Optimizing Sensitivity and Selectivity for the Analysis and Characterization of Synthetic Oligonucleotides via LC/MS

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Introduction

Interest in quality control analytical methods for oligonucleotides has grown rapidly in the last few years as several oligonucleotide therapeutic candidates are passing through the research phase and into clinical trials[1]. Traditionally, IEX-HPLC, CE, PAGE, and MALDI-MS have been used to characterize oligonucleotides. However, each method has unique difficulties in quantitating and characterizing minor impurities. Lately, manufacturers have looked to adopt reversed phase LC/MS (RP-LC/MS) for characterizing impurities in oligonucleotide products[2]. RP-LC/MS has unique advantages in that impurities can be chromatographically separated from a parent and then identified and quantitated based on MS or tandem MS/MS analysis.

However, the LC/MS method is not without its limitations. Modifications to the ion-pairing mobile phase to allow for MS compatibility often results in reduced resolution of oligonucleotides versus traditional ion-pairing RP-HPLC separation methods. Sensitivity can also be an issue in that ion-pairing reagents are known suppressors of electrospray mass spectrometry, however such reagents are needed when working with oligonucleotides to achieve retention on reversed phase columns[3,4]. Research was undertaken to improve LC/MS sensitivity by looking at different levels of ion-pairing buffers to achieve the best MS signal as well as maintain adequate selectivity between oligonucleotides that differ by only one residue in length. Different MS systems were used, as well, to determine if mobile phase conditions were MS dependant or specific to a particular interface. As TEA/ HFIP buffer mixtures are commonly used for oligo MS work, the ratio between the buffers was varied to determine the mixture that delivered the optimal MS signal while maintaining resolution.

Materials and Methods

Several different crude trityl-on ssDNA and ssRNA oligonucleotides were obtained from industrial and academic sources (IDT, Coralville, IA; USC DNA Core Lab, Los Angeles, CA). Complex oligonucleotide mixtures were obtained from crude oligonucleotides by collecting the flow-thru (n-1 contaminant fraction) from an oligonucleotide purification using a Clarity® QSP™ trityl-on purification cartridge. The crude mixture was directly injected on HPLC after concentration. Poly dT DNA oligonucleotide standards were purchased from GE Healthcare (Buckingham, UK); standard was diluted 10:1 prior to injection on HPLC. Chemicals and solvents were purchased from EMD Science (Madison, WI).

Oligonucleotides samples were run on an Agilent 1100 HPLC with quaternary pump module, autosampler, and a diode array detector. Data was collected using ChemStation version 9.2 software for UV data (monitored at 260 nm). Column oven was set at 50 °C and various gradients were used depending on the sample and mobile phase used for analysis. LC/MS analysis of oligonucleotides was performed using either the Applied Biosystems® API 3000™ or the Bruker Esquire™.

The API 3000 was run in ESI-MS mode with a Q1 scan from 500-2000 m/z over 2.2 seconds. Optimized conditions include a curtain and nebulizer gas of 10, an electrospray voltage of -4300 V, heater temperature of 425 °C, DP at -50, FP at -350 and a heater gas flow of 5000 cc/min. The Bruker Esquire was run in ESI-MS mode with MS trap scan from 400-2200 m/z with an accumulation time of 200 ms (2 x 10^4 ICC). The capillary was set at 33-67 nA, nebulizer gas was at 40 psi, and N_2 dry gas flow was at 8.3 L/min.

For UV studies a Clarity® 3 µm Oligo-RP™ 150 x 4.6 mm column was used as well as a competing 3 µm and 2.5 µm column of the same dimension for comparison. For MS studies a 150 x 2.0 mm Clarity 3 µm Oligo-RP was used. Mobile phase was varied run-to-run (see specific figures for composition), but were a combination of triethylamine (TEA) and hexafluoroisopropanol (HFIP) mixed in with 98 % water and 2 % methanol for the aqueous mobile phase. For the organic mobile phase TEA and HFIP were mixed in with 98 % methanol and 2 % water. The gradient used was ramped from 5 % to 10 % in 5 minutes followed by a ramp from 10 % to 30 % organic for 15 minutes. For the last set of chromatograms the second ramp was shallower using a 10 % to 30 % organic gradient in 60 minutes.

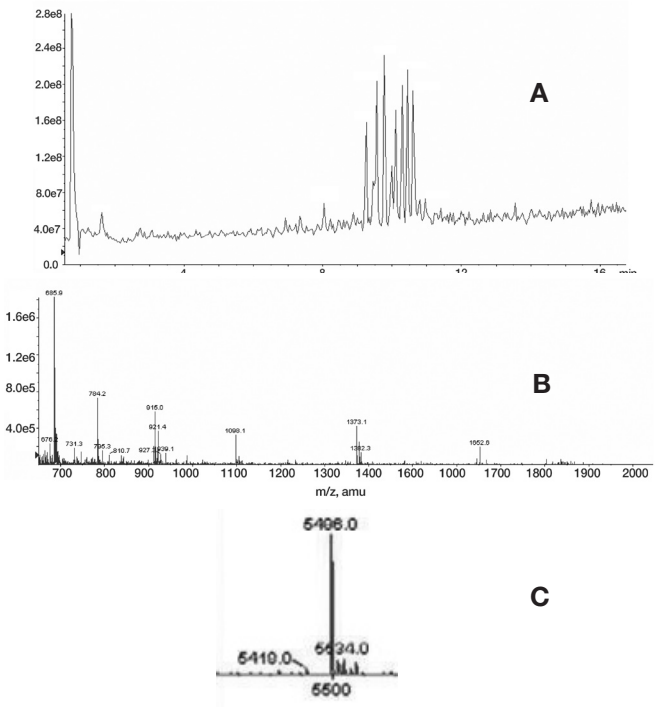
Results and Discussion

LC/MS of Oligonucleotides

For oligonucleotide purification, researchers generally use TEAA buffer in high concentrations (100 mM) for ion-pairing reversed phase purification of oligonucleotides. To adapt MS detection to reversed phase separations of oligonucleotides, others have shown applications using a combination of TEA and HFIP (generally 15 mM TEA/ 400 HFIP) for LC/MS separations[4]. This mobile phase combination of ion-pairing reagents TEA and HFIP is more compatible for MS sensitivity, however there is a concern that such conditions compromise both the separation of oligonucleotides as well as reduce the sensitivity of MS detection due to ion suppression. Efforts were undertaken to achieve greater LC/MS sensitivity in reversed phase QC methods, while maintaining enough ion-pairing reagent to maintain acceptable oligonucleotide selectivity. LC/MS separations were performed using reduced amounts of ion-pairing reagent similar to what has been shown in other studies.

An example of a typical LC/MS run using reduced ion-pairing reagent is shown in **figure 1**. The top chromatogram (**figure 1A**) shows a separation of a poly dT standard, the lower figure shows the MS spectra of the last eluting peak in the mixture (**figure 1B**). Note that in the case of most therapeutic oligonucleotides (18-27 mer in length) that -5 to -15 are the predominant ions detected in an ESI mass spectrometer. As such, reconstruction software (BioAnalyst is used with the API 3000) is required to generate a parent mass (**figure 1C**). Such information is necessary to detect minor modifications generated during synthesis. Data indicates that using a lower concentration of buffer salts (4 mM TEA/ 100 mM HFIP) with the Clarity Oligo-RP column is a good balance between separation and sensitivity for LC/MS applications.

Figure 1.
LC/MS of A Poly dT Standard



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Example LC/MS run of a poly dT oligonucleotide standard run on the Clarity® Oligo-RP™ column. A) is the TIC for the LC/MS run, B) is the MS spectra of the dT18 peak in the chromatogram, and C) shows with reconstructed mass based on the multiple charge states. ABI Bioanalyst software was used to generate the reconstructed parent mass.

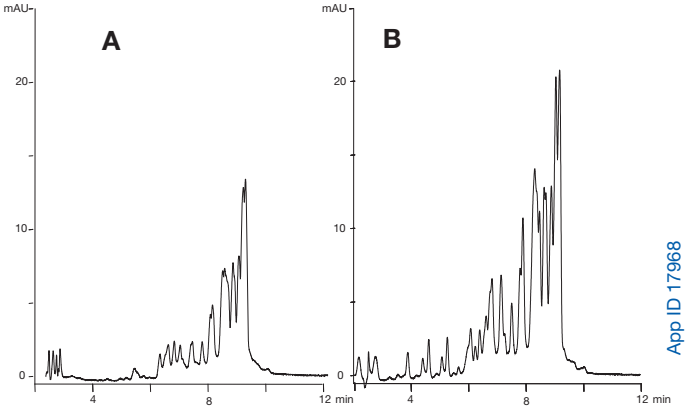
Selectivity Comparisons

While reduced ion-pairing reagent provides improved MS sensitivity, selectivity of oligonucleotides in MS compatible buffer can be a concern. Clarity Oligo-RP was developed specifically for separating oligonucleotides using standard purification conditions. It is assumed that this greater retention and selectivity for oligonucleotides would translate to MS compatible conditions, especially if lower ion-pairing concentrations were used. Examples of this high selectivity are shown in **figure 2** where the Clarity Oligo-RP is compared against a market-leading oligonucleotide-specific RP column. Unlike **figure 1**, where a standard mixture was used, this application used a highly complex mixture of failed oligonucleotide sequences to demonstrate sequence specific separations. This selectivity is believed to be related to enhanced polar selectivity of the TWIN™ particle media used in the manufacturing of the column. This improved selectivity is still seen when looking at very low buffer concentration (4 mM TEA/ 100 mM HFIP) when some additional applications were run using LC/MS (**Figure 3**) compared against a different column chemistry. Note the additional peaks resolved compared to the different chemistry.

LC-UV Data vs. LC/MS Data

When comparing LC-UV data (**figure 2**) to the LC/MS data **(figures 1 and 3**) it is easy to see the reduced separation with the MS data. Much of these differences are related to MS sampling rate versus UV. Furthermore, additional post column void volume introduced in the connection of the LC to the MS, can also contribute to this effect. This is not related to the LC column chemistry, an example of a UV chromatogram and MS TIC trace from the same run is shown in **figure 4**. As evident, the resolution of the UV trace is much higher than the MS trace. This emphasizes the importance of optimizing the MS conditions to maintain a high sampling rate as well as minimizing post column volume.

Figure 2.
LC/UV Comparison Between Oligonucleotide-specific Columns



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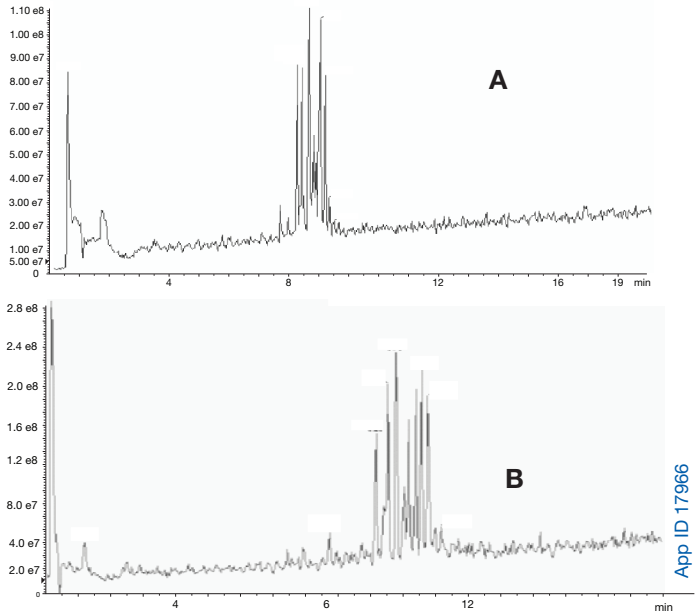
A flow-thru mixture of oligonucleotide degradation products from DNA sequence: GTGGATCTGCGCACTTCAGGCTCCTGGGCG were run on HPLC columns to evaluate selectivity at reduced ion-pairing concentrations (2.8 mM TEA/ 280 mM HFIP): A) Competitor X (3.5 µm C18), B) Clarity 3 µm Oligo-RP column. The Clarity Oligo-RP maintains good selectivity of complex mixtures of oligonucleotides in reduced ion-paring reagent.

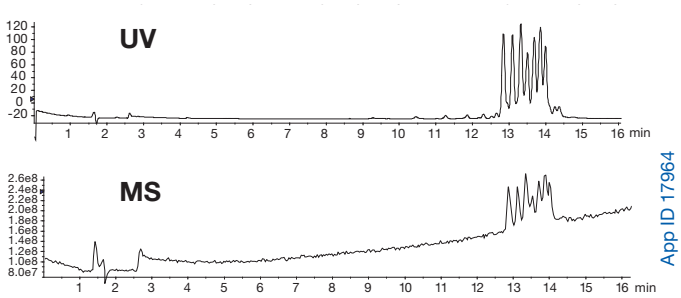
Figure 3.
LC/MS Comparison Between Oligonucleotide-specific Columns



App ID 17966

LC/MS separations of a 12-18 dT Oligonucleotide standard mixture. Ion-pairing reagent has been further reduced (4 mM TEA/ 100 mM HFIP). Columns used: A) Competitor Y (2.5 µm C18), B) Clarity 3 µm Oligo-RP. As is observed with the LC-UV separations, the Clarity Oligo-RP delivers good separation in LC/MS compatible conditions.

Figure 4.
Contrasting Resolution Between UV and MS Detection



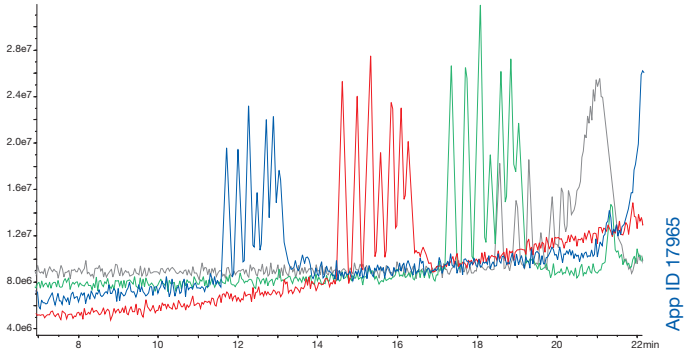
LC/MS TIC and UV-Vis Chromatogram from the same run shown together of a poly dT standard run on a Clarity® Oligo-RP™ column. Note the reduced resolution from the MS chromatogram. MS sampling rate and post column void volume can dramatically affect the efficiency and resolution of closely eluting oligonucleotide peaks.

Mobile Phase Optimization

While previous results show good selectivity and sensitivity using a reduced amount of ion-pairing reagent, further efforts were undertaken to determine the optimal buffer levels. The poly-dT standard was run on the Clarity Oligo-RP column at different mobile phase concentrations to determine which conditions gave the best selectivity and resolution. HFIP/TEA concentrations were varied between 50 mM HFIP/ 2 mM TEA to 400 mM HFIP/ 16 mM TEA. An overlay of such separations is shown in **figure 5**. The results are interesting in that mobile phase conditions using low amounts of ion-pairing buffer (50 mM HFIP/ 2 mM TEA) gave reduced MS signal and oligo retention (and selectivity). This is contrary to the hypothesis that reduced ion-pairing reagent delivers improved MS sensitivity, but is easily explained due the reduced retention of the oligonucleotide resulting in an elution in a lower percentage of organic mobile phase. It has been widely observed that increasing organic content in a electrospray interface results in improved MS sensitivity; such an observed decrease in MS signal using low amounts of ion-pairing reagent is easily explained. Further investigation of **figure 5** also reveals a reduction in MS signal intensity for higher levels of ion-pairing reagent despite an increase in retention, such observations match with expected results and suggests that ion suppression is occurring at high levels of ion-pairing reagent. These initial results suggest that a mobile phase between 4-8 mM TEA and 100-300 mM HFIP is required for optimal separation and signal for this application.

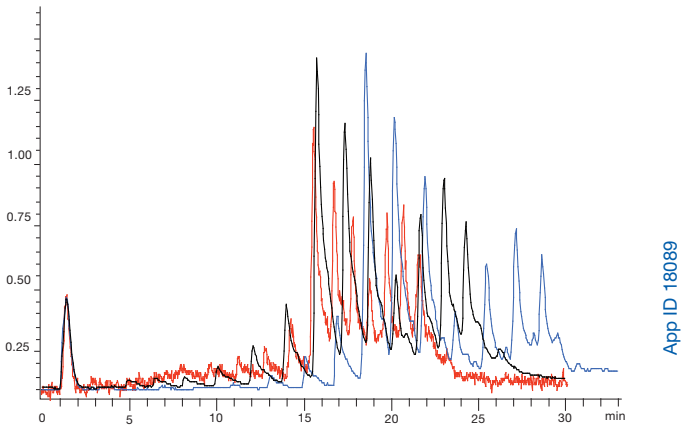
Additional studies were performed to determine if different ratios between TEA and HFIP provide differences in resolution and MS sensitivity. From the previous data, one aspect of obtaining maximum resolution and MS sensitivity revolved around maintaining good retention of the ion-paired oligonucleotide. Different ratios were investigated based on using a fixed amount of HFIP (200 mM). TEA concentrations from 1 mM to 14 mM were tested. Results are shown in **figure 6**. pH of the resultant mobile phase was tested and shown in the figure. Findings suggest that the ratio between HFIP and TEA is critical in obtaining the maximum retention, MS sensitivity and selectivity. Similar to what was observed in **figure 5**, results suggest that the optimal mobile phase for maximum MS sensitivity and oligonucleotide separation is a balance between obtaining maximum retention (via ion-pairing reagent) and minimizing MS suppression. Using the Clarity Oligo-RP column it appears that mobile phase conditions using 200 mM HFIP and 8 mM TEA (pH 8.0) provided the optimal results for LC/MS analysis of oligonucleotides.

Figure 5.
Ion Pairing Concentration Effect on LC/MS Resolution and Sensitivity



LC/MS separations of a 12-18 dT oligonucleotide standard using different ion-pairing concentrations. Concentrations used were: 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. Note the increasing MS signal with reduced ion-pairing buffer concentration.

Figure 6.
Buffer Ratio Effects on Oligonocleotide Separations



Different ratios of HFIP to TEA were investigated using the poly dT 12-18 standard mixture. HFIP was fixed at 200 mM and different levels of TEA were used between 1 and 20 mM (4 mM-Red trace {pH 7.6}, 8 mm-Blue trace {pH 8.0} and 14 mM–Black trace {pH 8.3}). Optimal retention, MS response, and resolution were observed for the 200 mM HFIP/ 8 mM TEA mobile phase mixture.

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Conclusions

Results in this study suggests that Clarity® Oligo-RP™ is an excellent media for LC/MS applications and its optimal operation conditions use lower mobile phase ion-pairing buffer than other specific products on the market. Further, comparisons with other oligonucleotide-specific products show better separation under LC/MS friendly conditions. Reducing extra column volume, by removing the UV detector from the flow path, is important for obtaining MS conditions better than the high resolution that UV detection demonstrates. Deconvolution software is also important for determining identity of the different modified oligonucleotides that elute in a QC sample or mixture.

Lowering the amount of ion-pairing reagent can improve MS signal for oligonucleotides but only to a point; loss of retention under conditions with low ion-pairing reagent can actually reduce the MS signal (due to earlier elution in lower organic mobile phase concentrations). Reduced ion-pairing reagent also reduces resolution of similar oligonucleotides. Using the Clarity Oligo-RP column, the optimal mobile phase conditions for good resolution and MS signal was found to be 200 mM HFIP/ 8 mM TEA. While not tested here, different types of oligonucleotides (RNA or phosphorothioate DNA) will likely have different optimums based on their different hydrophobicities.

References

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3. Hall, M.; Elliott, B.; Anderson, K.- American Biotechnology Laboratory-Application Supplement January 2004

4. Gilar, M; Fountain, K.; Bulman, Y; Holyoke, J.; Dovoudi, H.; Gebler, J.- Oligo-nucleotides 13: 229-243 (2003)

Ordering Information

Part No.	Description	Unit
00F-4441-B0	Clarity Oligo-RP 3 µm 150 x 2.0 mm	ea
00F-4441-E0	Clarity Oligo-RP 3 µm 150 x 4.6 mm	ea

TN65661108_L