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Note d'application

LC-MS Bioanalytical Quantification of a GalNAc-siRNA Conjugate Oligonucleotide Using Semi-Automated Solid Phase Extraction

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Abstract

The following work demonstrates the capabilities of the Pipette+ in combination with the Otto SPEcialist for semi-automated liquid handling and solid phase extraction (SPE) of a GalNAc-siRNA conjugated oligonucleotide. The Pipette+ was used to carry out liquid handling and the Otto SPEcialist was used to extract biological samples for subsequent LC-MS/MS analysis and quantification using the ACQUITY Premier Oligonucleotide BEH C_{18} Column, ACQUITY Premier LC System, and Xevo TQ-XS Mass Spectrometer.

Benefits

- Semi-automated sample preparation and extraction using Otto SPEcialist Positive Pressure Manifold and Pipette+ reduces variability between users and ensures accurate and reproducible results
- Transferrable methods on the easy-to-use Otto SPEcialist and OneLab Software allows for implementation of the same preparation procedure across users and labs
- Use of the ACQUITY Premier System and ACQUITY Premier Oligonucleotide BEH C_{18} Column with MaxPeak

High Performance Surfaces (HPS) Technology improves method detection limits and reproducibility of this developed method by mitigating metal adsorption of oligonucleotides

- Accurate and sensitive quantification of a GalNAc-siRNA conjugated oligonucleotide with LODs of 1 ng/mL from extracted urine and plasma

Introduction

With a rise in research and development efforts related to oligonucleotide therapeutics (ONTs), there has been a growing need for more robust, sensitive, and selective sample preparation and LC-MS methods for evaluating these therapeutics. This can be challenging due to the complex and diverse characteristics of ONTs. They are poly-anionic in nature and are known to non-specifically adsorb to metal surfaces and proteins in biological samples. This can lead to analyte loss during biological sample preparation as well as binding to metal surfaces during LC analysis. Furthermore, when manually preparing samples, mistakes can be made by the scientist which can lead to even greater loss of the analyte of interest.[1] All of these unwanted interactions can lead to poor, inconsistent chromatographic performance, and subsequent mass analysis.

The work described herein describes a semi-automated sample preparation and extraction method for a GalNAc-siRNA conjugated oligonucleotide (GalNAc) from various biological matrices. The use of the Pipette+ and the Otto SPEcialist to carry out sample preparation and solid phase extraction (SPE) on a mixed-mode weak anion exchange sorbent provides a semi-automated approach that can mitigate manual error and improve analytical recoveries. Semi-automated sample preparation combined with the ACQUITY Premier LC and Oligonucleotide BEH C_{18} Column for chromatographic separation improves analytical results by reducing metal adsorption, ultimately improving recovery of GalNAc. This developed method achieves high oligonucleotide SPE recovery, with low matrix effects (≤10%), and achieved low (ng/mL) levels of detection from neat and post-spiked extracted plasma and urine samples.

Experimental

Materials

A GalNAc-siRNA conjugated oligonucleotide was provided by Alnylam Pharmaceuticals, (Cambridge, MA), and the oligodeoxynucleotide phosphorothioate, Gem132, was custom synthesized by Nitto Denko Avecia (Milford, MA). Sample preparation was conducted using Sprague Dawley rat plasma, purchased from BIOIVT (Westbury, NY), or pooled human urine.

Sample Preparation

A stock solution of GalNAc and Gem132 was prepared at a concentration of 1.0 mg/mL in RNAse free H_2O. A working stock solution of Gem132, used as an internal standard (IS), was prepared with a final concentration of 250 ng/mL. A working stock solution of GalNAc was prepared at a concentration of 10 µg/mL in mobile phase A. The working stock solutions were used to prepare a calibration curve and quality control (QC) samples. The Pipette+ and OneLab Software was used to prepare post-spiked calibration curves with a final concentration upon analysis ranging from 2.0–1000 ng/mL (N=2), and quality control samples with a final concentration upon analysis at 7.5, 75, and 750 ng/mL (N=4) respectively, in mobile phase A. A neat recovery experiment was conducted at low and high concentrations of 10 ng/mL (N=4) and 1000 ng/mL (N=4) respectively in 50 mM ammonium acetate (pH 5.5). Information regarding the protocol for the SPE procedure using Oasis WAX µElution SPE and the pretreatment steps for liquid-liquid extraction (LLE) of 200 µL of blank plasma can be found in the Waters Application Note 720007019EN.[2] For sample pretreatment in urine, 200 µL of blank urine was diluted 50:50 with 50 mM ammonium acetate (pH 5.5). SPE samples were eluted into a 700 µL QuanRecovery with MaxPeak HPS 96-well plate and covered with a silicone PTFE cap mat, vortexed, and submitted for analysis by LC-MS. The full bioanalytical workflow from biological sample to analysis is illustrated in Figure 1.

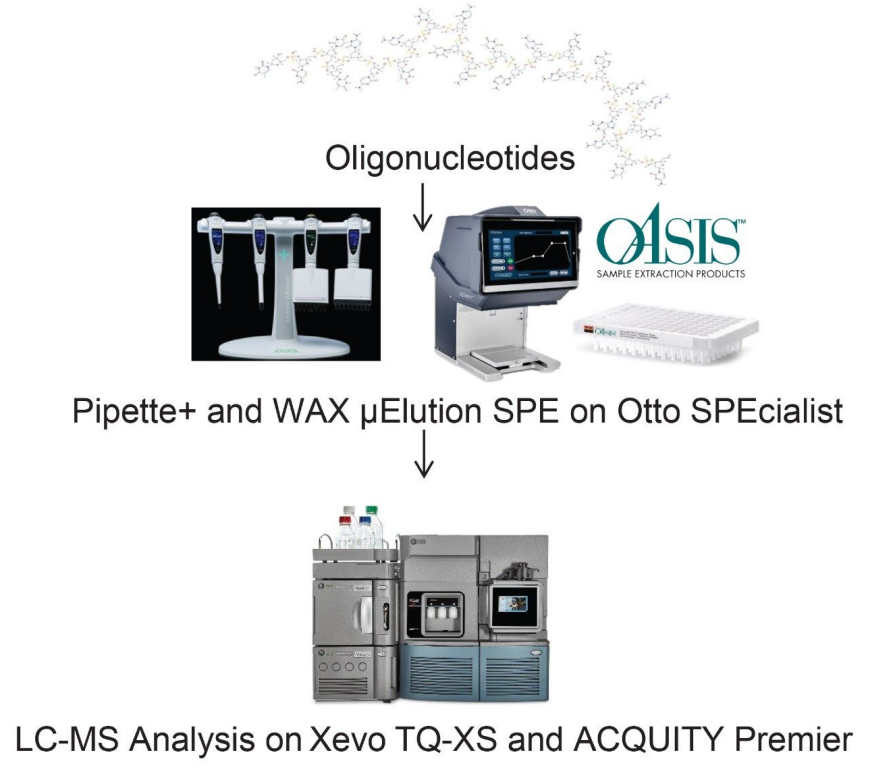

Figure 1. Bioanalytical sample preparation and LC-MS workflow of a GalNAc-siRNA conjugate oligonucleotide.

LC-MS Conditions

LC system:	ACQUITY Premier System
Mobile phase A:	1% Hexafluoroisopropanol (HFIP) + 0.1% N, N-Diisopropylethylamine (DIPEA) in Water
Mobile phase B:	0.75% Hexafluoroisopropanol (HFIP) + 0.0375% N, N-Diisopropylethylamine (DIPEA) in 65% Acetonitrile

Purge solvent:	Water:Methanol (90:10 v/v)
Wash solvent:	Acetonitrile:isopropanol:water:methanol (25:25:25:25 v/v/v/v)
Detection:	Xevo TQ-XS
Column(s):	ACQUITY Premier Oligonucleotide BEH C_{18}, 130 Å, 1.7 µm, 2.1 x 50 mm
Column temp.:	50 °C
Sample temp.:	8 °C
Injection volume:	10 µL
Flow rate:	0.6 mL/min

Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.600	95.0	5.0	6
3.50	0.600	75.0	25.0	6
4.00	0.600	10.0	90.0	6
4.50	0.600	95.0	5.0	6
5.00	0.600	95.0	5.0	6

MS Settings

Ionization mode:	ESI-
Acquisition range:	MRM

Capillary voltage:	2.00 kV
Cone voltage:	50 V
Desolvation temp.:	500 °C
Desolvation flow:	1000 L/Hr
Cone gas flow:	150 L/Hr
Collision gas flow:	0.2 mL/min
Nebulizer gas flow:	7 Bar

Data Management

Instrument control software:	MassLynx v4.2
Quantification software:	TargetLynx

Results and Discussion

LC-MS/MS quantification was performed using the Xevo TQ-XS MS (ESI-). Final MRM transitions used for detection and quantification, including precursors and fragments for GalNAc and Gem132 can be seen in Table 1. Gem132 was monitored during testing to ensure analytical performance throughout experimentation. The ACQUITY Premier Oligonucleotide BEH C_{18} Column with MaxPeak High Performance Surfaces (HPS) Technology was used for this analysis. HPS Technology was developed specifically to minimize non-specific metal interactions with analytes such as oligonucleotides. Using the ACQUITY Premier LC and Oligonucleotide BEH C_{18} Column for this work ensured analytical separation performance, improving oligonucleotide recovery, and limits of detection, while reducing time spent on system passivation and downtime.

Compound	Charge	Parent (*m/z*)	Daughter (*m/z*)	Cone (V)	Collision (eV)
GalNAc	(-11)	779.6	227.2	30	30
			430.1		18
Gem132	(-10)	659.3	94.80	30	30
			303.9		20
			319.0		20
			343.8		20

Table 1. Final MS conditions used for analysis of the GalNAc and Gem132 oligonucleotides.

The Otto SPEcialist is a compact and semi-automated sample preparation device that combines the efficient use of positive pressure with software that automatically changes the applied pressure to a sorbent bed through every step of the SPE process. SPE extraction performance using Otto SPEcialist and Oasis WAX µElution SPE for GalNAc is shown in Figure 2. For this, neat solution samples were prepared at 10 ng/mL and 1000 ng/mL, N=4 per concentration, and were extracted using the protocol described in the application note 720007019EN. Mean SPE recoveries for the low and high concentrations were 109.7% and 88.6% respectively with RSD values at 9.97% and 4.38%, respectively. These results show that the Oasis WAX µElution SPE plate and the developed method provides adequate retention of GalNAc to the stationary phase, while the Otto SPEcialist gives high recoveries with minimal variation between samples.

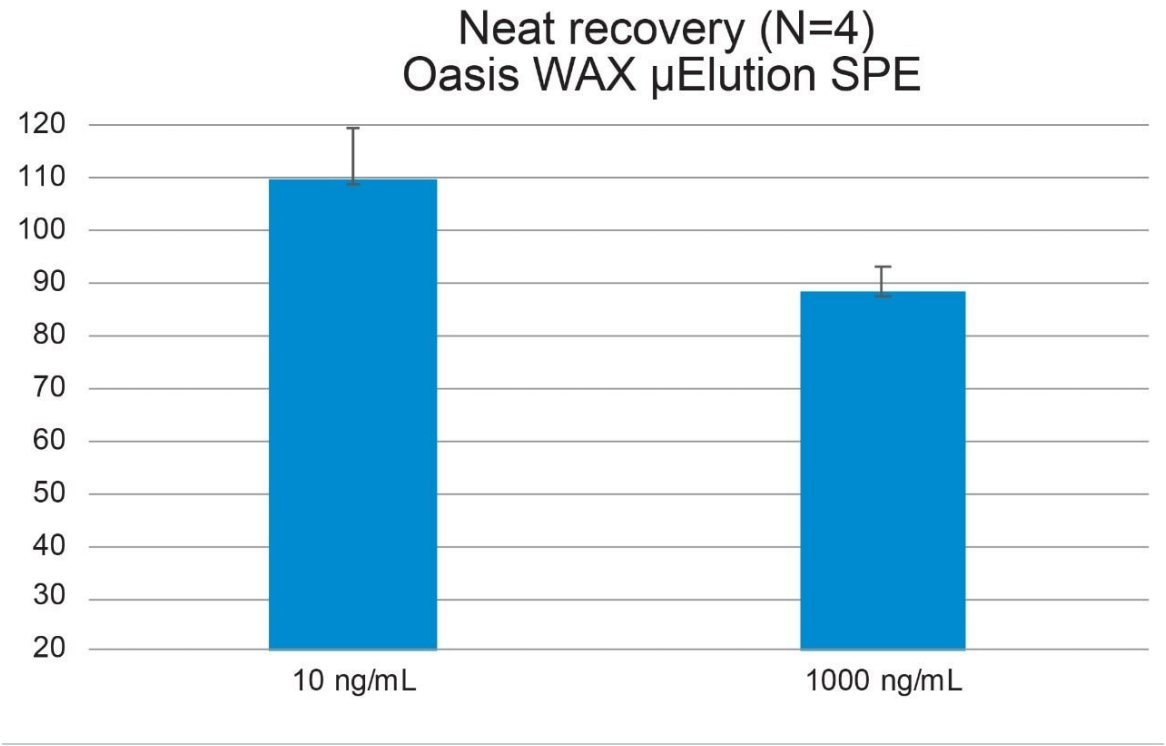

Figure 2. Neat solution SPE recoveries of GalNAc at 10 and 1000 ng/mL using an Oasis WAX µElution SPE in the 96-well format and performed with Otto SPEcialist.

The combination of the ACQUITY Premier HPS Technologies with semi-automated sample preparation and extraction of GalNAc from biological matrices ensured linear, accurate, and reproducible quantitative performance. Figure 3 represents quantitative performance of post-spiked extracted plasma samples, achieving an LOD of 1 ng/mL and a linear dynamic range between 2.0–1000 ng/mL (R^2 value of 0.996 1/x weighted regression). Accuracies ranged from 86.2–117%. Figure 4 represents quantitative performance of post-spiked extracted urine samples, achieving an LOD of 1.0 ng/mL and dynamic range between 5–1000 ng/mL (R^2 value of 0.996 1/x weighted regression). Accuracies ranged from 85.9–119%. This data meets regulatory requirements of accuracy (±20%) and precision (±20%) with R^2 values of ≥0.980.[3]

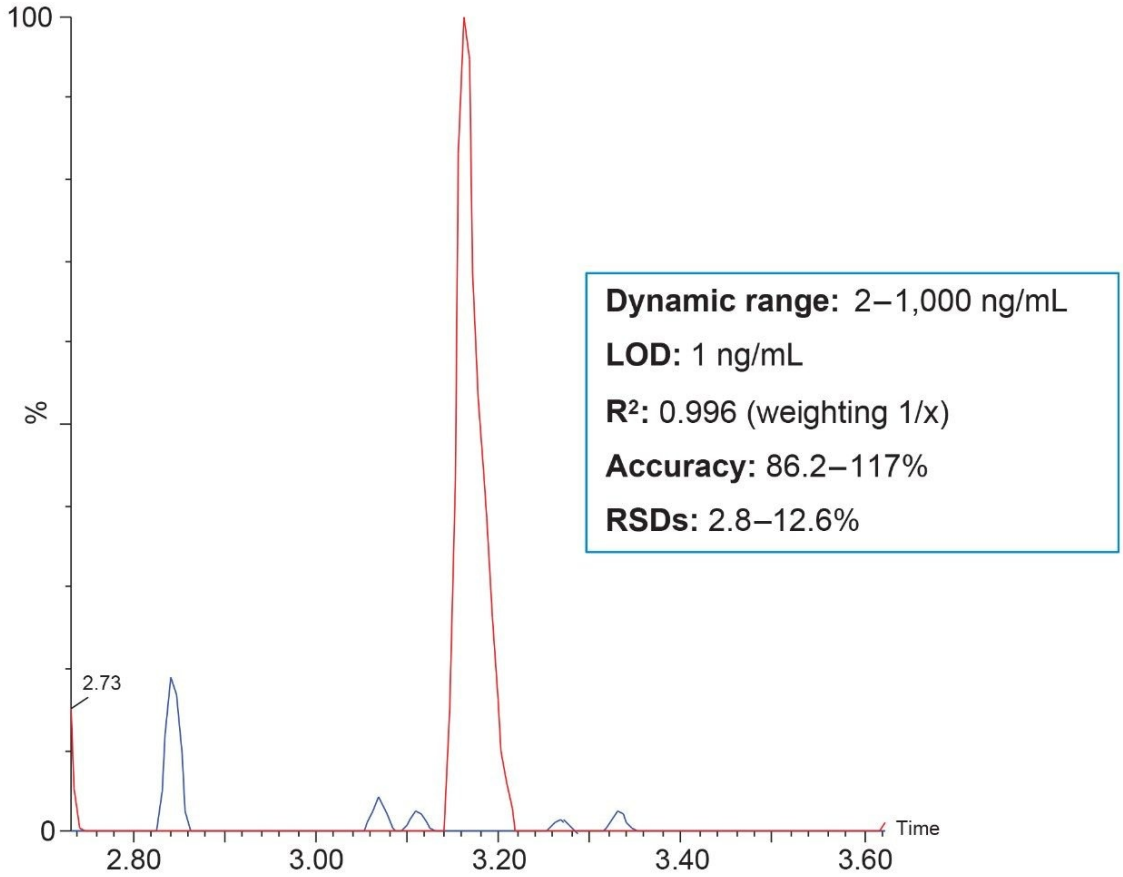

Figure 3. Quantitative performance of post-spiked extracted plasma, achieving 1 ng/mL LOD.

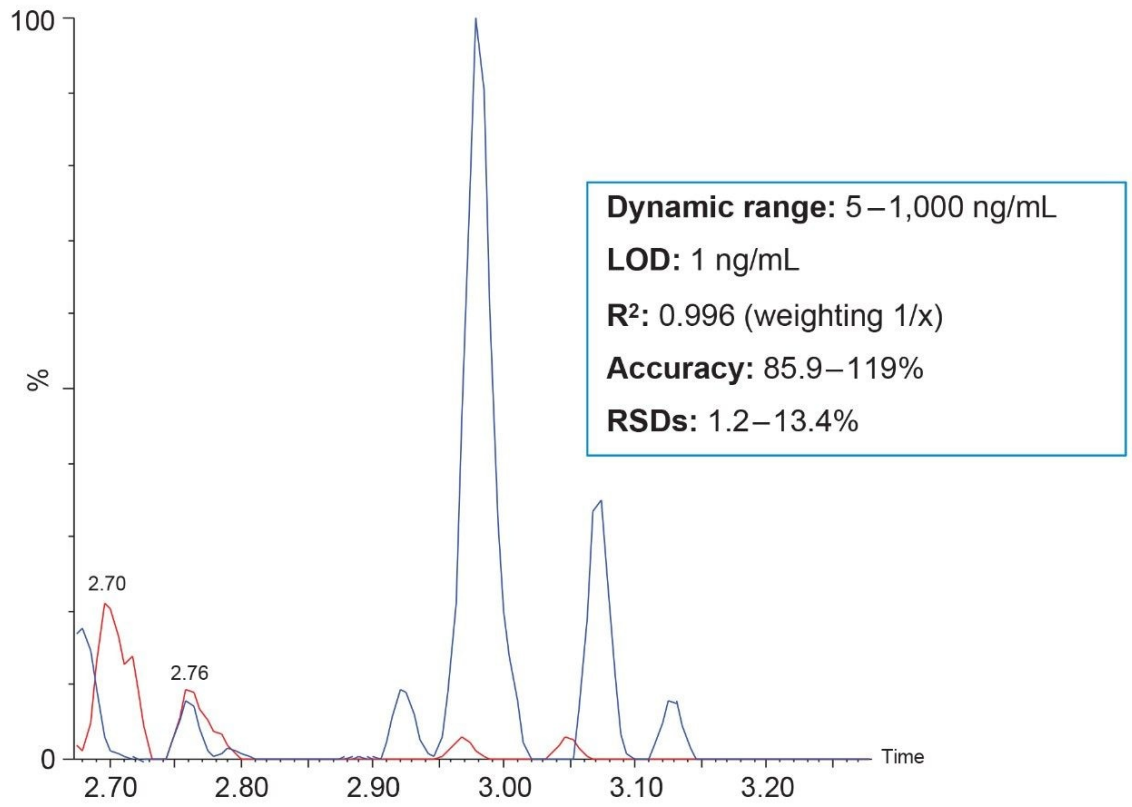

Figure 4. Quantitative performance of post-spiked extracted urine, achieving a LOD of 1 ng/mL.

Pipette+ is controlled through the user-friendly browser based OneLab Software. Creating and running pipetting methods through OneLab can ensure robust, reliable, and reproducible sample preparation day to day and user to user. The Pipette+ followed the pipetting steps in the serial dilution method created through OneLab. For this method, the Pipette+ was used for preparation of calibration curves and QCs in biological matrix. For sample extraction and analysis of rat plasma, calibration curves, and QCs were prepared across three different batches (inter-day). Inter-day and intra-day QC accuracies and RSDs can be seen in Table 2. Inter-day accuracies ranged from 90.8–106.8% with RSDs between 2.2–13.9%. Intra-day (Day 3) accuracies ranged from 92.4–113.7% with RSDs between 1.1–12.6%. For sample extraction and analysis of pooled human urine, inter-day calibration curves, and QCs were prepared across two different batches. Inter-day and intra-day QC accuracies and RSDs can be seen in Table 3. Inter-day accuracies ranged from 93.8–111.4% with RSDs between 3.3–13.4%. Intra-day (Day 1) accuracies ranged from 90.2–109.3% with RSDs between 2.7–11.9%. The use of the Pipette+ and OneLab for preparation of QCs ensured excellent performance, and accurate and reproducible results across batches for

post-spiked extracted samples.

Interday (N=3) plasma accuracy and RSDs				
ID	Conc. (ng/mL)	Calculated conc. (ng/mL)	% Accuracy	% RSD
QC1	7.50	7.60	101	13.9
QC2	75.0	68.1	90.8	3.69
QC3	750	801	107	2.23
Intraday (N=4) day 3 plasma accuracy and RSDs				
ID	Conc. (ng/mL)	Calculated conc. (ng/mL)	% Accuracy	% RSD
QC1	7.50	6.95	92.4	12.6
QC2	75.0	74.3	99.0	1.12
QC3	750	853	114	2.14

Table 2. Inter-day and Intra-day QC statistics from post-spiked extracted plasma samples prepared with Pipette+ and extracted using Otto SPEcialist.

Interday (N=2) Urine Accuracy and RSDs				
ID	Conc. (ng/mL)	Calculated conc. (ng/mL)	% Accuracy	% RSD
QC1	7.50	7.99	106	13.4
QC2	75.0	70.4	93.8	3.32
QC3	750	835	111	3.60
Intraday (N=4) day 3 plasma accuracy and RSDs				
ID	Conc. (ng/mL)	Calculated conc. (ng/mL)	% Accuracy	% RSD
QC1	7.50	7.18	95.6	11.9
QC2	75.0	67.7	90.2	2.73
QC3	750	819	109	4.10

Table 3. Inter-day and Intra-day QC statistics from post-spiked extracted urine samples prepared with Pipette+ and extracted using Otto SPEcialist.

Conclusion

This application highlights the successful SPE extraction and LC-MS/MS quantification of a GalNAc-siRNA conjugated oligonucleotide from neat solution and post-spiked extracted plasma and urine. Using Pipette+ and Otto SPEcialist simplified and streamlined sample preparation and extraction procedures, maximizing productivity, reducing errors, and ensuring overall analytical performance of the method. The ACQUITY Premier LC and Oligonucleotide BEH C_{18} Column, specifically designed to mitigate adsorption of metal sensitive analytes, ensured consistently high levels of oligonucleotide recovery. The analytical sensitivity of this method achieved a LOD of 1 ng/mL from extracted plasma and urine for a GalNAc-siRNA conjugated oligonucleotide.

Acknowledgement

The authors thank Greg Jones and Alnylam Pharmaceuticals for donation of 21 mer GalNac oligonucleotide for our experiments.

References

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720007418, October 2021