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アプリケーションノート

Elements of Robust SPE-Based Oligonucleotide Extraction

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研究目的のみに使用してください。診断用には使用できません。

Abstract

Synthetic oligonucleotide therapeutics can be used to target previously untreatable genetic diseases. Understanding their DMPK and ADME properties requires efficient extraction and recovery from biological matrices so that subsequent quantitation can be facilitated. A screening protocol involving two different oligomers, based on the Waters™ 20mer ssDNA and Lipid Conjugated ASO LC-MS Standards was developed to perform quality control and batch selection testing of a polymeric anion exchange SPE sorbent. Batches exhibiting highest recoveries for both oligomers were selected for use in new extraction devices developed specifically for oligonucleotide bioanalysis and included in the OligoWorks SPE Kits and Components portfolio. In addition, a novel elution solution was devised for maximum oligonucleotide recovery using OligoWorks SPE devices. The optimized eluent composition allows for direct injection of the eluate into LC-MS systems for quantitative analysis, saving time, and avoiding potential sample losses during evaporation and reconstitution. The eluted oligonucleotide (nucleic acid) components can be readily analyzed via standard ion-pairing reversed phase chromatography and UV/MS-based detection and quantitation. This application note also details the utility of RapiZyme™ Proteinase K for non-specific degradation of proteins in biological matrices to effectively disrupt oligonucleotide binding to endogenous protein and thus improve oligonucleotide recovery. As demonstrated, the OligoWorks SPE approach entailing the use of RapiZyme Proteinase K sample pre-treatment, OligoWorks SPE WAX sorbent, and OligoWorks SPE eluent has been found to yield highly

reproducible results.

Benefits

- Rigorous OligoWorks SPE WAX sorbent batch selection process provides for consistent, highly reproducible oligonucleotide recovery and quantitation
- OligoWorks SPE Eluent yields high recoveries across a diverse range of oligonucleotide therapeutics
- Reproducible recovery of a lipid conjugated ASO indicates the suitability of SPE for bioanalysis of oligonucleotide therapeutics designed to target the central nervous system (CNS)
- RapiZyme Proteinase K digestion effectively disrupts oligonucleotide binding to protein, enabling high oligonucleotide recovery and eliminating the adverse effects of detergent based workflows

Introduction

To successfully discern the Drug Metabolism and Pharmacokinetic (DMPK) properties of oligonucleotide therapeutics, analysts need to establish robust and efficient extraction procedures. Oligonucleotide-based drugs work at the level of gene transcription and translation and can provide therapeutic solutions to diseases that are not treated by traditional medications. The two primary modalities being developed today are antisense oligonucleotides (ASO’ s) and small interfering RNA (siRNA), both of which enable targeted degradation of mRNA transcripts, thus “silencing” or modulating gene expression. ASO’ s can also effect Exon-skipping during pre-mRNA splicing and thus yield alternate splicing variants (*e.g.,* Spinraza). Many of these drug candidates today are being conjugated with new types of moieties, such as a C_{16} alkyl chain, in order to effect targeted delivery into various tissues within the body. Through gene silencing and/or exon skipping they have been shown to facilitate the treatment of viral diseases, macular degeneration, cholesterol control as well as central nervous system (CNS) diseases.[1–2] Bioanalysis of oligonucleotide therapeutics has often involved hybridization enzyme-linked immunoassays (ELISA). However, ELISA lacks specificity to distinguish intact and truncated metabolites.[3] LC-MS is an alternative analytical method that can detect both intact, truncated and molecularly modified products. LC-MS provides three levels of discrimination - chromatographic separation, mass measurement of the intact oligo analyte or its metabolite and fragment/product ion detection for extra molecular discrimination.[3] Oligo analytes generally exhibit a propensity for non-specific binding to cationic materials such as metals. Hydrophobic modifications can further increase their non-specific binding to proteins.[4] Therefore, it is critical to minimize the non-specific adsorption to various surfaces during LC-MS analysis. Here, we report on several elements important to achieving robust extraction and quantitation of

modified oligonucleotides. These include using a carefully selected and rigorously batch tested polymeric ion exchange SPE sorbent, an optimized SPE eluent, and a high purity, high activity recombinant proteinase K reagent as a pretreatment step to disrupt protein binding interactions.

Experimental

Waters Sample Dilution/Treatment Solution: 200 mM ammonium acetate pH 5.5 prepared and adjusted the pH with dilute acetic acid (1M).

Waters SPE Equilibration Solution: 100 mM ammonium acetate pH 5.5, pH adjusted with dilute acetic acid (1M).

Waters Wash Solution 1: 100 mM ammonium acetate pH 5.5, pH adjusted with dilute acetic acid (1M).

Waters Wash Solution 2: 50% Methanol

Waters SPE eluent: 0.1M TEA in 50% Methanol, 0.3% ammonium hydroxide (28–30%) Aldrich, ACS Grade, PN 32,014–5

Waters OligoWorks SPE Test Mixture: Prepared by adding 1.7 µL of L-Tyrosine (Sigma p/n: 93829, 10 mg/mL, basic pH), ssDNA 20-mer LC-MS Standard (Waters p/n: 186009451 < https://www.waters.com/nextgen/global/shop/standards--reagents/186009451-ssdna-20-mer-lc-ms-standard.html> , 10 µg) and 87.5 µL of Lipid Conjugated ASO LC-MS Standard (Waters p/n: 186010747, dissolved in 20% acetone to yield a 100 pmol/µL stock concentration) to 1810.8 µL of 18.2 MΩ water to obtain a 2 mL final volume.

Screening Ion Exchange Sorbent Batches: 10 mg quantities of OligoWorks SPE WAX 30 µm sorbent were packed into 1 cc cartridges. This is a hydrophilic lipophilic balanced polymeric sorbent (comprised of vinylpyrrolidone and DVB) that is functionalized with piperazine anion exchange ligands. As such, OligoWorks SPE WAX sorbent exhibits mixed-mode (reversed-phase and a weak anion-exchange) functionalities suitable for selective adsorption and elution of oligonucleotides. The applied SPE protocol for a 1 cc cartridge included conditioning the sorbent with methanol, equilibrating with equilibration solution, loading a sample that has been diluted 1:1 with sample treatment solution, washing the sorbent with a SPE equilibration solution followed by a 50% methanol wash solution. Lastly, target oligos were collected in consecutive elution steps using OligoWorks SPE Eluent (Waters p/n: 186010610 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010610-oligoworks-spe-eluent-25-ml.html>). Eluates were then pooled and diluted 1:1:1 with sample dilution solution and water. The protocol is shown in Figure 1.

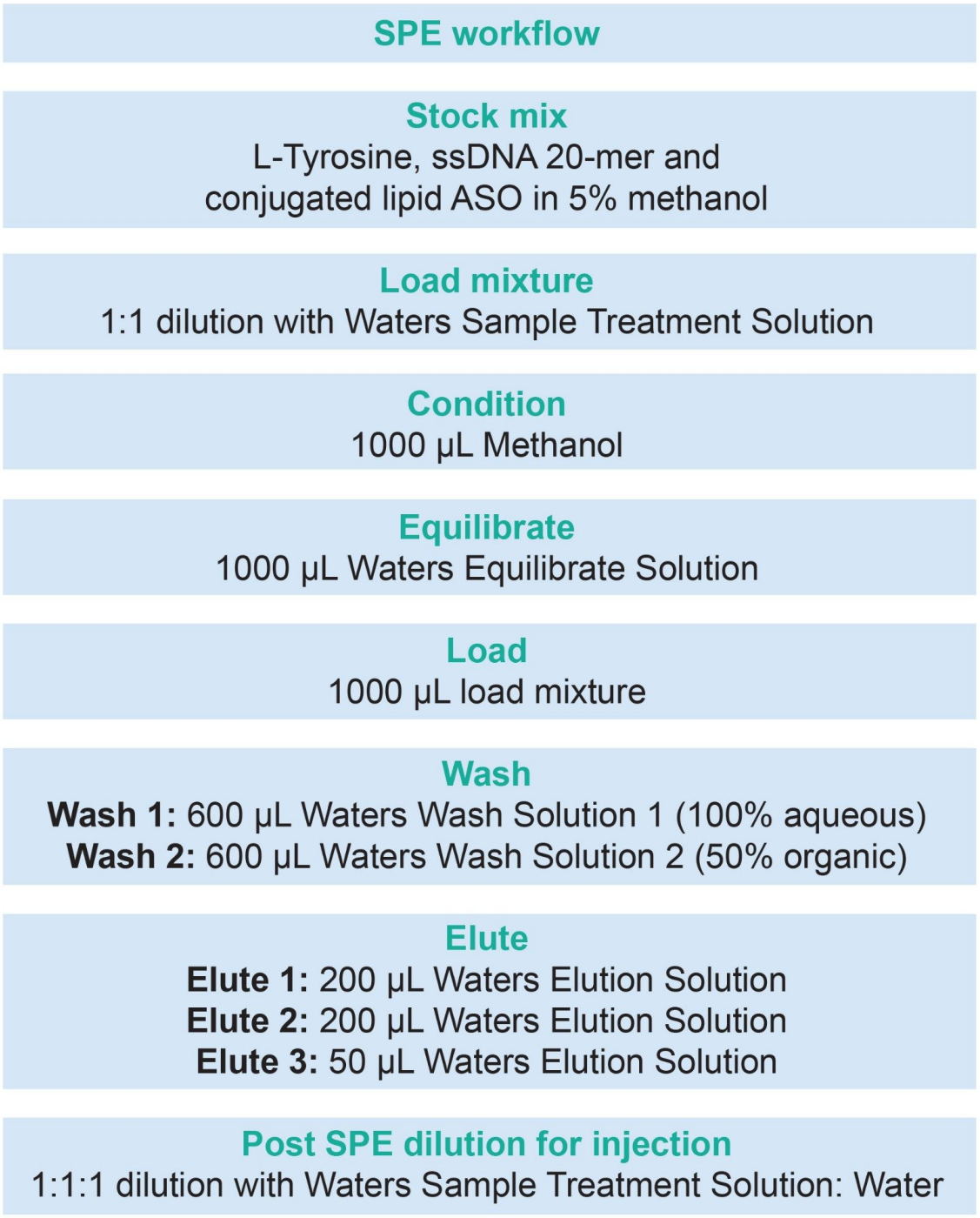

Figure 1. SPE Protocol.

IP-RP-LC-UV Assay for Sorbent Studies and Selection

Analyte mixture before and after SPE was subjected to ion pairing reversed phase liquid chromatography (IP-RP-LC-UV) analysis using the following LC conditions.

LC system:	ACQUITY™ UPLC™ I-Class PLUS or ACQUITY Premier UPLC – BSM System
Detection:	TUV detector
Wavelength:	260 nm
Vials:	Clear Glass 12 x 32 mm Screw Neck Qsert Vial, 300 µL Volume (p/n: 186002804)
Columns:	ACQUITY Premier Oligonucleotide BEH™ C_{18}, 130 Å , 1.7 µm, 2.1 x 50 mm (Waters, p/n: 186009484)
Column tempertaure:	60 °C
Sample temperature:	6 °C
Injection volume:	5 µL full loop
Flow rate:	0.6 mL/min
Mobile phase A:	0.1M TEAA in 18.2 MΩ water, pH 7.0
Solvent line B:	1:1 ratio of Mobile phase A:Acetonitrile pH 7.0
Weak wash solvent:	100% 18.2 MΩ water
Strong wash solvent:	10% Acetonitrile/ 90% 18.2 MΩ water
Seal wash:	20% Acetonitrile/ 80% 18.2 MΩ water

Gradient:	Isocratic for 7 min

Time	Flow rate	%A1	%B1	Curve
Initial	0.60	99.9	0.1	*
15.0	0.60	0	100	6
15.1	0.60	0	100	1
18.0	0.60	99.9	0.1	1

Table 2. RP-IP-LC gradient for detection of oligonucleotides.

IP-RP-LC-UV-MS assay of the Lipid Conjugated ASO LC-MS Standard

The Lipid Conjugated ASO LC-MS Standard was analyzed by IP-RP-LC-UV-MS using a BioAccord™ LC-MS System comprised of an ACQUITY Premier UPLC System and ACQUITY RDa mass detector.

LC system:	ACQUITY UPLC I-Class PLUS with RDa detector (BioAccord)
Detection:	ACQUITY TUV Detector for optical, ACQUITY RDA Detector for MS
Wavelength:	260 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Preslit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Columns:	ACQUITY Premier Oligonucleotide BEH™ C_{18}, 130 Å, 1.7 µm, 2.1 x 50 mm (Waters, p/n: 186009484)
Column temperature:	70 °C

Sample temperature:	6 °C
Injection volume:	5 µL full loop
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% N,N-diisopropylethylamine (DIPEA) as the IP reagent and 1% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in 18.2 MΩ water
Solvent Line B:	0.0375% DIPEA and 0.075% HFIP in 65:35 Acetonitrile: 18.2 MΩ water
Weak wash solvent:	100% 18.2 MΩ water
Strong wash solvent:	10% Acetonitrile/ 90% 18.2 MΩ water
Seal wash:	20% Acetonitrile/ 80% 18.2 MΩ water
Gradient:	See below

Gradient Table

Time (min)	mL/min	A (%)	B (%)	Curve
Initial	0.4	65	35	*
9.0	0.4	50	50	6
12.0	0.4	10	90	6
12.5	0.4	65	35	6
20.0	0.4	65	35	6
Run time = 20 min				

MS conditions

MS system:	BioAccord LC-MS System
Detector:	ACQUITY RDa Detector
Mode:	Full scan with fragmentation
Polarity:	Negative
Cone voltage:	40 V
Fragmentation cone voltage:	80–200 V
Mass range:	High (400–5000 *m/z*)
Scan rate:	2 *Hz*
Capillary voltage:	0.80 kV
Desolvation temperature:	400 °C

Informatics: The Intact Mass analysis application of the waters_connect™ platform was employed to compute the neutral mass values of oligonucleotides.

Sample Preparation: Biological fluids are rich in proteins that interfere with extraction. Using Rat plasma as an example, we subjected sample matrix to RapiZyme Proteinase K digestion (Waters, p/n: 186010601 < https://www.waters.com/nextgen/global/shop/standards--reagents/186010601-rapizyme-proteinase-k-digestion-module.html>) under denaturing conditions. The digestion contained 100 µL of rat plasma, 20 µL of 6 M Guanidine HCl, 10 µL of 0.5 M TCEP and 50 µL of RapiZyme Proteinase K (20 mg/mL). This mixture was incubated at 55 °C for a defined time period and immediately injected onto an ACQUITY Premier Protein SEC Column, 250 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186009963 < https://www.waters.com/nextgen/global/shop/columns/186009963-acquity-premier-protein-sec-column-250a-17--m-46-x-150-mm-1-pk.html>) for size-exclusion chromatography analysis.

SEC 2x Strength PBS Mobile Phase (*20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4)*: 1000 g of 18.2 MΩ water was added to a 1-liter borosilicate reservoir bottle. Two PBS (phosphate buffered saline) packets (Sigma p/n: P3813) were then dissolved into the water. The resulting mobile phase was filtered through a 0.2 µm nylon

membrane and stored at 4 °C.

SEC Column Storage Solvent (10% acetonitrile in 25 mM sodium phosphate + 100 mM KCl): SEC column storage solvent was prepared by adding 2.179 g of sodium phosphate dibasic anhydrous (Na_2HPO_4), 1.332 g of sodium phosphate monobasic monohydrate (NaH_2PO_4-H_2O), 7.455 g of potassium chloride (KCl) to 900 mL of 18.2 MΩ water until all solutes were dissolved. Subsequently, 77.7 g of acetonitrile was added before the solution was mixed and stored at 4 °C.

SEC-UV Analysis of Plasma Protein Digests

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS
Detection:	ACQUITY TUV
Wavelength:	280 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Preslit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Columns:	ACQUITY Premier Protein SEC Column, 250 Å, 1.7 µm, 4.6 x 150 mm (p/n: 186009963)
Column temp.:	35 °C
Sample temp:	6 °C
Injection volume:	50 µL
Flow rate:	0.4 mL/min
Mobile phase A:	2X PBS
Solvent line B:	Column Storage Solvent

Weak wash solvent:	100% Milli-Q water
Strong wash solvent:	100% Milli-Q water
Seal wash:	10%/90% Methanol/water
Gradient:	Isocratic for 7 min

Results and Discussion

We have screened WAX SPE ion exchange resin batches for efficient adsorption and elution of both an unmodified and C_{16} lipid-conjugated, 2’ -MOE-modified phosphorothioated oligonucleotide. To monitor the selectivity of the sorbent batches, these oligonucleotide test probes were also mixed with a charged non-nucleic acid molecule, tyrosine, and SPE was performed at optimal binding and elution conditions. Weakly bound tyrosine was washed away with a carefully selected combination of wash solutions, and oligo elution was achieved with an LC-MS compatible ion-pairing solvent. Through this process, excellent recoveries were achieved for the oligo analytes.

Figure 2 details the screening process that has been adopted to select OligoWorks SPE sorbent from the Waters Oasis WAX manufacturing process. Figure 2A illustrates the LC-UV profile of the oligonucleotide analytes before and after the described SPE protocol. Note the depletion of tyrosine from the eluate. Figure 2B details an analysis of various analyte recoveries as tested in the original Oasis WAX quality control procedure. Oasis WAX was first introduced in 2008 and has now been manufactured by Waters manufacturing in Taunton, MA for 15 years. A full view of the process control for 130 batches of 30 µm Oasis WAX sorbent is provided as has been monitored using a six analyte recovery test involving 2-naphthalene-sulfonic acid sodium salt, amitriptyline, salicylic acid, secobarbital, ketoprofen, and 4-propylbenzoic acid. OligoWorks WAX SPE has been created from this tightly controlled Oasis WAX manufacturing process by testing and selecting sorbent batches for their oligonucleotide recovery performance. Only batches that achieve precise and high recovery performance for oligo SPE are selected and certified for use in OligoWorks SPE devices and kits.

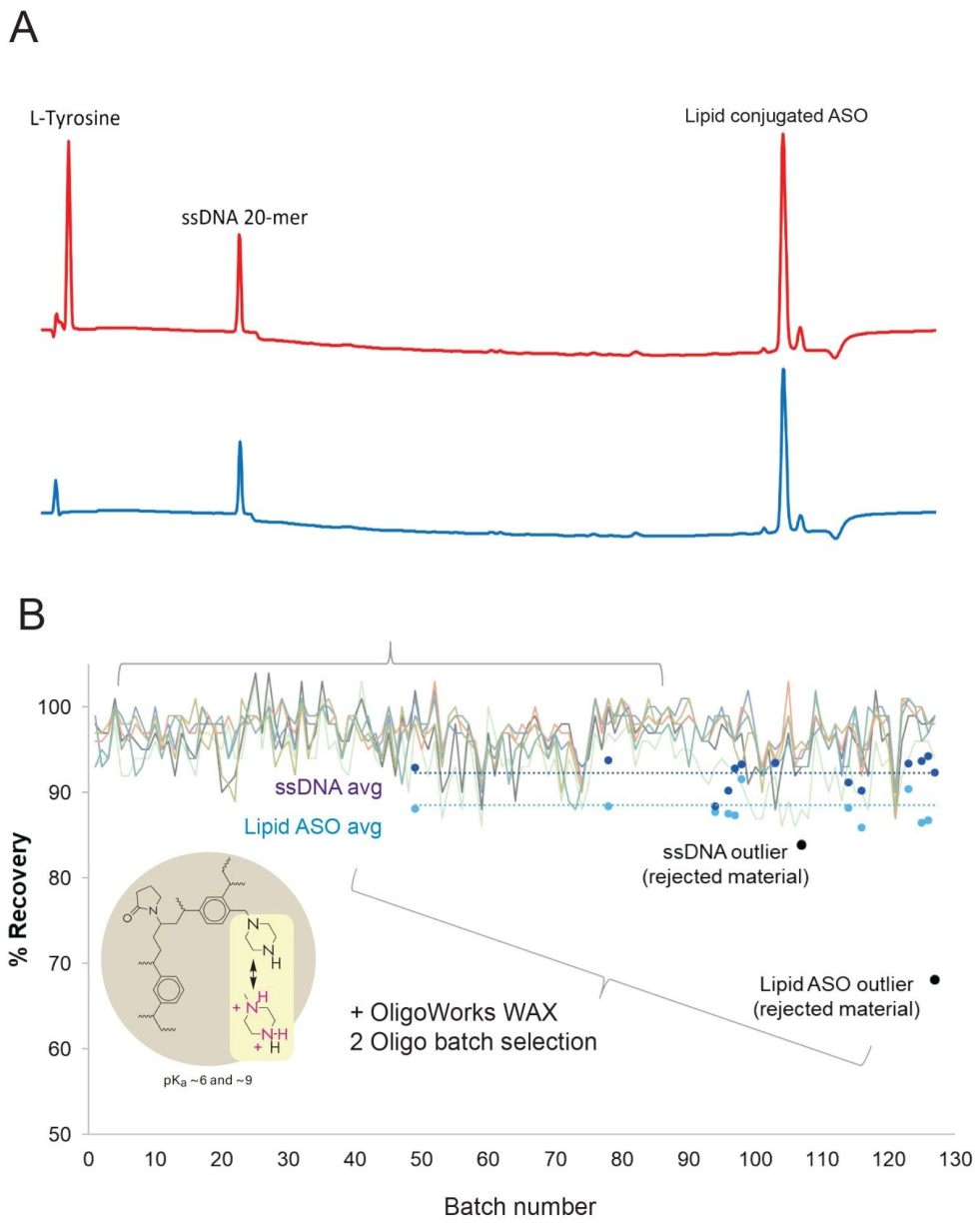

Figure 2. OligoWorks SPE WAX Sorbent Selection. A. IP-RP-LC-UV analysis of the OligoWorks SPE analyte test mixture before (red trace) and after (blue trace) performing SPE with the OligoWorks SPE Eluent. B. Control chart for Oasis WAX and examples of additional batch testing and selection that is applied to create OligoWorks SPE WAX sorbent. Percent recoveries of small molecule analytes is compared to the recoveries obtained for the 20mer ssDNA and Lipid Conjugated ASO LC-MS Standards. The WAX ligands and amine pKa of the

piperazine-based OligoWorks WAX sorbent is shown in the inset.

Lipid conjugated oligonucleotide therapeutics are being developed as a means to target the central nervous system (CNS) and to facilitate brain tissue trafficking. Such conjugates impart hydrophobicity to the molecule and can thus exhibit unique solubility properties. When starting with a lyophilized conjugate, it is necessary to first reconstitute with an organic solvent. It is recommended to first dissolve the Waters Lipid Conjugated ASO LC-MS Standard in 20% acetone to prepare a working stock solution that can be subsequently diluted with water or buffer (see the experimental section for test mixture preparation considerations). Figure 3 illustrates the LC-MS analysis of the Lipid /Conjugated ASO LC-MS Standard that is used in the OligoWorks WAX sorbent screening. As predicted, this reference material exhibits higher hydrophobicity compared to the 20mer ssDNA, and it therefore requires higher organic solvent to be eluted from a reversed phase column. This occurs with both TEAA (Figure 2) and DIPEA-HFIP based mobile phases. The chromatographic peak obtained in the DIPEA-based mobile phase exhibited partially resolved peaks. This is most likely due to the separation of diastereomers of the phosphorothioated backbone in the oligomer. Mass spectra collected across these chromatographic peaks showed multiply charged ions corresponding to the expected mass of 6047 Da for the phosphorothioated, 2’ MOE -modified, and lipid-conjugated reference material oligonucleotide.

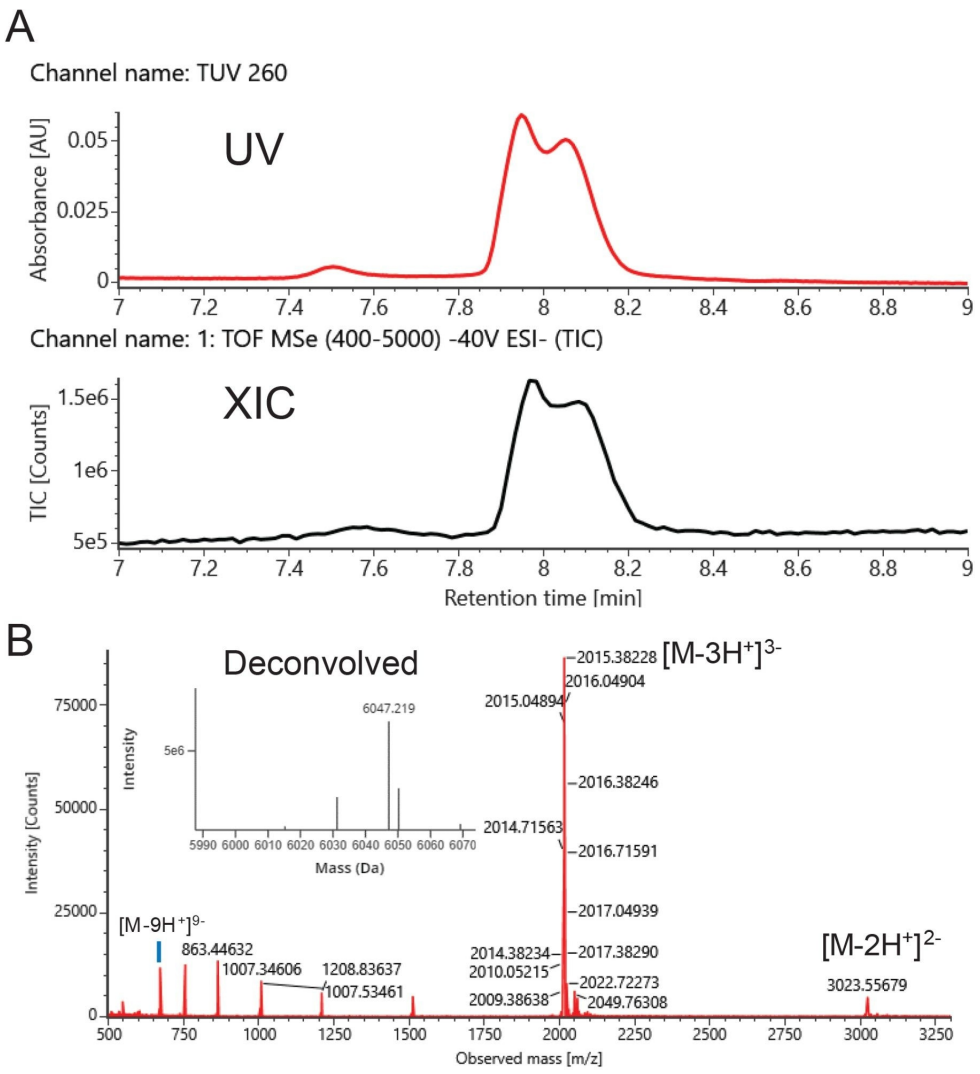

Figure 3. IP-RP-LC-MS analysis of the Waters Lipid Conjugated ASO LC-MS Standard. (A) UV and low energy extracted ion chromatograms are shown. (B) Mass spectrum of the Lipid Conjugated ASO LC-MS Standard. Deconvolution of the summed mass spectrum to yield neutral mass values of 6047 Da and 6031 Da, which correspond to the intact oligomer and a low amount of once oxygenated molecule.

Another component of oligonucleotide extraction that should be understood is the interference imposed by highly abundant proteins. Our studies identified optimal protein digestion conditions to remove the potential interference of endogenous proteins during the extraction of oligonucleotide from biological matrices. Figure 4 illustrates the effectiveness of RapiZyme Proteinase K to convert high molecular weight proteins into peptides

across a time course experiment aimed to track the digestion of plasma proteins. The plasma sample was tested as a negative control and the components of the mixture were separated based on size using an ACQUITY Premier Protein SEC 250 Å 1.7 µm column. The blue trace shows early eluting components with UV absorbance at 280 nm, which can likely be attributed to large protein species. Incubation with RapiZyme Proteinase K resulted in protein degradation with a corresponding increase in signal for late eluting species attributed to small peptides. A 40 to 60 min incubation at 55 °C is considered to be optimal for degrading the protein components of a mixture as well as RapiZyme Proteinase K itself. Time frames such as these can be achieved with RapiZyme Proteinase K because it is a high activity, highly purified recombinant form of the protease that was first discovered in *T. album*. Most importantly, RapiZyme Proteinase K is certified to be free of nuclease activity. It can accordingly be used at high molar ratios without any concern of introducing sample handling artifacts to the oligonucleotide analyte of interest.

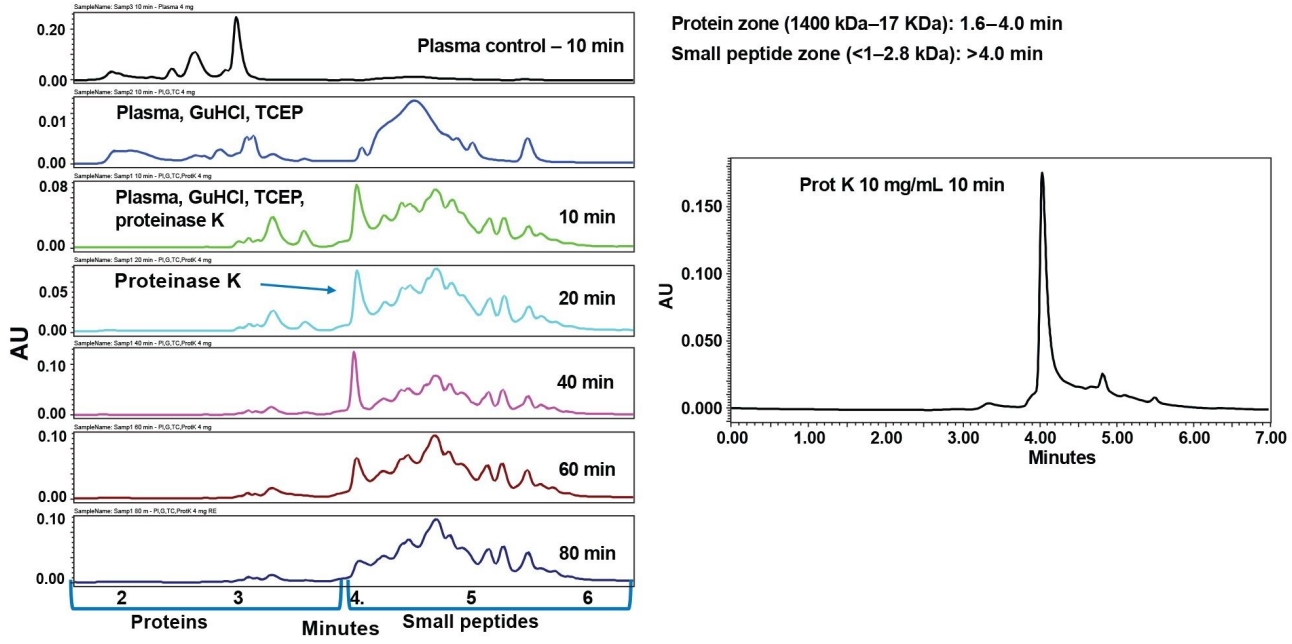

Figure 4. SEC profiles of plasma proteins following digestion with RapiZyme Proteinase K in a time course study. SEC profile of RapiZyme Proteinase K alone is shown on the right.

Thus, a combination of carefully batch selected anion exchange sorbent for OligoWorks SPE, an improved detergent-free sample preparation method and advanced analytical LC-MS technology can help expedite the bioanalysis of oligonucleotide-based drugs for accelerated therapeutic development.

Conclusion

OligoWorks SPE WAX sorbent has been created from an already rigorous manufacturing process that has for 15 years already produced 130 unique batches of Oasis WAX particles. Using two oligonucleotides test analytes, including a lipid conjugated ASO, Oasis WAX sorbent batches are subjected to a secondary quality control test. Only batches that yield high recoveries of both oligonucleotides are selected and certified for use in OligoWorks WAX SPE devices and kits. This provides rigorous screening and rejection of low-performing sorbents so as to ensure batch-to-batch consistency and reproducible recoveries for multiple types of oligonucleotides.

The development team behind this batch selection process found such value in the lipid conjugated ASO test analyte that it has now been commercialized as the Waters Lipid Conjugated ASO LC-MS Standard (p/n: 186010747). Owing to the inherent hydrophobicity of its lipid component, this conjugated ASO reference material requires higher organic solvent for its elution from an RPLC column. Moreover, it has been seen that its diastereomers can be partially resolved by certain mobile phase conditions. It is our hope that these example data for the 20-mer ssDNA and Lipid Conjugated ASO LC-MS Standard will make it possible for others to adopt them as proficiency and system suitability standards. One last insight was explored in this application note, namely the optimization of conditions for applying RapiZyme Proteinase K during a sample pre-treatment step. Use of RapiZyme Proteinase K leads to digestion of proteins in biological matrices. It is active and effective even under partially denaturing conditions. Through a peptide versus protein compositional study, final protocol parameters could be established for disrupting oligonucleotide protein binding. In all, the elements of oligonucleotide extraction that have been studied in this application note help give insights on how high recovery results can be achieved with OligoWorks SPE kits.

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720008141, December 2023

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