Ultra-sensitive quantitation of therapeutic oligonucleotide in human plasma by a high resolution accurate mass Orbitrap mass spectrometer

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Abstract

We developed a liquid chromatography coupled to high resolution accurate mass (HRAM) mass spectrometry (LC-HRAM-MS) method for quantifying two FDA-approved antisense oligonucleotide (ASO) drugs, Fomivirsen and Nusinersen. This method was developed using Thermo Scientific™ Vanquish™ Horizon UHPLC and Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer. This method can achieve a LLOQ of 0.05 ng/ml for both drugs in human plasma with excellent precision and accuracy. In addition, a fit-for-purpose report was created in the compliance-ready Thermo Scientific™ Chromeleon™ CDS software, allowing quick review of the results. This complete solution enables straightforward integration for automated data acquisition, processing and reporting within regulated bioanalysis laboratories that support the clinical trials of these emerging oligonucleotide therapeutics.

Introduction

Recent development in short ASO or small interfering ribonucleic acid (siRNA) that target RNA transcripts have shown great success for the treatment of rare diseases. As a result, highly selective and robust bioanalytical methods are desired to support routine characterization of key pharmacokinetic properties for these new modalities. While triple quadrupole systems are still the workhorse instruments for routine quantitation, high resolution mass spectrometers provide additional resolution to separate chemical interferences from complex biological matrices, thus accelerating method development with improved specificity. In this study, a LC-HRAM-MS method was developed to quantify Fomivirsen and Nusinersen in human plasma samples, and the sensitivity, linearity, specificity, accuracy and precision were evaluated.

Materials and methods

Sample Preparation

ASO standards were prepared in nuclease-free water at a stock concentration of 1 mg/mL, aliquoted, and stored in a -80ºC freezer. Working standards ranging from 0.5 ng/mL to 500.0 ng/mL were prepared by diluting the stock solution with 15 mM DIPEA, 25 mM HFIP in UHPLC-MS grade water.

A liquid-liquid extraction was performed on 200 µL aliquot of K2EDTA human plasma using phenol/chloroform/isoamyl alcohol (25:24:1) solution. The mixture was vortexed for 10 seconds at 2,000 rpm, and supernatants were extracted from the top layer and dispensed into a 0.5 mL DNA LoBind® microcentrifuge tube. Extracted human plasma samples were dried down in a speed vacuum for 30 min and reconstituted with 200 µL of standard solutions containing fomivirsen and nusinersen with a concentration ranging from 0.05 ng/mL to 50 ng/mL, and the internal standard (IS) with a concentration of 10.0 ng/mL.

Chromatography

A Vanquish Horizon UHPLC was used for ion-pair reversed-phase liquid chromatography (IPRP-LC) separation of the ASO from the plasma matrix. 20 µL samples were injected onto a C18 column and separated using an 8-minute gradient as outlined in Table 1.

Mass Spectrometry

The LC-HRAM-MS method, operated in tMS2 scan, was developed on the Orbitrap Exploris 480 mass spectrometer for quantitation of the ASOs. Table 2 outlines the ion source and scan experiment settings. Table 3 outlines the tMS2 scan mass list details.

Software

Chromeleon CDS version 7.3.2 was used for all data acquisition, processing, and reporting.

Table 1. LC and autosampler conditions.

HPLC column	C18 column, 2.1 x 50mm, 2.6µm	
Flow Rate	0.25 mL/min	
Solvent A	15mM DIPEA and 25mM HFIP in water	
Solvent B	15mM DIPEA and 25mM HFIP in 80/20 acetonitrile/water (v/v)	
Gradient	Time (min)	%B
	0	5
	1	5
	3.5	24
	4	80
	6	80
	6	5
	8	5
Injection Volume	20 µL	
Needle Wash	After draw, 30 µL/s for 10s with 10% methanol	
Thermostatting mode	Still Air	
Column oven temperature	50°C	
Divert to source	2.8 – 4.8 minutes	

Table 2. MS ion source and scan experiment settings.

Instrument	Orbitrap Exploris 480 mass spectrometer
MS source parameters	
Negative ion (V)	3000
Sheath Gas (Arb)	35
Aux Gas (Arb)	10
Sweep Gas (Arb)	0
Ion transfer tube temperature (ºC)	325
Vaporizer temperature (ºC)	350
tMS2 scan parameters	
Scan resolution	60000
Isolation Window (m/z)	2
Scan range (m/z)	300 – 800
RF Lens (%)	50
Polarity	Negative

Table 3. tMS2 scan mass list for fomivirsen, nusinersen, and IS. 23% HCD was applied for all precursors, and the quantifier product ions are shown here.

Standard	Start – end time (min)	Precursor (m/z)	Product (m/z)
Fomivirsen	3 – 3.8	834.19	319.0173
Nusinersen	3.8 – 4.6	889.78	393.0550
IS	3 – 3.8	820.85	374.0340

Results

ASOs were separated on a C18 column using the developed IPRP gradient shown in Table 1. Figure 1 showed an extracted ion chromatogram (XIC) of the quantifier product ions for the analysis of both ASOs and the IS in a 10 ng/mL spiked sample. With this method, both ASOs were baseline resolved chromatographically from the IS, and the resultant peak widths were less than 10s.

ASOs were analyzed by the Orbitrap Exploris 480 mass spectrometer operated in tMS2 mode. Using this mode, MS/MS scans with defined mass range were collected for target precursors as outlined in Table 3. A typical tMS2 scan of fomivirsen and nusinersen, obtained at peak apex for precursor ion with charge state of -8, were shown in Figure 2. The most intense product ion was selected (labeled in Figure 2) and screened against the matrix, extracted human plasma samples, for crosstalk. As illustrated in Figure 3, we did not observe any crosstalk in the extracted human plasma samples for this selected precursor-to-product transition. As a result, the analyses of fomivirsen and nusinersen in the 0.05 ng/mL spiked samples were interference-free.

Figure 1. IPRP-LC separation of fomivirsen, nusinersen, and IS at the 10.0 ng/mL level each using the IPRP-LC-HRAM-MS method.

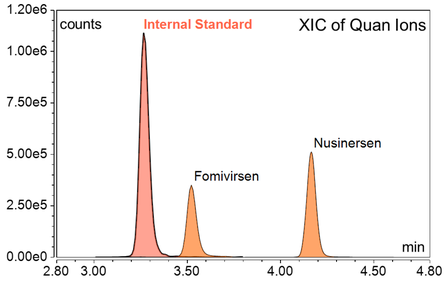


Figure 2. tMS2 scan of (left) fomivirsen and (right) nusinersen. For both analytes, -8 charge state was selected and fragmented with 23% HCD. The most intense fragment ion was selected as the quantifier product ion.

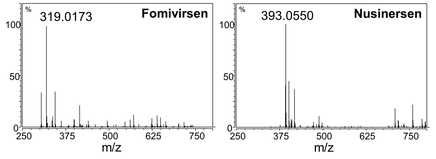
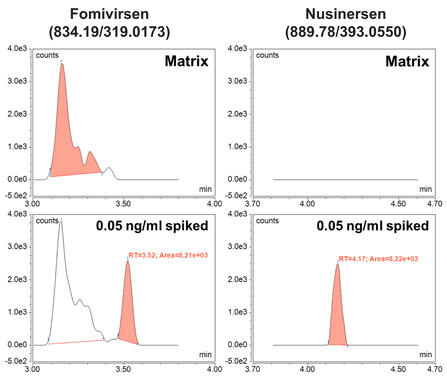


Figure 3. Analysis of (left) fomivirsen and (right) nusinersen in matrix and 0.05 ng/ml spiked sample using the IPRP-LC-HRAM-MS method.



With this method, we were able to quantify both fomivirsen and nusinersen at 0.05 ng/mL with greater than 80% accuracy and within 15% precision as shown in Table 4. The method also resulted in great consistency across five replicate injections per concentration level and exhibited a linear response over at least 3 orders of dynamic range with R2 values of greater than 0.998 for both calibration plots (Figure 4). All results were captured in a cGLP-compliance ready, fit-for-purpose Chromeleon report. An example is provided in Figure 5, consisting of two parts. The first part provides the information about injection details with integrated chromatographic peak results for each sample. The second part contains a full calibration plot and accuracy, precision, and other quantitative details for each calibration standard level.

Table 4. Precision and accuracy evaluation of the LC-HRAM-MS method. Values were averaged over five replicates.

Concentration (ng/ml)	Fomivirsen		Nusinersen	
	Precision (%RSD)	Accuracy (% Diff)	Precision (%RSD)	Accuracy (% Diff)
0.05	11.64	14.56	7.31	8.02
0.10	12.42	3.28	3.17	-3.41
0.25	4.68	4.01	2.28	-9.67
0.50	6.31	-8.99	1.75	1.65
1.0	4.41	-1.16	1.31	3.96
2.5	1.11	-5.15	1.52	-1.89
5.0	1.70	-3.25	0.45	2.37
10	0.82	-2.88	0.52	-0.57
25	3.08	-3.97	0.54	-0.93
50	0.87	2.89	1.13	0.31

Figure 4. Calibration plots for the quantitation of (left) fomivirsen and (right) nusinersen showing the linear signal response across the concentration range from 0.05 to 50 ng/mL. The linear fits were weighted with 1/X.

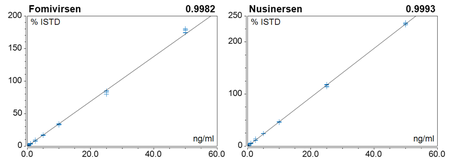
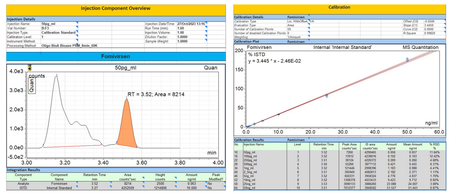


Figure 5. Example of summary report for the quantitation of fomivirsen generated by Chromeleon CDS. (Left) Integrated peak results per injection and (right) full calibration result summary for each analyte.



Conclusions

A highly sensitive IPRP LC-HRAM-MS method was developed on Vanquish Horizon UHPLC coupled to an Orbitrap Exploris 480 mass spectrometer for the quantitation of fomivirsen and nusinersen in human plasma. This method provides:

- Ultra sensitive quantitation of fomivirsen and nusinersen at an LLOQ of 0.05 ng/mL
- Accurate quantitation of fomivirsen and nusinersen with excellent precision and accuracy, and at least 3 orders of linear dynamic range
- A compliance-ready software solution, Chromeleon CDS, enables streamlined data acquisition, processing, to reporting within a regulated environment

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