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Characterization of Oligonucleotide Metabolism *In Vivo* via Liquid Chromatography/Electrospray Tandem Mass Spectrometry with a Quadrupole Ion Trap Mass Spectrometer

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The pattern of nuclease degradation observed for an antisense phosphorothioate oligonucleotide in pig kidney was determined using liquid chromatography/electrospray mass spectrometry (LC/ESI-MS) and LC/ESI-MS/MS with a quadrupole ion trap mass spectrometer. Metabolites were separated by length using reversed-phase high-performance liquid chromatography with aqueous hexafluoropropan-2-ol–triethylamine and a methanol gradient. The individual masses of metabolites in each LC peak were determined via deconvolution and converted into potential nucleotide compositions. The nucleotide composition was used to locate metabolites within the known oligomer sequence. The identity of metabolites was confirmed using on-line LC/MS/MS to generate fragment ions suitable for sequence verification. A limited number of shorter oligonucleotide fragments were observed, suggesting that metabolism in vivo may be sequence dependent. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) are currently in clinical trials for the treatment of viral infections, cancer and inflammation.[1,2] These trials are based on preliminary animal studies which show that phosphorothioate (PS) ODNs reach their target cells and work in a sequence-specific manner to reduce the level of the desired mRNA and protein.[3–7] Stability studies and pharmacokinetic analyses have demonstrated that PS ODNs are metabolized rapidly in serum and tissues by 3′- and 5′-exonucleases, although the action of endonucleases or preliminary oxidation of the P–S bond cannot be ruled out.[8–13]

Prior studies of oligonucleotide metabolism have been performed using high-performance liquid chromatography (HPLC) or slab gel electrophoresis for the separation and radiolabels for detection. Information on metabolites is limited by the chemical location and metabolic fate of the radiolabel, and alternative methods have been developed to follow ODN metabolism without labels. Capillary gel electrophoresis (CGE) rapidly separates ODNs based on length. The sensitivity of capillary electrophoresis allows detection of drug in plasma and tissues down to nanomolar levels.[14] However, CGE provides no information regarding the sequences of the ODNs or other metabolic alterations which may have occurred *in vivo*. Hence the development of additional analytical methods to obtain information on sequence and/or metabolic alterations is desirable.

Electrospray mass spectrometry (ESI-MS) has been proven to be a gentle and sensitive method for the analysis of natural and modified nucleic acids.[15–20] Determination of molecular mass is straightforward, since ESI mass spectra for each ODN contain a distribution of charge states which can be deconvoluted. Mass measurements are complicated by the solution affinity of the polyanionic backbone for ubiquitous solution cations. These cations lower the sensitivity for the analyte by dispersing the ion abundance among multiple adducted species. Cation adduction can be reduced by sample preparation or through addition of chelators or organic bases to the mobile phase.[17,18,21,22]

It has been demonstrated that strong anion-exchange chromatography combined with reversed-phase HPLC can be used to fractionate RNA and oligodeoxynucleotides.[13,23] However, the oligomer fractions have been pooled prior to ESI-MS analysis. Direct HPLC/ESI-MS has been used to separate and analyze oligodeoxynucleotide phosphodiesters, but length-based separation of modified ODNs such as methylphosphonates and phosphorothioates has proved elusive.[24,25] Phosphorothioate ODNs also have a greater avidity for cation adduction relative to phosphodiester ODNs.[21] Gaus and co-workers[13] have used LC/ESI-MS for the characterization of PS ODN metabolites. Limited

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length-based separation of metabolites was obtained with a C_{18} reversed-phase column, but >15 metabolites could be identified based on accurate measurement of molecular mass. However, significant overlap among the multiply charged ions from each species limited the quantitation and identification of many metabolites.

We have extended these prior studies of PS ODN metabolism in kidney using LC/ESI-MS and LC/ESI-MS/MS with a quadrupole ion trap mass spectrometer. Metabolites were fractionated and desalted using strong anion-exchange and reversed-phase chromatography. Two pooled fractions were separated and analyzed by reversed-phase LC/ESI-MS. The structures of metabolites were assigned from accurate measurements of molecular mass and via sequence confirmation from fragment ions.[26–31] The formation of fragment ions from collisionally activated precursor ions with low charge states benefits from the high transmission efficiency of the ion trap.[26] This feature, combined with the tandem in time nature of the ion trap, provides high sensitivity for the LC/ESI-MS/MS analysis of metabolites.[32] Sequence confirmation was obtained for many metabolites from only 480 pmol of injected sample. Masses of individual metabolites were determined from LC peaks estimated to contain <25 fmol μl^{-1} of material. LC/ESI-MS and -MS/MS provide a more detailed identification of PS oligonucleotide metabolism *in vivo* compared with methods using radiolabels or size-based separation techniques.

EXPERIMENTAL

Animal Studies

Animal studies were carried out by H.T.I. Bioservices (Ramona, CA, USA) and conducted entirely under the FDA Good Laboratory Practice Regulations (21 CFR Part 58). Female Yucatan minipigs (60–80 kg) were dosed subcutaneously with Isis 2302 (GCCCAAGCTGGCATCCGTCA; M_r 6369.2 Da) at 300 mg per day for 8 days. Organs were removed 24 h after the final dose and stored at −80 °C.

Oligonucleotide and metabolite extraction and purification

One pig kidney was homogenized using a commercial blender in 250 ml of 4 M guanidinium thiocyanate, 0.5% NP-40, 50 mM Tris–HCl (pH 9.0), 100 mM NaCl and 250 ml of phenol–chloroform (1:1). The aqueous layer was removed and the phenol–chloroform layer was re-extracted with 250 ml of 15% ammonia solution. The aqueous layers were combined, extracted with 250 ml of chloroform and precipitated with three volumes of ethanol. The precipitated material was dissolved in water and filtered through a 0.2 μm filter prior to HPLC purification.

Anion-exchange HPLC fractionation was accomplished using a BioCad HPLC system and a Poros II HQ/M 100 × 25.4 mm i.d. column (PerSeptive Biosystems, Framingham, MA, USA). Phosphorothioate ODN metabolites were separated using a linear gradient from 0 M NaBr, 10 mM Tris–HCl (pH 9.0) to 1.5 M NaBr, 10 mM Tris–HCl (pH 9.0) over 40 column volumes at a flow rate of 20 ml min^{-1}. Fractions containing phosphorothioate oligonucleotide and metabolites were collected (~45 ml each and brought to ~0.1 M triethylammonium actetate (TEAA) (pH 7.0) by the addition of 5 ml of 1 M TEAA. Each fraction was then desalted using a Poros II R/H 100 × 4.6 mm i.d. column. The column was equilibrated with 0.1 M TEAA (pH 7.0) and each anion exchange fraction was loaded manually using a 50 ml superloop (Pharmacia). After loading, the column was washed with water until the conductivity trace showed no further decrease. The column was then washed with 1 mM triethylamine (TEA) until the pH trace stabilized (between pH 10 and 11). Bound phosphorothioate oligonucleotide and metabolites were then eluted with 20% acetonitrile. The eluted material was evaporated to dryness and resuspended in water.

Capillary electrophoresis

Desalted metabolite fractions were analyzed using a Beckman P/ACE 5010 capillary electrophoresis instrument. Electrophoresis was carried out using an ssDNA-R kit (Beckman Instruments, Fullerton, CA, USA). Capillaries were cut to 27 cm total length (20 cm to the detector). Analyses were carried out with an applied voltage of 15 kV at 30 °C.

HPLC

An HPLC system composed of two Ultra Plus pumps and controller (Micro-Tech; San Jose, CA, USA) was interfaced to the mass spectrometer through 15 cm of fused-silica capillary column (150 μm i.d.). The resuspended oligonucleotide fractions were loaded on to a 150 × 1.0 mm i.d. column packed with Intertsil 5 μm C_{18} (Micro-Tech; San Jose, CA, USA). The samples were eluted from the column and infused directly into the electrospray ion source at 20 μl min^{-1} using aqueous 0.4 M hexafluoropropan-2-ol (HFIP) adjusted to pH 7.0 with triethylamine and a linear gradient of 20–60% methanol containing 0.4 M HFIP over 45 min.[33]

Electrospray ionization mass spectrometry

Mass spectra were acquired using an LCQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MATT; San Jose, CA, USA). Preliminary LC/MS studies demonstrated that no PS oligonucleotide products eluted during the first 25 min of the LC gradient. In subsequent experiments, acquisition of MS data was initiated 25 min into the HPLC gradient, and times shown on the LC/MS traces are relative to the beginning of acquisition. The spray needle voltage was set to −4.4 kV and the spray stabilized with a sheath flow at 70 psi and a 15 psi auxiliary gas flow (both 60:40 N_2–O_2). The stainless-steel inlet capillary was heated to 190 °C. A mass range of m/z 600–1850 was scanned in 0.15 Da steps over 5.0 s

and typically 12 averages were summed during the elution of each LC peak. Calculated molecular masses were accurate to ± 1.0 Da. In all cases, helium was admitted to the vacuum system to a pressure of 1 mTorr (uncorrected), and the instrument was operated at a background pressure of 2×10^{-5} Torr. The automatic gain control feature was used to set the ionization time (20–200 ms). In no case did an LC peak produce sufficient ions to 'fill' the ion trap. In MS/MS experiments, the most abundant 'low' charge state was isolated via resonance ejection. Following isolation, the ions of interest were subjected to collisional activation by applying an additional r.f. voltage to the end caps. This voltage was increased manually to a value which minimized the intensity of the parent ion and generated the highest abundance of fragment ions. The mass/charge scale was calibrated using the $[M - 11H]^{11-}$ through $[M - 4H]^{4-}$ charge states of Isis 2302.

Determination of oligonucleotide composition from molecular mass

A computer program was developed to generate lists of deoxyoligonucleotide base compositions for a given input molecular mass, mass tolerance and ODN backbone composition. The output base compositions are ordered via the calculated mass difference from the input mass. The program was written and compiled in C^{++} using MetroWerks CodeWarrior on a MacIntosh PowerPC 7200.

RESULTS

Following extraction from tissue, Isis 2302 and metabolites were fractionated by anion-exchange chromatography to remove contaminating RNAs and proteins prior to LC/MS analysis. Two fractions from the anion-exchange separation were desalted and concentrated, enriched with shorter (Fraction A) and longer metabolite sequences (Fraction B), respectively. Capillary gel electrophoresis studies were performed to confirm the lengths of the metabolites present in each fraction and the results are presented in (Fig. 1). A signal which comigrates with intact Isis 2302 is observed in both fractions. Fourteen-base oligonucleotides are the most abundant constituents in Fraction A, but 12–20mers are observed. Isis 2302 is the most abundant component of Fraction B, which contains ODNs ranging from 17–21 bases in length. The same concentrated fractions were used for LC/ESI-MS.

Fraction A

The LC/ESI-MS total ion current from an injection of 0.1 OD units of Fraction A as a function of retention time is presented in Fig. 2. The TIC is comprised of four major peaks, with smaller abundances of leading and trailing species. The extracted ion currents for m/z values generated by oligonucleotides of different lengths also are presented. The relative abundances of metabolites observed by CE and LC/ESI-MS are in qualitative agreement.

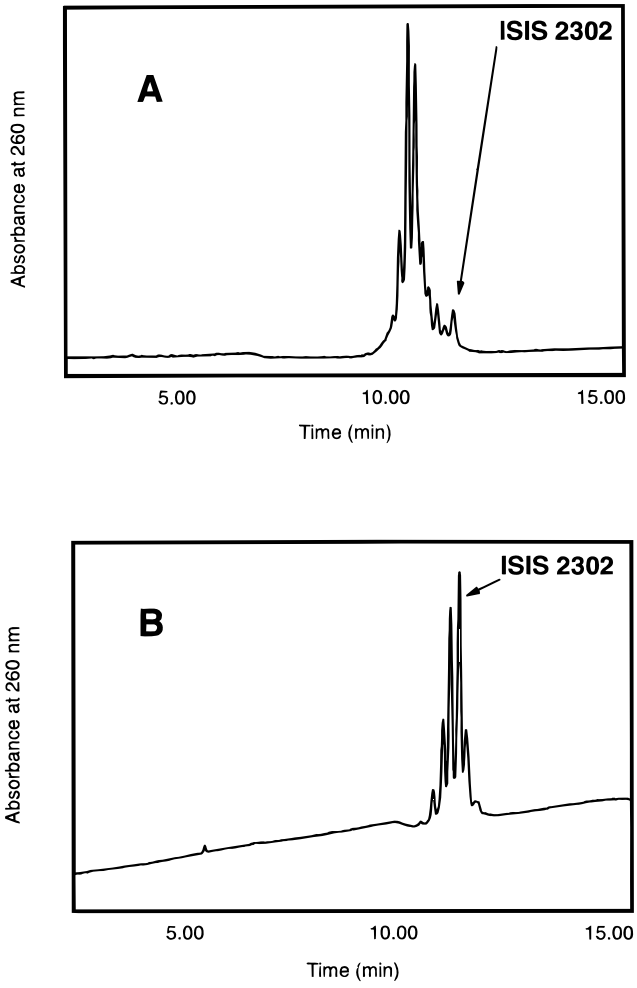

Figure 1. Capillary electrophoretic separations of oligonucleotide metabolites present in (A) Fraction A and (B) Fraction B isolated by strong anion-exchange chromatography.

Ions which correspond to a mass of 3477.4 Da are generated by an 11mer metabolite which elutes over a 1 min interval prior to the peak at 2.82 min. This peak comprises less than 0.1% of the injected material (0.06 ODs contain $\sim$480 pmol of '14mer') or $\sim$480 fmol of 11mer.

The ion current from 2.1 to 3.4 min in the TIC corresponding to the first major peak was summed to produce the mass spectrum shown in [Fig. 3(A)]. The m/z values of the four most abundant charge states were deconvoluted to give a molecular mass of 3814.5 Da. These charge states comprise $>90\%$ of the ion current in the LC peak. A search of all potential base compositions for this mass yields four 12mer PS candidates, listed in Table 1. Two potential compositions contain five and six dAs, while the starting Isis 2302 only contains four dA residues. Similarly, a mass of 3813.5 Da, within the experimental mass error for the metabolite, could only have been generated by a 12mer containing four dT residues. These base compositions are inconsistent with simple degradation from Isis 2302. The potential formulas containing a single PO linkage require either no dA or four dT residues, also inconsistent with Isis 2302. The formula $d(A_1G_4C_4T_3)$ most likely arises

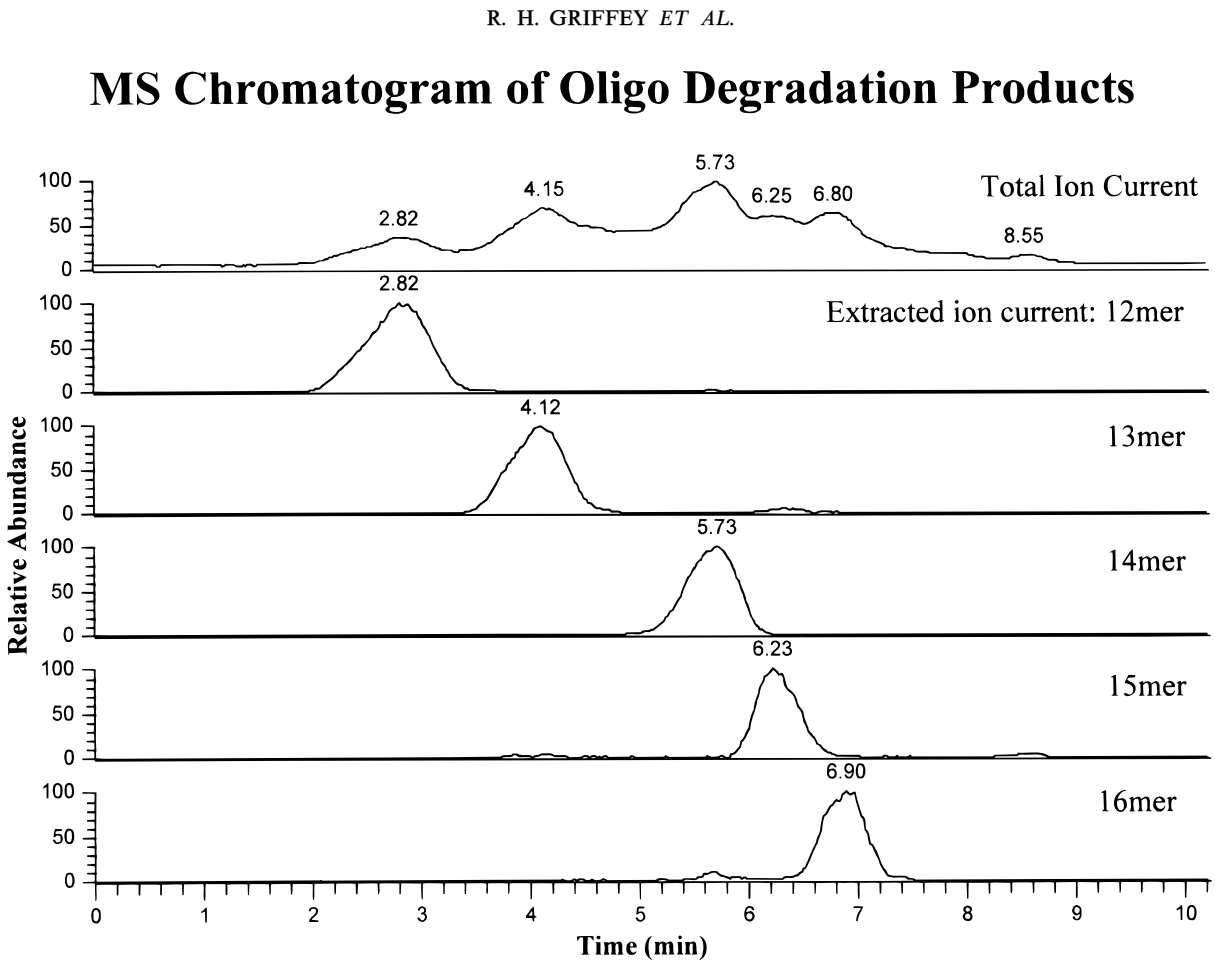

Figure 2. Total ion current and selected ion chromatograms measured for Fraction A as a function of LC retention time. A, Total ion current; B, ion current from *m/z* 762.0 produced by the n − 8 metabolite; C, ion currents from *m/z* 685.8, 683.1 and 689.9 produced by the n − 7 metabolites; D, ion current from *m/z* 740.8 produced by the n − 6 metabolite; E, ion current from *m/z* 793.1 and 795.5 produced by the n − 5 metabolites; F, Ion current from *m/z* 843.9 produced by the n − 4 metabolite.

from a 12mer produced by loss of two nucleotides from the 3′-end of Isis 2302 and six nucleotides from the 5′-terminus.

The sequence of this 12mer metabolite was confirmed using LC/MS/MS. The MS/MS experiment was performed by isolating the $[M - 3H]^{3-}$ charge state at *m/z* 1270.8 and the resulting spectrum is presented in Fig. 3(B). The most abundant fragment ion at *m/z* 1220.1 is generated by loss of neutral guanine from the oligonucleotide. Ions from loss of adenine and cytosine also are observed. Assignments and masses for the fragment ions are listed in Table 2. Following the accepted nomenclature, the most diagnostic ions arise from fragmentation which produces w series ions (used to determine sequence in the 3′ → 5′ direction; ions terminate with a 5′-phosphate) and a-Base series ions (used to determine sequence in the 5′ → 3′ direction; ions terminate with a furan on the 3′-side).[26,27] Eight w series ions and five a-Base series ions could be identified in the spectrum, along with several ions produced by secondary loss of a neutral base from w-series fragment ions. Further increases in the activating r.f. voltage decreased the abundance of the diagnostic fragment ions.

The most abundant ions in the LC peak at 4.15 min correspond to a mass of 4119.7 Da. The most likely base compositions are the 13mers $d(A_6GC_3T_3)$ and

Table 1. Potential base compositions for the metabolite with M_r 3814.5 Da

Mass (calc.)	ΔM_{obs}	Formula[a]
3814.5	0.03	**$d(A_1G_4C_4T_3)$**
3814.6	0.10	$d(\mathit{A}_6GC_2T_3)$
3813.5	1.00	$d(A_2G_3C_3\mathit{T}_4)$
3815.6	1.11	$d(\mathit{A}_5G_2C_3T_2)$
3813.4	1.14	$d(A_1G_4C_3\mathit{T}_4)$ (1 PO)

[a] The base compositions in italics are inconsistent with the base composition of Isis 2302.

Table 2. Assignment of fragment ions from MS/MS collision-induced dissociation of *m/z* 1270.8

m/z	Ion assignment	Sequence
681.8	$w_2^{(1-)}$	GT-3′
987.1	$w_3^{(1-)}$	CGT
642.1	$w_4^{(2-)}$	C CGT
805.7	$w_5^{(2-)}$	TC CGT
970.2	$w_6^{(2-)}$	ATC CGT
1123.2	$w_7^{(2-)}$	C ATC CGT
1295.1	$w_8^{(2-)}$	GC ATC CGT
1187.1	$w_{11}^{(3-)}$	CT GGC ATC CGT
1067.1	a_4-$B^{(1-)}$	5′-GCT G
705.1	a_5-$B^{(2-)}$	GCT GG
1030.7	a_7-$B^{(2-)}$	GCT GGC A
1261.4	a_8-$B^{(2-)}$	GCT GGC AT
1004.7	a_{10}-$B^{(3-)}$	GCT GGC ATC C
1220.1	-GH	GCT GGC ATC CGT

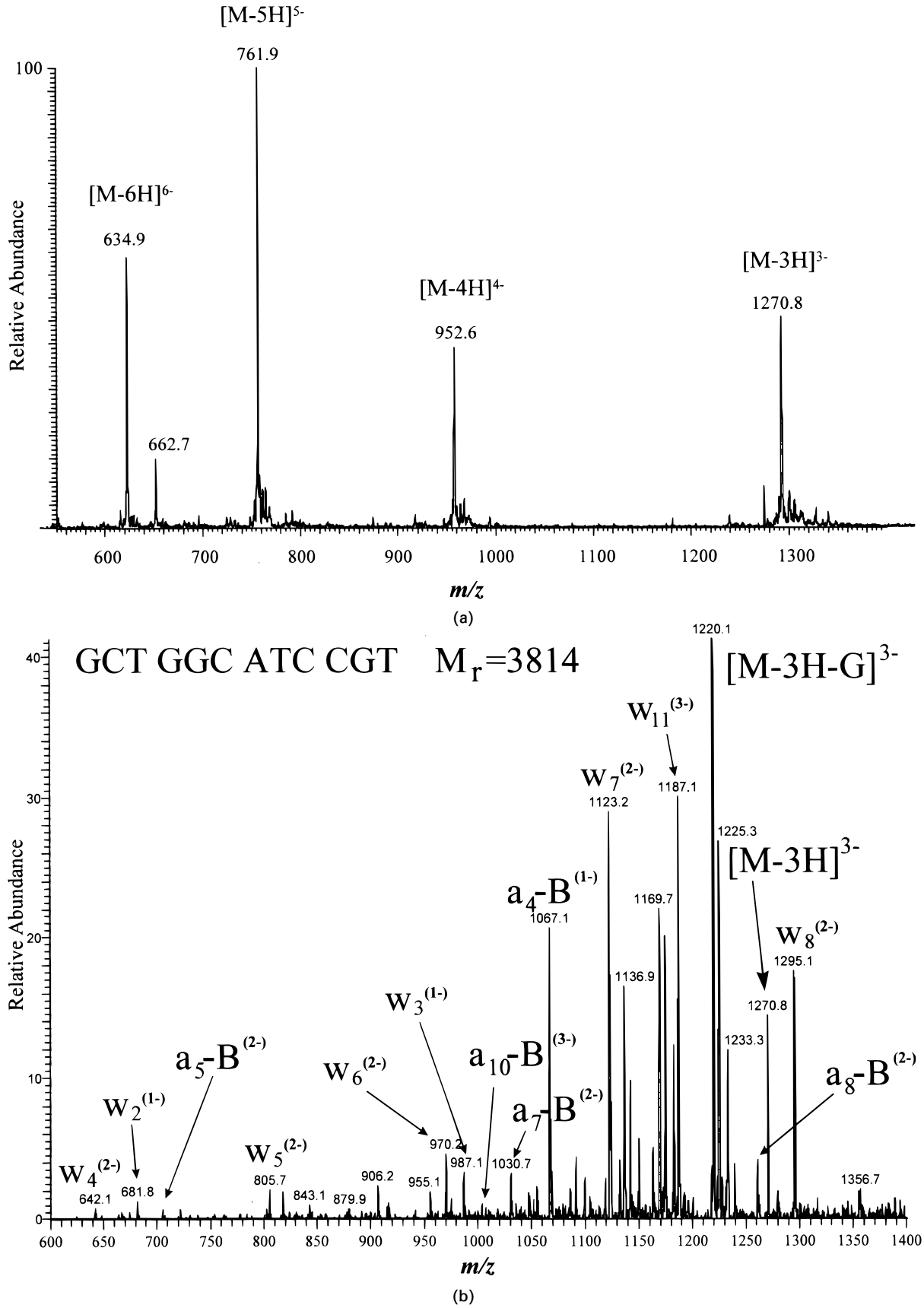

Figure 3. LC/ESI mass and tandem mass spectra obtained from a 12mer oligonucleotide. A, Raw mass spectrum obtained from 2.1 to 3.4 min in the liquid chromatogram; B. tandem mass spectrum produced following isolation of the ion at *m/z* 1270.8. A window of 1.5 Da was employed for ion isolation. A mass range of *m/z* 450–1750 was scanned in the second dimension. Ion masses and assignments are listed in Table 2.

Table 3. Observed masses and sequences of metabolites from Isis 2302 detected using LC/MS

Mass (obs.)	Sequence[a]
3477.7	GGC ATC CGT CA
3494.1	GCT GGC ATC CG
3814.5	**GCT GGC ATC CGT**
4119.7	GCT GGC ATC CGT C
4143.8	A GCT GGC ATC CGT
4103.8	CT GGC ATC CGT CA
4449.5	**GCT GGC ATC CGT CA**
4778.2	**A GCT GGC ATC CGT CA**
4763.3	**GCC CAA GCT GGC ATC**
5069.2	GCC CAA GCT GGC ATC C
5414.5	GCC CAA GCT GGC ATC CG
5734.7	GCC CAA GCT GGC ATC CGT
6039.9	GCC CAA GCT GGC ATC CGT C
6369.2 (Isis 2302)	GCC CAA GCT GGC ATC CGT CA
6716.4	GCC CAA GCT GGC ATC CGT CA*G*

[a] Italics base represents addition of a guanosine phosphate to the 5′- or 3′-terminus, or addition of a deoxyguanosine phosphorothioate to the 3′-terminus. Underline indicates two possible formulas, with indicated bold sequence confirmed by MS/MS experiments.

$d(AG_4C_5T_3)$, with the latter corresponding to addition of a dC to the 3′-terminus of the 12mer (Table 3). A second 13mer with mass 4143.8 Da results from elongation of the 12mer sequence by a dA in the 5′-direction. A third 13mer which elutes at 4.5 min in the chromatogram gives a mass of 4103.8 Da. This mass is consistent with the formula $d(A_2G_3C_5T_3)$, which could arise only from loss of seven bases exclusively from the 5′-end of the oligomer.

One 14mer having mass 4449.5 Da is observed with high abundance at 5.73 min, along with the potassium-adducted ion. This molecule, with a most likely base composition of $d(A_2G_4C_5T_3)$, could be produced by loss of six nucleotides from the 5′-terminus or five nucleotides from the 5′-terminus and the 3′-dA. LC/MS/MS was performed on the $[M - 3H]^{3-}$ charge state at m/z 1482.1. The $w_2^{(1-)}$ and $w_3^{(1-)}$ fragment ions corresponding to a sequence with TCA-3′ were detected along with the a_4-$B^{(1-)}$, a_5-$B^{(1-)}$ and a_6-$B^{(2-)}$ fragment ions, confirming the sequence listed in Table 3. None of the possible w series or a-Base series fragment ions expected from the alternative sequence with a 5′-dA residue were observed. A small amount of 14mer oligonucleotide generated by loss of five bases from the 5′-terminus and dA from the 3′-terminus elutes between the 13mer and 14mer signals at 5.25 min in the TIC.

The elution pattern for the 15mer metabolites is complex. A signal is observed at 6.2 min in the chromatogram which generates ions from an oligomer with a mass of 4763.3 Da. This could be generated by loss of five bases from the 3′-terminus, or by loss of three bases from the 3′-terminus and two bases from the 5′-

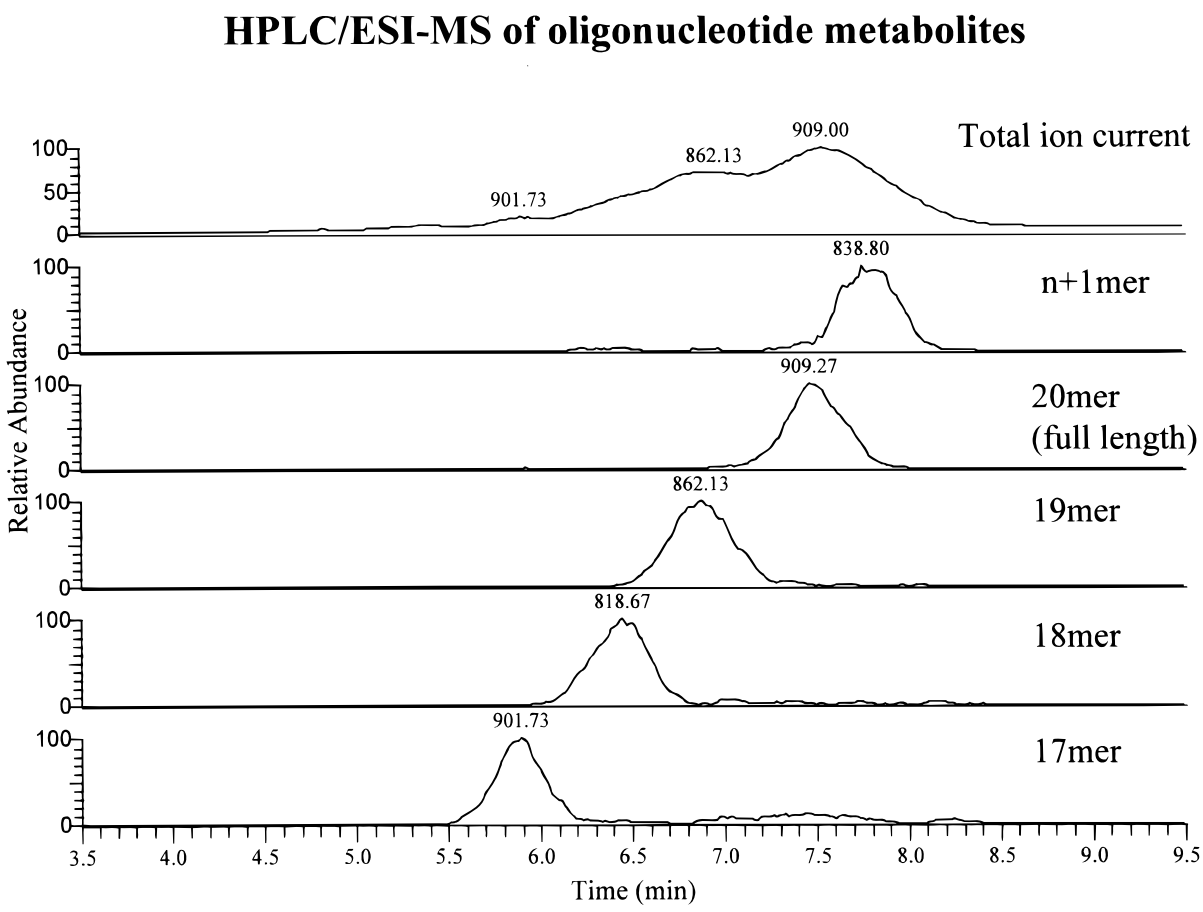

Figure 4. Total ion current and selected ion chromatograms obtained from Fraction B as a function of LC retention time. A, Total ion current; B, ion current from m/z 838.8 produced by the n + 1mer metabolite; C, ion current from m/z 909.3 produced by Isis 2302; D, ion current from m/z 862.1 produced by the 19mer metabolite; E, ion current from m/z 818.7 produced by the 18mer metabolite; F, ion current from m/z 901.7 produced by the 17mer metabolite.

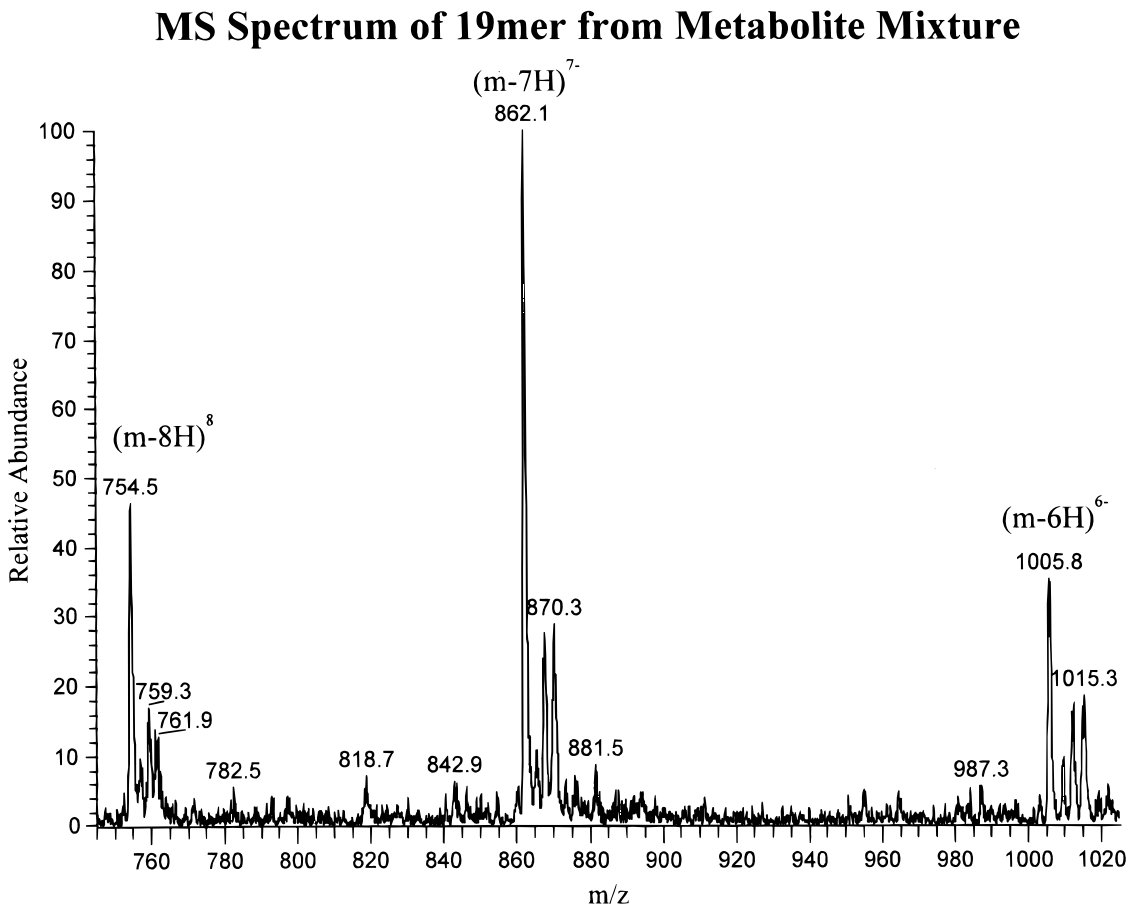

Figure 5. LC/ESI mass spectrum obtained for a 19mer metabolite with mass 6039.9 Da. Additional signals are observed from the sodium, potassium and iron adducts at higher values of *m/z*. However, no signals are observed at lower *m/z* values which might be expected if the PS linkages had been oxidized *in vivo*.

terminus. Additional ions which correspond to a mass of 4819.2 Da are detected. No known base composition for a simple phosphorothioate can generate this parent mass. However, a cleavage pattern which leaves a 5′- or 3′-thiophosphate and removes four bases from the 3′-terminus and one from the 5′-terminus could produce molecules with a mass of 4819.2 Da, as could an adducted iron atom. LC/MS/MS analysis of the $[M - 3H]^{3-}$ charge state generates a-Base series fragment ions corresponding to a 5′-terminus of 5′-GCCCA without a 5′-phosphorothioate. The w series fragment ions confirm a sequence of GCATC-3′ without a phosphorothioate at the 3′-terminus. Hence, the 'metabolite' with mass of 4819.2 Da may be generated by the iron adduct of the 15mer with mass 4763.3 Da, although addition of a fragment with mass 56 Da to an internal nucleotide unit cannot be ruled out. The electrospray ionization process is the most likely source of the adducted iron atoms.

An additional 15mer is detected at 4778.2 Da at 6.8 min in the chromatogram. This mass could originate from metabolites with a 3′-ACT or a 3′-CTG. LC/MS/MS of the $[M - 3H]^{3-}$ charge state at *m/z* 1592.1 was consistent with a 3′-terminus of 3′-ACT and a 5′-terminus of 5′-AGC. While the $w_1^{(1-)}$ fragment ion was not observed, the 3′-position of the dA could be inferred from the low abundance of the fragment ion from the $w_3^{(1-)}$ ion generated by loss of adenine. It has been noted previously that loss of adenine from the 3′-residue is unfavored.[26] No discrete ions are detected for metabolites produced by loss of four 3′-nucleotides and one 5′-nucleotide or loss of five 5′-nucleotides.

Among the 16mer metabolites, only ions corresponding to loss of four nucleotides from the 3′-terminus or loss of three nucleotides from the 3′-terminus and the 5′-dG (isobaric) were detected.

Fraction B

The ion chromatogram from fraction B containing longer metabolites (N + 1 to N − 3) is presented in Fig. 4. The length-based separation is not as great as observed for the shorter metabolites, but the resolution is adequate for identification of all masses listed in Table 3. Intact Isis 2302 is detected at 7.5 min and sequential loss of 3′-nucleotides can be observed as metabolites which elute at shorter retention times. The most abundant ion from Isis 2302 is the $[M - 7H]^{7-}$ charge state and $[M - 4H]^{4-}$ through $[M - 9H]^{9-}$ charge states are observed. Ions with adducted iron are observed at M + 56 Da for some of the charge states. The sole 19mer metabolite has a mass of 6039.9 Da, corresponding to loss of the 3′-terminal dA. The metabolite corresponding to loss of the 3′-C and A deoxynucleotides with mass of 5734.7 Da is observed at 6.5 min in the ion chromatogram. Loss of the three 3′-TCA residues produces ions from the $[M - 4H]^{4-}$ through

$[M - 7H]^{7-}$ charge states for a mass of 5414.5 Da. An expanded view of the spectrum is presented in (Fig. 5). No signal intensity is seen at M − 16 Da, which would be generated by metabolic desulfurization of the oligomer at a single site. No metabolites are detected which can be assigned to loss of any nucleotides from the 5′-terminus.

Additional metabolites can be observed at longer retention times in the ion chromatogram. These have been ascribed to 'longmer' metabolites corresponding to addition of nucleotide(s) to the starting oligomer.[13] The major N + 1 metabolite generates a mass of 6716.4 Da, resulting from addition of a riboguanosine phosphate to the 3′- or 5′-terminus, or possibly by addition of a deoxyguanosine phosphorothioate to the 3′-terminus. Attempts to fragment the $[M - 6H]^{6-}$ charge state of this species produced ions corresponding to loss of adenine, guanine and cytosine, with no sequence-specific w or a-Base ions observed. Other N + 1mers with masses of 6676.5 and 6699.6 Da are present, at about 50 and 30% of the intensity observed for the N + G oligonucleotide. These are consistent with addition of rC/rU and rA, respectively, to Isis 2302.

DISCUSSION

ESI-MS analysis of PS ODNs and their metabolites provides insight into the processing of this new class of therapeutic agent *in vivo*. The oligomers can be separated from tissue proteins and RNA using strong anion-exchange chromatography with some length-based separation. The collected fractions are desalted via a preliminary bulk HPLC elution. The concentrated oligonucleotide metabolites are ready for analysis using CE, MS or other methods. Conventional enzymatic sequencing of metabolites from PS ODNs is difficult, given the enhanced stability of the PS linkage relative to phosphodiesters. Hence, development of a rapid protocol which separates metabolites and provides sequence identification and confirmation would facilitate the pharmacokinetic characterization of this class of therapeutic agent.

A length-based resolution of the majority of PS ODN metabolites is obtained using a solvent system of aqueous hexafluoropropan-2-ol–triethylamine and a methanol gradient. The abundances of the peaks in the HPLC trace correlate well with the CGE profile. LC/ESI-MS provides information on length, sequence and chemical modification rather than length alone. Adduction of cations to the PS ODNs is not significant, even though the samples were eluted from the strong anion-exchange medium with 1.5 M NaBr.

Accurate mass measurement is useful for the direct determination of the nucleotide composition. For many of the metabolites, the potential sequences could be narrowed down to only a few possibilities based on the known sequence of the starting compound. Once a fixed sequence point has been established for one length, many longer sequences can be determined from base composition alone by extension in the 3′ or 5′ directions. This strategy has been used successfully for characterization of ribosomal RNA fragments obtained by digestion.[17,23]

The sequences of short (<16) PS oligonucleotides can be confirmed via MS/MS during the LC infusion. Previous studies indicate that PS ODNs generate fragment ions suitable for sequence confirmation.[31] Although a complete set of w or a-Base series ions were not generated in these experiments, enough unique product ions are obtained for sequence verification. More fragment ions have been obtained for shorter oligonucleotides using ion trap and tandem quadrupole instruments.[26,27,34] For longer oligonucleotides, the nucleotide composition of the 'missing' portion unavailable from fragment ions often can be established from the net unsequenced mass.[35] The MS/MS data differentiates among possible overlapping sequences which have the same base composition. In addition, MS/MS can be used to verify the identity of cation-adducted species which may co-elute with major metabolites.

LC/ESI-MS and -MS/MS studies highlight the complexity of PS ODN metabolism *in vivo*. Sequential 3′-degradation is detected among the metabolites present in Fraction B. These metabolites may be generated by the action of serum 3′-exonucleases prior to uptake by the kidney. No products from 5′-exonuclease activity are detected in this fraction. Only a few metabolites are observed in Fraction A from among the large number of possibilities which might be generated by the random action of 3′- and 5′-exonucleases. For example, only one 12mer and one major 14mer ODN metabolite are detected from pig kidney. Hence, renal nuclease activity may have some degree of sequence specificity. The paucity of sequences which have been partially degraded from the 5′-terminus in Fraction A suggests that 5′-endonuclease degradation may be significant.

CONCLUSIONS

LC/ESI-MS and LC/ESI-MS/MS with the quadrupole ion trap mass spectrometer provide a complete identification of oligonucleotide metabolites generated *in vivo*. The PS ODNs can be separated based on length using a C_{18} column with an aqueous solvent system containing 0.4 M hexafluoropropan-2-ol–triethylamine with a methanol gradient. The LC/ESI-MS analysis has good sensitivity for the less abundant species which are not observed using UV-based detection schemes. The identity of many metabolites can be established by comparing potential base compositions identified from the molecular mass with the base composition of the starting oligomer. In cases where ambiguity arises, LC/ESI-MS/MS can be used for sequence verification of oligomers up to 16 in length. As MS/MS methods are improved for longer oligomers, nearly every oligonucleotide metabolite separated in an LC chromatogram will be sequenced and identified unambiguously.

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