Preparation and LC/MS Analysis of Oligonucleotide Therapeutics from Biological Matrices

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Abstract

Due to their association with other biomolecules as well as their unique chemical properties, oligonucleotide therapeutics present unique challenges in their isolation and LC/MS analysis from biological matrices. A new isolation protocol using a mixed-mode SPE cartridge is presented that demonstrates high recovery and sensitivity into the low ng/mL range. Optimization of LC/MS mobile phase conditions as well as MS analysis parameters results in improved sensitivity and identification of oligonucleotides and their metabolites.

Introduction

Therapeutic uses of oligonucleotides are becoming an increasingly popular approach to treat disease. Since the human genome was fully decoded back in 2003, researchers are gaining a rapid and comprehensive understanding of which genes code for which processes in normal human physiology. Gaining knowledge of what is normal has allowed scientists to identify abnormalities in genes which lead to disease states. Such abnormalities manifest in the incorrect coding of proteins which then leads to downstream problems with cell physiology.

Gene therapy uses manufactured genetic material to correct for abnormalities brought on by the onset of a disease state. There are two main forms of gene therapy: classical and interference. The former differs from the latter in that complete genes coding for a normal protein are inserted into diseased cells. The cells then start to code the normal protein and cell function is returned back to a normal state. The second form of gene therapy is known as interference therapy it differs from classical gene therapy because the mode of action is to silence mutated genes or correct for reading frame errors. Interference therapy uses two different types of RNA: miRNA (micro RNA) and siRNA (short interfering RNA). Both are short strands of RNA (>22 nucleotides in length), are manufactured to code as a complementary antisense sequence to either silence or interfere with a specific mRNA and hence correct for errors.

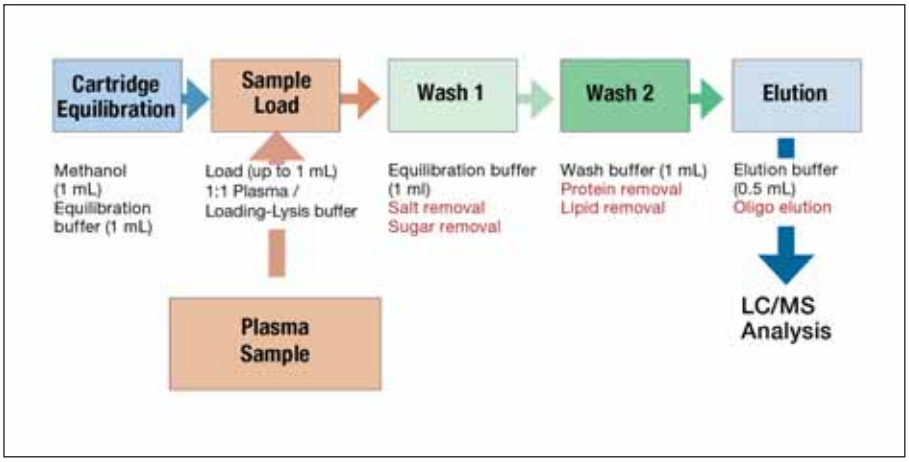

Figure 1: Oligonucleotide isolation protocol (Clarity OTX) is shown with individual isolation steps.

The technology for developing treatments using interference therapy is still in its infancy and faces many challenges. One such challenge is to manufacture the drug such that it is stable enough to survive nuclease activity such that its therapeutic action can be achieved. Another challenge is devising effective and targeted delivery systems. However a huge challenge facing scientists is to develop extraction protocols and analytical tools to identify metabolites and conduct pharmacokinetic studies to mirror those conducted for small molecules. The challenges are more difficult than small molecules for the following reasons. Interfering RNA molecules mirror physiological molecules so specific extraction protocols can be problematic. One such protocol developed by Zhang et al employs the use of LLE and RP SPE [1]. The drawbacks of this method are: that the preparation time is long (1-2h), there's significant sample manipulation risking degradation and the method is difficult to automate. The highly polar nature of RNA makes RP HPLC impossible without the use of ion pairing agents to increase the retention and resolution of oligonucleotides. A disadvantage of ion pairing reagents is that they tend to inhibit electrospray ionization resulting in reduced MS sensitivity. The addition of an agent such as HFIP (Hexafluoroisopropanol) acts to improve MS sensitivity when added as a modifier in the mobile phase. Ion exchange chromatography (IEX) can separate poly-anionic oligonucleotides based on their length because each nucleotide is linked via a negatively charged phosphodiester backbone. The strong anion exchange

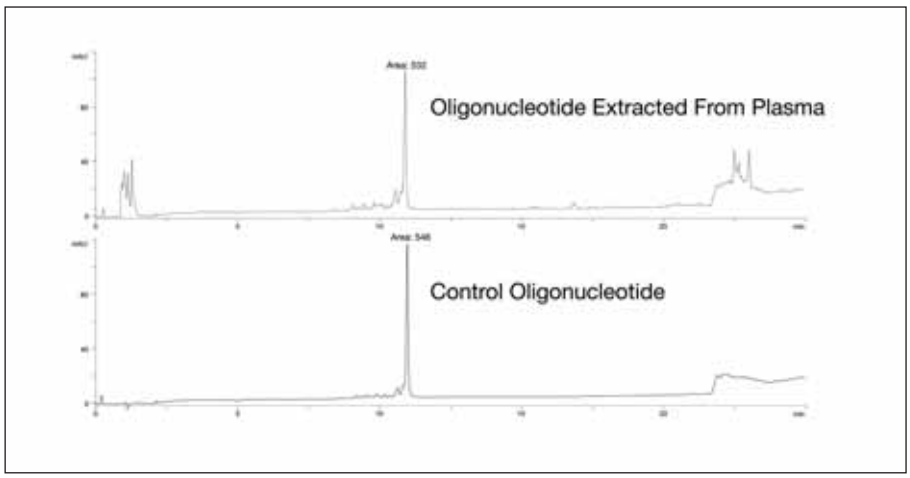

Figure 2: Recovery and cleanup of a 27mer DNA phosphorothioate oligonucleotide from plasma using the Clarity OTX protocol. Oligonucleotide was spiked into a plasma sample, extracted, and compared to a control. Recovery is estimated at 97 % with only minor plasma contaminants.

columns typically used for separating oligonucleotides require moderate amounts of salt to break up electrostatic interactions between the media and the oligonucleotide ligand; such salt completely suppresses electrospray ionization making IEX LC/MS impractical.

The work outlined in this paper details a number of solutions for the extraction and analysis of oligonucleotides. This includes use of a simple but quick and effective extraction kit based on mixed-mode ion exchange SPE. Moreover, work with augmenting the ratio of ion paring agents such as TEA and HFIP shows improvements to MS sensitivities compared to reported methods. Finally, the utility of using deconvolution software is demonstrated in identifying low-level metabolites which can be potentially overlooked in the raw spectra of an oligonucleotide.

Materials and Methods

All chemicals and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise stated. Oligonucleotide samples were either purchased from Integrated DNA Technologies (Coralville, IA, USA) or generously provided by various industry and academic sources (ISIS: Carlsbad, CA, USA; USC Oligonucleotide Laboratory, Los Angeles, CA, USA). HPLC solvents were purchased from EMD (San Diego, CA, USA). Serum and Plasma was purchased from Bioreclamation (Liverpool, NY, USA).

Oligonucleotide Isolation

Various oligonucleotides were spiked at different concentrations into serum and plasma to demonstrate the ability of developed isolation protocols to recover oligonucleotides and their metabolites from biological matrices. While various interactions were investigated, the general isolation protocol revolved around mixing equal aliquots of the Clarity OTX loading buffer (Phenomenex, Torrance, CA, USA) and serum/plasma samples together prior to loading on the SPE cartridge (loading volume is typically less than 2 mL of the mixture). The SPE isolation cartridge (Clarity OTX 100mg/ 3mL tube; Phenomenex) is first "wetted" with methanol then equilibrated with Clarity OTX equilibration buffer (10 mM Phosphate pH 5.5) prior to sample loading. After sample loading the cartridge is rinsed twice with equilibration buffer followed by rinses with Clarity OTX wash buffer (10 mM Phosphate pH 5.5/ 50% acetonitrile). The oligonucleotide is eluted from the cartridge using elution buffer (100 mM ammonium bicarbonate pH 8.0/ 40% acetonitrile/10% tetrahydrofuran). Samples can be either lyophilized or speed vac evaporated before reconstitution for LC analysis.

LC-UV and LC/MS Analysis

Isolated oligonucleotides were analyzed by either LC using UV detection or by LC/MS for more sensitive detection. For LC-UV analysis samples were injected on an Agilent HP1100 HPLC (Palo Alto CA, USA) using a Clarity 3 µm Oligo-RP or Clarity 2.6 µm Oligo-MS HPLC column (Phenomenex, Torrance, CA, USA; column dimension 50 x 2.0 mm). The HPLC has an autosampler, column oven, and UV detector. Data was analyzed using Chemstation version 8.0 software. For LC/MS analysis samples were either detected using a AB Sciex API 3000 (ABSciex, Foster City, CA, USA) at Phenomenex or was analyzed at Novatia using a Novatia Oligo HTCS HPLC system (Monmouth Junction, NJ, USA) connected to a LTQ Orbitrap mass spectrometer (San Jose, CA, USA). Oligonucleotide ion spectra were reconstructed using the proMass software (Novatia) to give parent mass information for specific oligonucleotide ions and their metabolites. HPLC conditions were as follows for most separations: a gradient separation method using an aqueous mobile phase A of 8 mM triethylamine/ 200 mM hexafluoroisopropanol pH 8.0 and organic mobile phase B of acetonitrile. Various gradients were used depending on the column and instrument being used as well as the specific oligonucleotide being analyzed. Most oligonucleotide gradient methods start low organic mobile phase B (around 3-5%B); oligonucleotide elutes during a shallow gradient up to 15-35%B. Specific conditions are listed with each figure.

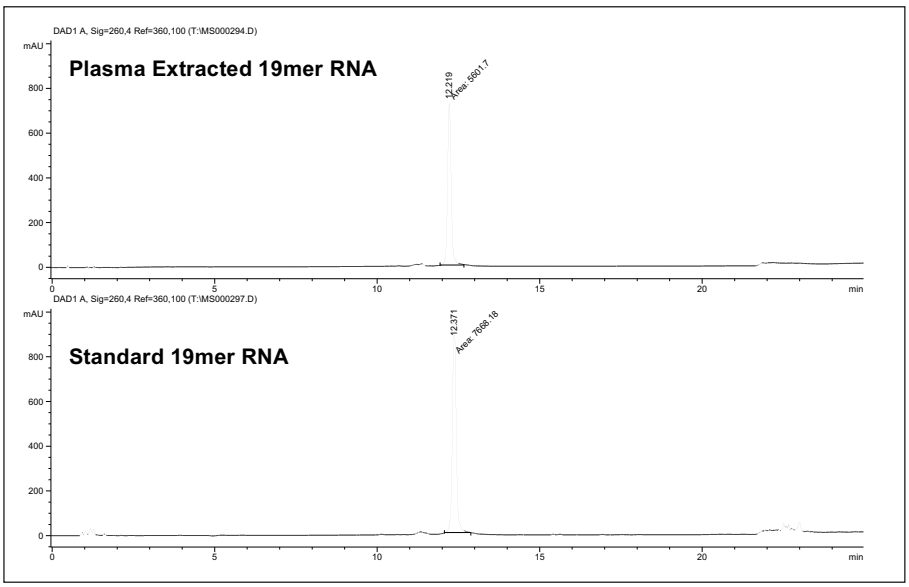

Figure 3: Recovery and cleanup of a 19mer RNA gapmer from plasma using the Clarity OTX protocol compared to a control. Minimal plasma contaminants are observed with 76% recovery.

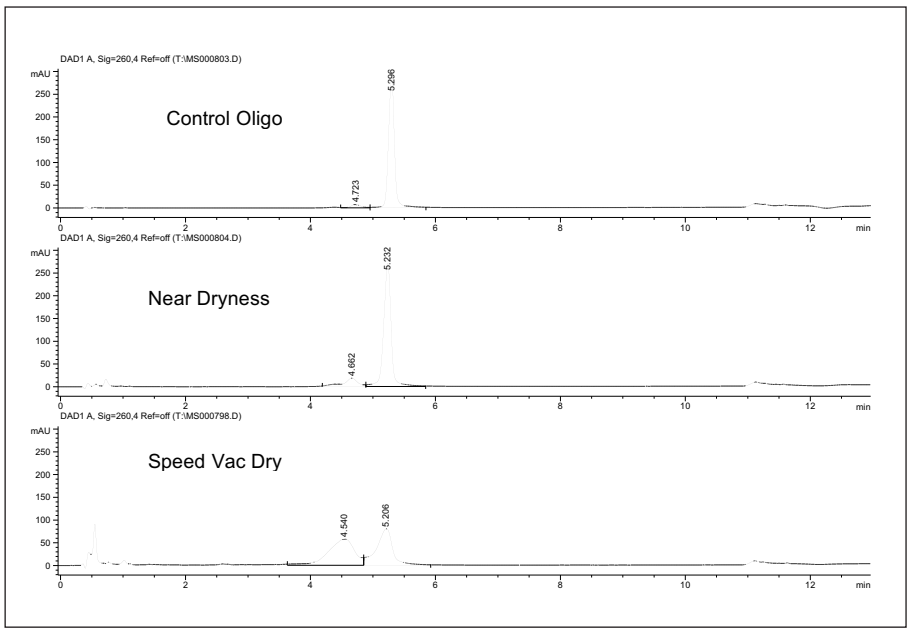

Figure 4: Speed-vac effect on oligonucleotide recovery. An oligonucleotide standard (top chromatogram) is compared to samples that are concentrated using a speed-vac evaporator. The middle chromatogram is of the oligonucleotide evaporated to near dryness and little loss in recovery is observed. In the bottom chromatogram the oligonucleotide is evaporated to dryness. Significant loss in recovery is observed when an oligonucleotide is speed-vac evaporated to dryness.

Results and Discussion

Oligonucleotide Isolation

Probably the most difficult aspect of monitoring the ADME characteristics of oligonucleotide therapeutics revolves around isolating a therapeutic oligonucleotide from biological matrices. Unlike most small molecule therapeutics that can be easily isolated from serum or plasma by a simple protein precipitation or SPE method, oligonucleotides tend to co-precipitate or co-migrate with plasma components during traditional sample clean-up steps. Several documented examples [2,3] show that short oligonucleotides tend to strongly associate with serum proteins and lipid, thus their intransigence in being isolated by simple separation protocols. A key requirement for therapeutic oligonucleotide isolation centers around disassociating binding to proteins and lipids before any isolation technique is employed. Other isolation techniques [1] employ liquid-liquid extraction (LLE) to disrupt oligonucleotide association; however, LLE is a cumbersome technique to automate or multiplex and still requires additional SPE steps to isolate oligonucleotides.

An alternate technique for disrupting associations between oligonucleotides and proteins and/or lipid revolves around using traditional protein unfolding agents like chaotropic salts and detergents to disrupt any binding. Once binding has been disrupted oligonucleotides, proteins, and lipids can be easily separated based on their chemical differences: proteins will tend to be neutral or basic and hydrophobic, lipids will tend to be very hydrophobic, and oligonucleotides will be acidic and highly polar. A new SPE-based protocol has been developed (Clarity OTX) that uses this concept to isolate therapeutic oligonucleotides (a schematic of the process is shown in figure 1). Oligonucleotide-containing serum or plasma samples treated with the chaotrope/detergent buffer are loaded on to a mixed-mode SPE sorbent (weak anion exchange and reversed phase) around pH 5.5 which is below the pKa of the anion exchange moiety resulting in a positively charged sorbent. The oligonucleotide binds to the sorbent as does much of the protein and lipid in the sample. Subsequent washes at low pH with water and organic removes most of the protein and lipid while the oligonucleotide is retained by ion exchange interactions. Elution of the oligonucleotide is achieved with moderate organic at an elevated pH (pH~8) where the weak anion exchange moiety on the sorbent is deprotonated resulting in an unchanged sorbent. Tight pH control is critical throughout the process; DNA extended exposure below pH 5 results in depurination and RNA exposure above pH 9 can lead to 2'-3' isomerization of the ribose sugar.

An example of the cleanup is shown in figure 2. HPLC chromatograms of a phosphorothioate 27mer DNA oligonucleotide spiked into plasma and purified using the Clarity OTX protocol is compared against a control oligonucleotide. Almost complete recovery (97%) of the oligonucleotide is obtained with only small amounts of matrix contaminant being seen in the chromatogram at retention times far away from most oligonucleotides and their metabolites. Another example is shown in figure 3 where a 19mer 2'methoyx-ethyl (2'MOE) gapmer RNA/DNA chimera was extracted from plasma with an approximate recovery of around 76% based on HPLC. Recoveries have ranged between 65-99% depending on the oligonucleotide and biological matrix used for the isolation with good linearity for a specific oligonucleotide and biological matrix type [4]. Being somewhat "cell free", serum and plasma require only disruption of protein and lipid binding to release the oligonucleotide into solution. Thus, high recoveries with a simplified protocol can be achieved for oligonucleotide therapeutics from such matrices. Isolating oligonucleotides from tissues is a much more

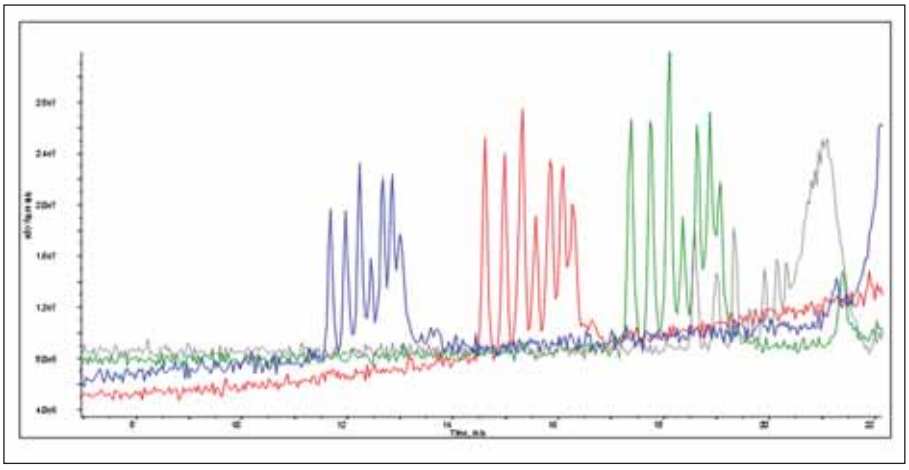

Figure 5: Ion-pairing concentration effects on LC/MS sensitivity and resolution. A 12-18 poly dT oligonucleotide standard is run on a Clarity Oligo-RP column using different ion pairing concentrations: Black trace = 15 mM TEA/ 400 mM HFIP, Green trace= 2.8 mM TEA 280 mM HFIP, Red trace = 4 mM TEA/ 100 HFIP, blue trace = 2 mM TEA/ 50 mM HFIP. Retention, resolution and MS signal intensity appear optimal between 4-8 mm TEA and 200-300 mm HFIP.

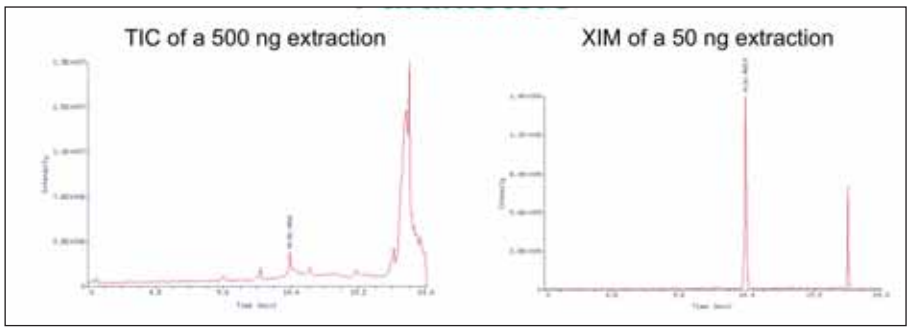

Figure 6: Protocol Sensitivity Studies. The total ion chromatogram of 500 ng/mL of a 19mer oligonucleotide extracted from plasma is shown in chromatogram 6A. Note the low level peak corresponding to the oligonucleotide at RT of 14.3 minutes. The extracted ion chromatogram (-7 charge state ion at m/z of 944) of a 50 ng/mL sample is shown in chromatogram 6B. This isolation protocol demonstrates good recovery well into low nanograms/ mL concentrations.

complex process in that cell membranes must be disrupted to release an oligonucleotide; as a result, recovery is often significantly lower with tissue samples without an optimized cell lysis methodology (data not shown).

Additional studies were undertaken to better understand some of the factors involved in sample recovery. While the subject is rather complex, one source of variability was identified that could potentially affect any isolation methodology used. A common step used in all oligonucleotide isolation methodologies is a sample concentration step. Such concentration typically uses a speed-vac evaporator to concentrate the oligonucleotide prior to injection on HPLC. Common practice is to evaporate a sample to dryness before reconstitution in mobile phase. However, this appears to affect the recovery of the oligonucleotide when compared to a sample that is only evaporated to near dryness. Figure 4 demonstrates this convincingly. An oligonucleotide isolated from plasma was either evaporated to "near-dryness" or to dryness using a speed-vac evaporator. Samples were reconstituted in mobile phase and run on HPLC. Profiles from both samples were compared to a standard; the sample evaporated to "full dryness" showed significant loss in recovery versus the "near-dryness" sample which demonstrated good recovery. The loss of recovery during speed-vac evaporation has been reported for proteins and is suspected to be due to irreversible adsorption to the surface of polypropylene tubes. [5] Based on such results, speed-vac evaporation should be avoided for low-level oligonucleotide isolation techniques. Lyophilization appears to be robust alternative for concentrating samples without similar deleterious effects.

LC/MS Analysis of Oligonucleotides

Isolation of oligonucleotides from biological matrices is the major challenge of any ADME/ pharmacokinetics study of oligonucleotide therapeutics, however optimal analysis conditions can play a major role in maximizing the sensitivity and information content of a study. Oligonucleotides are typically analyzed by LC/MS using an ion-pairing reversed phase method where a mixture of HFIP and TEA is used to elicit retention of the polar polyanionic molecule. [6,7] These ion-pairing interactions with the negatively charged phosphate backbone of oligonucleotides provide retention based on the size of the biopolymer which allows one to resolve smaller breakdown fragments from the full-length intact oligonucleotide. In LC/MS applications, ion-pairing retention must be balanced against ion suppression to maximize MS sensitivity. High concentrations of ion-paring buffer can suppress electrospray ion generation resulting in lower oligonucleotide sensitivity. An example of this effect is shown in figure 5 where different levels of ion-pairing buffer were used in the mobile phase of a LC/MS run of a mixture of oligonucleotide standards. Maximizing MS sensitivity becomes a balance between ion suppression and retention; higher concentrations of ion pairing buffer results in greater retention and resolution of oligonucleotides but only to a certain point. At higher concentrations, MS signal is suppressed resulting in lower sensitivity. Unlike previous results [8] which report an optimal mobile phase concentration of 16mm TEA/400 mm HFIP, our findings suggested that a lower concentration between 4-8mm TEA/ 200-300mm HFIP was optimal for the HPLC columns (Clarity Oligo-RP and Clarity Oligo-MS) used in this study.

When the mixed-mode SPE procedure (Clarity OTX) is combined with optimized LC/MS conditions very sensitive detection of oligonucleotide from biological matrices can be realized. In figure 6 different levels of a 19mer phosphorothioate RNA oligonucleotide isolated from plasma samples using the Clarity OTX protocol were run on the Oligo HTCS system connected to an Orbitrap MS. TIC mode was used in the analysis of a 500 ng/mL spiked sample in figure 6A to display the effectiveness of the protocol in removing matrix interference. In figure 6B the same acquisition method used on a 50 ng/mL spiked sample using extracted ion (XIC) is displayed for the -7 ion of the isolated oligonucleotide. Using extracted ions show that at the 50 ng/mL level is far from the detection limit of the cleanup and analysis methodology provided one is using a sensitive mass spectrometer. In addition, oligonucleotide analysis requires deconvolution software for the identification of oligonucleotides. This can be especially important when looking for low level metabolites on an oligonucleotide therapeutic which correspond to unique molecular weights. An example of this is shown in figure 7 where the spectrum of the 19mer P-S RNA oligonucleotide is displayed in raw and reconstructed mode. The raw spectra in figure 7A show the predominant -6, -7, and -8 of the expected oligonucleotide; however one is challenged in discerning the presence of any metabolites in the sample based on the spectra. However, when the spectra is reconstituted using the ProMass software (figure 7B), one can identify low level

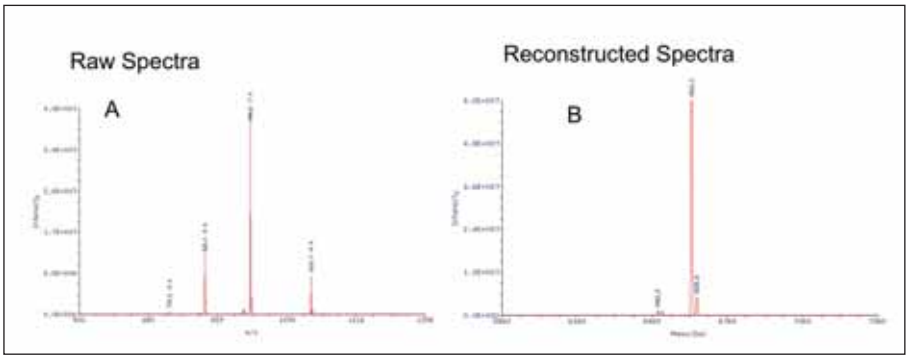

Figure 7: Using deconvolution software to identify metabolites. The raw spectra from 19mer oligonucleotide in A is compared to the reconstructed spectra in B. Note reconstructed masses for the oligonucleotide of interest at MW= 6616 as well as a minor component corresponding to a depurinated oligonucleotide at MW=6482. Deconvolution software is a useful tool in identifying low level metabolites of oligonucleotides.

masses that correspond to a salt adduct as well as a depurinated oligonucleotide. Such results demonstrate the utility of deconvolution software for oligonucleotide analysis.

Conclusion

Analyzing oligonucleotides and their metabolites from biological matrices presents significant challenges compared to small molecule therapeutics. Methodologies presented here provide unique solutions for the growing interest in oligonucleotide ADME/pharmacokinetics analysis. Isolation of oligonucleotides from biological matrices using mixed-mode SPE allow for a rapid and easily multiplexed methodology that can accommodate the large numbers of samples typically seen in a clinical trial of a therapeutic candidate. However, in any methodology one must ensure that sample manipulation does not contribute to recovery losses or chemical modification of the desired oligonucleotide.

While isolation methodology plays an important role in analyzing oligonucleotide therapeutics, of equal importance are the LC/MS analysis conditions used. Optimizing ion-pairing mobile phase conditions for a particular oligonucleotide and HPLC column can optimize resolution and retention while minimizing ion suppression effects. When this sample isolation methodology is combined with optimized LC/MS conditions and powerful mass spectrometry tools, quantitation and identification of oligonucleotides and their metabolites can be achieved in the low nanograms/ mL range.

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