DEVELOPMENT OF A BIOANALYTICAL SPE LC-MS/MS ASSAY FOR OLIGONUCLEOTIDES

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INTRODUCTION

Over the past decade, there has been a dramatic increase in research toward oligonucleotides therapies (ONTs). With improved target specificity of next-generation oligonucleotides, the demand for LC-MS bioanalytical assays in support of their research and development has also increased.

Developing robust, sensitive, and selective sample preparation and LC-MS methods for ONTs remains quite challenging, due to their size, poly-anionic nature, physiochemical diversity, stability, poor chromatographic retention, non-specific binding (NSB), and strong protein binding in biomatrices. In addition, the charged anionic backbones of oligonucleotides are prone to adsorb to electron deficient surfaces, such as the metal surface of LC columns. These challenges often result in low sample recovery from matrix, poor MS sensitivity due to limited ionization/fragmentation due to their size, lack of chromatographic selectivity or resolution from endogenous matrix interferences due to poor RP chromatographic retention, as well as adsorptive losses due to metal interaction, ultimately limiting overall limits of detection (LOD).

This work described herein demonstrates a simple and robust SPE LC-MS/MS method, for the quantification of various oligodeoxythymidines nucleotides standards (OSTs), 15-35 mer in length, and the fully thioated oligonucleotide, GEM91®. Using reversed-phase (RP) and mixed-mode µElution SPE sample preparation, analytical scale sub-2µm LC chromatographic separation with a prototype RP C18 column, coupled to a high sensitivity tandem quadrupole MS, facilitates high recovery, sensitivity, and specificity.

METHODS

Sample Preparation

Oligonucleotide SPE Method Development

Aqueous solutions of the Waters MassPREP OST standard (15-35 mer) and the oligodeoxynucleotide phosphorothioate, GEM91 were prepared at various concentrations in proteinase free water. SPE protocols using the Waters Oasis™ HLB (Figure 1A) and WAX (Figure 1B) µElution 96-well SPE extraction plate were evaluated and optimized for oligonucleotide recovery.

Various parameters of the SPE extraction, including: loading, wash and elution conditions, were evaluated and optimized to achieve maximum recovery and reduce matrix effects.

Oligonucleotide SPE & LC-MS/MS Quantification

To prepare calibrators, GEM91 was added to either water or commercially available plasma/sera (400 µL) at various concentrations (0.01– 100 µg/mL). A 2:1 ratio of denaturing buffer was then added to the prepared samples and mixed. To provide selective sample clean up and enrichment, samples were then extracted using the Waters WAX µElution 96-well SPE plate with the protocol shown in Figure 1B.

LC-MS/MS Conditions

LC-MS/MS analysis of the OSTs and GEM91 was performed with a Waters ACQUITY UPLC I-Class PLUS (FTN) coupled to a Waters Xevo TQ-XS tandem quadrupole mass spectrometer using negative electrospray ionization (ESI-) and multiple-reaction monitoring mode (MRM). Chromatographic separation was achieved using a Waters prototype sub-2 µm, 2.1 mm x 50 mm column, using a flow rate of 0.5 mL/min and the gradient is shown in Table 1. Mobile phases used were 150 mM hexafluoroisopropanol (HFIP) containing 5mM hexylamine (HA) in water (MP A) or methanol (MP B). MS conditions used for for the 15-35 mer OSTs and GEM-91 are summarized in Table 2. Total analysis time was 5 minutes. Injection volumes used for quantification were 10-20 µL.

The Waters MassPREP OST Standard (2 nmol/mL) was injected pre and post- analysis to verify UPLC/MS system performance.

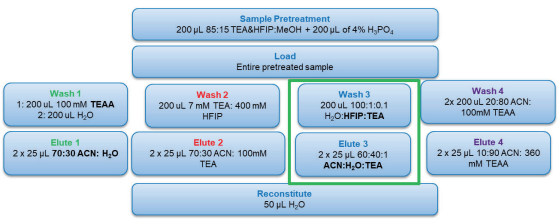
Table 1. LC Gradient

Time (min)	Flow Rate (mL/min)	% A	% B	Curve
0.0	0.5	90	10	6
1.0	0.5	90	10	6
1.5	0.5	50	50	6
3.0	0.5	40	60	6
3.1	0.5	5	95	6
3.5	0.5	5	95	6
3.6	0.5	90	10	6
4.25	0.5	90	10	6

Table 2. MRM conditions for the MassPREP OSTs (15-35 mer), GEM91 and GEM132 (IS).

Oligonucleotide	Charge State	Precursor (m/z)	Product (m/z)	Collision Energy (eV)	Cone Voltage (V)
GEM-91	6	1294.1	94.8	40	35
	6	1294.1	41.1	12	20
	5	1553.7	512.8	40	30
	5	1553.7	722	40	30
GEM-132 (IS)	6	1099.4	94.2	40	30
	5	1319.2	94.5	40	30
MassPREP OST 15 mer	4	1123.8	382.8	40	40
MassPREP OST 20 mer	4	1504.2	382.8	40	40
MassPREP OST 25 mer	4	1884.7	382.8	45	40
MassPREP OST 30 mer	5	1811.5	382.8	45	40
MassPREP OST 35 mer	6	1762.9	382.9	50	40

A Oasis Reversed-Phase HLB SPE Protocol



B Oasis Mixed-Mode WAX SPE Protocol

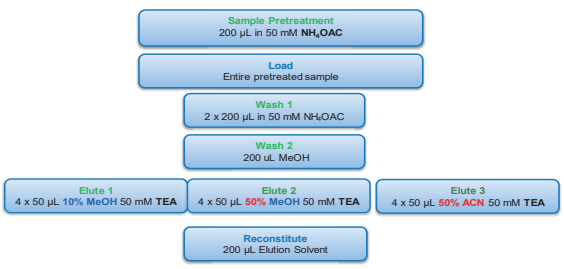


Figure 1. Oasis HLB (A) & WAX (B) SPE oligonucleotide sample extraction protocols.

RESULTS

I. SPE SAMPLE PREPARATION METHOD DEVELOPMENT & OPTIMIZATION

Figure 2. SPE HLB (A) and WAX (B) SPE Recovery for the various OSTs (15-35 mer) and GEM91 (C). Using an HFIP/TEA wash and elution with ACN/TEA yielded best oligonucleotide HLB recovery, while an ammonium acetate/MeOH wash and elution with MeOH/TEA yielded best oligonucleotide WAX recovery for the OSTs and GEM91.

II. OLIGONUCLEOTIDE CHROMATOGRAPHIC PERFORMANCE

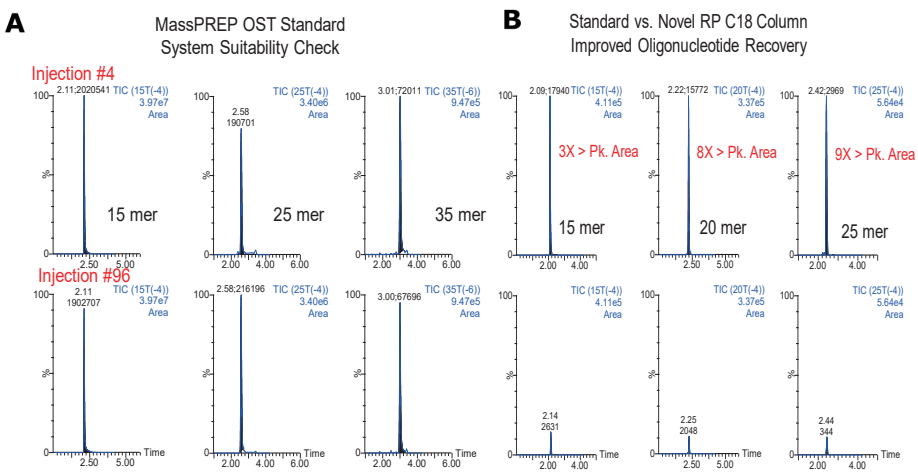


Figure 3A. UPLC/MS system performance verification (Retention Time/Peak Area) using the Waters MassPREP OST standard (2 nmol/mL). Comparison of injection #4 and 96, illustrating retention time/peak area (15, 25, and 35 mer) were maintained throughout analysis.

Figure 3B. Improved oligonucleotide chromatographic performance (Peak Height/ Area). Comparison of standard vs. prototype RP C18 column (1.7 µm, 2.1 mm x 50 mm) using the Waters MassPREP OST standard (2 nmol/mL).

III.MIXED-MODE WAX SPE AND LC-MS/MS QUANTIFICATION OF GEM91

LINEAR & ACCURATE QUANTIFICATION

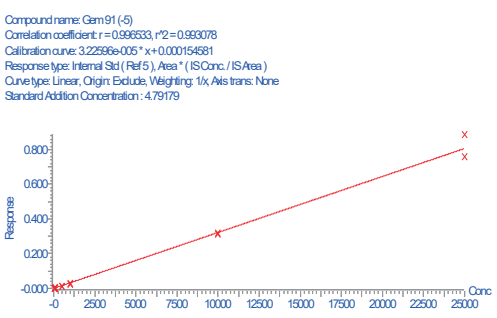


Figure 4. Representative standard curves (25-25,000 ng/mL) for GEM91 solution using mixed-mode WAX SPE in the 96-well µElution format and the described SPE protocol.

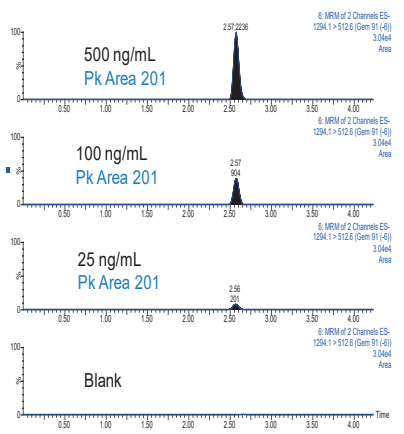


Figure 5. Representative chromatogram highlighting GEM91 extracted from 400 µL of sample using mixed-mode WAX SPE in the 96 -well format, achieving an LOD of 25 ng/mL.

APPLICATION HIGHLIGHTS

- A simple sample preparation and UPLC-MS/MS analytical method was developed for detection and quantification of the MassPREP OST, oligodeoxythimadine, standard and GEM91, 50-mer fully thioated oligonucleotide.
- A selective, yet simple extraction method, using both RP and mixed-mode SPE, achieving high oligonucleotide recoveries (Figures 2A-C), was developed for successful extraction of oligonucleotides. The µElution format 96-well SPE plate eliminated the need for evaporation, reducing oligonucleotide losses due to adsorption and NSB.
- The Waters MassPREP OST standard was used as a system suitability check (Figure 3A), pre and post-sample analysis, ensuring overall system health and performance of the UPLC-MS system .
- Use of a RP sub-2µm prototype column, improved oligonucleotide chromatographic recovery (Figure 3B), and ultimately improved LLOQs.
- Quantification limits of GEM91, following WAX SPE extraction, was 25– 10,000 ng/mL (Figures 4 &5).
- This method shows promise for high sensitivity quantification of synthetic oligonucleotides.

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