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Analysis of modified oligonucleotides by capillary electrophoresis in a polyvinylpyrrolidone matrix coupled with electrospray mass spectrometry

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Abstract

This paper describes the coupling of capillary electrophoresis (CE) with a polyvinylpyrrolidone (PVP) matrix and electrospray mass spectrometry for the analysis of a series of short modified oligonucleotides. The separation of modified oligonucleotides, four, five and six bases in length, was accomplished by CE in a coated fused-silica column filled with an aqueous solution of PVP. The resolved components were then identified by electrospray mass spectrometry with negative-ion detection. Importantly, oligonucleotides with the same length and sequence but differing only by the presence or absence of a small modification (such as a methyl group) on a single base were easily resolved by CE. This observation suggests that the matrix, PVP, is behaving as a pseudo-phase in which oligomers with hydrophobic modifications are retained longer than their normal unmodified analogs.

Keywords: Polyvinylpyrrolidone matrix; Oligonucleotides

1. Introduction

In recent years synthetic oligonucleotides have become increasingly important in the fields of molecular biology and medicine. They are used as probes in cDNA hybridization studies, as primers for sequencing or PCR amplification and in the diagnostic testing of target DNA. Antisense therapy also utilizes short strands of DNA or DNA analogues for the pharmacological control of a specific gene expression which may contribute to a disease state. The rapid growth in the use of synthetic oligonucleotides has spurred the development of analytical methodology for accurate and rapid determination of the integrity of the synthetic product.

As an analytical tool, mass spectrometry (MS) offers unparalleled capability for chemical detection and structure elucidation. In the last decade several new ionization techniques have been developed which permit routine analysis of proteins, oligonucleotides, oligosaccharides, etc., by mass spectrometry. For example, electrospray mass spectrometry (ES-MS) is now routinely used to determine the molecular mass of oligonucleotides up to 10 000 and even greater than that once the sample is thoroughly desalted [1,2]. In ES, analyte ions are produced from liquid solutions under the influence of an electric field [3]. Because oligonucleotides have

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an acidic proton at each equivalent position along the phosphodiester backbone, they typically form deprotonated molecule ions with a multiplicity of different charge states upon ES ionization. This envelope of charge states permits the accurate determination of molecular mass [4].

Capillary electrophoresis is a rapid, easy-to-use and high-efficiency separation technique which has been successfully coupled with mass spectrometry for the analysis of peptides, proteins, nucleosides-(tides), etc. [5–9]. In capillary zone electrophoresis (CZE) ionic species are separated as a result of differential migration in an electric field. To separate on the basis of hydrophobicity or size requires modification of the running buffer. There has been considerable interest in the use of water-soluble polymers to provide size discrimination [10–13] and even hydrophobic selectivity [14,15] in CE separations.

Polyvinylpyrrolidone (PVP) has been used as a buffer additive in CE for the separation of diastereomers [14,15]. In these instances PVP is believed to operate as a pseudo-phase in which diastereomers can effectively partition. PVP is thought to interact with organic anions through hydrogen bonding, dipole–dipole, dipole-induced dipole as well as by hydrophobic interactions [16]. Interestingly, oligonucleotides have been separated on the basis of size using PVP as a medium for size discrimination as well [17].

We have explored the coupling of CE in a polymer solution with electrospray mass spectrometry for the analysis of modified oligonucleotides. This type of CE–MS coupling combines powerful separation capability with extensive qualitative information for the analysis of oligonucleotides. In addition, on-line coupling decreases the total analysis time and reduces sample loss. This paper describes the coupling of CE in a PVP matrix with electrospray mass spectrometry for the separation and detection of short oligonucleotides. In this preliminary study we analyzed test solutions of short oligomers with slight structural differences (see Figs. 1 and 4).

Table 1
Calculated and observed molecular mass of the modified oligodeoxynucleotides (four-mers and five-mers)

Oligodeoxynucleotides	M_r	
	Calculated	Observed
TGCA	1173.8	1173.8
TG(O^6Me)CA	1187.8	1187.8
TG(O^6BU)CA	1229.8	1229.4
GCAGC	1488.0	1487.6
GU*AGC	1523.0	1522.6

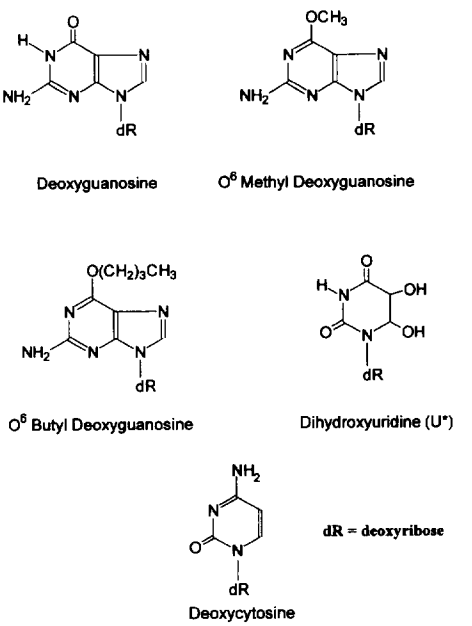

Fig. 1. Structures of the modified guanine and uracil bases.

2. Experimental

2.1. CE conditions

The polymer solution was prepared by dissolving polyvinylpyrrolidone (M_r 1 000 000) in the running buffer (20 m*M* NH_4OAc, pH 9.8) at a concentration of 14% (w/v). The matrix was stirred at room temperature for 12 h and degassed under vacuum. The solution was introduced into a coated silica capillary (50 cm×150 μm I.D.) by pressure with a syringe [18]. The coating utilized was covalently attached polyvinylalcohol [19]. The CE instrument was built in-house with a Spellman (Plainview, NY, USA) high-voltage power supply. Analytes were introduced into the capillary column electrokinetically (5 kV, 3 s). During the electrophoretic run, a constant 5-kV voltage was applied across the capil-

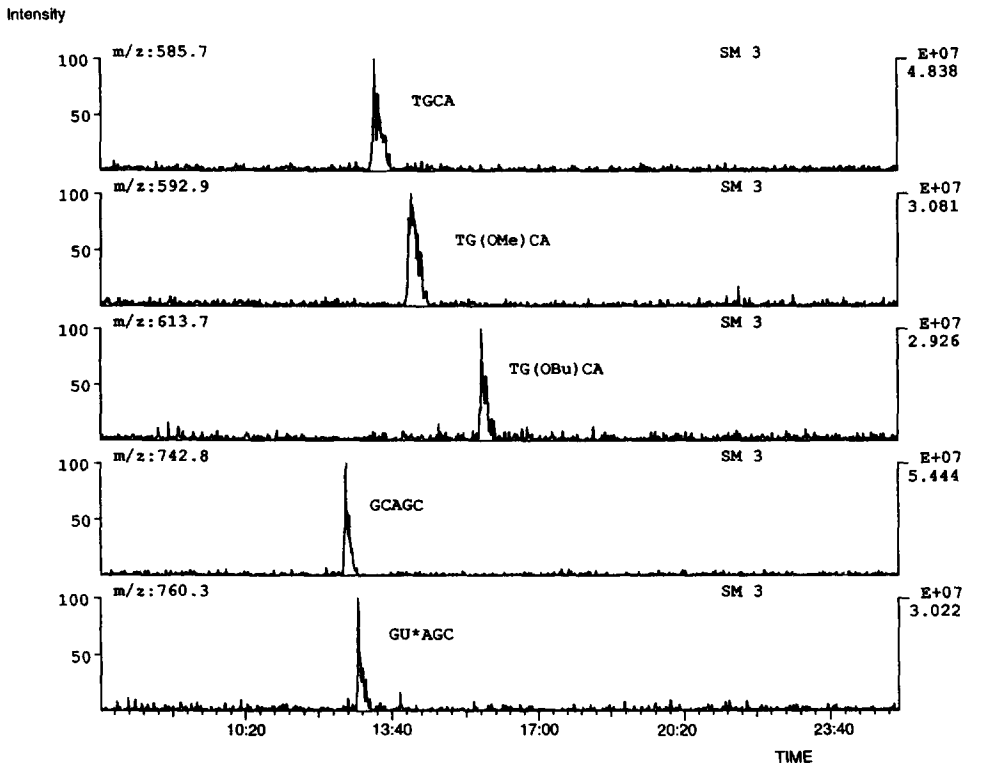

Fig. 2. Extracted mass electropherograms showing resolved oligonucleotides (electrokinetic injection: 5 kV, 3 s, 100 fmol/μl).

lary generating a current of approximately 20 μA. After each run the ES source was pulled back from the mass spectrometer and the polymer solution in the capillary replaced by pressure displacement. The capillary itself was not removed from the source. After the fresh matrix had been inserted, the source was placed into position and a new analysis begun. The entire process requires less than 5 min.

2.2. ES-MS conditions

All of the analyses were run on a triple quadrupole mass spectrometer, Finnigan MAT TSQ700 (Finnigan, San Jose, CA, USA), equipped with an electrospray ion source. The electrospray needle was maintained at −3000 V and the sampling orifice was set to ground potential, consistent with negative-ion formation. Calibration of the mass spectrometer was performed using a 5 pmol/μl of myoglobin solution in 5% acetic acid. The instrument was tuned on a series of standard oligonucleotides synthesized in-house. The electron multiplier was set to 1800 V, conversion dynode to 15 kV and the quadrupole manifold heated to 70°C. A makeup liquid was introduced to compensate for the near-zero volumetric flow-rate of the CE column. This sheath liquid consisted of 75% (v/v) running buffer and 25% (v/v) 2-propanol, infused at a rate of 3 μl/min. The third quadrupole was scanned from M_r 400 to 1000 in 1 s. The ES-MS and ES-MS–MS of these compounds have been discussed previously [20].

2.3. Chemicals

All solvents were HPLC grade and obtained from Fischer Scientific (Pittsburgh, PA, USA). PVP was purchased from Polysciences (Warrington, PA, USA). Myoglobin (used for tuning the MS) was obtained from Sigma (St. Louis, MO, USA). The synthesis of the modified oligonucleotides will be described elsewhere [21].

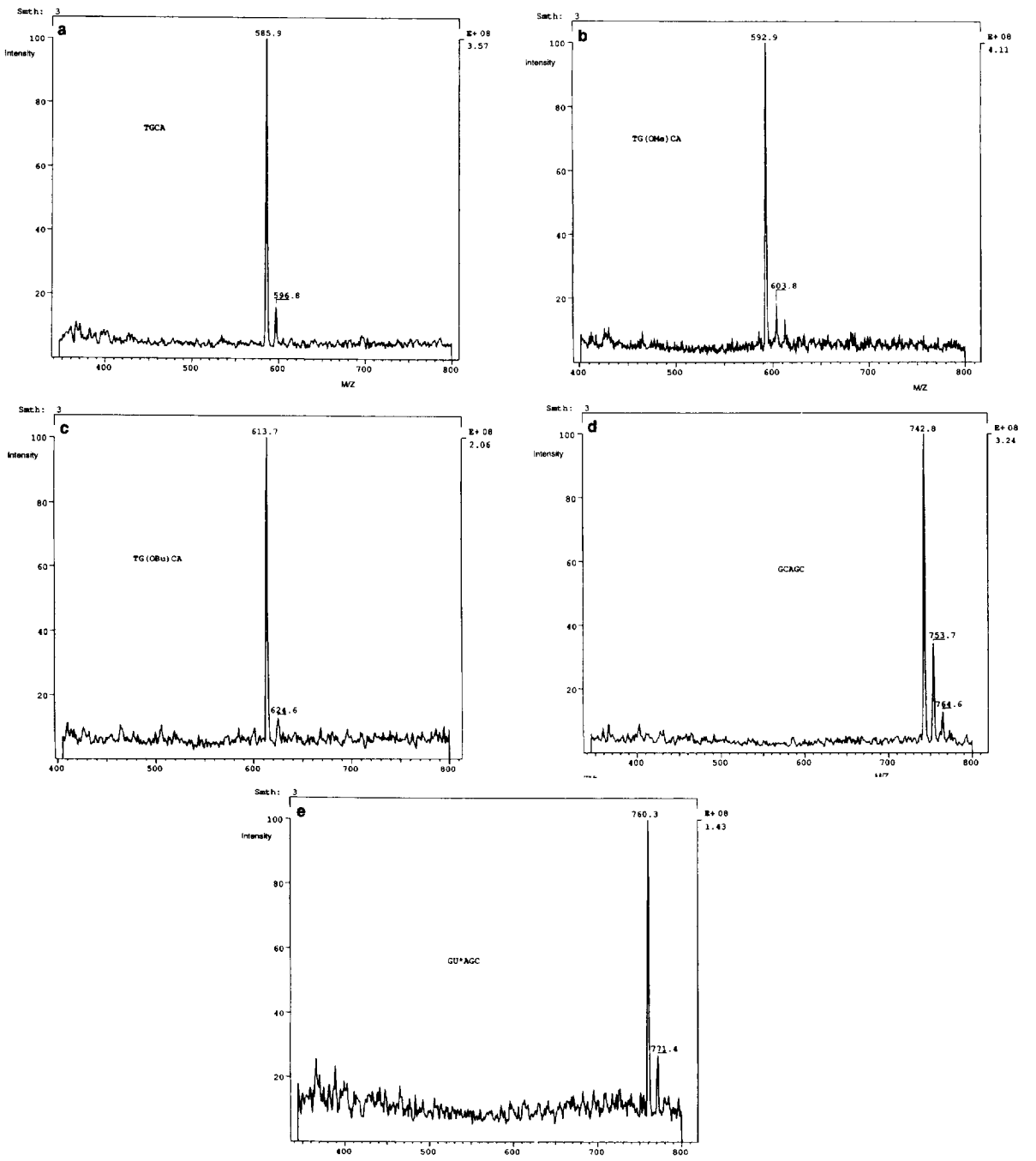

Fig. 3. (a) Negative-ion spectrum of TGCA. (b) Negative-ion spectrum of TG(O^6Me)CA. (c) Negative-ion spectrum of TG(O^6Bu)CA. (d) Negative-ion spectrum of GCAGC. (e) Negative-ion spectrum of GU*AGC.

Table 2
Calculated and observed molecular mass of the modified oligodeoxynucleotides (six-mers)

Substituent (X)	M_r	
	Calculated	Observed
Ethyldeoxyuridine	1806.3	1805.8
Bromovinyldeoxyuridine	1882.2	1882.2
Chlorodeoxyadenosine	1826.2	1826.0
Trifluorodeoxythymidine	1845.2	1802.4

3. Results and discussion

In the first experiment five oligonucleotides were mixed together in approximately equal quantities (100 fmol/μl). The resultant solution contained three four-mers and two five-mers. These compounds are listed in Table 1 and their structures shown in Fig. 1. Note that the three four-mers contain the sequence TG*CA and that the only difference between them is the nature of the modification on the O^6 oxygen of the guanine. The selected mass electropherograms are shown in Fig. 2 and the mass spectra obtained by combining the scans underneath each individual peak in Fig. 3. The calculated molecular masses are listed in Table 1 and compared with the experimentally determined values.

As seen in Fig. 2, all the oligonucleotides are baseline resolved. The five-mers, having a greater mobility towards the anode, elute more rapidly than the smaller four-mers. Interestingly, all three four-mers are easily resolvable due to their differences in hydrophobicity. It appears that the polymer behaves as a pseudo-phase on which oligomers with hydrophobic modifications are retained longer than their normal analogues. This observation is of considerable importance for the analysis of short oligomers: oligonucleotides of the same length and having only slight differences in mobility may thus be separated on the basis of their hydrophobicity.

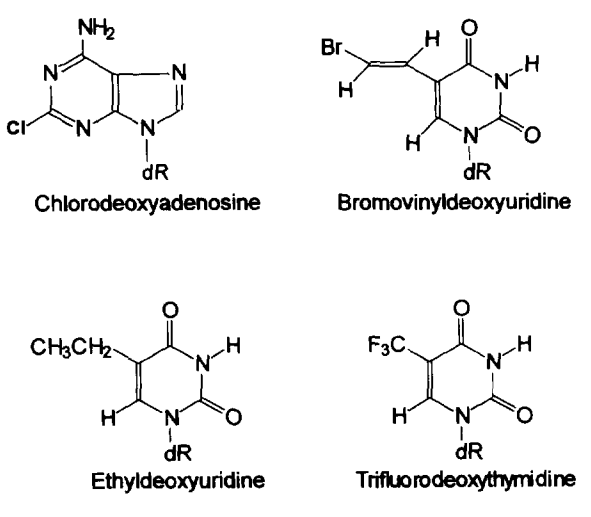

Fig. 4. Structures of modified bases in the six-mers.

The mass spectra were obtained by scanning from M_r 400 to 800 and summing the scans under each peak in the electropherogram. The doubly charged deprotonated ion is the most dominant ion observed in all the spectra. In addition to this ion, the sodiated ion(s) is observed in the ES spectra of these oligonucleotides. Although usually regarded as a persistent and deleterious contaminant, especially when analyzing larger oligonucleotides (M_r>10 000), this satellite ion may be used to determine the charge state of the deprotonated ion: if the m/z difference (Δm) between the molecule ion and the first sodiated ion is 22, then z=1 (Na^+ replaces an acidic phosphate H^+). If Δm is 11.0 or 7.3 then the charge states would be z=2 and z=3, respectively. Thus, accurate measurement of Δm provided a quick means for determining the ion charge state. In all of the displayed spectra the observed Δm value is 11.0, suggesting that the ion charge state is z=2. Thus, even in the absence of other charge states, the ion mass can be determined.

To investigate further the selective characteristics of the polymer, a test mixture of four six-mers in approximately equal quantities (100 fmol/μl) was analyzed by CE-MS. These compounds and their molecular mass are listed in Table 2. The oligomers have the same underlying sequence, CACGTG, but differ only in modifications on the adenine and thymidine bases. The structures of these bases are shown in Fig. 4. Three of the oligonucleotides have the sequence CACGXG, where X is a modified thymidine, and one has the sequence CXCGTG, where X is chloroadenosine.

For three of the four modified oligonucleotides, the experimentally determined molecular masses were in good agreement with the calculated masses (Table 2). Interestingly, the experimentally deter-

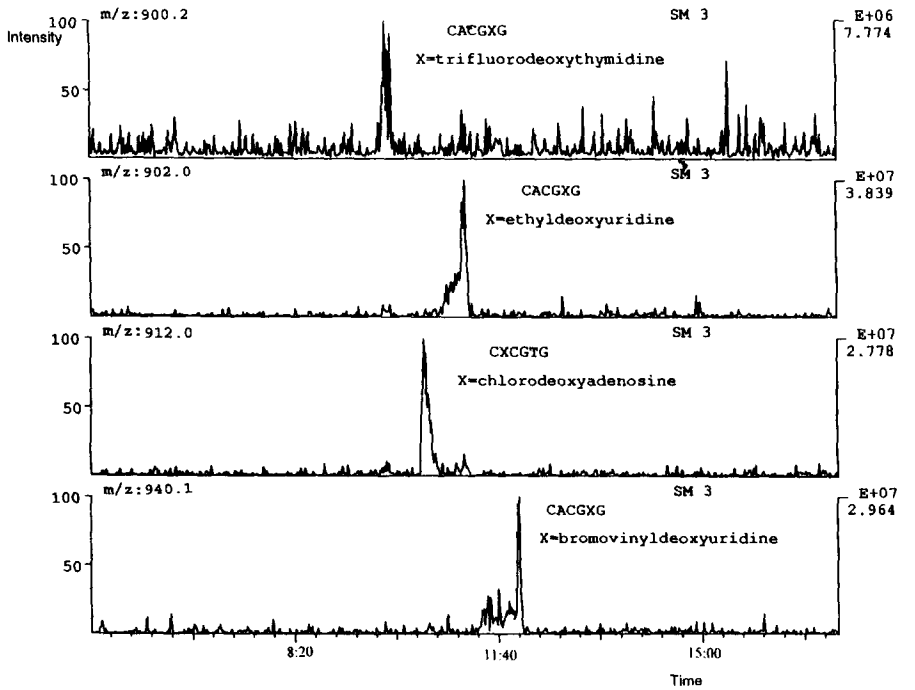

Fig. 5. Extracted mass electropherograms showing resolved oligonucleotides (electrokinetic injection: 5 kV, 3 s, 100 fmol/μl).

mined molecular mass of the trifluorodeoxythymidine analog differed from the calculated value by approximately 43. This discrepancy may have resulted from the elimination of HNCO from the modified base via a retro Diels–Alder reaction. The trifluoro modification has a large inductive withdrawing effect and should promote the retro Diels–Alder process. It is not completely clear whether the loss of HNCO is the result of a mass spectrometric process [22,23] or a degradation of the sample itself.

In the mass electropherograms shown in Fig. 5, all the oligomers were again baseline resolved. The observed order of elution was trifluoro-, chloro-, ethyl- and bromovinyl. From these limited studies it appears that oligomers with polar substituents are retained less on the non-polar polymer. It may also be possible that molecules with strong π-systems would exhibit higher migration times on PVP. The mass spectra obtained from combining the scans under each peak are displayed in Fig. 6 and the results tabulated in Table 2. It is significant that these compounds could not be resolved mass spectrometrically if directly infused into the ES-MS as the spectra have ions which are isobaric (see spectra in Fig. 6). Therefore, it is necessary to resolve these compounds by chromatography or by electrophoresis.

As a control, the analyses were repeated using UV detection under the same conditions (column dimensions, buffer, column coating, injection, etc.) but *without* the polymer matrix. The sample constituents were not resolvable by open-tube CE with these operating parameters. The UV electropherogram of the six-mer mix is shown in Fig. 7.

4. Conclusions

This report demonstrates the feasibility of coupling capillary electrophoresis filled with polymer solution with electrospray mass spectrometry. These

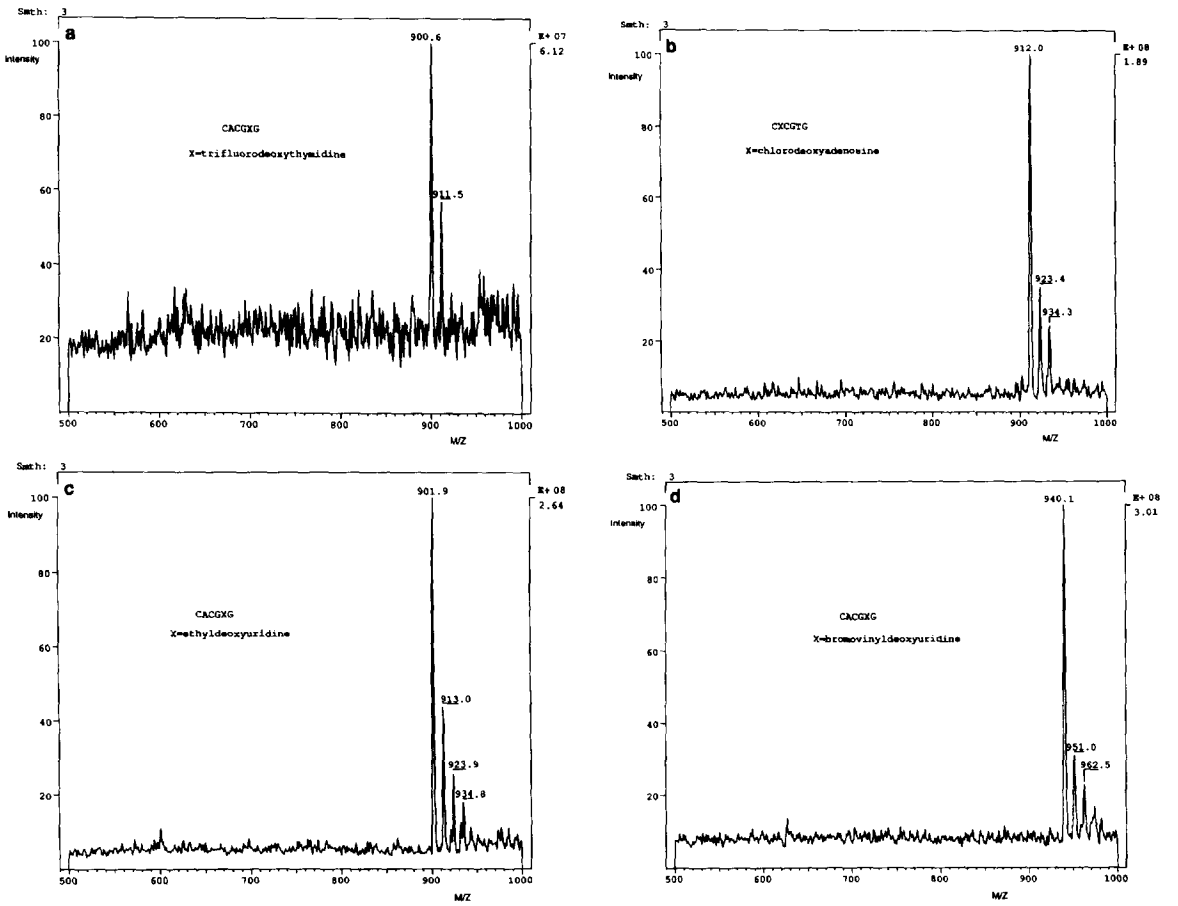

Fig. 6. (a) Negative-ion spectrum of CACGXG (X=trifluorodeoxythymidine). (b) Negative-ion spectrum of CACGXG (X= chlorodeoxyadenosine). (c) Negative-ion spectrum of CACGXG (X=ethyldeoxyuridine). (d) Negative-ion spectrum of CACGXG (X= bromovinyldeoxyuridine).

combined techniques should contribute to analytical methodology for the analysis of oligonucleotides and modified oligonucleotides. This approach provides the capability to rapidly separate and identify oligomers in a complex mixture. Results indicate that the polymer in solution retains components on the basis of hydrophobicity or other interactive mechanisms. Thus, in addition to separating oligonucleotides on the basis of size, polymer solutions may be used for the separation of short oligomers differentiated by subtle changes in nucleobase properties. We are currently investigating the utility of this methodology for the analysis of short oligonucleotides with covalent modifications caused by the presence of xenobiotics.

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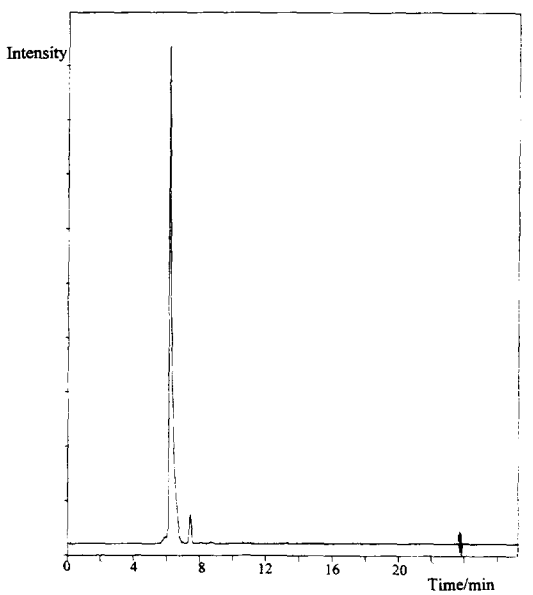

Fig. 7. CE–UV electropherogram run with no polymer matrix (electrokinetic injection: 5 kV, 3 s, 100 fmol/μl).

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