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Monitoring and analysis of antisense DNA by high-performance capillary gel electrophoresis

Maria Vilenchik, Alexei Belenky and Aharon S. Cohen*

Hybridon Inc., Worcester, MA 01605 (USA)

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ABSTRACT

The use of antisense DNA to modulate human immunodeficiency virus gene expression *in vitro* has increased the need for analytical methods to support clinical studies. High-performance capillary electrophoresis and slab gel electrophoresis are useful methods for the analysis. Especially important is low level and rapid monitoring of phosphorothioate DNA (SODNs) in biological fluids. The method presented here is based on Watson–Crick hydribization between phosphodiester and the target DNA. Various techniques of staining or labeling are investigated to improve detection limits. The sensitivity of this method is 0.1 ng/ml.

INTRODUCTION

Recent advances in antisense DNA analogues, especially phosphorothioates, have generated a tremendous demand for the characterization of phosphorothioate oligodeoxynucleotides (SODNs). As a result, new challenges for analytical research have surfaced including the need for rapid determination of molecular mass, purity, sequence and base composition.

SODNs have a non-bridging oxygen-to-sulfur substitution per phosphate in the oligomer chain (*i.e.*, P–O to P–S) and therefore are expected to behave very similarly to phosphodiesters. Unfortunately, this is not the case. Charge localization in the species is not the same. In aqueous solutions a negative charge localized on sulfur is far more stable than the same charge localized on an oxygen atom. The larger size and polarity of sulfur relative to oxygen allow the charge density of a thioate anion to be less than that of oxyanions. This difference contributes to the greater acid strength of thioates (P–O and P–S bond lengths are 1.48 and 1.95 nm, respectively) and changes their characters substantially [1]. As a result, analytical methods that were developed for phosphodiesters should be adapted, but in many cases new methods need to be developed for SODNs.

As we mentioned earlier, analysis of phosphorothioates needs to address the general issues of oligomer length, base composition, base sequence, chemical purity and stereochemical purity. The absolute length and the degree of length heterogeneity are currently assessed by ion exchange and reversed phase HPLC [2,3] though there can be separation problems due to hydrophobic interactions. Gel electrophoresis, either in the slab or capillary format, is an alternative method [3,4]. Both allow the achievement of single base resolution of phosphorothioates. Unfortunately none of these techniques has demonstrated quantitation capability. Slab gel electrophoresis coupled with laser densitometry is not a quantitative technique because there is no quantitative dye binding to SODNs; besides, slab gel procedures are long, tedious and labor ineffec-

* Corresponding author.

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tive. High-performance capillary gel electrophoresis (gel HPCE) is very promising since UV detection can be done on-line, and neither staining or destaining procedures are needed; however, quantitation has not been demonstrated thus far.

Clinical studies of SODNs antisense effects are close to being realized in humans. In providing successful therapy and proper patient care, the development of quantitative analysis becomes one of the most important issues. Pharmacokinetic measurements are often concerned with relatively low concentrations of a drug and its metabolites in a sample of blood, tissue, urine, etc. However, to the best of our knowledge, only one non-radioactive work on the quantitative analysis of SODNs has been reported thus far [5].

This paper addresses "on-line quantitative Southern blotting" using gel HPCE as a quantitative analytical tool for antisense DNA monitoring. Laser-induced fluorescence (LIF) detection coupled with gel HPCE demonstrates detection at the (ng/ml) level for the targeted phosphorothioate DNA (GEM).

EXPERIMENTAL

Chemicals and reagents

Sequagel Sequencing System (acrylamide–bisacrylamide, 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 the laboratory, 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-methylcarbocyanine bromide) was purchased from Eastman Kodak (Rochester, NY, USA) and ISS Oligo Staining System was received from Integrated Separation Systems (Natick, MA, USA).

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, Pittsburg, PA, USA). The applied voltage was 250 V, which corresponds to an electric field strength of 19.2 V/cm. Gels were stained with Stains-All or ISS Oligo Staining System and dried on a gel dryer (Buchler Instruments, Lenexa, KA, USA).

HPCE Apparatus

The CE apparatus with UV and LIF detection and the preparation of gel-filled capillaries for the separation of DNA molecules have been described previously [6]. 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 phosphorothioates at 270 nm was accomplished with a Spectra 100 (Spectra-Physics, San Jose, CA, USA). For LIF detection an argon ion laser (Model 543 100BS; Omnichrom, Chino, CA, USA) was employed. The data were acquired and stored on an Acer-Power 486/33 computer (Acer American Corp, 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 an inner diameter of 75 μm, an outer diameter of 375 μm, an effective length of 15–20 cm and a total length of 30–60 cm was treated with (methylacryloxypropyl)trimethoxysilane (Petrarch Systems, Bristol, PA, USA) and then filled with a degassed solution of polymerizing 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.

RESULTS AND DISCUSSION

Conventionally, Southern blotting is performed to identify the nucleotide sequence of a

DNA molecule [7]. The process involves the separation of DNA species by gel electrophoresis, transfer and immobilization onto a membrane support, and hydridization with a radioactively labeled probe. An autoradiogram is developed, and the molecules carrying the complementary sequence to the probe are identified (by Watson–Crick hybridization). Although this method is a powerful tool, it is a time-consuming and laborious process.

This study describes the analysis of GEM in human serum by gel HPCE coupled with LIF that significantly extends detection limits.

Antisense phosphorothioate oligomer was "fished" out from a sample solution by Watson–Crick hybridization and analyzed by gel HPCE. Alternatively, polyacrylamide gel electrophoresis (PAGE) was employed for the same analysis and used as an experimental backup to verify capillary results. The former was found to be more efficient and effective than the traditional PAGE.

The major advantage of using Watson–Crick hybridization for "fishing" is that the probe, a complementary DNA molecule, hybridizes very specifically with the DNA target molecule. The probe molecule tagged with the appropriate fluorescent dye allows very low detection limits. For example, gel HPCE coupled with LIF allows detection limits in the range of 10^{-21} mol (concentration of 10^{-12} M at the sample vial). Only the hybridized duplex and the probe molecule can be detected with LIF, *i.e.*, other DNA molecules present in the sample vial will not disturb the LIF electropherogram. "Fishing" GEM from physiological fluids adds another level of complexity, since for example, serum proteins clog gel pores in the capillary and consequently, prevent DNA injection. Therefore, a physiological sample preparation step is necessary to digest or eliminate proteins from the sample vial. A detailed description of the enzymatic digestion has been published earlier [5] and a schematic diagram of the present procedure is provided in Fig. 1.

Although at the concentrations needed for HPCE with UV detection DNA hybridization can be done in water, increased ionic strength is often used to enhance the yield of hybridization of DNA molecules. It is known that the presence of significant concentrations of salt in the sample preparation greatly reduces the amount of DNA sample introduced by electrokinetic injection. Since the molecule in question is a 25-mer phosphorothioate, we first turned to optimize hybridization at very low to almost zero ionic strength, using the traditional method of slab gel electrophoresis to screen the results.

Fig. 2A and B demonstrate that the complementary probe molecule COM does hybridize the target molecule GEM. Lanes 1 and 2 in Fig. 2A and B represent the probe COM, positioned at 1III and the GEM positioned at 2II respectively. Lanes 3 and 4 in Fig. 2B represent the

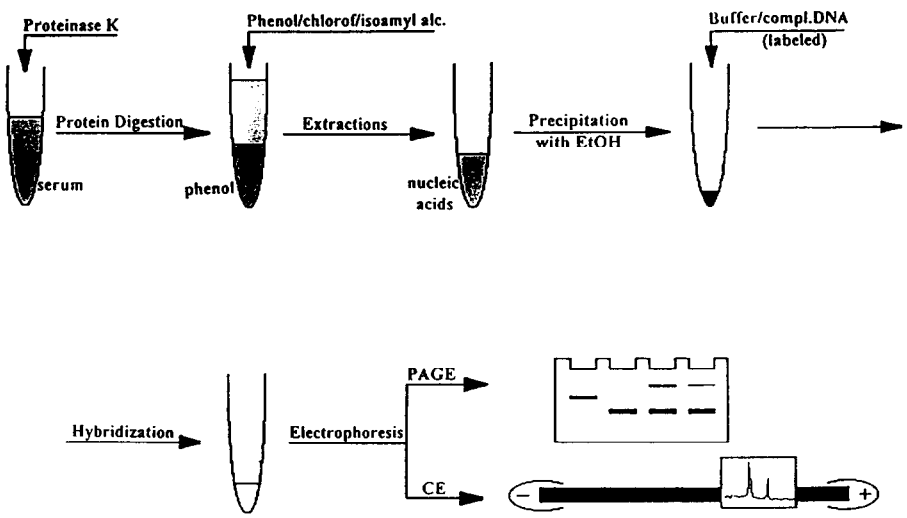


Fig. 1. Schematic diagram of analysis.

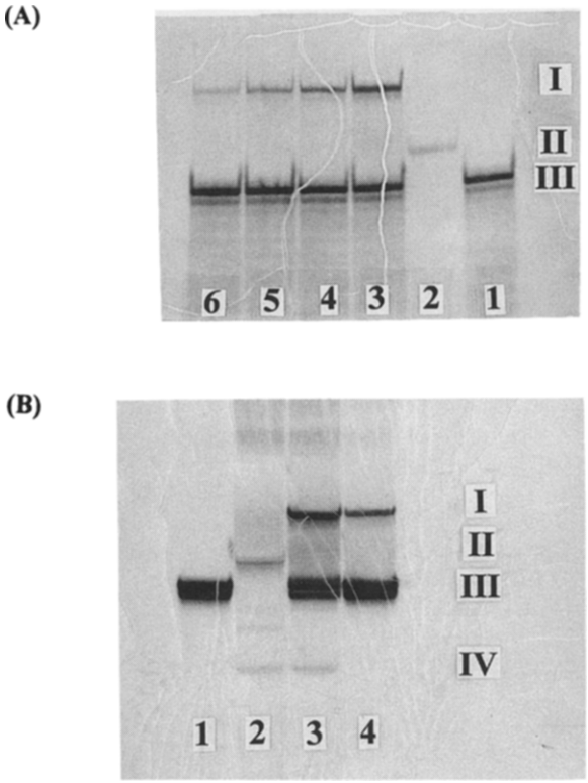


Fig. 2. Separation of analyzed GEM and COM mixture after hybridization using PAGE slab gel. (A) lanes: 1 = COM; 2 = GEM (dissolved in water); 3–6 = duplex of GEM and COM where concentrations of GEM were 100, 50, 25 and 2 μg/ml, respectively. (B) lanes: 1 = COM; 2 = GEM (extracted from human serum by enzymatic digestion); 3, and 4 = duplex of GEM and COM where concentrations of GEM were 100 and 50 μg/ml, respectively. See text for more details. Conditions: polyacrylamide gel (acrylamide–bisacrylamide, 19:1), 0.1 *M* TBE buffer, pH 8.3. Electrophoresis was performed at constant current 50 mA; stained with silver-based system. See text.

mixture of GEM and COM. The band positioned at 3III and 4III represents the excess of COM that was not hybridized while the bands positioned at 3I and 4I represent the GEM–COM duplex. Interestingly, the duplex migrates slower than the single-stranded GEM and COM under the experimental conditions. Also observed is a band positioned at 2IV and 3IV (Fig. 2B). This band represents an impurity in the serum, digested with protein kinase [5], that was not hybridized with GEM demonstrating high selective binding.

Fig. 2A demonstrates "titration" of COM with GEM in water. Differences in band intensities high to low, positioned at 3I to 6I respectively represent differences in the duplex product amount on the slab gel.

Next we turn to optimize the experimental conditions for the gel capillary. To prevent any discrepancies in sample preparation, we used the same samples as in the slab gel experiment described in Fig. 2. The samples were injected electrokinetically into two capillaries, one of which, for comparison, contained denaturing conditions. The results in a form of UV electropherogram are shown in Fig. 3. The same mixtures (1,2,3) were injected under non-denaturing (A) and denaturing (B) conditions. Three peaks are observed under non-denaturing conditions (mixtures 2 and 3). The first migrating peak was identified as COM, the last migrating peak was identified as GEM. The third peak between the first and the last was identified as the duplex. Identification was done by spiking. Since hybridization is an equilibrium process, increase in duplex peak area is well correlated with the increase in GEM concentration at a constant COM content in the injected sample mixture. GEM was completely hybridized in excess of COM (mixture 1) and therefore not observed in Fig. 3 (A1). As expected, only two peaks were observed under denaturing conditions that is illustrated on Fig. 3B. The absence of the duplex peak is not surprising, probably due to denaturation upon injection. Interestingly, the migration order of GEM and COM under non-denaturing conditions is reversed compared to denaturing conditions, most likely due to SODNs secondary structure that is well pronounced under nondenaturing conditions. With GEM hybridization under control, relative quantitation by capillary gel electrophoresis was possible. Fig. 4A illustrates a set of seven consecutive electropherograms, six of which were spiked with $pd(A)_{19-24}$ as an internal standard and titrated with known amounts of GEM (80–1330 ng/ml). Reproducibility of migration time can easily be observed. Also observed is the proportional change in duplex peak height with increased GEM amount. Fig. 4B shows a plot of the experimental results obtained from Fig. 4A.

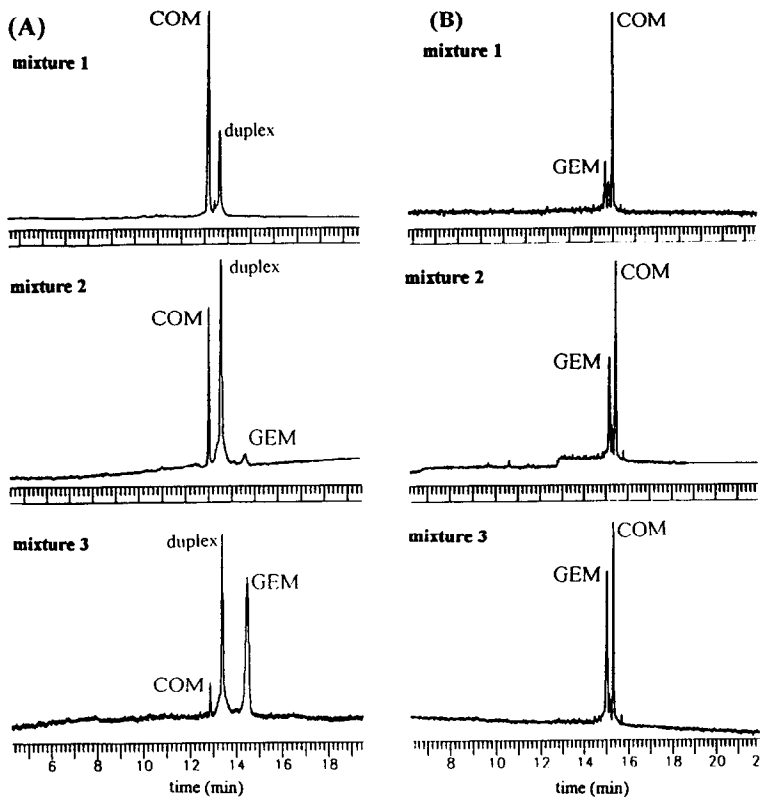
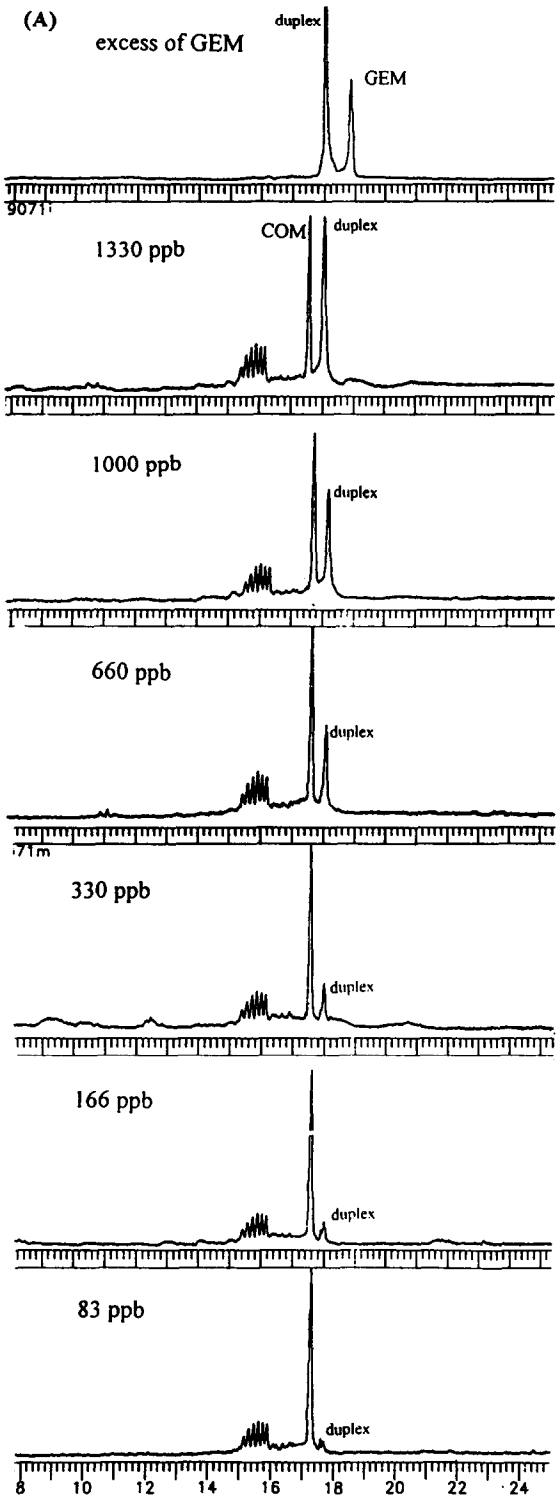


Fig. 3. The separation of GEM, COM and duplex by capillary electrophoresis using UV detection. Electropherograms 1, 2 and 3 show different amounts of GEM with constant COM concentration. (A) Non-denaturing conditions, (B) denaturing conditions. Conditions: (A) 9%T linear polyacrylamide column, effective length $l = 20$ cm, applied electric field $E = 200$ V/cm; (B) 13%T linear polyacrylamide, denaturing conditions, effective length $l = 15$ cm, applied electric field $E = 400$ V/cm.

A linear relationship between GEM concentration and normalized duplex peak height was observed over three orders of magnitude of GEM concentration. In our hands, under the experimental conditions, using UV detection and capillary gel electrophoresis the lowest detected amount of GEM (SODNs) was 80 ng/ml. The ultimate goal was to achieve low detection limits for GEM. Traditionally, slab gel and radioactive labeling by ^{32}P are used to achieve low detection limits. An alternative evolving method is LIF coupled with CE. The LIF optical setup was described previously [6]. The method requires a fluorescent dye to be chemically bound to a small complementary segment of DNA that is hybridized with the target DNA. This method is very selective because unlike UV detection, only labeled DNA or DNA that is hybridized to the labeled DNA will be detected by the LIF method. All other oligonucleotides which may be present in the sample vial will not be detected. In this experiment fluorescein was used as the dye chemically linked to COM (complementary segment of DNA). Unfortunately, the dyed COM co-migrated with the duplex DNA as shown in Fig. 5A. Using ethidium bromide (EtBr) as an additive, we were able to open a time window between the duplex and COM DNA's so that baseline separation was obtained. EtBr is known as a double-stranded DNA intercalator which induces conformational changes as well as charge changes, which result in mobility changes. Fig. 5A demonstrates the EtBr concentration effect on the separation. Since EtBr in the gel matrix can cause fluorescence interference, a concentration of 0.1 μM EtBr was found to be a good compromise as indicated by the sigmoidal curve in Fig. 5B where selectivity was



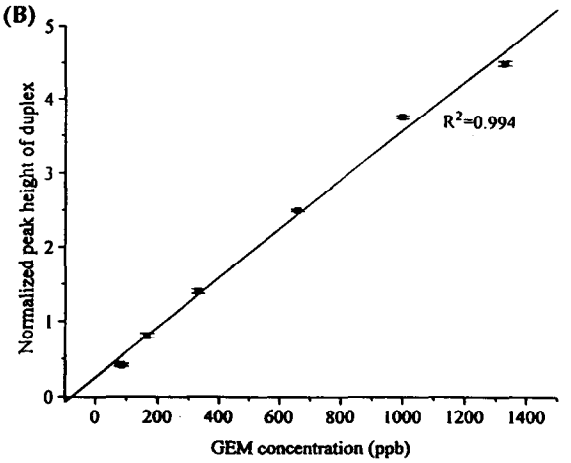


Fig. 4. Determination of GEM by capillary electrophoresis. (A) UV detection using [pd(A)$_{19-24}$] as an internal standard, numbers indicate GEM concentration; (B) calibration curve for determination of GEM concentration relative to internal standard. Electrophoresis conditions as in Fig. 3A. Annealing was performed at 65°C for 5 min followed by cooling down at room temperature at the rate of 1°C/min. ppb = ng/ml.

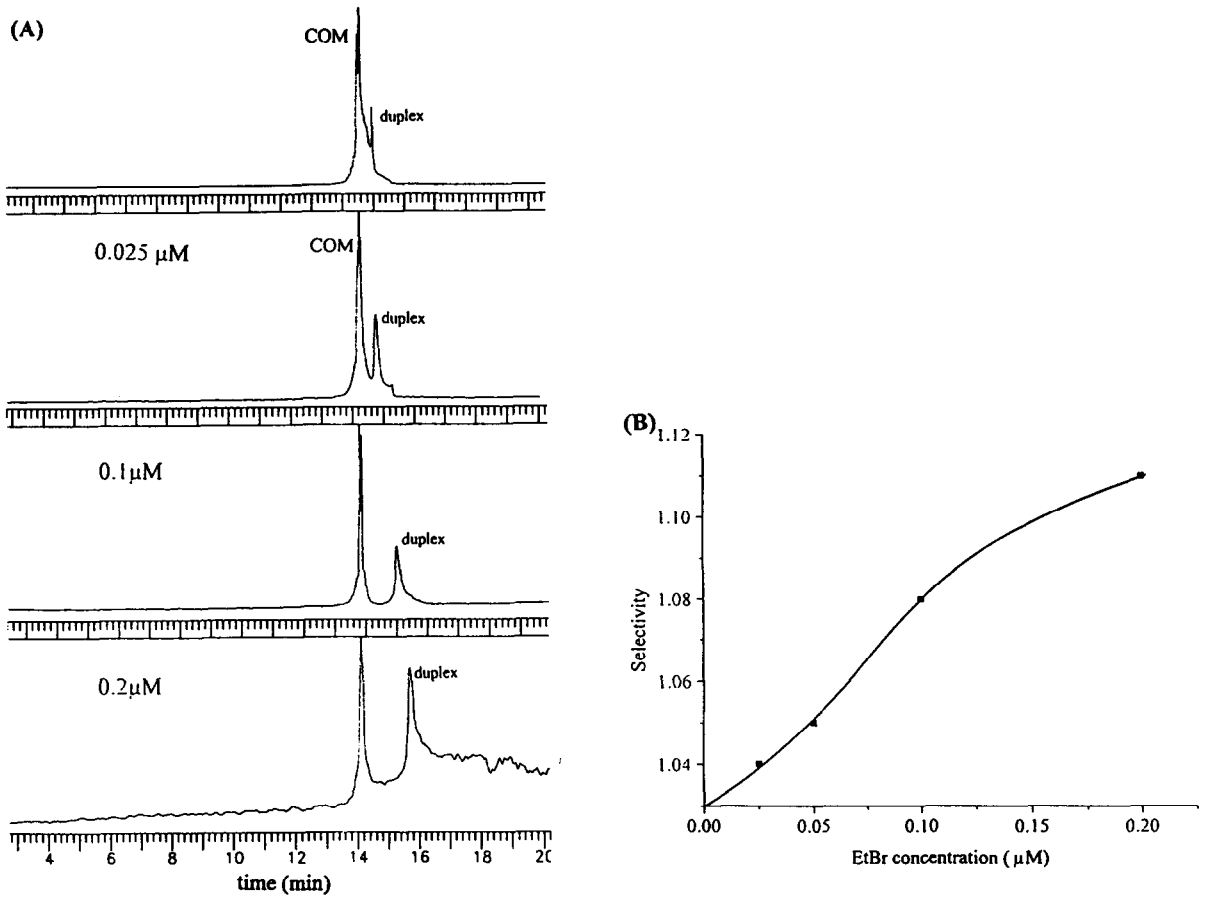


Fig. 5. Effect of intercalator concentration (EtBr) on the separation of single and double stranded oligonucleotides. (A) Electropherograms obtained using LIF detection, numbers indicate EtBr concentrations; (B) plot of selectivity *vs.* intercalator concentration; selectivity factor α is defined as $\alpha = t_B/t_A$, where t_B and t_A are the migration times of duplex and COM, respectively. Conditions as in Fig. 3A.

plotted against EtBr concentration. Experimentally, EtBr was added into the buffer reservoirs and the gel capillary was equilibrated for a few hours to distribute evenly along the capillary. GEM detection limits under these experimental conditions were found to be 2 ng/ml, and the calibration curve with a correlation coefficient of $R^2 = 0.994$ is shown in Fig. 6. Although we expect one or two orders of magnitude better detection limits, we have to remember that a good quantitative annealing reaction between COM and GEM at very low concentration is unlikely to happened unless high salt (at least 10 m*M*) is present in the sample matrix. This, of course, dramatically decreases the amount injected electrophoretically into the gel capillary as predicted by Kohlrauch's law.

Based on our experience, double-stranded DNA molecules usually migrate faster than single-stranded molecules of the same length in a capillary column [8]. However, the hybridized species observed in this study apparently migrated more slowly than the single-stranded oligonucleotide. This may be because the COM molecules are phosphodiesters and the duplex molecules is a hybrid of the COM strand and the GEM strand which is a phosphorothioate that normally migrates slower than the equivalent phosphodiester on non-denaturing polyacrylamide gels.

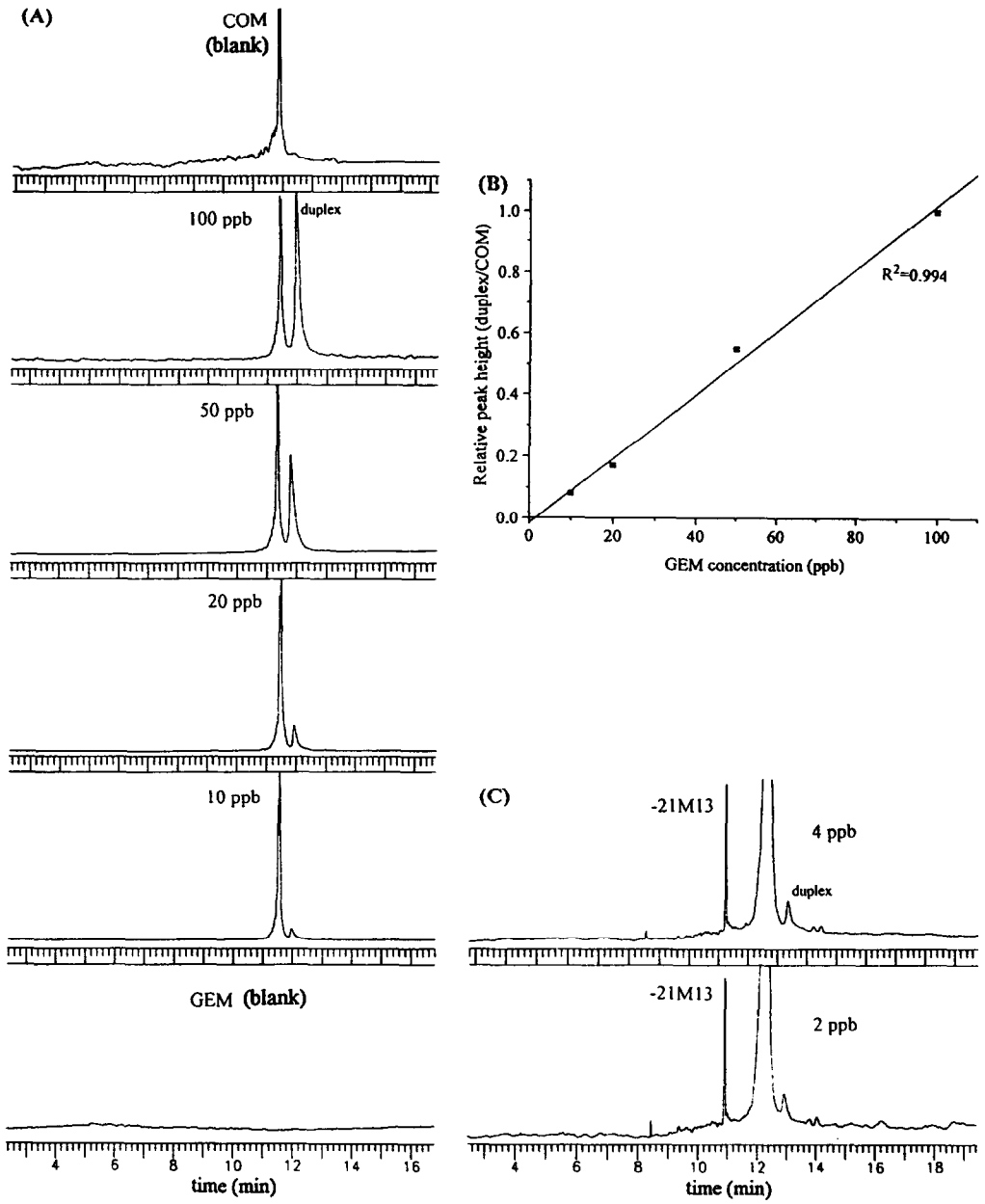


Fig. 6. (A) Low detection limits achieved by capillary electrophoresis using LIF detection, numbers indicate GEM concentrations, numbers indicate GEM concentrations; (B) plot of relative peak height duplex/COM *vs.* GEM concentration; (C) analysis under optimal conditions with the use of primer *−21M13* labeled with fluorescent dye as an internal standard, numbers indicate GEM concentrations. Conditions: 10 μM of $MgCl_2$ were added to the injected solution; electrophoresis was performed on 9%T linear polyacrylamide with 0.04 μM of EtBr, other conditions as in Fig. 3A. ppb = ng/ml.

It may be noteworthy that DNA molecules separated by capillary gel electrophoresis can also be collected and immobilized on a membrane. Additional confirmation with specific probes, similar to conventional Southern blotting could then be performed [9].

CONCLUSIONS

Conventional Southern blotting although a powerful tool, is time consuming and laborious. Previously [9] we have demonstrated how Southern blotting can be transferred from a slab gel to a capillary column with all the advantages provided by HPCE technique. If a capillary is coupled with a LIF detector, extremely low detection limits can be obtained. In this work we have demonstrated that HPCE can be used not only as new approach to Southern blotting with very high sensitivity, but also as a quantitative tool. The three elements (ease of handling, sensitivity and quantitation) which are so important for bioanalysis are combined. The extremely high efficiency and resolution obtained in capillary gel electrophoresis operation are advantageous and make this method unique. The presence of background DNA in the detection cell or sample vial, can be problematic in UV detection, but do not affect the analysis when LIF detection is used. This enables us to detect low concentrations of specific DNA fragments in large DNA bulk by "fishing" out the target oligonucleotide. The technique of capillary gel electrophoresis that is now widely accepted as a standard analytical tool for studying and separating biopolymers has much potential for as yet undiscovered applications. The invention of novel gel matrices and various detection systems opens new horizons for capillary electrophoresis in its role in biochemistry.

REFERENCES

1 M. Matsukura, A. Shinozuka, G. Zong, H. Mitsuya, M. Reitz, J.S. Cohen and S. Broder, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 7706–7710.
2 S. Agrawal, J.Y. Tang and D.M. Brown, *J. Chromatogr.*, 509 (1990) 396–399.
3 A.S. Cohen, M. Vilenchik, J.L. Dudley, M.W. Gemborys and A.J. Bourque, *J. Chromatogr.*, 638 (1993) 293–301.
4 L.A. DeDionisio, *J. Chromatogr. A*, 652 (1993) 101.
5 A.J. Bourque and A.S. Cohen, *J. Chromatogr.*, 617 (1993) 43–49.
6 A.S. Cohen, D.R. Najarian and B.L. Karger, *J. Chromatogr.*, 516 (1990) 49–60.
7 E.M. Southern, *J. Mol. Biol.*, 98 (1975) 503–517.
8 K. Krapko, J.S. Hanekamp, W.G. Thilly, A. Belenkii, F. Foret and B.L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, (1993) in press.
9 J.W. Chen, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 559 (1991) 295–305.