Improving LC-MS/MS Bioanalytical Quantitation of Oligonucleotides using New High Performance Tandem Quadrupole Mass Spectrometer

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Introduction

Improving LC-MS/MS bioanalytical performance especially for ESI negative analytes has been of high interest especially for the challenging analysis of ONTs (oligonucleotide therapeutics) in biological matrices. Developing low/sub ng/mL oligonucleotide therapeutic quantitation methods requires special attention to recovery, removal of protein/interference and losses due to non-specific binding.[1,2]

Here we demonstrate the performance capabilities of the high-performance tandem MS Waters™ Xevo™ TQ Absolute coupled to the Waters ACQUITY™ Premier UPLC system for the analysis of oligonucleotides in biological matrices. Quantification of oligodeoxy-thymidines standards and GEM91 (trecovirsen), a fully phosphorothioated antisense oligonucleotide were investigated.

Methods

Stock solutions of the Waters™ MassPREP™ Oligonucleotide Separation Technology (OST) Standard and GEM91 ([d(P-Thio) (C-T-C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T-T-C-T)-DNA]) were prepared at 10µM and 1 mg/mL respectively in TE buffer. Curves were prepared by serial dilution of stock solution in human plasma. GEM132 (100ng/mL) was used as internal standard for quantitation of GEM91 and OSTs (15-35mer). Plasma samples were extracted using liquid-liquid extraction method with phenol:chloroform:isoamyl alcohol 25:24:1 followed by a second extraction with chloroform, +99%. Finally, all aqueous extracts were dried and reconstituted in 100µM EDTA solution. Extracts were analyzed on the Xevo TQ Absolute coupled to the Waters ACQUITY Premier UPLC system using 100mM hexafluoroisopropanol (HFIP) + 15mM N, N-diisopropylethylamine (DIPEA) in water as mobile phase A and 100mM hexafluoroisopropanol (HFIP) + 15mM N, N-diisopropylethylamine (DIPEA) in 90% acetonitrile as mobile phase B with flow rate of 0.5mL/min. Waters ACQUITY PREMIER Oligonucleotide C18 column, 1.7 µm, 2.1 x 50 mm was used. A shallow LC gradient of 0-3.5min at 5-22% B was used for analyte separation and elution, followed by wash and column equilibration with a 5 min run time. TargetLynx XS was used for data acquisition and processing.

Results

To demonstrate reproducibility, duplicates of calibration standards and six replicates of QCs of GEM91 in each run were tested (table 1) in three runs on 3 separate days. The calibration curves were linear with r^2 values >0.99 ($1/x^2$ weighting) with >75% non-zero calibrator levels and QCs met acceptance criteria in each run i.e., Non-zero calibrators and QCs should be ±15%, except at LLOQ where the calibrator or QCs should be ±20% of nominal concentrations in each run as shown in table 2.

	GEM91			OSTs	15T	20T	25T	30T	35T
Std Conc (ng/mL)	Day 1 (n=2)	Day 2 (n=2)	Day 3 (n=2)	Std Conc (nM)	Day 1 (n=2)				
0.1	100.1	99.1	101.6	0.01	104.0	101.1	101.0	96.7	_
0.2	100.1	100.2	101.6	0.02	93.9	100.3	98.0	102.0	_
0.5	96.1	102.8	94.4	0.05	94.0	95.4	99.9	101.9	98.9
1	113.2	99.2	94.5	0.1	102.1	96.3	97.5	101.4	100.4
2	197.7*	98.5	101.2	0.2	101.4	101.2	104.6	103.1	103.5
10	105.7	96.7	102.2	1	103.1	102.6	101.0	100.8	101.7
100	101.0	99.5	103.3	10	100.7	102.1	100.1	95.7	94.8
1000	101.0	106.1	106.0	100	99.8	102.0	102.5	100.9	101.3
5000	92.9	99.8	94.9	500	100.8	100.2	99.2	98.3	99.0
10000	96.8	98.0	101.3	1000	100.4	98.9	96.3	97.7	100.6
r^2	0.9958	0.9981	0.9964	r^2	0.9968	0.9933	0.9958	0.9975	0.9966

*: Excluded from the curve

Table 1– Statistics for calibration standards

	LLOQ (ng/mL)
15T	0.045
20T	0.060
25T	0.075
30T	0.090
35T	0.529

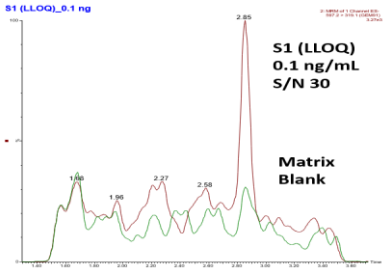

Figure 1 – GEM 91 (597.2 > 319.1) 0.1 ng/mL standard (LLOQ) overlaid with matrix blank (green trace)

OSTs were tested to ensure system performance over the characterized range of polythymidine oligonucleotide ladder standards (15-35mer). OST standard results show that smaller oligonucleotides typically have more sensitivity compared to longer ones and performance becomes more challenging at higher chain lengths, but sub nM or ng/mL is possible even for the 35mer. OST standards are provided in mM conc but LLOQs are also reported in ng/mL to the right of figure 1. GEM91 which is of similar length to 25T standard shows similar performance, whereas 15T standard showed 2x higher sensitivity at 0.05 ng/mL as LLOQ. The analyte response at LLOQ is >5x the analyte response in matrix blank (shown in figure 1). The lower limit of quantification (LLOQ) of 0.1 ng/mL was achieved over a calibration range of 0.1 to 10,000 ng/mL in human plasma and standard curve is shown in figure 2.

	GEM91						15T		20T		25T		30T		35T	
	Day 1 (n=6)		Day 2 (n=6)		Day 3 (n=6)		Day 1 (n=6)									
Std Conc (ng/mL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.1	111.0	7.3	106.7	5.8	97.5	13.6	101.0	13.9	104.1	13.9	101.6	13.9	94.2	14.1	_	_
0.5	110.7	2.3	102.1	5.4	88.7	4.2	102.3	3.7	101.0	5.7	92.0	5.9	104.1	4.6	91.2	11.0
50	98.3	2.7	107.4	1.7	108.4	1.9	102.4	2.1	105.6	2.1	104.3	3.6	102.8	2.9	106.2	4.1
8000	99.2	2.6	107.8	2.3	102.1	1.2	102.1	2.0	101.4	2.6	99.2	2.3	98.8	2.6	99.7	3.4

Table 2 – Statistics for QC samples

Name	Mol. Wt	Precursor m/z	Product m/z	Cone (V)	Collision (eV)
15T	4501	561.8	125.1	30	35
20T	6022	601.1	125.1	30	35
25T	7543	627.3	303.1	30	20
30T	9064	646.5	303.1	30	20
35T	10585	646.5	303.1	30	20
GEM91	7776	597.2	319.1	30	20
GEM132 (IS)	6600	732.7	319.0	30	40

Table 3 – List of oligonucleotides and transitions

Figure 2 – Standard curve for GEM 91 analyzed on TQ Absolute showing 5x linear dynamic range, inset shows 0.1 to 10 ng/mL standards.

Conclusions

- With enhanced sensitivity for challenging negative ionization compounds, the TQ Absolute tandem MS can generate high quality data for routine LC-MS/MS based quantitation of oligonucleotides in biological matrices.
- Low ng/ml levels of sensitivity, with good dynamic range performance for oligonucleotides was observed in human plasma for both an antisense oligonucleotide as well as oligonucleotide performance standards.
- The use of ACQUITY Premier with MaxPeak High Performance Surfaces (HPS) technology help to mitigate metal adsorption, ensuring robust and sensitive quantitation performance for quantitative bioanalytical assays.

References

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2. Jennifer M Nguyen, Martin Gilar, Brooke Koshel, Michael Donegan, Jason MacLean, Zhimin Li & Matthew A Lauber, 'Assessing the impact of nonspecific binding on oligonucleotide bioanalysis', Future Science, BIOANALYSIS VOL. 13, NO. 16.