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Effect of temperature on the separation of DNA fragments by high-performance liquid chromatography and capillary electrophoresis: a comparative study

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Abstract

This study investigates the effect of experimental temperature on the separation of DNA fragments, 21–587 bp, by both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The results show that the temperature plays an important role in the HPLC separation of DNA fragments. The optimum temperature was found to be between 40 and 50°C for HPLC, while 25°C was the optimum temperature for the CE separation. Also, although CE migration times became shorter, efficiency and resolution decreased with an increase in temperature from 25 to 50°C, but the separation was not significantly affected. Also, the optimum HPLC temperature might be different depending on the fragment sizes to be resolved.

Keywords: Temperature effects; DNA fragments

1. Introduction

Human diseases such as cancer, diabetes, cystic fibrosis, Alzheimer and others are the result of small alterations in DNA sequence. Therefore, early detection of these alterations in DNA sequence has very important implications in the diagnosis and treatment of these diseases.

Since its introduction by Tiselius [1] in 1930, electrophoresis has been utilized as an important analytical tool in molecular biology for the separation of proteins, DNA fragments, PCR products, polynucleotides, and others. Slab gel electrophoresis is the method most widely used for the separation of DNA fragments employing either polyacrylamide or agarose gels [2]. Although good separation of DNA fragments is achieved by slab gel electrophoresis, the method is tedious, time consuming, manual, and cannot be automated. However, recently some attempts were made to automate this technique [3]. Recently, two modern analytical techniques, capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), have been used for the separation of DNA fragments, polymerase chain reaction (PCR) products, and for mutant ion detection. DNA restriction fragments and PCR products were separated by ion-pair, reversed-phase HPLC on a column packed with alkylated non-

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porous poly(styrene–divinylbenzene) particles at 50°C [4]. Huber [5] reviewed the use of micropellicular phases for HPLC separation of double-stranded DNA fragments. Guttman et al. [6] used CE with polyacrylamide-filled capillaries for the separation of DNA fragments. Since then, CE has been widely and successfully used for the separation of DNA fragments and PCR products using a variety of approaches [6–23].

Issaq et al. [24] examined the effect of different parameters on migration time, resolution, and speed of CE analysis of DNA fragments and PCR products. These parameters included capillary length, applied voltage, gel type and concentration, and buffer ionic strength. The results indicated that resolution of large fragments is directly proportional to column length at the same field strength. Also, resolution of large fragments is higher (better) at lower field strength at constant column length. Analysis is fastest (high throughput) using a short capillary and moderate field strength (200 V/cm). Capillaries filled with either hydroxyethyl cellulose or non-crosslinked polyacrylamide replaceable gels gave comparable results for the separation of DNA fragments.

Huber et al. [4] determined the optimum temperature for the separation of DNA fragments by ion-pair HPLC, as mentioned above, to be 50°C. They also presented a figure comparing the separation of pBR322 DNA/Hae III digest at different temperatures (Ref. [4], Fig. 4), without an extensive discussion of the effect of temperature on the separation of different size fractions (from 21 to 587 base pairs). In this study, we discuss the effect of temperature on the separation of PBR322/Hae III digest by both HPLC and CE, which is quite different.

2. Experimental

2.1. Materials

CE: A replaceable DNA buffer was obtained from Sigma (St. Louis, MO, USA). The running buffer contains 1 μ*M* YO-PRO-1 dye (Molecular Probe, Eugene, OR, USA) for on-column fluorescent detection. An argon ion laser was purchased from Ion Laser Technology (Salt Lake City, UT, USA).

HPLC: Two different HPLC columns were used for this study: (a) DNA sep Column (Sarasep, Inc., San Jose, CA, USA), 4.6×50 mm, packed with nonporous polystyrene/divinylbenzene (PS/DVB), C_{18} alkylated, 2.2 μm particles; (b) Zorbax Eclipse dsDNA Analysis Column (Hewlett-Packard, Baltimore, MD, USA), 4.6×75 mm, packed with 3.5 μm alkylated wide-pore silica-based particles. Triethylammonium acetate (TEAA) from Perkin-Elmer (Foster City, CA, USA), EDTA from Fluka (Ronkonkoma, NY, USA) and acetonitrile from EM Science (Gibbstown, NJ, USA).

2.2. Apparatus and procedures

CE: A Beckman CZE Model P/ACE 5510 equipped with an automatic injector, a fluid-cooled cartridge, an argon ion laser, and a System Gold data station were used for this study. DNA separations were performed using a 27 cm×50 μm FC capillary (J and W, Folsom, CA, USA). The samples were injected electrokinetically, 3 s at 2 kV. The capillary was rinsed between runs with the gel buffer. Applied voltage was 7 kV.

HPLC: A Varian Prostar HPLC system equipped with Model 210 pumps, column heater, autosampler, UV detector and data station. Gradient elution at a flow-rate of 0.75 ml/min was used for the separation. Mobile phase A, 0.1 *M* TEAA+0.1 m*M* EDTA, pH 7.0; mobile phase B, 0.1 *M* TEAA+25% acetonitrile with 0.1 m*M* EDTA. The gradient was 40–80% B in 30 min [25].

3. Results and discussion

The effect of temperature on the HPLC separation of double-stranded DNA fragments was examined using two different columns. The first was packed with alkylated nonporous poly(styrene–divinylbenzene) particles similar to that used by Huber et al. [4]. The second column, an Eclipse dsDNA analysis column, was packed with a material made by chemically bonding a dense layer of an aliphatic organosilane stationary phase to a specially prepared silica support. These are two columns which are basically packed with different base materials. We were interested in comparing the effect of temperature on the fragment separation by both columns. Also, these

results (between 25 and 50°C) will be compared with those obtained by CE using replaceable gel buffer, as stated in the Experimental section. Comparison of the two HPLC chromatograms (Fig. 1a and b) shows that the HP column performs better in resolving the smaller DNA fragments at 25°C, but worse in resolving the large fragments. At the optimum separation temperature, 40–50°C, both columns resolve all the different DNA fragments equally well. It is believed that the separations are slightly different because the gradient selected was optimum for the HP column and may not be the optimum gradient for the other column. Also, the HP column is 7.5 cm long, while the other column is only 5.0 cm long.

Our HPLC results using both columns agree with those reported by Huber et al. [4] in that retention times increased with increasing column temperature from 25 to 50°C, but decreased when the column temperature was raised to 60°C, which is due to denaturing effects. The reasons for the increase in retention times of the fragments was discussed by Huber et al. [4]. The optimum temperature for the baseline separation of all the fragments using both columns, albeit using different mobile phase gradients, was found to be between 40 and 50°C. However, depending on the fragment size, i.e. the number of base pairs (bp), the optimum temperature may be different.

To simplify the discussion of the chromatogram, Fig. 1 is divided into four regions based on the

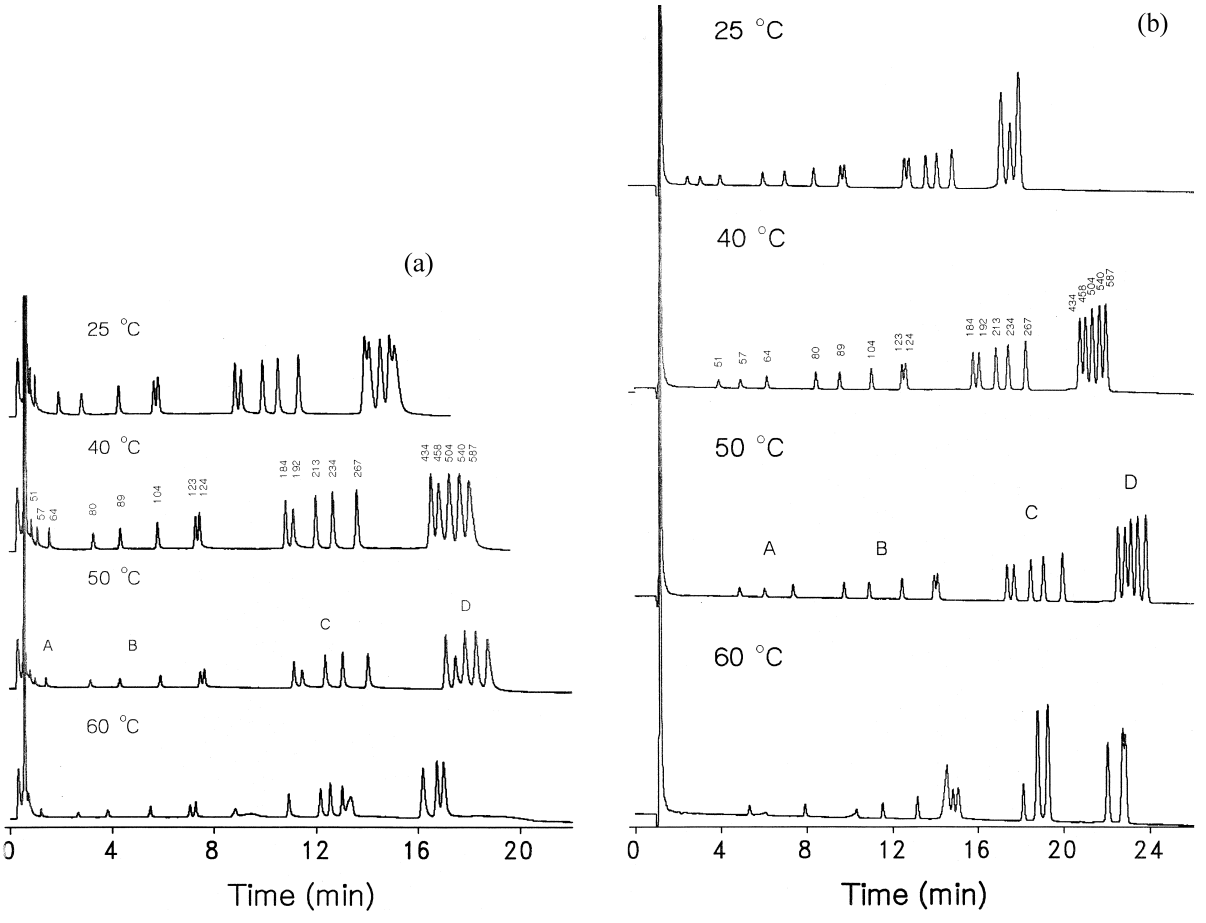

Fig. 1. Chromatograms of the separation of DNA digests at 25, 40 50 and 60°C using two different HPLC columns, a nonporous polystyrene/divinyl benzene column (a), and a silica-based wide-pore dsDNA analysis column (b). Other experimental conditions are as in text.

number of bp in each group. Group A includes fragments under 80 bp, Group B between 80 and 124 bp, Group C between 184 and 267 bp, and Group D between 434 and 587 bp. Comparison of the chromatograms (Fig. 1a and b) reveals that, at 25°C, Groups B and C are well resolved, however Groups A and D are not. Therefore, it is clear that the analysis of DNA fragments between 80 and 267 bp is possible at room temperature under the present experimental conditions. At 40°C, an overall improvement in resolution of all groups is obtained. This seems to be the optimum temperature. At 50°C, a slight improvement in the resolution of fragments above 80 bp is observed (Groups B, C, and D), however the resolution of those under 80 bp worsens. At 60°C, the resolution becomes worse. Group A merges together, the resolution of Groups B and C decreases, while Group D is split into two groups. Two of the five peaks in Group D become members of Group C. We suspect that these two fragments are the 458 and 587 bp fragments because of their relatively lower GC content (43%) than the other three fragments in Group D (≥53%), resulting in faster conformational change at high temperature. In addition, resolution of the other three fragments worsened, and they were not all baseline resolved, as can be seen from the chromatograms (Fig. 1a and b).

Although the retention times in HPLC increased with increasing temperature, up to 50°C, the CE migration times decreased with increasing temperature from 25 to 40 to 50°C (Fig. 2). This is due to the fact that the viscosity of the gel buffer decreases with increasing temperature, which contributes to faster migration times. This is clear from the relationship

$$\mu_{ep} = \frac{q}{6\pi\eta R}$$

where μ_{ep} is the electrophoretic mobility, q the net charge, R the Stokes radius and η the viscosity.

However, the decrease of migration times, i.e. increase in temperature, did not affect the separation of the DNA fragments as in the HPLC experiments (compare Fig. 2 with Fig. 1). Table 1 lists the peak efficiency and resolution of some of the DNA fragments separated by CE (Fig. 2). In general, the efficiency and resolution decreased with increasing temperature. The optimum CE separation was obtained at 25°C while, for HPLC, 40–50°C is the

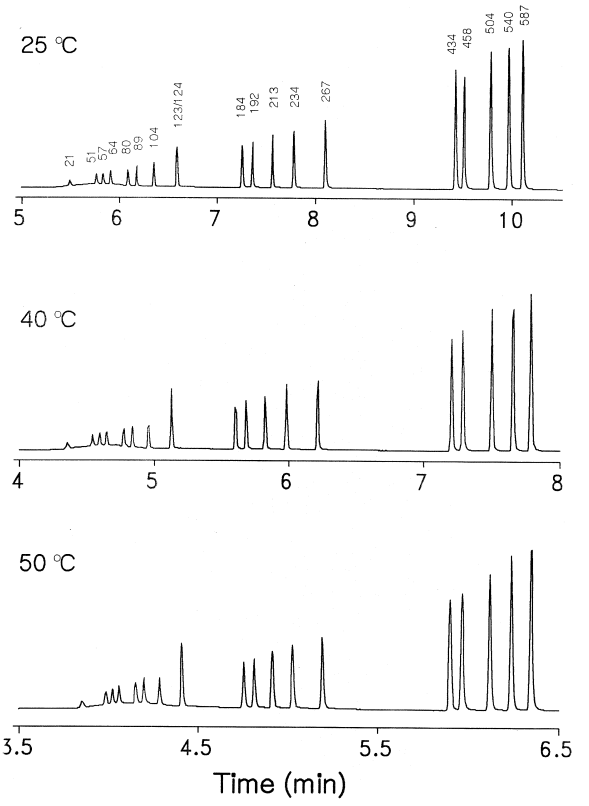

Fig. 2. Electropherograms of the CE separation of DNA digest at 25, 40 and 50°C. Other experimental conditions are as in text.

optimum separation range. Although the migration times became shorter with increasing temperature from 10.2 min for the 587 bp fragment at 25°C to 6.4 min at 50°C, baseline resolution of all the groups was possible (see Fig. 2), except for 123/124 bp. The 123/124 bp were not separated in this experiment simply because the separation conditions used were not optimized. These two peaks were separated as doublet peaks using a different type of coated capillary in our previous experiment [19]. Baseline separation of the 123/124 pairs was possible once the conditions were optimized, but this was not the main goal of this study. Also, it was observed, based on the migration time between 434/458/504 bp fragments, that the 458 bp fragment migrates faster than expected. This was because the cationic intercalating dye in the get buffer affects DNA migration which was sequence dependent [26]. Since the GC contents of the 434, 458 and 504 bp fragments are

Table 1
Peak efficiency and resolution of DNA fragments separated by CE at different temperatures

Base pairs	25°C		40°C		50°C	
	Efficiency	Resolution	Efficiency	Resolution	Efficiency	Resolution
80	919 000	10.6	650 000	5.8	430 000	3.8
89	1 260 000	3.8	967 000	3.0	420 000	1.8
104	1 070 000	7.4	1 050 000	6.2	453 000	3.5
192	1 220 000	3.9	1 220 000	3.8	488 000	2.1
213	1 370 000	7.7	1 360 000	7.0	410 000	3.5
234	1 400 000	8.5	1 350 000	7.8	423 000	3.7
458	1 620 000	2.9	1 560 000	3.4	556 000	2.2
504	1 410 000	8.8	1 340 000	8.7	602 000	4.9
540	1 840 000	5.9	1 390 000	6.0	629 000	3.8

58, 43, and 53%, respectively, less intercalating dye binds to the 458 bp fragment. Therefore, the 458 bp fragment was more negatively charged than the other two fragments, resulting in faster migration. No peaks merged together or shifted position as in the HPLC experiments. Therefore, it is concluded that the optimum CE separation temperature of the DNA fragments is room temperature. Also, increasing the column and buffer temperature to 50°C did not contribute to an appreciable worsening of the separation, as in HPLC.

4. Conclusions

Our results show that the experimental temperature plays a major role in the HPLC separation of DNA fragments between 50 and 600 bp; while fragments between 8 and 267 bp are resolved at room temperature, larger fragments are resolved at 40–50°C. At 60°C, the resolution of fragments larger than 434 bp and those smaller than 80 bp worsened. Changing the temperature from 25 to 50°C did not significantly affect the separation of CE, although the efficiency and resolution decreased with increasing temperature.

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