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Comparative study of capillary zone electrophoresis and high-performance liquid chromatography in the analysis of oligonucleotides and DNA☆

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ABSTRACT

Capillary zone electrophoresis and high-performance liquid chromatography were compared with regard to the separation of oligonucleotides and double-stranded DNA. Both anion-exchange and reversed-phase high-performance liquid chromatography on non-porous particles are considered to be superior to capillary electrophoresis in terms of speed and selectivity in the analysis of oligonucleotides up to 30 bases in length. Moreover, reversed-phase chromatography allows the simultaneous purification of detritylated oligonucleotides with recoveries >90%. Compared with anion-exchange chromatography, there is no need for a subsequent desaltation step because the volatile buffer system can be readily evaporated. With regard to dsDNA, however, the resolving power of capillary electrophoresis cannot be matched by anion-exchange chromatography at present. Moreover, the combined use of hydroxyethylcellulose and ethidium bromide not only yielded a separation efficiency equal to that achieved by means of gel-filled capillaries but also avoids some of their limitations such as the destruction of the gel matrix at high current densities and the bias involved in electrokinetic injection.

INTRODUCTION

The impressive advances made over the past few years in the fields of biotechnology and molecular biology, such as solid-phase DNA synthesis and polymerase chain reaction, have resulted in a growing demand for rapid and automated analytical methods for the separation of single- and double-stranded DNA molecules. Early attempts to replace slab-gel electrophoresis with high-performance liquid chromatography (HPLC) on porous stationary phases have not yielded the expected enhancement in speed and resolution owing to the restricted intraparticle diffusion of biopolymers [1]. Since the introduction of non-porous micropellicular packings, however, chromatographic analyses of biopolymers can be obtained within minutes owing to such properties as fast mass transfer kinetics, maximum surface accessibility and fast column regeneration [2]. Recently, a great challenge to HPLC in the analysis of oligo- and polynucleotides has arisen through the use of capillaries as the migration channel in electrophoresis. This has not only put electrophoresis on the same instrumental footing as HPLC

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but has also allowed the rapid analysis of sample volumes as small as a few picolitres with unprecedented resolution and sensitivity [3]. As HPLC and capillary electrophoresis have their own advantages and limitations, they may be more or less suited for specific applications. Therefore, we have undertaken the present comparison of the two techniques with regard to their speed and efficiency in separating synthetic oligonucleotides and double-stranded DNA.

EXPERIMENTAL

Chemicals

Non-porous, fused-silica microspheres (particle diameter, $d_p \approx 2$ μm) were obtained from Glycotech (Hamden, CT, USA). Styrene, divinylbenzene (DVB) and polyvinyl alcohol (PVA) were purchased from Riedel-de Haën (Seelze, Germany). Polyethyleneimine (PEI, molecular mass 600) and ethanediol diglycidyl ether were obtained from Polysciences (Warrington, PA, USA). HPLC gradient-grade acetonitrile, dioxane, 2-propanol and methanol were obtained from Merck (Darmstadt, Germany) and triethylammonium acetate (TEAA) from Applied Biosystems (San Jose, CA, USA). Hydroxyethylcellulose (HEC, viscosity of a 2% aqueous solution 0.3 Pa s) was purchased from Serva (Heidelberg, Germany). Tris, sodium dodecyl sulphate (SDS) and boric acid were of electrophoretic purity (Bio-Rad Labs., Richmond, CA, USA). Ammonium sulphate, ethidium bromide, EDTA and alkali metal salts were obtained from Sigma (St. Louis, MO, USA). Mesityl oxide, which served as electroosmotic flow-marker, was purchased from Merck. Buffer solutions were prepared with high-purity water (NANOpure; Barnstead, Newton, MA, USA) and filtered through a 0.2-μm pore size filter (Schleicher & Schuell, Keene, NH, USA).

Oligonucleotides

Standards of dephosphorylated and phosphorylated oligodeoxyadenylic acids [$d(A)_{12-18}$, $pd(A)_{16}$, $pd(A)_{12-18}$] were purchased from Pharmacia (Uppsala, Sweden). Heterooligonucleotides were synthesized on a DNA synthesizer (Model 381-A, Applied Biosystems) using phosphoramidite chemistry. Trityl-on oligonucleotides were purified by means of oligonucleotide purification cartridges (OPC, Applied Biosystems).

DNA size standards

Double-stranded DNA size standards were purchased from Bio-Rad Labs. (pBR322 DNA-*Ava* II/*Eco*R I Digest), Boehringer (Mannheim, Germany, pBR322 DNA-*Hae* III Digest and pBR328 DNA-*Bgl* I/*Hinf* I Digest) and Pharmacia (ϕ*x*174 DNA-*Hae* III Digest and λ-*Hind* III Digest). pBR322-*Msp* I was prepared by digesting 6 μg of pBR 322 (Boehringer) with the restriction endonuclease *Msp* I (Promega, Madison, WI, USA) for 60 min at 37°C using the buffer supplied with the enzyme.

Columns

A Progel-TSK DEAE-NPR column was purchased from Supelco (Bellefonte, PA, USA). PEI-silica was prepared according to the method reported by Regnier and co-workers [4,5]. Before packing into a stainless-steel column (30 × 4.6 mm I.D.), 1 g of the modified silica microspheres was suspended in 10 ml of 2-propanol and sonicated for 20 min, then the slurry was packed into the column at a pressure of 70 MPa with the use of an air-driven pump (Maximator MSF 111; Ammann Technik, Kölliken, Switzerland) and methanol as the driving solvent. Thereafter, methanol was replaced with water at the same inlet pressure. The same procedure was employed to pack a 50 × 4.6 mm I.D. column with 1.1 g of the highly cross-linked polystyrene (PS)–DVB particles [60% (v/v) DVB], which had been prepared by a two-step microsuspension method either in the absence or in the presence of 0.1% (w/v) PVA [6]. Instead of 2-propanol, however, 10 ml of dioxane were used to suspend the beads.

Instrumentation

Capillary zone electrophoretic analyses were performed on either an Applied Biosystems Model 270A or a Beckman (Palo Alto, CA, USA) P/ACE System 2100, equipped with a 72 cm × 50 μm I.D. or a 67 cm × 75 μm I.D. fused-silica capillary, respectively. Moreover, a phenylmethyl-deactivated fused-silica capillary (Restek, Bellefonte, PA, USA) of I.D. 100 μm was used for the separation of DNA restriction fragments. Detection was carried out by on-column measurement of UV absorption at 260 nm at 22 cm from the cathode in the case of the Applied Biosystems equipment or at 254 nm at 7 cm from the cathode in the case of the Beckman sys-

tem. Electropherograms were recorded either on a Shimadzu (Kyoto, Japan) Chromatopac C-R6A integrator or on a IBM PS/2 Model 70 with Beckman System Gold v. 6.01 software. Samples were loaded either by applying a vacuum at a pressure of 16.9 kPa (Applied Biosystems system) or by means of pressure injection at 3.45 MPa (Beckman system). The temperature was kept constant at 30 and 25°C, respectively.

The HPLC system consisted of two pumps (Model 114M; Beckman), a dynamic gradient mixer (Model 340; Beckman), a gradient controller (Model 421; Beckman), a sample injection valve (Model 7125; Rheodyne, Cotati, CA, USA) with a 20-μl sample loop, a variable-wavelength UV monitor (Model 484; Waters, Milford, MA, USA), a column oven (Model CTO-2A; Shimadzu) and an integrator (Model C-R6A; Shimadzu).

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a 1.5-ml microcentrifuge tube by adding 2 μl of cDNA, which had been obtained by reverse transcription of 1 μg of mRNA isolated from scrotal skin fibroblasts, to a total volume of 100 μl of 10 m*M* Tris–HCl (pH 9.0, 25°C)–50 m*M* KCl–1.5 m*M* $MgCl_2$–0.2 m*M* deoxynucleotide triphosphates (dNTPs)–0.1 mg/ml gelatin–0.1% Triton X-100–0.5 μ*M* of each oligonucleotide primer–2.5 U Taq polymerase (Promega). To prevent evaporation, the reaction mixture was sealed with 50 μl of mineral oil (Sigma). Thirty cycles of amplification were carried out in a thermocycler (BioMed, Theres, Germany) with a 95°C denaturation step for 25 s, a 57°C annealing step for 30 s and a 73°C extension step for 60 s. The first denaturation step was elongated for 2 min and the last synthesis step for 3 min to ensure completion of the final extension step. Beginning at cycle 16, each following DNA synthesis step was elongated for 5 s. Finally, samples were cooled to room temperature.

Purification of PCR products

PCR products were purified by means of size-exclusion chromatography. Briefly, 0.7 ml of Sephadex G-150 (Pharmacia) slurry was filled into a 1-ml syringe and washed twice with tris-EDTA (TE) buffer. Then, 50–70 μl of PCR sample were applied to the top of the gel bed. The column was centrifuged in a swinging bucket at 1100 *g* for 4 min. This procedure was repeated a second time with 75 μl of TE buffer. The eluted DNA was then precipitated with 0.1 volume of 3 *M* sodium acetate (pH 5.0) and 1 volume of 2-propanol, stored at −30°C for 1 h and centrifuged at 16 000 *g* for 30 min. The supernatant was discarded and the pellet was rinsed twice with 75% ethanol, dried under vacuum and usually resuspended in 10 μl of TE buffer prior to analysis.

Capillary conditioning

Every new fused-silica capillary was flushed with 1 *M* NaOH for 1 h followed by 0.001 *M* NaOH for 5 min. Between runs, the capillary was washed first with 1 *M* NaOH for 2 min and then with 0.001 *M* NaOH for 1 min. Finally, it was equilibrated with running buffer until the baseline remained stable. Overnight the capillary was stored in 0.001 *M* NaOH. Phenylmethyl-deactivated fused-silica capillaries were flushed with doubly distilled water only.

Chromatographic conditions

The gradient profiles used for anion-exchange and reversed-phase separations are given on each chromatogram. In order to keep the concentration of TEAA constant and unaffected by volume contraction during mixing of organic solvents with water, the mobile phase used for reversed-phase chromatography of oligonucleotides was prepared as follows: for a 10% solution of acetonitrile in 0.1 *M* TEAA, 50 ml of a 2 *M* TEAA stock solution were added to 100 ml of acetonitrile in a 1000-ml volumetric flask and the final volume was made up to 1000 ml with water.

RESULTS AND DISCUSSION

Fig. 1 shows the capillary zone electrophoretic separation of non-phosphorylated and phosphorylated oligodeoxyadenylic acids, 12–18 bases in length, in a 67-cm fused-silica capillary of I.D. 75 μm with the use of a borate buffer containing 25 m*M* of SDS, which had been found to enhance the selectivity [7]. It is evident that the additional phosphate group at the 5′-end of phosphorylated oligonucleotides allows them to migrate faster against the overwhelming electroosmotic flow, which car-

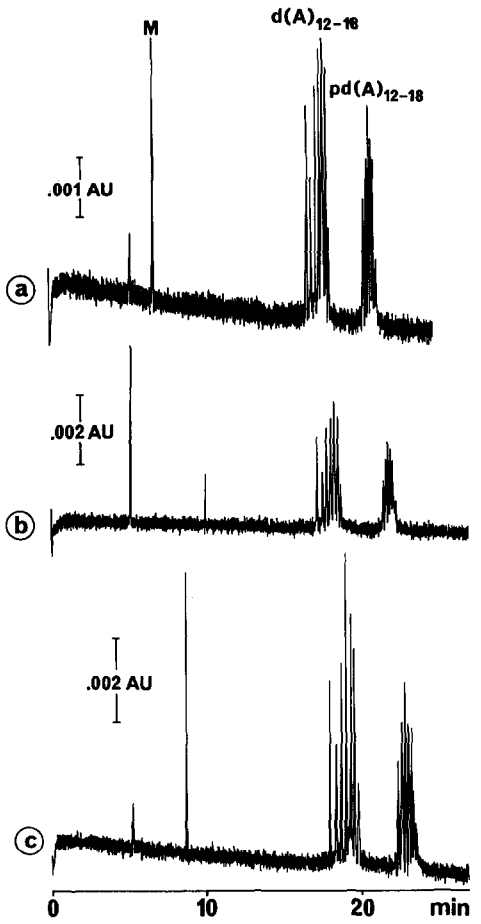

Fig. 1. Capillary zone electrophoretic separations of dephosphorylated and phosphorylated oligodeoxyadenylic acids. Capillary, fused silica, L = 67 cm, l = 60 cm, I.D. = 75 μm; carrier, 300 mM borate (pH 9.0)–(a) 25 mM SDS, (b) 25 mM NaCl and (c) 50 mM SDS; temperature, 25°C; voltage, 30 kV; current, 79, 99 and 89 μA, respectively; injection time, 7.5 s; detection, UV (254 nm); sample, $d(A)_{12-18}$ and $pd(A)_{12-18}$; M = mesityl oxide.

ries them past the detector toward the cathode. When SDS was replaced with an equivalent amount of sodium chloride, no significant change in resolution was observed. This indicates that the micelles are not required in order to obtain good resolution. However, as the sulphate moiety of SDS contributes less than chloride to the overall conductivity of the buffer system, higher concentrations of sodium in the carrier electrolyte can be realized without generating excess Joule heating. This allows a further enhancement in resolution, as can be seen in Fig. 1c.

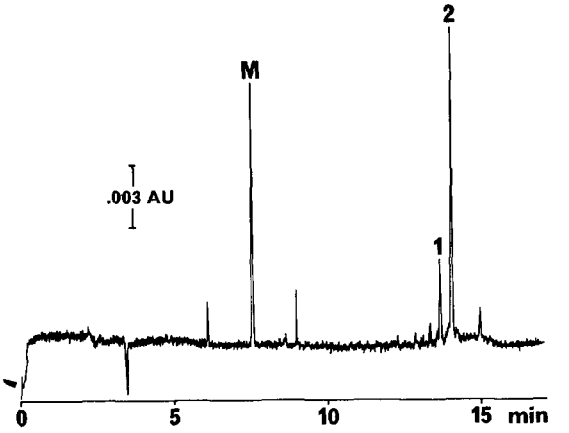

Fig. 2. Electropherogram of a trityl-on crude 21-mer heterooligonucleotide (GTGCTCAGTGTAGCCCAGGAT) and a 20-mer trityl-off sequence failure (M = mesityl oxide). Capillary, fused silica, L = 67 cm, l = 60 cm, I.D. = 75 μm; carrier, 300 mM borate (pH 9.0)–100 mM SDS; temperature, 25°C; voltage, 30 kV; current, 135 μA; injection time, 5 s detection, UV (254 nm).

Fig. 2 depicts the separation of a crude trityl-on

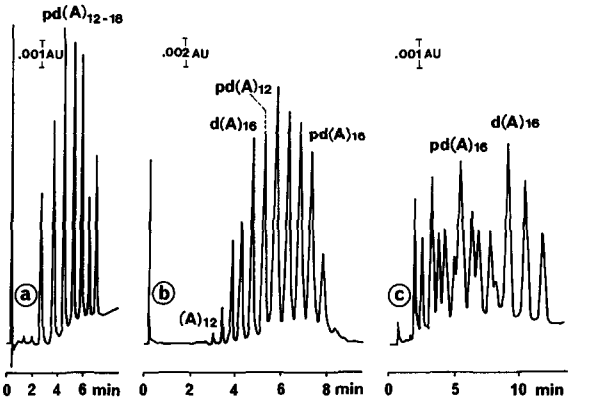

Fig. 3. HPLC separations of oligodeoxyadenylic acids. (a) Column Progel-TSK DEAE-NPR, 2.5 μm, 35 × 4.6 mm I.D.; buffer, 0.020 M Tris–HCl (pH 7.5); gradient, 0.15–0.5 M $(NH_4)_2SO_4$ in 15 min; flow-rate, 1 ml/min; sample, 0.75 μg of $pd(A)_{12-18}$. (b) Column, PEI-silica, 2 μm, 30 × 4.6 mm I.D.; buffer, 0.05 M phosphate (pH 5.9)–30% methanol; gradient, 0–0.5 M $(NH_4)_2SO_4$ in 10 min; flow-rate, 2 ml/min; sample, 1.75 μg pd $(A)_{12-18}$. (c) Column, PS-DVB-PVA, 2.3 μm, 50 × 4.6 mm I.D.; buffer, 0.1 M TEAA (pH 7.0); gradient, 12.5–20% acetonitrile in 20 min; flow-rate, 1 ml/min; sample, 0.5 μg $pd(A)_{12-18}$ and 0.083 μg $pd(A)_{16}$. All chromatograms were obtained by means of UV detection at 254 nm at room temperature.

21-mer heterooligonucleotide from a 20-mer sequence failure. However, with the chosen electrolyte system oligonucleotides cannot be separated according to size, because bases exhibit different electrophoretic mobilities in the order C < T < A. Therefore, this system is of limited value for the rapid characterization of synthetic oligonucleotides in comparison with capillary gel electrophoresis, which allows their strict separation according to size [8,9].

Fig. 3 shows the comparison of three different non-porous packing materials used for the analysis of oligodeoxyadenylic acids by means of anion-exchange and reversed-phase chromatography. The separation depicted in Fig. 3a was performed on a Progel-TSK DEAE-NPR stationary phase which consists of a non-porous hydrophilic resin covalently derivatized with diethylaminoethyl groups. The advantage of the hydrophilic resin is that the totally organic polymer is operable over a wide pH range, typically 1–13, without any damage occurring to the packing. This results in a long column lifetime and allows the regeneration of a deteriorated column with aqueous sodium hydroxide, which is of great advantage in the analysis of biopolymers. The number of functional groups that can be covalently attached, however, is limited by the Van der Waals radius of the molecules used for derivatization. In order to increase the ion-exchange capacity, a durable hydrophilic anion-exchange material was synthesized by the adsorption of polyethylenimine to non-porous silica particles with a diameter of 2 μm. Subsequently, the polyethylenimine layer was cross-linked into a stable layer by ethanediol diglycidyl ether. The resulting pellicular coating is more stable in aqueous media than the underlying silica and can be operated over the pH range 2–9.2 with no change in efficiency [4]. The higher ion-exchange capacity is reflected in the higher concentration of ammonium sulphate required to elute the oligodeoxyadenylic acids (Fig. 3b). However, the charge content has been found not to influence the resolution significantly, which is in accordance with a previous study on the ion-exchange chromatographic separation of double-stranded DNA fragments [10].

As analytes are separated according to charge in ion-exhange chromatography, it is not suprising that non-phosphorylated oligonucleotides are eluted earlier than phosphorylated ones. The presence of dephosphorylated oligodeoxyadenylic acids in a commercial sample of phosphorylated oligodeoxyadenylic acids is due to the incomplete phosphorylation of the oligonucleotides by polynucleotide kinase on completion of solid-phase synthesis. The relative content of dephosphorylated oligonucleotides has been found to vary considerably from batch to batch to batch, as can be seen in Fig. 3.

In order to prevent the corrosive action of halogen-containing eluents on the stainless-steel components of conventional HPLC equipment, an ammonium sulphate gradient was employed instead of the usual sodium chloride gradient. Moreover, ammonium sulphate improved significantly the resolution of oligonucleotides.

For many years anion-exchange chromatography of oligonucleotides has been considered superior to reversed-phase chromatography in terms of resolution and speed. Recently, however, we have succeeded in obtaining a baseline resolution of oligodeoxyadenylic acids up to 30 nucleotides in length on highly cross-linked PS–DVB–PVA particles using a

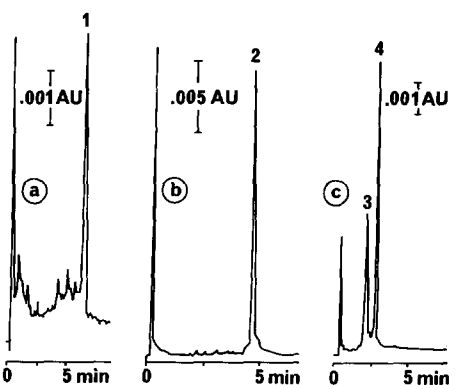

Fig. 4. HPLC analyses of synthetic phosphodiester heterooligonucleotides. (a) Column, Progel-TSK DEAE-NPR, 2.5 μm, 35 × 4.6 mm I.D.; buffer, 0.02 *M* Tris–HCl (pH 8); gradient, 0.4–1.0 *M* $(NH_4)_2SO_4$ in 10 min; flow-rate, 1 ml/min; temperature, ambient; sample, 0.36 μg of GTGCTCAGTGTAGCCCGTGATGCC, crude (1). (b) Column, PEI-silica, 2 μm, 30 × 4.6 mm I.D.; buffer, 0.05 *M* phosphate (pH 5.9)–30% methanol; gradient, 0–0.35 *M* $(NH_4)_2SO_4$ in 10 min; flow-rate, 2 ml/min; temperature, ambient; sample, 0.23 μg of TTGAAGTCACAGGAGACAACCTGGT, OPC-purified (2). (c) Column, PS–DVB–PVA, 2.3 μm, 50 × 4.6 mm I.D.; buffer, 0.1 *M* TEAA (pH 7); gradient, 6.5–8% acetonitrile in 2 min; flow-rate, 1 ml/min; temperature, 40°C; sample, GTGCTCAGTGTAGCCCAGGAT (3) and CTGTTGAACTCTTCTGAGCAA (4), 0.32 μg each. All chromatograms were obtained by means of UV detection at 254 nm.

volatile triethylammonium acetate buffer and a linear gradient of acetonitrile [11]. The particles were synthesized by a two-step microsuspension technique. Improved mass transfer and, hence, high resolution were obtained by adding PVA during polymerization, which yielded a more homogeneous and a less hydrophobic surface in comparison with PVA-free particles. As reversed-phase chromatography separates molecules based on their hydrophobicity, phosporylated oligonucleotides are eluted earlier than their dephosphorylated analogues (Fig. 3c).

Fig. 4a shows the analysis of a crude 24-mer oligonucleotide primer on a DEAE-bonded hydrophilic resin. Almost no failure sequences are detectable in addition to the desired, full-length oligomer. This corroborates the high efficiency of phosphoramidite chemistry [12] in the rapid preparation of oligonucleotides by means of an automated synthesizer, with the average yield per cycle being typically higher than 99%. From the chromatographic conditions chosen it is also evident that in comparison with homooligonucleotides higher concentrations of ammonium sulphate are required to elute heterooligonucleotides. This may be the result of additional hydrophobic interactions of the sample molecules with the stationary phase.

The efficacy of oligonucleotide purification cartridges [13] to purify the desired product from truncated oligomers and various organic salts contained in the crude synthesis mixture is demonstrated by the anion-exchange chromatographic analysis of a purified oligonucleotide on PEI-silica (Fig. 4b).

As illustrated in Fig. 4c, reversed-phase chromatography even permits the separation of isomeric oligonucleotides which differ only with regard to their base composition. The difference between the two oligonucleotides has been the replacement of three guanine residues with one adenine and two thymidine residues in the later eluting oligonucleotide. This retention behaviour is in accordance with our previous findings that the retention times of homooligonucleotides increase in the order C < G < A < T [11]. In addition to base composition, sequence was observed to exert an even greater influence on retention, especially in the presence of clusters of certain bases.

The polymerase chain reaction has revolutionized the identification of disease-associated genes,

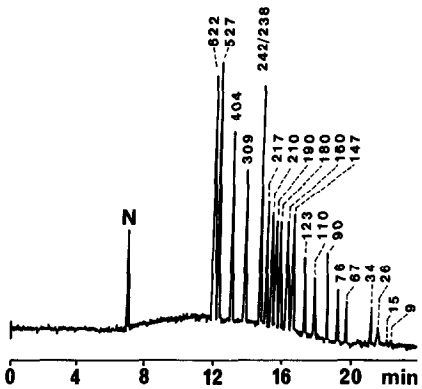

Fig. 5. Electropherogram of a pBR322-*Msp* I digest. Capillary, fused silica, L = 72 cm, l = 50 cm, I.D. = 50 μm; buffer, 0.01 M Tris–borate (pH 8.7)–0.1 mM EDTA–25 mM NaCl–0.5% (w/v) HEC–1.27 μM ethidium bromide; voltage, 15 kV; current, 16 μA; temperature, 35°C; detection, UV (260 nm); injection, vacuum, 5 s; sample concentration, 0.1 μg/μl.

because only a few copies are required to achieve the selective enrichment of DNA sequences of up to 6000 base pairs in length by a factor of 10^6 [14]. Recently, great interest has arisen in the quantification of PCR products to determine, for instance, the methylation of DNA or the rate of expression of certain genes. Quantification has been accomplished by various means such as liquid scintillation counting of excised gel bands, densitometry of photographic negatives of ethidium bromide-stained gels, hydridization with ^{32}P-labelled probes and

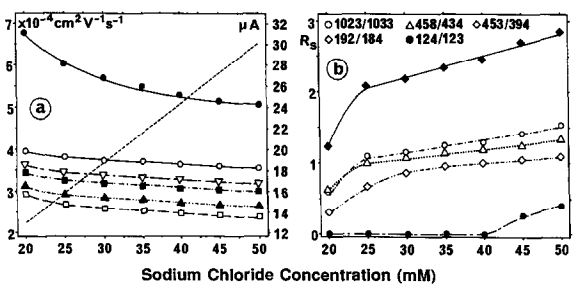

Fig. 6. Effect of sodium chloride concentration on (a) current (- - -), electroosmotic flow (●) and electrophoretic mobilities and (b) resolution (R_s) of DNA restriction fragments: ○ = 154 base pairs; ▽ = 298 base pairs; ■ = 394 base pairs; ▲ = 653 base pairs; □ = 1230 base pairs. Buffer, 0.01 M Tris–borate (pH 8.7)–0.1 mM EDTA–5–50 mM NaCl–0.5% (w/v) HEC; sample, pBR328 DNA-*Bgl* I/*Hinf* I digest, 0.25 μg/μl. Other conditions as in Fig. 5.

autoradiography, or high-performance liquid chromatography. However, these techniques are, for the most part, manually intensive, time consuming and prone to irreproducibility and poor quantitative accuracy.

Recently, we have established a capillary electrophoretic method that allows the rapid quantitative analysis of PCR products with a coefficient of variation as small as 3.4% by determining the molar ratio–peak area ratio of the amplified DNA segment with respect to a restriction fragment of known concentration [15]. Fig. 5 shows the separation of a pBR322 DNA-*Msp* I digest in a fused-silica capillary with an effective length of 50 cm and I.D. 50 μm. Hydroxyethylcellulose was added at a concentration of 0.5% to the running buffer as a sieving agent in order to permit the separation of nucleic acids according to size. Another prerequisite was the addition of sodium chloride, which not only reduced electroosmotic flow by increasing the thickness of the diffusion double layer at the inner capillary wall but also altered the mobility of DNA fragments in a variety of ways which affect charge, shape and size. Fig. 6a depicts the decrease in true electrophoretic mobilities of DNA restriction fragments observed with increasing concentration of sodium ions in the running buffer. The improvement in resolution (Fig. 6b) which can be noted when the sodium chloride concentration is increased from 20 to 25 m*M* is mainly due to the spread in electrophoretic mobilities between the largest and the smallest DNA fragment. Above that, no further increase in selectivity is observed. Hence the slight and linear improvement in resolution seen at even higher concentrations of sodium chloride may be a consequence of the increase in current density which is known to cause a proportional increase in the number of theoretical plates.

The influence of various alkali metal ions, namely lithium, sodium, potassium, rubidium and cesium, on the capillary electrophoretic separation of DNA restriction fragments is depicted in Fig. 7. It is evident that as the cation size increased the migration times increased. This is mainly due to a decrease in ζ potential, which causes a lower electroosmotic flow. The ζ potential is linearly proportional to the charge density, which decreases as the atomic weight of the cation increases. Another observation is that the spread in electrophoretic mobilities between the largest and the smallest fragments increased considerably when lithium or sodium chloride was replaced with potassium chloride. While caesium chloride yielded the best separation of DNA fragments, the migration times were five times longer than when lithium chloride was used. Generally, an increase in resolution of all DNA fragments was achieved with an increase in the size of the cation, but the concomitant increase in migration times might be unacceptable in routine analysis.

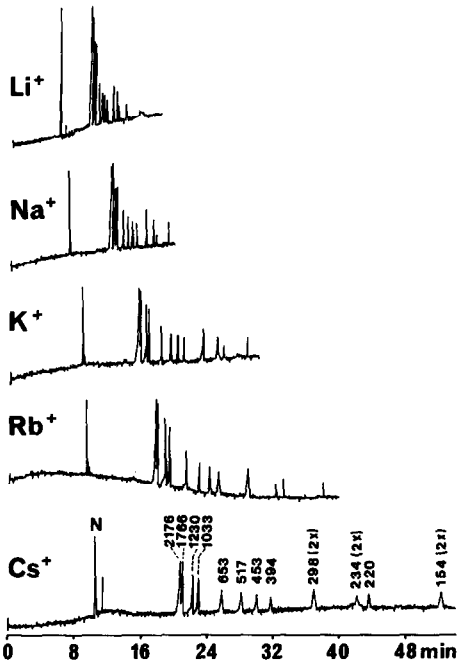

Fig. 7. Effect of various alkali metal cations on the separation of dsDNA. Capillary, fused silica, L = 72 cm, l = 50 cm, I.D. = 50 μm; buffer, 0.01 *M* Tris–borate (pH 8.7)–(0.1 m*M* EDTA–0.5% (w/v) HEC–25 m*M* LiCl, NaCl, KCl, RbCl or CsCl; voltage, 17, 15, 13, 12 and 12 kV, respectively; current, 16 μA; temperature, 35°C; detection, UV (260 nm); injection, vacuum, 8 s; sample, pBR328-*Bgl* I/*Hinf* I digests, 0.25 μg/μl.

A further improvement in resolution was obtained by the addition of ethidium bromide to the running buffer. Ethidium bromide minimizes variations in electrophoretic mobility caused by the metastability of DNA by the way of intercalation between two adjacent GC pairs. Optimum resolution in comparison with a buffer system containing no ethidium bromide (Fig. 8a) was obtained at a

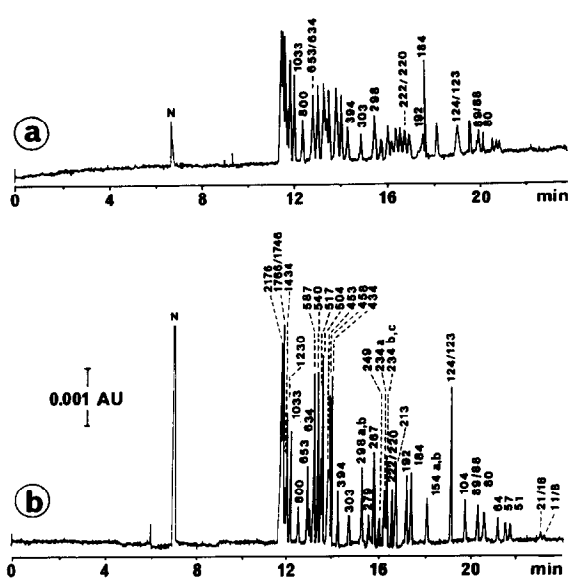

Fig. 8. Effect of ethidium bromide on the capillary electrophoretic separation of DNA restriction fragments. Capillary, fused silica, L = 72 cm, l = 50 cm, I.D. = 50 μm; buffer, 0.01 M Tris–borate (pH 8.7)–0.1 mM EDTA–25 mM NaCl–0.5% (w/v) HEC, (a) without and (b) with 1.27 μM of ethidium bromide; voltage, 15 kV; current, 16 μA; temperature, 35°C; detection, UV (260 nm); injection, vacuum, 8 s; sample, a mixture of pBR322 DNA-*Ava* II/*Eco*R I (0.2 μg/μl), pBR322 DNA-*Hae* III (0.25 μg/μl) and pBR328 DNA-*Bgl* I/*Hinf* I (0.25 μg/μl) digests in a ratio 1:2:2.

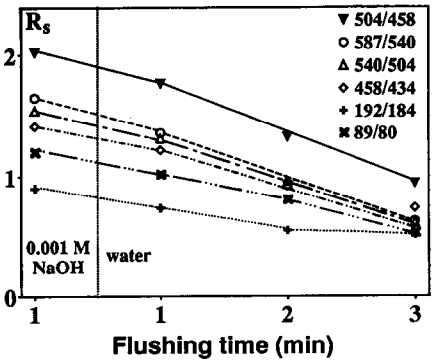

Fig. 9. Effect of flushing fused-silica capillaries with dilute sodium hydroxide or water on resolution of DNA fragments. Capillary, fused silica, L = 72 cm, l = 50 cm, I.D. = 50 μm; buffer, 0.01 M Tris–borate (pH 8.7)–0.1 mM EDTA–25 mM NaCl–0.5% (w/v) HEC; temperature, 35°C; voltage, 12 kV; current, 13 μA; detection, UV (260 nm); injection, vacuum, 5 s.

concentration of 1.27 μM (Fig. 8b). The addition of even more ethidium bromide up to a concentration of 7.6 μM only enhanced the resolution of shorter DNA fragments, whereas the resolution for DNA molecules longer than 400 base pairs started to decline again. The tremendous improvement in efficiency is further endorsed by a fourfold increase in the number of theoretical plates. At 1.27 μM of ethidium bromide up to $2 \cdot 10^6$ plates per metre could be realized for fragments less than 300 base pairs in length. On addition of even more ethidium bromide the number of theoretical plates declined again. Finally, at 7.6 μM the same number of plates per metre was observed as without ethidium bromide, namely $2 \cdot 10^5$–$4 \cdot 10^5$ for DNA restriction fragments ranging from 89 to 1230 base pairs in length.

The physical and the chemical states of the inner capillary surface exert a major impact on the resolution of dsDNA fragments. Hence, capillaries of fused silica must be prepared for use prior to any analytical runs by washing for at least 30 min with 1 M NaOH to remove any adsorbed impurities or traces of old sample and to ensure maximum ionization of silanol groups. Following a flush with 0.001 M NaOH, the capillary has to be equilibrated with the running buffer until the baseline remains stable. This procedure has to be repeated between runs whenever loss of reproducibility and resolution is observed. Otherwise capillaries are flushed first with 1 M NaOH for 2 min, then with 0.001 M NaOH for 1 min. The 2-min rinse with 1 M NaOH yielded optimum resolution, whereas shorter or longer washing periods reduced the separation efficiency. The dilute sodium hydroxide flush was preferred to water, which exerted a deteriorating effect on resolution due to reprotonation of the charged silanols on the inner capillary wall (Fig. 9). Moreover, it is recommended to replace the cathode reservoir during the sodium hydroxide wash in order to avoid a loss of reproducibility and resolution due to a change in buffer composition. Taking these precautions, it suffices to renew the buffer in both reservoirs every 3–4 runs.

Using a phenylmethyl-deactivated fused-silica capillary, theoretical plates in excess of $4 \cdot 10^6$ plates per meter could be realised for restriction fragments ranging from 220 to 1766 base pairs in length when the aforementioned buffer system was replaced with 100 mM Tris–borate (pH 8.7)–0.5% hydroxyethylcellulose–0.653 μM ethidium bromide (Fig. 10). Al-

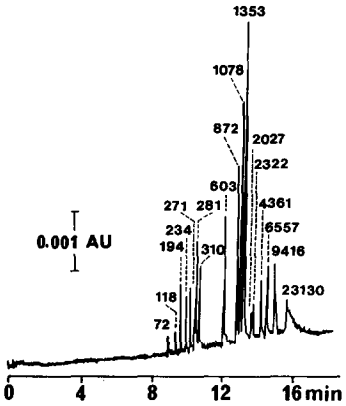

Fig. 10. Electropherogram of a λ-*Hind* III/φX174-*Hae* III digest. Capillary, phenylmethyl-deactivated fused silica, L = 72 cm, l = 50 cm, I.D. = 50 μm; buffer, 0.1 *M* Tris–borate (pH 8.7)–1 m*M* EDTA–0.5% (w/v) HEC–0.635 μ*M* ethidium bromide; voltage, −20 kV; current, 26 μA; temperature, 35°C; detection, UV (260 nm); injection, vacuum, 2 s; sample concentration, 0.5 μg/μl.

though separation is achieved basically according to size, minor discrepancies may be observed on addition of ethidium bromide to the buffer system, because its cationic-charged nitrogens interact electrostatically with the phosphate groups of DNA, which slows the migration rate depending on the content of adjacent GC pairs.

Resolution in capillary electrophoresis is also influenced significantly by sample composition. The salts, nucleotides and primers contained in PCR samples have been found to cause considerable band broadening owing to the conductivity difference between the sample zone and the surrounding buffer (Fig. 11a). Hence, purification of PCR products prior to capillary electrophoretic analysis is required. It has been observed that neither ethanol precipitation nor polyacrylamide size exclusion gel chromatography with an exclusion limit of 20 base pairs removes all unincorporated nucleotides and

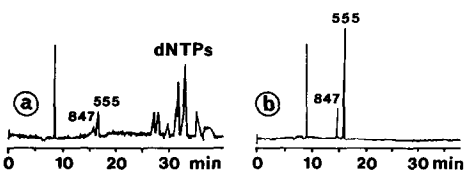

Fig. 11. Electropherograms of two (a) unpurified and (b) Sephadex G-150-purified PCR products. Buffer, 0.01 *M* Tris–borate (pH 8.7)–0.1 m*M* EDTA–0.5% (w/v) HEC–25 m*M* NaCl–1.27 μ*M* ethidium bromide. Other conditions as in Fig. 8.

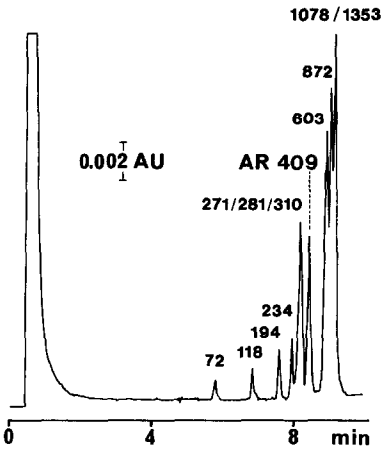

Fig. 12. Anion-exchange chromatographic analysis of a PCR-amplified 409-base pairs fragment of the androgen receptor spiked with a φX174-*Hae* III digest. Column, Progel-TSK DEAE-NPR, 2.5 μm (35 × 4.6 mm I.D.); eluents, (A) 0.025 *M* Tris–borate (pH 8.6)–0.25 m*M* EDTA, (B) 1 *M* NaCl in A, linear gradient from 45 to 75% B in 15 min; flow-rate, 1 ml/min; temperature, 25°C; detection, UV (260 nm).

primers. Only Sephadex G-150 retained all nucleotides and primers, hence ensuring optimum resolution of the PCR products (Fig. 11b).

The concentration sensitivity of capillary electrophoresis using on-column UV detection equals that obtained by ethidium bromide-stained gels. However, owing to the lower mass detection limit, which is *ca.* 3 pg for a 72 base pair restriction fragment at a signal-to-noise ratio of 3, only 3–5 nl of sample need to be injected in contrast to the 10 μl required for an agarose slab-gel separation.

Even with the best non-porous packing materials availaible, anion-exchange chromatography fails to provide the same degree of resolution as that obtained by means of capillary electrophoresis (Fig. 12). Moreover, retention times of DNA fragments do not always correlate with their size, because AT pairs interact more strongly than GC pairs with the stationary phase [10]. Therefore, fragments with a relatively high AT content are retarded more than others.

CONCLUSIONS

It is concluded that HPLC on non-porous particles is superior to capillary electrophoresis in terms

of speed and selectivity in the analysis of oligonucleotides up to 30 bases in length. Moreover, reversed-phase chromatography of detritylated oligonucleotides permits their simultaneous purification without the need for subsequent desaltation because the volatile buffer system can be readily evaporated. With regard to dsDNA, however, the resolving power of capillary electrophoresis cannot be matched by anion-exchange chromatography at present. Moreover, the combined use of hydroxyethylcellulose and ethidium bromide not only yields a separation efficiency equal to that achieved by means of gel-filled capillaries but also avoids some of their limitations such as the destruction of the gel matrix at high current densities and the bias involved in electrokinetic injection which does not allow the same amount of each DNA species to be delivered.

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REFERENCES

1 W. Haupt and A. Pingoud, *J. Chromatogr.*, 260 (1983) 419.
2 G. Jilge, K. K. Unger, U. Esser, H. J. Schäfer, G. Rathgeber and W. Müller, *J. Chromatogr.*, 476 (1989) 37.
3 M. J. Gordon, X. Huang, S. L. Pentoney and R. N. Zare, *Science*, 242 (1988) 224.
4 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
5 K. Kopaciewicz, M. A. Rounds and F. E. Regnier, *J. Chromatogr.*, 318 (1985) 157.
6 S. Wongyai, J. M. Varga and G. K. Bonn, *J. Chromatogr.*, 536 (1991) 155.
7 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, *Anal. Chem.*, 59 (1987) 1021.
8 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.
9 A. Paulus and J. I. Ohms, *J. Chromatogr.*, 507 (1990) 113.
10 E. Westman, S. Eriksson, T. Låås, P. A. Pernemalm and S. E. Sköld, *Anal. Biochem.*, 166 (1987) 158.
11 C. G. Huber, P. J. Oefner and G. K. Bonn, *J. Chromatogr.*, 599 (1992) 113–118.
12 S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.*, 22 (1981) 1859.
13 L. J. McBride, C. McCollum, S. Davidson, J. W. Efcavitch, A. Andrus and S. J. Lombardi, *BioTechniques*, 6 (1988) 362.
14 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim, *Science*, 230 (1985) 1350.
15 S. Nathakarnkitkool, P. J. Oefner, G. Bartsch, M. A. Chin and G. K. Bonn, *Electrophoresis*, 13 (1992) 1.