Sub-ng/mL quantification of antisense oligonucleotides in rat plasma using trap and elute with microflow LC

Increasing sensitivity for oligonucleotide quantification using the SCIEX QTRAP® 6500+ LC-MS/MS System and M5 MicroLC System

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This technical note describes how the lower limits of quantification (LLOQs) for antisense oligonucleotides (ASOs) in rat plasma are improved by using a microflow trap and elute method. Compared to previously published data acquired using analytical flow LC on a SCIEX QTRAP 6500+ System[1], sensitivity was increased by a factor of approximately 10 (see Table 1 for achieved LLOQs).

Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as their potency improves and delivery challenges are addressed. Modalities such as ASOs are becoming more important due to their high specificity and ability to reach formerly undruggable targets.

Sensitive and robust methods for quantitative analysis of oligonucleotides are needed to support the development of ASO therapeutics. LC-MS/MS has been employed routinely for studies of oligonucleotides. It provides excellent selectivity, sensitivity and the ability to analyze multiple oligonucleotides in a single assay. However, for studies where sample is limited, such as preclinical pharmacodynamic studies in rats or mice, sensitivity can still be insufficient.

Published data shows microflow LC lowered the lower limit of quantification for the ASO fomivirsen in an LC-MS/MS assay.[2] This technical note presents an expanded version of that microflow method. The expanded workflow uses a trap and elute microflow approach that allows injection of larger volumes of extracted plasma samples. The expanded method further lowered the LLOQ for fomivirsen by a factor 3. It also achieved similar LLOQs for two other ASO drugs, nusinersen and eluforsen, and a 20-mer model ASO that was both fully phosphorothioated and 2'O-methylated (methyl-oligo).

Table 1. Lower limits of quantification achieved for different ASOs in rat plasma using a trap and elute microflow method and the SCIEX QTRAP 6500+ System.

Analyte	LLOQ (ng/mL)	ULOQ (ng/mL)	LDR (orders)
20-mer phosphorothioated and 2'O-methylated ASO	*0.03*	*300*	*4.0*
Fomivirsen	*0.03*	*300*	*4.0*
Nusinersen	*0.03*	*100*	*3.5*
Eluforsen	*0.10*	*300*	*3.5*

Key features of the trap and elute workflow for oligonucleotide quantification

- Increased sensitivity is achieved over standard HPLC, resulting in sub-ng/mL LLOQs of ASOs in rat plasma
- Large sample volumes can be injected without increasing the method run time while improving overall method robustness.
- Easy switch between analytical flow and microflow setup without the need for electrode position optimization using the OptiFlow® Turbo V Ion Source used with the QTRAP 6500+ System
- Reduced consumption of LC-MS grade ion-pairing reagents provides significant savings and increases robustness

Methods

Samples and reagents: Fomivirsen, nusinersen, eluforsen, a model fully phosphorothioated 2'O-methylated oligonucleotide with the sequence mU*mA*mU*mC*mC*mG*mC*mC*mU*mC*mG*mU*mG*mA*mG*mA*mA*mG*mA*mU and an internal standard with the sequence CATGGTCCTGCTGGAAGTTCGTG were all purchased from IDT. The ion-pairing reagents 1,1,3,3,3-hexafluoroisopropanol (HFIP, ≥ 99.8%) and diisopropylethylamine (DIEA, ≥ 99.5%,) were purchased from Sigma Aldrich. Ethylenediaminetetraacetic acid (EDTA) was also purchased from Sigma Aldrich.

Sample preparation: Calibration curves were prepared by spiking analytes and internal standard (500 ng/mL) in extracted rat plasma (Sprague Dawley, K2 EDTA, BioIVT). 1 mL plasma samples were extracted using Clarity OTX solid phase extraction (SPE) cartridges (Phenomenex) following the manufacturer's protocol for extracting oligo therapeutics from biological samples. After drying with nitrogen gas at 40°C, the plasma extract was reconstituted in 1 mL mobile phase A containing 100 µM EDTA.

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 and columns are summarized in Tables 2 and 3. 30 µL of reconstituted samples were loaded onto the trap column. Loading flow rate was 35 µL/min for 2 minutes after which the trap valve was switched and the sample eluted onto the analytical column. The analytical gradient was ramped from 5% to 60% mobile phase B in 3 minutes at a flow rate of 5 µL/min. The trap valve was switched back to the load position 3 minutes after the start of the analysis for washing at the loading flow rate.

Table 2. LC method.

Time (min)	Loading pump %B	Analytical pump %B	Trap valve position
0.0	*0*	*5*	*LOAD*
2.0	*0*	*5*	*INJECT*
3.0	*60*		
4.0			
5.0		*60*	*LOAD*
7.0	*60*		
7.2	*90*		
8.0	*90*		
8.2	*5*		
9.0		*60*	
9.2		*5*	
11.0	*5*	*5*	

Table 3. Chromatographic conditions.

Parameter	Setting
Mobile phase A	*Water + 15 mM N,N-diisopropylethylamine + 35 mM hexafluoroisopropanol*
Mobile phase B	*90/10 methanol/water + 15 mM N,N-diisopropylethylamine + 35 mM hexafluoroisopropanol*
Trap column	*0.3 x 5 mm, 3 µm, 120 Å, YMC Triart C18*
Trap column temperature	*80°C*
Analytical column	*0.3 x 50 mm, 3 µm, 120 Å, YMC Triart C18*
Analytical column temperature	*80°C*
Injection volume	*30 µL*

Mass spectrometry: A SCIEX QTRAP 6500+ System with an OptiFlow® Turbo V Ion Source and a SteadySpray™ low-micro electrode was used in MRM mode. All source and MS parameters were optimized (Tables 4 and 5). The MS method included two MRM transitions for each analyte and one MRM transition for the internal standard.

Table 4. Source conditions.

Parameter	Setting
Polarity	*Negative*
Curtain gas	*20 psi*
Gas 1	*30 psi*
Gas 2	*20 psi*
CAD gas	*12*
Ion spray voltage	*-3500 V*
Source temperature	*100°C*

Table 5. MRM transitions and MS parameters.

ID	Q1 mass (*m/z*)	Q3 mass (*m/z*)	Dwell time (ms)	DP (V)	CE (V)	CXP (V)	EP (V)
*Fomivirsen 1**	*667.0*	*319.0*	*20*	*-50*	*-35*	*-12*	*-10*
Fomivirsen 2	*741.0*	*319.0*	*20*	*-50*	*-39*	*-12*	*-10*
Methyl-oligo 1	*693.8*	*358.0*	*20*	*-80*	*-37*	*-12*	*-10*
*Methyl-oligo 2**	*693.8*	*374.1*	*20*	*-80*	*-37*	*-12*	*-10*
Nusinersen 1	*790.7*	*402.0*	*20*	*-60*	*-38*	*-12*	*-10*
*Nusinersen 2**	*790.7*	*393.2*	*20*	*-60*	*-38*	*-12*	*-10*
*Eluforsen 1**	*715.4*	*358.1*	*20*	*-40*	*-35*	*-12*	*-10*
Eluforsen 2	*673.3*	*358.1*	*20*	*-40*	*-32*	*-12*	*-10*
IS 1	*786.1*	*303.1*	*20*	*-80*	*-43*	*-12*	*-10*

**** Used for quantification.***

Data processing: MRM data were processed with SCIEX OS Software 2.0 using the MQ4 integration algorithm.

Results and discussion

Figure 1 shows baseline separation was achieved for the four oligonucleotides and internal standard. MRM transitions used for the quantification of oligonucleotides and their metabolites/impurities often share the same fragment, and there is possibly also some overlap in the precursor because of the large number of charge states with similar intensities. Therefore, it is important to have good chromatographic separation of all analytes and the internal standard.

Calibration curves were measured in triplicate for all of the analytes by spiking the analytes into plasma extract in the concentration range between 0.03 and 300 ng/mL. Excellent precisions and accuracies were achieved for all analytes (Table 6). LLOQs and calibration curve ranges were determined based on the requirements that the %CV of the calculated mean is below 20% at the LLOQ and below 15% for all higher concentrations while the accuracy of the calculated mean is between 80% and 120% at the LLOQ and between 85% and 115% for the higher concentrations.

The LLOQ for eluforsen was found to be 0.1 ng/mL, while the LLOQs for the other oligonucleotides were 0.03 ng/mL. For methyl-oligo, the LLOQ was >10x lower compared to what was previously determined using analytical flow LC[1], demonstrating the sensitivity improvements achieved by using a trap-and-elute microflow setup. The calibration curves showed excellent linearity up to 300 ng/mL: 4 orders of linear dynamic range (LDR) for each compound except nusinersen, which had a linear response up to 100 ng/mL and 3.5 orders of LDR (Figure 2).

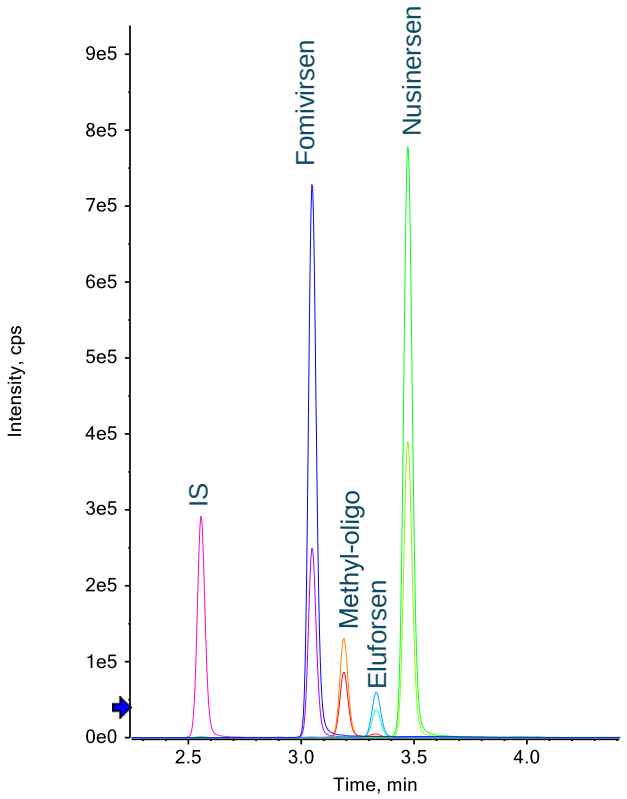

Figure 1. XICs for the MRMs of four ASOs and the IS. Two MRM transitions were used for each analyte, one for the IS. Baseline separation was achieved using a 3 min gradient.

Table 6. Precision and accuracy for each oligonucleotide.

	Fomivirsen			Methyl-oligo			Nusinersen			Eluforsen		
Actual (ng/mL)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)	Mean (ng/mL)	CV (%)	Accuracy (%)
0.03	*0.03059*	*6.45*	*102.0*	*0.03078*	*2.43*	*102.6*	*0.03001*	*5.03*	*100.1*	*N/A*	*N/A*	*N/A*
0.1	*0.09358*	*6.44*	*93.58*	*0.09451*	*2.75*	*94.51*	*0.09986*	*3.20*	*99.86*	*0.09717*	*9.58*	*97.17*
0.3	*0.2904*	*1.48*	*96.81*	*0.2695*	*3.77*	*89.84*	*0.2947*	*10.07*	*98.22*	*0.3177*	*4.20*	*105.9*
1	*1.067*	*3.09*	*106.7*	*1.021*	*4.40*	*102.1*	*1.042*	*2.12*	*104.2*	*1.074*	*1.18*	*107.4*
3	*3.212*	*1.79*	*107.1*	*2.971*	*1.17*	*99.04*	*3.136*	*2.59*	*104.5*	*3.126*	*5.92*	*104.2*
10	*10.43*	*1.56*	*104.4*	*10.18*	*5.08*	*101.8*	*10.26*	*3.27*	*102.6*	*9.900*	*4.26*	*99.00*
30	*30.69*	*3.71*	*102.3*	*30.22*	*3.04*	*100.7*	*30.11*	*1.67*	*100.4*	*29.02*	*5.14*	*96.73*
100	*100.9*	*0.639*	*101.0*	*107.5*	*2.04*	*107.5*	*90.20*	*0.706*	*90.20*	*97.07*	*2.43*	*97.07*
300	*259.2*	*0.735*	*86.38*	*305.6*	*1.66*	*101.8*	*N/A*	*N/A*	*N/A*	*277.5*	*1.39*	*92.51*

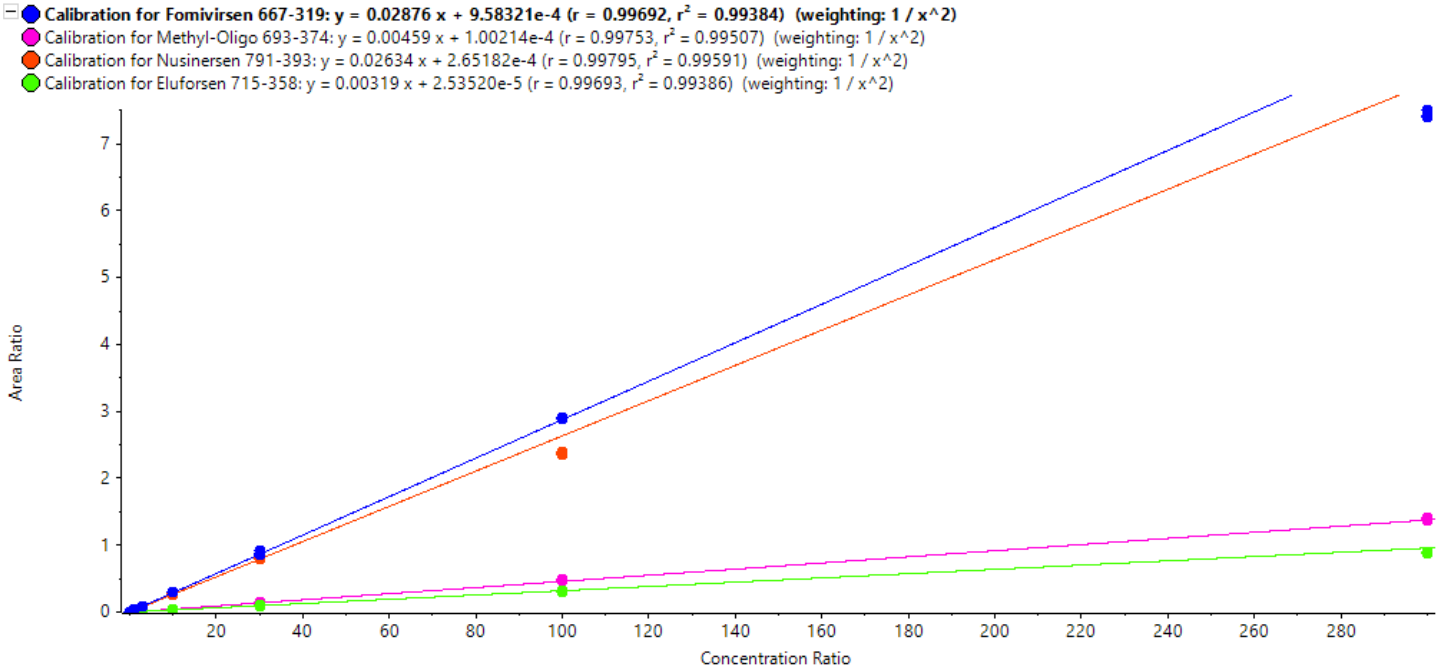

Figure 2. Calibration curves based on IS-adjusted XIC areas for each of the oligonucleotides.

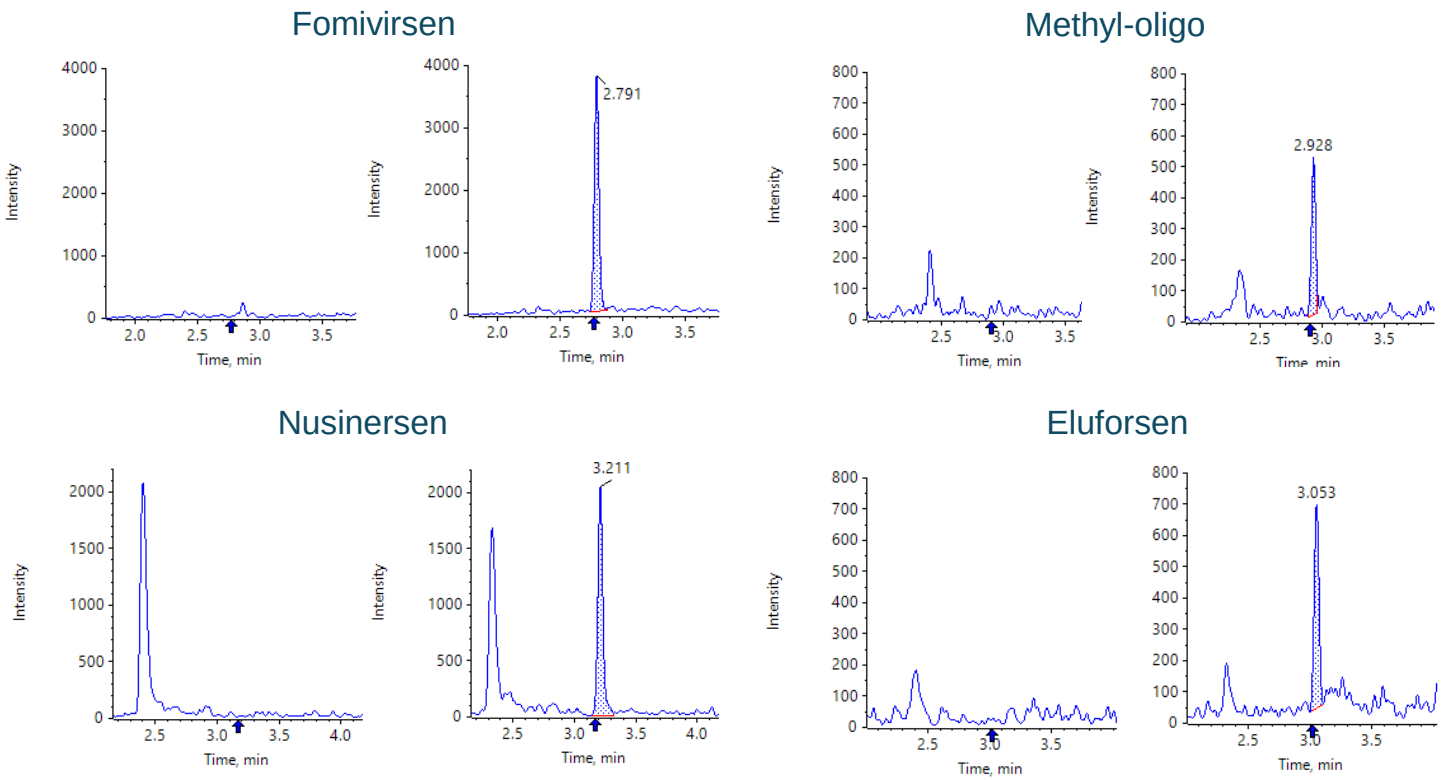

Figure 3. XICs for the transitions of each analyte. Blanks from extracted plasma (left) and ASOs at their LLOQs (right). No noticeable interference was observed in the matrix blank.

Figure 3 shows the XICs for all analytes for a blank extracted plasma injection (left) and at their LLOQ levels (right). No significant interferences were seen, illustrating efficient clean-up of the plasma matrix using the Clarity OTX SPE cartridges. Carryover was determined to be around 0.5% in the first blank after injecting the upper limit of quantification (ULOQ). This could be further reduced by adding longer column and trap washes if the full linear range is required.

Conclusions

- Ultra-low LLOQs (0.03–0.1 ng/mL) for ASOs in rat plasma were achieved using a trap-and-elute microflow method
- Greater than 10x improvement in sensitivity was achieved compared to previous analyses done at analytical flow rates[1]
- Sample throughput using the trap and elute microflow method with 30 µL extracted samples was comparable to throughput using the previous analytical flow LC-MS method
- 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 also promotes robustness by reducing the amounts of ion-pairing reagents introduced into the mass spectrometer

References

1. Development of an ultra-sensitive assay for anti-sense oligonucleotide quantification. SCIEX technical note, RUO-MKT-02-12551-A. https://sciex.com/content/dam/SCIEX/pdf/tech-notes/all/RUO-MKT-02-12551-A-ASO-Quant-7500.pdf

2. Improve sensitivity for antisense oligonucleotide quantification in plasma using microLC-MRM methodology. SCIEX technical note, RUO-MKT-02-10992-A. http://sciex.com/documents/tech%20notes/Improve-Sensitivity-for-Antisense-Oligonucleotide-Quantification-in-Plasma%20Using-MicroLC-MRM-Methodology.pdf

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