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Biopharma

Sensitive LC-MS/MS quantitation of antisense oligonucleotides in plasma using the TSQ Altis Plus mass spectrometer

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Keywords

Oligonucleotide bioanalysis, ASO, liquid-liquid extraction (LLE), ion-pairing reversed phase liquid chromatography (IPRP-LC), LC-MS/MS, selected reaction monitoring (SRM), Vanquish Horizon UHPLC, TSQ Altis Plus mass spectrometer, Chromeleon CDS

Application benefits

Sensitive and reproducible quantitation of ASOs with human plasma matrix by IPRP-LC-MS/MS using the Thermo Scientific™ TSQ Altis™ Plus mass spectrometer coupled to the Thermo Scientific™ Vanquish™ Horizon UHPLC system

Goal

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

Introduction

Over the last decade, antisense oligonucleotide (ASO)-based therapeutics have gained tremendous interest due to their ability to regulate gene translation through complementary hybridization to their mRNA targets, which can trigger inadvertent cleavage of DNA-RNA heteroduplex by RNase H endonucleases.[1] Since the first approval of fomivirsen in 1998, nine additional ASO-based drugs have been approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA), and 38 more are currently undergoing phase III clinical trials.[2] Given the number of drug candidates entering clinical trials, there has been an increased demand for highly sensitive and robust quantitative bioanalytical methods to evaluate their pharmacokinetic and metabolic profiles.

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Although ligand-binding assays (LBA) and fluorescence-based detection are still the primary methods for quantitative oligonucleotide bioanalysis due to their low limits of detection, mass spectrometric approaches have become an alternative technique for providing high-throughput analysis of target oligonucleotides and their impurities with comparable limits of detection and high specificities, which the other two methods lack. Herein, we report the use of an LC-MS/MS method developed on the TSQ Altis Plus mass spectrometer for quantitative analysis of fomivirsen and nusinersen, two FDA-approved ASO-based drugs, with human plasma matrix. Using this method, we can achieve an LLOQ of 0.10 ng/mL for both drugs with excellent reproducibility, accuracy, and linearity. In addition, a fit-for-purpose report under the CFR 21 Part 11-ready Chromeleon CDS was developed, allowing for quick review of the results. This complete solution enables straightforward integration with regulated bioanalysis laboratories.

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 – 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 samples were dried down in a speed vacuum for 30 min and reconstituted with 200 µL of standards such that the final concentrations of fomivirsen and nusinersen ranged from 0.10 ng/mL to 100 ng/mL, and the internal standard (IS) concentration was 10.0 ng/mL.

Table 1. Antisense oligonucleotide 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'-O-methoxyethylated ribose; "m" denotes 2'-O-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-MS/MS method was developed on the TSQ Altis Plus mass spectrometer for quantitation of the ASOs. Table 3 outlines the source and scan settings. Table 4 outlines the SRM transitions.

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. A linear regression curve was fitted to the calibration plot with 1/x weighting.

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 temperature	50 °C
Divert to source	2.8–4.8 min

Table 3. MS source and scan settings

Parameter	Value
TSQ Altis Plus MS source parameters	
Negative ion	3,000 V
Sheath gas	50 Arb
Aux gas	10 Arb
Sweep gas	1 Arb
Ion transfer tube temperature	325 °C
Vaporizer temperature	350 °C
SRM parameters	
Q1 resolution (FWHM)	1.2 Th
Q3 resolution (FWHM)	1.2 Th
CID gas	2 mTorr
Source fragmentation	0
Points per peak	10

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, targeted MS processing, and reporting.

Results and discussion

Human plasma samples were 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. This solution has been widely used for molecular diagnostic applications in forensic sciences by promoting the partitioning of lipids and cellular debris into the organic phase, leaving isolated DNA in the aqueous phase.[3] As a result, we observed minimal matrix interferences for the analysis of the purified ASOs using the LC-MS/MS method. As shown in Figure 1, both ASOs were baseline-resolved chromatographically from the

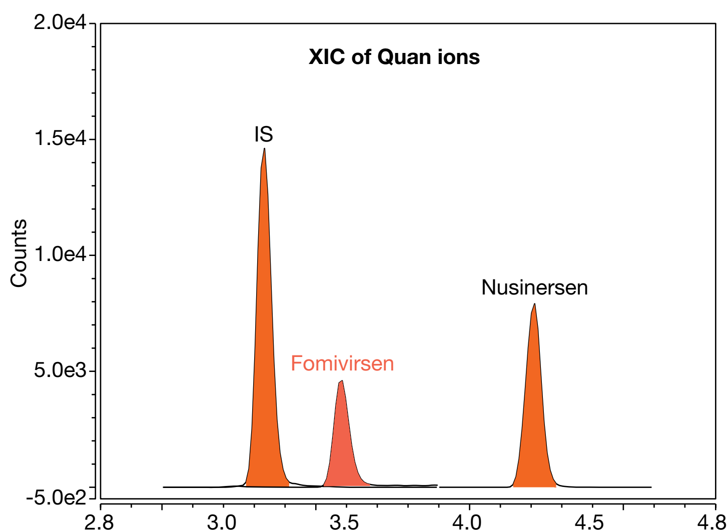

Figure 1. Separation of fomivirsen, nusinersen, and IS at the 10.0 ng/mL level each using the IPRP-LC-MS/MS method

Table 4. 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*)	Collision energy (V)	RF lens (V)
Fomivirsen (Quan)	3–3.9	834	319.2	35	175
Fomivirsen (Qual)		741.3		28	
Nusinersen (Quan)	3.9–4.6	889.8	393.05	32	200
Nusinersen (Qual)		1017		28	
IS (Quan)	3–3.9	820.8	374.03	40	200
IS (Qual)		938.1		40	

IS. The resulting peak areas of the ASOs in both the matrix (Figure 2a) and matrix with IS (Figure 2b) were less than 10% compared to the peak areas of the ASOs in the 0.10 ng/mL spiked sample.

The LC-MS/MS method was operated in SRM mode for the quantitation of ASOs in human plasma. We were able to quantify both standards at 0.10 ng/mL with greater than 80% accuracy and within 10% precision as shown in Table 5. The method also demonstrated a linear signal response from 0.10 to 100 ng/mL with great consistency across five replicate injections per concentration level. As shown in Figure 3, both calibration plots had coefficients of determination or R^2 values of 0.998 for the linear regression fit with 1/x weighting.

All results were captured in a cGLP compliant, fit-for-purpose report. An example is provided in Figure 4, 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.

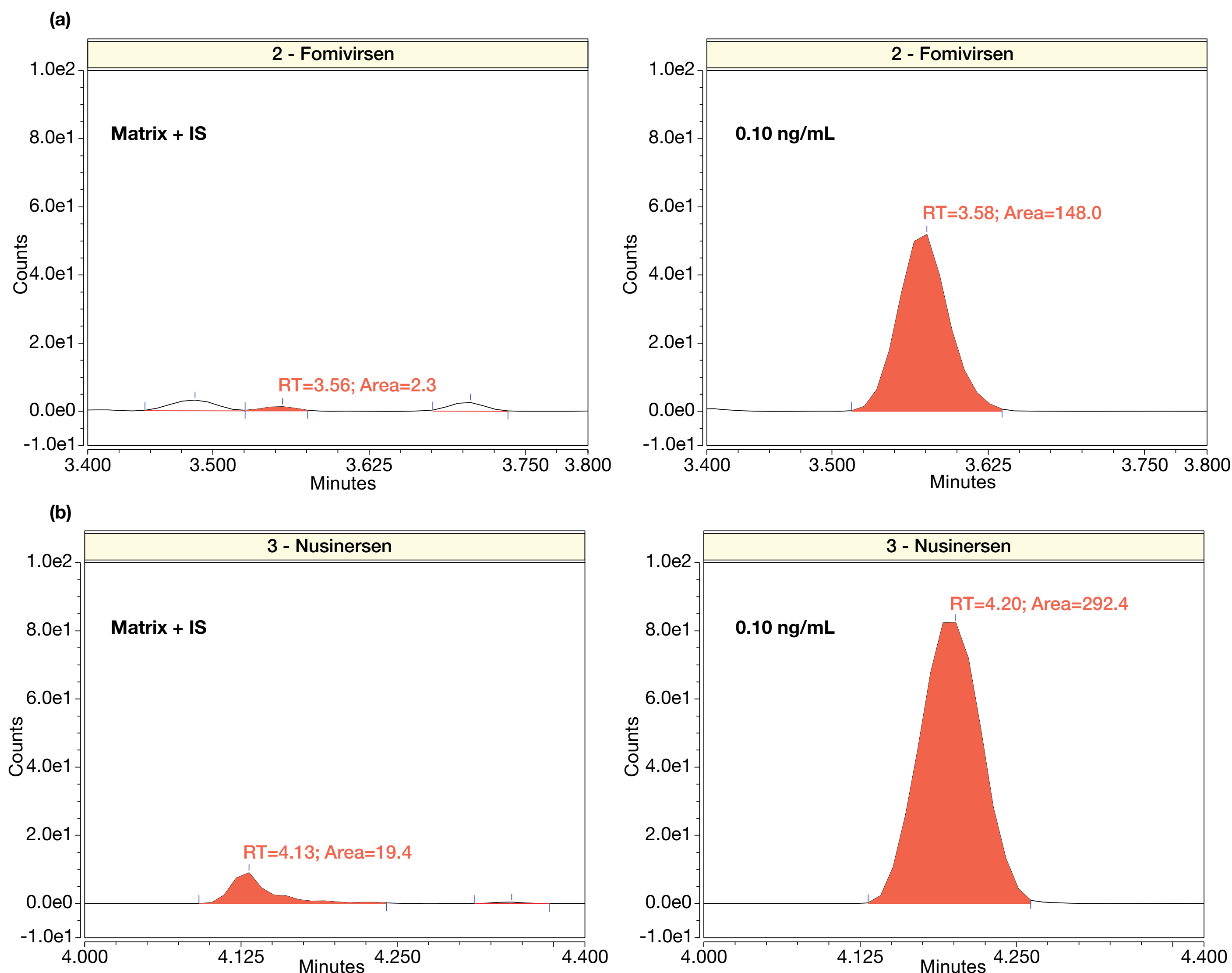

Figure 2. Peak area comparison between the matrix with IS and 0.10 ng/mL spiked sample for the analysis of fomivirsen (a) and nusinersen (b) using the quantifier ion. Minimal interferences were observed.

Table 5. Precision and accuracy evaluation of the LC-SRM-MS method. Values were averaged over five replicate injections at each calibration level.

	Fomivirsen		Nusinersen	
Concentration (ng/mL)	Precision % RSD	Accuracy % Diff	Precision % RSD	Accuracy % Diff
0.10	10.7	15.2	9.8	19.6
0.20	5.4	14.9	3.9	16.4
0.50	8.9	8.3	3.8	0.1
1.0	8.6	1.3	3.0	4.2
2.0	6.1	8.6	2.7	10.5
5.0	4.2	6.3	2.2	8.6
10	3.0	1.5	2.8	5.5
20	4.0	1.9	4.3	2.3
50	4.6	2.4	3.0	1.5
100	3.6	1.5	0.9	2.4

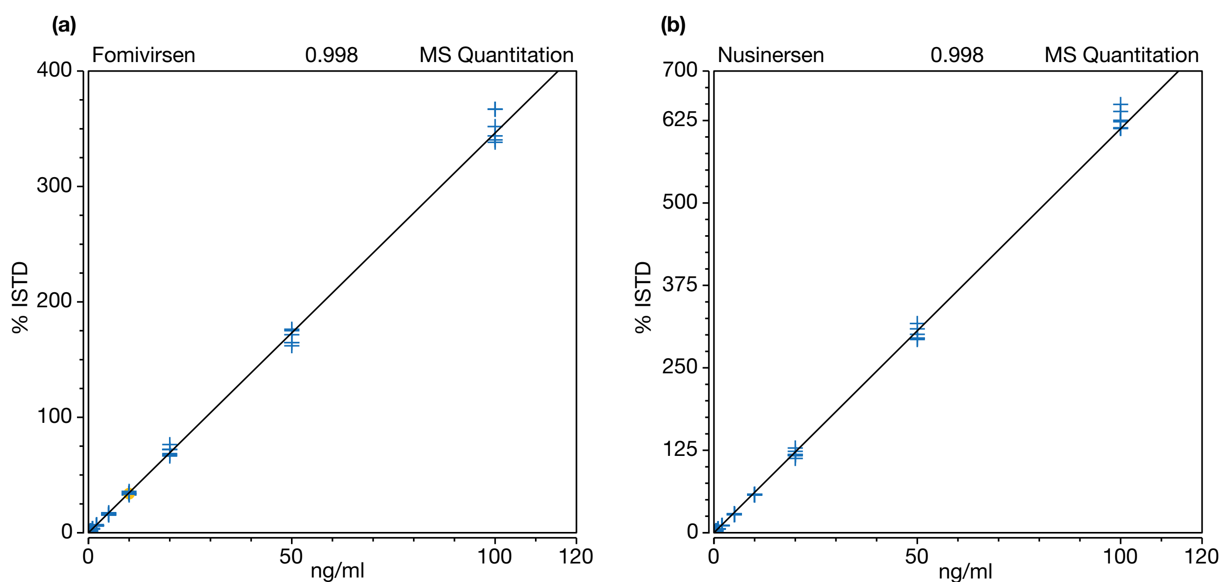

Figure 3. Calibration plots for the quantitation of (a) fomivirsen and (b) nusinersen showing the linear signal response across concentrations ranging from 0.10 to 100 ng/mL. Values were averaged over five replicate injections at each calibration concentration.

Figure 4. Example of a summary report for the quantitation of fomivirsen generated by Chromeleon CDS. (a) Sample injection information and integrated peak details, (b) calibration plot and the results for each calibration level (n=5)

Conclusions

A selective and sensitive LC-MS/MS method was developed on the TSQ Altis Plus mass spectrometer for the quantitation of fomivirsen and nusinersen with human plasma matrix. This method provides:

- Interference-free quantitation of formivirsen and nusinersen with human plasma matrix extracted from a single LLE extraction step
- A sensitive detection and quantitation of fomivirsen and nusinersen with human plasma matrix at an LLOQ of 0.10 ng/mL
- A consistent and accurate quantitation of fomivirsen and nusinersen in human plasma matrix with greater than 80% accuracy, within 10% precision, and linear response over 0.995 R^2 across the concentration range from 0.10 to 100 ng/mL
- A cGLP compliant report, capturing all the results for every single injection, that is suited for a regulated environment

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