Sensitive Quantification of Therapeutic Oligonucleotides Extracted from Plasma

Featuring the SCIEX QTRAP® 6500+ LC-MS/MS System

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Oligonucleotide therapeutics and gene therapies are rapidly gaining attention. Advances in chemical modifications particularly to the antisense oligonucleotides (ASO) and small interfering RNA (siRNA) therapeutics continue to improve target binding, exonuclease resistance and delivery to target organs. Furthermore, the potential disease areas in which oligonucleotide based therapies are being developed is rapidly expanding due to their potential for treating previously undruggable targets.

The increased interest in these modalities necessitates the development of suitable bioanalytical approaches to understand both the pharmacokinetic profiles and metabolism. Multiple bioanalytical methods have been employed for quantitative bioanalysis of therapeutic oligonucleotides with hybridization-based ELISA (HELISA) and liquid chromatography coupled with mass spectrometry (LC-MS) being the two most commonly used. HELISA assays have been widely used in support of toxicokinetic/pharmacokinetic evaluations primarily due to the low detection limits that can be achieved. However, hybridization assays lack the specificity to distinguish between full length

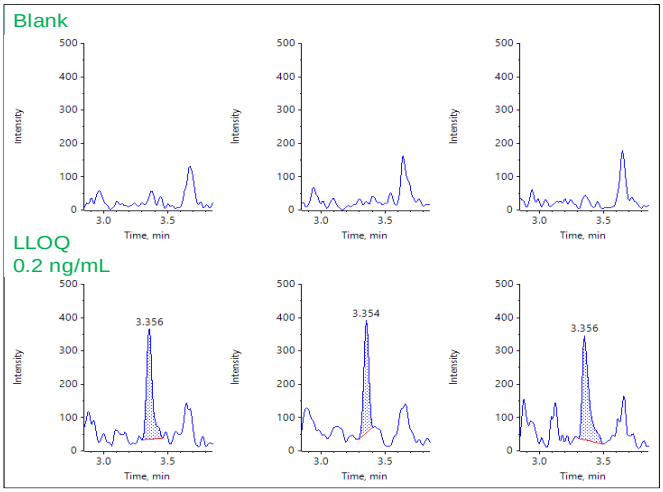

Figure 1. The LLOQ of 0.2 ng/mL and the blank.

SCIEX QTRAP® 6500+ LC-MS/MS system

oligonucleotide products and their metabolites. Furthermore, while the hybridization assay is highly sensitive, the linear dynamic range of the assay can be limiting. As such, LC-MS is an attractive technique for the bioanalysis of oligonucleotide therapeutics as the assay has the potential to discriminate and quantify not only the full length product (FLP) but also impurities and metabolites. However, LC-MS presents its own unique challenges at each point in the analytical workflow. Sample preparation often yields low recoveries and extracted samples still suffer from matrix effects. Additionally, a combination of the use of necessary ion- pair (IP) reagents, wide charge state distributions and the potential for adduct formation can further reduce the ability to reach the required levels of quantification. As such, a robust and highly sensitive MS is essential to combat the chromatographic challenges and achieve low limits of quantification (LLOQ). In this work, we present a highly sensitive LC-MS/MS assay for the quantification of an ASO extracted from plasma. Data was acquired using the QTRAP 6500+ LC-MS/MS system.

Key Features of the QTRAP 6500+ LC-MS/MS System for Oligonucleotide Quantification

- Exceptional negative ion performance for the highly sensitive analysis of the ASO extracted from plasma.
- The IonDrive™ QJet Ion Guide for efficient ionization and transmission and reduced contamination
- The enhanced speed, sensitivity and resolution of the Linear Ion Trap (LIT) functionality to facilitate on-column method development

Methods

Samples and Reagents: All reagents were purchased from Sigma Aldrich. This includes the 1,1,1,3,3,3-hexafluoro isopropanol ≥ 99.8% (HFIP), diisopropylethylamine 99.5 % (DIEA). Ethylenediaminetetraacetic acid (EDTA), phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), chloroform, Tris-HCl pH 8 and concentrated ammonium hydroxide. The ASO was provided by Wave Life Sciences and was a stereopure fully phosphorothioated 20 mer with additional chemical modifications for enhanced stability.[1] Human plasma was employed as the matrix.

Sample Preparation: Plasma samples were prepared using a double liquid-liquid extraction (LLE). An equal 50 µL volume aliquot of plasma samples was combined with an aliquot of 2 µg/mL of the 20 mer oligonucleotide internal standard (IS) in water. Aliquots of 60 mM Tris-HCl pH 8, 10 mM EDTA and concentrated ammonium hydroxide were added. The samples were vortexed and phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and water were added. The samples were further vortexed. Following centrifugation the upper aqueous layer was transferred to a new tube, the bottom layer was discarded. A second LLE was then performed with chloroform. The samples were then vortexed and centrifuged again. The upper layer was removed and dried down to near dryness. The samples were reconstituted to 100 µL of water containing 100 µM EDTA.

Chromatography: The separation was accomplished using an Agilent 1290 Infinity UHPLC fitted with a Phenomenex Clarity Oligo-XT Column, 2.1 mm x 50 mm with 2.6 µm particle size. Mobile phase A and B were 15 mM DIEA with 100 mM HFIP in water and methanol, respectively. The gradient used is shown in Table 1. The column temperature was held at 60ºC. An injection volume of 15 µL was employed.

Mass Spectrometry: A SCIEX QTRAP 6500+ LC-MS/MS System fitted with an IonDrive™ Turbo V Source was utilized. The source conditions employed are listed in Table 2. The QTRAP System functionality was leveraged for rapid method development using an information dependent acquisition (IDA) workflow, the criteria can be seen in Table 3. An Enhanced Mass Spectrometry (EMS) scan was employed to determine the precursor masses while an Enhanced Product Ion (EPI) scan was used for determination of product ions specific for the ASO. Following determination of the precursor and product ions an MRM workflow was employed for quantification, the conditions are listed in Table 4. All data was acquired using Analyst® Software 1.7.1.

Data Processing: Method development data was visualized using Explorer and Bio Tool Kit Sofware within SCIEX OS-Q Software. The quantification data was processed using Analytics also within SCIEX OS-Q Software.

Table 1. LC Gradient.

Time (min)	%A	%B	Flow Rate (µL/min)
Initial	*85*	*15*	*300*
4.5	*55*	*45*	*300*
4.6	*10*	*90*	*300*
5.6	*10*	*90*	*300*
5.7	*85*	*15*	*300*
8.0	*85*	*15*	*300*

Table 2. Source Parameters.

	Value
Polarity	*Negative*
GS1 (psi)	*80*
GS2 (psi)	*80*
Curtain Gas	*40*
Temperature (°C)	*400*
Spray Voltage (V)	*-4500*
CAD Gas	*12*

Table 3. IDA Parameters.

	Value
EMS mass range (m/z)	*600-2000*
EMS scan rate (Da/s)	*10000*
LIT fill time (ms)	*Dynamic*
IDA Criteria	
Trigger for EPI	*2 most intense ions*
Mass range (m/z)	*600-2000*
Threshold (cps)	*1000*
Exclude former target ion	*After 3 occurrences for 1 sec*
Rolling collision energy	*On*
EPI mass range (m/z)	*300-800*
EPI scan rate (Da/s)	*1000*
LIT fill time (ms)	*2*

Table 4. MRM Conditions for Analysis.

	Q1	Q3	CE	DP
-10_1	*716*	*392*	*-39*	*-50*
-10_2	*716*	*319*	*-29*	*-50*
-10_3	*716*	*733*	*-35*	*-50*
-9_1	*796*	*392*	*-45*	*-50*
-9_2	*796*	*319*	*-35*	*-50*
-9_3	*796*	*733*	*-45*	*-50*
IS MRM_1	*739*	*709*	*-29*	*-50*
IS MRM_2	*739*	*374*	*-34*	*-50*

Results

Method Development: Infusing oligonucleotides for method optimization remains a challenge. Despite purification small traces of salts in the sample can degrade the oligonucleotide signal. Method optimization is therefore completed using an on-column approach, the method optimization to quantification workflow is presented in Figure 2. The enhanced speed of the linear ion trap (LIT) was leveraged for rapid method development. An information dependent acquisition (IDA) workflow was employed to determine the precursor and product ions simultaneously. A 10 µg/mL sample of the analyte was prepared in water containing 100 µM EDTA and 20 ng was injected onto the column for method optimization. The chromatographic and source conditions described in Table 1 and Table 2 above were employed.

The precursor mass can be determined using an EMS scan by scanning from 600-2000 m/z. This mass range for the Q1 scan allows most of the charge state envelope of an ASO of typical lengths to be visualized. Figure 3 shows the charge state envelope and the reconstructed mass of the analyte. The charge state envelope distributes the signal from the -11 to the -4 charge state. The -10 and the -9 were the two most abundant charge states and these were selected as the precursor masses for the MRM transitions.

In addition to the precursor mass selection the raw spectra can be reconstructed using Bio Tool Kit Software within SCIEX OS-Q Software. From this, the level of adducts and degradation products can be determined, additionally the presence of any impurities can be understood. As is widely known, the formation of adducts are an inherent challenge for LC-MS oligonucleotide analysis as the negatively charged phosphodiester or phosphorothioate backbone electrostatically attracts the positively charged alkaline metal salts which are commonly present in the LC system and columns. The presence of adducts, commonly the sodium and potassium adducts, further distributes the oligonucleotide signal which compromises the targeted quantitative abilities of the assay. As seen above the presence of the sodium adduct was approximately 2% indicating the level of cleanliness of the LC system and column are suitable for the quantitative assay. In addition to the sodium adduct, a small impurity peak can be seen with a – 16 Da shift, which indicates the presence of a small amount of the phosphodiester (PO) backbone.

An IDA workflow was employed to trigger the EPI scan, the IDA criteria are listed in Table 3. The sensitivity and resolution of the product ion spectra is dramatically improved over a conventional product ion (PI) scan generated using the triple quadrupole functionality. Figure 4 demonstrates selected product ions obtained from the -10 charge state, 716.6 m/z. On the left, an EPI scan was employed and on the right a PI scan. Figure 2 reveals the improved sensitivity and resolution that can be obtained when leveraging an EPI scan. The signal improvements demonstrated were as great as 15x for the phosphorothioate 94.9 m/z product ion while 133.0 and 733.3 m/z revealed signal improvements of 6x and 8x, respectively. Additionally, resolution

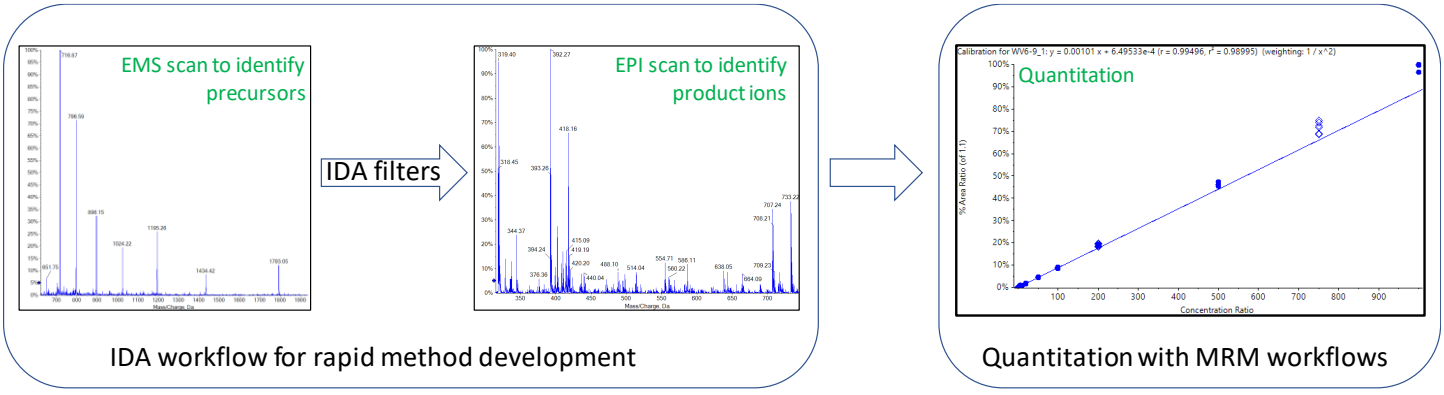

Figure 2. The method optimization workflow utilizing the LIT followed by quantification with MRM.

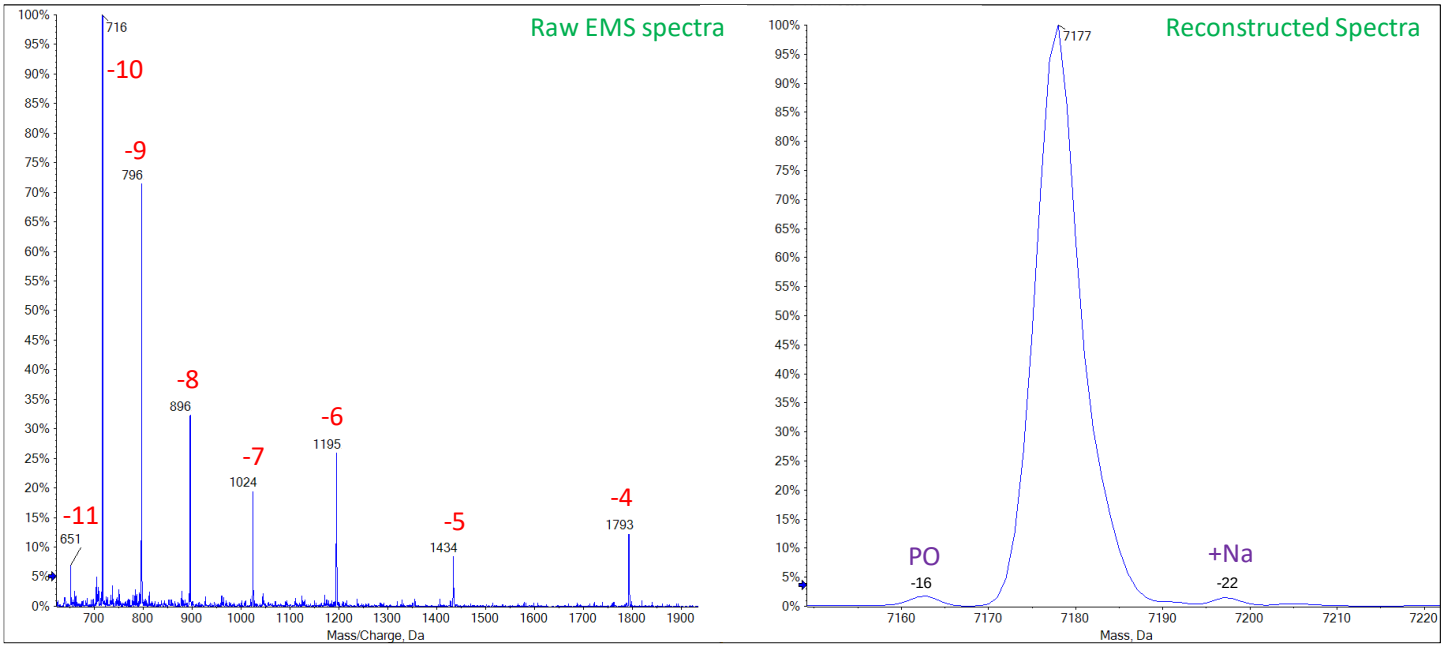

Figure 3. The Q1 scan of the phosphorothioated therapeutic oligonucleotide.

of the product ions was dramatically improved as can be seen by the reduction of the peak width at 50%.

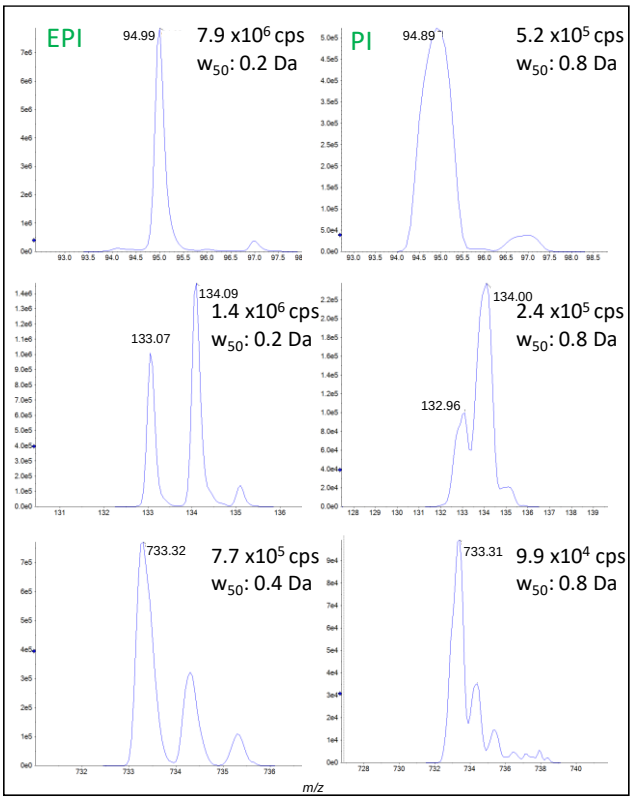

Figure 4 Selected product ions of the EPI and PI scans for the -10 charge state 716 m/z. A collision energy of -40 V and a declustering potential of -50 V.

The full EPI scan of the 716 m/z precursor ion, the -10 charge state, is presented in Figure 5.

To ensure assay specificity only product ions with a m/z greater than 300 m/z were selected for quantification. The -9 charge state revealed the same product ions (data not shown). Three product ions were selected to populate the MRM table for the -10 and -9 charge states (Table 4). The collision energy was optimized for both the -10 and -9 charge states by assessing signal response over the range of -25 to 45 V in 2 V increments. Finally, the source parameters were optimized. While most of the source parameters in Table 2 can be used as default source parameter for this assay, source temperature must be optimized

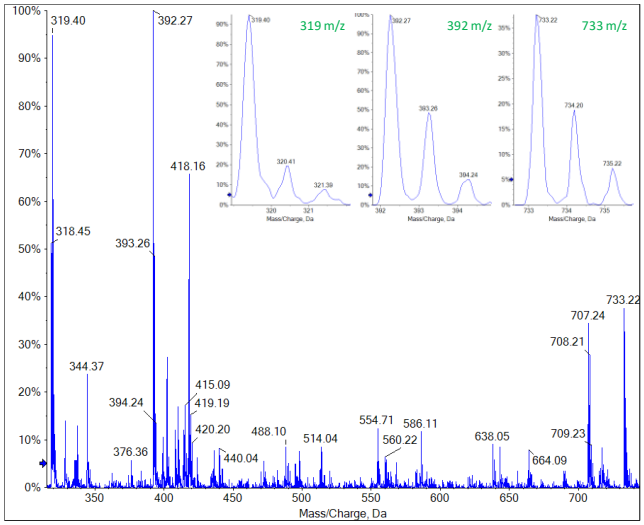

Figure 5 Full EPI scan of 716 m/z, the -10 charge state of the analyte.

for the ASO being analyzed to prevent in-source degradation. This was done by assessing 300 to 500°C in 50°C increments.

Quantitative Results: The optimized MRM transitions and source parameters for quantification can be seen in Table 2 and Table 4, respectively. A set of calibrants and quality controls (QCs) in human plasma were prepared using an LLE extraction, the concentration ranged from 0.2 to 1000 ng/mL. The transition of 716.6 → 392.1 m/z was selected for quantification as it provided the best signal to noise at the low end of the quantitative curve. The assay was considered acceptable if at least three-quarters (75%) of the standards as well as two-thirds (67%) of total QCs for the run were within 15% of deviation (20% at LLOQ) from nominal concentration. Data was processed using Analytics within SCIEX OS-Q Software. A linear regression with $1/x^2$ weighting was applied to the data with r = 0.99446. Figure 6 demonstrates the linear calibration curve ranging from 0.2 to 1000 ng/mL. While Figure 1 displays triplicate injections of the blank and 0.2 ng/mL.

Table 5 and Table 6 highlight the figures of merit of the assay for both the calibration curve and the QCs, respectively. The results generated demonstrate that the system was able to quantify an ASO extracted from human plasma down to the sub ng/mL level (i.e., 200 pg/mL).

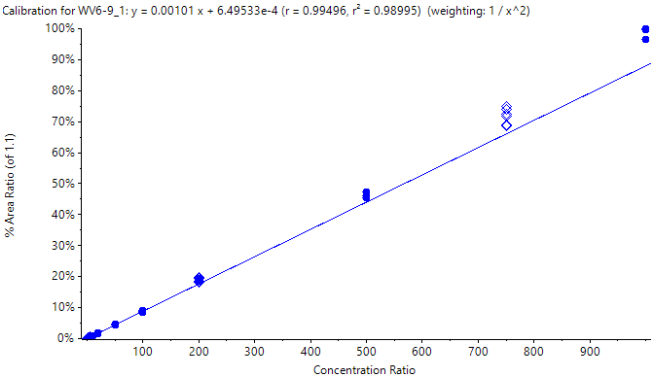

Figure 6. The calibration curve for 0.2-1000 ng/mL of a therapeutic oligonucleotide LLE extracted from plasma. The filled blue circles represent calibrants while the open diamonds represent the QCs.

Table 5. The calibration curve MRM transition 716.6 → 392.1 m/z of the oligonucleotide extracted from plasma, r = 0.99496 weighting $1/x^2$.

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)	CV (%)
0.2	*0.21*	*105.62*	*11.73*
0.5	*0.46*	*89.49*	*12.05*
1	*0.93*	*91.21*	*0.77*
5	*4.95*	*100.02*	*3.88*
10	*9.31*	*90.39*	*4.82*
20	*18.36*	*93.00*	*6.06*
50	*51.12*	*100.45*	*4.50*
100	*99.51*	*100.13*	*4.42*
200	*214.40*	*108.14*	*2.44*
500	*525.50*	*106.65*	*2.26*
1000	*1122.00*	*111.98*	*2.43*

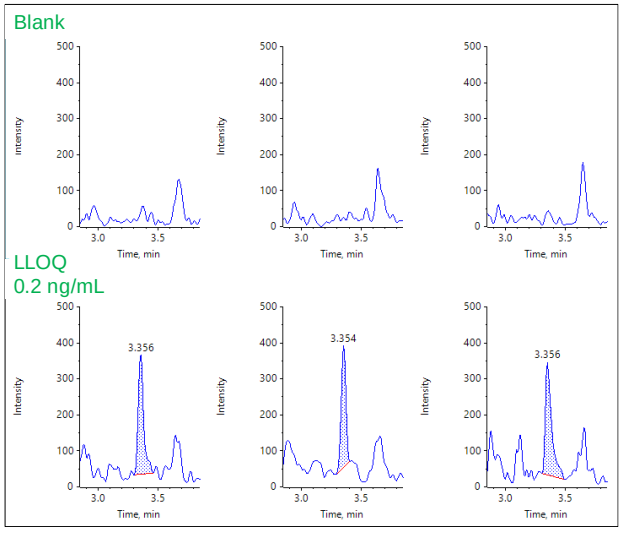

Figure 1. The LLOQ of 0.2 ng/mL and the blank.

Table 6. The QCs of MRM transition 716.6 → 392.1 m/z of the oligonucleotide extracted from plasma.

Actual Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)	CV (%)
0.2	*0.17*	*85.68*	*18.29*
0.6	*0.68*	*104.15*	*7.61*
1.5	*1.36*	*94.09*	*8.95*
5	*4.92*	*93.68*	*8.72*
200	*215.90*	*108.44*	*4.15*
750	*815.00*	*110.98*	*2.91*

Conclusions

- The LIT functionality of QTRAP 6500+ LC-MS/MS System was leveraged for rapid on-column method development of the MRM workflow.
- A high flow MRM based MS/MS assay was developed for an ASO extracted from human plasma.
- A linear range of quantification was established from 0.2 to 1000 ng/mL covering 3.5 orders of magnitude.
- The highly sensitive quantitative assay yielded an LLOQ in matrix at the sub ng/mL level (i.e., 200 pg/mL).

References

1. Iwamoto, N. et al. Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides. *Nat. Biotechnol.* (2017) **35(9)**, 845-851.

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