BIOANALYTICAL QUANTIFICATION OF A LIPID CONJUGATED ANTI-SENSE OLIGONUCLEOTIDE ON A HRMS SYSTEM

Authors: Nikunj Tanna, Joe Fredette and Mary Trudeau
Affiliations

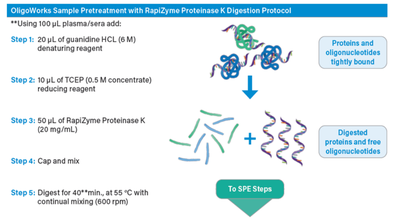
INTRODUCTION

Oligonucleotide therapeutics (ONTs) are a key focus area for many drug developers today given their powerful ability to address disease biology at the level of gene transcription and translation, and for their high target specificity and low toxicity. As the pipeline for this class of nucleic acid therapeutics continues to expand, so does the need for sensitive, accurate and robust bioanalytical assays to support their discovery and advancement through each stage of development.
Discovery bioanalytical laboratories strive to streamline most aspects of analytical testing to increase efficiency as they are required to screen large numbers of potential candidates. A standardized sample preparation approach that works across a wide variety of oligonucleotides can be extremely useful in this regard and can save significant method development time and cost in these laboratories. Additionally, high resolution mass spectrometry (HRMS) is often used to collect data in an untargeted manner, allowing for a single, generic method that can be deployed across a wide variety of analytes. Another critical advantage of the information-rich data obtained from HRMS systems is that it can be re-investigated at later stages to better understand analyte ADME profiles and screen for metabolites of interest, which can accelerate the progression of drug candidates.

In this work, we describe an analytical workflow used for the extraction of a lipid conjugated antisense oligonucleotide (ASO) from rat plasma using the OligoWorks™ SPE Microplate Kit (OligoWorks Kit) followed by quantification on a LC-HRMS system. The OligoWorks Kit offers a simple, broadly applicable sample preparation solution that provides high recoveries and low matrix effects across a diverse set of therapeutic oligonucleotides from biomatrices. Extracted samples were then subjected to LC-MS analysis using an ACQUITY™ UPLC™ H-Class Plus Bio System combined with a Xevo™ G3 Quadrupole-time of flight (QToF) HRMS instrument along with a generic data acquisition method that facilitates sensitive, robust and efficient quantitative analysis of the analyte; demonstrating a standardized analytical workflow that can enhance productivity in discovery bioanalytical laboratories performing therapeutic oligonucleotide analysis.

Figure 1. OligoWorks SPE MicroPlate kit

METHODS



LC System: ACQUITY H-Class PLUS Bio System	
Column	ACQUITY Premier Oligonucleotide BEH™ C18, 130Å, 1.7 µm, 2.1x50 mm Column
Column Temperature (°C)	55°C
Sample Temperature (°C)	10°C
Mobile Phase A	1% HFIP (Hexafluoro-2-propanol) 0.1% DIPEA (N,N-Diisopropylethylamine) in H2O
Mobile Phase B	0.75% HFIP (Hexafluoro-2-propanol), 0.0375% DIPEA (N,N-Diisopropylethylamine, 65% ACN 35% H2O
Purge Solvent	25:25:25:25 Methanol:Acetonitrile:Isopropanol:Water
Injection volume (µL)	10 µL

MS System: Xevo G3 QToF MS	
Ionisation mode	ESI Negative
Acquisition mode	**MS Full Scan**
Capillary Voltage (kV)	2.0
Desolvation temperature (°C)	600
Desolvation gas flow (L/Hr)	1000
Cone gas flow (L/Hr)	150
Collision gas flow (L/Hr)	0.2
Nebulizer (Bar)	7

SPE METHOD OPTIMIZATION

OligoWorks SPE Protocol Optimization:

Initial plasma recovery and matrix experiments for the lipid conjugated ASO were performed using the starting sample preparation and extraction protocol described in the OligoWorks SPE Care and Use Manual. LC-MS analysis for these experiments were performed using an ACQUITY Premier LC System and Xevo TQ Absolute Tandem Quadrupole (TQ) Instrument and MRM analysis using the $[M\text{-}9H^+]^-$ precursor 671.1 m/z and fragment ion of 95.1 m/z and the same chromatographic conditions and column as described in the methods section. Using the starting protocol resulting recoveries were ≈80% and 60% for the lipid conjugated ASO and IS, respectively. Matrix effects were <15% (Data not shown). Although these recovery values are acceptable, we attempted to further optimize the SPE protocol to enhance the recoveries and maximize sensitivity.
The OligoWorks SPE WAX Sorbent is a polymeric reversed-phase, weak anion exchange mixed-mode sorbent. This bi-modal functionality can be leveraged for protocol optimization in the load, wash and elution steps. We started with optimizing the Wash 2 steps as this step can affect retention of the analyte of interest while also impacting removal of unwanted matrix components. A reduction in the organic composition of Wash 2 from 30% methanol (recommended Wash 2 composition in the Care & Use manual) to 10% greatly improved recovery for the target lipid conjugated ASO, but decreased recovery for the IS. In this specific instance, the target lipid conjugated ASO while loaded at a pH of 5.5, was partially eluting in the wash 2 step due to the increase in the methanol composition. In contrast, lower recoveries were seen for the IS when wash 2 methanol composition was reduced. This performance is shown in Figure 2 & 3. As evident from this data, analytes closely related in sequence can still show differential recoveries based on percent organic solvent in Wash 2 step of the SPE.

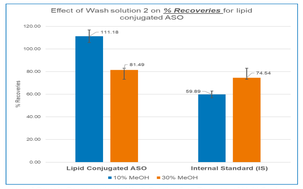


Figure 2 – Impact of Organic Concentration in Wash Solution 2 on Recovery of Lipid Conjugated ASO LC-MS standard and Internal Standard (IS) extracted from rat plasma using the OligoWorks SPE Kit

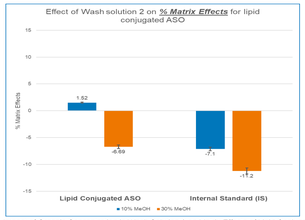


Figure 3 – Impact of Organic Concentration in Wash Solution 2 on Matrix Effects of Lipid Conjugated ASO LC-MS standard and Internal Standard (IS) extracted from rat plasma using the OligoWorks SPE Kit

RESULTS AND DISCUSSION

The Xevo G3 QToF MS maximizes sample information for analytes of interest, from detailed characterization to accurate quantitation. A full MS scan experiment (m/z 50-2000, scan time 0.2 sec) was set up using the UNIFI™ application in waters_connect™ software to collect data in an untargeted manner. This approach allows for a single acquisition method that can be used across a diverse set of analytes without the need for added method development. Figure 4 shows the MS spectra for precursor $[M\text{-}9H^+]^-$ at m/z 671.2297 (Figure 4A) and precursor $[M\text{-}7H^+]$ at m/z 863.2976 (Figure 4B) for a 100 pmol/mL standard of the lipid conjugated ASO extracted from rat plasma.

In addition to the ease of data acquisition, the high resolution of the Xevo G3 QToF MS allows for a deeper investigation of the data and enables the use of narrow mass extraction (XIC) windows to achieve maximum signal-to-noise (S/N). A TQ instrument is routinely operated at unit resolution of +/- 0.7 Da. The high resolving power of the HRMS system can consistently differentiate masses separated by m/z of 0.001 Da. While processing HRMS data, different XIC windows can be evaluated to eliminate MS signal from unwanted matrix components, while focusing on the m/z of the analyte of interest to ensure sufficient signal. This allows the users to minimize the background noise while maintaining the analyte signal, thereby increasing S/N. However, after a certain point, tightening the XIC window starts to negatively impact the analyte peak area counts, without having a significant impact on the background peaks in the chromatogram. Finding the ideal XIC window is therefore a balance between minimizing signal from unwanted background components without compromising the analyte peak area counts.
For the lipid conjugated ASO, XIC windows of 0.7 to 0.01 Da were evaluated. As evident from Figure 5, as the XIC window narrows the non-specific background peaks from matrix components continue to get smaller, providing a cleaner chromatographic background and improving assay selectivity. For final quantification, we chose a XIC window of 0.025 Da as it gave the best balance between a clean background and sufficient analyte peak area counts.

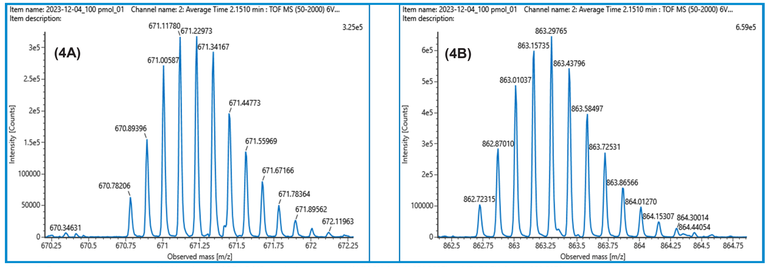


Figure 4 – Representative mass spectra of Lipid conjugated ASO LC-MS standard precursors at (A) [M-9H+]- (m/z 671.2297) and (B) [M-7H+]- (m/z 863.2976)

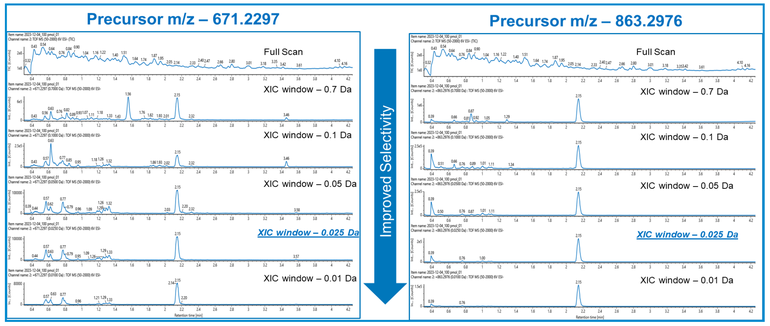


Figure 5 – Effect of XIC window on background signal and analyte peak area counts for lipid conjugated ASO LC-MS standard extracted from rat plasma using the OligoWorks SPE Microplate Kit

QUANTIFICATION DATA

The lipid conjugated ASO spiked in rat plasma, extracted using the OligoWorks Kit and analyzed on the Xevo G3 QToF MS system was linear from 1-1,000 pmol/mL, with a linear fit (r^2) of 0.991 using a 1/x weighting. The % accuracy range across all points on the calibration curve was 87.8-114.2 and % CV range across all points on the curve was 1.29-10.61. (Table 1)

The mean % accuracy and mean % CV for the LQC (5 pmol/mL), MQC (50 pmol/mL) and HQC (750 pmol/mL) were 98.19% and 3.78%, 113.18% and 0.85% and 106.24% and 3.85% respectively (Table 1). The area counts for the calibration curve and QC points increased linearly with an increase in concentration, as highlighted in the representative QC chromatograms in *Figure 4*

Calibration Curve Statistics				
Calibration range (pmol/mL)	Weighting	r^2	% Accuracy range	% CV Range
1-1,000	1/x	0.991	87.8-114.2	1.29-10.61

QC Stastistics				
QC Level	Expected Concentration (pmol/mL)	Observed concentration (pmol/mL)	Mean % Accuracy	Mean % CV
LQC	5.00	4.91	98.19	3.78
MQC	50.00	56.59	113.18	0.85
HQC	750.00	796.81	106.24	3.85

Table 1 - Calibration curve and QC statistics for lipid conjugated ASO LC-MS standard extracted from rat plasma using the OligoWorks SPE Microplate Kit

CONCLUSION

Discovery bioanalysis laboratories are often looking for standardized, streamlined workflows to support the large number of projects and analytes they are required to support. To facilitate greater efficiency and productivity in this regard, we have demonstrated a simple, easy-to-implement workflow using the OligoWorks SPE Microplate Kit for extracting the analyte of interest from biological matrix, followed by untargeted quantification of the analyte on the Xevo G3 QToF HRMS Instrument.

For the lipid conjugated ASO LC-MS standard, we were able to achieve a linear calibration curve from 1-1000 pmol/mL, with all calibration curve and QC points passing the FDA bioanalytical method validation guidelines.

References

1. OligoWork SPE Kits and Components—Care and Use Manual
2. Quantification of a Lipid Conjugated Antisense Oligonucleotide (ASO) Extracted From Rat Plasma Using the OligoWorks™ SPE Microplate Kit on a HRMS System
3. An Automated, Standardized, Kit-Based Sample Preparation Workflow for Bioanalytical Quantification of Therapeutic Oligonucleotides
4. Development of a Standardized, Kit-Based Approach for Selective and Reproducible Sample Preparation and Extraction for Therapeutic Oligonucleotides from Biological Matrices

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS