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Analysis of native and chemically modified oligonucleotides by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry

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Ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) was utilized in tandem with negative-ion electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) for the analysis of native and chemically modified oligonucleotides. Separation was performed on a 1.0 × 50 mm column packed with porous C_{18} sorbent with a particle size of 2.5 μm and an average pore diameter of 140 Å. A method was developed which maximizes both chromatographic separation and mass spectrometric sensitivity using an optimized buffer system containing triethylamine and 1,1,1,3,3,3-hexafluoro-2-propanol with a methanol gradient. The ESI-TOFMS tuning parameters were also optimized in order to minimize in-source fragmentation and achieve the best sensitivity. Analyses of native, phosphorothioate, and guanine-rich oligonucleotides were performed by LC/MS. Detection limits were at sub-picomole levels with an average mass accuracy of 125 ppm. The described method allowed for the LC/MS analysis of oligonucleotides up to 110mer in length with little alkali cation adduction. Since sensitive detection of oligonucleotides was achieved with ultraviolet (UV) detection, we utilized a combination of UV-MS for quantitation (UV) and characterization (MS) of oligonucleotides and their failure sequence fragments/metabolites. Copyright © 2003 John Wiley & Sons, Ltd.

Synthetic oligonucleotides are utilized for diagnostic as well as therapeutic purposes.[1] The synthesis of native and chemically modified oligonucleotides typically yields a product of limited purity.[2,3] Consequently, oligonucleotides used for diagnostic and therapeutic purposes must be purified prior to use. Quality control methods are needed to verify the product purity and identify the nature of the contaminants. This is most important in clinical trials and metabolism studies in which drug metabolites must be characterized.

Both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS)[4,5] and electrospray ionization mass spectrometry (ESI-MS)[6] have proved to be useful tools in the sequencing, identification, and characterization of oligonucleotides, as well as for genotyping applications.[7,8] A modest number of publications describing the MS analysis of DNA has been presented when compared with the number of publications dealing with protein/peptide MS analysis. This can be explained primarily by two factors: (i) limited ionization efficiency (detection sensitivity) of DNA molecules, and (ii) alkali cation adduction, which complicates the interpretation of the spectra.[9,10] Therefore, sample desalting is an integral part of the MS analysis. Both MALDI-TOFMS and ESI-MS are typically able to detect picomole amounts of oligonucleotides,[10,11] although lower detection limits have been reported.[12]

Reversed-phase HPLC (RP-HPLC) has been coupled online to ESI-MS and used for preclinical and clinical trials,[13] pharmacokinetic and toxicological studies, and oligonucleotide quality control. An advantage of liquid chromatography/mass spectrometry (LC/MS) is that it combines desalting, separation, and characterization of oligonucleotides. The LC/MS analysis of oligonucleotides is important for (i) investigation of therapeutic oligonucleotides and their metabolism, (ii) quality control of large synthetic oligonucleotides (>60 nucleotides) which are difficult to analyze by MALDI-TOFMS, and (iii) single nucleotide polymorphism (SNP) genotyping and genotype-discovery applications.

Chemically modified oligonucleotides are employed as antisense drugs and require strict quality control. The analysis of modified oligonucleotides was found to be more challenging than the analysis of native ones, in part due to the complexity of the sample (one or more of backbone, nucleobase, and carbohydrate moieties can be modified). LC/MS was applied for characterization[14] and analysis of *in vivo* metabolism[13] of antisense oligonucleotides; however, separation was not optimal. Gaus *et al*.[13] and Griffey *et al*.[15] were able to successfully determine the pattern of nuclease degradation of phosphorothioate (PS) oligonucleotides. In this case, ion-pair (IP) RP-HPLC was utilized only as a desalting technique prior to analysis by MS.

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Long oligonucleotides (>60mer) are important in the areas of diagnostics, genotyping, and oligonucleotide array design. For instance, use of longer oligonucleotides in gene-targeting experiments will, to some extent, eliminate cross-hybridization and the need to amplify large fragments of DNA. Quality control (and purification) of these oligonucleotides is highly desirable. The analysis of long oligonucleotides by LC/MS has been found to be substantially more challenging than the analysis of short ones. This is because the probability of cation adduction to the oligonucleotide backbone increases with increasing oligonucleotide length, due to the presence of a larger number of negatively charged phosphate groups.[11,12,16–20]

Several laboratories have utilized MS-friendly mobile phases consisting of ammonium acetate or ammonium formate with an acetonitrile gradient for the LC/MS analysis of DNA. However, chromatographic resolution was limited to short (<30mer) oligonucleotides.[17–21] The use of ion-pairing buffers, usually triethylammonium acetate (TEAA), enhances the separation, but is not compatible with ESI-MS detection. An alternative MS-friendly ion-pairing buffer, comprised of triethylammonium bicarbonate with an acetonitrile gradient, was introduced by Huber and Krajete[11] for the analysis of up to 40mer oligonucleotides. This buffer system was later used for analysis of longer DNA fragments with post-column addition of acetonitrile, which improved the MS signal. Apffel *et al*.[22] introduced an ion-pairing buffer system comprised of triethylamine (TEA) as the ion-pairing agent and hexafluoroisopropanol (HFIP) as the buffering acid. Although these ion-pairing systems improved LC/MS performance, there was a compromise between separation quality and MS sensitivity.

In this paper we describe a method for the LC/MS analysis of oligonucleotides using an ion-pairing mobile phase comprised of TEA/HFIP. The concentration of HFIP as well as the concentration of the ion-pairing reagent (TEA) were adjusted so that neither chromatographic resolution nor MS sensitivity was compromised. The method was utilized for the separation and characterization of native and chemically modified oligonucleotides (phosphorothioates).

The MS ionization conditions were tuned to minimize in-source fragmentation (depurination)[23] and eliminate mobile phase background. Although the column effluent in this method is comprised of only ~18–23% methanol, no sheath liquid was required to enhance ESI efficiency and MS sensitivity. The method was successfully employed for the LC/MS analysis of oligonucleotides up to 110mer without extensive alkali cation adduction.

EXPERIMENTAL

Chemicals and oligonucleotide samples

Triethylamine (TEA, 99.5%), glacial acetic acid (99.99%), and HPLC-grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water (18 MΩcm) was purified in-house using a Milli-Q system (Millipore, Bedford, MA, USA). Bovine intestinal mucosa phosphodiesterase I (3′-exonuclease) and 1,1,1,3,3,3-hexafluoro-2-propanol were purchased from Sigma (St. Louis, MO, USA). Oligonucleotides were obtained from the following sources: 10–30mer homooligodeoxythymidines, 80mer, 90mer, 100mer and 110mer heterooligonucleotides were from Midland Certified Reagents (Midland, TX, USA); 25mer phosphorothioate (PS) was provided by Hybridon, Inc. (Hopkinton, MA, USA); 25mer homooligodeoxyguanidine was supplied by Ransom Hill Bioscience (Ramona, CA, USA); 70mer heterooligonucleotide sample was purchased from Biosynthesis Incorporated (Lewisville, TX, USA). The sequences are as follows (5′-3′): 10mer TTT TTT TTT T; 15mer TTT TTT TTT TTT TTT; 30mer TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT; 25mer (PS) CTC TCG CAC CCA TCT CTC TCC TTC T; 25mer GGG GGG GGG GGG GGG GGG GGG GGG G; 70mer GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC T; 80mer GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GA; 90mer GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA; 100mer GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC T; 110mer GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GAC TTA GA.

LC/MS analysis

A capillary HPLC system (CapLC™; Waters Corporation, Milford, MA, USA) equipped with a photodiode array detector was connected to an ESI-TOF mass spectrometer (Micromass LCT™, Waters Corporation) using a 30-cm fused-silica capillary, 50 μm i.d. A 1.0 × 50 mm Xterra® MS C_{18} column packed with 2.5 μm particles (average pore diameter 140 Å) was used for all LC/MS experiments. For gradient, mobile phase, and other conditions, see figure captions. The built-in column heater of the HPLC system was set to 50 or 60°C; mobile phase flow rate was 23.6 μL/min. The LC/MS system was operated by MassLynx™ software, version 3.5 (Waters Corporation). LC/MS chromatograms were acquired in negative ion mode using an ESI-MS capillary voltage of 2.0–2.5 kV, a sample cone voltage of 18–25 V, an extraction cone voltage of 1 V, and an MCP detector voltage of 2700 V. Desolvation gas flow rate was maintained at 410 L/h. Cone gas flow rate was set to 30 L/h. Desolvation temperature and source temperature were set to 120 and 100°C, respectively. The acquisition range was m/z 510–1000; the 1.1 s cycle consisted of a 1.0 s acquisition time and a 0.1 s delay. Typically, 5–10 individual acquisitions were summed in order to generate a spectrum. Instrument calibration was performed routinely in negative ion mode prior to LC/MS experiments by direct infusion of poly-DL-alanine (Sigma) in 50% methanol/50% water. The mass range for calibration was as specified above.

HPLC mobile phase preparation

The triethylammonium acetate (TEAA) buffer (0.1 M, pH 7) was prepared by mixing appropriate volumes of TEA and acetic acid in water. Because of the limited solubility of TEA in water, we used the following protocol (1 L of 0.1M

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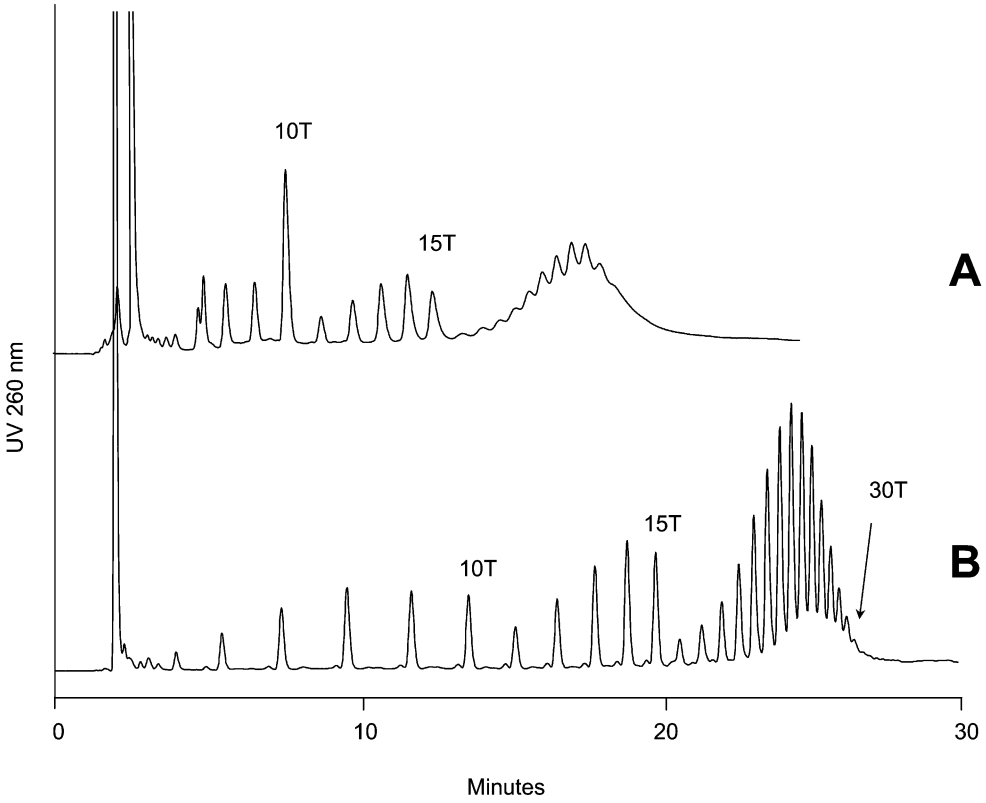

Figure 1. Separation of a 5–30mer oligodeoxythymidine ladder in 0.56 mM TEA/100 mM HFIP, pH 7 (A) and 7.0 mM TEA/100 mM HFIP, pH 8.25 (B). Xterra® MS C_{18}, 4.6 × 50 mm, 2.5 μm column. Mobile phase A: one of two above; B: methanol. Gradient from 10% to 22% B in 30 min, 0.5 mL/min, 50°C, UV 260 nm.

buffer): 5.6 mL of glacial acetic acid were placed in ~950 mL of water. While mixing, 13.86 mL of TEA were slowly added; the pH of the resulting solution was typically between 5 and 9. The pH was carefully adjusted to 7 by the addition of either TEA or acetic acid. Since the desired pH of 7 is more than 1 pH unit apart from the pK_a values of TEA (10.72) and acetic acid (4.71), the amount of acid or base needed to adjust the pH is very small. The volume was adjusted to 1 L with water, bringing the final concentration of TEA and acetic acid to ~0.1 M.

Similarly, the TEA/HFIP buffers were prepared by titrating the acid solution (HFIP) with TEA. To prepare 400 mL of 16.3 mM TEA/400 mM HFIP buffer, pH 7.9, we dissolved 16.85 mL of HFIP in ~350 mL of water and slowly added 0.91 mL of TEA. The solubility of TEA in HFIP solution is poor, and the last addition of TEA usually dissolves after adjusting the solution to a final volume of 400 mL. The final pH is approximately 7.9. In our experience it is possible to prepare a higher concentration of TEA in 400 mM HFIP only if methanol is added to the buffer to improve TEA solubility. Alternatively, a buffer consisting of 8.6 mM TEA and 100 mM HFIP, pH 8.3, was used in some experiments (4.2 mL of HFIP and 0.48 mL of TEA dissolved in water; final volume of solution was 400 mL).

RESULTS AND DISCUSSION

Optimization of ion-pairing buffer for efficient HPLC separation

In our evaluation, the TEA/HFIP buffer system proposed by Apffel *et al.*[22] performs well for the separation of short oligonucleotides (<30mer). However, separation rapidly deteriorates for oligonucleotides greater than 30mer in length. This is likely due to a suboptimal concentration of pairing triethylammonium ion in the mobile phase (~2 mM). The impact of pairing ion concentration on separation is demonstrated in Fig. 1 using a 5–30mer homooligonucleotide ladder. It is the TEA concentration rather than the concentration of HFIP[24] that plays a principal role in the separation. We investigated the effect of TEA concentration in the range 0.56–31.4 mM (pH 7–9) while keeping the concentration of HFIP constant (100 mM). In addition, we also varied the concentrations of HFIP in the range 12.5–400 mM. The results are summarized in Table 1. The separation performance was evaluated using 10-, 15- and 30mer standards. We calculated the column peak capacity (P) value for 10/15mer and 10/30mer pairs according to Eqn (1):

$$P = 1 + \frac{t_2 - t_1}{w_{4\sigma}} \quad (1)$$

where t_1 and t_2 are retention times (minutes) of the earlier- and later-eluting peaks, respectively, and $w_{4\sigma}$ is the average peak width (min) at 13.4% of peak height. Therefore, the peak capacity value P represents the number of peaks that can be theoretically separated in a given gradient time window. For example, a P value greater than 5 for the 10/15mer pair suggests the capability of the chromatographic system to theoretically resolve all 10–15mer peaks in the ladder. Similarly, a peak capacity value of 15 or greater for the 15/30mer pair predicts baseline separation of all 15–30mer peaks.

Table 1. Ion-pairing mobile phase optimization for the separation of oligonucleotides

Buffering acid	Acid conc. (mM)	TEA conc. (mM)	Measured pH	10mer retention time (min)	15mer retention time (min)	30mer retention time (min)	10/15 peak capacity	15/30 peak capacity	Note
HFIP	12.5	1.83	8.6	11.0	12.1	13.7	6.2	6.5	[a]
HFIP	50	15.7	9	10.1	12.0	13.9	10.2	10.0	[a]
HFIP	100	1.15	7	11.6	13.4	17.8	7.1	3.7	[a]
HFIP	100	8.6	8.3	13.8	15.9	18.2	11.4	12.4	[a]
HFIP	100	14.64	8.5	13.5	15.7	18.3	11.9	13.4	[a]
HFIP	100	31.4	9	7.4	10.3	13.3	13.7	13.6	[a]
HFIP	400	2.25	7	16.4	18.8	21.0	10.9	8.7	[a]
HFIP	400	16.3	7.9	18.1	22.3	25.5	19.4	19.6	[a]
AcOH	100	100	~7	21.3	22.1	23.1	4.9	5.5	[a]
AcOH	100	100	~7	14.3	14.9	15.7	4.1	5.2	[b]
AcOH	100	100	~7	16.5	17.7	19.6	7.1	10.2	[c]

[a]Gradient starts from 5% methanol, slope 1%/min, XTerra® MS C_{18}, 2.5 μm, 50×4.6 mm, 0.5 mL/min, 50°C.
[b]Gradient starts from 2% acetonitrile, slope 1%/min, other conditions as above.
[c]Gradient starts from 5% acetonitrile, slope 0.5%/min, other conditions as above.

Table 1 suggests that a greater concentration of TEA in 100 mM HFIP enhances the ion-pairing capability of the buffer and improves the separation performance. The retention increases with the addition of TEA, but as the pH of the buffer reaches ~9, we observe a retention decrease. This may be attributed to the incomplete protonation of the triethylammonium ion as the pH approaches the pK_a of TEA (10.7). It has been reported that the non-protonated TEA is capable of displacing DNA from the C_{18} surface and thus reduce its retention.[25] We found that the buffer system comprised of 8.6 mM TEA and 100 mM HFIP, pH 8.3, gave a separation comparable to that traditionally found with the 100 mM TEAA, pH 7, ion-pairing system.

Interestingly, we found that, besides the concentration of TEA, HFIP also has a significant impact on ion-pairing efficiency and separation performance. Table 1 shows that the 400 mM HFIP buffer with 16.3 mM TEA allows for a dramatically better separation than the buffer consisting of a similar concentration of TEA in 50 mM HFIP. The contribution of HFIP to the separation is probably linked to the solubility of TEA in the HFIP buffer. This was discussed in earlier reports.[24–26] We observed that TEA is poorly soluble in HFIP aqueous buffers, especially at higher concentrations of HFIP. In fact, 16.3 mM of TEA is the highest concentration we were able to dissolve in 400 mM HFIP at room temperature. We believe that the limited solubility affects the distribution of the triethylammonium ion between the mobile and stationary phases, and favors the adsorption of TEA^+ on the reversed-phase sorbent. This mechanism enhances the ion-pairing efficiency of the buffer.

Increasing the concentration of TEA in the optimized buffer also has an impact on the final pH of the buffer. Since the hybrid-silica column employed was designed to tolerate a wide pH range, the elevated buffer pH does not have a detrimental effect on column lifetime. We used the column over several hundred injections without noticeable deterioration of oligonucleotide separation.

We compared the separation achieved with TEA/HFIP buffers with the commonly used TEAA buffer using both methanol and acetonitrile gradients (Table 1). A shallower gradient was required for the TEAA ion-pairing system to match the oligonucleotide separation performance of the TEA/HFIP buffer system. We also investigated the impact of pH on the separation performance of the TEAA ion-pairing system. Since the pK_a values of acetic acid (4.7) and TEA (10.7) are more than one pH unit from the evaluated pH, a very small amount of TEA was needed to adjust the TEAA buffer to pH 8–9. Besides a small decrease in retention time, no impact on oligonucleotide resolution was observed.

Evaluation of ion-pairing buffer compatibility with ESI-MS analysis

The compatibility of optimized ion-pairing buffers was investigated for ESI-MS oligonucleotide analysis. Figure 2 shows a direct infusion of a 25mer PS oligonucleotide in two different solvents. It is clear that the infusion performed in the TEA-HFIP/methanol solvent gives a greater MS signal than the one performed in 100 mM TEAA/methanol. Interestingly, there is a shift to higher charged states with the TEA/HFIP buffer (Fig. 2(A)). The infusion of sample in the TEA/HFIP buffers (mixed 1:1 with acetonitrile or methanol) gave an MS signal comparable to that of an infusion in 50% methanol/50% water (data not shown). While acetic acid seems to be the principal component in the TEAA buffer responsible for ion suppression,[11] HFIP is apparently more MS-friendly. Replacing the acetic acid with HFIP increases MS signal intensity ~20- to 100-fold.

Comparing the ESI-MS signal of the 25mer oligonucleotide infused in TEA/HFIP buffers versus pure water, we observed a substantially reduced number of sodium adducts for the former experiment. The TEA^+ effectively displaces alkali cations on the DNA backbone and is partially stripped in the gas phase in the electrospray source. The extent of TEA^+ adduction (MW 101.2) detected depends on the ESI source temperature and the selected ionization conditions.

In order to optimize the MS signal, we evaluated the source cone voltage and desolvation temperature. However, these parameters were found to have an impact on in-source depurination (loss of guanosine or adenosine base) of oligonucleotides. A decrease in both cone voltage and desolvation temperature resulted in a decrease of the depurinated 25mer PS oligonucleotide. We chose the tuning

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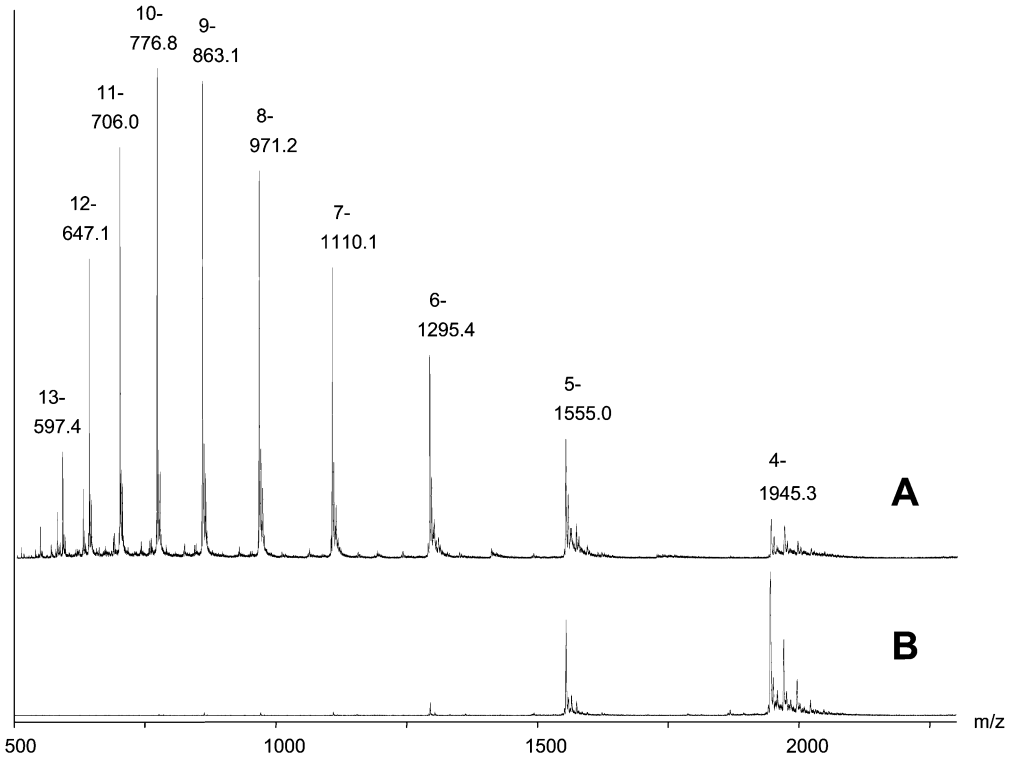

Figure 2. ESI-MS infusion of a 25mer phosphorothioate oligonucleotide in 50% methanol/50% 8.6 mM TEA/100 mM HFIP, pH 8.3 (A) or 50% 100 mM TEAA, pH 7 (B). Triethylamine (MW 101.2) and one sodium molecule were the observed adducts. Infusion performed at 10 μL/min. For ESI-TOFMS conditions, see Experimental section.

parameters appropriately to minimize in-source fragmentation while still obtaining a significant MS signal of the target species. This reduced the chance of mischaracterizing oligonucleotide failure fragments and metabolites.

HFIP appears to create a significant background at *m/z* 336.08 and 504.12 (dimer, trimer), and there is some unidentified background probably originating from either HFIP or TEA at *m/z* values greater than 1100. Since most of the signal from different oligonucleotide charged states appears in the region *m/z* 510–1100, we limited data collection to this range. Using this approach, we were able to eliminate mobile phase background and acquire high-quality total ion current (TIC) MS chromatograms.

LC/MS applications for oligonucleotide analysis

It has previously been shown that the TEA/HFIP buffer can be used for routine LC/MS analysis of oligonucleotides less than 60mer in length.[24] Due to the enhanced ion-pairing efficiency, this buffer practically eliminates the impact of the oligonucleotide sequence[26] on retention and allows for charge-dependent (length-dependent) retention. In addition, HFIP effectively disrupts the oligonucleotide secondary structure, and intra- and intermolecular complexes. This is demonstrated by the separation of a 25mer oligodeoxyguanosine from the failure products from its synthesis (Fig. 3). G-Rich oligonucleotides are known to form very stable quadruplex complexes as well as inter- and intramolecular structures, which makes their analysis by RP-HPLC extremely difficult. Either high pH (~12) or high temperature (94°C) was required to successfully analyze G-rich oligonucleotides.[27–29] We found that the 16.3 mM TEA/400 mM HFIP buffer is an efficient denaturant and allows for an efficient separation of G-rich oligonucleotides. Figure 3 shows that this synthesis of 25mer oligodeoxyguanosine results in large amounts of shorter failure products. This may suggest that the secondary structure of oligonucleotides has an impact on the synthesis quality.

Based on peak area (Fig. 3), we were able to detect down to 4 pmol (19mer) of sample. The target 25mer oligonucleotide was identified with a mass accuracy of 300 ppm (theoretical MW 8168.3 Da). The deconvoluted spectrum (Fig. 3, inset) shows a small amount of sodium/potassium adducts and of a cyanoethyl protection group ($\Delta = +53.0$ Da) not cleaved after synthesis.

A TEA/HFIP ion-pairing buffer has unique separation performance for the analysis of chemically modified oligonucleotides, such as phosphorothioates. We were not able to achieve sufficient resolution of PS oligonucleotides with traditional TEAA (or other) buffers, most probably due to the partial separation of multiple diastereomeric forms. We successfully performed analysis on a 25mer PS oligonucleotide digested *in vitro* with 3′-exonuclease using the LC/MS-compatible buffer (Fig. 4). The deconvoluted spectra of the target product and 17mer metabolite are shown as insets in the figure. Total sample load injected on-column was 206 pmol. The resolution of metabolites and limit of detection are suitable for studies of *in vivo* antisense drug degradation. Based on peak area, it was calculated that the 25mer comprised 91 pmol of the sample and the 17mer metabolite was detected at a level of 660 fmol. UV has a lower detection

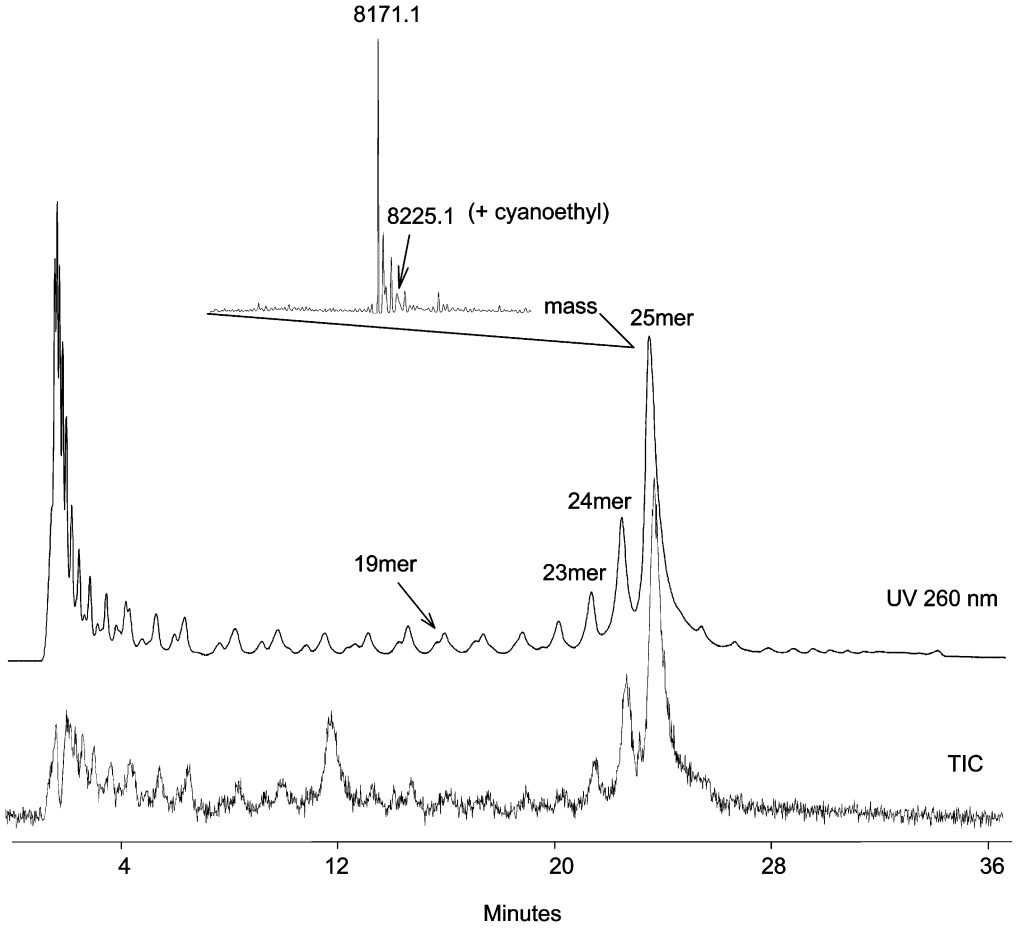

Figure 3. LC/MS analysis of a crude synthetic 25mer homooligodeoxyguanidine. Xterra® MS C_{18}, 1.0 × 50 mm, 2.5 μm column. Mobile phase A: 5% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9; B: 60% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9. Gradient from 14.5% B (13% MeOH) to 30.9% B (22% MeOH) in 36 min, 23.6 μL/min, 60°C, UV 260 nm. For ESI-TOFMS conditions, see Experimental section. Inset to figure shows deconvoluted spectrum of 25mer target product.

limit than MS, and the 17mer peak is clearly detectable. Despite low signal-to-noise of the MS TIC signal, we were able to extract a correct 17mer molecular mass. The target product and its metabolites were identified with an average mass accuracy of 300 ppm (theoretical MW of 25mer 7776.4 Da). Few sodium or potassium adducts were observed in the mass spectra. The peak eluting after the main peak has the molecular mass of the target product +53.0 Da. This suggests the presence of a cyanoethyl group used in synthesis. There is also a small depurination peak ($\Delta = -135.0$ Da) in the target spectrum (inset in Fig. 4), which represents the loss of an adenosine base in-source. Although conditions were optimized to reduce in-source fragmentation, there is still some present (<5% of main peak). An unknown impurity that was 106.4 Da greater than the expected MW of each of the metabolites was also detected. An example of this can be seen in the inset in Fig. 4 for the 17mer.

LC/MS analysis of long oligonucleotides

ESI-MS seems to be a more suitable technique for the analysis of >100mer oligonucleotides than MALDI-TOFMS. However, ESI-MS is dramatically less tolerant to sample contamination with alkali cations. The LC/MS method developed here could provide an efficient sample desalting/analysis tool.

We challenged the method for the LC/MS analysis of 70–110mer oligonucleotides. Table 2 shows the mass accuracy, number of adducts, % purity, and amount of target oligonucleotide detected by MS for five different crude synthetic oligonucleotides. Percent purity and limit of MS detection were estimated from UV peak area. Note that, with the exception of the 90mer heterooligonucleotide, the number of sodium/potassium adducts increases as oligonucleotide length increases.

Figure 5 shows the LC/MS analysis of the crude synthetic 100mer heterooligonucleotide from Table 2. The inset in the figure shows the deconvoluted spectrum of the target oligonucleotide, which was identified with a mass accuracy of 29 ppm. Several sodium and potassium adducts were observed in this spectrum, but the level of adduction is low and does not obscure data interpretation. Unknown adducts observed at 30992.8 Da ($\Delta \sim +167$ Da) and at 31159.8 ($\Delta \sim +2 \times 167$ Da) could be HFIP adducts (deprotonated form of HFIP has molecular mass ~167 Da). This is presumably due to its interaction with positively charged nucleobases. We again detected the presence of one and two

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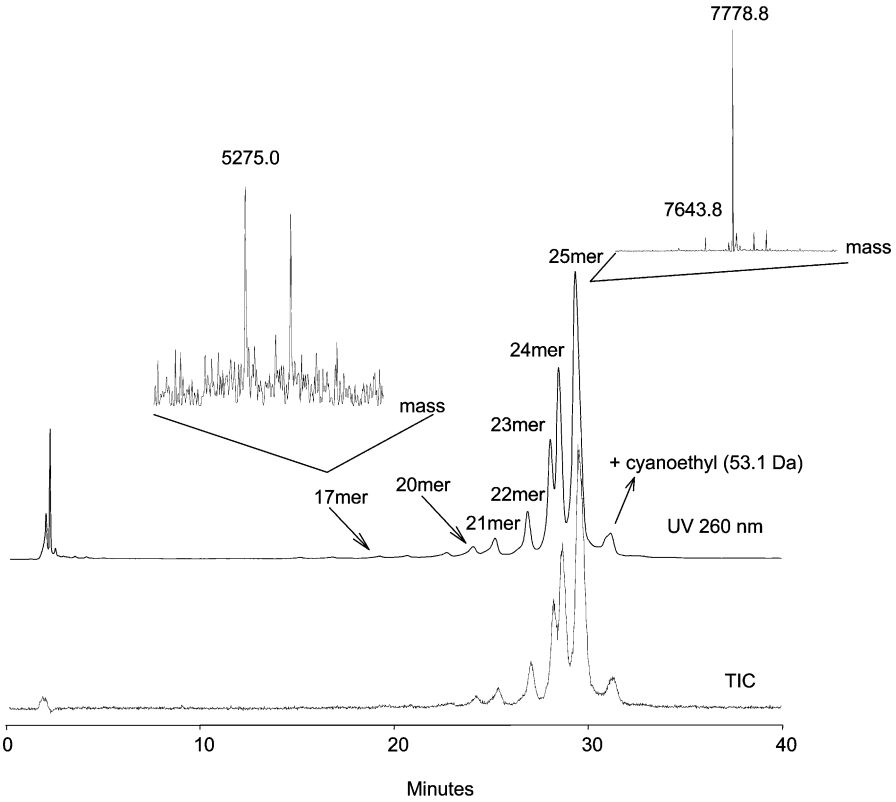

Figure 4. LC/MS analysis of a 25mer PS oligonucleotide digested *in vitro* with 3′-exonuclease. Xterra® MS C_{18}, 1.0×50 mm, 2.5 μm column. Mobile phase A: 5% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9; B: 60% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9. Gradient from 18.2% B (15% MeOH) to 36.4% B (25% MeOH) in 40 min, 23.6 μL/min, 60°C, UV 260 nm. For ESI-TOFMS conditions, see Experimental section. Inset to figure shows deconvoluted spectra of 25mer target oligonucleotide and 17mer metabolite.

cyanoethyl protecting groups ($\Delta = +53.0$ Da, +106.0 Da). These are identified on the UV trace of the figure. For some of the other oligonucleotides in Table 2, one, two, three, and sometimes four isobutyryl groups ($\Delta = +70.1$, +140.2, +210.3, +280.4 Da) were observed (data not shown). These were previously reported by Huber.[1]

CONCLUSIONS

Ion-pair RP-HPLC was coupled on-line to ESI-TOFMS for the characterization and identification of native and chemically modified oligonucleotides up to 110mer in length. This method is useful for antisense oligonucleotide quality control, metabolic studies, and clinical trials. It allows for the identification of failure sequences and can be easily used for oligonucleotide purity determination. MS detection limits were as low as 660 fmol for digestion products of a 25mer PS oligonucleotide using a 1.0×50 mm column. Mass determination was performed with an average accuracy of 125 ppm. The optimized buffers were efficient for high-performance separation of oligonucleotides and compatibility with MS detection. LC provides for efficient desalting; alkali cation adduction was greatly reduced or eliminated, allowing for the routine analysis of oligonucleotides up to 110mer in length.

Table 2. LC/MS analysis of 70–110mer oligonucleotides

Oligonucleotide	70mer	80mer	90mer	100mer	110mer
Theoretical MW (Da)	21559.1	24672.1	27736.1	30825.1	33938.1
Experimental MW (Da)	21560.6	24668.5	27736.2	30826.0	33939.1
Mass Accuracy (ppm)	70	146	4	29	27
# Na^+, K^+ adducts[a]	3	5	3	4–5	5–6
Estimated % Purity[b]	8.7	6.9	18.3	17.3	16.0
LOD (pmol)[b]	1.9	1.3	4.8	5.4	6.4

[a]Represents the total number of adducts observed in the deconvoluted spectrum of each target oligonucleotide.
[b]Amount of the total mass injected that is target oligonucleotide. Purity was estimated based on UV peak area.

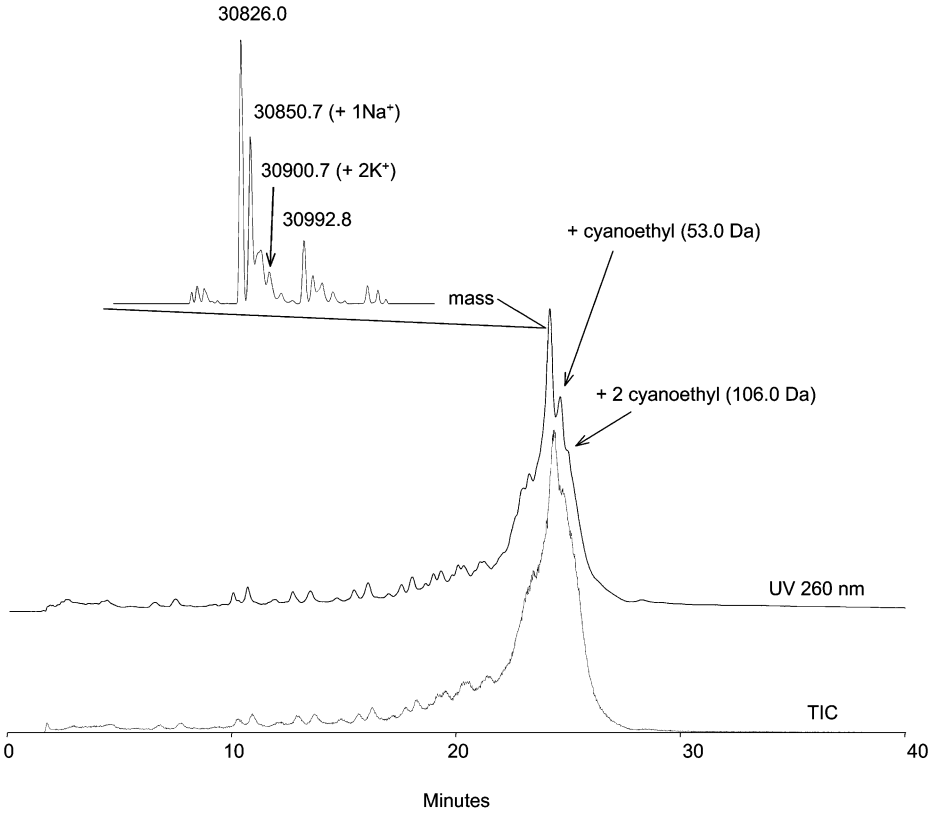

Figure 5. LC/MS analysis of a crude synthetic 100mer heterooligonucleotide. Xterra® MS C_{18}, 1.0 × 50 mm, 2.5 μm column. Mobile phase A: 5% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9; B: 60% MeOH in 16.3 mM TEA/400 mM HFIP, pH 7.9. Gradient from 26.5% B (19.6% MeOH) to 44.7% B (29.6% MeOH) in 40 min, 23.6 μL/min, 60°C, UV 260 nm. For ESI-TOFMS conditions, see Experimental section. Inset to figure shows deconvoluted spectrum of 100mer target oligonucleotide.

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