

Analysis of Oligonucleotides by HPLC–Electrospray Ionization Mass Spectrometry

Alex Apffel,^{*,†} John A. Chakel,[†] Steven Fischer,[‡] Kay Lichtenwalter,[†] and William S. Hancock[†]

Biomeasurements Group, Hewlett-Packard Laboratories, Palo Alto, California 94304, and California Analytical Division, Hewlett-Packard Company, Palo Alto, California 94304

A new interface procedure has been developed that allows, for the first time, the high-efficiency analysis of synthetic oligonucleotides up to 75 bases by reversed-phase HPLC and on-line electrospray ionization mass spectrometry. For oligonucleotides up to 30 bases in length, single-base resolution can be obtained with low levels of cation adduct formation in the negative ion electrospray mass spectra. A key part of the method uses 1,1,1,3,3,3-hexafluoro-2-propanol as an additive to the HPLC mobile phase, adjusted to pH 7.0 with triethylamine. This novel additive results in both good HPLC separation and efficient electrospray ionization. The broad potential of this new method is demonstrated for synthetic homopolymers of thymidine (PolyT), fragments based on the pBR322 plasmid sequence, and phosphorothioate ester antisense oligonucleotides. This approach will be of particular utility for the characterization of DNA probes and PCR primers and quality control of antisense compounds such as phosphorothioates and their metabolites, as well as of materials used in clinical trials.

The rapid growth of the human genome projects as well as novel therapeutics based on either recombinant DNA technology or direct gene therapy has resulted in widespread use of synthetic oligonucleotides in methodologies such as PCR, in situ hybridization, and in vivo genetic manipulations. In each case, the successful application of the technology requires extensive analytical characterization of the starting oligonucleotide substrate as well as the biological products. In addition, the therapeutic use of antisense oligonucleotides such as phosphorothioate derivatives requires a high degree of quality control and identification of minor impurities, as well as monitoring of metabolic products. This has resulted in expanded analytical demands for the characterization of these compounds. Until recently, applicable analytical tools have been limited to high-performance liquid chromatography (HPLC) and gel electrophoresis. While the separating power of these techniques, particularly the new format of capillary gel electrophoresis (CGE), is very impressive, the qualitative conclusions are based on retention or migration times and are not sufficient for absolute identification.

Previously, a number of high-resolution separation systems have been developed for oligonucleotides, but none has been directly compatible with electrospray ionization mass spectrometry. Ion pair reversed-phase HPLC is usually performed using a triethylammonium acetate buffer/acetonitrile gradient mobile phase

at pH 7.0, either on conventional silica-based ODS packing material^{1,2} or on polymeric materials³ such as alkylated polystyrene divinylbenzene materials. Under ideal conditions, oligonucleotides can be separated with 1–2 base resolution out to several hundred bases in length.⁴ Anion exchange chromatography using non-volatile salt gradients on DEAE materials has also been used for smaller fragments, although the resolution is generally poorer than that of reversed-phase separations.⁵ Either methodology uses mobile phases that contain salts which greatly reduce the efficiency of any subsequent mass spectrometric detection.

The advent of electrospray ionization mass spectrometry (ESI) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) for the analysis of large biopolymers has had a significant effect on the analyses of proteins and peptides and has begun to be more widely used for the analysis of oligonucleotides. Early studies with both techniques were somewhat hampered by ubiquitous cation adduction due to the high-affinity binding of Na⁺ and K⁺ to the polyanionic phosphate backbone of the nucleic acids. It has been subsequently shown,⁶ however, that the addition of strong bases such as triethylamine (TEA) or piperidine significantly suppresses adduct formation while dramatically increasing sensitivity for electrospray ionization. According to the most common ionization model for ESI, it is critical that the analytes exist as ions in solution; consequently, for the negative ion formation of oligonucleotides, pH 10 is near optimal. Furthermore, it has been shown⁶ that the presence of a strong organic base such as TEA effectively suppresses the sodium and potassium adduct formation via a displacement mechanism. ESI/MS has been shown with quadrupole analyzers to be useful for the analysis of oligonucleotides of length up to 120 bases,⁷ although most work to date has been performed on smaller fragments of 10–75 residues for naturally occurring and synthetic oligonucleotides,^{8–13} modified oligonucleotides,¹⁴ PCR

- (1) Kamel, A. M.; Brown, P. R. *Am. Lab.* **1996**, February, 40–46.
- (2) Fritz, H. J.; Belagaje, R.; Brown, E. L.; Fritz, R. H.; Jones, R. A.; Lees, R. G.; Khorana, H. G. *Biochemistry* **1978**, *17*, 1259.
- (3) Huber, C. G.; Oefner, P. J.; Bonn, G. K. *Chromatographia* **1993**, *37*, 653–658.
- (4) Oefner, P. J.; Bonn, G. K. *Am. Lab.* **1994**, June, 28C–28J.
- (5) Warrent, W. K.; Stanic, W. A.; Gorenstein, M. V.; Young, P. M. *Biotechniques* **1995**, *18* (2), 282–287.
- (6) Greig, M.; Griffey, R. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 97–102.
- (7) Limbach, P. A.; Crain, P. F.; McCloskey, J. A. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 27–39.
- (8) Bleicher, K.; Bayer, E. *Biol. Mass Spectrom.* **1994**, *23*, 320–322.
- (9) Bayer, E.; Bauer, T.; Schmeer, K.; Bleicher, K.; Maier, M.; Gaus, H.-J. *Anal. Chem.* **1994**, *66*, 3858–3863.
- (10) Ding, J.; Anderegg, R. J. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 159–164.
- (11) Cheng, X.; Gale, D. C.; Udseth, H. R.; Smith, R. D. *Anal. Chem.* **1995**, *67*, 586–593.

[†] Hewlett-Packard Laboratories.

[‡] California Analytical Division.

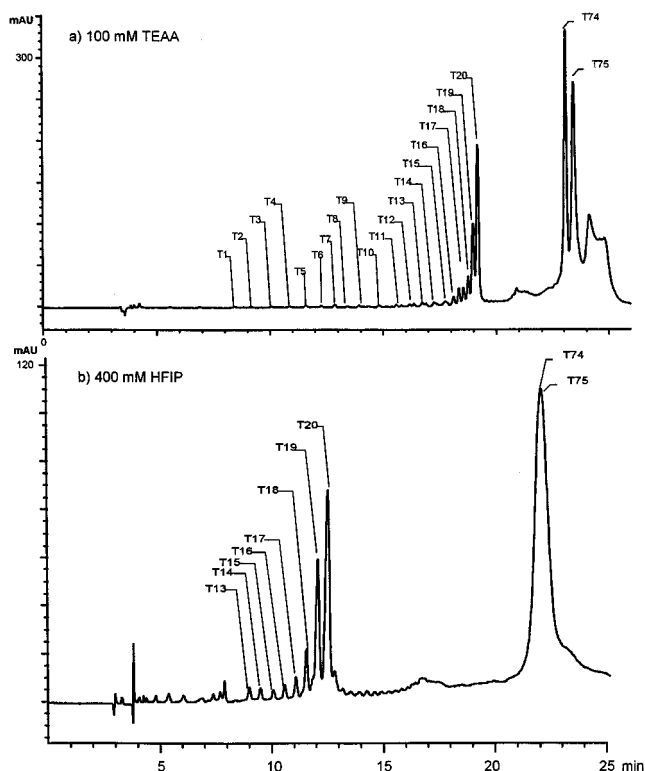


Figure 1. Typical separation of oligonucleotides using TEAA- and HFIP-based mobile phases. Synthetic mixture of PolyT oligonucleotides (19, 20, 74, 75) at 50 pmol/ μ L each component. UV detection at 269 nm. (a) 200 mM TEAA, pH 7.0, HPLC gradient 12–18% acetonitrile/30 min at 200 μ L/min, 35 $^{\circ}$ C. (b) 400 mM HFIP adjusted to pH 7.0 with TEA, HPLC gradient 25–40% methanol/30min at 200 μ L/min, 50 $^{\circ}$ C.

products,¹⁵ and antisense oligonucleotides.¹⁶ Direct ESI/MS can be used without initial separation to determine the molecular weight of oligonucleotides of small to moderate size as well as simple mixtures to a mass accuracy of approximately 0.02%. More complex mixtures, especially with extensive adduct formation and the presence of oligonucleotides of identical composition but different sequence, however, require an additional dimension of separation. Approaches to the combination of HPLC and ESI/MS have been reported^{17–19} but have recognized that the optimal mobile phases for HPLC result in drastic reduction in ion production for electrospray ionization, while the optimal solvents for electrospray ionization with minimization of adduct formation are not suitable HPLC separation. For example, approaches have focused on the combination of reversed-phase HPLC using triethylammonium acetate (TEAA) or similar ion pairing buffers which are not particularly volatile. The alternative use of ion exchange chromatography is even less amenable to electrospray ionization due to the presence of nonvolatile inorganic salts.

- (12) Limbach, P. J.; Crain, P. F.; McCloskey, J. A. *Curr. Opin. Biotechnol.* **1995**, *6*, 96–102.
- (13) Reddy, D. M.; Iden, C. R. *Am. Lab.* **1995**, June, 15–20.
- (14) Coleman, R. S.; Kesicki, E. A.; Arthur, J. C.; Cothman, W. E. *Bioorg. Med. Chem. Lett.* **1994**, *4* (15), 1869–1872.
- (15) Naito, Y.; Ishikawa, K.; Koga, Y.; Tsuneyoshi, T.; Terunuma, H.; Arakawa, R. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1484–1486.
- (16) Baker, T. R.; Keough, T.; Dobson, R. L. M.; Riley, T. A.; Hasselfield, J. A.; Hesselberth, P. R. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 190–194.
- (17) Bleicher, K.; Bayer, E. *Chromatographia* **1994**, *39* (7/8), 405–408.
- (18) Glover, R. P.; Sweeman, G. M. A.; Farmer, P. B.; Roberts, G. C. K. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 897–901.
- (19) Bothner, B.; Chatman, K.; Sarkisian, M.; Siuzdak, G. *Bioorg. Med. Chem. Lett.* **1995**, *5* (23), 2863–2868.

This report describes a method using a HPLC mobile phase containing 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) adjusted to pH 7.0 with triethylamine, which results in efficient HPLC separation and high-sensitivity electrospray ionization with a minimum of cation adduction. A mechanism is proposed that is based on the dynamic adjustment of the pH in the electrospray droplets due to a rapid removal of the volatile HFIP during desolvation.

EXPERIMENTAL SECTION

HPLC. The HPLC separation was performed on a Hewlett-Packard 1090 liquid chromatography system equipped with a DR5 ternary solvent delivery system, a diode array UV/visible detector (DAD), an autosampler, and a heated column compartment (Hewlett-Packard Co., Wilmington, DE). All HPLC separations were done using a YMC, Inc. (Wilmington, NC) 3 μ m particle, 120 \AA pore size ODS-AQ C18 reversed-phase column, 250 mm \times 2.1 mm i.d. Flow rates were 0.2 mL/min. Injection volumes were 1–2 μ L. The column temperature was maintained at 35 $^{\circ}$ C throughout the separation. UV absorbance was monitored at 269 nm with a 10 nm slit width, and the reference was at 480 nm with an 80 nm slit width. As described below in the discussion, two main gradient solvent systems were used: 100 mM TEAA pH 7.0/acetonitrile and 400 mM hexafluoro-2-propanol/methanol. The TEAA mobile phase was prepared with a dilution of a 1 M preformulated commercial buffer (Fluka BioChemika, Buchs, Switzerland) to 100 mM. The pH was measured to ensure pH 7.0. For this solvent system, the B buffer consisted of 50% acetonitrile and 100 mM TEAA. The HFIP mobile phase was prepared as a stock solution of 800 mM, adjusted to pH 7.0 with triethylamine. Approximately 1.2 mL of TEA is needed to titrate 1 L of 800 mM HFIP to pH 7. This stock solution was diluted to 400 mM with water for the A solvent and with methanol for the B solvent. All solvents were degassed ultrasonically.

ESI/MS. Mass spectrometry was done on a Hewlett-Packard 5989B quadrupole mass spectrometer equipped with an extended mass range, high-energy dynode detector (HED) and a Hewlett-Packard 59987A API electrospray source with high-flow nebulizer option. Both the HPLC and MS were controlled by the HP Chemstation software, allowing simultaneous instrument control, data acquisition, and data analysis. The high-flow nebulizer was operated in a standard manner, with N_2 as nebulizing (1.5 L/min) and drying (15 L/min at 300 $^{\circ}$ C) gases. The system was operated in negative ion electrospray mode. The use of the high-flow nebulizer negates the need for flow splitting or for scavenger gases such as oxygen or SF_6 . Typical source high-voltage settings were $V_{\text{cap}} = 4000$ V, $V_{\text{cyl}} = 3500$ V, and $V_{\text{end}} = 4000$ V. Typical source internal lens voltages were $\text{CapEx} = -191$ V, $\text{ESSkim1} = -30$ V, ion guide $V_b = -5$ V, ion guide $V_e = 46$ V, and entrance lens = -75 V. The HED detector was utilized with 10 kV bias voltage and electron multiplier voltage of 2500 V. MS data were acquired in raw scan mode scanning from 500 to 2000 Da at an acquisition rate of 1.0 Hz at 0.15 Da step size. Data were filtered in the mass domain with a 0.5 Da Gaussian mass filter and in the time domain with a 0.05 min Gaussian time filter. Unit mass resolution was maintained for all experiments, allowing unambiguous identification of -1 and -2 charge states. Unit resolution also allows high-charge states to be identified as >-2 .

Chemicals. HPLC grade water was purified using a MilliQ system (Millipore, Bedford, MA). HPLC grade methanol and acetonitrile were obtained from J. T. Baker (Phillipsburg, NJ). Triethylammonium acetate buffer was obtained from Fluka BioChemika, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was

Table 1. Oligonucleotide Samples

oligonucleotide	sequence	mass (Da)		error (%)
		calcd	exptl	
PolyT				
dT15		4500.98	4499.80	0.03
dT19		5717.76	5716.90	0.02
dT20		6021.96	6020.80	0.02
dT25		7542.94	7541.80	0.02
dT74		22448.55	22461.31	0.06
dT75		22752.75	22764.31	0.05
pBR322 Plasmid				
pBR322(10)	GGCATCGTGG	3084.05	3083.50	0.02
pBR322(11)	GGCATCGTGGT	3388.25	3387.50	0.02
pBR322(12)	GGCATCGTGGTG	3717.46	3716.75	0.02
pBR322(13)	GGCATCGTGGTGT	4021.65	4020.90	0.02
pBR322(14)	GGCATCGTGGTGTC	4310.84	4310.10	0.02
pBR322(15)	GGCATCGTGGTGTC	4624.05	4623.25	0.02
pBR322(19)	GGCATCGTGGTGTCACGCT	5835.82	5834.80	0.02
pBR322(20)	GGCATCGTGGTGTCACGCTC	6125.01	6123.40	0.03
pBR322(29)	GGCATCGTGGTGTCACGCTCGTCGTTTGG	8947.81	8945.90	0.02
pBR322(30)	GGCATCGTGGTGTCACGCTCGTCGTTTGGT	9252.01	9250.01	0.02
pBR322(39)	GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCA	12027.80	12034.53	0.06
pBR322(40)	GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT	12332.00	12337.12	0.04
Antisense Compounds				
B2A2	CGCTGAAGGGCTTCTTCCTTATGAT	8343.97	8342.90	0.01
B3A2	CGCTGAAGGGCTTTTGAACCTCTGCTT	8368.98	8367.88	0.01
LR3523	CCCTGCTCCCCCTGGCTCC	6206.38	6206.30	0.00
LR3280	AACGTTGAGGGGCAT	4881.52	4880.65	0.02
LR3001	TATGCTGTGCCGGGTCTTCGGGC	7776.52	7774.95	0.02

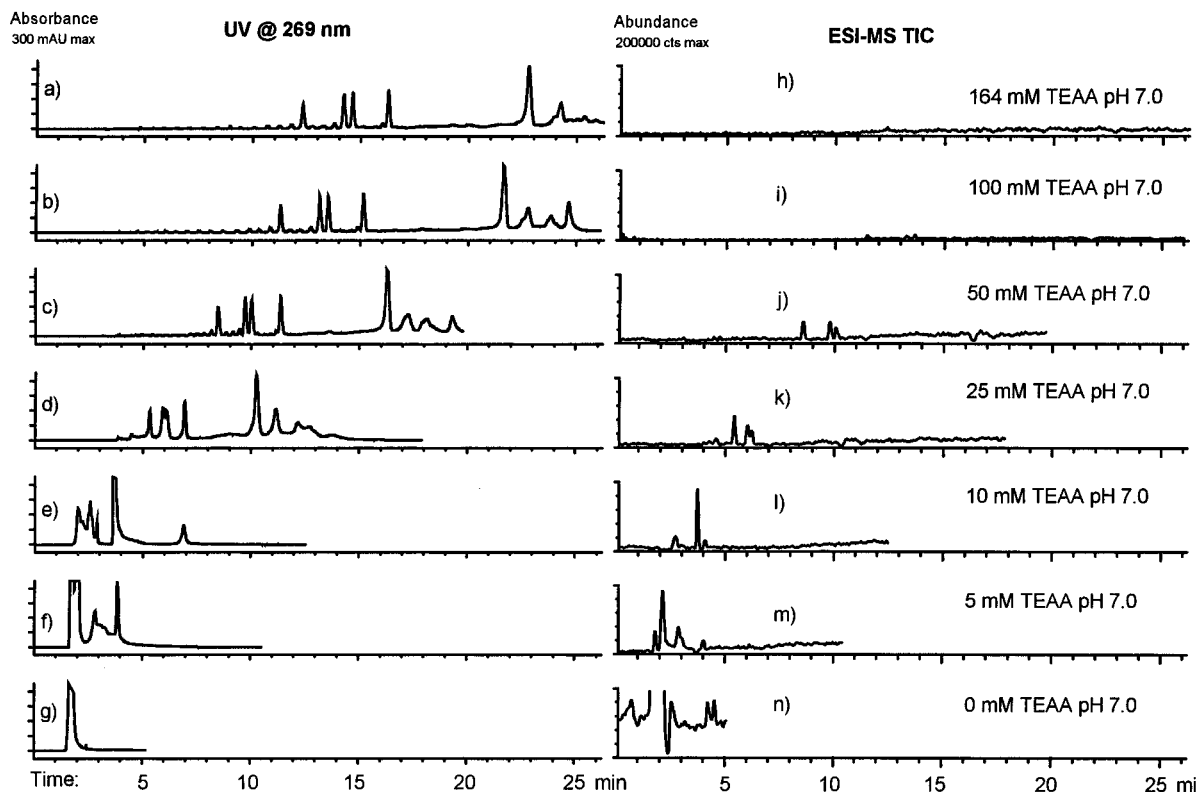


Figure 2. Effect of [TEAA] on chromatographic resolution. PolyT mix (15, 19, 20, 25, 74, 75) at 100 pmol/component. HPLC gradient 10–20% acetonitrile/30 min at 200 μ L/min, 35 $^{\circ}$ C. (a–g) UV detection at 269 nm. (h–n) ESI total ion current. (a, h) 164 mM TEAA, pH 7.0; (b, i) 100 mM TEAA, pH 7.0; (c, j) 50 mM TEAA, pH 7.0; (d, k) 25 mM TEAA, pH 7.0; (e, l) 10 mM TEAA, pH 7.0; (f, m) 5 mM TEAA, pH 7.0; (g, n) 0 mM TEAA, pH 7.0.

obtained from Sigma Chemical Co. (St. Louis, MO). The synthetic oligonucleotides listed in Table 1 were obtained from Cruachem, Inc. (Dulles, VA) as lyophilized triethylammonium salts. The two series of oligonucleotides consisted of PolyT homopolymer series (dT15, dT19, dT20, dT25, dT74, and dT75) and a series based on

the pBR322 plasmid sequence (pBR322(10)–pBR322(40)). The synthetic pBR322 plasmid sequence polymer was created such that the first bases (10, 11, 12, etc.) are identical to those in the naturally occurring plasmid. Although the samples from both of the sequences were synthetic, they were not further purified, and

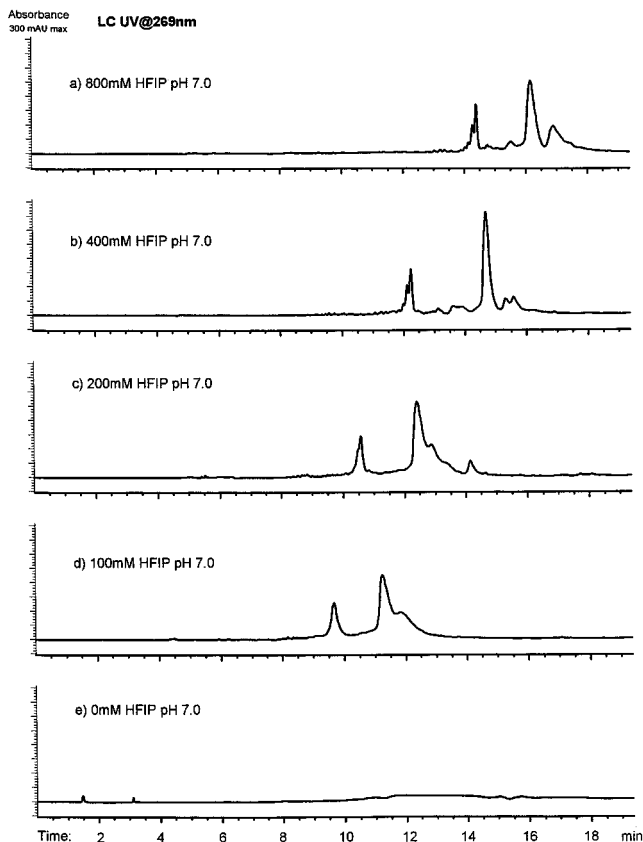


Figure 3. Effect of [HFIP] on chromatographic performance. PolyT mix (15, 19, 20, 25, 74, 75) at 100 pmol/component. HPLC gradient 0–30% methanol/30 min at 200 μ L/min, 35 $^{\circ}$ C. UV detection at 269 nm. (a) 800 mM HFIP, pH 7.0 (adjusted with TEA); (b) 400 mM HFIP, pH 7.0 (adjusted with TEA); (c) 200 mM HFIP, pH 7.0 (adjusted with TEA); (d) 100 mM HFIP, pH 7.0 (adjusted with TEA); (e) 0 mM HFIP.

analysis by MALDI-TOF indicates a considerable amount of adduct formation. In addition, a series of phosphorothioate compounds were obtained courtesy of Karen Fearon at Lynx Therapeutics (Hayward, CA).

RESULTS AND DISCUSSION

Analytical Context. The stimulus for this study was the seeming incompatibility of high-resolution separations and electrospray ionization mass spectrometry as demonstrated from the literature. While a clear need exists, previous attempts required a compromise in either one system or the other. Chromatographic compromises typically were loss of resolution by use of insufficiently high buffer levels to maintain the appropriate pH or substitution of a more volatile but less effective ion pairing reagent. Electrospray performance was compromised either with excessive adduct formation with the result of poor sensitivity or with the inability to detect impurities due to the lack of a high-resolution separation.

Mobile Phases Containing TEAA. TEAA is the most common buffer system used for reversed-phase ion pair separations of oligonucleotides. A typical separation of a mixture of PolyT oligonucleotides using a standard TEAA-based separation system is shown in Figure 1a. One approach to the on-line combination of LC and ESI/MS is to find a TEAA concentration that would represent a compromise and yield both adequate chromatographic resolution and electrospray signal intensity. To this end, the effects of TEAA concentration on separation and

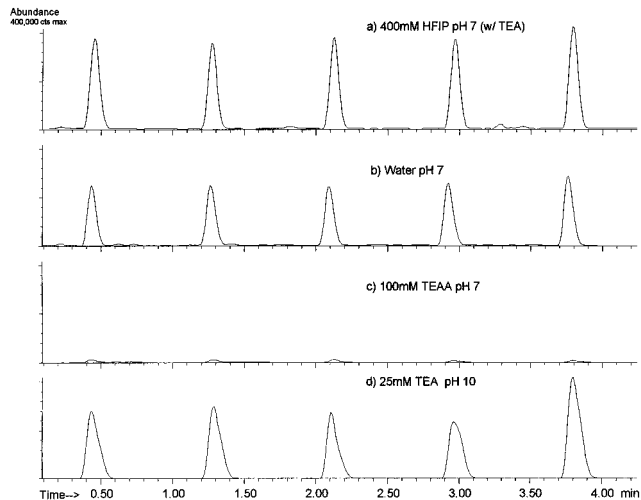


Figure 4. Flow injection comparison of ESI solvent additives: 50 pmol flow injections of dT₂₀ at 200 μ L/min. ESI total ion current. (a) 400 mM HFIP, pH 7.0 (adjusted with TEA); (b) water, pH 7.0; (c) 100 mM TEAA, pH 7.0; (d) 25 mM TEA, pH 10.

electrospray performance were evaluated. The aim was Figure 2 shows the effect of [TEAA] on the resolution (a–g) and electrospray signal intensity (h–n) for a standard mixture of synthetic dT15, dT19, dT20, dT25, dT74, and dT75. As can be seen, for mobile phases of less than 50 mM, the retention and resolution of the sample decrease rapidly. The data shown in Figure 2a–g are UV chromatograms at 269 nm. Although these data were obtained with a single gradient which was not reoptimized at each TEAA concentration, below 25 mM TEAA there was insufficient retention to obtain adequate resolution using any gradient evaluated. The opposite effect is seen for the effect of TEAA concentration on electrospray performance, as shown in Figure 2h–n, which shows the ESI total ion chromatograms (TIC) for the same separations shown as UV chromatograms on the left. As can be seen, as the TEAA concentration is reduced, the electrospray signal increases. Although it is possible to see some separation of the oligonucleotides with electrospray detection at 50 mM TEAA, this compromise condition represents a significant reduction in both potential chromatographic resolution and electrospray signal intensity.

Mobile Phases Containing HFIP. In an effort to overcome the limitations of the TEAA separation system, investigations were conducted into the use of 1,1,1,3,3,3-hexafluoro-2-propanol as a mobile phase additive. The effect of the HFIP concentration on chromatographic retention and resolution is shown in Figure 3. At the 400 mM level, when the solution was adjusted to pH 7.0 with triethylamine, separations could be obtained comparable to those obtained using TEAA mobile phases. See Figure 1b for a typical separation of PolyT oligonucleotides. The HFIP-based separation is based on a gradient using methanol as an organic modifier. Interestingly, HFIP is miscible with water, methanol, 2-propanol, and hexane but immiscible with acetonitrile.

ESI performance using HFIP adjusted to pH 7.0 with TEA as a solvent was found to be superior to that obtained with the currently optimum modifier, TEA. This is demonstrated in Figure 4 in which results for five replicate injections of 50 pmol of dT₂₀ are shown with the dilution and carrier solvents consisting of 400 mM HFIP adjusted to pH 7.0 with TEA, water, 100 mM TEAA, and 25 mM TEA. The intensity of the peaks for the 400 mM HFIP/TEA solution is approximately 2 \times greater than in the 25

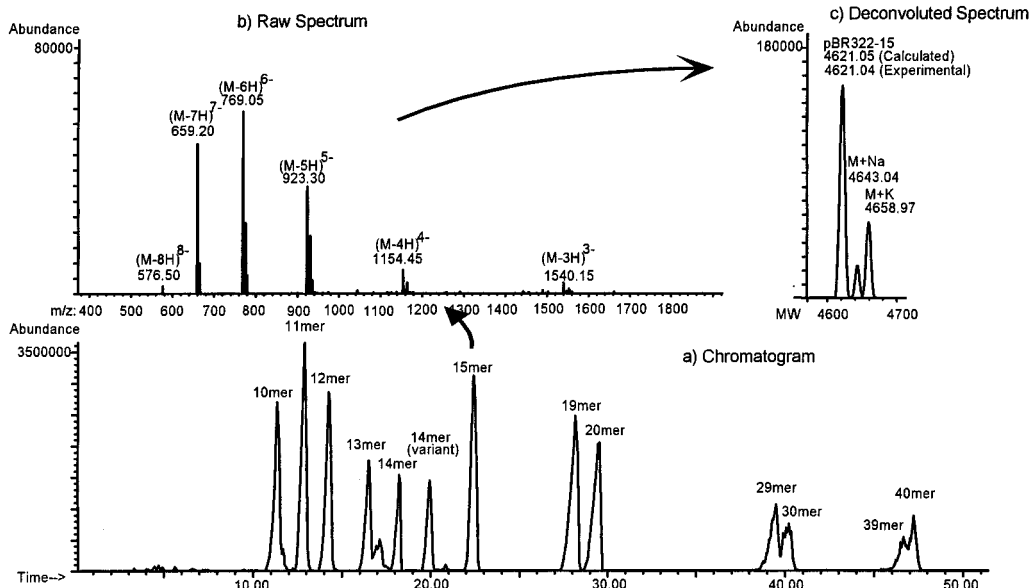


Figure 5. Analysis of synthetic pBR322 oligonucleotides by HPLC-ESI/MS. Conditions shown in figure. (a) Total ion chromatogram, (b) raw mass spectrum, (c) deconvoluted mass spectrum.

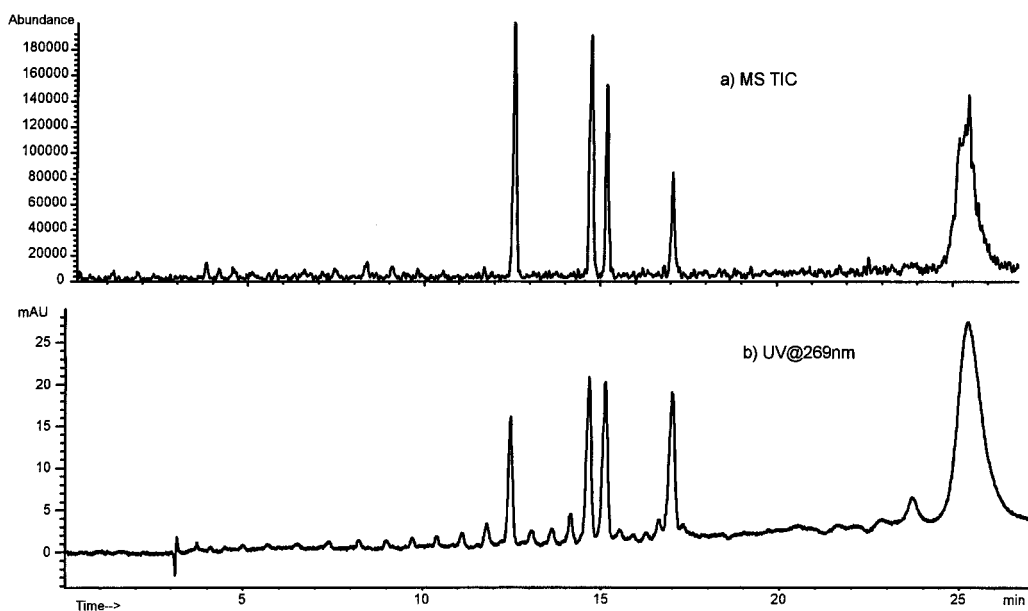


Figure 6. Sensitivity of HPLC-ESI/MS analysis of PolyT oligonucleotides. Conditions shown in Figure 1b. (a) Total ion chromatogram and (b) UV at 269 nm.

mM TEA case, while the spectra (not shown) are nearly identical in terms of adduct formation and ion envelope distribution. In contrast, the signal intensity using TEAA is substantially lower, while the spectral characteristics when using pure water show a high degree of adduct formation.

The mechanism proposed for the behavior of this system is based on the dynamic adjustment of the pH in the electrospray droplet as a function of the preferential removal of anionic counterion from the droplet by evaporation. Comparing the two solvent systems aqueous triethylamine/acetic acid and aqueous triethylamine/hexafluoro-2-propanol, the key physicochemical parameters involved are the relative volatilities of the species and the relative dissociation constants. HFIP (bp = 57 °C) is more volatile than TEA (bp = 89 °C), while acetic acid is much less volatile (bp = 118 °C). As a buffer system for HPLC, the weak acid/weak base system of HFIP/TEA maintains a stable pH at around 7.0. During the separation, the TEA ions ion-pair with

the negatively charged phosphate groups of the oligonucleotide backbone. However, as the column effluent is electrosprayed and desolvated, the volatile HFIP is depleted at the droplet surface (if not the bulk), and the pH at the surface rises toward 10. At the higher pH, the oligonucleotide-TEA ion pairs dissociate, and the oligonucleotides can be desorbed into the gas phase. The other important parameter involved is the dissociation constants in the two buffer systems. The pK_a values of acetic acid, hexafluoro-2-propanol, and triethylamine are 4.75, ~9, and 11.01, respectively. Thus, at pH 7.0, acetic acid is completely dissociated and consequently cannot be removed by evaporation. The HFIP is not charged at pH 7.0 and can be evaporated freely.

Applications. As an example of the potential use of mass spectrometry in plasmid mapping, Figure 5 shows the analysis of a mixture of synthetic oligonucleotides based on the pBR322 plasmid sequence. Figure 5a shows the total ion chromatogram, Figure 5b shows the raw electrospray negative ion spectrum for

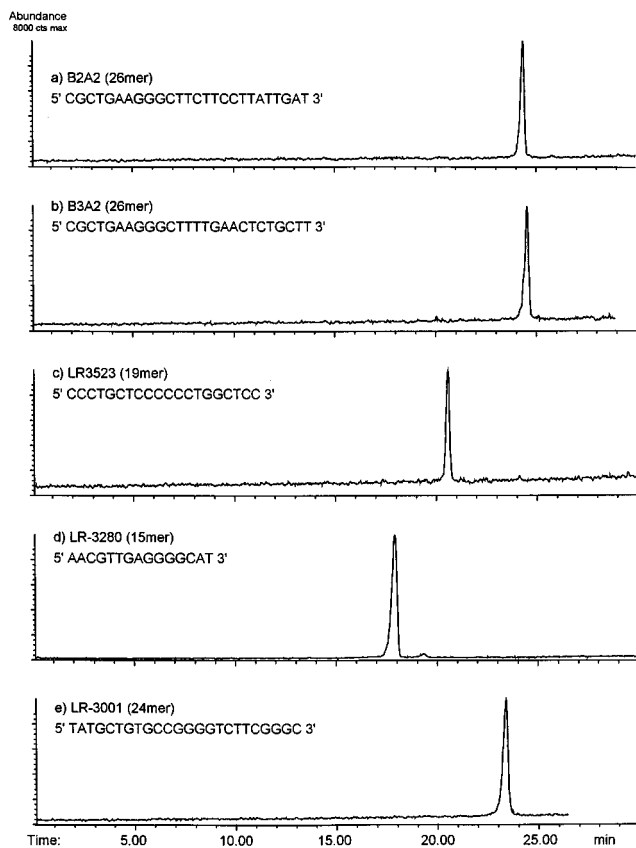


Figure 7. Analysis of phosphorothioate ester antisense oligonucleotides by HPLC-ESI/MS: 50 pmol/component, 400 mM HFIP adjusted to pH 7.0 with TEA, HPLC gradient 35–65% methanol/30 min at 200 μ L/min. (a) B2A2 (26-mer), (b) B3A2 (26-mer), (c) LR3523 (19-mer), (d) LR3280 (15-mer), (e) LR3001 (24-mer).

pBR322(15), and Figure 5c shows its deconvoluted spectrum. The experimental and calculated masses for the oligonucleotides determined in this study are shown in Table 1. Note that, in this sample, there is a low level of Na^+ and K^+ adduct formation, but it does not interfere with the accurate mass determination. Also note the presence of two components with the mass of the 14-mer (4310.84 Da) but with differing retention times. Although it cannot be determined directly from this data, these differences may be due to variations in the sequence or conformational differences. Other applications of these types of analyses lie in the better characterization of synthetic oligonucleotides for probes and PCR primers.

Similar studies have been conducted with synthetic mixtures of a PolyT homopolymer. More extensive dilution and linearity studies demonstrate that the method can be used at levels below 10 pmol. Sensitivity appears to be relatively length independent, with changes in peak height due to increased peak width. Figure 6 demonstrates the sensitivity of the analysis with a sample

consisting of 10 pmol/component of a poly T sample. Although minor peaks, presumably incomplete synthetic fragments, that were observed in the UV chromatogram can also be detected in extracted ion chromatograms, they are below the signal-to-noise level for the ESI total ion current.

As a final example, Figure 7 shows the analysis of a series of phosphorothioate oligodeoxynucleotides targeted toward treatments of either acute or chronic myelogenous leukemia.²⁰ The backbone of these oligonucleotides differs from that of the naturally occurring biopolymers by the substitution a sulfur atom for one of the nonbridging oxygen atoms in each phosphate group. The absence of significant impurity peaks is consistent with the high purity of these samples. This example has clear application for the improved characterization of synthetic purity and product identification for quality control, as well as for the characterization of in vivo metabolic processes for these antisense pharmaceuticals, e.g., detecting oxidative and other degradative processes such as depurination reactions and hydrolysis by nucleases.

CONCLUSIONS

In conclusion, a method has been demonstrated for the analysis of oligonucleotides of up to 75 bases in length by combined HPLC-ESI/MS, which does not require compromise in the performance characteristics of either the HPLC separation or the electrospray mass spectra. The on-line combination of HPLC and ESI/MS with a conventional quadrupole analyzer allows a rapid, robust, and easy-to-use method for characterizing short oligonucleotides. In addition to high-resolution separations, the technique provides effective suppression of cation adduct formation. The combination of HPLC and ESI/MS allows the identification of oligonucleotides of similar composition but varying sequence or conformation. The method is useful below the 10 pmol level. Applications have been demonstrated for synthetic oligonucleotides as well as phosphorothioate antisense compounds. The method is particularly useful and robust for oligonucleotides up to 30 bases in length in which single base resolution can be obtained with essentially adduct-free mass spectra. Future developments are under way to extend the size range, and consequently the mass range, of this technique through the use of nonporous polymeric supports for the reversed-phase separation as well as to apply this method to the characterization of metabolic products of antisense oligodeoxynucleotide pharmaceuticals.

ACKNOWLEDGMENT

The authors gratefully acknowledge Karen Fearon of Lynx Therapeutics for the supply of phosphorothioate oligonucleotide samples.

Received for review September 11, 1996. Accepted January 15, 1997.[⊗]

AC960916H

(20) Skorski, T.; Nieborowska-Skorska, M.; Nicolaidis, N. C.; Szczylik, C.; Iversent, P.; Iozzo, R. V.; Zon, G.; Cacibretta, B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4504–4508.

[⊗] Abstract published in *Advance ACS Abstracts*, February 15, 1997.