Extraction of 2'-MOE Phosphorothioate from Plasma Using Clarity® OTX™

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Overview

Oligonucleotides represent an emerging therapeutic modality. Because they can modulate both gene expression and post-transcriptional splicing of introns and exons, oligos can be used to treat a wide variety of indications, including previously "undruggable targets." However, there are many challenges associated with oligos. For analytical chemists involved in bionanalytical workflows, the challenges are several fold, though particularly, the extraction of the oligo is imperative for good assay specificity, sensitivity and linearity.

Because of therapeutic oligos have unique physicochemical properties due to their myriad modifications for nuclease resistance, protein precipitation or other traditional sample preparation approaches can prove quite challenging. Further, because of their relatively low dosing (lower than 1mg/kg at times), obtaining relevant lower limits of quantitation (LLOQ) and good linearity for oligo pharmacokinetic assays is particularly challenging.

One common approach to using the novel, one step solid phase extraction (SPE) Clarity™ OTX. Here we present the extraction of a, 2'-methoxyethyl phosphorothioate splice switching oligo (SSO) from monkey plasma.

Materials and Methods

All samples and analytes (Fully thioated, 2'-MOE Gapmer and internal standard) were purchased from Integrated DNA Technologies (Coralville, IA). All experiments were performed by a third-party contract laboratory. Analysis was performed using a Shimadzu Nexera HPLC and SCIEX Triple Quad 6500+ nominal mass spectrometer. Data processing was performed on SCIEX Analyst software. LC and MS Conditions are noted in Tables 1 and 2, respectively.

Monkey plasma was spiked with calibration standards, QCs (Low, Mid, High), internal standards (IST) and QCs (Low, Mid, High), Spiked samples were then processed using Clarity OTX , 100 mg/well, 96-well plates, using a Tomtec automated workstation. Calibration curve was generated, and accuracy and recoveries determined for QCs as a proof of concept of using this workflow to facilitate pharmacokinetic studies for oligo-based therapeutics.

Extraction Protocol

1. Aliquot 75 µL calibration standards, QC samples, study samples onto 2 mL 96-well round bottom plate
2. Add 30 µL IS (1 µg/mL) to all wells except blank controls (add 30 µL water to blanks)
3. Condition 96-well plate with 1 mL MeOH
4. Equilibrate with 1 mL Equilibration Buffer (50 mM Ammonium Acetate, pH 5.5)
5. Add 600 µL of Lysis Loading Buffer to all samples
6. Vortex mix samples for ~5 min; incubate at RT for additional 5 minutes
7. Transfer solution to TOMTEC or equivalent
8. Wash OTX plate 3X with 1 mL Equilibration Buffer
9. Wash plate 3X Wash Buffer (50 mM Ammonium Acetate, pH 5.5: ACN, 50:50)
10. Add 0.5 mL Elution Buffer (100 mM Ammonium Bicarbonate, pH 9.5:ACN:THF (50:40:10, v/v/v) and elute into collection plate
11. Dry samples to ~400 µL by Nitrogen
12. Prior to analysis, vortex-mix samples for ~1 minute.

LC-MS Optimization

The LC method utilized for this application is representative of most LC methods for optimizing sensitivity for oligos in high flow. That is, it uses ion pairing agents to facilitate the retention time of the otherwise polar oligo. Additionally, combined with the fluorinated alcohol HFIP, signal can be enhanced to ensure optimal electrospray ionization. Other notable mobile phase additive is EDTA; this is used to minimize any chelation that may effect sample recovery and improve robustness. Finally, the method implements two separate column washes after the gradient program has been run; this is to ensure no memory effect of oligo contributes to column carryover.

Direct infusion is not commonly recommended for tuning MRM transitions for quantitation. Trace salt and other interferences are typically present in oligonucleotide samples. Figure 1 1 shows the Q1 and Q3 scans on column for the SSO. The Q1 scan from 300-1200 m/z identifies the different precursor ions in the multiply charge for the SSO; the -7 charge state was chosen both for its response and specificity.

Other considerations include product ion and optimization of collision energy (CE). It is critical that CE is not set too high as interferences may impact both specificity and sensitivity; here a CE of -50 was selected. Final MRM conditions are shown in Table 4.

Table 1: HPLC Conditions

Column:	bioZen™ 2.6 µm Oligo
Dimensions:	100 x 2.1 mm
Part No.:	00D-4790-AN
Mobile Phase A :	1.0 % HFIP, 0.1% DIPEA in Water with 10 µm EDTA
Mobile Phase B:	0.075% HFIP, 0.0375% DIPEA in Water:ACN (35:65) with 10 µm EDTA
Gradient Program:	Gradient Slope: 20-60% B in 1.5 minutes Column Wash: 20-95% B (2X)
Flow-rate:	0.5 mL/min
Temperature:	80°C
Injection:	2 µL

Figure 1. Q1 and Q3 Scans for SSO

Q1 scan showing the precursor ions and oligo multiply charge. Q3 shows CE at -50 giving optimal response for product ion at 393 m/z

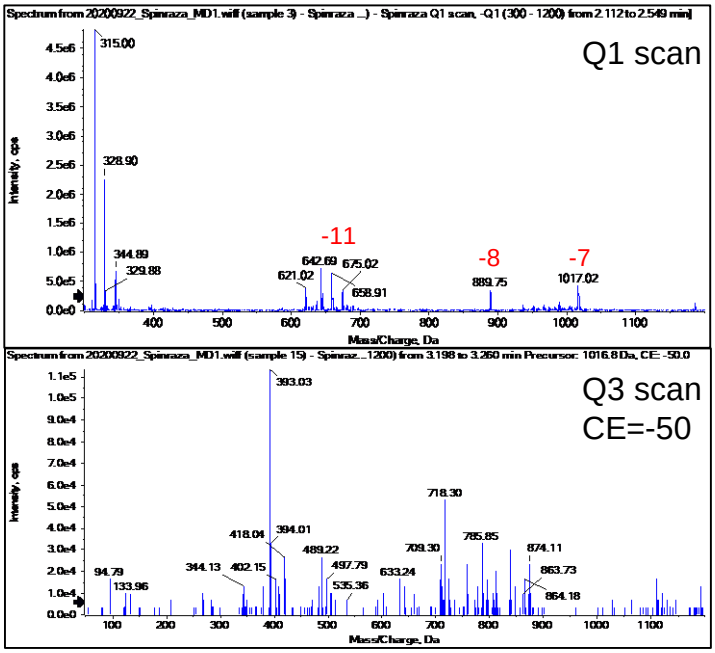

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Table 2: MS Conditions

Polarity:	(-)
CAD Gas:	10
Curtain Gas:	20
GS1 (psi):	50
GS2 (psi):	50
Spray Voltage (V):	-4000
Temperature (C°):	500

Table 3. MRM Conditions

	Q1	Q3	Dwell	CE	DP
Sample	1017.0	393.2	50	-60	-50
IST	828.70	811.8	50	-25	-75

Results

Table 4: Plasma Standard Curve

Summary of spiked calibration standards, extracted from monkey plasma using Clarity® OTX™ SPE. All replicates report good repeatability and accuracies are within +/- 15%. One replicate for high standard did not come into range and was omitted.

ng/mL	Name	Replicates	Mean	%CV	Accuracy
5	Std-1	5 of 5	5.33	5.8	106.6
10	Std-2	5 of 5	8.88	5.5	88.8
50	Std-3	5 of 5	44.9	3.3	89.9
200	Std-4	5 of 5	201	3.8	100.4
800	Std-5	5 of 5	833	6.0	104.1
1500	Std-6	5 of 5	1606	3.8	107.0
2000	Std-7	5 of 5	2109	2.9	105.5
2500	Std-8	4 of 5	2431	0.8	97.2

Figure 2: Calibration Curve

Calibration curve for spiked standards and replicates. Correlation coefficient is <0.99, indicating good linearity from 5-2500 ng/mL.

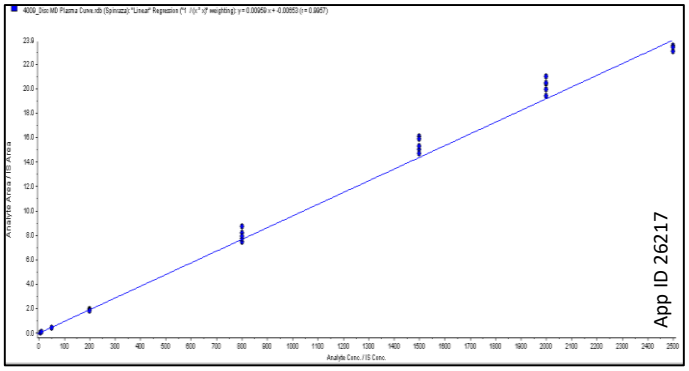

Figure 3: Standards and IS

Examples of SSO standards, showing extracted ion chromatograms for standards 1 and 8. IS chromatograms are shown as well.

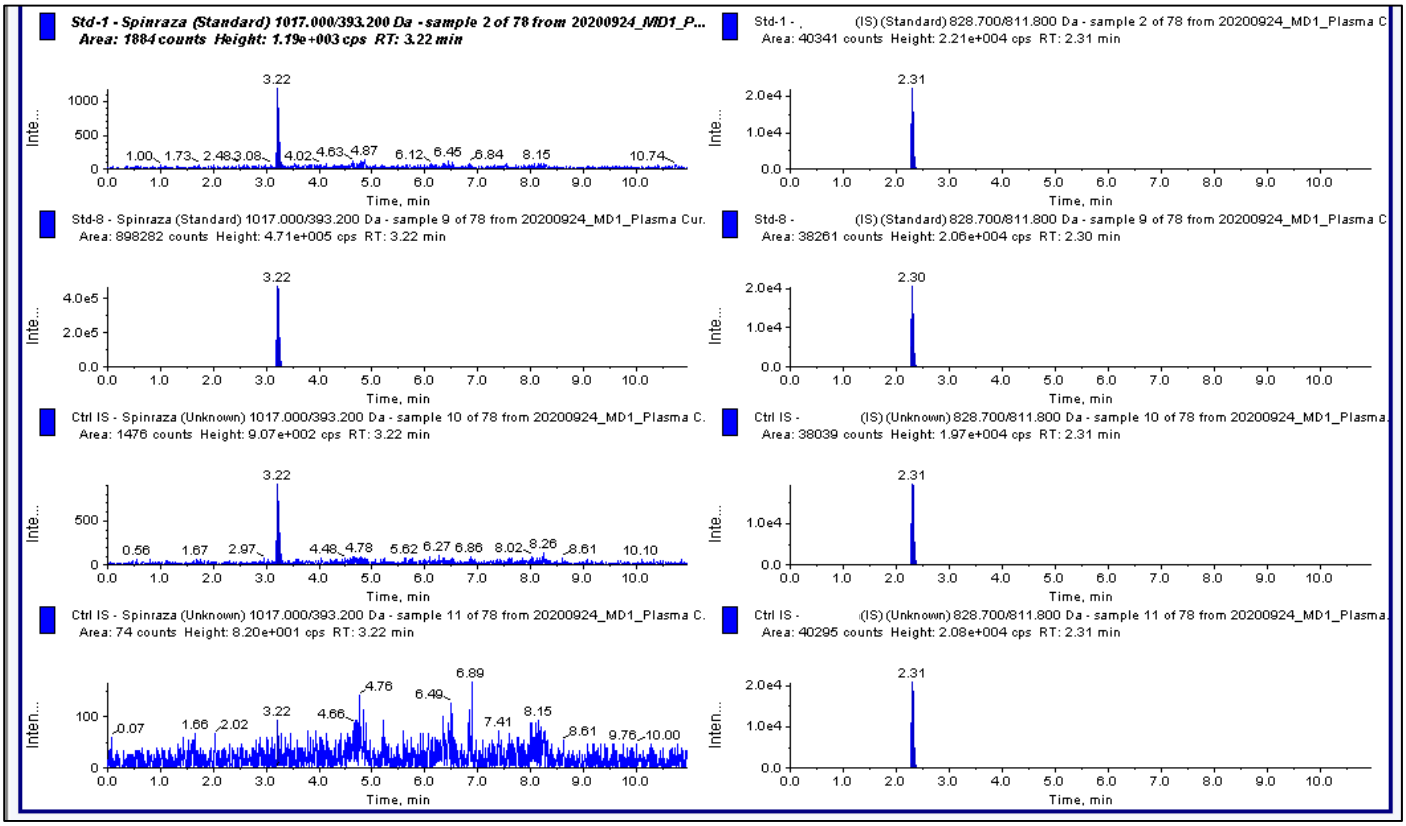

Table 5: Summary of QCs

Summary of spiked QC samples , extracted from monkey plasma using Clarity® OTX™ SPE. IS peak areas are consistent across QC levels. Additionally, recoveries are within acceptable ranges.

Sample Name	Analyte Peak Area (counts)	IS Peak Area (counts)	Area Ratio	Average	% Recovery
QC-Low AB/IA	2090	241000	0.00867		
QC-Low AB/IA	2500	244000	0.0102		
QC-Low AB/IA	2430	276000	0.00880	0.00924	
QC-Low AA/IA	2180	240000	0.00908		98.9%
QC-Low AA/IA	2590	264000	0.00981	0.00935	
QC-Low AA/IA	2470	270000	0.00915		
QC-High AB/IA	489000	297000	1.65		
QC-High AB/IA	487000	277000	1.76		
QC-High AB/IA	388000	221000	1.76	1.72	
QC-High AA/IA	468000	263000	1.78		90.0%
QC-High AA/IA	474000	232000	2.04	1.91	
QC-High AA/IA	426000	223000	1.91		

Conclusion

Although the extraction of oligos presents many challenges, implementing a sample strategy streamlines the workflow considerably and here, using the Clarity OTX extraction protocol vendor recommendations, we have demonstrated its use to develop a method for the extraction of therapeutic oligos from plasma. The 2'-MOE Gapmer SSO used in this study is a common therapeutic platform, though not representative of all oligo or nucleic acid modalities. Consequently, the solid phase extraction may need adjusted appropriately, depending on the physicochemical properties of the sample.

Linearity of the assay, as demonstrated by calibration curve and returning of the QCs, is acceptable for this study, at 5-2500 ng/mL. Depending on the oligo and dosing, LLOQs may need to be lower. In these instances, sample preparation may need to be optimized. In this study, 100mg/well plates were used; microelution formats may be another approach to minimize dry down times and improve recovery. Further optimization of wash steps in the SPE may also incrementally improve sensitivity.

Other nominal improvements to the sensitivity to the method might come with slight improvements in chromatographic method. It has been noted that modulation of HFIP and alkylamine can improve electrospray efficiency. For example, simple adjustment to the method might decrease the HFIP concentration from 1% to 0.1-0.2% HFIP might improve sensitivity.

Finally, although a nominal mass triple quadrupole was used in this assay, using a high-resolution instrument might also allow for biotransformation analysis. This may be at the expensive of sensitivity, as nominal mass instruments are commonly more sensitive.

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