Rapid and accurate sizing of DNA fragments by ion-pair chromatography on alkylated nonporous poly(styrene-divinylbenzene) particles

ARTICLE · JANUARY 1995

CITATIONS
22

READS
8

3 AUTHORS, INCLUDING:

Christian G Huber
University of Salzburg
191 PUBLICATIONS **5,002** CITATIONS
SEE PROFILE

Guenther Karl Bonn
University of Innsbruck
346 PUBLICATIONS **7,078** CITATIONS
SEE PROFILE

Anal. Chem. **1995**, *67*, 578–585

Rapid and Accurate Sizing of DNA Fragments by Ion-Pair Chromatography on Alkylated Nonporous Poly(styrene–divinylbenzene) Particles

Christian G. Huber,*,† Peter J. Oefner,‡ and Günther K. Bonn†

Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens-University, Innrain 52a, A-6020 Innsbruck, Austria, and Department of Genetics, Stanford University, Stanford, California 94305-5120

Ion-pair reversed-phase high-performance liquid chromatography on nonporous alkylated poly(styrene–divinylbenzene) particles allowed the separation of double-stranded DNA fragments in a few minutes with a resolution comparable to that obtained in slab or capillary gel electrophoresis. Using gradients of acetonitrile in 0.1 M triethylammonium acetate, DNA fragments differing in length only by 2–3% could be resolved. Synthesis of the stationary phase was very reproducible, and equilibration of a freshly packed column for at least 2 h proved to be necessary for optimum column performance, whereas column regeneration for 1–5 min was adequate between gradient runs. Various DNA molecular weight markers ranging from 51 to 2176 base pairs in length were used to study the size dependence of retention. A plot of capacity factors versus logarithm of molecular weights revealed a clear correlation between size and retention. Both a local and a global approximation method, which differed with regard to the number of known standard fragments used below and above the fragments with unknown length to create a sizing curve based on simple linear regression, gave essentially identical results, with the inaccuracy of length measurement being 0.05–3.2%. This compares favorably with the accuracy obtained in gel electrophoresis.

The introduction of type II restriction endonucleases about 25 years ago by Smith exerted a tremendous impact on molecular biology and gene technology.[1] Restriction endonucleases recognize specific nucleotide sequences and cleave double-stranded DNA into fragments of equimolar amount, which are then usually separated by means of agarose or polyacrylamide slab gel electrophoresis, exploiting the strictly length-dependent polyanionic properties and the considerable shape regularity of DNA.[2] But gel or electric field inhomogeneity often result in inconsistent migration of sample zones among lanes or even in a single lane in a slab gel. Further, detection and quantitation of the separated species is unreliable, as the intercalation of the mutagenic reagent ethidium bromide, which is usually employed for staining gels, between adjacent GC base pairs is very sensitive to experimental conditions. Although the latter problem has overcome by the introduction of high-performance capillary electrophoresis (HPCE), which put electrophoretic separations on the same instrumental footing as high-performance liquid chromatography (HPLC) and allowed the detection of nucleic acids by on line UV absorbance[3–5] as well as direct and indirect fluorescence[6] detection, the technique still suffers from several drawbacks such as capillary and gel instability as well as the need for samples to be desalted prior to analysis.

Liquid chromatographic separations of DNA restriction fragments are primarily based on principles of ion-exchange (IEC),[7] ion-pair reversed-phase (IP-RPC),[8,9] or size exclusion (SEC) chromatography.[9] Chromatography of DNA fragments on the porous anion-exchange supports Mono Q and Mono P allowed the separation of DNA restriction fragments within a wide range of size,[10] but resolution of fragments larger than 200 base pairs (bp) generally declined rapidly and separations of DNA molecules up to a length of 500 bp took almost 4 h. A considerable reduction in analysis time to less than 15 min as well as a significant enhancement in resolution was achieved by the introduction of nonporous particles to which diethylaminoethyl groups (DEAE) had been bonded chemically[11] (a comparison of different porous and nonporous anion-exchange packings for the separation of restriction fragments is given in ref 12).

A major disadvantage of anion-exchange chromatography is the sequence-dependent retention behavior of DNA fragments, because high AT contents tend to cause double-stranded DNA to bind to anion exchangers more tightly than expected simply on the basis of their molecular size.[7,13,14] This effect makes a separation according to molecular size difficult. Bloch showed that, to a certain extent, a length-relevant separation of DNA fragments is feasible on a nonporous DEAE anion exchanger with

† Leopold-Franzens-University.

‡ Stanford University.

* Internet: Christian.Huber@uibk.ac.at.

(1) Smith, H. O.; Wilcox, K. W. *J. Mol. Biol.* **1970**, *51*, 379–91.
(2) Southern, E. M. *Anal. Biochem.* **1979**, *100*, 319–23.
(3) Heiger, D. N.; Cohen, A .S.; Karger, B. L. *J. Chromatogr.* **1990**, *516*, 33–48.
(4) Nathakarnkitkool, S.; Oefner, P. J.; Bartsch. G.; Chin, M. A.; Bonn, G. K. *Electrophoresis* **1993**, *13*, 18–31.
(5) McGregor, D. A.; Yeung, E. S. *J. Chromatogr. A* **1993**, *652*, 67–73.
(6) Chan, K. C.; Whang, C.-W.; Yeung, E. S. *J. Liq. Chromatogr.* **1993**, *16*, 1941–62.
(7) Kato, Y.; Sasaki, M.; Hashimoto, T.; Murotsu, T.; Fukushige, S.; Matsubara, K. *J. Chromatogr.* **1983**, *265*, 342–6.
(8) Eriksson, S.; Glad, G.; Pernemalm, P.-A.; Westman, E. *J. Chromatogr.* **1986**, *359*, 265–74.
(9) Thompson, J. A. *BioChromatography* **1987**, *2*, 4–18.
(10) Westman, E.; Eriksson, S.; Låås, T.; Pernemalm, P.-A.; Sköld, S.-E. *Anal. Biochem.* **1987**, *166*, 158–71.
(11) Kato, Y.; Yamasaki, Y.; Onaka, A.; Kitamura, T.; Hashimoto, T.; Murotsu, T.; Fukushige, S.; Matsubara, K. *J. Chromatogr.* **1989**, *478*, 264–8.
(12) Strege, M. A.; Lagu, A. *J. Chromatogr.* **1991**, *555*, 109–24.
(13) Patient, R. K; Hardies, S. C.; Larson, J. E.; Inman, R. B.; Maquat, L. E.; Wells, R. D *J. Biol. Chem.* **1979**, *254*, 5548–54.
(14) Hecker, R.; Colpan, M.; Riesner, D. *J. Chromatogr.* **1985**, *326*, 251–61.

0003-2700/95/0367-0578$9.00/0

eluents containing tetramethylammonium chloride (TMAC).[15] However, while the 434 bp fragment could be resolved from a 458 bp fragment, the latter coeluted with a 504 bp fragment despite the 46 bp difference in size. Moreover, the addition of TMAC caused a significant, general decrease in chromatographic performance.

Ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) is a well-established technique for separating mono- and oligonucleotides.[16,17] The principal feasibility of separating DNA restriction fragments by means of IP-RP-HPLC on uncoated poly(chlorotrifluoroethylene) powder has been demonstrated as early as 1979,[18] but attempts to transfer the results to commercial packing materials were not successful till 1986, when Eriksson et al.[8] published the separation of DNA molecules on a PepRPC (C_2/C_{18}) column, the packing of which is based on porous silica. However, complete separation of restriction fragments up to a size of 600 bp took more than 3 h, which makes the method impractical for both analytical and preparative purposes. Nevertheless, both studies confirmed that the separation of restriction fragments in IP-RP-HPLC depends mainly on chain length. Recently, we have demonstrated the applicability of nonporous alkylated poly(styrene–divinylbenzene) particles for high-resolution separations of oligonucleotides,[19] DNA fragments,[20] and polymerase chain reaction (PCR) products[21,22] by IP-RP-HPLC. The present study shows not only the high batch-to-batch consistency in preparation of the stationary phase but also its suitability for the rapid and accurate sizing of DNA fragments by IP-RP-HPLC.

EXPERIMENTAL SECTION

Materials. HPLC gradient-grade acetonitrile, analytical grade tetrahydrofuran, methanol, sodium chloride, magnesium chloride, tin tetrachloride, tris(hydroxymethyl)aminomethane (TRIS), and hydrochloric acid were obtained from Merck (Darmstadt, Germany). A 1 M stock solution of triethylammonium acetate (TEAA), pH 7.0, was prepared from triethylamine (Fluka, Buchs, Switzerland) and glacial acetic acid (Merck). High-purity water (Epure, Barnstead Co., Newton, MA) was used for preparation of the buffers.

Instumentation. The HPLC system consisted of a high-precision low-pressure gradient pump (Model 480 GT, Gynkotek, Germering, Germany), a degasser (Liliput, Gynkotek), a column oven (Model STH 585, Gynkotek), a diode-array UV detector (Model UVD 320, Gynkotek), a biocompatible sample injection valve (Model 9125, Rheodyne Inc., Cotati, CA) with a 10 μL sample loop, and a PC-based data system (GynkoSoft, Version 5.12, Gynkotek).

Chromatography. Columns (50 × 4.6 mm i.d.) packed with alkylated poly(styrene–divinylbenzene) (PS–DVB-C_{18}) beads were prepared according to the previously published protocol[19,23,24] except that tin tetrachloride was used instead of aluminium chloride in the Friedel–Crafts reaction, as the former can be easier removed after alkylation. In Figures 1–8, eluent A was 0.1 M TEAA, pH 7.0, and elutent B was 0.1 M TEAA, pH 7.0, 25% acetonitrile, whereas eluent A was 0.1 M TEAA, pH 7.0, 8% acetonitrile and elutent B was 0.1 M TEAA, pH 7, 20% acetonitrile in Figures 9–11. Gradient profiles are given on each chromatogram. In Figures 1–8, the linear gradient program was started before injection of the sample in order to account for the 1 mL dead volume of the low-pressure gradient system.

DNA Size Standards and PCR Products. Size standards of double-stranded DNA restriction fragments were purchased from Boehringer Mannheim (pBR322 DNA *Hae*III digest and a mixture of a pBR328 DNA *Bgl*I and a pBR328 DNA *Hinf*I digest, Mannheim, Germany) and USB (ΦX174 DNA *Hinc*II digest and pBR322 DNA *Msp*I digest, United States Biochemical, Cleveland, OH). DNA sequences and restriction sites were obtained from the EMBL+GenBank Release 81. The separated DNA fragments are identified in chromatograms and tables by their respective length in base pairs (1 bp = 660 Da). PCR products of different chain length (120, 167, 198, and 257 bp, respectively) were prepared according to standard PCR protocols as described elsewhere[20–22] and injected without prior purification.

DNA Digestion. A pBR322 *Hae*III digest (Boehringer Mannheim) was further digested with the restriction endonuclease *Eco*RV. Briefly, 2.5 μg of pBR322 *Hae*III digest in 12 μL of 20 mM Tris-HCl, pH 8.0, 5 mM magnesium chloride, 100 mM sodium chloride, and 1 mM EDTA were digested for 2 h at 37 °C with 0.5 unit of *Eco*RV (Boehringer Mannheim). After digestion, the reaction mixture was heated to 80 °C for 3 min, then cooled on ice, and injected without prior purification.

Calculation of Fragment Length from Capacity Factors. The logarithmic relationship between capacity factors and fragment length was used to convert capacity factors to molecular size. This relationship is $k = b \log \text{bp} + a$ where k is capacity factor, bp is length of DNA fragments in base pairs, and a and b are constants. Least-squares linear regression analysis was applied to calculate the constants a and b. For method 1, a linear regression curve was created by using two standard points below and one point above the fragment and the size is determined. Another curve was created from one standard point below and two standard points above and a second value is assigned. The two size values were averaged to determine the unknown fragments length. With this method, only the region of the size ladder near the fragment of unknown length is analyzed. For method 2, a set of 10 standard points (5 points below and 5 points above the unknown fragment) was analyzed for linear regression, therefore utilizing a larger range of fragment sizes for calibration.

RESULTS AND DISCUSSION

Reproducibility of Alkylation. Alkylation of monodisperse nonporous PS–DVB particles (2.1 μm particle diameter, 0.12 μm standard deviation) is essential for obtaining high-resolution analysis of nucleic acids.[20] Figure 1 shows the separation of DNA fragments on three different batches of alkylated PS–DVB particles. The essentially identical chromatograms corroborate the high batch-to-batch consistency of the stationary phase. The cleanup of the alkylation reaction was made significantly easier

(15) Bloch, W. European Patent Application EP 0 507 591 A2, 1992.
(16) Jost, W.; Unger, K.; Schill, G. *Anal. Biochem.* **1982**, *119*, 214–23.
(17) Haupt, W.; Pingoud, A. *J. Chromatogr.* **1983**, *260*, 419–27.
(18) Usher, D. A. *Nucleic Acids Res.* **1979**, *6*, 289–306.
(19) Huber, C. G.; Oefner, P. J.; Bonn, G. K. *Anal. Biochem.* **1993**, *212*, 351–8.
(20) Huber, C. G.; Oefner, P. J.; Preuss, E.; Bonn, G. K. *Nucleic Acids Res* **1993**, *21*, 1061–6.
(21) Huber, C. G.; Oefner, P. J.; Bonn, G. K. *Chromatographia* **1993**, *37*, 653–8.
(22) Oefner, P. J.; Huber, C. G.; Puchhammer-Stöckl, E.; Umlauft, F.; Grünewald, K.; Bonn, G. K.; Kunz, C. *BioTechniques* **1994**, *19*, 898–908.
(23) Bonn, G. K.; Huber, C. G.; Oefner, P. J. Austrian Patent Application A 2285/92, 1992.
(24) Bonn, G. K.; Huber, C. G.; Oefner, P. J. International Patent Application PCT/US93/10975, 1993.

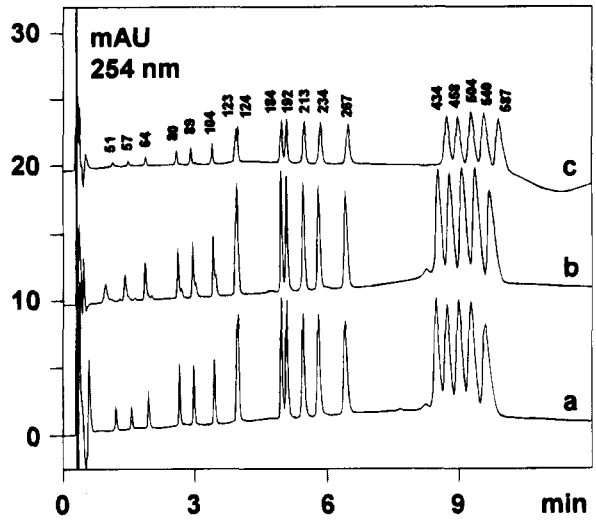

Figure 1. Reproducibility of Friedel–Crafts alkylation of poly(styrene–divinylbenzene) particles: columns, three different batches of PS–DVB-C_{18}, 2.1 μm, 50 × 4.6 mm i.d.; mobile phase, (A) 0.1 M TEAA, pH 7.0, (B) 0.1 M TEAA, pH 7.0, 25% acetonitrile; linear gradient, 30–55% B in 4 min, 55–65% B in 6 min; flow rate, 1 mL/min; temperature, 50 °C; detection, UV, 254 nm; sample, 0.75 (a, b) and 0.5 μg (c) of pBR322 DNA-*Hae*III digest.

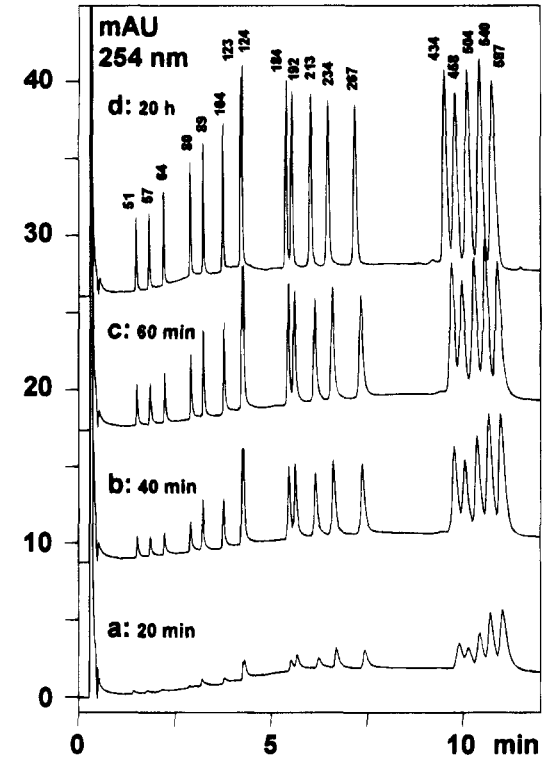

Figure 2. Equilibration of a newly packed column: column, PS–DVB-C_{18}, 2.1 μm, 50 × 4.6 mm i.d.; mobile phase: (A) 0.1 M TEAA, pH 7.0, (B) 0.1 M TEAA, pH 7.0, 25% acetonitrile; linear gradient, 35–55% B in 4 min, 55–67% B in 8 min; flow rate, 1 mL/min; temperature, 50 °C; detection, UV, 254 nm; sample, 1.25 μg of pBR322 DNA *Hae*III digest injected after 20 min (a), 40 min (b), 60 min (c), and 20 h (d) of equilibration time.

by replacing aluminium chloride with tin tetrachloride as the Friedel–Crafts catalyst, as the latter can be extracted more readily with water resulting in a higher degree of batch reproducibility and efficiency of nucleic acid separations.

Equilibration of a Newly Packed Column. Sufficient equilibration of newly packed columns is also an important determinant for obtaining high-performance separations. The time course of equilibration is summarized in Figure 2. The newly packed column was washed with pure water at a flow rate of 1 mL/min and a temperature of 50 °C in order to achieve an equilibrium of column temperature. Then the column was eqilibrated for 7 min with 0.1 M TEAA, pH 7.0, containing 18.75% acetonitrile. The mobile phase was adjusted to the starting conditions of the linear gradient (see legend of Figure 2) for 3 min, before a pBR322 DNA *Hae*III digest was injected. The resultant chromatogram (not shown) revealed only an injection peak. Separations of DNA fragments after 20, 40, and 60 min as well as 20 h of total equilibration time, respectively, are depicted in Figure 2a–d. It is evident that retention times remained constant already after 20 min. Separation efficiency, on the other hand, increased significantly between 20 and 60 min, with a further increase in resolution being observed after overnight equilibration. Both resolution R_s of selected pairs of DNA fragments and peak asymmetry factors As are given in Table 1. Therefore, overnight equilibration of a newly packed column is recommended for maximum performance, though an equilibration time of 1–2 h is sufficient for most applications. Nonetheless, once a newly packed column column has been adequately equlilibrated, regeneration and equilibration of the stationary phase after separation under gradient conditions is accomplished within less than 1–5 min, which makes fast and repetitive injections practicable for routine analyses.

Optimized Ion-Pair Chromatographic Analysis of DNA Restriction Digests. Some of the chromatographic conditions important for nucleic acid separations such as temperature, concentration of ion-pair reagent, flow rate, and gradient slope have already been discussed elsewhere[20–22] and should only be summarized here briefly. The influence of temperature was investigated between 20 and 70 °C, and optimum resolution was achieved at 50 °C. Retention times and resolution of DNA fragments were found to increase with increasing concentrations of TEAA as ion-pair reagent ranging from 25 to 125 mM. Gradient slopes between 0.05 and 5% acetonitrile in 0.1 M TEAA per minute were used for elution, depending on difference in length and overall length of DNA fragments to be separated. Flow rate also exerts a considerable effect on resolution of DNA fragments. Small restriction fragments (<200 bp) are separated most efficiently at flow rates between 0.5 and 1.2 mL/min, whereas flow rates smaller than 0.5 mL/min should be used for high resolution of larger DNA molecules (>500 bp).

Table 1. Resolution and Peak Asymmetry of Selected DNA Fragments

equilibration time	R_s 184/192 bp	R_s 540/587 bp	As 213 bp
20 min	0.88	0.90	1.45
40 min	1.22	0.98	1.86
60 min	1.35	1.00	1.63
20 h	1.52	1.12	1.36

Several DNA restriction digests are commercially available as DNA size standards for calibration in electrophoresis and HPLC. DNA from different sources, mainly bacterial plasmids, is digested with restriction enzymes, resulting in digests covering a certain size range. The plasmid pBR322 is an *Escherichia coli* cloning vector of 4363 bp. A digest with the restriction enzyme *Hae*III yields a total of 22 fragments (8, 11, 18, 21, 51, 57, 64, 80, 89, 104,

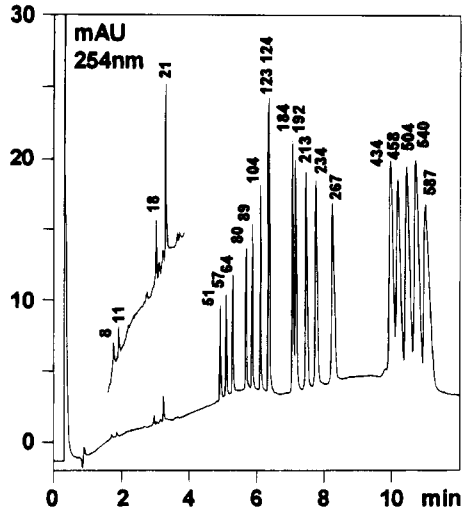

Figure 3. Separation of DNA restriction fragments ranging in length from 8 to 587 bp: linear gradient, 10–55% B in 6 min, 55–65% B in 6 min; flow rate, 1 mL/min; sample, 1.5 μg of pBR322 DNA *Hae*III digest. Other conditions as in Figure 2.

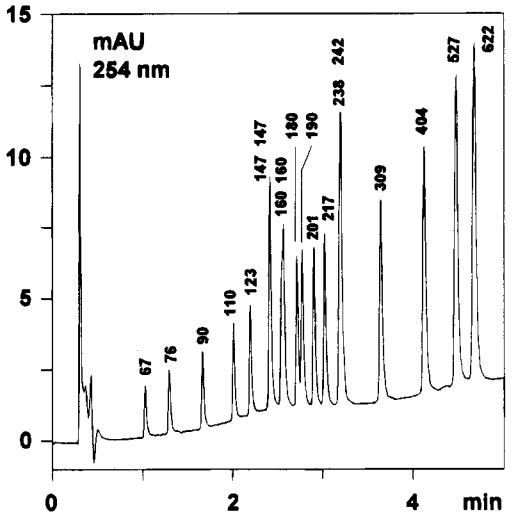

Figure 4. Separation of DNA restriction fragments from pBR322 DNA cleaved with *Msp*I on alkylated PS–DVB: linear gradient, 40–58% B in 2 min, 58–70% B in 3 min; flow rate, 1 mL/min; sample, 1.5 μg of pBR322 DNA *Msp*I digest. Other conditions as in Figure 2.

123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540, 587 bp) and is distributed as DNA molecular weight marker V by Boehringer Mannheim. After digestion, all fragments are present in equimolar amounts, with the UV absorbance of a linear DNA fragment being approximately directly proportional to the number of bases it contains.[25] Because of the wide dynamic range of on-column UV detection in HPLC and HPCE, fragments with a large difference in size can be visualized in a single run. In gel electrophoresis, on the other hand, small fragments are only detected on overloaded gels, because only few GC base pairs are available for intercalation with ethidium bromide. Paulus and Hüsken[26] showed in a comparison of agarose slab gel electrophoresis and polyacrylamide capillary gel electrophoresis that visualization of a 72 bp fragment from the ΦX174 *Hae*III digest was only possible at a 5 μg per lane loading whereas resolution of larger fragments decreased drastically because of overloading of the gel.

The chromatogram of a pBR322 *Hae*III digest with fragments from 8 to 587 bp illustrates (Figure 3) that chromatographic separation and quantitation of DNA fragments over a wide range is feasible without overloading the column. The loading capacity for analytical applications is about 1 μg for a single DNA fragment. For preparative isolation and purification as much as 4–5 μg of DNA fragments can be loaded onto a 50 × 4.6 mm i.d. column with only little loss in resolution.

Digestion of the pBR322 plasmid with the restriction enzyme *Msp*I produces 26 fragments ranging in length from 9 to 622 bp (9, 9, 15, 26, 26, 34, 34, 67, 76, 90, 110, 123, 147, 147, 160, 160, 180, 190, 201, 217, 238, 242, 307, 404, 527, 622 bp). In this example, the gradient was optimized to resolve fragments ranging from 67 to 622 bp within 5 min (Figure 4). Sixteen out of 17 fragments of different chain length were separated, and only the two fragments with 238 and 242 bp coeluted.

ΦX174 DNA originates from a small *E. coli* bacteriophage and consists of 5382 bp. A digest with the restriction enzyme *Hinc*II consists of 13 fragments ranging between 79 and 1057 bp (79, 162, 210, 291, 297, 335, 341, 345, 392, 495, 612, 770, 1057 bp).

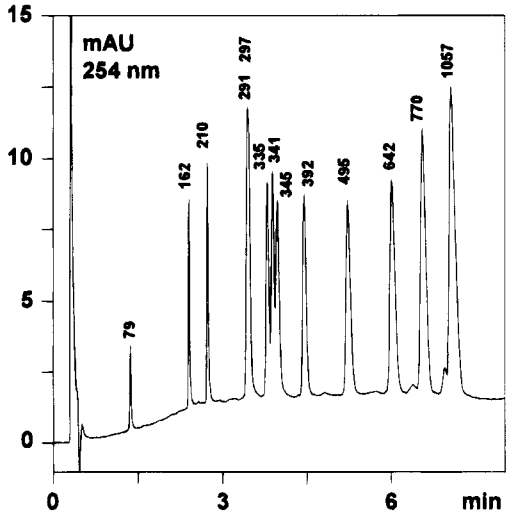

Figure 5. Separation of DNA restriction fragments from ΦX174 DNA cleaved with *Hinc*II on alkylated PS–DVB: linear gradient, 40–60% B in 2 min, 60–64% B in 3 min, 64–70% B in 3 min; flow rate, 1 mL/min; sample, 0.75 μg of ΦX174 DNA *Hinc*II digest. Other conditions as in Figure 2.

Figure 5 shows the chromatogram of ΦX174 DNA *Hinc*II restriction fragments consisting of 12 peaks with the 291 and 297 bp fragments coeluting. The Boehringer Mannheim DNA molecular weight marker VI is a mixture of two separate restriction digests of the pBR328 plasmid (4907 bp), a derivative of the pBR322 plasmid, with the restriction enzymes *Bgl*I and *Hinf*I, respectively. This sample is composed of 15 fragments, three of which are of identical length, covering a size range from 154 to 2176 bp (154, 154, 220, 234, 234, 298, 298, 394, 453, 517, 653, 1033, 1230, 1766, 2176 bp); 0.4 μg of this mixture was injected onto the column and eluted at a flow rate of 0.5 mL/min in order to achieve better resolution of large DNA fragments (Figure 6).

The high-resolution separation of 37 DNA fragments in less than 20 min is depicted in Figure 7. The sample consisted of a mixture of a pBR322 *Hae*III and a pBR322 *Msp*I digest. From the 37 fragments detected in the chromatogram, 34 fragments are of different chain length and could be resolved at least partially into 32 peaks. In can be seen clearly that, by using gradient slopes

(25) Merion, M.; Warren, W.; Stacey, C.; Dwyer, M. E. *BioTechniques* **1988**, *6*, 246–51.
(26) Paulus, A.; Hüsken , D. *Electrophoresis* **1993**, *14*, 27–35.

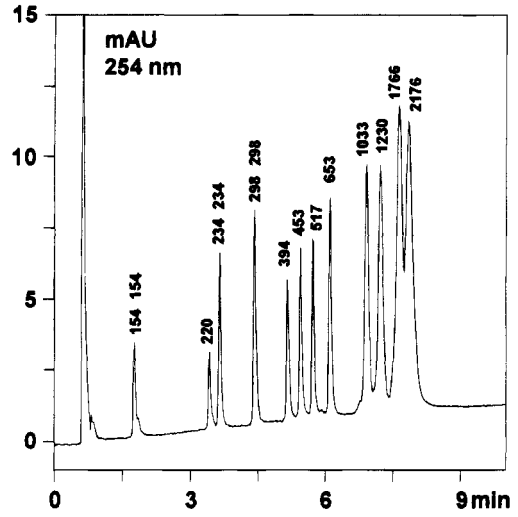

Figure 6. Separation of DNA restriction fragments from pBR328 DNA cleaved with *Bgl*I and pBR328 DNA cleaved with *Hin*fI on alkylated PS–DVB: linear gradient, 50–65% B in 5 min, 65–70% B in 5 min; flow rate, 0.5 mL/min; sample, 0.4 μg of pBR328 DNA *Bgl*I/ pBR328 DNA *Hin*fI digest. Other conditions as in Figure 2.

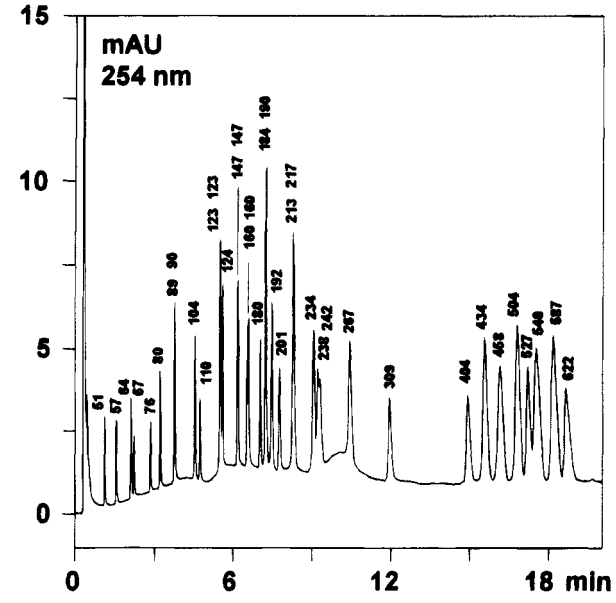

Figure 7. High-resolution chromatographic separation of DNA restriction fragments: linear gradient, 37–55% B in 6 min, 55–65% B in 14 min; flow rate, 1 mL/min; sample, 0.75 μg of pBR322 DNA *Hae*III digest and 0.4 μg of pBR322 DNA *Msp*I digest. Other conditions as in Figure 2.

between 0.75 and 0.18% acetonitrile per minute, DNA fragments can be separated if their overall length differs by 2–3%.

All previous examples demonstrate the high resolving power and the high speed of separations on alkylated PS–DVB, which is a consequence of the lack of internal pore structure of the nonporous particles and their narrow size distribution. The major advantage of micropellicular sorbents stems from the rapid mass transfer for eluite exchange between the thin layer of the stationary phase at the surface and the mobile phase in the column.[27] Moreover, fast mass transfer also enables fast regeneration and equilibration of the column after gradient runs.

Micropreparative Isolation of DNA Restriction Fragments. Figure 8a shows the micropreparative fractionation of 4 μg of a pBR322 *Hae*III digest in less than 15 min. The eluted peaks were collected and some of the fractions were rechromatographed

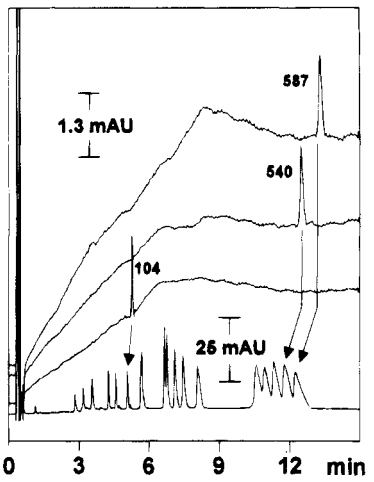

Figure 8. Micropreparative fractionation (a; from bottom to top) and rechromatography (b–d) of some of the isolated fragments: linear gradient, 30–55% B in 6 min, 55–65% B in 9 min; flow rate, 1 mL/ min; sample, 4 μg of pBR322 DNA *Hae*III digest (a) and isolated fragments 104 (b), 540 (c), and 587 (d) bp in length.

under identical conditions. As shown in the chromatograms of Figure 8b–d, no cross contamination of eluted fractions could be detected. In contrast to ion-exchange chromatography,[28] it is not necessary to desalt the eluted fractions by ethanol precipitation, size exclusion chromatography, or ultrafiltration, all of which result in low sample recovery, as the volatile compounds of the mobile phase in IP-RP-HPLC can be removed by evaporation only. Since the amounts of DNA available in molecular biological or biochemical experiments are usually much less than 1 μg, loading capacity of analytical 50 × 4.6 mm i.d. columns is enough for most applications. High separation efficiency, high speed, high sample recovery (>95% for a 404 bp DNA fragment[20]), the absence of cross contamination, and simple post-HPLC processing of isolated fractions thus render IP-RP-HPLC a highly valuable method for micropreparative isolation of DNA fragments.

Size Determination of DNA Restriction Fragments. In gel electrophoresis, the Southern method can be used to determine the size of DNA fragments by using the reciprocal relationship between fragment length and mobility.[2,29] Because the electrophoretic mobility of a DNA fragment is affected by its sequence-dependent secondary structures, local sizing accuracy will be compromized by standard fragments with anomalous mobility.[30] Anomalous migration behavior is also detected by capillary gel electrophoresis. The electropherogram of a pBR322 *Msp*I digest in a non-cross-linked polyacrylamide capillary[26] reveals for instance equal migration differences between the peaks of 217/238 bp fragments and 238/242 bp fragments, respectively, whereas the migration difference between the 180/190 bp fragments is significantly greater than the migration difference between the 190/201 bp fragments. The influence of AT content on retention of DNA fragments resulting in occasional inversions of eluted peaks as a function of molecular size prevented also the employment of anion-exchange chromatography for size-accurate fragment identification.[7,14]

(27) Kalghatgi, K.; Horváth Cs. In *Analytical Biotechnology*; Horváth, Cs., Nikelly, J. G., Eds.; ACS Symposium Series 434; American Chemical Society: Washington, DC, 1990; Chapter 10.
(28) Pietta, P.; Mauri, P.; Appierto, V.; Mostardini, M.; Biun, I. *Appl. Biochem. Biotech.* **1994**, *44*, 119–24.
(29) Elder, J. K.; Southern, E. M. *Anal. Biochem.* **1983**, *128*, 227–31.
(30) Stellwagen, N. C. *Biochemistry* **1983**, *22*, 6186–93.

So far, only few data have been published about the influence of fragment size and base composition on retention of double-stranded nucleic acids in ion-pair chromatography. Eriksson et al.[8] reported that DNA retention on PepRPC was generally a function of molecular size. However, ion-pair chromatography on PepRPC also showed inversion of some of the eluted peaks, e.g., the 281 and 271 pb fragments from the ΦX174 RF *Hae*III digest or the 517 and 506 bp fragments from the pBR322 *Hinf*I digest. Although we were not able to detect retention inversions of fragments on alkylated PS–DVB, a closer examination of the chromatogram in Figure 7 reveals some slight details of fragment retention.

Fragments 89 and 90 coelute whereas fragments 123 and 124 are nearly baseline separated. Looking at the separation of DNA fragments of similar length, e.g., 104/110, which elute relatively closely together, the separation of the 123 and 124 fragments seems to be too good. No correlation between this observed anomalous retention behavior and base composition could be detected. This finding is corroborated by the coelution of both 123 bp fragments with significantly different base compositions (33.3 and 43.9% AT, respectively) and the separation of fragments 123 and 124 with very similar base compositions (33.3 and 34.7% AT, respectively). Other examples of unequal differences in retention are the coeluting 213 and 217 bp fragments (38.5 and 40.1% AT, respectively) and the separated 234 and 238 bp fragments (41.0 and 37.4% AT, respectively). Nevertheless, in most cases, fragments of identical size coelute (123 and 147 bp in Figure 7; 154, 234, 298 bp in Figure 6) with the only exception the two 160 bp fragments in Figure 7, which are partially separated despite having the same length and almost identical base composition (38.1 and 39.4% AT, respectively).

Elution order of DNA fragments was confirmed by isolation and subsequent polyacrylamide gel electrophoresis of individual peaks.[20] The correct elution order of the 123 and 124 bp fragments was determined by treatment of the pBR322 *Hae*III digest with another restriction enzyme, *Eco*RV. There is only one restriction site for *Eco*RV in the pBR322 plasmid at position 187, which is within the 123 bp fragment of the *Hae*III digest cutting this fragment into one 13 bp and one 110 bp fragment. Figure 9 reveals that in fact the earlier eluting peak corresponds to the 123 bp fragment. The length of the newly formed fragment was calculated according to method 1 (see Experimental Section) from the average retention times of three injections using the capacity factors of 89, 104, 124, and 184 bp fragments as standards, resulting in a measured length of 110.2 bp. This example clearly demonstrates the high accuracy of length measurement by IP-RP-HPLC.

From this observations it can be concluded that although the high separation efficiency of the alkylated PS–DVB stationary phase allows the detection of minute influences of DNA sequence and ensuing structure on retention behavior, the separation is size dependent to a very high degree. Moreover, unlike in anion-exchange chromatography, no specific influence of AT content on retention behavior was detected. This is also a contrast to IP-RP-HPLC of single-stranded oligonucleotides,[19,31] where retention times of homooligonucleotides increased in the order of C < A < T. Concerning double-stranded native DNA molecules, it has been known for quite some time that tetramethyl-, triethyl-, and tetraethylammonion ions are capable of binding preferentially

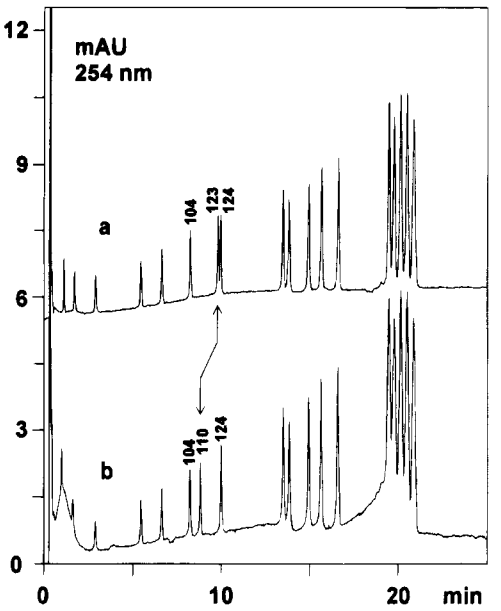

Figure 9. Identification of 123 and 124 bp fragments of the pBR322 *Hae*III digest by further digestion with *Eco*RV: mobile phase, (A) 0.1 M TEAA, pH 7.0, 8% acetonitrile, (B) 0.1 M TEAA, pH 7.0, 20% acetonitrile; linear gradient, 15–85% B in 25 min; flow rate, 1 mL/min; sample, 0.75 μg of pBR322 DNA *Hae*III digest (a) and 0.75 μg of pBR322 DNA *Hae*III digest treated with *Eco*RV (b). Other conditions as in Figure 2.

to AT base pair-rich sequences in aqueous solutions, while no such preferential binding has been observed for tetrapropyl- and tetrabutylammonium ions presumably due to a steric factor, as the latter molecules are too large to fit into the large groove of the double helix.[32–34]

However, if the binding of alkylammonium cations to the DNA polyanion in the aqueous mobile phase and the subsequent adsorption of the hydrophobic DNA–alkylammonium complex to the hydrophobic column matrix were the predominating mechanism in the separation of double-stranded DNA fragments, one would expect AT-rich sequences to be retained longer than GC-rich sequences. But since the elution behavior of DNA restriction fragments does not reveal any dependence of retention behavior on base composition, one may assume that the separation of double-stranded DNA fragments is achieved by both ion pairing and dynamic anion exchange, with the ion-pairing reagent suppressing the preferential binding of AT-rich sequences to the alkylammonium salts adsorbed on the stationary phase. Interestingly, the same mechanism was already held responsible for eliminating the base composition-dependent retention of DNA restriction fragments in anion-exchange chromatography on a nonporous organic polymeric stationary phase derivatized with diethylaminoethyl groups.[15]

Confident DNA fragment identification on the basis of position in a chromatographic elution profile depends on the precision with which that position can be reproduced. For this purpose, a set of four different commercially available DNA size standards (pBR322 *Hae*III, pBR322 *Msp*I, ΦX174 *Hinc*II, pBR328 *Bgl*I–*Hinf*I) was injected sequentially four times in order to check reproduc-

(31) Huber, C. G.; Oefner, P. J.; Bonn, G. K. *J. Chromatogr.* **1992**, *599*, 113–8.

(32) Shapiro, J. T.; Stannard, B. S.; Felsenfeld, G. *Biochemistry* **1969**, 8, 3233–41.

(33) Melchior, W. B.; von Hippel, P. H. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 298–302.

(34) Orosz, J. M.; Wetmur, J. G. *Biopolymers* **1977**, *16*, 1183–99.

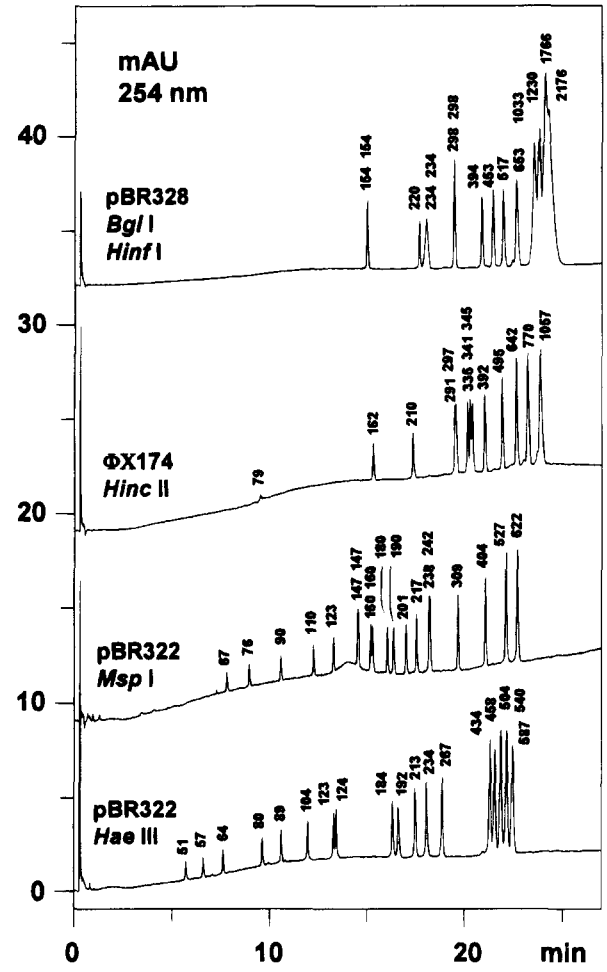

Figure 10. Comparative separation of four DNA length standards under identical gradient conditions: linear gradient, 0–100% B in 30 min; equilibration, 1 min hold at 100% B, 100–1% B in 1 min, 8 min hold at 0% B; flow rate, 1 mL/min; sample, pBR322 DNA *Hae*III digest, pBR322 *Msp*I digest, ΦX174 DNA *Hinc*II digest, pBR328 DNA *Bgl*I, and pBR328 DNA *Hinf*I digest, 0.75 μg each. Other conditions as in Figure 9.

ibility of retention. The respective chromatograms of one set of size standards eluted under linear gradient conditions are illustrated in Figure 10. Average retention times and relative standard deviations of 57 fragments for size calibration in the range between 51 and 2176 bp are given in Table 2. It can be seen clearly that retention of DNA fragments is highly reproducible with relative standard deviations between 0.15 and 0.60% and the average standard deviation being 0.31%.

Figure 11 shows the semilogarithmic plot of capacity factors obtained from the chromatograms in Figure 10 against the number of base pairs. An almost linear correlation between capacity factors and log bp is observed up to 400–500 bp. In order to check accuracy of size measurements, DNA restriction fragments of known length were treated as unknown samples and their length was calculated according to the local and global methods (see Experimental Section). Method 1, using local calibration points, gave maximal errors between −3.2 and +2.8%, the average error being ±1.1% (Table 3). Application of method 2, using a greater range of calibration points, resulted in maximal errors between −2.3 and +2.8% with an average error of ±1.1% (Table 4). These figures compare very well with the results obtained by Elder and Southern[29] for the gel electrophoretic measurement

Table 2. Average Retention Times and Relative Standard Deviations for DNA Restriction Fragments

size (bp)	t_R[a] (min)	RSD[b] (%)	size(bp)	t_R[a] (min)	RSD[b] (%)
51	5.66	0.568	291	19.48	0.153
57	6.58	0.434	297	19.54	0.161
64	7.62	0.600	298	19.55	0.378
67	7.81	0.382	307	19.68	0.258
76	8.97	0.240	335	20.12	0.220
79	9.51	0.211	341	20.26	0.249
80	9.64	0.451	345	20.36	0.271
89	10.59	0.412	392	20.99	0.376
90	10.58	0.344	394	20.96	0.379
104	11.92	0.291	404	21.07	0.293
110	12.25	0.380	434	21.26	0.167
123	13.25	0.285	453	21.53	0.374
124	13.37	0.319	458	21.51	0.162
147	14.50	0.330	495	21.87	0.441
154	15.07	0.384	504	21.81	0.173
160	15.23	0.226	517	22.06	0.389
162	15.33	0.349	527	22.11	0.268
180	16.06	0.174	540	22.10	0.199
184	16.33	0.270	587	22.40	0.223
190	16.38	0.183	612	22.63	0.426
192	16.63	0.281	622	22.67	0.230
201	17.04	0.184	653	22.74	0.404
210	17.35	0.171	770	23.24	0.424
213	17.50	0.337	1033	23.65	0.416
217	17.58	0.165	1057	23.88	0.360
220	17.75	0.378	1230	23.90	0.391
234	18.10	0.349	1766	24.18	0.342
240	18.23	0.165	2176	24.36	0.335
267	18.87	0.186			

[a] Average retention times of four runs, conditions as in Figure 10. [b] Relative standard deviation.

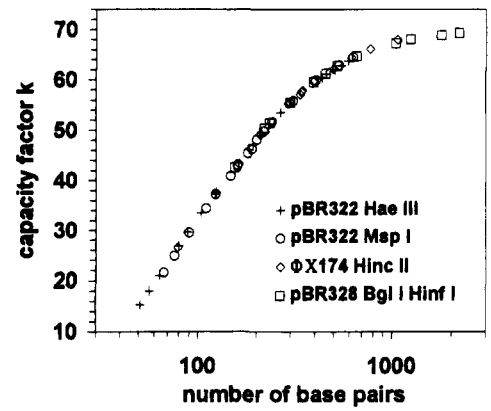

Figure 11. Semilogarithmic plot of capacity factors of DNA fragments ranging from 51 to 2176 bp in length. Chromatographic conditions as in Figure 10.

of DNA fragments in the kilobase range. They reported errors in length estimates of λ-DNA restriction fragments between −0.8 and −3.3%. Since there is no correlation between base composition and anomalous retention, these errors are due to the effect of nucleotide sequence on secondary structures of DNA fragments,[30,35] and studies employing electron microscopy have shown[36] that some of the anomalously migrating DNA fragments are macroscopically curved.

Reproducibilities of size measurement by capillary electrophoresis[4] and IP-RP-HPLC are compared in Table 5. Since the mobility curve (Figure 2 in ref 4) and the plot of capacity factors

(35) Stellwagen, A.; Stellwagen, N. C. *Biopolymers* **1990**, *30*, 309–24.
(36) Muzard, G.; Théveny, B.; Révet, B. *Embo J.* **1990**, *9*, 1289–98.

Table 3. Errors in Length Estimates of DNA Restriction Fragments Measured by Method 1

size (bp)	measd size (bp)	rel error (%)	size (bp)	measd size (bp)	rel error (%)
79	79.59	0.75	220	222.23	1.01
80	80.41	0.51	234	234.45	0.19
89	89.88	0.98	240	239.22	−0.33
90	88.82	−1.31	267	265.09	−0.72
104	105.58	1.52	291	294.57	1.23
110	108.70	−1.18	297	296.37	−0.21
123	123.29	0.23	298	297.53	−0.16
124	125.84	1.48	309	306.26	−0.89
147	143.73	−2.22	335	332.70	−0.69
154	157.39	2.20	341	340.82	−0.05
160	158.63	−0.86	345	348.77	1.09
162	161.88	−0.07	392	396.51	1.15
180	178.72	−0.71	394	390.06	−1.00
184	187.25	1.76	404	406.41	0.60
190	185.85	−2.19	434	424.73	−2.14
192	193.48	0.77	453	461.81	1.94
201	202.27	0.63	458	451.89	−1.33
210	209.07	−0.44	495	508.65	2.76
213	214.43	0.67	504	487.78	−3.22
217	215.31	−0.78			

Table 4. Errors in Length Estimates of DNA Restriction Fragments Measured by Method 2

size (bp)	measd size (bp)	rel error (%)	size (bp)	measd size (bp)	rel error (%)
79	79.66	0.84	220	223.87	1.76
80	80.97	1.21	234	235.59	0.68
89	90.36	1.53	240	240.14	0.06
90	90.34	0.38	267	267.22	0.08
104	105.77	1.70	291	297.47	2.22
110	109.75	−0.23	297	299.85	0.96
123	124.20	0.97	298	300.34	0.79
124	126.33	1.88	309	307.68	−0.43
147	145.46	−1.05	335	333.51	−0.45
154	157.45	2.24	341	343.30	0.68
160	160.21	0.13	345	352.01	2.03
162	162.08	0.05	392	402.81	2.76
180	178.27	−0.96	394	400.22	1.58
184	184.58	0.32	404	410.71	1.66
190	185.25	−2.50	434	428.11	−1.36
192	191.97	−0.01	453	457.93	1.09
201	202.56	0.78	458	454.84	−0.69
210	211.09	0.52	495	457.93	1.09
213	216.48	1.63	504	492.56	−2.27
217	218.94	0.90			

(Figure 11) have different slopes at each point, uncertainties in the electrophoretic mobilities and capacity factors correspond to an uncertainty in fragment length, which depends on fragment size. It is obvious that reproducibility of size measurement by HPLC is superior to HPCE for DNA fragments up to 300 bp due to the lager slope of the calibration plot in this range. Nevertheless, errors increase for larger fragments as the calibration curve flattens above 600 bp, but this drawback could be reduced by the application of shallower gradients for the elution of larger fragments. As far as accuracy of size measurements by HPCE is concerned, Applied Biosystems/Perkin Elmer reports 96% sizing accuracy out to 700 bp in a 30 min run.[37] This again compares very well with the results obtained by IP-RP-HPLC. Finally, method 1 was applied to the measurement of the size of PCR fragments. Four different PCR-amplified DNA fragments with

(37) *Sizing of DNA Fragments by Capillary Electrophoresis*; Applied Biosystems Application Note 127450, Foster City, CA, 1994.

Table 5. Comparison of Size Measurement by HPLC and HPCE

HPCE		HPLC	
size (bp)	SD[a] (bp)	size (bp)	SD[b] (bp)
118	6	123	1.16
194	7	192	2.00
234	7	234	5.00
271	7	267	2.94
281	6	291	2.84
310	6	309	6.22
603	16	612	33.72

[a] Standard deviations of electrophoretic mobilities converted to base pairs (data from ref 4). [b] Standard deviations of retention times converted to base pairs (method 1, data from Table 2).

Table 6. Size Measurement of PCR Products

size of PCR fragment[a] (bp)	120	167	198	257
measured size[b] (bp)	122.7	162.6	201.9	259.2
relative error (%)	2.21	−2.64	1.99	0.85

[a] According to oligonucleotide primers. [b] Method 1.

nominal sizes of 120, 167, 198, and 257 bp were analyzed under the same gradient conditions as the DNA length standards. Table 6 lists the results of three averaged analyses and shows that relative errors of measured sizes are in the same range as for DNA restriction fragments.

CONCLUSIONS

IP-RP-HPLC on alkylated nonporous PS–DVB represents the first true chromatographic alternative to slab and capillary gel electrophoresis for the separation of DNA restriction fragments and PCR products up to 1000 bp in length with high resolution and size accuracy. Small influences of fragment sequence on migration and retention behavior, respectively, are observed with all three methods and are caused by differences in the secondary structures of DNA fragments such as DNA curvature. Retention times of eluted fragments are highly reproducible, and a wide range of gradient slopes can be varied to obtain the desired resolution of DNA samples and to optimize analysis times. Moreover, fast and automated analyses as well as micropreparative isolations of DNA digests and PCR products with sample loadings in the microgram range can be performed without prior purification.

ACKNOWLEDGMENT

The authors are indebted to Dr. E. Preuss who provided the PCR products for size measurement and for his help in DNA digestion and to Mag. G.-N. Berti for his help in synthesis of alkylated particles. Part of this work was supported by a grant from the Austrian National Bank (Project 4621).

Received for review August 8, 1994. Accepted November 22, 1994.[⊗]

AC940786H

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1995.