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New procedure for the use of high-performance liquid chromatography–electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides

Alex Apffel[a,*], John A. Chakel[a], Steven Fischer[b], Kay Lichtenwalter[a], William S. Hancock[a]

[a]*Biomeasurements Group, Hewlett–Packard Laboratories, Palo Alto, CA 94304, USA*
[b]*California Analytical Division, Hewlett–Packard Company, Palo Alto, CA 94304, USA*

Abstract

A method is described which allows the combination of high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides without compromising the performance of either technique. The essential feature of the method uses 1,1,1,3,3,3-hexafluoro-2-isopropanol as an additive to the HPLC mobile phase. This novel additive results in both good HPLC separation and efficient negative ion mode electrospray ionization. The method is demonstrated for oligonucleotides samples such as synthetic homopolymers of thymidine (PolyT), of fragments based on the pBR322 plasmid sequence and analysis of phosphorothioate ester antisense oligonucleotides. The differentiation of phosphorylation states of natural nucleotides as well as nucleotide analogues, such as ziduvodine (AZT), is also demonstrated. © 1997 Elsevier Science B.V.

Keywords: Mobile phase composition; Nucleotides; Oligonucleotides; AZT; Ziduvodine

1. Introduction

Just as requirements of bioanalytical methods for recombinant protein pharmaceutical products has generated a rapid growth in the methodology of combined high-performance separations and mass spectrometry, similar needs are seen in the biotechnology industry in the analysis of nucleotides and oligonucleotides. The development of methods for these DNA components has lagged somewhat behind the methods for the analysis of proteins largely because the incompatibility of their conventional separation methods and mass spectral detection. Nonetheless, the need for these methods has grown due to the progress of the human genome projects as well as the progress in molecular biology for the development of recombinant DNA derived protein pharmaceuticals and direct gene therapy. The wide use of synthetic oligonucleotides range from polymerase chain reaction (PCR), in situ hybridization and in vivo genetic manipulation as well as the therapeutic use of antisense oligunucleotides. Similarly, the therapeutic use of nucleotide analogues such as ziduvodine (AZT) for the treatment of viral infections such as human immunodeficiency virus (HIV), have placed demands on analytical technology for the analysis of pharmaceutical product purity and metabolic studies.

*Corresponding author.

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Conventional reversed-phase separation methods for oligonucleotides are based on the use of triethylammonium acetate (TEAA) containing mobile phases at pH 7.0 [1–3]. Unfortunately, these methods are not compatible with electrospray ionization mass spectrometry (ESI-MS). Anion-exchange chromatography using non-volatile salt gradients on DEAE materials have also been used for smaller fragments, although the resolution is generally poorer than reversed-phase separations [4] and the mobile phases are even less compatible with electrospray ionization mass spectrometry.

ESI-MS of infusions of oligonucleotides without separation have been somewhat hampered by ubiquitous cation adduction due to the high affinity binding of Na^+ and K^+ to the polyanionic backbone of the nucleic acids. Recently, Greig and Griffey [5] have demonstrated 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, and consequently for the negative ion formation of oligonucleotides, pH 10 is near optimal. ESI-MS has been shown with quadrupole analyzers to be useful for the analysis of oligonucleotides of length up to 120 bases [6], as well as smaller fragments of 10–75 residues for naturally occurring oligonucleotides [7–12], synthetic and modified oligonucleotides [13–17], PCR products [18] and antisense oligonucleotides [19].

For simple mixtures, ESI-MS can be used to determine the molecular mass of oligonucleotides of small to moderate size (up to 75 mers) to a mass accuracy of approximately 0.02%. More complex mixtures, extensive adduct formation and the presence of oligonucleotides with identical composition but different sequence require an additional dimension of separation. Approaches to the on-line combination of HPLC and ESI-MS have been reported [20–23], but have recognized that the optimal mobile phases for HPLC result in drastic reduction in ion production for electrospray ionization. Conversely, the optimal solvents for electrospray ionization with minimization of adduct formation are not suitable for use as mobile phases in HPLC separations.

An analogous situation exists for the analysis of nucleotides and analogues. Due to the low hydrophobicity of these monomeric compounds relative to oligonucleotides, the conventional reversed-phase separation relies on the use of a phosphate buffered ion pairing reagent containing a more hydrophobic tetrabutylammonium (TBA) phosphate. While it is possible to substitute more volatile buffer systems, the presence of TBA results in substantially reduced performance in ESI-MS. From the MS point of view, the analysis of nucleotides is somewhat less demanding than oligonucleotides due to the low amount of cationic adduct formation. The combination of HPLC and ESI-MS has been demonstrated [23] using ion suppression HPLC combined with negative ESI-MS, but this approach shows some degree of compromise between ideal HPLC and ESI-MS conditions. The method described here is the preliminary method development phase for the analysis of metabolic products of the nucleotide analogue antiviral agent ziduvodine (AZT) and transformation to its phosphorylated forms in biological fluids. This enzymatically mediated process is the limiting factor in the clinical effectiveness of AZT to inhibit HIV reverse transcriptase for the treatment of AIDS.

This report describes a method using a HPLC mobile phase containing 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) adjusted to pH 7.0 with triethylamine which results in efficient HPLC separation and high sensitivity electrospray ionization with a minimum of adduct formation. 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.

2. Experimental

2.1. HPLC

The HPLC separation was performed on a Hewlett–Packard 1090 liquid chromatography system equipped with DR5 ternary solvent delivery system, diode array UV–Vis detection (DAD), autosampler and heated column compartment (Hewlett–Packard, Wilmington, DE). All HPLC separations were done using a YMC (Wilmington, NC, USA) 3 μm particle, 120 Å pore size ODS-AQ reversed-phase column 250×2.1 mm I.D. Flow-rates were at 0.2

ml/min. Injection volumes were 1–2 μl. UV absorbance was monitored at 269 nm with 10 nm slit width and reference at 480 nm with an 80 nm slit width.

For the analysis of oligonucleotides, two main gradient solvent systems were used: 100 m*M* TEAA pH 7.0–acetonitrile and 400 m*M* HFIP–methanol. The TEAA mobile phase was prepared with a dilution of a 1 *M* preformulated commercial buffer (Fluka BioChemika, Buchs, Switzerland) to 100 m*M*. The pH was measured to ensure pH 7.0. For this solvent system, the "B" buffer consisted of 50% acetonitrile, 100 m*M* TEAA. The HFIP mobile phase was prepared as a stock solution of 800 m*M* adjusted to pH 7.0 with triethylamine. This stock solution was diluted to 400 m*M* with water for the "A" solvent and with methanol for the "B" solvent. The solvents were degassed ultrasonically. The column temperature was maintained at 50°C throughout the separation. For the analysis of nucleotides and nucleotide analogues, the same solvents A and B of 400 m*M* HFIP adjusted to pH 7.0 with TEA were used. For the separation of nucleotides the gradient was 0–10% B/30 min and for the separation of AZT samples the Gradient was 0–30% A/45 min. The column temperature was maintained at 30°C throughout the separation.

2.2. ESI-MS

MS was done on a Hewlett–Packard 5989B quadrupole mass spectrometer equipped with extended mass range, high energy dynode detection (HED) and a Hewlett–Packard 59987A API-electrospray source with high-flow nebulizer option. All electrospray analysis was performed in negative mode. Both 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°C) gases. The system was operated in negative ionization electrospray mode. The use of the high-flow nebulizer negates flow splitting or the need for scavenger gases such as oxygen or SF_6. Instrumental settings are shown in Table 1. For the analysis of oligonucleotides, MS data were acquired in raw scan mode scanning from 500 to 2000 u at an acquisition rate of 1.0 Hz at 0.15 u stepsize. Data were filtered in the mass domain with a 0.05 u 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. For the analysis of nucleotides and nucleotide analogues, MS data were acquired in both scan and SIM mode. For scan acquisition, data was acquired for 70–600 u at an acquisition rate of 1.3 Hz at 0.15 u stepsize. For SIM acquisition ions were acquired for the $(M-H)^{-1}$ ions listed in Table 2, in addition to *m/z* 79 for the pyrophosphate anion. The dwell time for each ion was 50 ms. To allow the collisionally induced dissociation (CID) generation of the PO_3^- fragment, the capillary exit (CapEx) potential was dynamically

Table 1
Mass spectrometry instrumental settings

Setting	Value oligonucleotides	Value nucleotides
Vcap	4000	4000
Vcyl	3500	3500
Vend	4000	4000
CapEx	−191	*m/z* 70–100: −300 V *m/z* 100–600: −100 V
ESSkim1	−30	−30
Ion guide V_b	−5	−5
Ion guide V_c	46	46
Entrance lens	−75	−75
High energy dynode	10 000	10 000
EMV	2500	2500

Table 2
Nucleotides

Nucleotides	Abbr.	M_r	Parent ion $(M-H)^{-1}$
Deoxyguanosine 5′-monophosphate	dGMP	363.2	362.2
Deoxyguanosine 5′-diphosphate	dGDP	443.2	442.2
Deoxyguanosine 5′-triphosphate	dGTP	523.2	522.2
Deoxycytidine 5′-monophosphate	dCMP	323.2	322.2
Deoxycytidine 5′-diphosphate	dCDP	403.2	402.2
Deoxycytidine 5′-triphosphate	dCTP	483.2	482.2
Deoxyadenosine 5′-monophosphate	dAMP	347.2	346.2
Deoxyadenosine 5′-diphosphate	dADP	427.2	426.2
Deoxyadenosine 5′-triphosphate	dATP	551.2	550.2
Deoxyuridine 5′-monophosphate	dUMP	324.2	323.2
Deoxyuridine 5′-diphosphate	dUDP	404.2	403.2
Deoxyuridine 5′-triphosphate	dUTP	484.1	483.1
Deoxythymidine 5′-monophosphate	dTMP	322.2	321.2
Deoxythymidine 5′-diphosphate	dTDP	402.2	401.2
Deoxythymidine 5′-triphosphate	dTTP	482.2	481.2
Nucleotide analogues			
Ziduvodine	AZT	267.2	266.2
Ziduvodine 5′-monophosphate	AZT-MP	346.8	345.8
Ziduvodine 5′-diphosphate	AZT-DP	426.8	425.8
Ziduvodine 5′-triphosphate	AZT-TP	506.6	505.6

Table 3
Oligonucleotide samples

Oligonucleotide	Sequence	Calculated mass
Poly T		
dT15		4500.98
dT19		5717.76
dT20		6021.96
dT25		7542.94
dT74		22 448.55
dT75		22 752.75
pBR322 plasmid		
pBR322(10)	GGCATCGTGG	3084.05
pBR322(11)	GGCATCGTGGT	3388.25
pBR322(12)	GGCATCGTGGTG	3717.46
pBR322(13)	GGCATCGTGGTGT	4021.65
pBR322(14)	GGCATCGTGGTGTC	4310.84
pBR322(15)	GGCATCGTGGTGTCA	4624.05
pBR322(19)	GGCATCGTGGTGTCACGCT	5835.82
pBR322(20)	GGCATCGTGGTGTCACGCTC	6125.01
pBR322(29)	GGCATCGTGGTGTCACGCTCGTCGTTTGG	8947.81
pBR322(30)	GGCATCGTGGTGTCACGCTCGTCGTTTGGT	9252.01
pBR322(39)	GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCA	12 027.80
pBR322(40)	GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT	12 332.00
Antisense compounds		
B2A2	CGCTGAAGGGCTTCTTCCTTATTGAT	8343.97
B3A2	CGCTGAAGGGCTTTTGAACTCTGCTT	8368.98
LR3523	CCCTGCTCCCCCCTGGCTCC	6206.38
LR3280	AACGTTGAGGGGCAT	4881.52
LR3001	TATGCTGTGCCGGGGTCTTCGGGC	7776.52

set to −300 V for m/z 70–100 and −100 V for m/z 100–600.

2.3. Chemicals

HPLC-grade water was purified using a Milli-Q System (Millipore, Bedford, MA, USA). HPLC-grade methanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Triethylamonium acetate buffer was obtained from Fluka Biochemika and HFIP was obtained from Sigma (St. Louis, MO, USA). The synthetic oligonucleotides listed in Table 3 were obtained from Cruachem (Dulles, VA, USA). The two series of oligonucleotides consisted of PolyT homopolymer series (dT15, dT19, dT20, dT25, dT74 and dT75) and a series based on the sequence of the pBR322 plasmid [pBR322(10)–pBR322(40)]. In addition, a series of phosphorothioate compounds were obtained courtesy of Karen Fearon at Lynx Therapeutics. Nucleotides standards were obtained from Sigma while ziduvodine (AZT) and its mono-, di- and triphosphates were obtained courtesy of Dr. Terrence Blashke of Stanford Medical Center.

3. Results and discussion

3.1. Analytical background and context

The need for an on-line combination of HPLC and MS for the analysis both of nucleotides and oligonucleotides has become increasingly clear as the use of these compounds has become more widespread as tools in the development of recombinant DNA derived protein pharmaceuticals, as well as direct uses as gene therapeutics. Unfortunately, in previous work, the combination of the two techniques has involved significant performance compromises in either one technique or the other.

The requirements for the analysis of oligonucleotides are similar in many ways to the analysis of

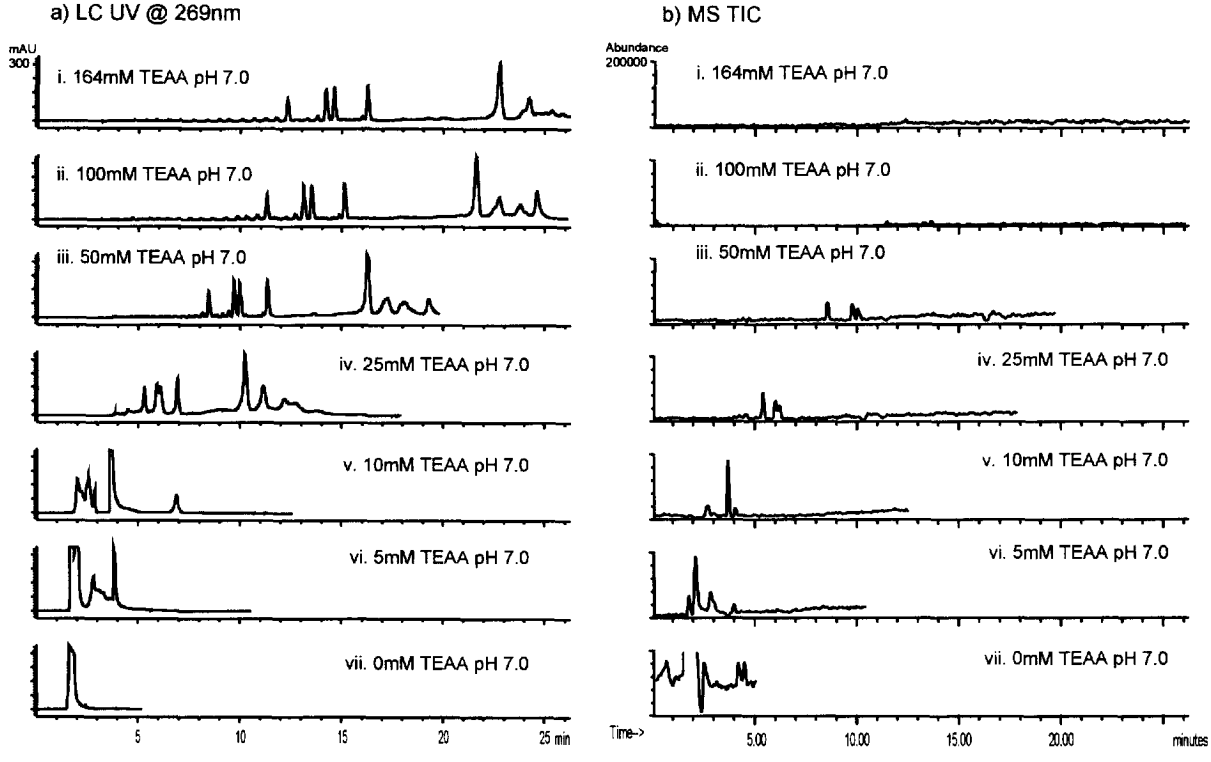

Fig. 1. Effect of (TEAA) on electrospray signal intensity. PolyT mix (15, 19, 20, 25, 74, 75) 100 pmol/component. HPLC gradient 10–20% acetonitrile/30 min at 200 μl/min, 35°C. (a) UV signal. (b) ESI total ion current. (i) 164 m*M* TEAA pH 7.0; (ii) 100 m*M* TEAA pH 7.0; (iii) 50 m*M* TEAA pH 7.0; (iv) 25 m*M* TEAA pH 7.0; (v) 10 m*M* TEAA pH 7.0; (vi) 0 m*M* TEAA pH 7.0.

other bioploymers such as proteins or peptides. In both cases, the large polymeric analytes which may exhibit transient secondary structure (even after denaturation), are separated by reversed-phase HPLC with characteristics dominated by controlled desorption from a hydrophobic surface rather than continuously variable partitioning between mobile and stationary phases. The ESI-MS spectra are also similar, although generated as negative ions, characterized by multiply charged ion spectra which can be deconvoluted to yield molecular masses of the parent molecules, which may far exceed the mass range of the mass spectrometer.

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 (as present in conventional HPLC separation systems for oligonucleotides) and aqueous triethylamine–HFIP (used here), the key physicochemical parameters involved are the relative volatilities of the species and the relative dissociation constants. HFIP (b.p.= 57°C) is more volatile than TEA (b.p.=89°C), while acetic acid is less volatile (b.p.=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 towards 10. At the higher pH, the oligonucleotide–TEA ion pairs dissociate and the oligonucleotides can be desorbed into the gas phase.

3.2. ESI-MS of oligonucleotides

As a consequence of the compromises observed above, studies were performed to evaluate optimal

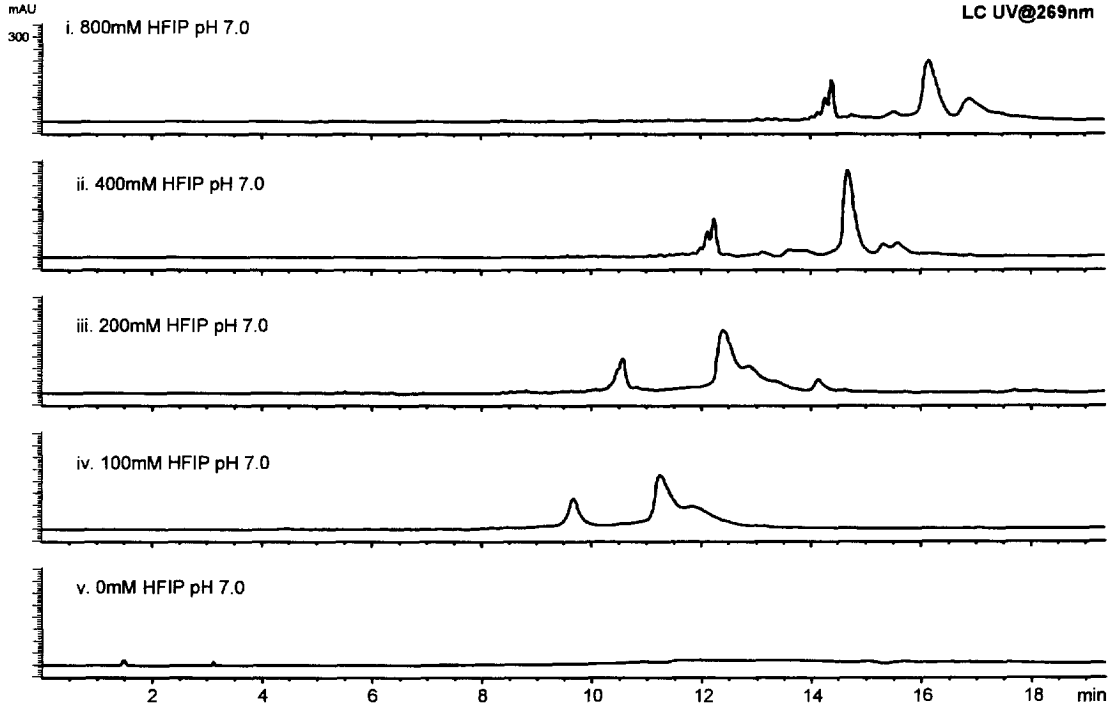

Fig. 2. Effect of (HFIP) on chromatographic performance. PolyT mix (15, 19, 20, 25, 74, 75) 100 pmol/component. HPLC gradient 0–20% methanol/30 min at 200 μl/min, 35°C. UV Detection at 269 nm. (i) 800 m*M* HFIP pH 7.0; (ii) 400 m*M* HFIP pH 7.0; (iii) 200 m*M* HFIP pH 7.0; (iv) 100 m*M* HFIP pH 7.0; (v) 0 m*M* HFIP pH 7.0; (vi) 0 m*M* TEAA pH 7.0.

conditions for electrospray ionization of oligonucleotides. The approach was aimed at adjusting HPLC mobile phase conditions by post-column addition of the appropriate modifiers prior to introduction into the electrospray source. Although no combination of TEAA containing mobile phase and post-column addition of modifying reagents resulted in satisfactory results, the effectiveness of the addition of triethylamine or piperidine [5] as electrospray solvent additives for infusions of single oligonucleo-

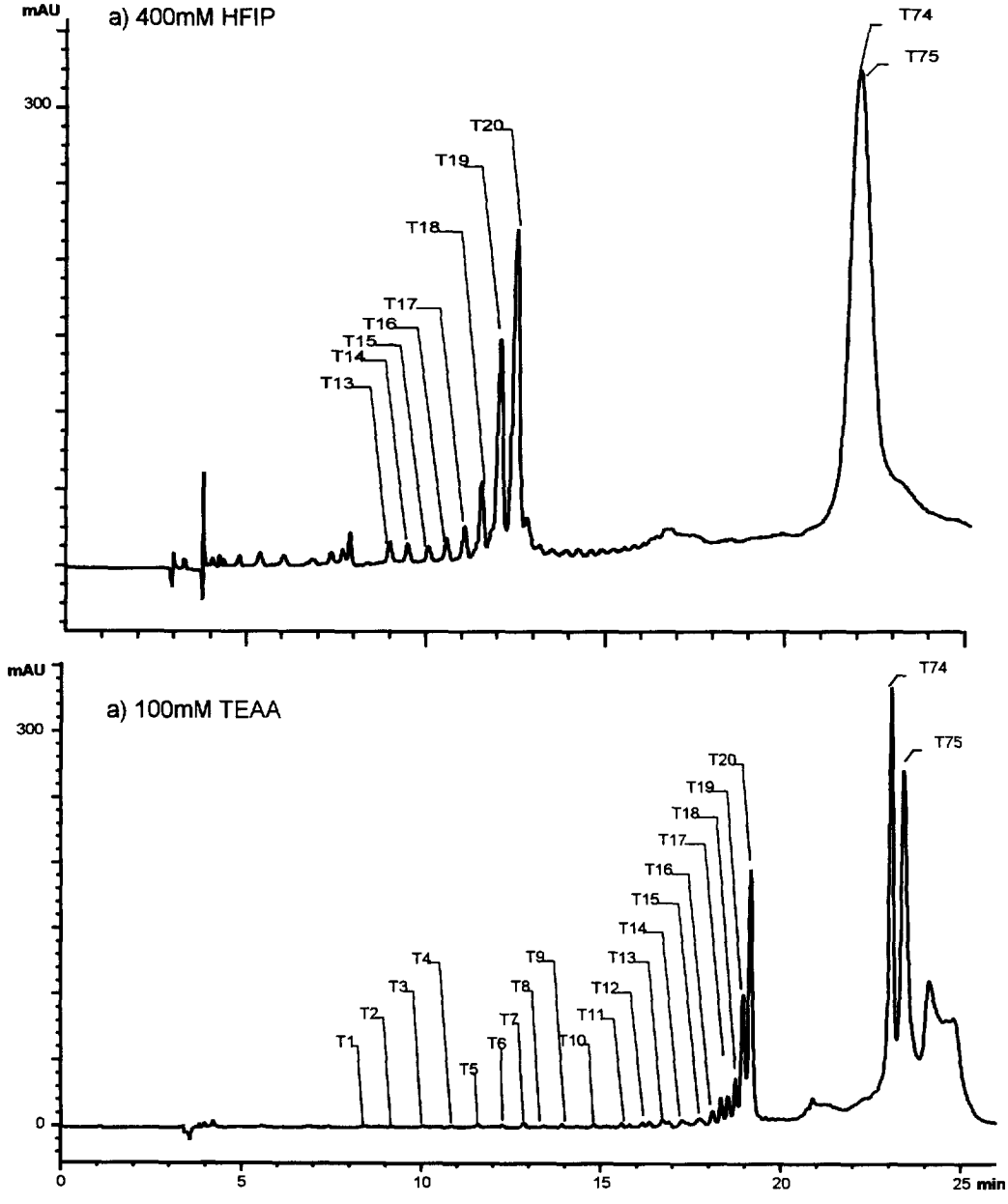

Fig. 3. Comparison of typical separations of oligonucleotides using HFIP and TEAA. Synthetic mixture of PolyT oligonucleotides (19, 20, 74, 75) at 50 pmol/μl each component. (a) 400 m*M* HFIP pH 7.0 at 200 μl/min, 50°C UV at 269 nm. (b) 100 m*M* TEAA pH 7.0 at 200 μl/min, 50°C UV at 269 nm.

tides or simple mixtures was experimentally confirmed. However, for mixtures of five or more components, the deconvolution of the multiply charged ion spectra becomes too complex to deconvolute reliably.

3.2.1. Mobile phases containing TEAA

Since TEAA is the most common buffer system used for reversed-phase ion pair separations of oligonucleotides, initially the effect of TEAA concentration on separation and electrospray performance was evaluated. The aim was to find a concentration which would represent a compromise and yield both adequate chromatographic resolution and electrospray signal intensity. Fig. 1a shows the effect of (TEAA) on the resolution for a synthetic mixture of dT15, dT19, dT20, dT25, dT74 and dT75. As can be seen, for mobile phases of less than 50 m*M*, the retention and resolution of the sample degrade rapidly. The data shown in Fig. 1a 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 m*M* TEAA there was insufficient retention to obtain adequate resolution using reoptimized gradients. Evaluation of the effect of pH on separation showed best performance at a pH of 7.0, which was used in subsequent experiments.

Although the chromatographic resolution is improved by higher concentrations of TEAA, exactly the opposite is the case for the electrospray performance as shown in Fig. 1b, in which the total ion chromatograms (TIC) for the same separations are shown as UV chromatograms in Fig. 1a. 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 m*M* TEAA, this compromise con-

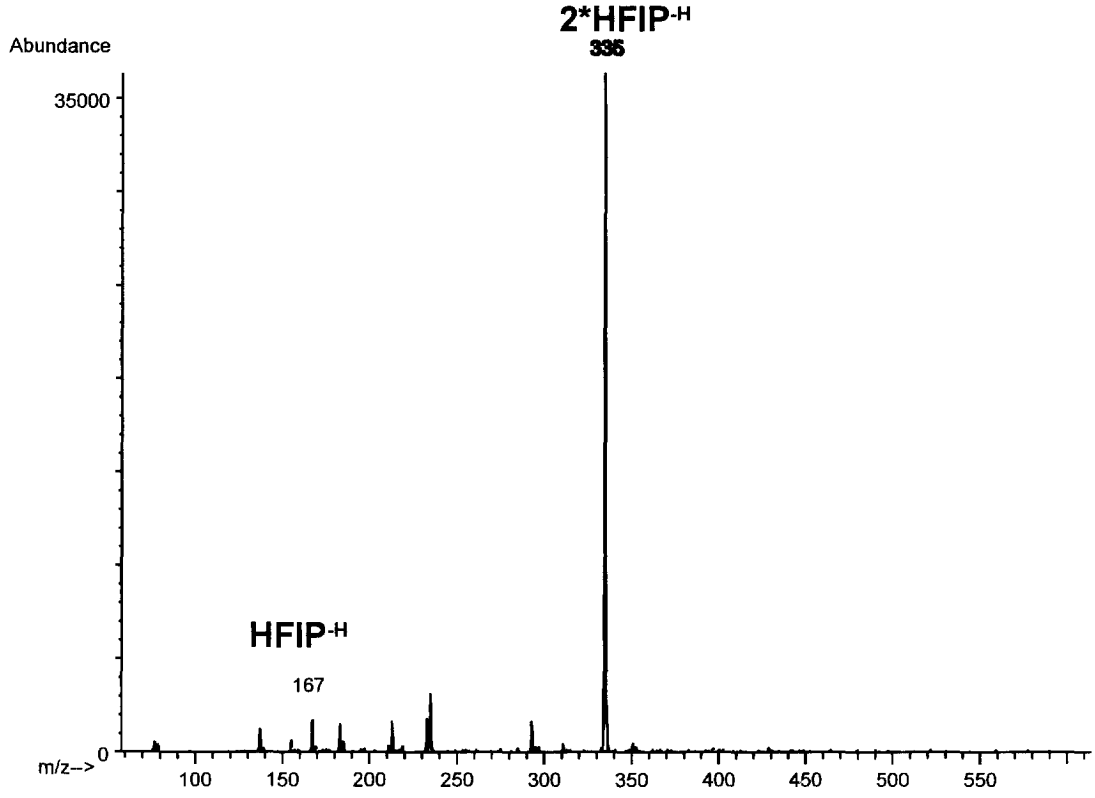

Fig. 4. HFIP background spectrum. Infusion of 400 m*M* HFIP pH 7.0, 50% methanol at 200 μl/min.

dition represents a significant reduction in both potential chromatographic resolution as well as electrospray signal intensity.

3.2.2. Mobile phases containing HFIP

As an alternative to TEAA mobile phases, HFIP was evaluated as a mobile phase additive. The effect of the HFIP concentration on chromatographic retention and resolution is shown in Fig. 2. For subsequent analyses, HFIP was used at the 400 m*M* level, adjusted to pH 7.0 with triethylamine. As demonstrated by the separation of synthetic PolyT oligonucleotides, as shown in Fig. 3, separations can be obtained comparable to those using TEAA containing mobile phases. The HFIP based separation is based on a gradient using methanol as an organic modifier. Interestingly, HFIP is miscible with water, methanol, isopropanol and hexane, but immiscible with acetonitrile.

3.3. Analysis of nucleotides and nucleotide analogues

In contrast to the analysis of oligonucleotides, the analysis of monomeric nucleotides is more similar to the characterization of small molecule pharmaceuticals than that of biopolymers. While the chromatography is somewhat more challenging due to their weak retention characteristics, for many applications, the MS is less demanding, based on singly charged $(M-H)^{-1}$ ions in targeted analysis.

3.3.1. Mobile phases containing tetrabutylammonium hydroxide

High hydrophylicity due to the ionic character of nucleotides is usually mediated through the use of ion pairing reagents such as tetrabutylammonium (TBA) salts. Unfortunately, neither the conventional non-volatile phosphate buffers nor the ion pairing reagents are compatible with ESI-MS. Ammonium

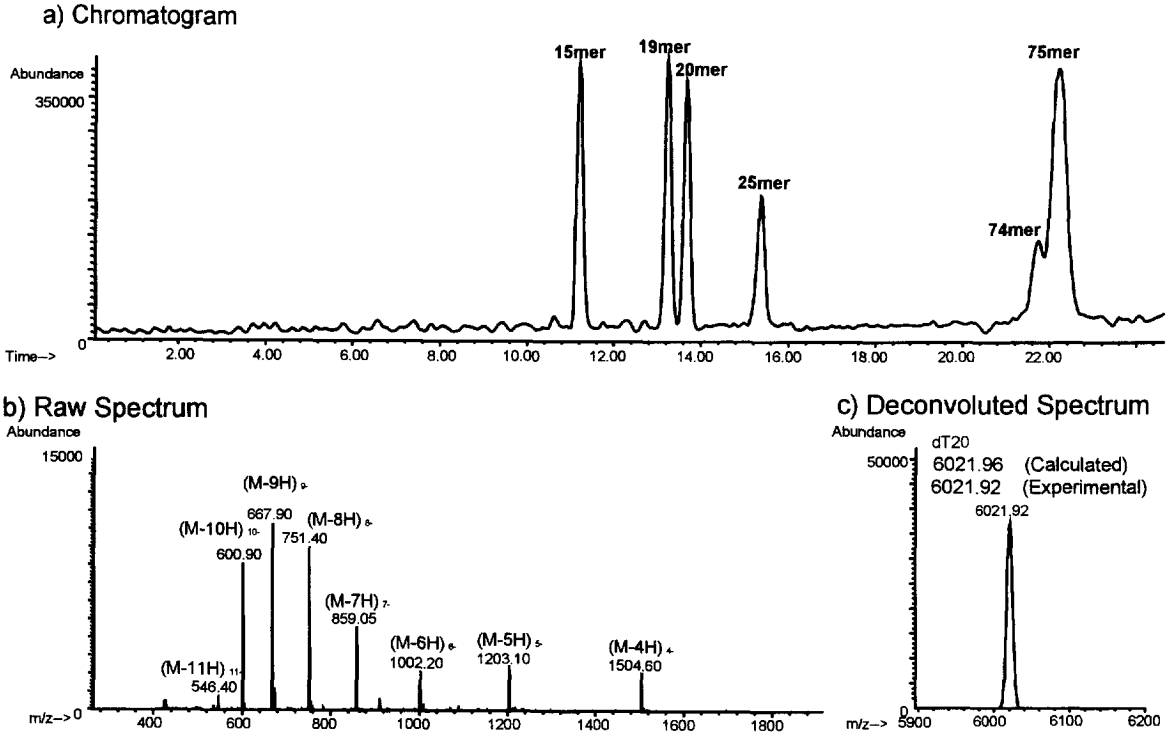

Fig. 5. Analysis of PolyT oligonucleotides by HPLC–ESI-MS. (a) Total ion chromatogram. (b) Raw mass spectrum. (c) Deconvoluted mass spectrum.

acetate buffers can be substituted for phosphate buffers, although these may result in reduced ionization efficiency at higher concentrations. More importantly, however, the presence of ion pairing reagents such as TBA result in substantial signal suppression in electrospray ionization due to the formation of strong and stable ion pairs with nucleotide analytes.

3.3.2. Mobile phases containing HFIP

The use of HFIP as a mobile phase additive with the pH adjusted to 7.0 with triethylamine allows the efficient separation of both nucleotides and nucleotide analogues. In contrast with conventional TBA based ion pairing separations, the TEA serves as a much weaker and less hydrophobic ion pair reagent. Consequently, much lower levels of organic modifier are needed to elute the analytes. In the case of nucleotides, different phosphorylation states for given nucleotides are easily separated, while for a given phosphorylation state not all of the different nucleotides are separated with baseline resolution. The higher hydrophobicity of the nucleotide analogues, AZT and its mono-, di- and triphosphates, allows greater retention and more easily optimized resolution.

Using the HFIP–TEA solvent system generates efficient electrospray ionization conditions comparable to analysis of the nucleotides using solvents containing TEA alone and superior, in terms of signal intensity, to simple aqueous samples. One disadvantage of the HFIP–TEA mobile phase is the background signal generated by the reagents. Fig. 4 shows the background spectrum of the mobile phase which is dominated by $HFIP^{-1}$ and the dimer $(2HFIP)^{-1}$ at m/z 167 and 335, respectively. In contrast with the oligonucleotide application which utilizes a higher mass range for data acquisition, the target nucleotide parent masses range between m/z 322 and 523 resulting in substantial overlap. As demonstrated below in specific applications, this can be mediated either through the use of selected ion monitoring methods or background subtraction for scan acquisition.

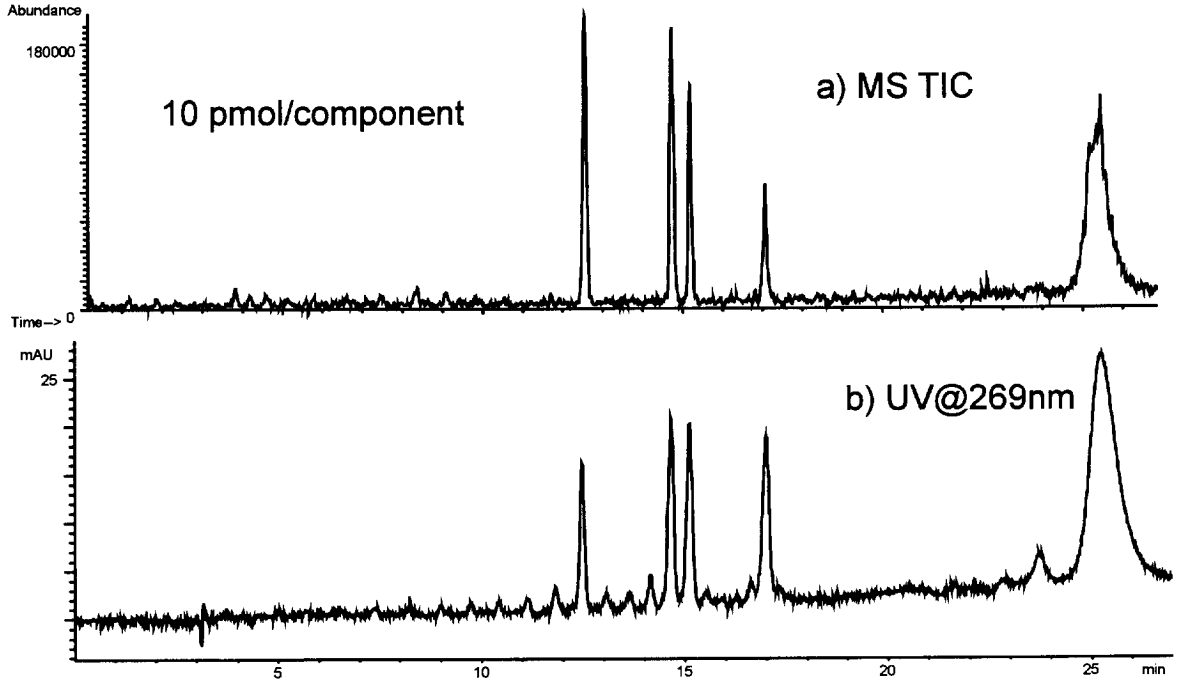

Fig. 6. Sensitivity of HPLC–ESI-MS analysis of PolyT oligonucleotides. (a) Total ion chromatogram. (b) UV at 269 nm.

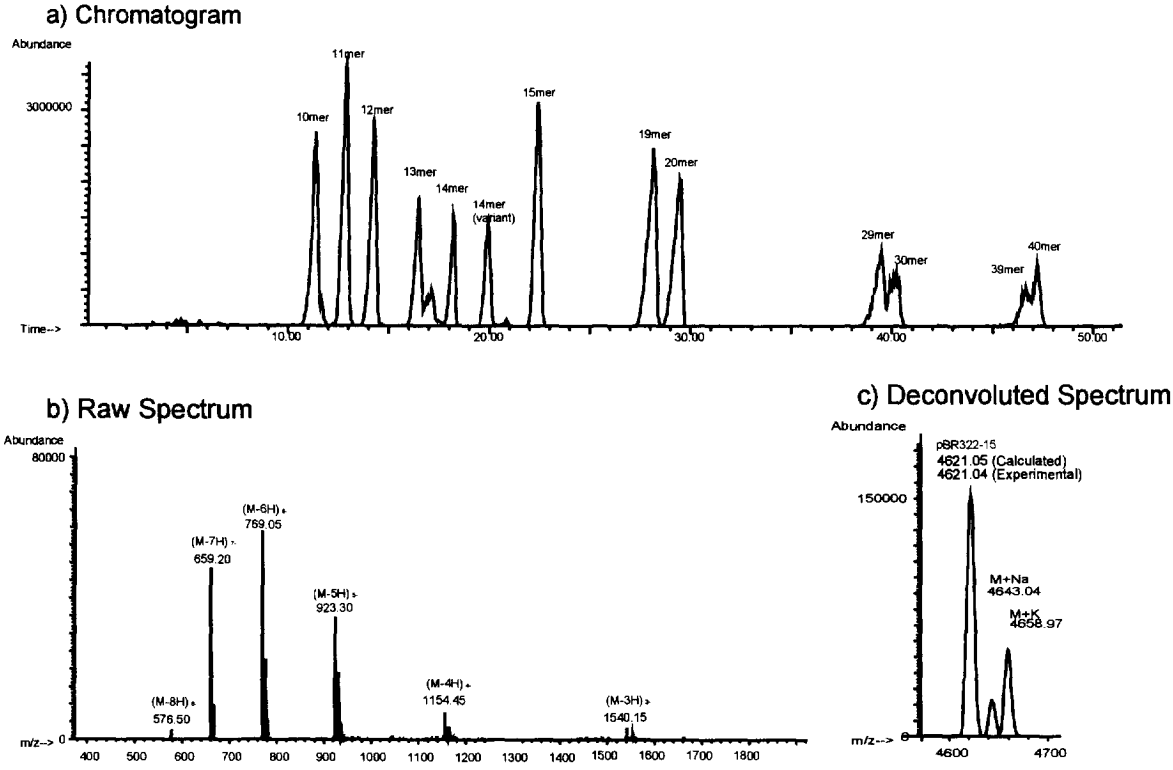

Fig. 7. Analysis of synthetic pBR 322 oligonucleotides by HPLC–ESI-MS. (a) Total ion chromatogram. (b) Raw mass spectrum. (c) Deconvoluted mass spectrum.

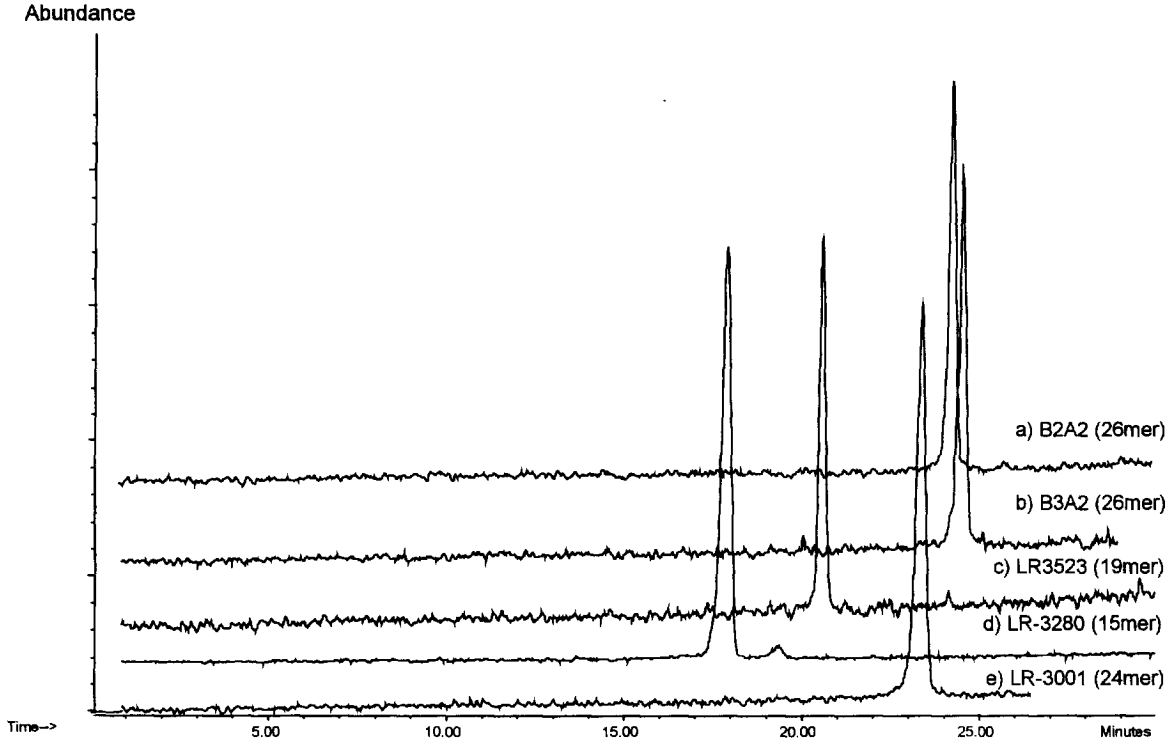

Fig. 8. Analysis of phosphorothioate ester antisense oligonucleotides by HPLC–ESI-MS. (a) B2A2. (b) B3A2. (c) LR3523. (d) LR3280. (e) LR3001.

3.4. Applications

3.4.1. Oligonucleotides

The application of the method to the analysis of synthetic PolyT oligonucleotides is shown in more detail in Fig. 5. Fig. 5a shows the HPLC–ESI-MS analysis of a mixture of synthetic PolyT oligonucleotides consisting of dT15, 19, 20, 24, 25, 74 and 75. The raw ESI spectrum consisting of multiply charged ions for the dT20 compound and shown in Fig. 5b and Fig. 5c, shows the deconvoluted spectrum with experimentally determined mass of 6021.92, which compares well with the calculated value of 6021.96. Note the very low level of adduct formation in both the raw spectrum and the deconvoluted spectrum. As an indication of sensitivity, Fig. 6a shows 10 pmol/component of a polyT sample with the simultaneously acquired UV chromatogram in Fig. 6b.

Fig. 7 shows the analysis of a mixture of synthetic oligonucleotides based on the pBR322 plasmid. Although synthetic, these heteropolymers of lengths 10, 11, 12, 13, 14, 15, 19, 20, 29, 30, 39 and 40 are based on the first 10–40 bases in the pBR 322 plasmid. The use of this sample demonstrates the performance of the system for analytes of mixed base composition as well as the potential application of the method PCR primer analyses. Fig. 7a shows the total ion chromatogram, Fig. 7b shows the raw spectrum for pBR322(15) and Fig. 7c shows its deconvoluted spectrum. Experimental and calculated

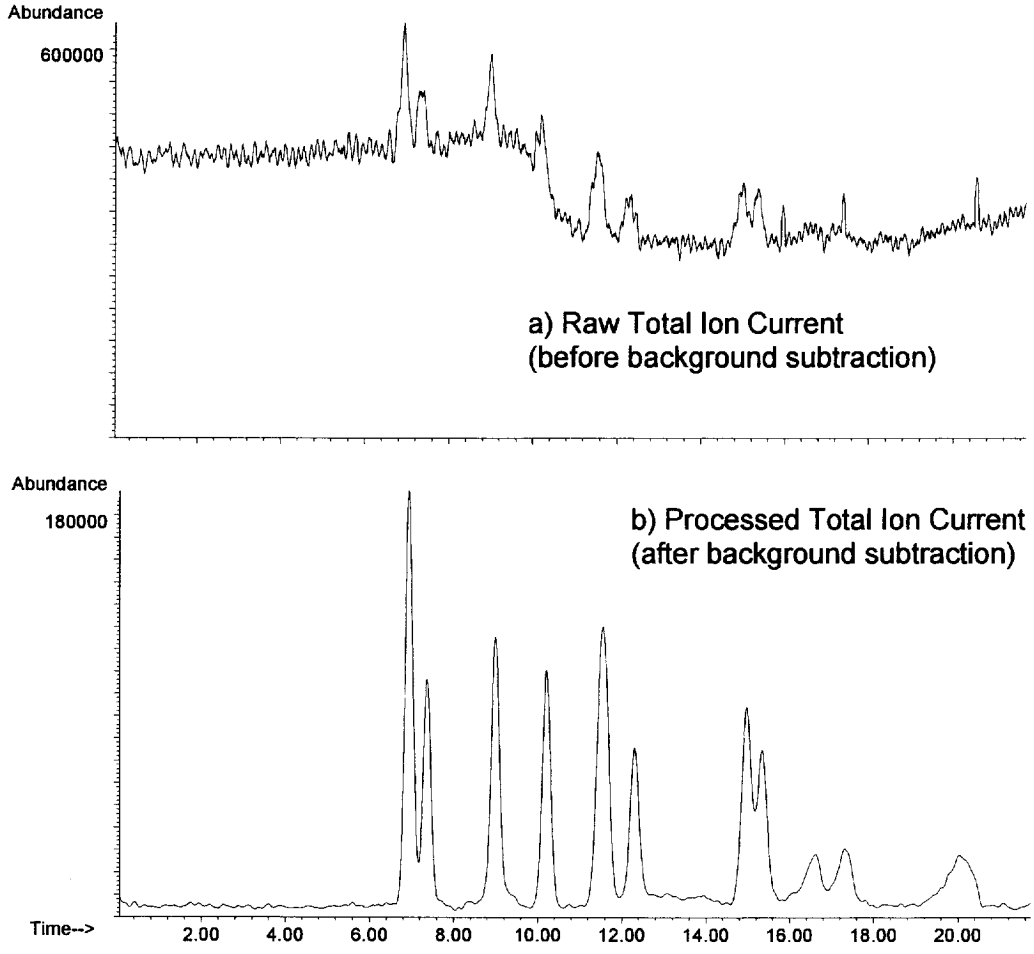

Fig. 9. Effect of background subtraction on HPLC–ESI-MS of nucleotides. (a) Before background subtraction. (b) After background subtraction.

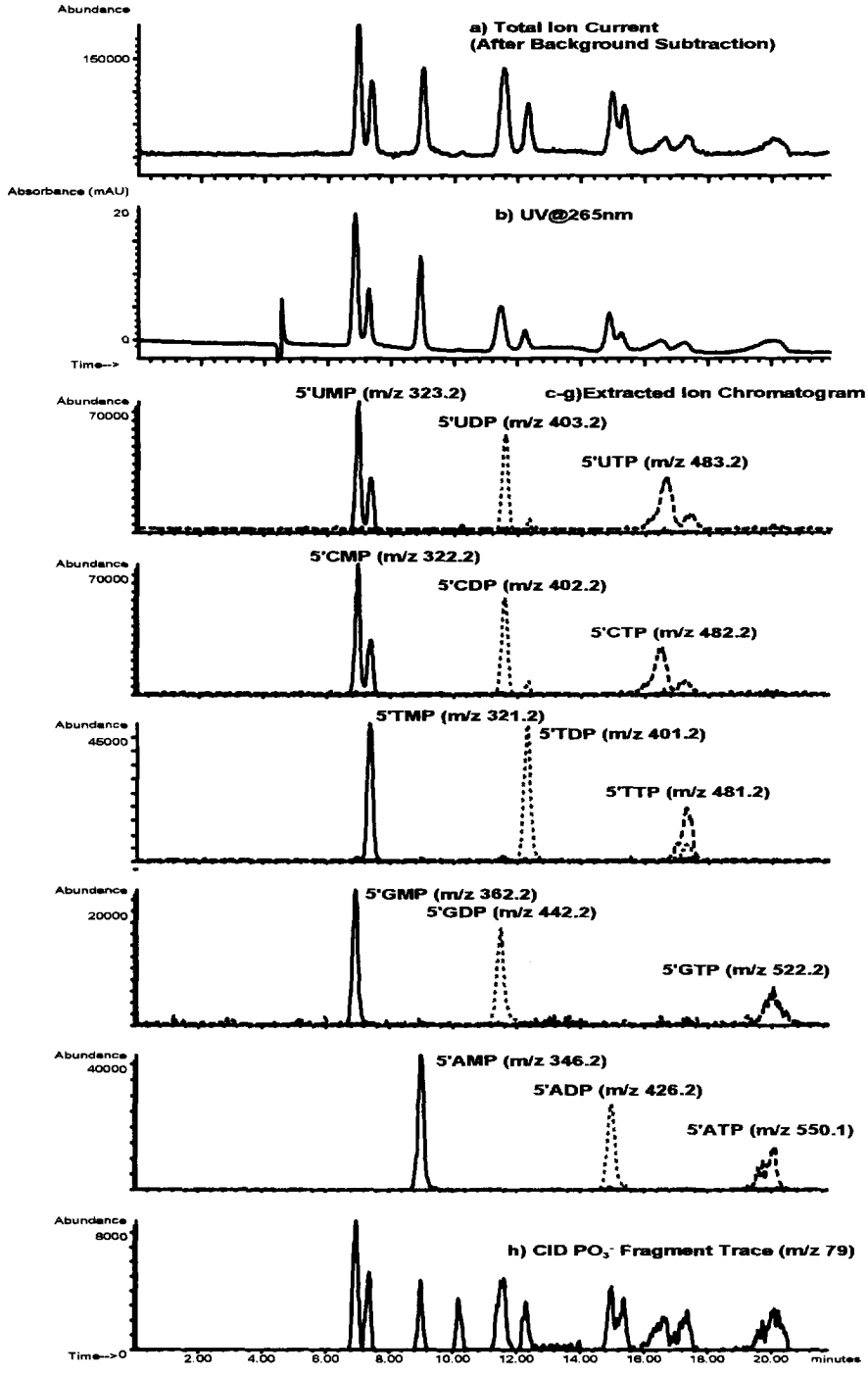

Fig. 10. Scan mode HPLC–ESI-MS analysis of nucleotide standard. (a) Total ion current. (b) UV at 269 nm. (c) Extracted ion chromatograms for deoxyuridine phosphates. (d) Extracted ion chromatograms for deoxycytidine phosphates. (e) Extracted ion chromatograms for deoxythymidine phosphates. (f) Extracted ion chromatograms for deoxyadenosine phosphates. (g) Extracted ion chromatograms for deoxyguanosine phosphates. (h) Extracted ion chromatogram for m/z 79: PO_3^-.

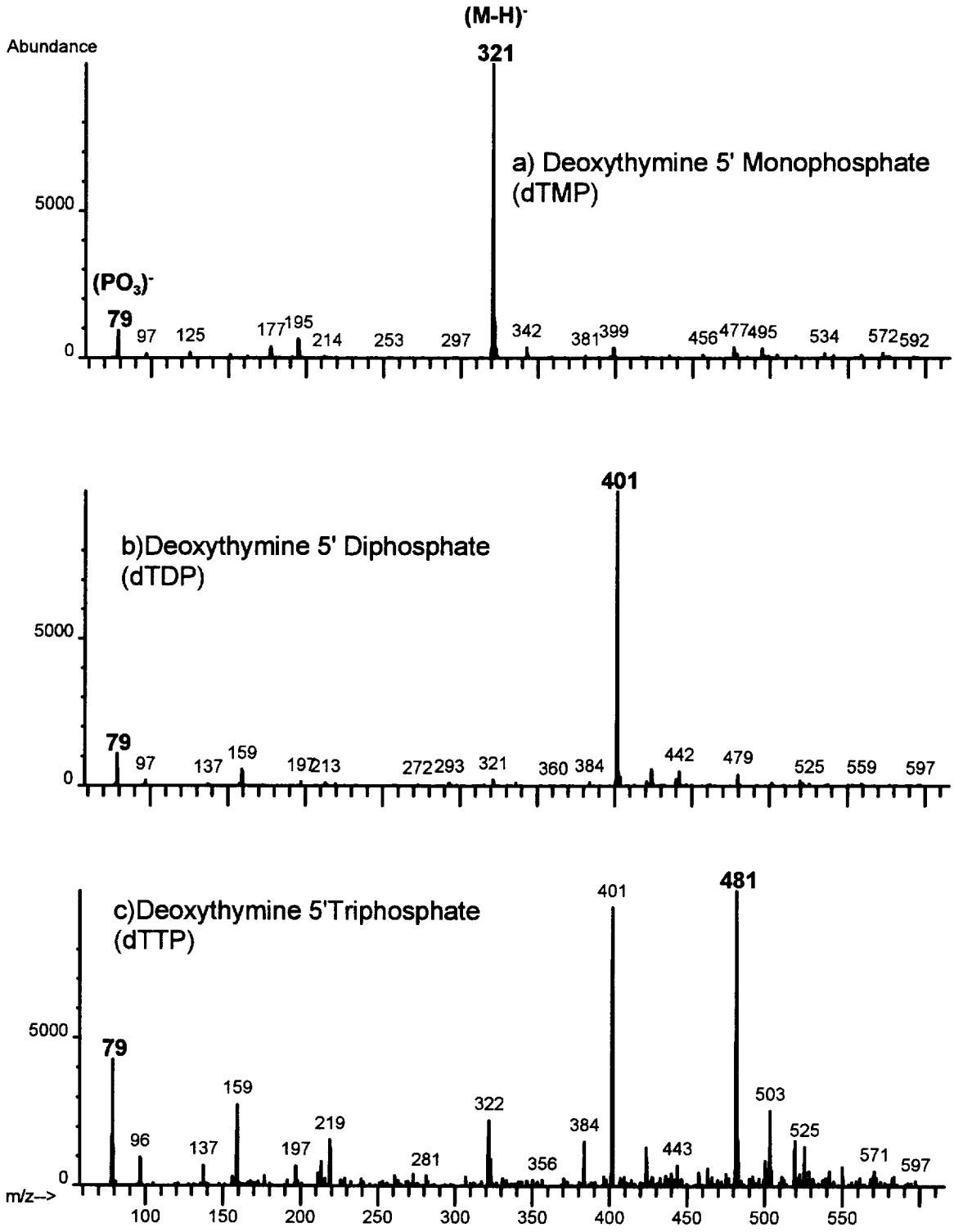

Fig. 11. ESI-MS Spectra of deoxythymidine nucleotides (after background subtraction). (a) deoxythymidine 5′-monophosphate. (b) Deoxythymidine 5′-diphosphate. (c) Deoxythymidine 5′-triphosphate.

masses compare very well. 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). Although it cannot be determined directly from this data, these differences may be due to variations in the sequence or conformational differences.

Fig. 8 shows the analysis of oligodeoxynucleotides targeted toward treatments of either acute or chronic myelogenous leukemia [24]. The backbone of these phosphorothioate oligonucleotides differs from the naturally occurring biopolymers by the substitution a sulfur atom for one of the non-bridging oxygen atoms in each phosphate group. This example has clear application for the improved characterization of synthetic purity and product identification for quality control, as well as the characterization of in vivo metabolic processes for these antisense pharmaceuticals.

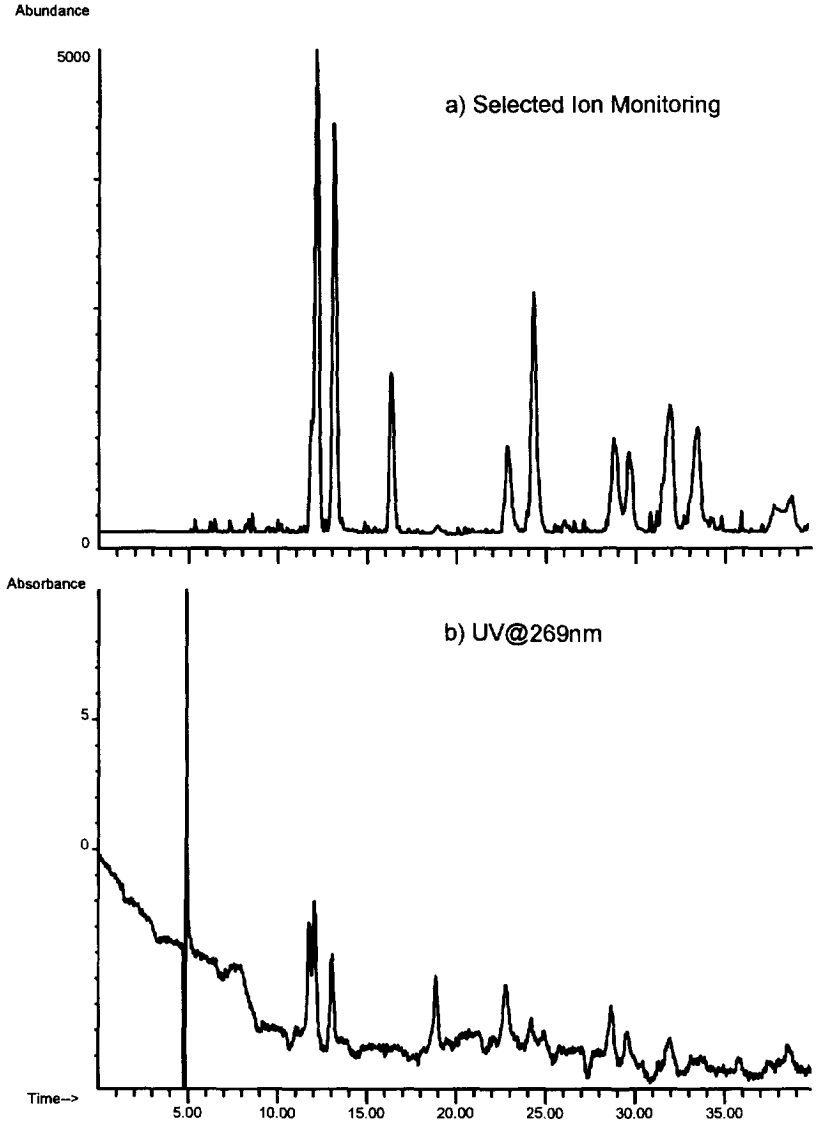

Fig. 12. SIM mode HPLC–ESI-MS of nucleotide standards. (a) Total ion current. (b) UV at 269 nm.

3.4.2. Nucleotides and nucleotide analogues

As mentioned above, the HFIP–TEA mobile phase background spectrum interferes with spectral acquisition of nucleotides in the useful mass range. Fig. 9 shows the scan acquisition TIC of a mixture of nucleotides at the 100 ng level before (a) and after (b) a post-acquisition background subtraction algorithm in which a single spectrum is subtracted from the entire data set. Fig. 10 shows the TIC and extracted ion chromatograms (EIC) for each of the nucleotides as well as the simultaneous UV signal. In addition, the m/z 79 ion is present as a CID fragment for each of the nucleotides. The spectra obtained from these data include the parent ion $(M-H)^{-1}$ and the PO_3^- as shown in Fig. 11. After background subtraction, scan detection limits are in the 10–100 pmol range. In selected ion monitoring (SIM) mode, it is not necessary to perform background subtraction and strong signals can be obtained as shown in Fig. 12. Detection limits in SIM mode are in the 1–10 pmol range. The unidentified peaks at 10 and 15 min in Fig. 10 and Fig. 12 appear to be degradation products with m/z values of 305 and 385, which suggests the loss of (deoxythimidine–OH) or dideoxythymidine. Since these peaks are in the UV chromatograms, they are not due to electrospray processes and can be attibuted to impure standards or degradation of the standard solution.

Similar results can be obtained for phosphorylated forms of AZT. As shown from the scan mode data in Fig. 13, a weak signal is present from the non-phosporylated AZT while the mono-, di- and triphosphates elute somewhat later in the chromatogram under higher organic mobile phase conditions.

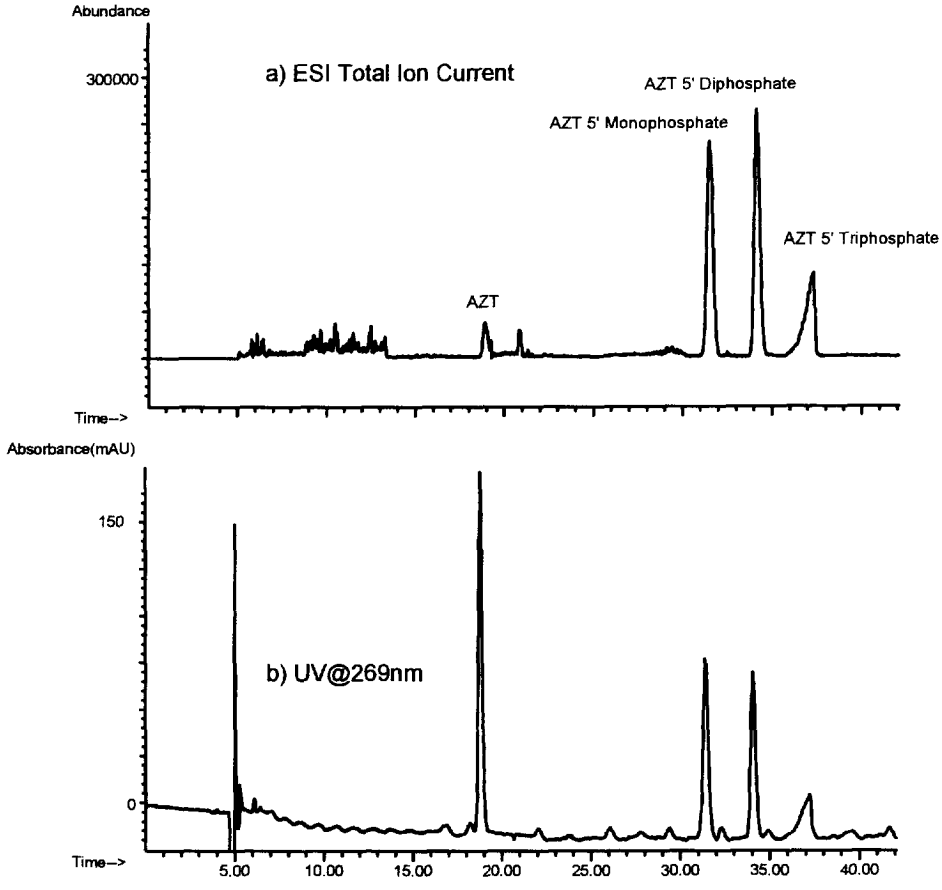

Fig. 13. Scan mode acquisition of AZT phosphates. (a) Total ion current. (b) UV at 269 nm.

Fig. 14 shows the spectra of each of the AZT compounds. Note the absence of the m/z 79 PO_3^- ion for the non-phosphorylated AZT. Detection limits for the scan acquisition are in the 10–50 pmole range. Improved specificity and detection limits can be obtained in SIM mode as shown in Fig. 15, with detection limits in the 1–10 pmol range. In future work, the combination of the SIM sensitivity with the possibility of injection of large volumes of biological fluid extracts with concomitant on-column preconcentration should allow the target detection limits of 10–100 pmol/ml.

4. Conclusions

In conclusion, a method has been demonstrated for the analysis of oligonucleotides by combined HPLC–ESI-MS which does not require compromised performance of either the HPLC separation or the electrospray mass spectra. 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 phosphorothio-

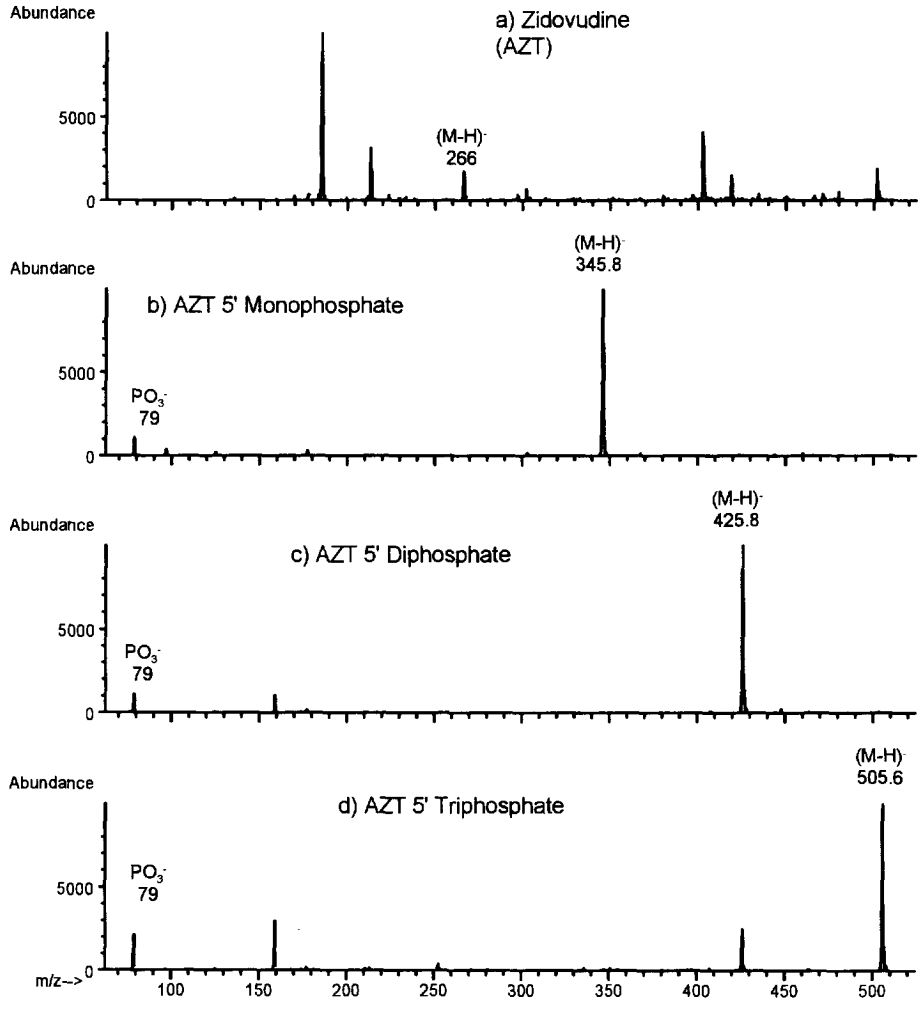

Fig. 14. ESI-MS spectra of AZT phosphates. (a) Ziduvodine (AZT). (b) AZT 5′-monophosphate. (c) AZT 5′-diphosphate. (d) AZT 5′-triphosphate.

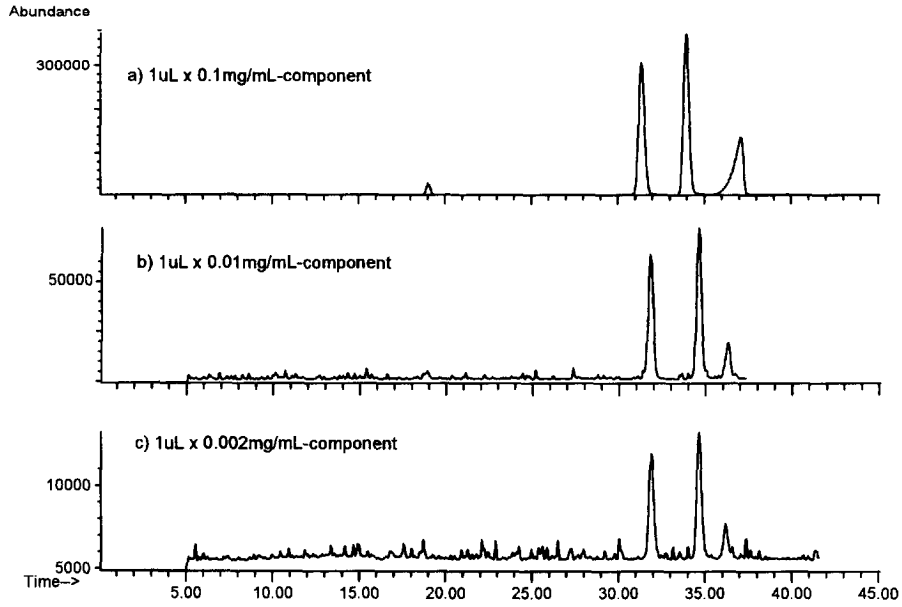

Fig. 15. SIM mode acquisition of AZT phosphates. (a) 1 μl×0.1 mg/ml. (b) 1 μl×0.01 mg/ml. (c) 1 μl×0.002 mg/ml.

ate antisense compounds. Future developments are under way to extend the mass range of this technique through the use of non-porous polymeric supports for the reversed-phase separation as well as to apply this method to the characterization of metabolic products of antisense oligodeoxynucleotide pharamaceuticals.

In addition, the method has been used for the analysis of nucleotides and nucleotide analogues. In scan acquisition, detection limits in the 10–100 pmol range are obtained while improved SIM detection limits are in the 1–10 pmol range. Future studies will apply this method to the characterization of metabolic phosphorylation of AZT in biological systems.

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