Sub ng/mL quantification and characterization of oligonucleotides in plasma using microflow LC coupled to a novel QTOF mass spectrometer

Remco van Soest, Eshani Nandita, Kerstin Pohl, Elliott Jones
SCIEX, USA

ABSTRACT

Oligonucleotide therapeutics such as antisense oligonucleotides (ASOs) and siRNA are becoming increasingly important for therapeutic applications involving untreatable diseases. Oligonucleotides require high performance and robust quantitative methods for pharmacokinetic analysis. Triple quadrupole mass spectrometers using multiple reaction monitoring (MRM) mode have been routinely employed for bioanalytical studies of ASOs, as they offer excellent sensitivity and overall quantitative capability. Quadrupole time-of-flight (QTOF) mass spectrometers are very well suited for characterization, but historically have not been used for quantification due to limited sensitivity. In this study, a highly sensitive workflow was developed for the quantification and characterization of several ASOs in extracted rat plasma using microflow LC coupled to a novel QTOF mass spectrometer.

INTRODUCTION

- Oligonucleotide therapeutics and gene therapies are rapidly gaining attention
- Modalities such as antisense oligonucleotides (ASOs) are becoming more important 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 ASO therapeutics
- Sensitivity using high resolution time-of-flight mass spectrometry (TOF MS) is typically low compared to using a triple quadrupole system because of duty cycle limitations inherent to the mass analyzer design
- In this study, a novel TOF mass spectrometer, the ZenoTOF 7600 system, with a novel ion trap (Zeno trap) to achieve significantly improved MS/MS sensitivity, was used for the quantification of ASOs in plasma
- Microflow LC was used to further improve sensitivity

MATERIALS AND METHODS

Sample preparation:
Rat plasma was extracted using solid phase extraction (SPE) cartridges (Phenomenex) following the manufacturer's protocol. Extracted plasma samples were spiked with a mixture of fomivirsen, nusinersen, eluforsen, a fully phosphorothioated 2'O-methylated 20-mer model oligonucleotide and an internal DNA standard.
Calibration curves were measured in triplicate for all analytes by spiking the analytes into a plasma extract in the concentration range between 0.01 ng/mL and 300 ng/mL.

Microflow LC conditions:
Samples were analyzed using a trap and elute microflow LC system, which allowed for the injection of 30 µL extracted plasma on the trap column. Reversed-phase ion pairing (RP-IP) with 15 mM diisopropylethylamine (DIEA) and 35 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in water (A) and 10/90 v/v water/methanol (B) was used.
A trap-and-elute microLC method was used. 30 µL sample was loaded for 2 minutes at 35 µL/min onto a 0.3 x 5 mm YMC-Triart 3 µm C18 trap held at 80ºC. Gradient microflow LC was performed at 5 µL/min using water and methanol with the ion-pairing reagents N,N diisopropylethylamine (15 mM) and hexafluoroisopropanol (35 mM) and a 0.3 x 50 mm YMC-Triart 3 µm C18 column at 80ºC.

MS conditions:
A ZenoTOF 7600 system (SCIEX) with an OptiFlow ion source was used in Zeno MRM^{HR} mode, and SCIEX OS software and Molecule Profiler software were used for data analysis. MRM^{HR} transitions were selected, and all parameters were optimized for best sensitivity. An XIC peak width of 0.05 Da was used for quantification. A 1/x^2 weighting was used for quantification.

RESULTS

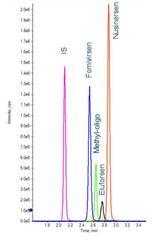


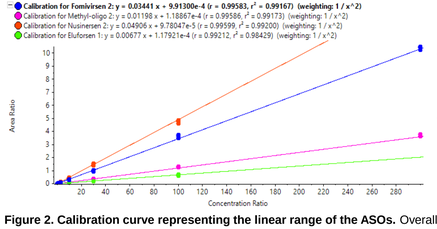
Figure 1. XICs of the four ASOs and IS.

A quantitative method was developed to quantify ASOs in rat plasma including fomivirsen, nusinersen, methyl-oligo, and eluforsen. The XICs of the 4 ASOs and the IS were extracted from the acquired MS/MS spectra using the fragment *m/z* values listed in Table 1.

All oligonucleotides were baseline separated within a 3 min gradient (Figure 1). Baseline separation is important because overlap among the precursors can often occur due to the large number of charge states of each oligonucleotide.

Table 1. MRM^{HR} parameters and fragments for quantification.

Analyte	Precursor ion (m/z)	Fragment ion (m/z)	DP (V)	CE (V)
Methyl-oligo	694.2	374.03	-135	-39
Fomivirsen	741.4	319.02	-125	-33
Nusinersen	790.9	393.05	-135	-40
Eluforsen	715.8	358.04	-135	-39
IS	786.3	303.04	-135	-49



Linear range:
Fomivirsen: 0.03 ng/mL to 300 ng/mL
Methyl-oligo: 0.03 ng/mL to 300 ng/mL
Nusinersen: 0.01 ng/mL to 100 ng/mL
Eluforsen: 0.03 ng/mL to 100 ng/mL

Figure 2. Calibration curve representing the linear range of the ASOs. Overall, the linear dynamic range (LDR) was greater than 3.5 orders of magnitude.

Calibration curves were measured in triplicate for all of the analytes by spiking the analytes into plasma extract in the concentration range between 0.01 ng/mL and 300 ng/mL. Exceptional linearity (Figure 2) and accuracy and precision (Table 2) were achieved for all analytes.

Table 2. Quantitative summary of the ASOs. The LLOQ, ULOQ, linearity, CV and accuracy at LLOQ demonstrated excellent quantitative performance.

Analyte	LLOQ (ng/mL)	ULOQ (ng/mL)	Linearity (orders)	CV at LLOQ (%)	Accuracy at LLOQ (%)
20-mer phosphorothioated and 2'-O-methylated antisense oligonucleotide	0.03	300	4.0	16.2	98.3
Fomivirsen	0.03	300	4.0	11.2	96.4
Nusinersen	0.01	100	4.0	3.69	96
Eluforsen	0.03	100	3.5	18.7	95.7

Exceptional linearity and accuracy and precision were achieved for all analytes.

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 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 nusinersen was 10 pg/mL, while the LLOQs for the other ASOs was 30 pg/mL (Figure 3). LDR was 3.5 to 4 orders of magnitude (Figure 2). No significant interferences were seen, illustrating efficient clean-up of the plasma matrix using the Clarity OTX SPE cartridges (Figure 3).

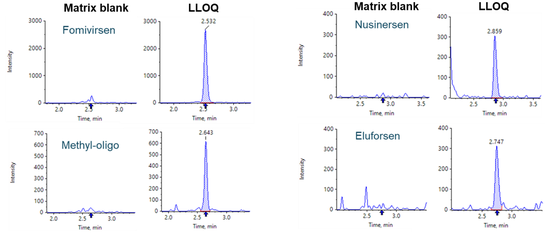
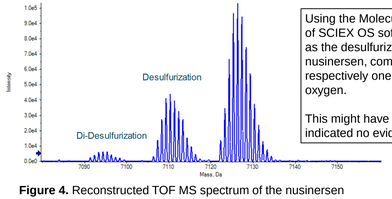


Figure 3. XICs of the matrix blank and LLOQs of fomivirsen, nusinersen, methyl-oligo, and eluforsen.
No matrix interferences were observed for any of the ASOs measured.

The TOF MS spectra can provide valuable information about non-targeted impurities. After reconstruction, using the Bio Tool Kit option in SCIEX OS software, two additional species were found in the nusinersen peak. Using the SCIEX Molecule Profiler software, they were identified as the desulfurized products of the main sequence. Exchange of a sulfur with an oxygen atom is a common occurrence during manufacturing or storage of phosphorothiated oligonucleotides.



Using the Molecule Profiler software, an optional component of SCIEX OS software, the additional species were identified as the desulfurization and di-desulfurization products of nusinersen, commonly found impurities that have respectively one or two sulfur atoms exchanged with an oxygen.

This might have occured during synthesis or storage as data indicated no evidence of in-source fragmentation.

Figure 4. Reconstructed TOF MS spectrum of the nusinersen chromatographic peak. A significant amount of the desulfurization and di-desulfurization products were apparent.

Figure 5 shows excellent correlation of the desulfurization impurity response from the TOF data with the standard concentrations of nusinersen. For this curve, the most intense isotope of the TOF MS spectrum was used.

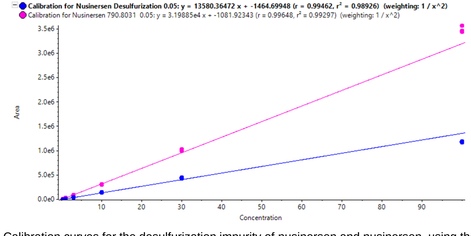


Figure 5. Calibration curves for the desulfurization impurity of nusinersen and nusinersen, using the most intense isotope of the TOF-MS spectrum.

The ratio of the calculated area of this isotope, using the curve shown in Figure 5, with the calculated area of the most intense isotope of nusinersen, was found to be on average 41% with a CV of 3.2% over the range of 1 to 100 ng/mL of nusinersen.

This indicates that using the TOF MS data, a good estimate of the level of an impurity that was not targeted with an MRM^{HR} experiment can still be determined.

CONCLUSIONS

- Low-pg/mL LLOQs for ASOs in rat plasma were achieved in MRM^{HR} mode using the Zeno trap function of the ZenoTOF 7600 system, allowing for pharmacokinetic studies with limited sample availability that require quantification at very low levels
- The TOF MS data acquired as part of the MRM^{HR} quantification workflow can be used for the identification and relative quantification of additional impurities or metabolites
- 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
- Reduced consumption of LC-MS grade ion-pairing reagents provides significant savings and increases robustness

TRADEMARKS/LICENSING