Novel Isolation and Analysis Method for Oligonucleotide Therapeutics and their Metabolites from Biological Matrices by LC/MS

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Analyzing oligonucleotides by LC/MS for ADME/pharmacokinetic studies has become a critical challenge in the commercialization of oligonucleotide therapeutics. Oligonucleotides are difficult to isolate from biological matrices (serum, plasma, and tissue) and existing methods (two step LLE/SPE) provide poor recovery and are too laborious to multiplex in an automated format, as is needed for clinical studies where thousands of samples are analyzed.

In addition, LC/MS analysis of oligonucleotides suffers from sensitivity limitations due to the ion-pairing reversed phase methods that must be used to retain and separate oligonucleotides by LC/MS. Efforts were undertaken to develop new sample preparation and analysis methods for oligonucleotides from biological matrices with a focus on improving sample preparation throughput as well as increasing LC/MS sensitivity.
Materials and Methods

Phosphorothioated oligonucleotides of 21-40 bases in length were ordered from Integrated DNA Technologies (IDT) or donated from ISIS pharmaceuticals. Mouse livers and plasma were obtained from Bioreclamation, Inc. Solvents and reagents were from Sigma. Oligo isolation was performed with 100 mg/3mL Clarity® OTX™ cartridges using a standard SPE vacuum manifold from Phenomenex. Plasma samples are diluted 1:1 with loading buffer prior to loading on cartridge. Cartridge is washed with equilibration buffer and wash buffer then oligonucleotide is eluted from the cartridge with an Acetonitrile/THF mixture. Samples are either speed-vac evaporated or lyophilized to near dryness then reconstituted and injected on LC/MS.

LC/MS analysis of purified samples was performed on a Novatia HTCS LC system connected to a Thermo Orbitrap™ mass spectrometer. Spectral deconvolution software that linked in with the Xcalibur™ software was provided by Novatia. HPLC columns used include Clarity® Oligo-RP™, Clarity® Oligo-MS™ and Luna® HILIC (all 50 x 2.1 mm dimension). RP gradient methods using Clarity columns used modified TEA/HFIP mobile phase system, HILIC separations used a NH₄OAc buffer system and an inverse organic gradient with Acetonitrile.
**Figure 1a. LLE/ SPE Method**

- 1-2 hour SPE cleanup procedure
- Significant sample manipulation
- Difficult to automate or multiplex


**Figure 1b. SPE Only Method (Clarity OTX)**

- 15 minute SPE cleanup procedure
- Available in tube or 96-well plate format
- Minimal sample manipulation
- Easy to automate and multiplex
Figure 2. Recovery and extraction effectiveness studies of the single-step SPE protocol using Clarity OTX for a 27mer phosphorothioate oligonucleotide. An aliquot of 12 µg was spiked into a plasma sample, extracted, and compared to a control. Recovery of 97% is observed with only minor plasma contaminants that elute far away from the main oligonucleotide peak on the HPLC run.
Figure 3. Sensitivity Studies with RNA

Figure 3: Aliquots of 500 ng and 50 ng spikes in plasma were extracted using SPE-only method (Clarity OTX) to determine sensitivity limits. A TIC of the 500 ng load is shown on the left. The 14.3 min peak corresponding to the 19mer P-S can still be quantitated. While a peak is not observed for the TIC of the 50 ng load, using a XIC at m/z of 944 (the -7 charge state) as is shown on the right, one can still readily quantitate the oligonucleotide at even lower levels. In most studies it appears that the sensitivity of the MS used determines absolute sensitivity, not the sample preparation protocol.

MS data courtesy of Mark Hail at Novatia
Figure 4: UV Chromatograms of oligonucleotide extracted from liver tissue with the SPE-only protocol. The 19mer extracted phosphorothioate oligonucleotide was spiked with 10 µg of a oligonucleotide internal standard before LC/MS analysis. The top two chromatograms represent different levels of the incubated P-S oligo. The bottom chromatogram is an external standard of equal amounts of the 19mer oligo and internal standard. Note the high recovery of the oligonucleotide and low level of tissue contaminants from the incubated samples and a linear response from different levels of spiked oligonucleotide.

Data courtesy of Hans Gaus at ISIS
Figure 5. Protocol-induced Modification of P-S Oligonucleotides (speed-vac oxidation)

Figure 5: The MS spectra on the left is a control 19mer P-S oligonucleotide directly injected on LC/MS while the MS spectra on the right is dried down (No SPE) using a speed vac evaporator (with heat) and reconstituted before LC/MS injection. Numerous MS peaks corresponding to multiple oxidations/ P-S to P-O desulfurizations are observed. Care must be taken with some modified oligonucleotides to avoid protocol-induced modifications (especially due to dry-down/reconstitution steps). In many cases, oligonucleotide lyophilization avoided oxidation.
Figure 6. Balancing LC Retention versus Ion-pairing Ion Suppression

Figure 6: LC/MS separation of a 12-18 poly dT oligonucleotide at different ion-pairing concentrations using Clarity Oligo-RP. Different concentrations of ion-pairing mobile phase are noted. Increasing ion-pairing concentration enhances resolution and sensitivity only to a point; concentrated ion-pairing reagent suppresses MS signal despite increasing retention.
**Figure 7. Increasing Throughput with Core-Shell HPLC Columns**

Figure 7: Poly dT standard is separated in under 5 minutes using a core-shell media based Clarity 2.6 µm Oligo-MS column. High efficiency core-shell columns allow for high-throughput LC/MS separations of oligonucleotides improving overall process throughput while maintaining good resolution.
Figure 8. **Alternate Separation Mode using HILIC**

Figure 8: Poly dT standard run using a highly-optimized HILIC method for a separation around 10 minutes. While HILIC methods offer some promise in potentially reducing ion suppression due to ion-pairing reagents, the limited resolution and ruggedness of oligo HILIC methods hamper their utility in developing high-throughput LC/MS methods for ADME/Pharmacokinetics studies.

Conditions: Luna 3 µm HILIC, 15 mM NH₄OAc, gradient 70 to 50 % Acetonitrile in 10 minutes.
Previous oligo isolation methods used a labor-intensive combination of LLE and SPE (Figure 1a) to solubilize and isolate oligonucleotides from proteins and lipids in plasma and tissue. A new SPE-only methodology (Clarity® OTX™) was developed that involved using chaotropes and detergents to break up interactions in plasma prior to loading on a mixed-mode SPE cartridge. For tissue samples, homogenization buffer can be diluted and directly loaded. The cartridge is washed and eluted using a mixture of Acetonitrile and THF, a schematic is shown in Figure 1b.

The isolation method works equally well with DNA and RNA samples including oligonucleotides that are phosphorothioated. As shown in Figure 2, Figure 3, and Figure 4; the methodology delivers good sample cleanup and recovery, can isolate very low levels of oligonucleotide from biological fluids and tissues, and demonstrates good linearity from the µg to ng ranges tested.

While the method works well for “standard” oligonucleotides, some method optimization is typically needed for more “esoteric” modified oligonucleotides. Key points in the method revolve around neutralizing nuclease activity and minimizing desulfurization/oxidation of P-S oligonucleotides. One troubling source of modification and loss of recovery for all methods is speed-vac evaporation (Figure 5), lyophilization and low-temperature speed-vac evaporation minimizes modification and improved oligo recovery.
While oligonucleotide isolation from biological fluids and tissue is one aspect of the challenges in performing ADME/PK studies of oligonucleotide therapeutics, obtaining accurate and sensitive LC/MS data present an equal set of challenges. Oligonucleotides are highly-anionic biopolymers that require the use of ion-pairing reagent to obtain retention on reversed phase columns; optimal oligo MS sensitivity and LC resolution require a balance between ion-pairing retention and ion suppression. An example of this balance is shown in Figure 6 where the optimal level of TEA/HFIP (around 4 mM TEA, 200 mM HFIP) was found to be much lower than previously published results.

In an attempt to increase the throughput of the LC/MS method, LC was investigated using recently introduced high-efficiency core-shell media (Clarity® Oligo-MS™) columns. Figure 7 shows an example of a 5 minute separation of an oligonucleotide standard with good efficiency and resolution. Such results suggest the core-shell media is a good solution for improving LC/MS throughput using existing HPLC systems.

Recent reports from other groups suggest that HILIC might be a potential solution for LC/MS of oligos. After significant optimization using several different classes of HILIC columns it was found that diol-based or amide-based provided some mediocre separation of oligonucleotides (Figure 8). However, in these studies each oligo required significant optimization to provide separation inferior to current LC methods making it unsuitable for ADME/PK methods.
Previous methods for oligonucleotide isolation and analysis provided useful solution for research setting but have serious throughput and automation limitations that make them impractical for clinical and animal studies where 1000's of samples potentially need to be analyzed. The new SPE-only oligonucleotide isolation method provides a new direction for high-throughput isolation of oligonucleotides from biological fluids and tissues that can be easily automated and multiplexed. Breaking up oligo interactions with matrix, removing LC/MS interfering matrix components, and preventing post collection modification of the oligonucleotide are all critical aspects of any methodology.

Maximizing sensitivity of LC/MS analysis of oligonucleotides requires balancing ion-pairing buffers based on the oligo being separated and the LC column being used. Moving to core-shell media offers new high-throughput LC/MS solutions for analyzing oligo samples which needed to match the presented throughput improvements in oligo isolation. While other separation modes (HILIC) offer the possibility of improving sensitivity, investigated methodologies cannot currently match the performance and reliability of ion-pairing reversed phase LC/MS methods.