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High-performance liquid chromatography and capillary gel electrophoresis as applied to antisense DNA

Aharon S. Cohen*, Maria Vilenchik, Judith L. Dudley, Mark W. Gemborys and André J. Bourque

Hybridon Inc., Worcester, MA 01605 (USA)

ABSTRACT

Reversed-phase and ion-exchange HPLC are compared with both slab gel and capillary gel electrophoresis for the separation of antisense phosphorothioate oligomers. The chromatographic separations were found to be markedly inferior to the electrophoretic separations, especially for oligomers greater than 20 bases in length. The potential of gel high-performance capillary electrophoresis for the analysis of phosphorothioate analogues is shown.

INTRODUCTION

A new field, "antisense DNA", was born following the discovery that a synthetic 13-mer DNA selectively inhibited gene expression in *Rous Sarcoma* virus [1]. Oligodeoxynucleotides (ODNs) that are complementary or "antisense" to a specific gene or mRNA sequence are potential chemotherapeutic agents [2,3]. Initially, this concept was accepted with skepticism. The half-life of short single-stranded DNA (ssDNA) in a living organism (and *in vitro*) is short; before it penetrates the cell membrane and reaches its target, most of it is digested by exonucleases. Therefore ssDNA is not effective as a targeted therapeutic drug at low dosages. To diminish enzymatic hydrolysis, synthetic analogues of oligonucleotides were synthesized. It is important to the antisense strategy to minimize disruption of the normal formation of hydrogen bonded Watson–Crick base pairs. Thus, antisense DNA modifications should be conservative. The substitution of a sulfur atom for an oxygen atom on the phosphate group is a conservative substitution which increases nuclease resistance without significantly impairing the hybridization of the antisense with the target mRNA [4]. These ssDNA analogues are known as phosphorothioates (SODNs).

SODNs have been successfully used as antiviral agents [5,6] and gene inhibitors [5]. Toxicity and pharmacokinetics of SODN oligomers have been conducted successfully on animals with promising preliminary results [7]. The principle by which such a compound is used is rather simple. Antisense DNA, the complement to a specific segment of mRNA, hybridizes to the target sequence and inhibits the gene's expression. If the product of the inhibited gene (*e.g.*, a protein) is essential to the cell, the cell will perish. Antisense DNA analogues are small synthetic molecules (*i.e.*, M_r *ca.* 10 000). The development and manufacture of antisense pharmaceuticals must address the issues of oligomer length, base composition, base sequence and chemical as well as stereochemical purity.

RP-HPLC is of limited use for the resolution of modified DNA because of the small differences in hydrophobicity with increasing chain length [8]. In addition, the resolution of SODNs may be complicated by stereochemical effects.

* Corresponding author.

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The substitution of a sulfur for an oxygen atom on the phosphate group results in the formation of a chiral center. Thus, for an oligomer with n SODN linkages, there can be 2^n stereoisomers only one of which may represent the most active molecule [4]. The reversed-phase partitioning is complicated by the nature of the DNA backbone, which requires low pH for suppression of SODN ionization. The addition of a cationic ion-pairing reagent increases the efficiency of reversed-phase separations and ODNs with up to 20 bases have been resolved [9]. However, these data were generated with phosphodiester DNA, not the sulfur analogue. Ion-exchange HPLC appears to offer the highest chromatographic resolution of modified oligomers. Polymer based columns yield more predictable behavior and increased longevity over silica based columns [10]. This is probably due to secondary interactions with silanol groups and the chemically labile nature of silica bonded phases. A potential benefit of polymer based columns is the ability to utilize pH extremes to differentiate between oligomers of equal length, but different base sequence [11]. The use of weak or strong anion-exchange columns (WAX/SAX) yielded comparable separations although the selectivity of the WAX column could be modified by pH. The advantage of ion-exchange HPLC over ion-pair HPLC is selectivity; ion-exchange HPLC resolution of SODNs is sequence *and* length dependent while ion-pair HPLC is only length dependent [9].

The length and heterogeneity of natural or modified DNA are best assessed by polyacrylamide gel electrophoresis (PAGE). There is a great deal of interest in the separation and characterization of SODN analogues for use as antisense pharmaceuticals. Synthetic SODNs have become standard reagents in most antisense laboratories using molecular biology techniques. When synthesized, the product is a mixture of truncated oligomers and the desired oligomer. Purification usually involves PAGE and RP-HPLC [12]. The task of purification is a challenging one, because failure sequences differ only in base number and possibly in the sequence of bases. A technique for the rapid separation of SODNs could result in important advances in the molecular biology of antisense pharmaceuticals.

We have demonstrated the high resolution power of PAGE filled capillaries for the separation of low-molecular-mass DNA [13] and enzymatic sequencing reaction mixtures [14]. Capillary gel columns with low monomer concentrations (less than 3% T) generate large numbers of theoretical plates; however, they are not suitable for SODN separations.

In this paper we compare and contrast the separation of SODNs via chromatographic and electrophoretic methods. Several factors which control efficiency and resolution of SODNs by HPCE are explored. The separation conditions for SODNs are significantly different from those recommended for phosphodiesters [14]. The results were used to define conditions where separation of SODNs can be achieved with single-base resolution up to 50 bases in length.

EXPERIMENTAL

Chemicals and reagents

Water and acetonitrile (ACN) were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Tetrabutylammonium phosphate (TBAP) was obtained from Fluka (Ronkonkoma, NY, USA). Sequagel Sequencing System (acrylamide–bis-acrylamide, 19:1) purchased from National Diagnostics (Manville, NJ, USA) was used for slab gel preparations. Ultra-pure Tris base, urea, acrylamide, and EDTA were purchased from Schwartz/Mann Biotech (Cleveland, OH, USA). N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Bio-Rad (Richmond, CA, USA). Boric acid was obtained from Sigma (St. Louis, MO, USA). All SODNs were synthesized in-house, desalted, lyophilized and reconstituted in sterile water for injection (Lyphomed, a division of Fujisawa USA, Deerfield, IL, USA). Stains-All (4,5,4′,5′-dibenzo-3,3′-diethyl-9-methylthia-carboncyanine bromide) was purchased from Eastman Kodak (Rochester, NY, USA).

HPLC apparatus

Two HPLC systems were employed in this work: an HP 1090m (Hewlett-Packard, Burlington, MA, USA) and a Waters system consisting of a 600E system controller, a 717 autosampler and a 490E UV detector (Waters, a division of

Millipore, Milford, MA USA). The reversed-phase resolution of SODNs was performed on a NovaPak C_{18}, 4 μm, 150 × 3.9 mm I.D. column (Waters) using the following mobile phases: (I) A: 0.1% formic acid, B: ACN containing 0.1% formic acid; (II) A: 0.1 *M* ammonium acetate (NH_4Ac), B: ACN–0.1 *M* NH_4Ac (80:20); (III) A: 50 m*M* KH_2PO_4 (pH 4.3), 10 m*M* TEAP, B: ACN–water (80:20) containing 10 m*M* TBAP. Ion-exchange separations were performed on a Partisphere WAX, 5 μm, 110 × 4.7 mm I.D. GENPAK column (Whatman, Clifton, NJ, USA), a GEN-PAK FAX, 2.5 μm, 100 × 4.6 mm I.D. column (Waters) and a NucleoPac PA-100, 13 μm, 250 × 4.0 mm I.D. column (Dionex, Sunnyvale, CA, USA). The mobile phases used for ion-exchange HPLC were (IV) A: 20 m*M* KH_2PO_4, 100 m*M* $(NH_4)_2SO_4$ (pH 6.3)–ACN–MeOH (8:2:1, v/v/v), B: 20 m*M* KH_2PO_4, 1 *M* $(NH_4)_2SO_4$ (pH 6.3)–ACN–MeOH (8:2:1, v/v/v) and (V) A: 25 m*M*, Tris 1 m*M* EDTA (pH 8.0)–ACN (9:1, v/v), B: 25 m*M* Tris, 1 m*M* EDTA, 2 *M* NH_4Cl (pH 8.0)–ACN (1:9, v/v). All effluents were monitored at 270 nm and gradients were as defined in Table I.

Slab gel apparatus

Electrophoresis was carried out in a vertical slab gel apparatus (Model V16, GIBCO BRL, Gaithersburg, MD, USA). The electric field was supplied by a regulated power supply (Model FB 400, Fisher, Pittsburgh, PA, USA). The applied voltage was 250 V, which corresponded to an effective field strength of 19.2 V/cm. Gels were stained with Stains-All and dried on a gel dryer (Buchler Instruments, Lenexa, KA, USA). Electropherograms were obtained on a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

HPCE apparatus

The capillary electrophoresis apparatus with

TABLE I

GRADIENT PROGRAMS USED FOR HPLC ANALYSES

Gradient program	Column	Flow-rate and temperature (ml/min, °C)	Time (min)	Mobile phase composition (%)	
				A	B
I	NovaPak C_{18}	1.5 at 50°C	0	94	6
			3	94	6
			13	26	76
II	NovaPak C_{18}	1.5 at 50°C	0	100	0
			2	100	0
			12	0	100
III	NovaPak C_{18}	1.5 at 50°C	0	50	50
			2	50	50
			8	50	100
IV	Partisphere WAX	1.5 at room temperature	0	100	0
			2	100	0
			25	40	60
			30	40	60
V	GenPak FAX	0.75 at 65°C	0	40	60
			15	5	95
			15.1	0	100
			30	0	100
VI	Nucleopak PA-100	2.0 at 70°C	0	90	10
			6	0	100
			10	0	100

UV detection and the preparation of gel-filled capillary for the separation of DNA molecules have been described previously [13,15]. A 30 kV, 500 μA direct current high-voltage power supply (Model ER/DM; Glassman, Whitehouse Station, NJ, USA) was used to generate the potential across the capillary, UV detection of SODNs at 270 nm was accomplished with a Spectra 100 (Spectra-Physics, San Jose, CA, USA). The data were acquired and stored on an AcerPower 486/33 computer (Acer American, San Jose CA, USA) through an analog-to-digital converter (Model 970, Nelson Analytical, Cupertino, CA, USA).

Gel-filled capillaries

Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm I.D., 375 μm O.D., effective length of 15–20 cm and the total length of 30–60 cm was treated with (methylacryloxypropyl)trimethoxysilane (Petrarch Systems, Bristol, PA, USA) and then filled with a degassed solution of 13–18% polymerizing linear acrylamide in aqueous or formamide media (0.1–0.3 *M* Tris-borate, 2–6 m*M* EDTA TBE buffer, pH 8.3 containing 7–8.3 *M* urea). Polymerization was achieved by adding ammonium persulfate solution and TEMED. An electric field of 400 V/cm was normally applied unless otherwise is indicated.

RESULTS AND DISCUSSION

The analysis of SODN antisense compounds is in its infancy. There are few published chromatographic or electrophoretic methods for the baseline resolution of phosphorothioate homologues beyond 20 bases.

Reversed-phase liquid chromatography

The separation of large SODN oligomers by RP-HPLC was unsuccessful. The resolution of SODNs of less than 7 bases was possible, but larger oligomers coeluted. Reversed-phase supports are useful for separation of dimethoxytrityl-blocked *vs*. deblocked species. Decreasing the pH of the mobile phase increased the interaction of the phosphorothioates with a hydrophobic stationary phase; however, the peak shape was skewed *vs*. chromatography at pH 6.5 (Fig. 1A and B). In both cases no resolution of individual oligomers was achieved. Aliquots from RP-HPLC (Fig. 1B) were collected and analyzed by PAGE. The aliquots from the front, middle and end of the peak showed the same ratio of oligomers indicating no resolution of bases had occurred. The addition of TBAP to the mobile phase increased the interaction with the hydrophobic support, but the resolution of

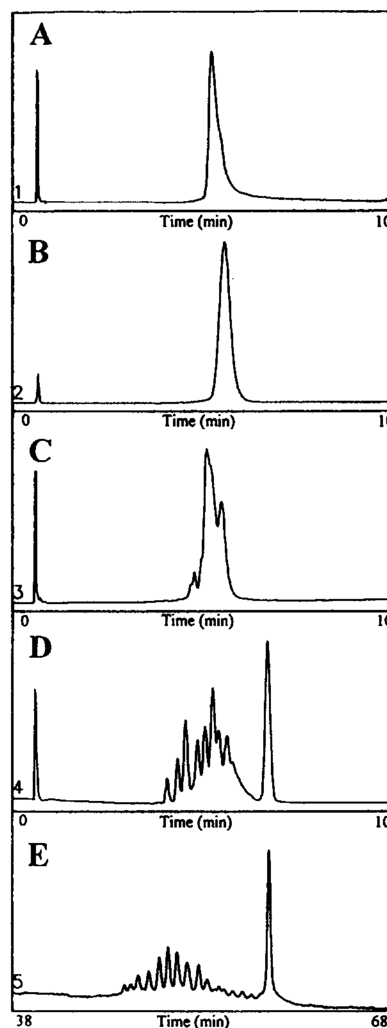

Fig. 1. Resolution of phosphorothioates via HPLC and HPCE. The sample is a crude 25-mer SODN containing failure sequences. (A) RP-HPLC at low pH, Table I, gradient I, (B) RP-HPLC at pH 6.5, gradient II, (C) ion-pair HPLC, gradient III, (D) ion-exchange HPLC, gradient VI, and (E) capillary gel HPCE conditions as in Fig. 4.

the 25-mer from the failure sequences was poor (Fig. 1C). The most effective chromatographic resolutions were obtained on anion-exchange HPLC supports (Fig. 1D). The best separation of this mixture was obtained with capillary gel HPCE (Fig. 1E). The reasons for such high efficiency will be discussed later.

Anion-exchange chromatography

Anion-exchange HPLC of the SODNs was possible on weak and strong anion-exchange supports. Reproducibility and efficiency were better with polymer-based supports *vs.* silica-based supports. The chemical stability of the polymer based columns and the absence of secondary effects from residual silanol groups were likely factors in their improved performance. The Partisphere column, a weak anion exchanger based on diethylaminoethyl groups covalently bonded to 10 μm silica, required several injections of concentrated phosphorothioate before reproducible recoveries were obtained. This presumably covered active sites in the column. The column efficiently resolved over 20 failure sequences from a 25-mer synthesis. However, the gradient program required daily modification because the retention of oligomers decreased by *ca.* 0.5 min per day. The GenPak FAX column and the NucleoPac PA-100 are polymer based pellicular supports. Although the GenPak FAX column contained weak anion-exchange groups and the NucleoPac PA-100 strong anion-exchange groups, the resolutions were virtually identical. Both polymer based columns yielded reproducible separations over several hundred injections. The ability to work at elevated temperature (60–70°C, *i.e.*, denaturing conditions) also reduced the possibility of secondary structure. The interaction of the SODNs increased with increasing temperature due to exposure of more ion-exchange sites on the SODN as the conformation became more relaxed [16]. The resolution of oligomers greater than 23 bases in length proved difficult. This appeared to be exacerbated when the components were present in unequal ratios (Fig. 2A–F). A spiking study was performed to determine the ability to separate oligomers of 23 and 25 bases in length. The retention times of the 23 and 25 mers appeared to be dependent on the relative ratios of the components (Fig. 2A–F). Since the mass load on the column was within the linear range, the reason for this phenomenon is uncertain.

Slab gel electrophoresis

Separations of SODN and other DNA analogues have been achieved by flat bed or slab gel electrophoresis [17]. Normal sequencing gel was used to separate SODN analogues with up to 50 bases [17]. We first examined the separation of a mixture of failure sequences ranging from 1 to 50 bases on a sequencing slab gel (Fig. 3). Although

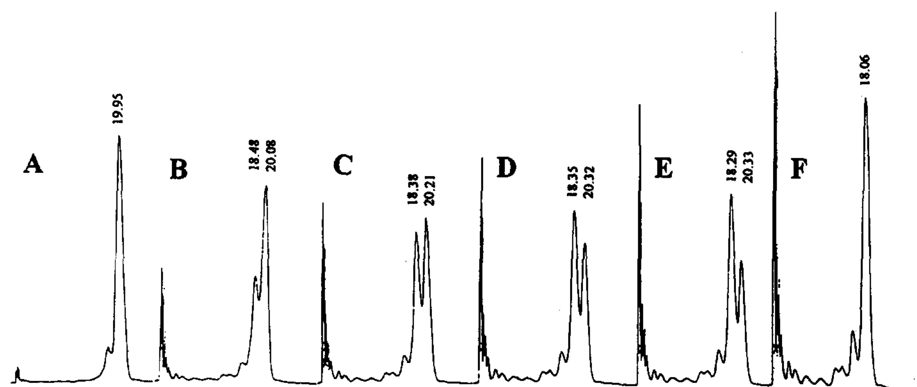

Fig. 2. Ion-exchange HPLC of 23- and 25-mer SODNs. The retention behavior was investigated *vs.* relative concentration of each oligomer. (A) 25-mer, (B) 1:3 (25/23), (C) 2:3, (D) 3:3, (E) 4:3 and (F) 23-mer. The analyses were performed on a GenPak FAX column, gradient V. Injection volume: 20 μl. Peak numbers indicate retention times in min.

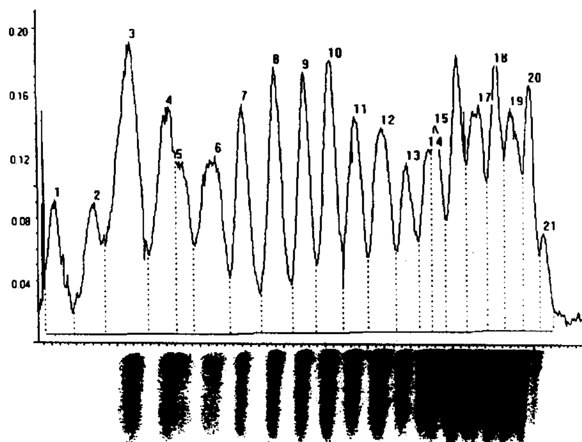

Fig. 3. Separation of failure sequence on a polyacrylamide slab gel. Conditions: 19% T, 1% C containing 8.3 *M* urea, 0.1 *M* TBE (pH 8.3); applied electric field 19.2 V/cm, voltage 250 V. Electropherogram was obtained by laser densitometry, peak numbers indicate delineation of bands by densitometer.

25 distinct bands could be seen on the gel, baseline separation was not obtained by densitometry because of the background from the Stains-All reagent (figure not shown). A laser densitometer gave better results under the exact same conditions (Fig. 3). Although the laser densitometer had better performance than the conventional densitometer, only 21 out of the expected 50 bands were detected. In addition to the problems of background absorbance and reproducibility of the staining, the binding of Stains-All to single-stranded phosphorothioate (ssSODN) was not a linear function of base length. Thus, quantitation or even relative quantitation, was not possible using this approach.

Gel HPCE separations

The separations of individual SODNs on gel matrices were investigated. The migration times of the SODNs were longer than the phosphodiester analogues. Electropherograms indicated no linear correlation between migration and SODN length (Fig. 4). Mobility and thus migration time were sensitive to temperature changes (*e.g.*, *ca.* 2%/°C) due to the sensitivity of the gel's viscosity to temperature. Therefore

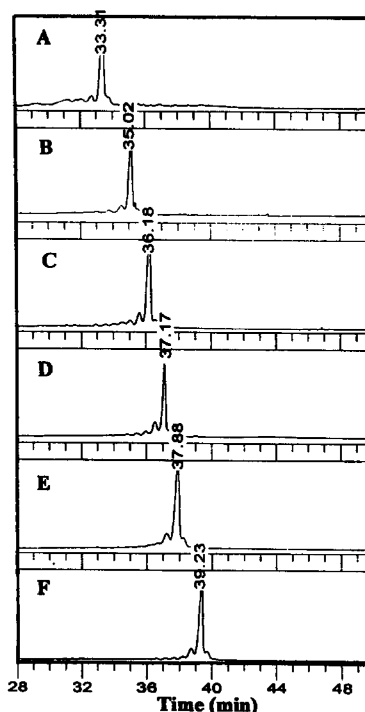

Fig. 4. Gel HPCE migration of SODNs on a capillary gel column. (A) 15-mer; (B) 19-mer; (C) 23-mer; (D) 25-mer; (E) 27-mer and (F) 29-mer. Conditions: 12% T, 0% C, 7 *M* urea, 0.1 *M* TBE (pH 8.3); capillary dimensions: 75 μm I.D., total length 30 cm (effective length 20 cm); applied electric field 300 V/cm, current 10 μA.

care was exercised to keep the Joule heating in the HPCE system below 0.5 W/m.

We approximated the amount of sample injected in Fig. 5 (5 kV for 2 s) by calibrating the capillary detector response in the absence of gel with known concentrations of sample. A linear plot was observed ($r^2 = 0.998$). The analyte concentration in each band passing through the detector was interpolated from this calibration curve. The average concentration of an individual band was determined from the average peak height of the bands in the electropherogram (Fig. 5). The volume of the band was calculated from the baseline peak width and the migration velocity of the species. The estimated peak volume was *ca.* 5 nl and the average mass per peak was 375 pg.

We have used the conventional sequencing gel formulation [14] which was found to have low resolving power (Fig. 4). A higher density gel

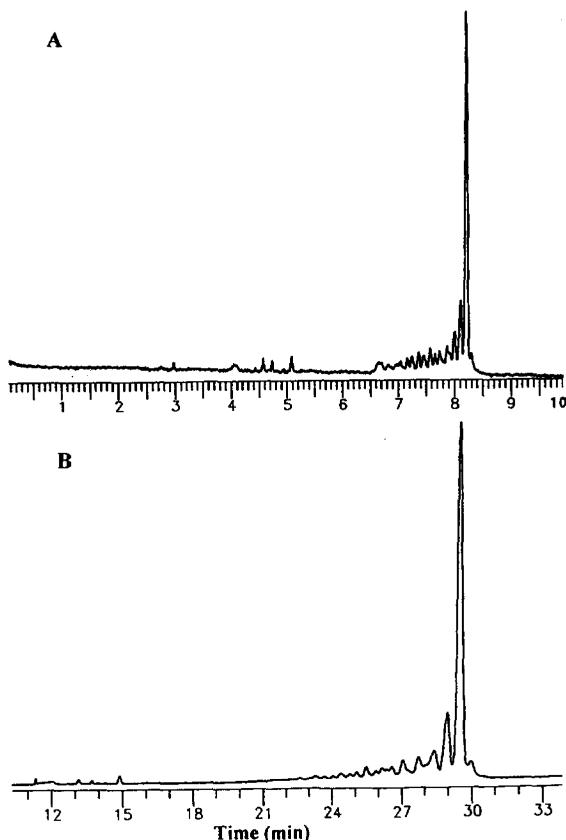

Fig. 5. Gel HPCE separation of a mixture of 23-, 24- and 25-mers of SODNs. (A) effective length 8 cm and (B) effective length 25 cm. Conditions: 13% T, 0% C, 8.3 *M* urea, 0.2 *M* TBE (pH 8.3); applied electric field 400 V/cm.

(*i.e.* higher %T) yielded a better separation (Fig. 5A and B). Artificial mixtures of 23-, 24-, 25-mers of SODNs were separated on a denaturing gel (7 *M* urea, 12% T) in 30 min (Fig. 5B). To reduce analysis time, the column was shortened from 25 to 8 cm (Fig. 5A). The ability of the 8-cm column to maintain high resolution with short run times was remarkable. The calculated efficiency for the 25-mer was $1.9 \cdot 10^6$ plates/m *vs.* $0.34 \cdot 10^6$ plates/m for the longer column. Thus, the shorter columns was three times as efficient in one third the time. Though the efficiency was not as high as expected based on known efficiencies for phosphodiester DNA, the number of possible diastereomers which migrate with the same effective charge was 2^{24} for the SODN 25-mer. As expected, the mass sensitivity also increased in the shorter column due to decreased band broadening. Since no efforts were made to thermostat the capillaries, a longer capillary may have experienced more temperature inhomogeneity than a short capillary. This phenomena will be the topic of future investigations.

An artificial mixture of 24- and 25-mers of SODNs was resolved on the 28-cm column, Fig. 6. The time window between the oligomers is large enough to accommodate an additional peak. This peak is presumed to be a failure sequence of the synthesized 25-mer and therefore a 24-mer. Since this peak is migrating right from the main 25-mer under denaturing conditions, we assume that the two 24-mers (possibly two 25-mers) are separated based on the difference in base sequence.

An improved version of the electrophoresis column is currently being developed for the separation of SODN analogues. The gel composition was modified with formamide [18]. Using this modified gel formulation, improved resolution of bases 1 through 50 was obtained (Fig. 7A and B) [18]. When migration time was examined with respect to fragment length, a linear relationship ($r^2 = 0.999$) was observed (Fig. 8). The linear behavior of SODNs ranging from 1 to 50 bases in length (or longer) is an important feature for SODN analyses. It is important to recognize that under these experimental conditions, no peak compression was observed in the

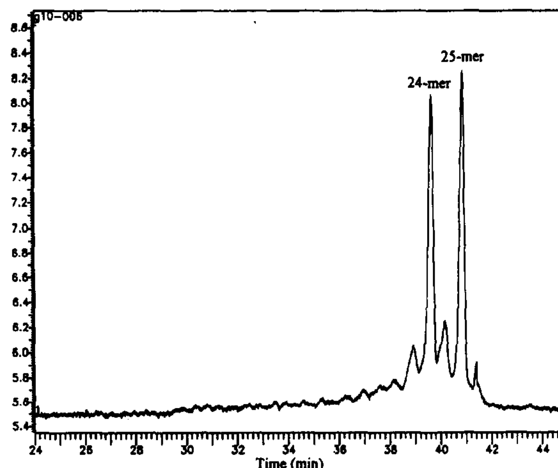

Fig. 6. HPCE separation of an artificial mixture of 24- and 25-mers of SODNs. For experimental conditions see Fig. 5.

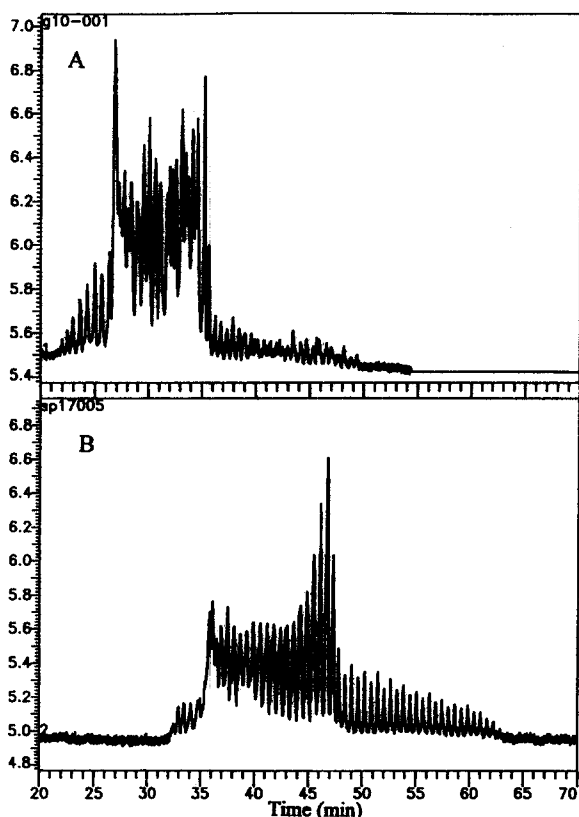

Fig. 7. Electropherograms of failure sequence of SODNs from 1 to 50 bases on (A) regular sequencing gel column and (B) modified sequencing gel column. Conditions: effective length 20 cm, applied electric field 400 V/cm.

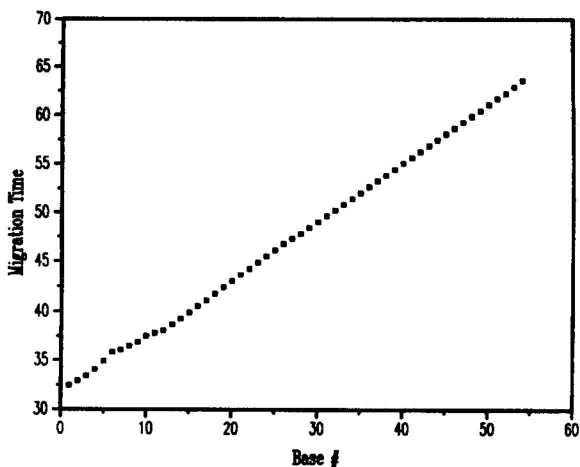

Fig. 8. Calibration plot of migration time *vs.* base length. All conditions identical to those of Fig. 7B.

range of 1 to 50 bases. We are currently investigating resolutions of SODNs beyond the 50 base range.

CONCLUSIONS

The analysis of SODNs via chromatographic means was possible for short oligomers with less than 30 bases. The highest resolutions were obtained using polymer based pellicular anion-exchange resins. Gel HPCE provides a facile approach to separation, analysis and purity determination of SODNs. High resolving power was possible with these capillary gel columns, permitting rapid separation of SODNs that differ by one base. A significant potential exists for sequencing of SODNs with HPCE gel columns. In addition, rapid assessment of the purity of synthesized oligonucleotides is possible by gel HPCE.

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REFERENCES

1 P. Zamecnik and M. Stephenson, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 280.
2 P.S. Miller and P.O.P. Ts'o, *Ann. Rep. Med. Chem.*, 23 (1988) 295.
3 G. Zon, *Pharm. Res.*, 5 (1988) 539.
4 F. Eckstein, *Anal. Biochem.*, 54 (1985) 367.
5 S. Agrawal, T. Ikeuchi, D. Sun, P.S. Sarni, A. Konopka, J. Maizel and P. Zamecnik, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (189) 7790.
6 J.M.E. Leiter, S. Agrawal, P.C. Zamecnik and P. Plese, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 3430.
7 T.M. Woolf, C.G. Jennings, M. Rebagliatti and D.A. Melton, *Nucleic Acids Res.*, 18 (7) (1990) 1763.
8 S. Agrawal, J.Y. Tang and D.M. Brown, *J. Chromatogr.*, 509 (1990) 396.
9 K. Makino, H. Ozaki, T. Matsumoto, H. Imaiishi and T. Takeuchi, *J. Chromatogr.*, 400 (1987) 271.
10 V. Metelev and S. Agrawal, *Anal. Biochem.*, 200 (1992) 342.
11 Y.-Z. Xu and P.F. Swann, *Anal. Biochem.*, 204 (1992) 185.
12 M.D. Edge, A.R. Greene, G.R. Heathcliffe, P.A. Meacock, W. Schuck, D.B. Scanlon, D.B. Atkinson, T.C.

Newton, C.R. Markham and A.F. Markham, *Nature (London)*, 292 (1981) 756.
13 A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith and B.L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9660.
14 A.S. Cohen, S. Carson, A.B. Belinkii and B.L. Karger, *Anal. Chem.*, in press.
15 D.N. Heiger, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.
16 R. Wiem, in E. Heftmann (Editor), *A Laboratory Handbook of Chromatography and Electrophoresis Methods*, Van Nostrand, New York, 3rd ed., 1975, p. 228.
17 D. Rickwood and B.D. James (Editors), *Gel Electrophoresis of Nucleic Acids: A Practical Approach*, IRC Press, Washington, DC, 1983.
18 A.S. Cohen and M. Vilenchik, Patent filed by Hybridon Inc., USA, March 16, 1993.