Sensitive quantitation of antisense oligonucleotides in plasma using high-resolution, accurate-mass (HRAM) mass spectrometry

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Keywords

Oligonucleotide bioanalysis, antisense oligonucleotides (ASO), liquid-liquid extraction (LLE), ion-pairing reversed phase liquid chromatography (IPRP-LC), liquid chromatography high-resolution accurate-mass mass spectrometry (LC-HRAM-MS), targeted MS/MS (tMS2), Vanquish Horizon UHPLC, Chromeleon CDS

Application benefits

Sensitive and reproducible quantitation of antisense oligonucleotides with human plasma matrix by an IPRP-LC-HRAM-MS method using the Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled with the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer

Goal

Demonstrate the use of an LC-HRAM-MS method with Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) for sensitive quantitation of synthetic oligonucleotide therapeutics in human plasma

Introduction

Antisense oligonucleotides (ASOs) are short synthetic oligonucleotides, with typical lengths ranging from 15 to 25 nucleotides, that specifically bind to mRNA via Watson-Crick base pairing to alter gene expression and translation of target protein.[1] This type of drug has shown promising results for treatment of rare neurodegenerative diseases. Since the first approval of fomivirsen in 1998, nine more ASO-based drugs have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), and 38 more are currently undergoing phase III clinical trials.[2] This surge of potential drug candidates has triggered strong demand for highly sensitive and robust quantitative bioanalytical methods to evaluate their pharmacokinetic and metabolic profiles.

High-resolution accurate-mass methods have gained tremendous interest for quantitative analysis of target oligonucleotides and their impurities as alternatives to ligand binding assays and fluorescent detection methods. This wider adoption was partly due to faster method development using parallel reaction monitoring mode, low detection limits, and high specificities in various complex matrices such as plasma, tissue, and other biological fluids. Herein, we report the use of an LC-HRMS-MS method developed on the Orbitrap Exploris 120 mass spectrometer for quantitative analysis of fomivirsen and nusinersen, two FDA-approved ASO-based drugs, in human plasma matrix. Using this method, we can achieve an LLOQ of 0.20 ng/mL for fomivirsen, and 0.10 ng/mL for nusinersen with excellent reproducibility, accuracy, and linearity. In addition, a fit-for-purpose report in the compliance-ready Chromeleon CDS was developed, allowing for quick review of the results. This complete solution enables straightforward integration with regulated bioanalysis laboratories that support the clinical trials of these emerging oligonucleotide therapeutics.

Experimental

Reagents and consumables

- Antisense oligonucleotide standards (see Table 1 for details)
- K2EDTA Human plasma lot# BRH945594 (BioIVT)
- Phenol/chloroform/isoamyl alcohol (25:24:1), stabilized, saturated with 100 mM Tris-EDTA to pH 8.0, for molecular PCR, Thermo Scientific Chemicals (Fisher Scientific, P/N AC327111000)
- Eppendorf™ DNA LoBind™ Deepwell plates 96, 1 mL (Fisher Scientific, P/N E951032808)
- Eppendorf™ DNA LoBind™ microcentrifuge tubes, 0.5 mL (Fisher Scientific, P/N 13-698-790)
- Thermo Scientific™ SUN-SRi™ MicroMat™ PTFE-Coated Silicone Sealing Mats (P/N 14-823-265)
- N,N-Diisopropylethylamine (DIPEA), 99.5+%, Thermo Scientific Chemicals (P/N 367841000)
- 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 99.9%, Thermo Scientific Chemicals (P/N 293410500)
- Nuclease-free water (not DEPC-treated), Invitrogen™ (P/N AM9938)
- Water, UHPLC-MS grade, Thermo Scientific (P/N W81)
- Acetonitrile, UHPLC-MS grade, Thermo Scientific (P/N A9561)

ASO sample preparation – liquid/liquid extraction (LLE)

Reference standards were prepared in nuclease-free water at a stock concentration of 1 mg/mL, aliquoted, and stored in a -80 °C freezer. Working standards ranging from 1.0 ng/mL to 1,000 ng/mL were prepared by diluting the stock solution with 15 mM DIPEA, 25 mM HFIP in UHPLC-MS grade water.

A 100 µL aliquot of K2EDTA human plasma was mixed with 100 µL of phenol/chloroform/isoamyl alcohol (25:24:1) solution. The mixture was vortexed for 10 s at 2,000 rpm. 25 µL of 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 0.10 ng/mL to 100 ng/mL, and the internal standard (IS) with a concentration of 10.0 ng/mL.

Table 1. Antisense oligonucleotide reference and internal standards and their sequences

ASO name	Sequence
Fomivirsen	G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G
Nusinersen	Te*Se*Ae*Se*Te*Te*Te*Se*Ae*Te*Ae*Ae*Te*Ge*Se*Te*Ge*Ge
Internal standard	mG*mC*mG*mA*mC*T*A*TACGCGCAmA*mU*mA*mU*mG

Note: * denotes phosphorothiolate nucleotide; “e” denotes 2’-0-methoxyethylated ribose; “m” denotes 2’-0-methylated ribose; “S” denotes 5-methylcytosine. For the internal standard, all the nucleotides are deoxyribonucleotides except for U which is ribonucleotide.

Acquisition method – chromatography

A Vanquish Horizon UHPLC system consisting of:

- Vanquish System Base F/H (VF-S01-A-02)
- Vanquish Binary Pump F (VH-P10-A-01)
- Vanquish Split Sampler FT (VH-A10-A-02)
- Vanquish Column Compartment H (VH-C10-A-03)

was used for 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 2.

Acquisition method – mass spectrometry

The LC-HRMS-MS method, operated in tMS2 scan, was developed on the Orbitrap Exploris 120 mass spectrometer for quantitation of the ASOs. Table 3 outlines the ion source and scan experiment settings. Table 4 outlines the tMS2 scan mass list details.

Table 2. LC and autosampler conditions

Parameter	Value
HPLC column	C18 column, 2.1 × 50 mm, 2.6 µm
Flow rate	0.25 mL/min
Solvent A	15 mM DIPEA and 25 mM HFIP in water
Solvent B	15 mM DIPEA and 25 mM 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 10 s with 10% methanol
Thermostatting mode	Still air
Column oven and pre-heater temperature	50 °C
Divert to source	2.8–4.8 min

Table 3. MS ion source and scan experiment settings

Parameter	Value
Orbitrap Exploris 120 MS ion source parameters	
Negative ion (V)	3,000
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temperature (°C)	325
Vaporizer temperature (°C)	350
tMS2 scan parameters	
Scan resolution	60,000
Isolation window (*m/z*)	2
Scan range	300–800
RF lens (%)	70%
Polarity	Negative

Table 4. tMS2 scan mass list for fomivirsen, nusinersen, and IS. Both quantifier (Quan) and qualifier (Qual) ion transitions are shown.

Standard	Start – end time (min)	Precursor (*m/z*)	Product (*m/z*)	HCD collision energy (%)
Fomivirsen (Quan)	3–3.9	834.19	319.0173	23
Fomivirsen (Qual)		741.30		
Nusinersen (Quan)	3.9–4.6	889.78	393.0550	23
Nusinersen (Qual)		1017.03		
IS (Quan)	3–3.9	820.85	374.0340	23
IS (Qual)		938.12		

Processing method – MS quantitation

The processing method for quantitation of ASOs was based on the default MS quantitative method with a few minor adjustments. The retention time window was set to 0.5 min and centered around the retention time of each analyte. The Genesis peak integration algorithm with a minimum peak signal over noise ratio set to 3 was used for peak integration. The area ratios, fomivirsen and nusinersen signal responses divided by IS signal response, are plotted against the concentration of the standards. Linear regression curve was fitted to the calibration plot with 1/x weighting.

Summary report

A fit-for-purpose summary report containing the quantitative results for the analysis of ASOs was generated using the default bioanalysis report in Chromeleon software. It contains sample injection details and integration peak results for each sample and a full calibration plot showing the linear dynamic range and R^2 value. Additionally, a calibration result table showing measured retention time, precision, accuracy, and other quantitative results per analyte concentration level is reported.

Software

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

Results and discussion

The quantitative performance of the LC-HRAM-MS method for the analysis of fomivirsen and nusinersen in human plasma samples was evaluated. Human plasma samples were first extracted using a simple, one-step LLE using the solution containing phenol/chloroform/isoamyl alcohol (25:24:1), stabilized and saturated with 100 mM Tris-EDTA to pH 8.0. Fomivirsen and nusinersen standards ranging from 0.10 ng/mL to 100 ng/mL with 10 ng/mL of IS were spiked into the extracted human plasma to mimic 100% recovery.

The spiked samples were separated on a C18 column using the developed IPRP-LC gradient and analyzed by the Orbitrap-based MS method operated in tMS2 mode. Using this mode, MS/MS scans with defined mass range were collected for target precursors as outlined in Table 4. For example, tMS2 of fomivirsen and nusinersen precursors ion with charge state of -8 are shown in Figure 1. The three most abundant product ions were selected (labeled in Figure 1) and screened against the extracted human plasma samples for crosstalk. As illustrated in Figure 2, we did not observe any crosstalk in the extracted human plasma sample for these selected precursor-to-product transitions. As a result, the analyses of fomivirsen and nusinersen in the 0.10 ng/mL spiked human plasma samples were interference-free. Consequently, *m/z* 834.19/319.0173 and *m/z* 889.78/393.0550 precursor-to-product transitions were chosen as the quantifiers for fomivirsen and nusinersen, respectively.

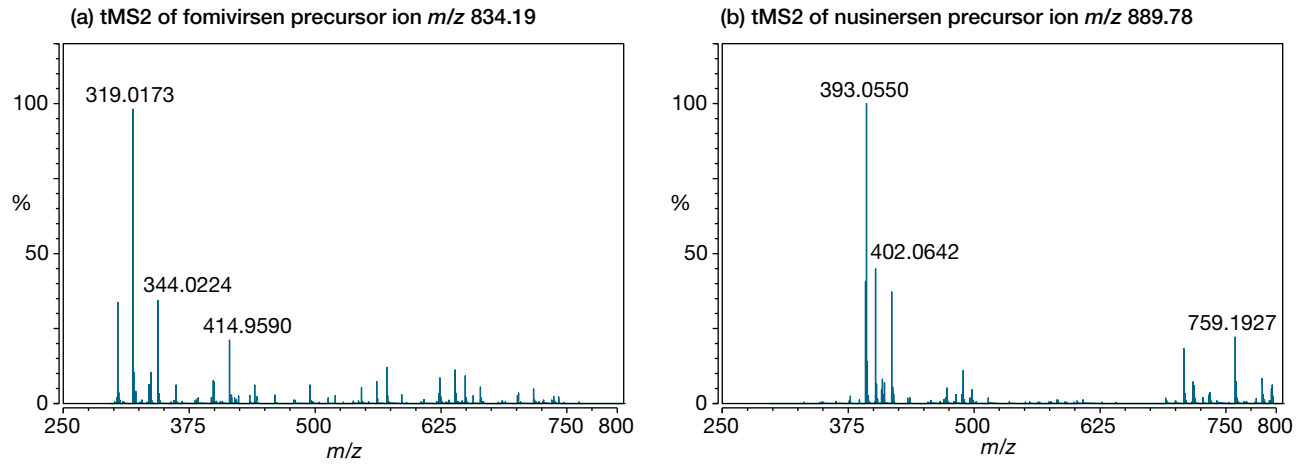


Figure 1. tMS2 scan of (a) fomivirsen and b) nusinersen. The most intense charge state, -8 for both analytes, was selected and fragmented with 23% HCD.

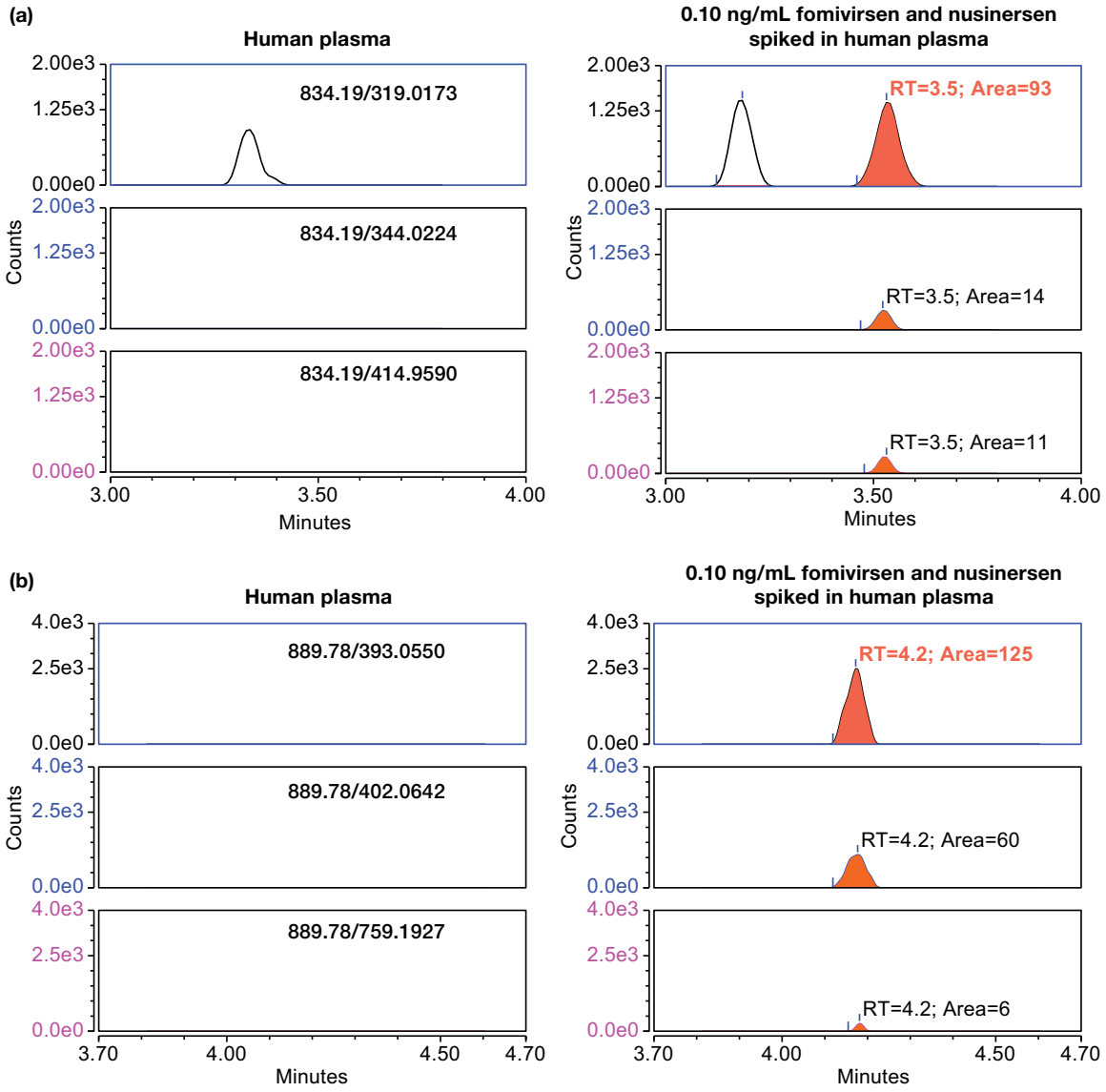


Figure 2. Crosstalk evaluation. (a) Analysis of fomivirsen in human plasma, and 0.10 ng/mL spiked human plasma. (b) Analysis of nusinersen in human plasma, and in 0.10 ng/mL spiked human plasma.

Figure 3 shows an extracted ion chromatogram of the quantifiers for the analysis of both ASOs and the IS in a 10 ng/mL spiked matrix sample. In this example, both ASOs were baseline-resolved chromatographically from the IS, and the resultant peak withs of the ASOs were less than 10 s. With this method, we were able to quantify fomivirsen at 0.20 ng/mL and nusinersen at 0.10 ng/mL with greater than 80% accuracy and within 10% precision as shown in Table 5. The method also resulted in data showcasing great consistency across five replicate injections per concentration level and exhibited a linear response across the tested concentration range from 0.10 to 100 ng/mL with R^2 values of greater than 0.994 for both calibration plots (Figure 4).

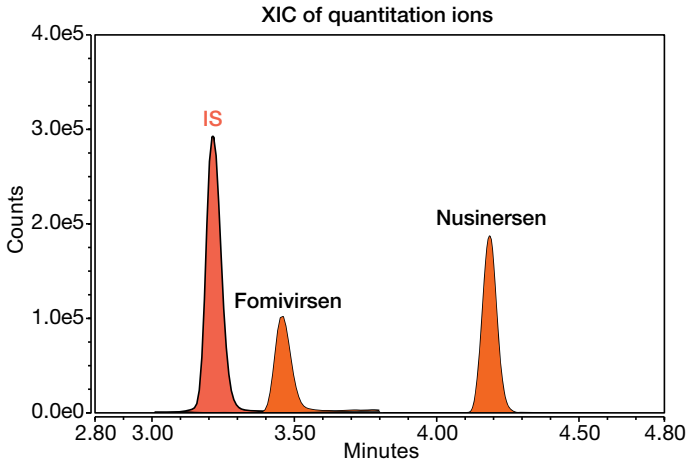


Figure 3. IPRP-LC separation of fomivirsen, nusinersen, and IS at the 10.0 ng/mL level each using the IPRP-LC-HRAM-MS method

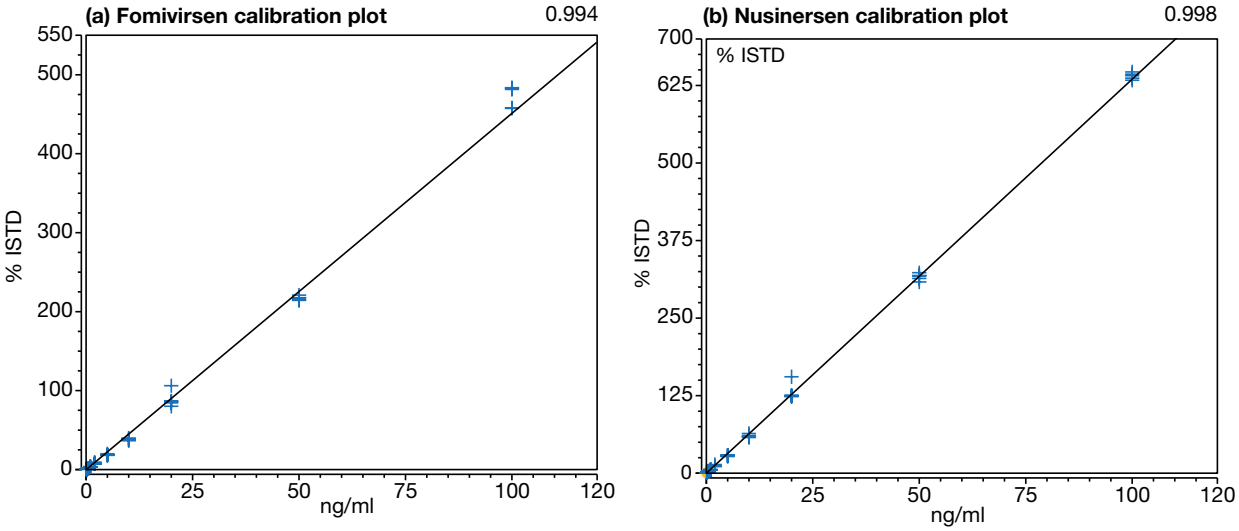


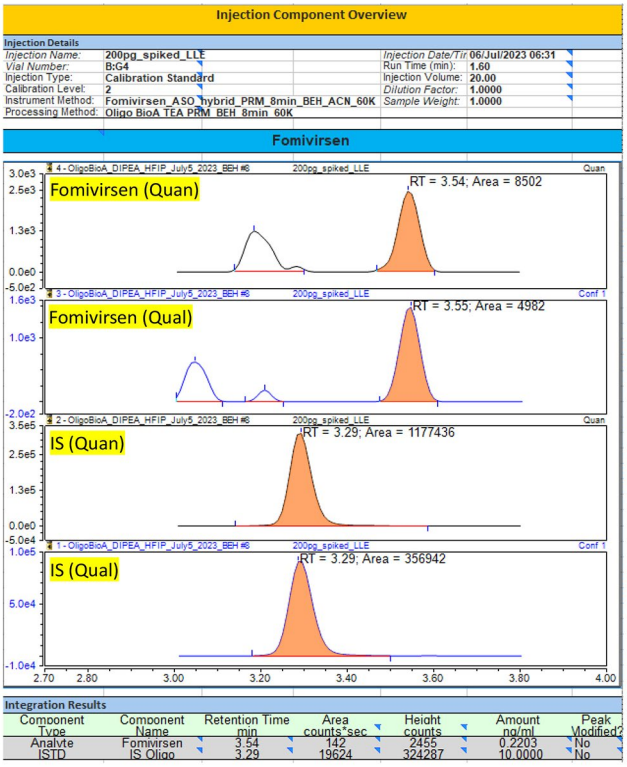
Figure 4. Calibration plots for the quantitation of (a) fomivirsen and (b) nusinersen showing the linear signal response across the concentration range from 0.10 to 100 ng/mL. Values were averaged over five replicate injections at each calibration concentration.

Table 5. Precision and accuracy evaluation of the LC-HRAM-MS method. Values were averaged over five replicate injections at each calibration level. The 0.10 ng/mL calibration standard was not removed during weighting for the evaluation of fomivirsen.

	Fomivirsen		Nusinersen	
Concentration (ng/mL)	Precision % RSD	Accuracy % diff	Precision % RSD	Accuracy % diff
0.10	13.7	65.5	3.0	13.3
0.20	14.6	5.7	5.1	13.1
0.50	8.3	13.3	3.1	1.5
1.0	5.0	15.8	2.5	8.7
2.0	1.2	11.1	7.3	8.3
5.0	4.1	16.0	2.6	11.1
10	2.5	14.6	2.8	4.2
20	2.2	1.4	1.6	4.2
50	4.6	3.7	4.3	0.1
100	4.0	4.8	2.4	0.4

All results were captured in a cGLP-compliant, fit-for-purpose 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. Not only are the results for every single injection captured, but any manual data integration was also recorded, an important software feature that is required to preserve the data integrity in a regulated environment.

(a)



(b)

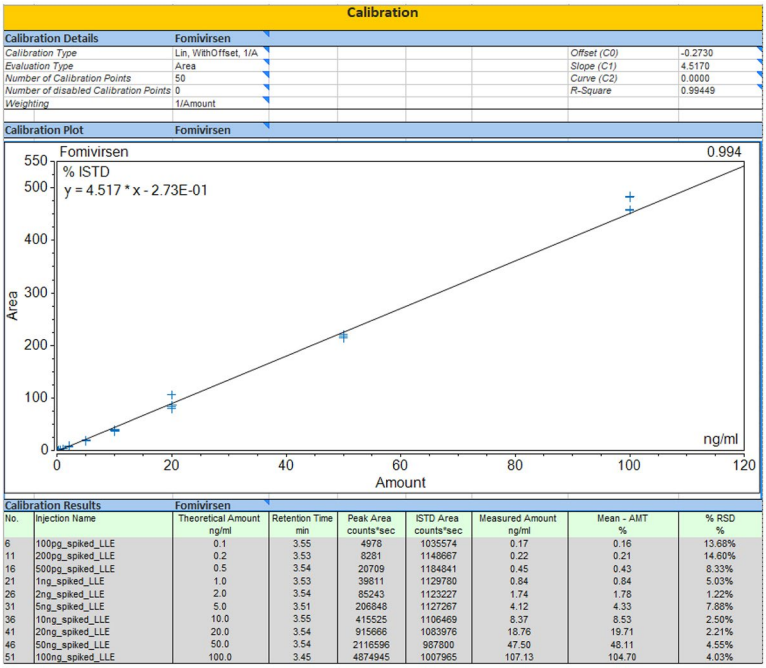


Figure 5. Example of summary report for the quantitation of fomivirsen generated by Chromeleon CDS. (a) Sample injection information and integrated peak details. (b) Calibration plots and calibration results for 10 calibration levels, with 5 replicates per each level.

Conclusions

A selective and sensitive LC-HRAM-MS method was developed on the Orbitrap Exploris 120 mass spectrometer and Chromeleon CDS for the quantitation of fomivirsen and nusinersen spiked into human plasma matrix. This method provides:

- Sensitive and selective quantitation of fomivirsen and nusinersen in human plasma matrix at an LLOQ of 0.20 ng/mL and 0.10 ng/mL, respectively
- Consistent and accurate quantitation of fomivirsen and nusinersen in human plasma matrix with excellent precision and accuracy, and at least 3 orders of linear dynamic range
- Quick method development that allows selection of target precuror-to-product transitions against any matrices with ease
- A compliant software solution from data acquisition and processing to reporting that is suited for a regulated environment

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