma injection at the LLOQ level (right) are shown. Only the sense strand of the lipid-conjugated siRNA showed some interference, which was not observed in the double blank sample. This suggests that the interference observed was not chromatographically separated from the IS. The absence of other interferences illustrates the efficient clean-up of the plasma matrix using the Clarity OTX SPE cartridges.

After injecting the upper limit of quantification (ULOQ), carryover was 1% for both the antisense and sense strand of the GalNAc-conjugated siRNA in the first blank and 0.2% in the second blank. For the lipid-conjugated siRNA, the carryover was significantly higher at 3% for the antisense strand and 6% for the sense strand in the first blank, and 1% and 2% respectively in the second blank. This carryover might be reduced, for example, by adding longer column and trap washes or by using higher flow rates. Further work on reducing the carryover will be required to fully utilize the demonstrated dynamic range.

Conclusions

- The sense and antisense strands of lipid-conjugated and GalNAc-conjugated siRNAs were quantified in rat plasma with a LLOQ of 0.05 ng/mL. This was accomplished using a trap-and-elute microflow method coupled to the SCIEX 7500 system that permits pharmacokinetic studies with limited sample availability and that require quantification at trace levels.
- The analysis time using the trap-and-elute microflow method was comparable to that using an analytical flow LC-MS method, resulting in similar sample throughput
- The 10–50x lower solvent consumption of the trap-and-elute microflow method can result in significant savings in expensive, high-purity modifiers such as hexafluoroisopropanol
- The lower solvent consumption of the trap-and-elute microflow method promotes robustness by reducing the level of ion-pairing reagents introduced into the mass spectrometer

References

1. Sub-ng/mL quantification of antisense oligonucleotides in rat plasma using trap and elute with microflow LC. SCIEX technical note, RUO-MKT-02-13015-A.
2. Achieving best in class sensitivity for antisense oligonucleotides in plasma using trap-and-elute microflow LC. SCIEX technical note, RUO-MKT-02-13206-A.

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