Rapid Extraction of Therapeutic Oligonucleotides from Primary Tissues for LC/MS Analysis Using Clarity® OTX™, an Oligonucleotide Extraction Cartridge

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Abstract

In the development of therapeutic oligonucleotides, researchers need to analyze the target tissues where oligonucleotide action occurs. This need to quantitate synthetic oligonucleotides and determine their chemical structure is especially of interest in pharmacokinetics studies of animal models during drug development. Until recently, most methods for quantitating oligonucleotides in tissues have been indirect because of the difficulty in isolating and quantitating oligonucleotide therapeutics by LC/MS. A new method of performing rapid oligonucleotide extraction from serum and plasma has been recently introduced. This method uses a newly released extraction product, Clarity® OTX™, and utilizes SPE to isolate oligonucleotides with greater than 80 % recovery and good reproducibility from plasma samples. While isolating oligonucleotides and metabolites from primary tissues is a much more complex procedure than from biological fluids, efforts were undertaken to evaluate the utility of using this Clarity OTX oligo cleanup methodology for LC/MS of tissue samples. Several candidate therapeutic oligonucleotides were isolated from primary tissues using the Clarity OTX cartridge with its standard purification protocol. Isolated oligonucleotides were analyzed by LC/MS. Results for oligonucleotides isolated from tissues closely resembled results obtained from biological fluids; little or no MS-interfering contaminants were observed and reproducible recoveries were 80 % or greater when analyzing oligonucleotides in the low nanomole to micromole range.

Introduction

As research for a potential oligonucleotide therapeutic moves past *in vitro* studies, there is a need to identify target tissues where the oligonucleotide may trigger a biological effect, whether it be from the desired oligonucleotide therapeutic or any breakdown product generated during distribution from injection site to target. Entry into the cell can also elicit changes, especially to RNAi-based therapeutics that often need to be processed to form their active “gene-silencing” form that binds to dicer. PCR analysis can reveal sequence information, but cannot identify any chemical modifications to the oligonucleotide therapeutic. Nor can PCR directly quantitate the different metabolites generated during processing. As such, other analysis techniques are needed.

The primary tool for high sensitivity quantitative and qualitative analysis of chemical compounds is LC/MS/MS, where complex mixtures of analytes are separated by HPLC then quantitated and identified by tandem mass spectrometry. LC/MS analysis of oligonucleotide therapeutics in primary tissues has been a difficult process due to the large number of MS- interfering substances in a cell lysate. Proteins, lipids, and salts are just a few interfering compounds that can mask MS signal of small oligonucleotides; such compounds must be removed to obtain useful information. A fundamental problem with LC/MS analysis of primary tissues is that all of the current extraction methods (the most popular uses a combination of liquid-liquid extraction {LLE} and solid phase extraction {SPE} together to isolate oligonucleotides) lack reproducibility and demonstrate poor recovery of oligonucleotide. As such, routine analysis of oligonucleotides in primary tissues is not currently practical.

A new product and protocol for extracting therapeutic oligonucleotides from serum and plasma, Clarity OTX, has been recently introduced that delivers high recovery and reproducibility and a simple purification protocol that is amenable to high-throughput automation. Efforts were undertaken to assess the feasibility of using the Clarity OTX product to analyze oligonucleotides from primary tissues.

Materials and Methods

Phosphorothioate oligonucleotides were kindly donated from ISIS Pharmaceuticals. Mouse livers were obtained from Bioreclamation, Inc. Solvents and reagents were from Sigma. Oligo isolation was performed with 100 mg / 3 mL Clarity OTX cartridges and buffer system using a standard SPE vacuum manifold from Phenomenex.

Figure 1. Extraction of Therapeutic Oligonucleotides from Tissue Sample Using the Clarity OTX Procedure

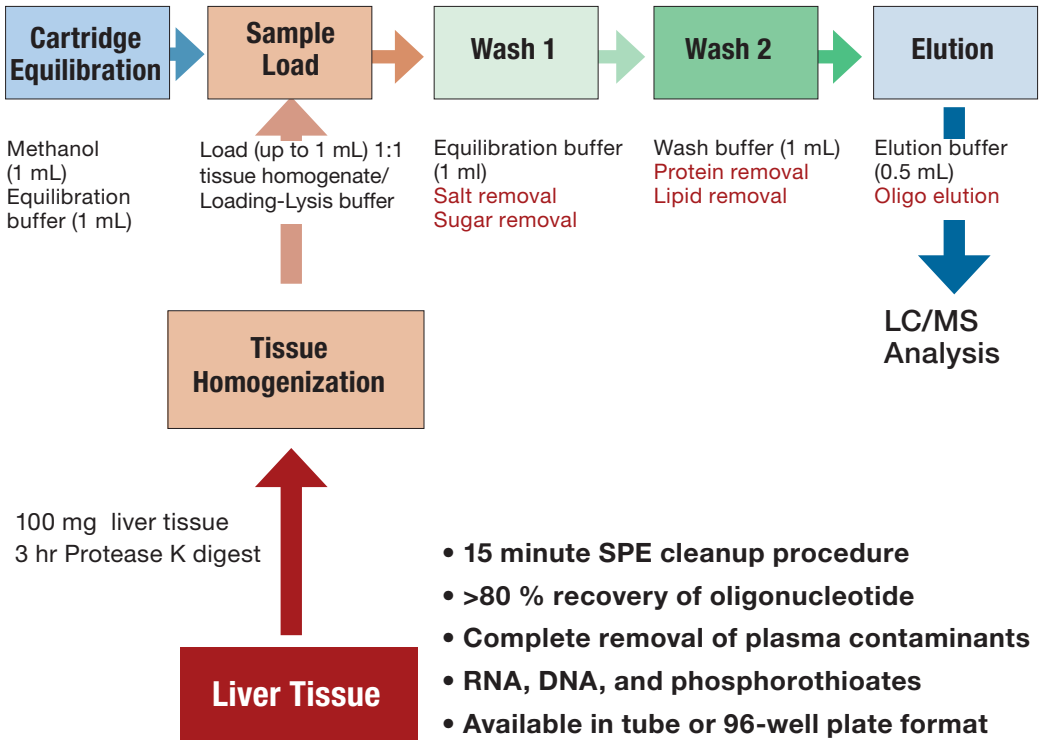

Figure 2. Oligonucleotide Extracted from Liver Tissue Using Clarity OTX

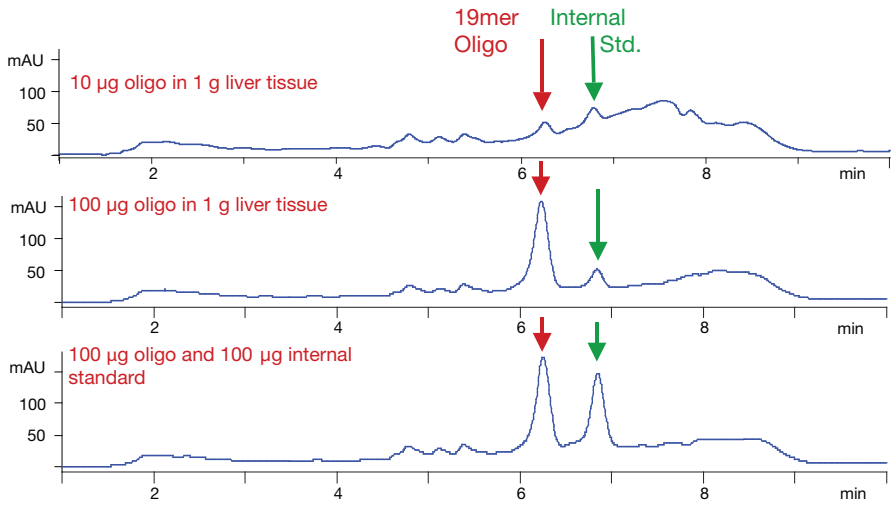

Figure 2: UV chromatograms of oligonucleotide extracted from liver tissue using Clarity OTX. The 19mer extracted phosphorothioate oligonucleotide was spiked with 10 µg of a oligonucleotide internal standard before LC/MS analysis. The top two chromatograms represent different levels of the incubated P-S 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 plasma contaminants from the incubated samples.

Figure 3. Spectra of Extracted P-S Oligonucleotide

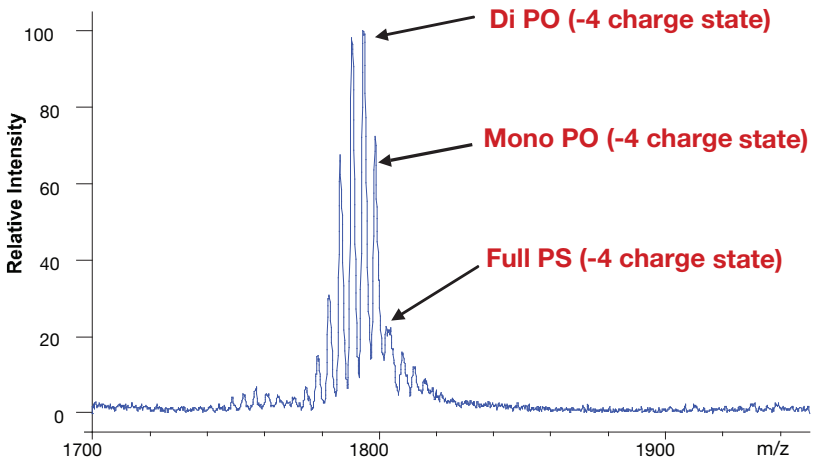

Figure 3: Zoom in spectra of the "-4" charge state of the 19mer P-S oligonucleotide spiked into liver tissue at the 50 ug/g level and extracted using Clarity OTX. Note the individual ions corresponding to degrees of desulfurization of the oligonucleotides. Rapid extraction, low MS background, and high recovery make the Clarity OTX protocol amenable for performing ADME / PK studies of tissue samples.

Figure 4. Oligonucleotide Calibration Curve for Liver Tissue Sample Using LC/MS Area Data

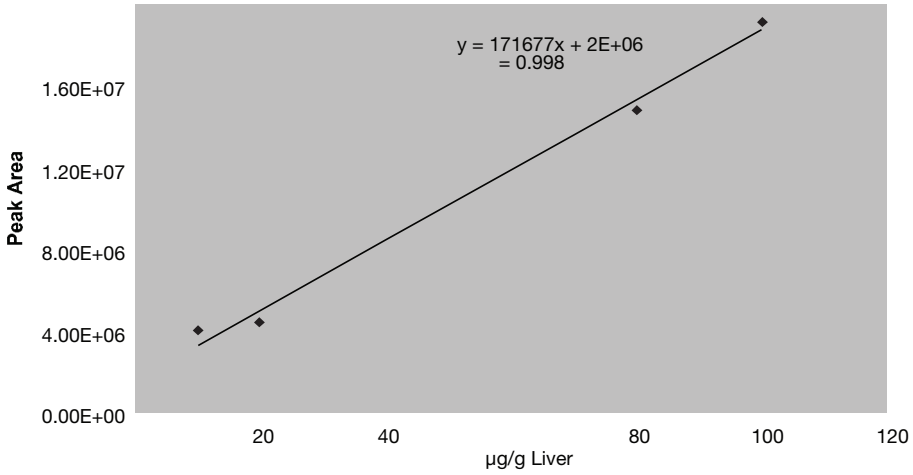

Figure 4: Linearity data for 19mer P-S oligonucleotide based on MS peak area. Four different oligo concentrations in liver tissue from 100 µg to 10 µg in 1 g of liver tissue as shown here. High recovery and good linearity is seen across physiologically relevant concentrations for this initial study.

Results & Discussion

A separate poster presented here at TIDES demonstrates the utility of Clarity OTX for isolating short chain oligonucleotides from serum and plasma samples similar to what one would expect during performing an adsorption, distribution, metabolism, excretion (ADME) / pharmacokinetics study. The key to obtaining quantitative LC/MS data from biological fluids is to remove all contaminants that can interfere with electrospray efficiency during the LC/MS analysis of the oligonucleotide. Typical interfering compounds from all biological sources include salts, proteins, and lipids; Clarity OTX uses a proprietary mixed-mode SPE sorbent to isolate short-length (less than 100 mer) oligonucleotides from other components. A representation of the procedure is shown in **Figure 1** The Clarity OTX uses buffers that maintain a physiological pH throughout the process to avoid unwanted damage to the oligonucleotide (depurination for DNA below pH 5 and 2'-3' isomerization for RNA above pH 8).

While serum and plasma samples are difficult sample matrices in which to analyze therapeutic oligonucleotides and their metabolites, analysis of oligonucleotides from tissues adds an additional set of complications. The relative concentrations of proteins and lipids are orders of magnitude higher than what is seen in serum or plasma. Tissue samples require digestion or homogenization to disperse extracellular matrix proteins. In addition, cells from tissue must be lysed and solubilized.

Experiments to assess oligonucleotide recovery and sample cleanup were performed. Duplicate analyses were performed on a mouse liver sample to see if some degree of reproducibility was obtainable from analyzing tissue sample with Clarity OTX. As recovery would be difficult to determine for an oligonucleotide delivered in vivo, a fixed dosage of oligonucleotide was injected into each liver sample, digested, then extracted. 100 mg of oligo spiked tissue was treated with 500 µL digestion buffer containing 20 µL / mL of Protease K for 3 hours at 50 °C. Samples were then diluted 1:1 with Clarity OTX lysis-load buffer and administered on Clarity 100 mg / 3 mL cartridges. Samples were then washed with equilibration buffer, followed by wash buffer, then eluted from the cartridge with elution buffer and lyophilized. A standard of the spiked oligonucleotide was used to determine recovery of the oligonucleotide. Examples of two different analyses are show in **Figure 2** and **Figure 3**. Results show excellent reproducible recovery along with very low level of MS - (or UV-) interfering contaminants. In research studies we have repeatedly seen greater than 80 % recovery of spiked oligonucleotide with minimal interference. This trend had been seen in samples from the 1 µg / mL to 50 ng / mL level regardless of the type of oligo (DNA, RNA, etc.) tested.

Conclusion

Isolating short oligonucleotides from biological fluids and analyzing them by LC/MS is difficult due to the complexity of the samples being analyzed as well as the low levels of oligo in vivo. Existing extraction methods are laborious and give poor recoveries, thus limiting useful information about generated metabolites from oligonucleotide therapeutics. Clarity OTX (Oligonucleotide Therapeutic eXtractor) delivers a new paradigm for analyzing oligonucleotides from serum and plasma (see the other Clarity OTX poster presented here).

While analyzing and quantitating plasma concentrations of oligonucleotides are important, another application with even greater challenges is analyzing primary tissues. Our data using the Clarity OTX protocol for extracting oligonucleotides from spiked tissue indicates that this protocol can eliminate unwanted contaminants and yield excellent recovery of the target sequence from tissue samples. Clarity OTX is a new product enabling fast and effective isolation of therapeutic oligonucleotides from tissue.