

Characterization of Therapeutic Oligonucleotides Using Liquid Chromatography with On-line Mass Spectrometry Detection

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ABSTRACT

A method for the analysis and characterization of therapeutic and diagnostic oligonucleotides has been developed using a combination of liquid chromatography and mass spectrometry (LC-MS). The optimized ion-pairing buffers permit a highly efficient separation of native and chemically modified antisense oligonucleotides (AS-ODNs) from their metabolites or failure synthetic products. The mobile phases were MS compatible, allowing for direct and sensitive analysis of components eluting from the column. The method was applied for the quantitation and characterization of AS-ODNs, including phosphorothioates and 2'-O-methyl-modified phosphorothioates. Tandem LC-MS analysis confirmed the identity of the oligonucleotide metabolites, failure products, the presence of protection groups not removed after synthesis, and the extent of depurination or phosphorothioate backbone oxidation.

INTRODUCTION

ADVANCES IN THE SYNTHESIS of chemically modified oligonucleotides have permitted the development of novel strategies in genetic medicine. Oligonucleotide modification assures the *in vivo* stability needed for successful therapeutic use. The most common chemical modification includes sulfurization, modification of the deoxyribose ring, or alteration of the entire oligomer backbone (Braasch and Corey, 2002). These modifications make analysis and characterization of therapeutic oligonucleotides more challenging than their native counterparts (Agrawal et al., 1990; DeDionisio and Lloyd, 1996; Gilar et al., 2000).

Analytical techniques traditionally used for the analysis of oligonucleotides include polyacrylamide gel electrophoresis (PAGE), capillary gel electrophoresis (CGE) (Cohen et al., 1997; Gilar et al., 1997), and anion-exchange high performance liquid chromatography (AX-

HPLC) (Bourque and Cohen, 1994; Srivatsa et al., 1997; Warren and Vella, 1995). Mass spectrometry (MS) is becoming a popular tool for analysis because of its capability to identify oligonucleotides. In principle, MS provides information about the molecular weight of the analyzed molecule, which reflects its elemental composition. The theoretical mass values of interrogated oligonucleotide(s) can be compared with measured mass spectral data and used to confirm their presence or the presence of parent molecule fragments. Accurate molecular mass measurement can be used to characterize synthesis failures or postsynthesis modification of molecules. Deviations from the theoretical molecular mass suggest modification of structure, such as depurination, oxidation, phosphorylation, or backbone fragmentation. Advanced MS methods are primarily useful for characterization of oligonucleotide modifications but can also directly provide sequence information.

The quantitation and characterization of oligonu-

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cleotides often combines separation methods with MS analysis. Off-line methods consist of slab gel band excising or HPLC fraction collection, followed by desalting of isolated oligonucleotides prior to MS analysis. Such an approach is tedious and difficult to automate. The current trend is to interface the separation methods on-line with MS. Interfacing CGE with electrospray ionization mass spectrometry (ESI-MS) has been described (Freudemann et al., 2001), but it is not trivial.

Because of its compatibility with MS, reversed-phase HPLC (RP-HPLC) is often employed for oligonucleotide analysis. However, several difficulties are associated with designing an on-line liquid chromatography-mass spectrometry (LC-MS) method: (1) MS signal intensity for DNA is often not sufficient for sensitive analysis, (2) alkali cation adduction complicates the interpretation of the spectra, and (3) HPLC separation of oligonucleotides with MS-compatible mobile phases is difficult.

The limited ionization efficiency of oligonucleotides is apparent from comparison of the limits of detection (LOD) typically reported in literature. The LOD of oligonucleotides for MALDI-MS is on the order of 0.1–1 picomole (Gilar et al., 2001), whereas the LODs for peptides are in the femtomole range. Similar trends are observed for LC-MS, although the LOD depends also on the column dimensions. Subpicomole levels of detection have been reported for LC-MS of DNA with capillary columns (Huber and Krajete, 1999; Premstaller et al., 2000), and values of two or more orders of magnitude lower can be achieved for peptides with a similar experimental setup. Because of the large extinction coefficient of DNA, ultraviolet (UV) detection provides for similar or more sensitive detection than ESI-MS. Therefore, a combination of UV and MS was proposed for quantitation and characterization of oligonucleotides (Gilar, 2001).

ESI-MS has low tolerance to salt contamination, and it is, therefore, a good practice to use deionized water for mobile phases (Gilar et al., 2001). Basic additives to the mobile phase can partially alleviate alkali cation adduction (Edmonds and Smith, 1990; Gaus et al., 1997). Early LC-MS methods used RP-HPLC as a tool for on-line sample desalting prior to MS analysis rather than as a separation technique (Deroussent et al., 1995). Various mobile phases or postcolumn additives, such as triethylamine (Bleicher and Bayer, 1994), imidazole (Deguchi et al., 2002; Greig and Griffey, 1995), or piperidine (Greig and Griffey, 1995), were reported to eliminate residual cation adduction and improve the quality of the MS signal. Other approaches for sample desalting include on-line postcolumn microdialysis (Liu et al., 1996) or the use of on-line postcolumn cation exchange suppressors (Huber and Buchmeiser, 1998), which are scavenging ions from the mobile phase. Notwithstanding the usefulness of these methods for on-

line MS sample preparation, the chromatographic separation was not adequate for more complex samples.

Optimizing mobile phase composition in order to improve the HPLC separation typically compromises the MS signal, and *vice versa* (Gaus et al., 1997). Volatile ion-pairing additives were employed to enhance the separation performance of RP-HPLC. The traditionally used triethylammonium acetate (TEAA) buffers, however, are detrimental to ESI-MS detection. It was found that the acid component caused MS signal suppression (Huber and Krajete, 1999). Reduction in TEAA concentration from 100 mM to 25 mM improved buffer compatibility with MS, but the lower concentration reduced the ion-pairing efficiency and compromised HPLC separation performance (Apffel et al., 1997a). Recent progress in the LC-MS analysis of oligonucleotides includes the use of more MS-compatible ion-pairing additives. Huber et al. investigated triethylammonium bicarbonate (TEAB) (Huber and Krajete, 1999, 2000; Premstaller et al., 2000), with postcolumn addition of a sheath liquid (acetonitrile or other organic solvents), and achieved better detection limits. Apffel et al. (1997a) suggested an ion-pairing mobile phase consisting of ~2.1 mM triethylamine (TEA) buffered with aqueous 400 mM hexafluoroisopropanol (HFIP, weak acid) solutions to pH 7. The authors observed little MS signal suppression by the mobile phase components and achieved acceptable separation of native oligonucleotides. More hydrophobic ion-pairing agents, such as the dimethylbutylammonium (DMBA) ion, offer enhanced ion-pairing at low concentrations. Therefore, some improvement in separation and compatibility with MS was achieved with a 25 mM DMBA-bicarbonate buffer, pH 7 (Oberacher et al., 2001).

Ion-pair RP-HPLC is well suited for the separation of native oligonucleotides (Huber et al., 1992). However, separation of phosphorothioate (PS) oligonucleotides generally fails (Agrawal et al., 1990; Metelev and Agrawal, 1992). This is because sulfuration of the oligonucleotide backbone creates multiple diastereomers with heterogeneous retention behavior, causing substantial peak broadening (Gilar et al., 2000; Mayr et al., 2002). To date, LC-MS methods have achieved limited success for the separation and characterization of PS therapeutic oligonucleotides.

In this work, an improved ion-pairing RP-HPLC system combined with a high-resolution ESI-TOF mass spectrometer was applied for the LC-MS analysis and quality control of antisense oligonucleotides (AS-ODN). A combination of UV and MS was used for quantitation and characterization of oligonucleotide impurities/metabolites. Comparison of calculated theoretical and experimentally observed molecular masses was used for peak assignments of eluting mixture components. The high mass accuracy of the ESI-TOF instrument allowed for identification of oligonucleotides with high confidence.

MATERIALS AND METHODS

Chemicals and reagents

TEA, 99.5%, glacial acetic acid, 99.99%, and HPLC grade acetonitrile (ACN) and methanol were purchased from J.T. Baker (Phillipsburg, NJ). Water (18 M Ω cm) was purified in-house using a Milli-Q system (Millipore, Bedford, MA). Bovine intestinal mucosa phosphodiesterase I (3'-exonuclease) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma (St. Louis, MO). Oligonucleotides were obtained from the following sources: 10–30-mer homooligodeoxythymidines were purchased from Midland Certified Reagents (Midland, TX); 21-mer PS G-rich oligonucleotide (>50% of G content, sequence does not contain GGGG stretches; the full sequence is proprietary), and the 20-mer PS and 20-mer PS/PO GGT TTG CTG TGT ATG GGA GG were provided by Omega PharmServices (Milford, MA); the 25-mer PS CTC TCG CAC CCA TCT CTC TCC TTC T and 21-mer PS with 2'-O-methyl-modified terminal nucleotides (four from each end) *UCG CAC CCA TCT CTC TCC UUC* were obtained from Hybridon (Cambridge, MA). All sequences are given from 5'-end to 3'-end.

LC and electrophoresis

An analytical HPLC system (Alliance[®] 2695 separation module) or a capillary HPLC system (CapLC[®]) was used for chromatography (both Waters, Milford, MA). Separation was carried out using XTerra[®] MS C₁₈ columns (Waters) packed with 2.5- μ m hybrid-silica sorbent. The 50 \times 4.6 mm columns were used for conventional chromatography, and 50 \times 1.0 mm columns were used on the capillary HPLC system. Chromatographic conditions are specified in the figure captions. The 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, the following protocol was used (1 L of 0.1 M buffer): 5.6 ml of glacial acetic acid was placed in 950 ml of water while mixing, then 13.9 ml of TEA was 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. As the desirable pH 7 is more than 1 pH unit apart from the pK_a 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 \sim 0.1 M.

Similarly, the TEA-HFIP buffers were prepared by titrating the acid solution (HFIP) with TEA. To prepare 1 L of 16.3 mM TEA-400 mM HFIP buffer, pH 7.9, we dissolved 41.5 ml HFIP in 950 ml water and slowly added 2.3 ml TEA. The solubility of TEA in HFIP solu-

tion is poor, and the last addition of TEA usually dissolves after adjusting the solution to a final volume of 1 L. The final pH is approximately 7.9. Alternatively, a buffer consisting of 8.6 mM TEA and 100 mM HFIP, pH 8.3, was used in some experiments (10.5 ml HFIP and 1.2 ml TEA dissolved in water, final volume of solution was 1 L).

CGE was performed on a Quanta[®] 4000 (Waters) using a BioCAP[™] DNA Analysis Capillary (Bio-Rad Laboratories, Hercules, CA), 375/75 μ m OD/ID, 34.5 cm length (effective capillary length to detector was 27.5 cm). The capillary was filled with sieving polymer matrix prior to each run. Replaceable sieving matrix was 1.8 g of polyethylene glycol (MW 35,000) dissolved in 7.2 ml of 0.1 M Tris-boric acid, 1.25 mM EDTA buffer (0.1 M TBE) with 1 M urea. The 0.1 M TBE, 1 M urea buffer was prepared fresh prior to use by dissolving 1.8 g urea in 3 ml of 1 M TBE buffer, pH 8.3, and diluting the solution with deionized water up to a final volume of 30 ml. Separation was performed at 15 kV for 40 minutes at 30°C. Samples were desalted prior to analysis using drop dialysis for 30 minutes on a floating 0.025- μ m nitrocellulose membrane (Millipore Corporation). Samples were injected onto CGE by applying a potential of 5 kV for 3–5 seconds. Sample data were acquired and processed using Millennium32[®] software, version 3.05 (Waters).

LC-MS

A capillary HPLC system was connected to an ESI-TOF mass spectrometer (Micromass[®] LCT[™], Waters) using a 30-cm fused silica capillary, 50 μ m ID. The LC-MS system was operated by Micromass MassLynx[™] software, version 3.5 (Waters). 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/hour. Cone gas flow rate was set to 30 L/hour. Desolvation temperature and source temperature were set to 120°C and 100°C, respectively. The scanning range was 400–2500 m/z with a 1.1-second scanning cycle (1.0 second scan time and a 0.1 second interscan delay).

RESULTS AND DISCUSSION

Ion-pairing buffer optimization

Because the relative differences in the length (hydrophobicity) or charge of N and N-1 oligonucleotides are minute, HPLC separation is difficult and becomes more challenging as N increases. In addition, slow mass transfer (diffusion) of high molecular weight analytes within sorbent pores further complicates the separation

prospect due to peak broadening. For that reason, the best resolution of biopolymers has been achieved with non-porous (Huber, 1998) or superficially porous (Kirkland et al., 2000) chromatographic sorbents.

The presented method developed for oligonucleotide separation employs an XTerra MS C₁₈ column packed with 2.5 μm porous sorbent. The small particle size of the packing material decreases the diffusion path of molecules and provides for high chromatographic performance. In conjunction with high separation temperatures (typically 60°C), relatively slow flow rates, and shallow gradients, a separation of N from N-1 for up to 60-mer oligonucleotides was routinely achieved (Gilar, 2001). Figure 1 illustrates that the performance of HPLC (Fig. 1B) is similar to CGE (Fig. 1A) for the separation of 7–30-mer oligodeoxythymidines (Gilar et al., 2002). Because the hybrid-silica sorbent is stable under the separation conditions and has high mass load capacity (unlike nonporous sorbents), the method is also useful for oligonucleotide purification (Gilar, 2001).

In a previously published work, the impact of the mobile phase on the selectivity of oligonucleotide separation was investigated (Gilar, 2001; Gilar et al., 2002). The TEAA ion-pairing system was evaluated along with the

TEA-HFIP buffers suggested for LC-MS analysis by Apffel et al. 1997a). Subsequently, the latter buffer composition was optimized to achieve the best possible resolution. Separation performance was characterized by peak capacity (P), which is defined as the number of peaks that can be resolved ($R_s = 1$) within a given separation time (equation 1) (Neue, 1997). The P value was calculated from the separation of 15-mer and 30-mer oligodeoxythymidine peaks using the indicated buffer and a gradient elution with methanol (1% per minute). The t_{30} and t_{15} are retention times (minutes) of the 30-mer and 15-mer, respectively, and $w_{4\sigma}$ is the peak width at 13.4% of peak height.

$$P = \frac{1 + t_{30} - t_{15}}{(w_{4\sigma(30)} + w_{4\sigma(15)})/2} \quad [1]$$

For example, if the averaged peak width ($w_{4\sigma}$) is 1 minute, and the difference in retention time of 15/30-mer is 15 minutes, the P value of 16 suggests that 15–30-mer oligodeoxythymidines will be nearly baseline separated. Experimentally, this situation ($P > 16$) is illustrated in Figure 1. In the case of wider peaks or lower separation selectivity (smaller difference in retention times between

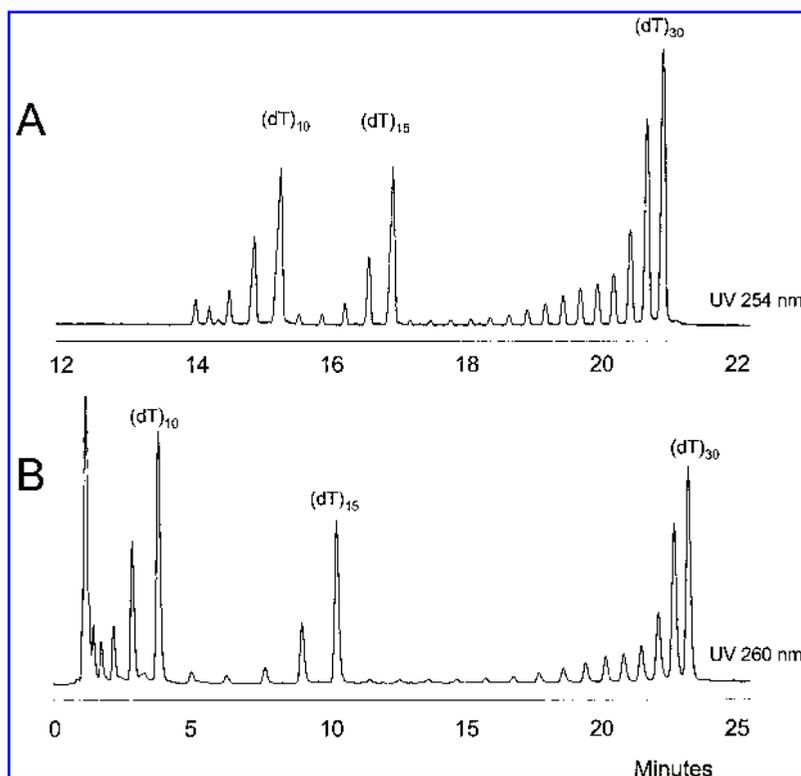


FIG. 1. Comparison of CGE (A) and RP-HPLC (B) for the separation of 7–30-mer oligodeoxythymidines. CGE conditions: BioCAP™ 75 μm, capillary was 34.5 cm (27.5 cm to detection window), temperature 30°C, polyethylene glycol sieving matrix, 15 kV run, 4 second injection at 5 kV. HPLC conditions: 50 × 4.6 mm column; 60°C, 0.5 ml/min, Mobile phase A: 10% MeOH in 16.3 mM TEA, 400 mM HFIP buffer, pH 7.9. Mobile phase B: 40% MeOH in 16.3 mM TEA, 400 mM HFIP buffer, pH 7.9, linear gradient from 0 to 10 minutes was 16.7% to 30% B, then from 10 to 30 minutes 30% B to 47.15% B.

15-mer and 30-mer), the P value decreases, indicating only partial oligonucleotide resolution. In other words, a higher P value indicates better separation.

Results from the evaluation of several ion-pairing systems are summarized in Table 1. Interestingly, the concentration of both TEA and HFIP is crucial for the success of oligonucleotide separation. The role of the triethylammonium cation (the active ion-pairing agent) is well understood. An increase in TEA concentration improves ion-pairing efficiency and, consequently, the separation selectivity. A more efficient ion-pairing mechanism demonstrates itself also as an increase in retention time (Table 1). The side effect of the increased TEA concentration is the rise of mobile phase pH (TEA pKa is 10.7), which may reduce the lifetime of silica-based columns. However, the hybrid-silica sorbent used in this method is highly stable up to pH \sim 12.

The impact of HFIP on the ion-pairing efficiency of the buffer is less clear. Increasing the HFIP concentration from 100 mM to 400 mM has a positive effect on separation (Table 1), but because HFIP is not an active ion-pairing agent, its effect could only be indirect. The most likely explanation is that the limited solubility of TEA in aqueous HFIP solutions changes the distribution of TEA between the mobile and stationary phases and forces the triethylammonium ion adsorption on the sorbent surface. This, in turn, enhances the ion-pairing retention mechanism and improves the separation performance. In fact, the most successful ion-pairing system (Table 1) represents the maximum concentration of TEA (16.3 mM) that is soluble in 400 mM HFIP aqueous solution at ambient temperature. This buffer provides for more efficient separation than traditional TEAA ion-pairing buffers, in which longer gradients were required to achieve similar column peak capacity (at 0.5% ACN per minute, $P \sim$ 10).

A very important feature of HFIP is that it reduces the impact of oligonucleotide hydrophobicity on retention

(Gilar et al., 2002), which appears to be crucial for the separation success of phosphorothioate oligonucleotides (PS-ODN). Earlier attempts to resolve PS-ODN in HPLC were obscured with the partial separation of numerous diastereomers (sulfurization of backbone creates multiple new chiral centers), causing massive peak broadening (Agrawal et al., 1990; Gilar et al., 2000; Metelev and Agrawal, 1992). Figure 2 clearly illustrates this situation for the separation of a 25-mer PS-ODN and its 3'-end truncated metabolites. RP-HPLC with the TEAA ion-pairing system (Fig. 2B) shows no useful separation (presumably due to an overlap of partially separated diastereomeric oligonucleotides), whereas baseline resolution of all 19–25-mer peaks was obtained with the TEA-HFIP mobile phase (Fig. 2A). Griffey et al. (1997) have used TEA-HFIP for LC-MS analysis of 12–20-mer PS-ODNs. However, LC resolution suffered from nonoptimal ion-pairing buffer composition. Separation performance of an optimized TEA-HFIP buffer (Fountain et al., 2003) makes it an ideal ion-pairing system for the analysis of therapeutic PS-ODNs.

Mobile phase compatibility with MS detection

For successful LC-MS method performance, the mobile phases have to be compatible with MS analysis. The compatibility of ion-pairing mobile phases with ESI-MS was investigated using direct infusion of a 25-mer PS. Figure 3 compares the signals from an infusion of 500 μ g/ml of oligonucleotide (desalted prior to experiment) in three mobile phases: 50% acetonitrile in water (Fig. 3A), 50% acetonitrile in 100 mM TEAA (Fig. 3B), and 50% acetonitrile in 16.3 mM TEA-400 mM HFIP (Fig. 3C). Whereas the 0.1 M TEAA buffer severely reduces the signal, TEA-HFIP does not appear to suppress oligonucleotide ionization. The best signal-to-noise ratio (S/N) was achieved with TEA-HFIP buffer (Fig. 3C) even when compared with the oligonucleotide infusion in

TABLE 1. IMPACT OF ION-PAIRING BUFFER COMPOSITION ON OLIGONUCLEOTIDE SEPARATION^a

TEA concentration (mM)	HFIP concentration (mM)	Buffer pH	Retention time t_{15} (min)	Retention t_{30} (min)	Peak capacity ^b
1.2	100	7.0	13.4	17.8	3.7
8.6	100	8.3	15.9	18.2	12.4
14.6	100	8.5	15.7	18.3	13.4
2.3	400	7.0	18.8	21.0	8.7
16.3	400	7.9	22.3	25.5	19.6

^aGradient starts at 5% MeOH, gradient slope was 1% MeOH per minute. HPLC conditions: 50 \times 4.6 mm column, flow rate 0.5 ml/min, temperature 50°C.

^bPeak capacity was calculated according to equation 1.

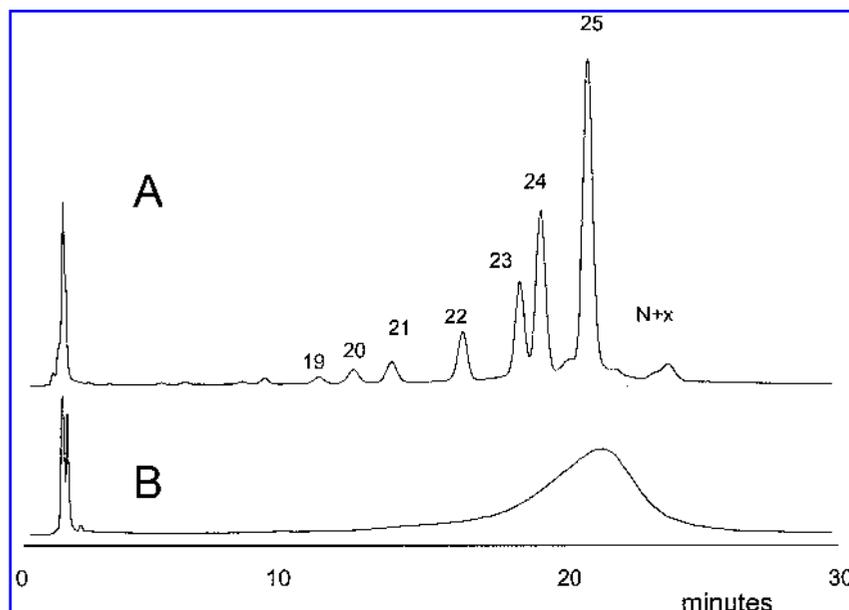


FIG. 2. Impact of ion-pairing buffer on the separation of PS-ODNs. (A) TEA-HFIP buffer. (B) Triethylammonium acetate. The 18–25-mer ladder was prepared by digesting the 25-mer with 3'-end exonuclease. HPLC conditions: 50×4.6 mm column; 60°C , 0.5 ml/min. Conditions (A): Mobile phase A: 8.6 mM TEA, 100 mM HFIP buffer, pH 8.6; B: methanol, linear gradient from 16% B, 0.15% per minute. Conditions (B): Mobile phase A: 0.1 M TEAA, pH 7. Mobile phase B: acetonitrile, gradient starts at 12% B, slope 0.125% B per minute.

50% acetonitrile/water (Fig. 3A). Similar results were obtained when using appropriate buffers with methanol instead of acetonitrile (data not shown). Interestingly, the buffers also cause a shift of the charged-state envelope. The most abundant signals from the infusion done in the TEAA buffer are the 4-charged and 5-charged states (Fig. 3B), and the envelope shifted toward higher charged states for the infusion done in the TEA-HFIP buffer (Fig. 3C). Both spectra in Figures 3B and 3C show some triethylammonium cation adducts ($\Delta = 101$ Da). The adduction of volatile TEA^+ can be reduced by elevating the electrospray probe temperature or increasing the cone voltage or both (Fountain et al., 2003).

Overall, by increasing the TEA concentration, the separation power of the TEA-HFIP ion-pairing system was dramatically improved compared with published reports (Apffel et al., 1997a,b; Griffey et al., 1997), without compromising the sensitivity of MS detection. Compared to other acids employed for pH adjustment of the TEA mobile phases, HFIP is more volatile (b.p. 59°C) and, at pH 7–8.3, is only partially ionized (pK_a 8.25). Its interference with oligonucleotide ionization appears to be minimal.

LC-MS analysis of AS-ODNs

AS-ODNs represent a class of therapeutic molecules. Therefore, they have to be well characterized before administration. Quality control includes quantitation and

identification of the active component and main contaminants (failure sequences), monitoring of *in vitro* drug stability (Gilar et al., 1998), and oligonucleotide sequence confirmation (Froim et al., 1997).

The LC-MS method was used for analysis of a 21-mer PS G-rich oligonucleotide. Figure 4A shows the LC-MS analysis of the crude synthetic 21-mer. The separation performance was adequate to partially resolve two N-1 failure oligonucleotides. If more complete separation is required, a shallower gradient can be used (Gilar et al., 2002). The target product molecular weight detected by MS was 6956 Da. Using ESI-TOF MS, 100 ppm or greater accuracy was routinely achieved. This means that a 30-mer oligonucleotide mass measured (10,000 Da) is typically within ± 1 Da of the actual value. Multiple impurities eluting prior to the main peak were identified as failure sequences (N-x fragments). The deconvoluted mass spectra indicate the presence of impurities with smaller molecular weights. The peaks eluting prior to and after the target 21-mer PS-ODN are identified in Table 2. Figure 4B illustrates the LC-MS analysis of the 21-mer PS purified by the supplier. The purity estimated as peak area at UV 260 nm is about 90%. The N-1 peak (5.7%) is mostly G-truncated 20-mer with some unidentified trace impurities. The later-eluting peak (4.3%) is a 22-mer longer (the mass of 7301.7 Da indicates the addition of a G mononucleotide to the parent oligonucleotide). Although described in the literature, the presence of long-

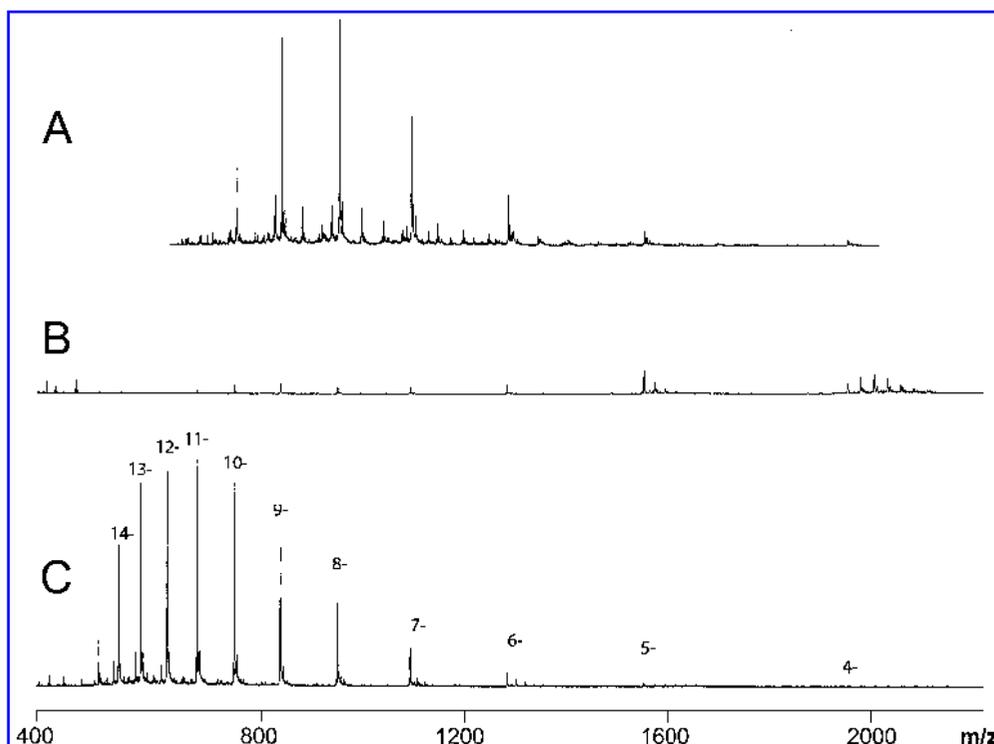


FIG. 3. Effect of HPLC ion-pairing buffer on ESI-MS signal. The 25-mer PS, 500 $\mu\text{g}/\text{ml}$, was infused in 50% acetonitrile in (A) water, (B) 100 mM TEAA, pH 7, and (C) 16.3 mM TEA, 400 mM HFIP buffer, pH 7.9.

mers in synthetic oligonucleotides is surprising. In our experience, the $N+x$ peak is often a full-length product with a cyanoethyl protecting group attached to the phosphate backbone (see, e.g., Figure 2A) or cyanoethyl rearranged during deprotection to a thymidine base (Wilk et al., 1999). We often detect a mass addition of 53 or 106 Da, which represents one or two cyanoethyl functionalities, as well as other protection groups used in oligonucleotide synthesis (Fountain et al., 2003). Analyzing 25–110-mers by LC-MS, it was found that the incomplete postsynthesis deprotection commonly gives rise to additional peaks eluting after the target product (data not shown) (Fountain et al., 2003; Fu et al., 2002; Gilar, 2001).

The LC-MS method was further applied for the analysis of *in vitro* generated 3'-truncated metabolites of a 21-mer PS with 4×4 2'-OMe modified termini. Good separation was achieved on a 50×1.0 mm capillary column connected in series to UV and ESI-TOF MS (Fig. 5). All metabolites were identified by their corresponding molecular mass (Table 3). Figure 5 insets from 3–6 minutes illustrate the differences in S/N ratios for UV and total ion count (TIC) MS chromatograms. UV detection is more sensitive and, therefore, more suitable for quantitation of minor metabolites (Fig. 5A) than the TIC chromatogram (Fig. 5B). Figure 6 shows the selective extraction of the most intense mass ions for the N-10 metabolite (m/z 583.1) and 21-mer oligonucleotide (m/z

671.5). Interestingly, the extraction provided for only moderate improvements in S/N (compare Fig. 5B and Fig. 6A). This is due to the fact that MS signals of oligonucleotides are divided into multiple charged m/z states, each of them proportionally reduced in intensity. It is also apparent that quantitation using extracted mass ions may be complicated by overlapping signals of charged states from different oligonucleotides. This is demonstrated in Figure 6C, where the extracted chromatogram of the 671.5 ion shows three peaks. Incidentally, N, N-2, and N-4 oligonucleotides exhibit an MS signal in the m/z 671.5 \pm 0.5 range. Although the 21-mer signal can be selectively extracted at m/z 610.3 (Fig. 6B), the previously shown example (Fig. 6C) demonstrates that chromatographic separation is beneficial for both UV and MS quantitation of oligonucleotides. In other words, in the case that N-mer, N-2 and N-4 coelute, the extraction of m/z 671.5 would give false quantitation results. Considering that each oligonucleotide in ESI-MS exhibits ~ 10 charged states (Fig. 3) and that the mass chromatogram is extracted in a ± 0.5 Da window, the probability of signal overlap is high even for moderately complex oligonucleotide samples.

The 21-mer peak in Figures 5 and 6 represents the injection of 0.45 μg (67 pmole) on the HPLC column. From the area of the N-10 peak and the knowledge of the molar extinction coefficients of N and N-10, it was estimated that the N-10 metabolite is present at the 10 ng

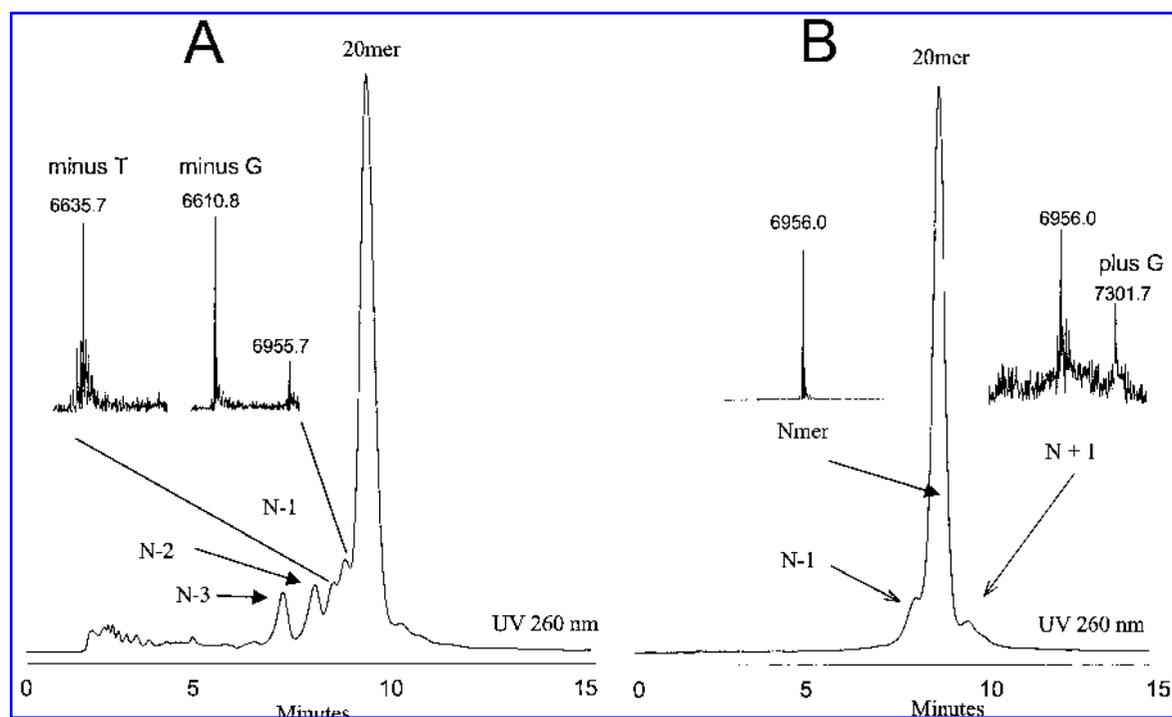


FIG. 4. LC-MS analysis of a G-rich 21-mer PS antisense oligonucleotide. Crude synthetic sample (**A**), quality control of the oligonucleotide after purification (**B**). Conditions: 50×1.0 mm column; 60°C , $23.6 \mu\text{l}/\text{min}$. Mobile phase A: 5% MeOH in 16.3 mM TEA, 400 mM HFIP, pH 7.9. Mobile phase B: 60% MeOH in 16.3 mM TEA, 400 mM HFIP, pH 7.9; gradient from 21.8% to 28.6% B in 15 minutes (0.225% methanol/min); detection UV 260 nm and ESI-MS. The mass injected on column was $0.6 \mu\text{g}$ (86 pmol) for **A** and $0.5 \mu\text{g}$ (72 pmole) for **B**.

(2.9 pmole) level. Based on the S/N, one could draw the conclusion that UV detection is capable of quantitation of subpicomole amounts of oligonucleotides, whereas the limit of quantitation (LOQ) of MS (either TIC or extracted ion) is between 3 and 5 picomoles (for the given HPLC setup).

The LC-MS data were useful for the confirmation of the 3'-end sequence of the 21-mer parent oligonucleotide (Table 3). The mass of the peak labeled "N+x" shown in

Figure 5 suggests the addition of a cyanoethyl protection group that was not cleaved from the target oligonucleotide (mass addition of 53 Da). Few minor peaks were observed that do not match the expected masses of 3'-end truncated metabolites (e.g., eluting between N-11 and N-12). A more detailed characterization of unknown components would require MS/MS analysis (Griffey et al., 1997).

Besides the separation and characterization of failure

TABLE 2. LC-MS ANALYSIS OF CRUDE 21-MER PS G-RICH OLIGONUCLEOTIDE^a

Peak assignment	Measured mass Da	Δ Mass ^b Da	Note
21-mer PS	6956.0	0	Parent drug
20-mer PS	6610.8	-345.2	N-1, loss of G
20-mer PS	6635.7	-320.3	N-1, loss of T
19-mer PS	6265.6	-690.4	N-2, loss of G, G
18-mer PS	5920.4	-1035.6	N-3, loss of G, G, G
22-mer PS	7301.7	+345.3	N+1, addition of G

^aFor chromatogram, see Figure 4.

^bLoss of G, A, T, and C nucleotides in PS-ODNs is indicated by the mass differences of 345.2, 329.2, 320.2, and 305.2 Da, respectively.

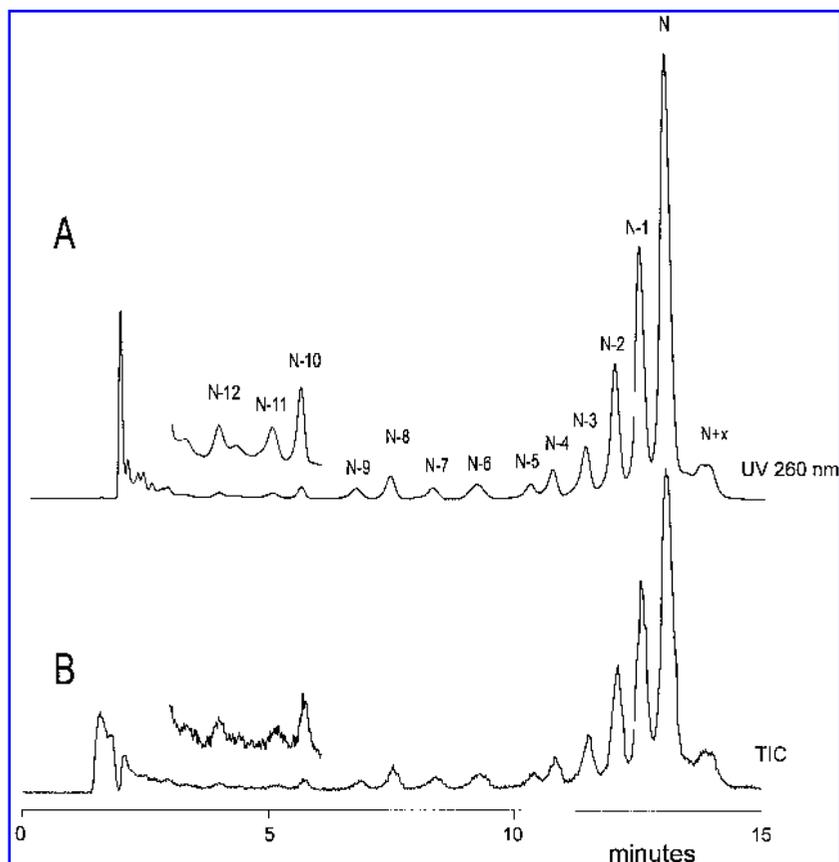


FIG. 5. LC-MS analysis of a 21-mer PS-ODN with 4×4 2'-OMe termini. **(A)** UV260 nm chromatogram. **(B)** TIC MS chromatogram. Conditions: 50×1.0 mm column, 60°C , $23.6 \mu\text{l}/\text{min}$. Mobile phase A: 16.3 mM TEA, 400 mM HFIP, pH 7.9. Mobile phase B: 30% MeOH in buffer A. Gradient from 53.3% to 73.3% B in 6 minutes (1% MeOH per minute), then from 73.3% to 93.3% B from 6 to 30 minutes (0.25% MeOH per minute).

products/oligonucleotide metabolites, the information about oxidation of the PS backbone is also highly desirable. Oxidation of the PS backbone to the phosphodiester (PO) form undermines antisense drug stability *in vivo*. Separation of the PS-ODN from its partially oxidized forms is typically performed by ion-exchange HPLC (Bourque and Cohen, 1993; Deshmukh et al., 2000; Srivatsa et al., 1997). The HPLC method used here did not match the separation performance of ion-exchange HPLC for the separation of PO/PS mixed backbone oligonucleotides. However, the mass difference of 16 Da (sulfur is replaced with an oxygen atom in the phosphate backbone) between all PS oligonucleotides and their monophosphodiester forms can be readily detected by MS. To evaluate the potential of the LC-MS method for identification of PS-ODN backbone oxidation, an all-PS 20-mer was spiked with monophosphodiester PO/PS 20-mer (Fig. 7). The total ion count was generated by all ions detected in the range of 400–2000 m/z (Fig. 7A). Both oligonucleotides eluted at approximately 6 minutes. The inorganic salts and other components eluting from

the column within the first 2 minutes of analysis were diverted from the mass spectrometer source by a switching valve. Deconvoluted mass spectrum of the entire TIC peak confirms the presence of two components (Fig. 8), all-PS 20-mer (MW 6580 Da) and 20-mer monophosphodiester (MW 6564 Da). The performance of the mass spectrometer is more than adequate for resolution of a mass difference of 16 Da, even when analyzing longer oligonucleotides. In order to quantify the monophosphodiester content in the all-PS-ODN, one can selectively extract the mass of the characteristic ion for a given component (Fig. 7B,C). However, each oligonucleotide produced at least 10 charged states of various intensities. We selected the (-5) charge state and extracted the masses of 1312.09 Da (oxidized form, Fig. 7B) and 1314.9 Da (all-PS, Fig. 7C). Integration of the area under the peak provides for quantitative data. As seen from Figure 7, the S/N ratio of the minor component was quite poor ($S/N=3$) and did not allow for accurate measurement of its content, despite the fact that the all-PS-ODN was spiked with 5% of PO/PS 20-mer. By extracting and

TABLE 3. LC-UV-MS CHARACTERIZATION OF 21-MER PS-ODN WITH 4 × 4 2'-OMe MODIFIED TERMINI: COMPARISON OF UV AND MS QUANTITATION METHODS

Retention time (minutes)	Peak assignment	Oligonucleotide sequence	Measured mass (Da) ^a	Δ mass (Da) ^b	UV peak area (N-x/N) × 100 (%) ^c	TIC MS peak area (N-x/N) × 100 (%) ^c	Deconvoluted MS peak area (N-x/N) × 100 (%) ^d
12.96	N	UCG CAC CCA TCT CTC TCC UUC	6725.3	1.8	100.0	100.0	100.0
12.46	N-1	UCG CAC CCA TCT CTC TCC UU	3689.5	1.6	49.6	54.1	53.5
11.95	N-2	UCG CAC CCA TCT CTC TCC U	6053.7	1.8	27.1	32.6	31.3
11.37	N-3	UCG CAC CCA TCT CTC TCC	5717.2	1.5	11.8	15.6	12.9
10.70	N-4	UCG CAC CCA TCT CTC TC	5381.8	1.4	5.7	8.2	6.4
10.25	N-5	UCG CAC CCA TCT CTC T	5076.2	1.1	3.0	4.0	1.9
9.17	N-6	UCG CAC CCA TCT CTC	4756.1	1.1	4.4	5.2	6.9
8.27	N-7	UCG CAC CCA TCT CT	4450.7	1.0	3.1	3.8	5.7
7.40	N-8	UCG CAC CCA TCT C	4130.6	1.1	4.5	5.3	8.4
6.70	N-9	UCG CAC CCA TCT	3825.1	0.9	2.6	2.4	4.1
5.60	N-10	UCG CAC CCA TC	3504.8	0.9	2.4	2.0	3.2
5.01	N-11	UCG CAC CCA T	3199.4	0.7	1.7	—	—
3.91	N-12	UCG CAC CCA	2879.0	0.6	1.0	—	—
13.72	N+1	UCG CAC CCA TCT CTC TCC UUC	6778.0	—	15.0	17.0	—

^aLos G, A, T, C, and U phosphorothioate mononucleotides represent mass loss of 345.2, 329.2, 320.2, 305.2, and 306.2 Da, respectively. For 2'-OMe modified mononucleotides, add 30.0 Da.

^bDifference between measured and theoretical mass of oligonucleotide.

^cSee also Figure 5.

^dSee also Figure 9.

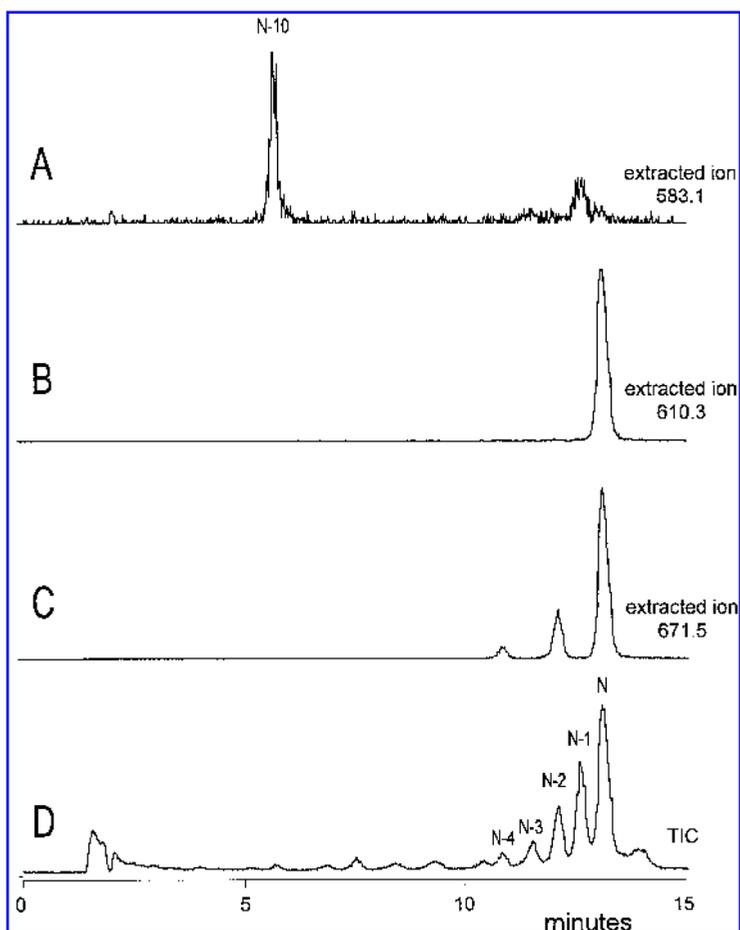


FIG. 6. Extracted MS chromatograms (A,B,C) and total ion count MS chromatogram (D) from analysis of 3' digested 21-mer PS-ODN with 4×4 2'-OME termini. Extracted ion 583.1, N-10 metabolite (A). Extracted ions 610.3 and 671.5 for 21-mer oligonucleotide are shown as chromatograms B and C, respectively. HPLC conditions are shown in Figure 5.

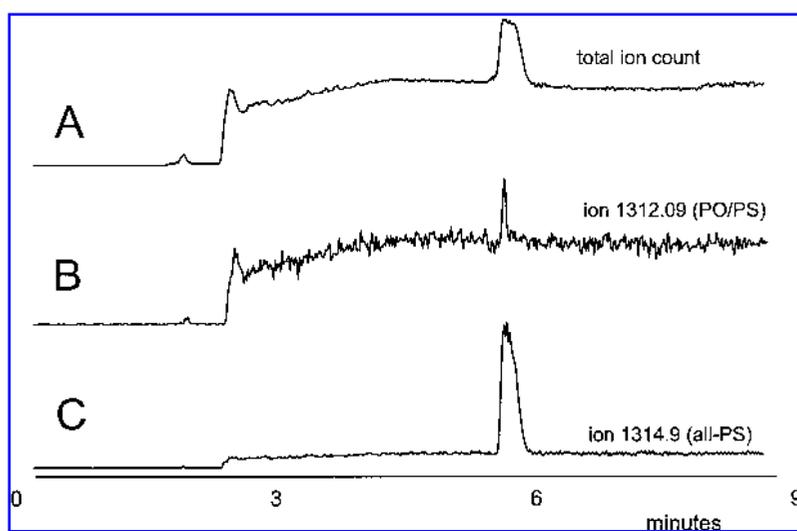


FIG. 7. LC-MS analysis of 20-mer PS-ODN backbone oxidation. (A) Total ion count MS signal; 20-mer peak elutes at ~ 6 minutes. (B) Extracted ion chromatogram corresponding to monophosphodiester and (C) to parent 20-mer PS. For HPLC conditions, see Figure 5. Gradient from 16.7% to 50% B in 10 minutes.

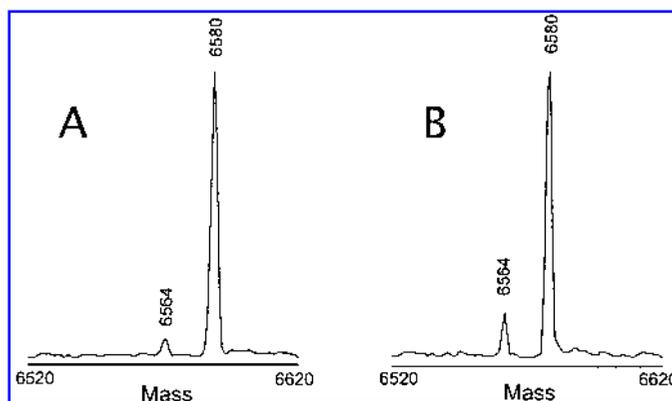


FIG. 8. Analysis of 20-mer PS oxidation using the deconvoluted ESI-MS signal. Spectrum (A) is 20-mer PS-ODN. (B) The same sample spiked with 5% of 20-mer monophosphodiester. The signal at 6580 Da represents the mass of all-PS 20-mer; 6564 Da is the signal of oxidized product (monophosphodiester).

summing more than one charge state, one can improve the limit of quantitation. Even then, <1% of the monophosphodiester form in the 20-mer PS was not detected. This makes analysis of trace level PO/PS contaminants by LC-MS impractical.

As an alternative approach, the entire peak (Fig. 7A) in the TIC chromatogram (5.4–6.1 minutes) was deconvoluted. Because all mass/charge states are employed in deconvolution, substantially better S/N ratios were obtained (Fig. 8). The area under the peaks representing molecular masses of the interrogated molecules was integrated, and the percentage of the minor component was calculated (all-PS area was considered to be 100%). A similar approach was applied by other authors for the characterization and quantitation of antibody conjugation (Adamczyk et al., 2000). Figure 8A shows the deconvoluted MS spectrum of the parent all-PS 20-mer oligonucleotide. Apparently, it contains a small amount of oxidation product that originates either from incomplete sulfurization during synthesis or from postsynthesis oxidation in solution. In a following experiment, the all-PS 20-mer was spiked with 1%, 5%, and 10% of synthetic monophosphodiester, and the mixtures were analyzed by LC-MS.

The deconvoluted spectra of the eluting peaks were used for quantitation of the oxidation product (Table 4). After subtraction of the background level of monophosphodiester (4.1%) present in the original all-PS-ODN, a good match was found between the expected and measured level of oxidation product. The practical LOQ can be enhanced by injecting greater amounts of oligonucleotide on the column or by directly infusing the well-desalted sample into the mass spectrometer and summing multiple spectra over a longer acquisition time. The LC-MS method is expected to be applicable as a quality control method for analysis of <1% of oxidation product in PS-ODNs. This is similar to or better than the LOQ of anion-exchange HPLC methods that are currently used.

One may notice that the 20-mer monophosphodiester is slightly less retained than the all-PS 20-mer (Fig. 7). Partial separation of di-, tri-, tetra- (etc.) phosphodiesters from the all-PS-ODN was observed. It is important to include the front part of the TIC chromatogram into deconvolution to compensate for the retention shift of higher oxidation products, whenever their quantitation is desirable (biologic samples). A sharper gradient will help to eliminate the retention time shift. In addition, well-de-

TABLE 4. LEVEL OF PS-ODN BACKBONE OXIDATION MEASURED BY MS

Sample	Monophosphodiester content (%)	Monophosphodiester content (%) after background subtraction ^a
PS 20-mer	4.1	0
PS 20-mer + 1% PO/PS	5.4	1.3
PS 20-mer + 5% PO/PS	10.1	6.0
PS 20-mer + 10% PO/PS	14.9	10.8

^aThe monophosphodiester (PO/PS) background level present in all PS 21-mer was subtracted (4.1%).

salted synthetic oligonucleotides can be directly infused into the mass spectrometer, which eliminates any retention shift.

The relative quantitation of a more complex oligonucleotide mixture using the deconvoluted spectrum is shown in Figure 9. The 21-mer PS 4×4 2'-OMe digested oligonucleotide (same sample as in Fig. 5) was injected on column and eluted with a ballistic gradient. All oligonucleotides eluted within a single peak. MS spectra were summed into one rather complicated spectrum. Despite the spectrum complexity, the deconvolution was successful and yielded a mass spectrum with oligonucleotide signals in similar ratios as seen in the chromatograms in Figure 5. The mass spectrum was integrated, and normalized peak areas were compared (Table 3). The results suggest that it is possible to use deconvoluted spectra for relative quantitation of oligonucleotides even for complex mixtures. However, the results need to be interpreted with caution, as the deconvolution algorithm may generate artificial harmonic noise and doubly charged signals.

As suggested earlier, using the resolving power of MS for selective extraction of m/z signals of chromatographically coeluting components may provide for false quantitation results (Fig. 6C). Extraction of specific components also requires the knowledge of their corresponding molecular masses, which is rarely the case in real-life analysis. The combination of efficient HPLC separation and UV/MS detection provides for a more robust method

for quantitation and characterization of therapeutic oligonucleotides.

Limits of LC-UV-MS detection for analysis of AS-ODNs

The use of a 50×1.0 mm microcolumn on a capillary HPLC system was suitable for routine analysis of 50–100 pmole of oligonucleotides. UV detection provides for significantly better S/N ratios than total ion or extracted ion mass chromatograms. Therefore, a tandem LC-UV-MS system was used to improve the limits of quantitation. The estimated LOQs by UV were <1 pmole of oligonucleotide injected on-column (S/N = 10). At that mass load, MS did not afford a detectable peak in TIC mode, but we were able to obtain a peak (S/N ~ 5) with a correct molecular mass by deconvoluting an MS signal in the appropriate peak elution time window.

The accuracy of quantitation using TOF-MS for oligonucleotides is comparable to quadrupole ESI-MS. However, we achieved better S/N with ESI-TOF MS. The relative quantitation was comparable to UV measurements (Table 3), despite the fact that shorter oligonucleotides have lower UV absorbance (smaller molar extinction coefficient) but greater MS signal (improved ionization efficiency).

Hypothetically, the LOQ of 1 pmole for 20-mer (MW ~ 6500 Da) represents an injection of 6.5 ng oligonucleotide on-column. Assuming 100% recovery in the

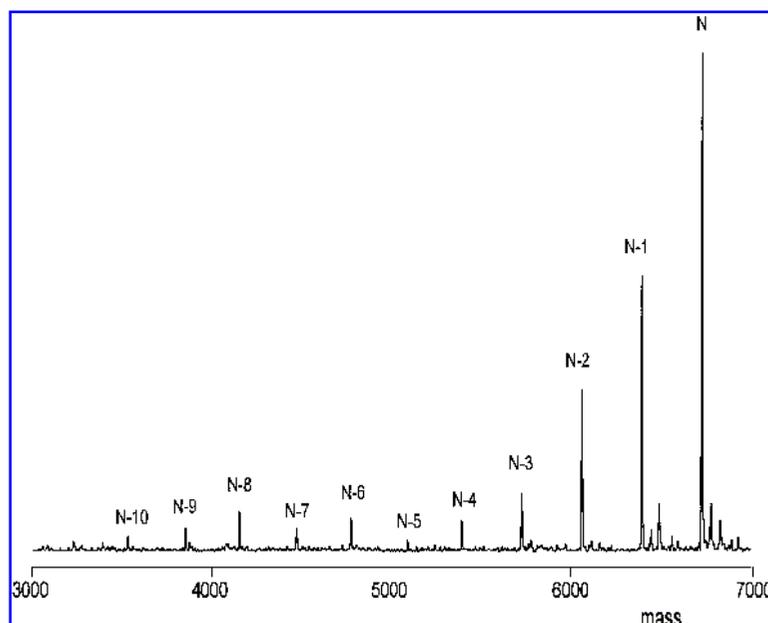


FIG. 9. MS spectrum for digested 21-mer PS-ODN oligonucleotide with 4×4 2'-OMe termini. The same sample was injected on column as in Figure 5. All oligonucleotides were eluted with ballistic gradient in a single peak. ESI-MS spectra were summed and deconvoluted. For relative oligonucleotide quantitation, see Table 3. HPLC conditions as in Figure 5; isocratic elution for 5 minutes at 0% B, then step gradient to 100% B.

sample preparation step for 1 ml of biologic liquid, the calculated LOQ is 6.5 ppb of oligonucleotide (concentration of 6.5 $\mu\text{g/L}$ or 1 nM). Realistically, 100% recovery is rarely achieved, and the amount of sample is usually limited. The use of smaller ID columns will provide a lower LOQ that may be required for clinical studies.

In conclusion, the LC-UV-MS method was applied for the quantitation and characterization of chemically modified AS-ODNs. To our knowledge, this is the only method suitable for highly efficient LC separation with sensitive on-line MS analysis of PS-ODNs. Accurate molecular weight determination of eluting components allows confirmation of the identity of oligonucleotide failure products and metabolites. The sensitivity of LC-MS is sufficient for clinical studies, although sample preparation and concentration are required. The use of capillary HPLC columns further improves the method sensitivity when desired. In the case of a complex pattern of failure products or metabolites, the molecular mass is not sufficient to characterize truncated oligonucleotides. For analysis of complex oligonucleotide mixtures, LC-MS/MS may be required for the identification of unknown components.

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