Extending the Lower Limits of Quantification of a Therapeutic Oligonucleotide Through Microflow LC-MS/MS

Featuring SCIEX QTRAP® 6500+ LC-MS/MS System with OptiFlow™ Turbo V source and the SCIEX M5 MicroLC System

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Introduction

Over the past 30 years oligonucleotide therapeutics have occupied a significant space in clinical drug development pipelines offering the exciting potential to target and modulate gene expression. Antisense RNA strands were initially identified as candidates for therapeutic promise in 1998. Fomivirsen, a phosphorothioate antisense oligonucleotide, received the first FDA approval as an oligonucleotide therapeutic. Since this milestone, antisense oligonucleotides still play an important role in modern drug development strategies. The development of ion-pairing reversed phase liquid chromatography (IP-RP LC) methodologies have paved the way for mass spectrometers to become a viable platform for oligonucleotide analysis by providing a strategy for sample introduction and orthogonal front-end separation that is compatible with electrospray ionization techniques common to modern mass spectrometers. IP-RP LC-MS is often the preferred solution for both qualitative and quantitative oligonucleotide analysis; however, several fundamental challenges remain with this approach. The concentrations of ion pairing reagents needed to adequately retain and separate polar oligonucleotides on a reversed phase column, often an alkylamine paired with a fluorinated alcohol, contribute to charge competition resulting in electrospray ion suppression. This phenomenon compromises the mass spectrometer response and may prevent desired limits of detection and quantification from being achieved. The routine use of ion pairing reagents pose another challenge as their accumulation within the mass spectrometer contributes to contamination which accelerates front end cleaning and maintenance intervals. Here a solution is presented to these widely recognized challenges through a novel microflow LC-MS strategy. The sensitivity improvements that result from reduced flow rates entering the MS and the associated enhancement in ionization efficiency have been well documented and prompted microflow LC-MS assays to be deployed for myriad applications. In this instance the reduction of contaminants entering the mass spectrometer is proportional to the mobile phase flow offering an additional advantage of microflow LC-MS. This study was designed to characterize the improvement of quantification for a phosphorothioate antisense oligonucleotide assay scaled down to microflow LC-MS.

Key Features of the SCIEX M-Series and Optiflow™ TurboV Ion source

- M5 MicroLC system provides:
  - Microfluidic flow control for accurate flow rates down to 1 µL/min
  - Direct Inject and trap-elute capabilities for fast and large volume sample loading
  - Flexibility to couple with any microLC column
  - Significantly reduced solvent consumptions and system contamination from ion paring reagents
- OptiFlow™ Turbo V Source on the QTRAP 6500+ LC-MS/MS system provides:
  - Easy setup with no probe or electrode position optimization required
  - Robust performance and long electrode lifetime

Materials and Methods

Samples and Reagents: All materials were purchased from Sigma Aldrich including HFIP (≥99.8%), DIEA (99.5%), Acetonitrile, Ethylenediaminetetraacetic acid (EDTA) and a custom oligonucleotide standard designed to be structurally equivalent to fomivirsen. Fomivirsen is a synthetic antisense oligonucleotide comprising of 21 bases (5'-G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G-3') linked by phosphorothioate bonds. Despite its molar mass of 6,682.4 g/mol, fomivirsen can be detected within a mass/charge range suitable for quadrupole analysis as a multicharged species in negative mode electrospray ionization (ESI).

Chromatography: A mobile phase system of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and N,N-Diisopropylethylamine (DIEA) was chosen to achieve adequate chromatographic retention and resolution when paired with a reversed phase C18 column. These mobile phases are also favored due to compatibility and enhanced ionization efficiency when coupled to MS. The formation of alkali metal adducts are an intrinsic challenge with an LC-MS oligonucleotide analysis as the negatively charged phosphodiester backbone electrostatically attracts positively charged alkali metal salt cations often ubiquitous to HPLC systems and columns. The formation of alkali metal adducts, most commonly but not exclusively Na+ and K+ cations, distribute the peak area across an envelope of adduct peaks compromising the targeted oligonucleotide peak intensity. The extent of passivation and materials present in the HPLC system are significant contributors to alkali metal adduct ratios and may bias a performance comparison between two unique HPLC systems, adduct formation was assessed for both conventional flow and microflow systems to ensure less than 10% adduct formation for both flow regimes. Both conventional 2.1mm ID and microbore 0.3 mm ID columns were purchased from Phenomenex, the stationary phases selected primarily on pH and temperature stability. A set of calibrators was prepared from 0.5 - 1000 ng/mL in a solution of 100µM EDTA in mobile phase A. An oligonucleotide internal standard was added to each calibrator at a concentration of 500 ng/mL.

Table 1. MS Conditions Common to Both Analysis.

Name	Q1	Q3	DP	CE	CXP	EP
Fomivirsen [M-10H] -10 MRM-1	667	319	-80	-35	-12	-10
Fomivirsen [M-10H] -10 MRM-2	667	304	-80	-45	-12	-10
Fomivirsen [M-9H] -9 MRM-1	741	319	-80	-40	-12	-10
Fomivirsen [M-9H] -9 MRM-2	741	344	-80	-40	-12	-10
Fomivirsen [M-8H] -8 MRM-1	834	319	-80	-45	-12	-10
Fomivirsen [M-8H] -8 MRM-2	834	649	-80	-35	-12	-10

LC-MS Conditions for Comparative Analysis: To identify the sensitivity difference between the LC-MS and microflow LC-MS analysis, each sample was analyzed in triplicate on the same QTRAP 6500+ LC-MS/MS system without modification to the instrument parameter settings.

As the observed charge state for oligonucleotides may be influenced by the reversed phase (RP-IP) mobile phase system and LC-MS conditions, charge state characterization and MRM method development were performed by injecting standards on column rather than a more conventional infusion approach. Low molecular weight fragment ions (< 200 Da m/z) such as phosphate ions were not selected during MRM method development due to poor specificity despite often offering superior intensity. The MRM parameters described in Table 1 were kept consistent between the two methodologies while Tables 2 and 3 contain the source and liquid chromatography settings that were optimized for each specific hardware configuration and flow rate. First, the mass spectrometer was coupled to an Exion™ UHPLC system and Ion Drive Turbo V™ ion source to benchmark the LC-MS performance then outfitted with a SCIEX M-series µUHPLC system and an OptiFlow™ Turbo V Source with a 25 µm SteadySpray™ probe and electrode for microflow LC-MS analysis. To attain a consistent linear velocity between the two experiments, the flow rate used for the microflow was simply scaled proportionately to the reduced column volume, the flow profile was slightly adjusted to account for the unique C18 stationary phases.

Assessment of Chromatographic Performance

Chromatographic resolution and peak shape play a critical role in the performance of any quantitative LC-MS assay. Peak symmetry and width are important factors that contribute to analyte signal to noise (S/N) and therefore influence the sensitivity and lower limits of quantification (LLOQ) of an assay. To properly evaluate the improved ionization efficiency at low microliter flow rates, it was essential to design microflow methodology with comparable chromatography to that of common conventional flow UHPLC results. In the absence of an LC system designed specifically for microflow separations, excessive dead volume and extra column peak band broadening frequently results in decreased throughput and poor peak shapes which prevent the benefits of microflow LC-MS from being realized. The low dead volume and precise solvent delivery of the SCIEX M-series system permitted throughput and chromatographic resolution to be equivalent to that typical of LC-MS result performed above 200 µL/minute (Figures 1 and 2).

Table 2. Conventional LC-MS Method Parameters.

Source/Gas Parameter	Value	Source/Gas Parameter	Value
Curtain gas:	30	CAD gas:	*High*
Ion source gas 1:	70	*Ion spray voltage:*	*-4500*
Ion source gas 2:	*70*	*Source temperature:*	*500*

Parameter	Value
Stationary phase	*Phenomenex Clarity® Oligo-MS 50 x 2.1 mm; 2.6µm particle; 100Å pore*
Mobile phase A	*15mM DIEA 100mM HFIP in water*
Mobile phase B	*15mM DIEA 100mM HFIP in 50% methanol*
Flow rate	*300 µL/min (3.6 mm/sec linear velocity)*
Column temperature	*60º*
Injection volume	*5 µL*

Time	Flow Rate (µL/min)	%A	%B
0	300	70	*30*
0.5	300	*70*	*30*
3.5	300	*10*	*90*
4.0	300	*10*	*90*
4.1	300	*70*	*30*
5.0	300	*70*	*30*

Table 3. Microflow LC-MS Method Parameters.

Source/Gas Parameter	Value	Source/Gas Parameter	Value
Curtain gas:	25	CAD gas:	*High*
Ion source gas 1:	40	*Ion spray voltage:*	*-4500*
Ion source gas 2:	*40*	*Source temperature:*	*350*

Parameter	Value
Stationary phase	*Phenomenex Gemini® C18 50 x 0.3 mm; 3µm particle; 100Å pore*
Mobile phase A	*15mM DIEA 100mM HFIP in water*
Mobile phase B	*15mM DIEA 100mM HFIP in 50% methanol*
Flow rate	*6 µL/min (3.5 mm/sec linear velocity)*
Column temperature	*60º*
Injection volume	*5 µL*

Time	Flow Rate (µL/min)	%A	%B
0	6	80	*20*
0.5	6	*80*	*20*
3.0	6	*10*	*90*
4.0	6	*10*	*90*
4.1	6	*80*	*20*
5.0	6	*80*	*20*

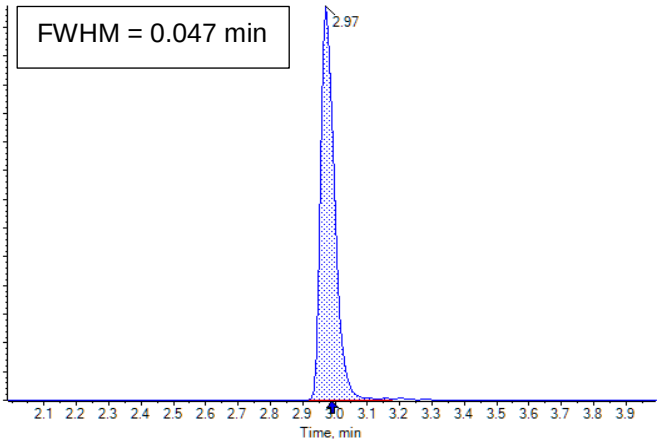

Figure 1. Example Chromatography Performance Achieved for Fomivirsen at 300 µL/minute.

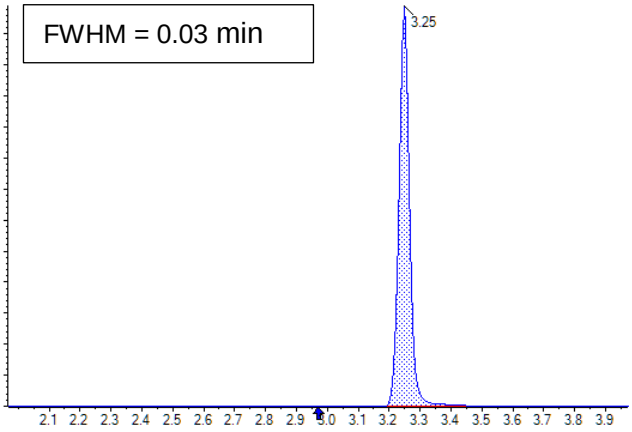

Figure 2. Example Chromatography Performance Achieved for Fomivirsen at 6 µL/minute.

Quantitation Results

Acceptance criteria of +/-15% accuracy and CV of <15% (<20% at LLOQ) was used to assess the linear range of quantification for both LC-MS and microflow LC-MS experiments. A linear regression with 1/x weighting was applied to the peak areas for both data sets. Data processing was performed using MultiQuant Software 3.0. A linear concentration range from 5 -1000 ng/mL was achieved on the conventional LC-MS configuration with an LOD of 2 ng/mL. Figure 3 describes the LC-MS calibration curve results and provides representative LOD and LLOQ peaks.

The set of calibrators prepared from 0.5 - 1,000 ng/mL was re-acquired on the QTRAP 6500+ system coupled with microLC. The presented microflow assay for fomivirsen demonstrated the significant improvement upon the LLOQ of 5 ng/mL achieved using the conventional flow LC-MS configuration. Using the same processing parameters and quantitative MRM transition, an LLOQ of 1 ng/mL was attained with an LOD of 0.5 ng/mL. The linear range of quantification from 1- 1,000 ng/mL. Figure 4 depicts the LOD and LLOQ XICs attained through microflow as well as the calibration curve results.

Table 4 provides a full summary of the quantitative performance of the microflow LC-MS configuration.

To further assess the improved ionization efficiency and the associated improvements in peak area and signal to noise, Figure 5 offers a comparison of peak intensity, area, and signal to noise for the LLOQ and ULOQ achieved at conventional flow rates, 2 ng/mL and 1,000 ng/mL. Figure 6 plots a comparison of the internal standard peak area across the duration of each data sample set.

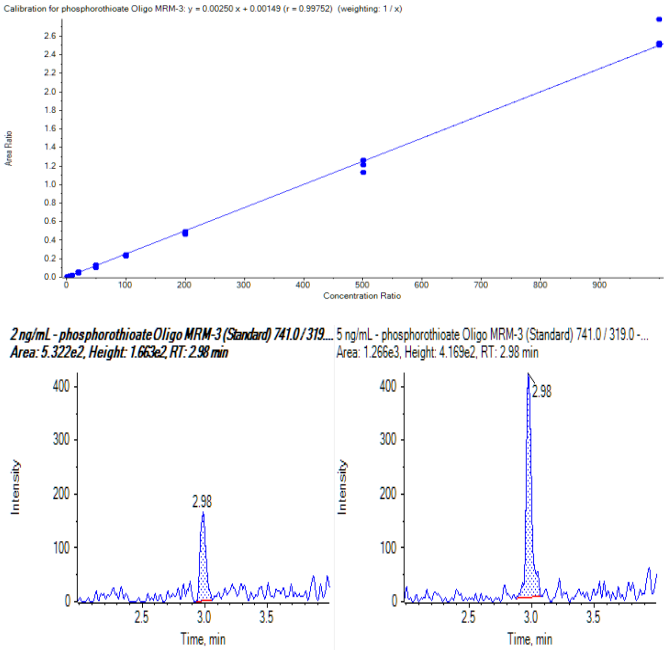

Figure 3. Quantitation Results for the Conventional Flow Experiment (300 µL/min). (Top) Calibration Curve for fomivirsen with r = 0.99752 and weighting of 1/x. (Bottom) Extracted ion chromatograms (XICs) of selected MRM for fomivirsen for the LOD of 2 ng/mL (left) and the LLOQ of 5 ng/mL (right).

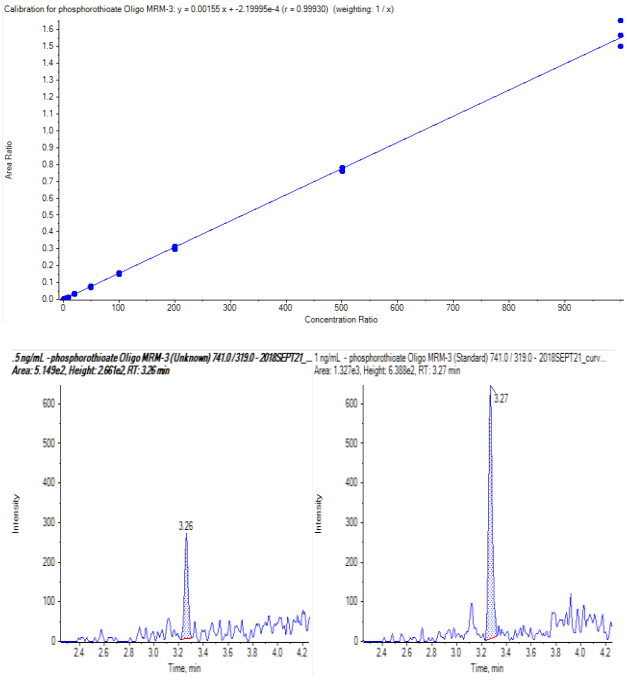

Figure 4. Quantitation Results for the Microflow Experiment (6 µL/min). (Top) Calibration Curve for fomivirsen with r = 0.99930 and weighting of 1/x. (Bottom) Extracted ion chromatograms (XICs) of selected MRM for fomivirsen for the LOD of 0.52 ng/mL (left) and the LLOQ of 1 ng/mL (right).

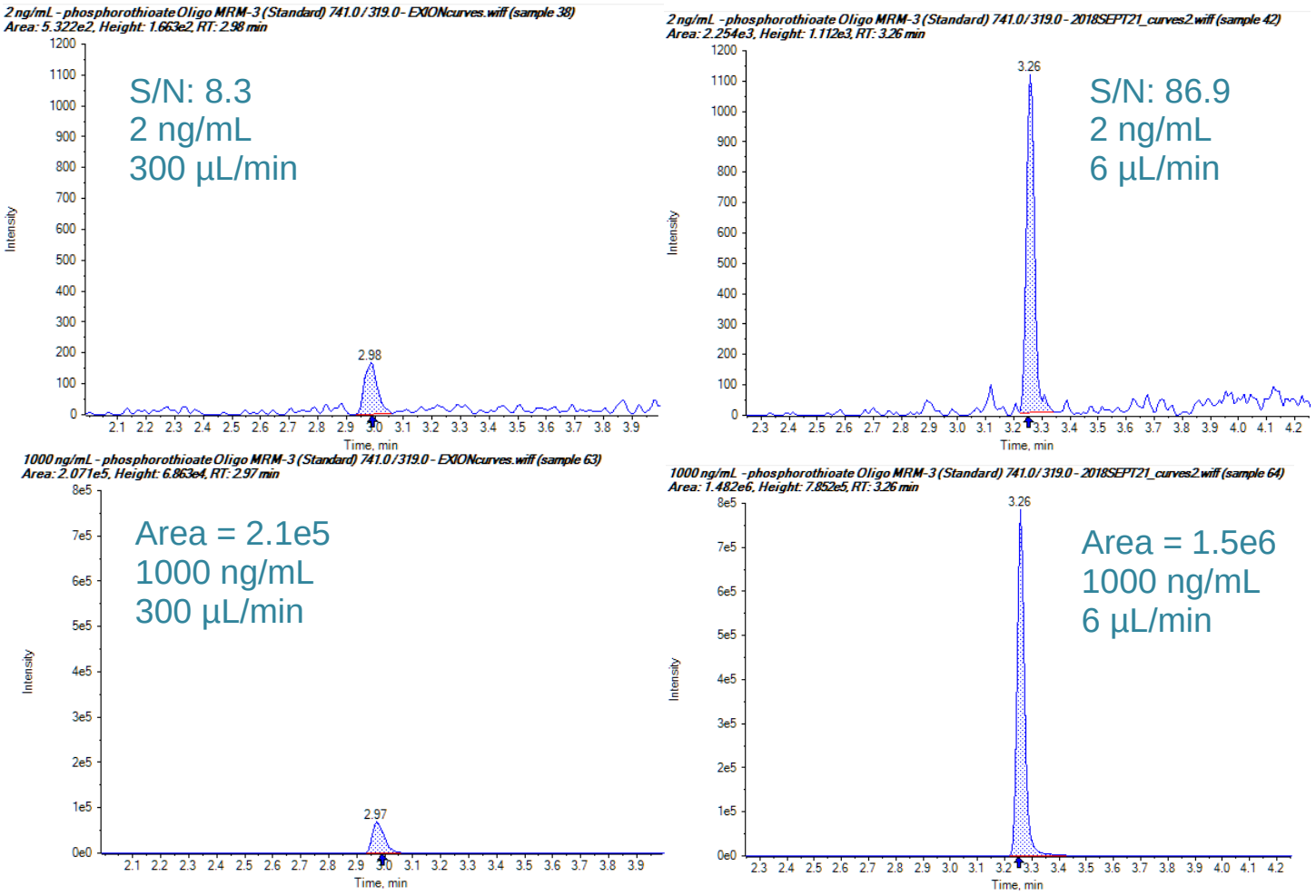

Figure 5. Extracted Ion Chromatograms (XICs) of Selected MRM Transitions. Data is shown for fomivirsen using conventional LC-MS (left) and microflow LC-MS (right) at 2 ng/mL (top) and 1000 ng/mL (bottom). Signal to noise calculations performed using MultiQuant software.

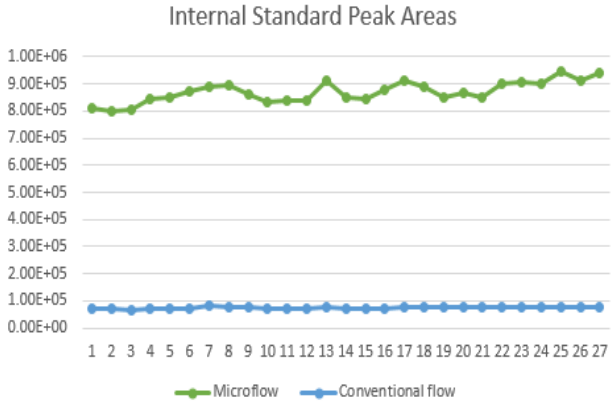

Figure 6. Metric Plot of Oligonucleotide Internal Standard Peak Areas for Microflow (green) and Conventional Flow (blue) Analysis.

Table 4. Microflow LC-MS Calibration Curve for fomivirsen at 6 µL/minute; r = 0.99930 (weighting: 1/x).

Actual Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)	CV (%)
0.5	0.62	123.80	16.07
1	1.02	101.87	14.87
2	1.92	96.19	0.89
5	4.59	91.78	8.96
10	9.06	90.64	6.81
20	20.61	103.04	3.65
50	47.96	95.91	3.94
100	98.47	98.47	2.85
200	196.08	98.04	2.91
500	494.46	98.89	1.70
1000	1013.72	101.37	4.82

Conclusions

MRM based LC-MS and microflow LC-MS assays were developed to assess the quantitative performance of a therapeutic oligonucleotide acquired at varied HPLC flow rates. The results presented indicate a significant improvement in peak area, peak intensity, and signal to noise when a dedicated microflow HPLC system running at 6 µL/minute is utilized compared to a conventional LC-MS system running 300 µL/minute. In addition to the improvement in sensitivity and overall quantitative performance, it is also notable that the microflow LC-MS assay dramatically reduced the required mobile phase additives that inherently contribute to front end contamination of the mass spectrometer.

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