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

M.McGinley, J. Rudge, *M. Hail, et al.

Phenomenex, Inc. 411 Madrid Avenue Torrance, CA 90501 USA

*Novatia LLC. Monmouth Junction, NJ, USA

PO86140311_L

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 Solid Phase Extraction (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

A huge challenge facing scientists developing oligonucleotide therapeutics is the difficult extraction protocols and analytical methods needed to identify metabolites and conduct pharmacokinetic studies to mirror those conducted for small molecules. Interfering RNA molecules often co-migrate physiological molecules so specific extraction protocols can be problematic. One such protocol developed by Zhang et al employs the use of Liquid-Liquid Extraction (LLE) and Reversed Phase (RP) SPE[1]. The drawbacks of this method include long preparation time and significant sample manipulation which can lead to oligo degradation and limited ability to multiplex the method in an automated format. After isolation, the highly polar nature of RNA makes RP HPLC impossible without the use of ion-pairing agents, which reduce MS sensitivity. Nonetheless, an agent such as Hexafluoroisopropanol (HFIP) acts to improve MS sensitivity when added as a modifier in the mobile phase, but requires optimization to maximize sensitivity.

The work outlined in the poster 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-pairing agents, such as Triethylamine (TEA) and HFIP, show improvements to MS sensitivities compared to reported methods. Finally the use of deconvolution software has demonstrated better sensitivity and specificities for analysing this most challenging family of analytes.

Materials

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 were purchased from Bioreclamation (Liverpool, NY, USA).

Methods

Oligonucleotides were spiked into serum and plasma to show the utility of the protocol. Equal aliquots of the Clarity® OTX™ loading buffer (Phenomenex, Torrance, CA, USA) and serum/plasma samples were mixed together prior to loading on the SPE cartridge. The SPE isolation cartridge (Clarity OTX 100 mg/ 3 mL tube) is first "wetted" with methanol then equilibrated with Clarity OTX equilibration buffer (10 mM Phosphate buffer, 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 buffer, 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.

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). For LC/MS analysis samples were either detected using a AB Sciex™ API 3000™ (AB Sciex, 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 (Thermo, San Jose, CA, USA). Oligonucleotide ion spectra were reconstructed using the ProMass® software (Novatia). A gradient separation method was developed 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 employed depending on the column and instrument being used as well as the specific oligonucleotide being analyzed.

Figure 1. SPE Only Method (Clarity OTX)

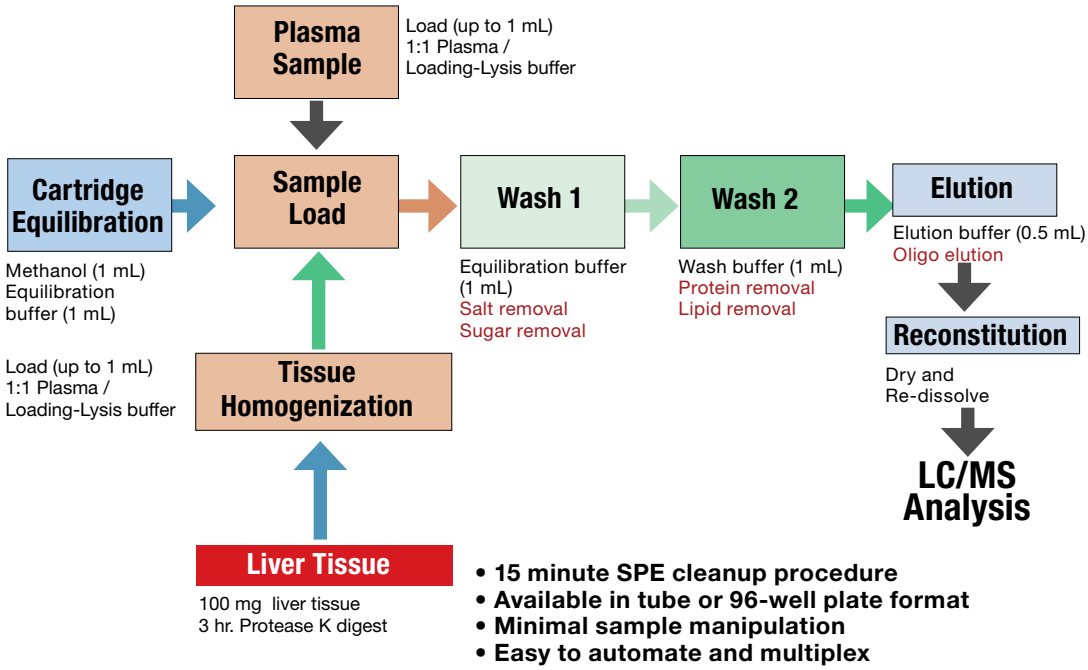


Figure 1: Oligonucleotide isolation protocol (Clarity OTX) is shown with individual isolation steps.

Figure 2. Recovery Studies from Plasma

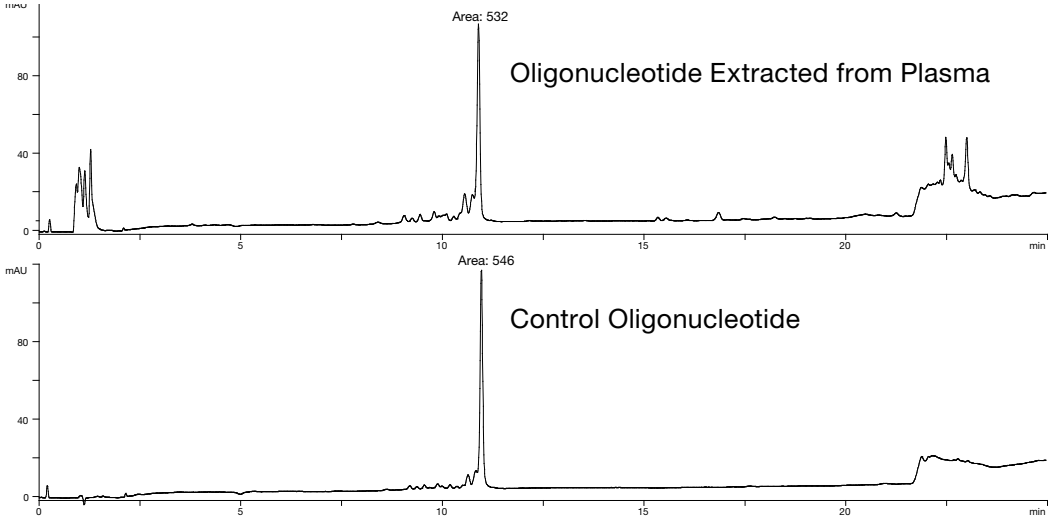
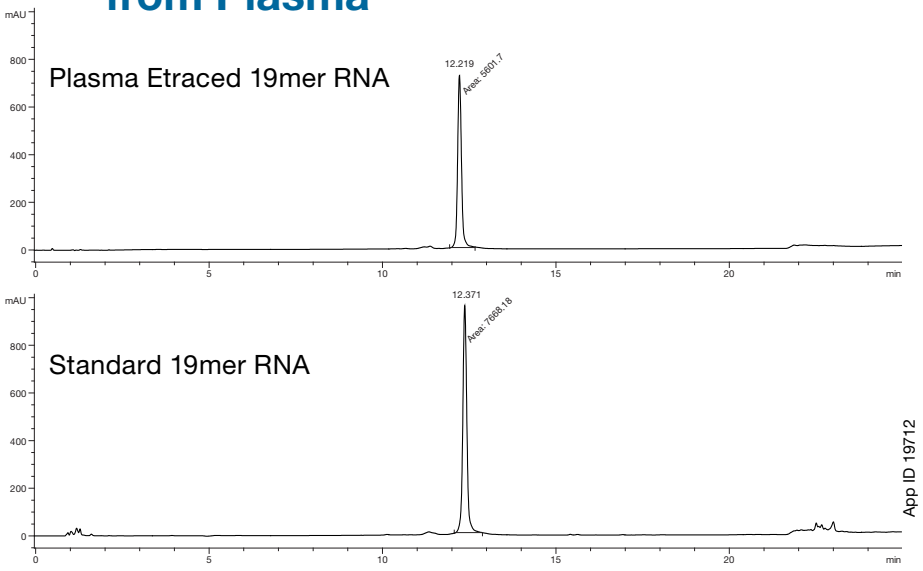


Figure 2: Recovery and cleanup of a 27mer DNA phosphorothioate oligonucleotide from plasma using the Clarity OTX protocol. Oligonucleotide was spiked into a plasma sample, extracted, and compared to a control. Recovery is estimated at 97 % with only minor plasma contaminants.

Figure 3. Clarity OTX: Recovery of a 19mer RNA from Plasma



Column: Clarity 3 µm Oligo-RP
Dimensions: 50 x 2.0 mm
Part No. 00B-4441-B0
Mobile Phase: A: 8 mm Tetraethylammonium / 200 mm HFIP
B: Acetonitrile

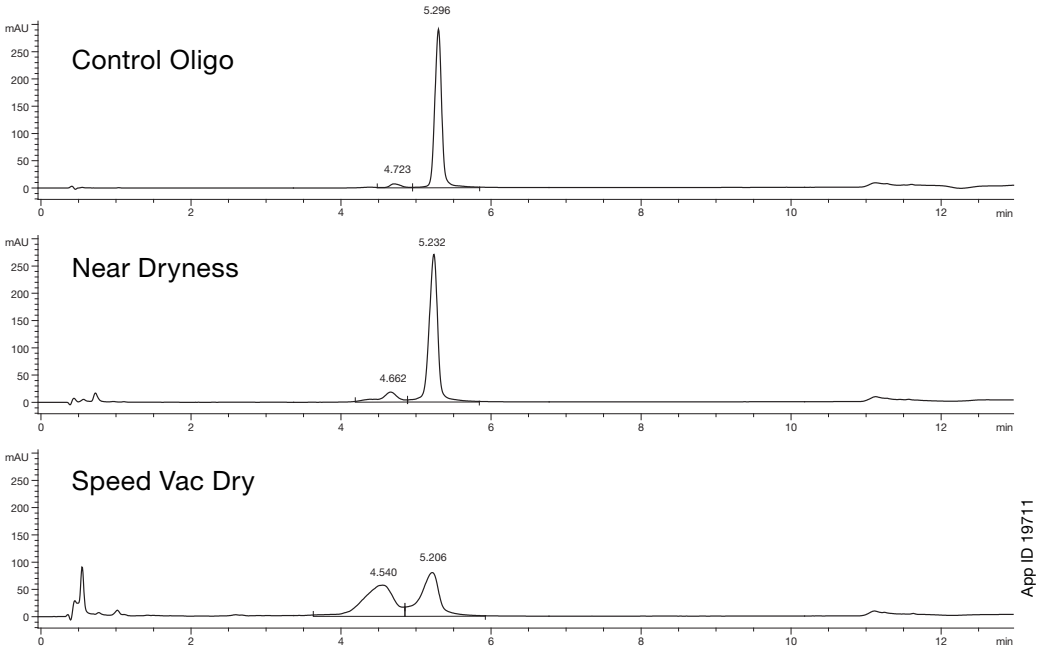
Gradient:

Time (min)	% B
0	5
0	5
10	0

Flow Rate: 0.3 mL/min
Temperature. Ambient
Detection: UV @ 260 nm
Backpressure: 200 bar
Sample: 19 mer RNA olibonucleotide

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. Clarity OTX: Speed-Vac Oligo Recovery 19mer



Column: Clarity 3 µm Oligo-RP
Dimensions: 50 x 2.0 mm ID
Part No. 00B-4441-B0
Mobile Phase: A: 8 mm Tetraethylammonium / 200 mm HFIP
B: Acetonitrile
Gradient:

Time (min)	% B
0	5
10	35

Flow Rate: 0.3 mL/min
Temperature. Ambient
Detection: UV @ 260 nm
Backpressure: 200 bar
Sample: 19 mer RNA oligonucleotide

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.

Figure 5. Balancing LC Retention Versus Ion-pairing Ion Suppression

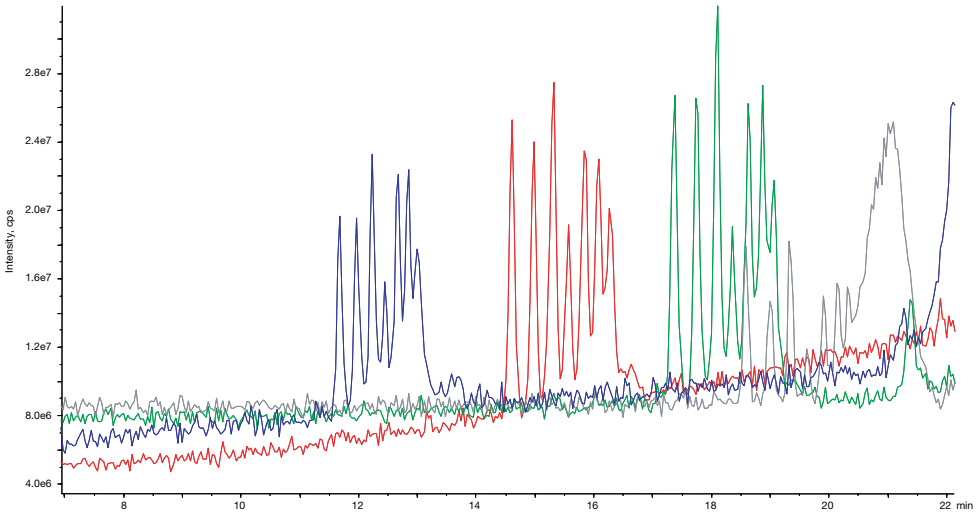
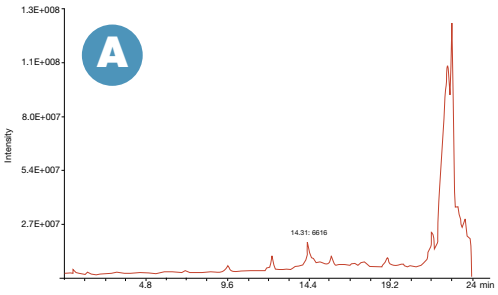


Figure 5: Ion-pairing concentration effects on LC/MS sensitivity and resolution. A 12-18 poly dT oligonucleotide standard is run on a Clarity Oligo-RP column using different ion-pairing concentrations: Black trace = 15 mM TEA/ 400 mM HFIP, Green trace= 2.8 mM TEA/ 280 mM HFIP, Red trace = 4 mM TEA/ 100 mm 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. Sensitivity Studies with RNA

TIC of a 500 ng extraction



XIC of a 50 ng extraction

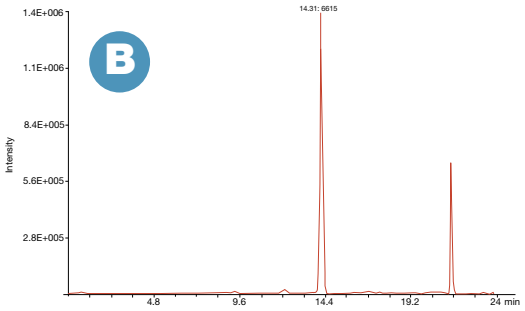


Figure 6: Sensitivity and MS Analysis Parameters. The total ion chromatogram of 500 ng/mL of a 19mer oligonucleotide extracted from plasma is shown in chromatogram 6A. Note the low level peak corresponding to the oligonucleotide at RT of 14.3 minutes. The extracted ion chromatogram (-7 charge state ion at m/z of 944) of a 50 ng/mL sample is shown in chromatogram 6B. Selected analysis of specific ions can realize large increases in sensitivity compared to standard MS methods.

Figure 7. Spectra of Extracted P-S Oligonucleotide

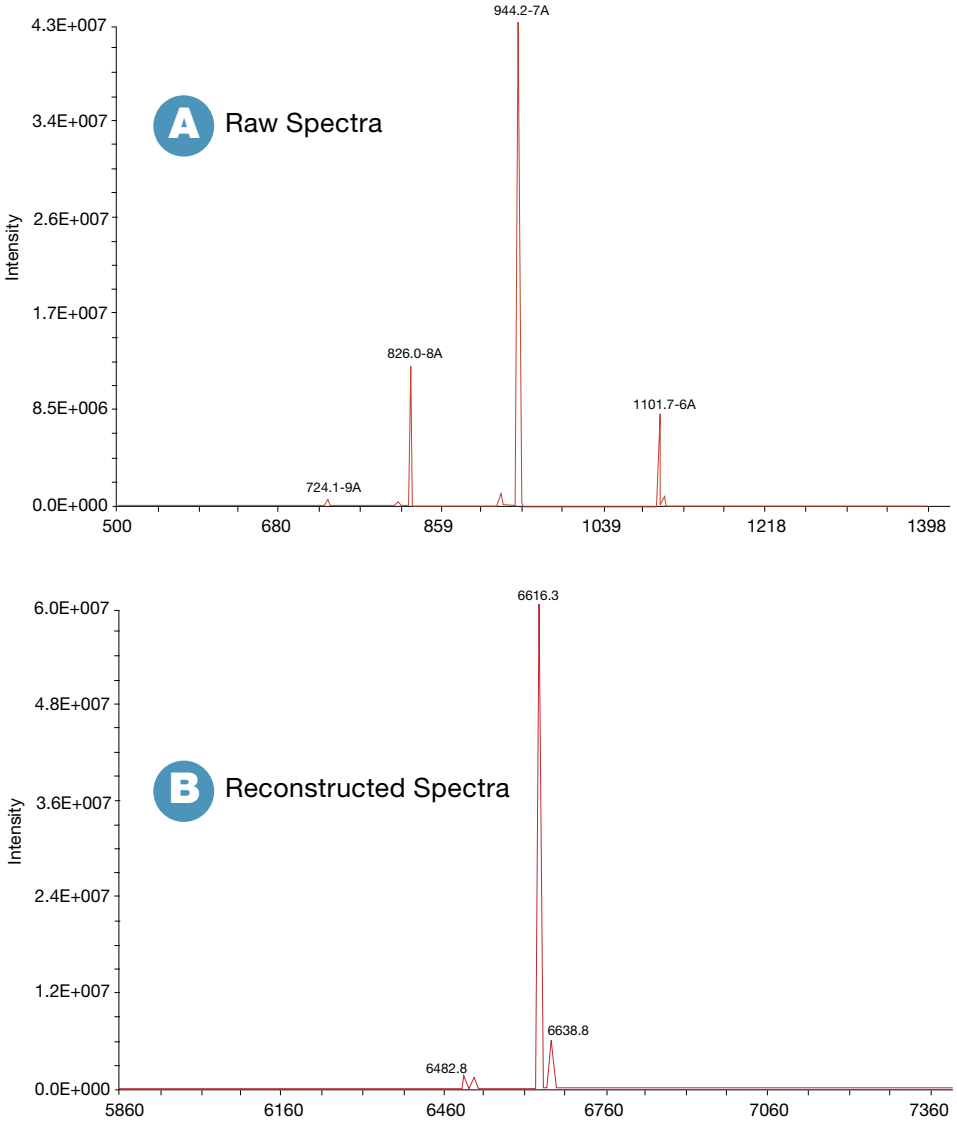


Figure 7: Using deconvolution software to identify metabolites. The raw spectra from 19mer oligonucleotide in A is compared to the reconstructed spectra in B. Note reconstructed masses for the oligonucleotide of interest at MW= 6616 as well as a minor component corresponding to a depurinated oligonucleotide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

Results and Discussion

Oligonucleotide Isolation

A simple mixed-mode SPE-based protocol has been developed (Clarity OTX) to isolate therapeutic oligonucleotides from biological matrices (**Figure 1)**[2,3]. Oligonucleotide-containing serum or plasma samples treated with the chaotrope/detergent buffer are loaded onto a mixed-mode SPE sorbent around pH 5.5. After low pH washes, elution of the oligonucleotide is achieved with moderate organic at an elevated pH (pH~8). 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 chromatogram of the effectiveness of 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].

Additional studies were undertaken to better understand some of the factors in sample recovery. A speed-vac evaporator is typically used 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. Sample evaporated to "full dryness" showed significant loss in recovery versus the "near-dryness" sample which demonstrated good recovery[5]. Based on such results, speed-vac evaporation should be avoided for low-level oligonucleotide isolation techniques. Lyophilization appears to be a robust alternative for concentrating samples without similar deleterious effects.

LC/MS Analysis of Oligonucleotides

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[6,7,8]. In LC/MS applications, ion-pairing retention must be balanced against ion suppression to maximize MS 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.

While optimizing mobile phase conditions is important, the mass spectrometer and data collection parameters can have a much larger influence. An example of this is shown in **figure 6** where 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. Using extracted ions shows that at the 50 ng/mL level is far from the detection limit for this application.

Oligonucleotide analysis requires deconvolution software for the identification of oligonucleotides. This can be especially important when looking for low level metabolites of oligonucleotide therapeutics 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 cannot discern 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 masses that correspond to a salt adduct as well as a depurinated oligonucleotide. Such results demonstrate the utility of deconvolution software for oligonucleotide analysis.

Conclusions

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. Focusing on specific groups and ranges of ions as well as using deconvolution software can significantly increase the sensitivity of any developed method.

References

1. G. Zhang, J. Lin, K Srinivasan, O. Kavetskaia, and J. Duncan. Journal of Analytical Chemistry 79(9) p3416-3424, 2007

2. Tanya A. Watanabe, Richard S. Geary, Arthur A. Levin. Oligonucleotides 16(2): p169-180, 2006

3. Antisense drug technology: principles, strategies, and applications. Edited by Stanley T. Crooke, CRC Press, p189-199, 2007

4. G. Scott, H. Gauss, B. Rivera, and M. McGinley. "Rapid Extraction of Oligonucleotides from Primary Tissues for LC/MS Analysis…" Poster Presentation from Tides 2009 Conference, Las Vegas, NV, May 17-20, 2009.

5. K. Speicher, O. Kolbas, S. Harper, and D. Speicher. Journal of Biomolecular Techniques 11:p74-86, 2000

6. A. Apffel, J. Chakel, S. Fisher, K. Lichtenwalter, W. Hancock. Analytical Chemistry 69 (7) p1320-1325, 1997

7. R. Griffey, M. Greig, and H. Gaus. Journal of Mass Spectrometry 32: p305-313, 1997

8. M.Gilar. Journal of Analytical Biochemistry 298 (2) p196-206, 2001

Trademarks

Clarity is a registered trademark of Phenomenex, Inc. OTX, Oligo-RP, and Oligo-MS are trademarks of Phenomenex, Inc. LTQ and Orbitrap are registered trademarks of Thermo Finnigan, LLC. ProMass is a registered trademark of Novatia, LLC. Agilent is a registered trademark of Agilent Technologies. AB Sciex and API 3000 are trademarks of AB Sciex Pte. Ltd. AB SCIEX™ is being used under license.

© 2011 Phenomenex, Inc. All rights reserved.