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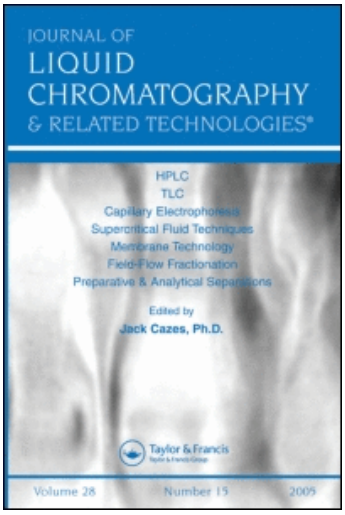

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A STUDY OF PARAMETERS THAT INFLUENCE THE HPLC AND CE SEPARATION OF DOUBLE STRANDED DNA FRAGMENTS AND DNA MUTANTS

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A STUDY OF PARAMETERS THAT INFLUENCE THE HPLC AND CE SEPARATION OF DOUBLE STRANDED DNA FRAGMENTS AND DNA MUTANTS

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ABSTRACT

The present study evaluates HPLC and CE experimental parameters, such as column packing properties, mobile phase pH, column temperature, and gel type on the separation of DNA fragments and heteroduplexes. The results of this study show that both HPLC and CE are useful techniques for the separation of DNA fragments and for the detection of DNA mutants.

Not all HPLC columns tested resolved the DNA fragments or detected the mutation. The packing material type and physical and chemical properties play a significant role. It was found that ion-pair DHPLC is easier and faster than single capillary CE for detecting DNA mutants at their melting temperature (5 min. vs 25 min.). However, CE is faster for the separation of DNA fragments (6.5 min. vs 20 min.). Most HPLC column packing materials tested resolved small DNA fragments, less than 200 bp, but not large fragments, greater than 200 bp. DNA fragments, 21-587 bp,

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are resolved by DHPLC at 40-50°C, but not at room temperature, using special columns; ultra pure, large pore, high coverage C-18-silica, nonporous C-14-silica or nonporous polystyrene/ divinylbenzene and ion-pair gradient elution.

CE can resolve the 21-587 bp fragments at room temperature, and at 30 to 50°C. It was also found that fluorocarbon coated capillary filled with polyacryl amide or hydroxycellulose liquid polymers, was more stable than DB-17, however, both capillaries gave good and reproducible CE results.

INTRODUCTION

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Cancer, diabetes, cystic fibrosis, Alzheimer, and other human diseases are the result of small alterations in DNA sequence. Therefore, early detection of theses alterations has very important implications in diagnosis and treatment of these diseases. Although, the determination of the nucleotide sequence of genomic DNA is the most direct approach for the detection of mutations, direct sequencing is not convenient for the screening of large numbers of subjects. Various techniques have been developed to detect sequence variations, particularly in polymerase chain reaction (PCR) products. The most commonly used techniques are RNase A cleavage,(1) denaturing gradient gel electrophoresis,(2) chemical mismatch cleavage,(3) single stranded conformation polymorphism,(4) and heteroduplex analysis.(5) Double-stranded mismatched DNA structures are called heteroduplexes. They are generated during polymerase chain reaction (PCR) amplification in late cycles when two homologous DNA segments, or alleles, which differ in sequence, are amplified (Figure 1). Heteroduplex analysis has been applied to the discovery of new mutations in disease genes and to the detection of new alterations.

Since its introduction by Tiselius in 1937, electrophoresis has been utilized as an important analytical tool in molecular biology for the separation of proteins, DNA fragments, PCR products, single nucleotide polymorphism (SNPs), polynucleotides, and others. Slab gel electrophoresis is the method most widely used for the separation of single and double stranded DNA fragments employing either polyacrylamide or agarose gels. Although, good separation of DNA fragments is achieved by slab gel electrophoresis, the method is tedious, time consuming, manual, and not easily automated.

High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used for the separation of DNA fragments, polymerase chain reaction products (PCR) and for mutant ion detection. Huber *et al.*(6) resolved DNA restriction fragments and PCR products by ion pair, reversed phase HPLC on a column packed with alkylated nonporous polystyrene/divinyl-

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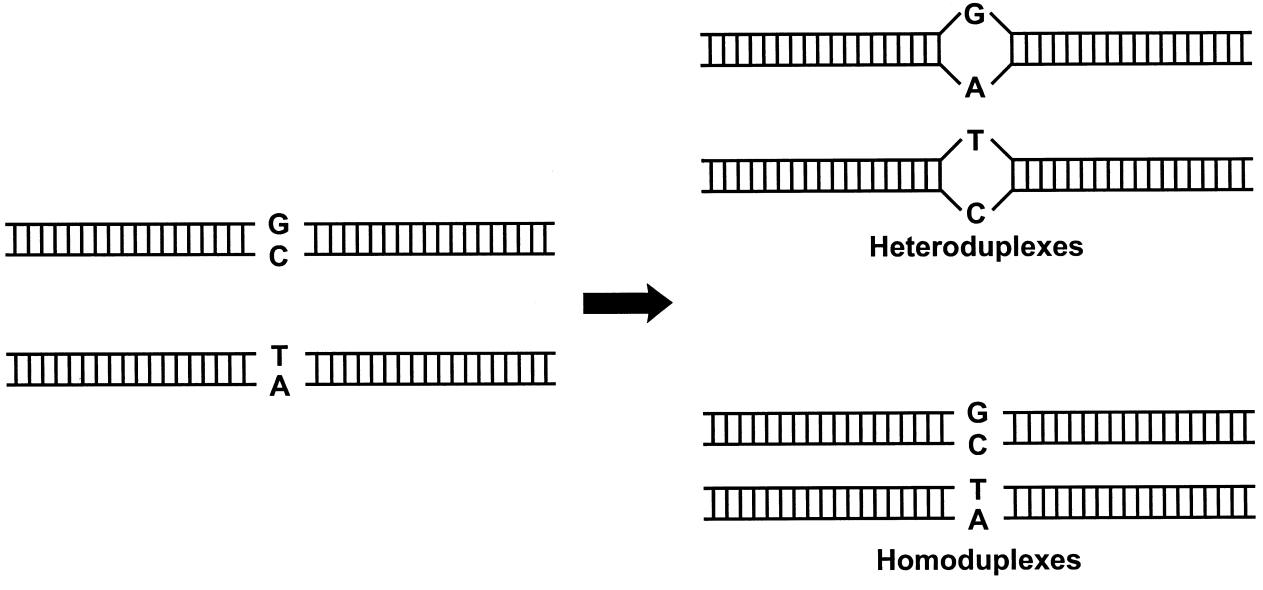

Figure 1. The principle of PCR heteroduplex analysis. Heteroduplexes are formed by the denaturation and annealing of two DNA molecules that differ in DNA sequence. Four species of molecules are generated, two heteroduplexes and two homoduplexes. Mismatched bases cause a bulge or bend in the DNA at the position of the mismatch and retard the heteroduplex molecules relative to the homoduplexes. (Reprinted with permission from reference 22.)

benzene (PS/DVB) particles, while Kirkland *et al.*(7) used a column packed with wide pore C-18 derivatized silica particles. Heteroduplex analysis has been done by denaturing HPLC (DHPLC), which is a rapid and efficient method for detecting variation in DNA heteroduplexes.(8) When the HPLC column is run at an elevated temperature, approximately 60 ± 3°C, depending on fragment size and sequence, partial denaturation of the dsDNA occurs. Formed heterozygotes denature at lower concentration of acetonitrile and are detected as a peak, or peaks, with shorter retention times than the homozygotes. So, DHPLC can be used to map human and murine genes and to validate single-nucleotide polymorphism (SNP). This procedure has been used in our laboratory for the identification of polymorphism of hundreds of PCR products of a wide range of fragment sizes.

Denaturing CE was used for the detection of p53 mutation.(9) Righetti and coworkers reported the use of denaturing gel(10) and temperature gradient(11) CE for the separation of DNA mutants. Gao and Yeung, used array CE with poly(vinylpyrrolidone) solution and gradient temperature for the detection of mutations.(12) Guttman *et al.*(13) used CE with polyacrylamide-filled capillaries for the separation of DNA fragments. The effect of column length, applied voltage, gel type (entangled polymers), and concentration on the CE separation of DNA fragments and PCR products was reported.(14) Issaq *et al.*(15) studied the effect of temperature on the separation of DNA fragments by both HPLC and CE. The results show that optimum separation of the DNA fragments was achieved at 40-50°C, while by CE no significant effect of temperature, from 20-50°C, on the separation was observed. Different CE approaches have been used for the analysis of DNA fragments, PCR products, and single-strand conformational polymorphism (SSCP) using gel filled capillaries(16) and microfluidics.(17)

The effect of temperature and pH on the CE analysis of SSCP was investigated using short-chain linear polyacrylamide as the sieving medium.(18) It was found that low temperature in general increased the number of detectable single-strand conformations (SSC) and thereby, the sensitivity of the analysis.(18) The pH effects of the separation matrix on the migration pattern, and thus, the assay sensitivity, varied markedly between the different DNA fragments. The pH range was found to be between 6.4 and 9.0.(18) Larsen *et al.*(19) reported the use of a high-throughput SSCP multiplexed CE. The temperature range used was between 14-45°C, and two mutants could not be detected at temperatures above 20°C, which confirms the findings of others that low temperature increased the number of detectable SSC,(18) and improved the separation of DNA fragments.(15) The tested fragments ranged in size from 166-1,223 bp. For information about SSCP analysis by CE the reader is referred to a review by Ren.(20)

The aim of this study is to investigate the effect of HPLC and CE experimental parameters on the separation of DNA fragments and DNA mutants.

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These include HPLC column packing material and porosity, mobile phase composition, pH, organic modifiers, and experimental temperature. Where applicable the HPLC results will be compared to published CE data.

EXPERIMENTAL

HPLC

Separations were carried out using a Pro Star HPLC instrument from Varian (Palo Alto, CA) equipped with a Varian auto sampler Model 430, a Rainin UV detector model UV-C and a Rainin column heater.

Data were collected using a computer and an XY recorder.

A series of HPLC columns packed with different materials were purchased from different manufacturers. These columns are: DNA Sep column, nonporous PS/DVB, C-18, 2.2 μm, 4.6 x 50 mm, from Sarasep, Inc. (San Jose, CA), Zorbax Eclipse dsDNA, wide pore, dense coverage aliphatic, 99.995% SiO2, 3.5 μm, 4.6 x 75 mm, and 2.1 x75 mm columns from Hewlett Packard (Agilent Technologies) (Baltimore, MD), Kovasil MS-C14, nonporous, C-14 spherical silica, 1.5 μm, 33 x 4.6 mm, from Phenominex (Torrance, CA), Jupiter, C-18 (13.25 ± 0.70), porous silica (320 ± 40 Å), 5.15 ± 0.30 μm, 4.6 x 50 mm, from Phenominex (Torrance, CA), Poros R/H, 4.6 x 100 mm, Perseptive Biosystems (Boston, MA), Symmetry, C-18, 5 μm, 4.6 x 250 mm, Waters Associates (Milford, MA), and Vydac C-18 polymeric column, 218 TP 54, 4.6 x 250 mm from Vydac (Hesperia, CA).

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HPLC Mobile Phase Composition

i) Gradient elution: Solvent A: 0.1 M TEAA + 0.1mM EDTA, pH 7.0.
Solvent B: Solvent A + 25%v/v acetonitrile.
Gradient: 40% to 80% B in 30 min.

ii) Isocratic: Different combinations (v/v percentages) of solvent A and acetonitrile.

Sample

pBR 322/Hae III digest (21 - 587 base pair) purchased from Sigma Chemicals (St. Louis, MO). PCR products for mutant detection were obtained from Dr. Mike Dean, National Cancer Institute at Frederick, Frederick, MD.

Capillary Electrophoresis

CE separations were performed using a Beckman P/ACE 5200 instrument equipped with an autosampler, liquid cooled cartridge, and laser-induced fluorescence detection. Fluorescence excitation was provided by an argon ion laser (ILT, Salt lake City, UT) operated at 5 mW. The fluorescence light was detected after passing through 488 nm cutoff and 520 nm interference filters.

Two different capillary columns were used: 27 cm x 50 μm i.d., fluorocarbon (FC) coated fused silica capillary, and 27 cm x 50 μm i.d., DB-17 fused silica capillary, both from J&W Scientific (Folsom, CA). The capillaries were pressure filled with DNA replaceable gel (Sigma Chemicals, St Louis, MO), pH 8.6 to which was added 1 μL/mL of an intercalating dye, YO-PRO-1 (Molecular Probes, Eugene, OR). Samples were injected using electrokinetic injection: 3s at 2 kV, and separation was carried out at an applied voltage of 7 kV.

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RESULTS AND DISCUSSION

Many parameters influence the separation process in both HPLC and CE. In HPLC these include, but not limited to, type of packing material (silica or polymeric), size, porous or non porous, pore size, groups attached to the base material, mobile phase composition, temperature, flow rate, etc. In CE, the buffer type, concentration and pH, organic modifiers, gel type, applied voltage, Joule heating, and column size and physical properties; coated or bare silica, and type of coating play an important role in the separation process.

Effect of Temperature

In a previous study from this laboratory(15) on the effect of temperature on the separation of DNA fragments, we reported that the optimum temperature for HPLC separations is between 40-50°C for both silica and PS/DVB packed columns, while for CE the optimum temperature was 25°C. 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. The temperature also plays an important role in the separation of DNA mutants where a few degrees change in the experimental temperature affect the separation, Figure 2; this is related to the melting temperature of the heterozygote pair, which is 63°C in this case.(6)

The heteroduplex analysis by both techniques should be carried out at the melting temperature. Figure 3 is a chromatogram of the DHPLC separation of a wild and mutant PCR product at 58°C. Note, that the separation is accomplished in less than 4 minutes. However, since DHPLC can only be used to analyze one

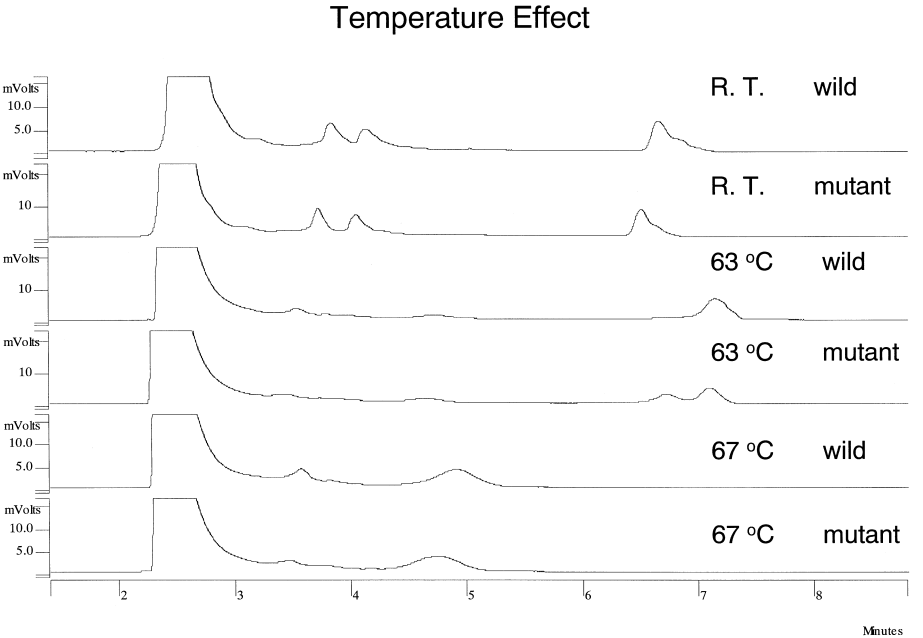

Figure 2. Effect of temperature on the DHPLC separation of mutants.

sample at a time, it cannot be classified as a high throughput technique. On the other hand, while a CE analysis takes about 20 minutes, Figure 4, it can be classified as a high throughput technique since 96 samples can be analyzed simultaneously using array CE. Gao and Yeung(12) achieved good and fast results using array CE, and temperature gradient (10°C), which covers the entire range of melting temperatures, to detect DNA mutations of 96 samples simultaneously in about 20 min., using a 75 μm i.d. fused silica capillaries (96 capillaries) filled with poly(vinylpyrrolidone) as the sieving matrix.(12)

It is worth mentioning here, that a few °C change in temperature in the melting temperature range will affect the detection of the mutation, Figure 2. We tried to use gradient temperature in the range of the melting point, but we were not successful due to the large internal diameter of the HPLC column. We believe that in order for gradient temperature to detect the mutation, a column diameter of under 1 mm should be used. These experiments are under study.

Column Packing Effects

Different column packing materials were selected in order to evaluate the effects of (a) type of packing material; and (b) porosity; and (c) to optimize the

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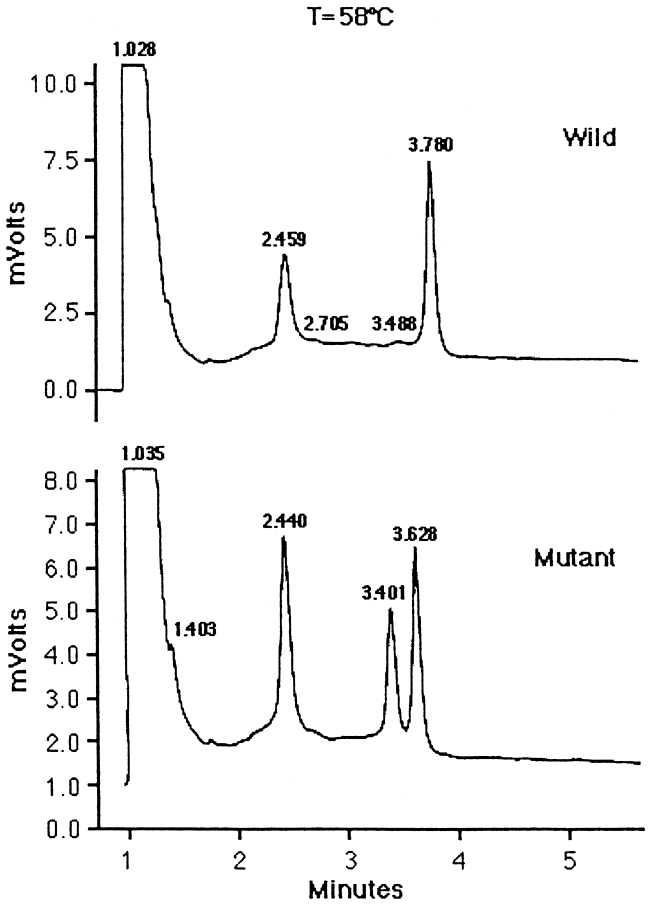

Figure 3. Chromatograms of the DHPLC detection of DNA mutation; wild type (upper trace) and mutant type (lower trace) at 58°C. Experimental conditions: 4.6 mm i.d. x 75 mm Zorbax dsDNA. HPLC column packed with 3.5 μm particle size. Gradient elution, solvent A: 0.1 M TEAA + 0.1mM EDTA, pH 7.0, solvent B: solvent A + 25%v/v acetonitrile, 40% to 80% B in 30 min, at a flow rate of 1 mL/min, at a temperature of 45°C.

separation of DNA fragments and mutants (heteroduplexes). HPLC packing materials are both silica based and polymer based to which different aliphatic organic groups are attached. Also, the base material could be porous or non-porous. In this study, we selected a series of columns packed with materials that are both silica based and polymer based derivatized with long aliphatic chains, some are porous, while others are not, as specified in the Experimental section.

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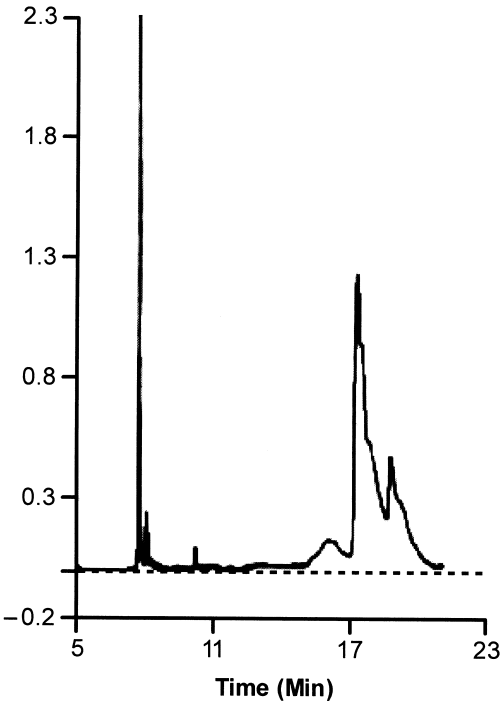

Figure 4. An electropherogram of mutation detection by CE. Experimental conditions: 27 cm x 50 μm i.d., DB-17 fused silica capillary pressure filled with DNA replaceable gel, pH 7 to which was added 1 μL/mL of an intercalating dye. YO-PRO-1 samples were injected using electrokinetic injection:3s at 2 kV, and separation was carried out at an applied voltage of 7kV at 61°C.

Two columns were polymer based, polystyrene/divinyl benzene, non porous, while the other one, Poros, was porous. Figure 5 is a series of chromatograms of the separation of DNA fragments obtained using five different HPLC columns packed with porous silica particles derivatized with C-18 and C-14 from different manufacturers.

The results show that although most of the columns resolved the small DNA fragments, 200 to 267 bp, only the HP column resolved all the fractions, small as well as large, up to 587 bp. Note that the Zorbax Eclipse column is packed with ultra high purity silica, 99.995% SiO2, with a pore size in excess of 300 nm,(21) that is densely covered with aliphatic groups, which is designed to deactivate the silica surface and to reduce or eliminate strong adsorption of basic and highly polar compounds.(20)

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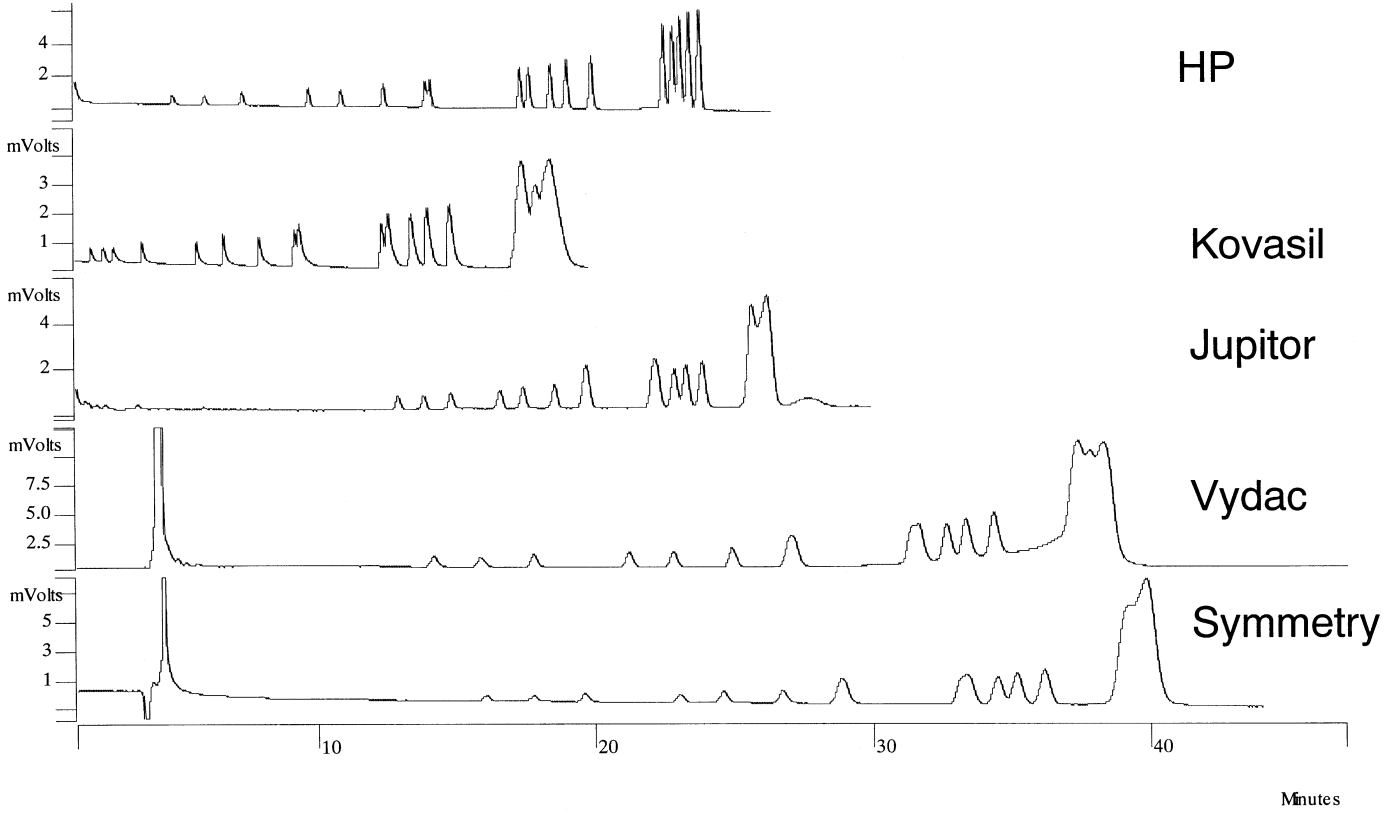

Figure 5. A chromatogram showing the effect of HPLC silica based columns from different suppliers on the separation of pBR 322/Hae III digest. Other experimental conditions as in Figure 3.

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It should be mentioned here, that no effort was made to optimize the mobile phase composition for each specific column. All columns were run under the same gradient conditions described in the Experimental section. When the PS/DVB columns were tested, the Transgenomic (Sarasep) column gave base line resolution of all the DNA fragments at the optimum temperature, 40-50°C. The Perceptive Poros column gave good resolution of the small fractions up to 267 bp, Figure 6. We also tested the effect of column i.d., 2.1 x 75 mm, and 4.6 x 75 mm on the DHPLC separation of DNA fragments and mutants. No difference in resolution was observed (results not shown).

Denaturing HPLC was used for the separation of heteroduplexes at their melting point, 58-65°C, depending on the fragment size and composition. Of all the tested columns, the Sarasep and the HP columns resolved the mismatched heteroduplex and detected the mutation, Figure 7. The Sarasep column, which is packed with PS/DVP, was more durable than the silica based HP column. The Poros column detected the mutation of some of the samples. A further examination of the results using these HPLC differently packed columns, indicate that the purity, porosity, and aliphatic coverage play a significant role in the detection of mutation. It seems that the porosity should be larger than 300 nm, but less than that of the Poros column, which is in excess of 1000 nm. Also, the results indicate, that when silica based materials with a porosity of less than 300 nm are employed, no mutation is detected. Comparison of the Kovasil column with the Eclipse column showed that although, the Kovasil column detected the mutation, Figure 8, the Eclipse column detected the mutation more consistently. The non-porous PS/DVB packed column gave excellent reproducibility and durability due to its polymeric nature, which can withstand the relatively harsh experimental conditions.

It was also found that in order to achieve mutation detection, gradient elution, as specified in the Experimental section, rather than isocratic elution should be used. We tried different combinations of solvents A and B isocratically, but were unable to detect the mutation.

Mobile Phase pH

It was found that the mobile phase pH plays a role in the separation of DNA mutants. We tested 3 different pH buffers, 6, 7, and 8. Mutation detection was achieved at pH greater than 6 but not at pH 6, Figure 9.

Capillary Electrophoresis

CE was used for mutation detection in our laboratory. We used CE with single capillary for mutation detection, but we found out that HPLC is simpler,

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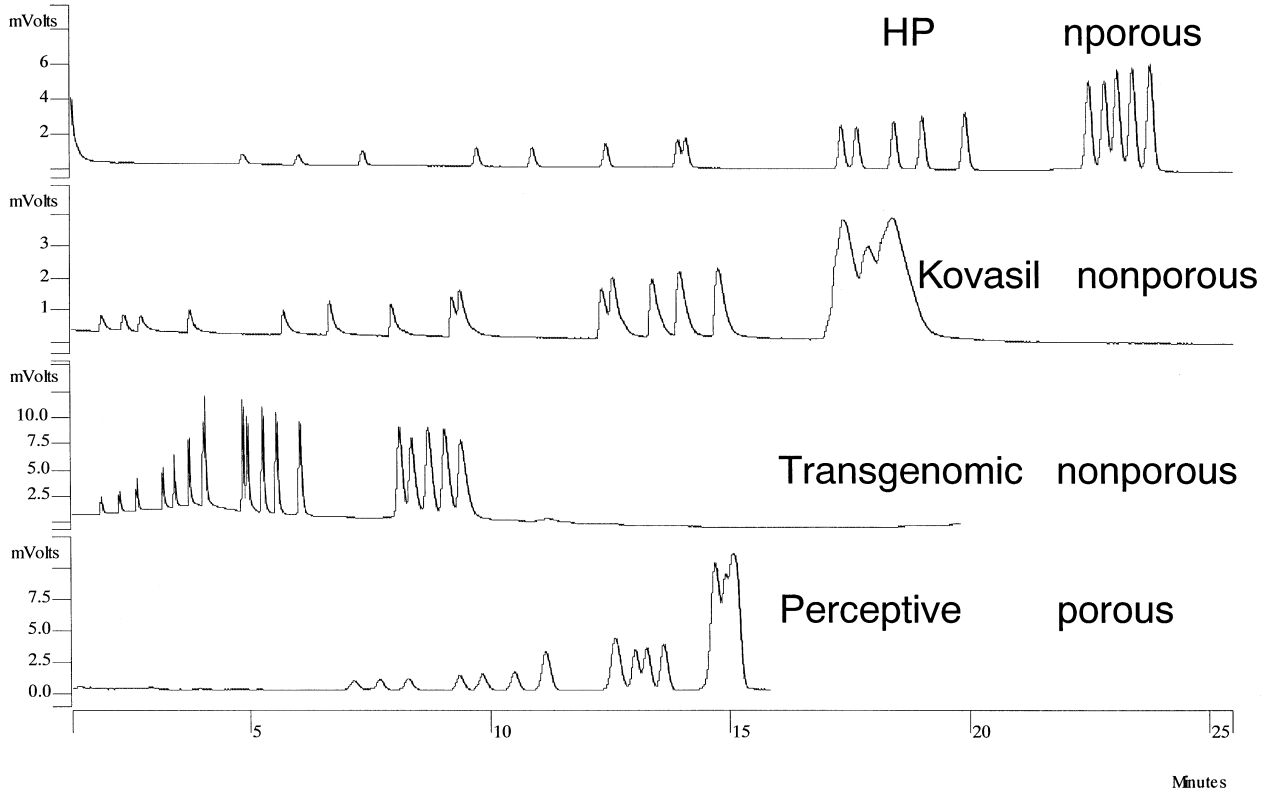

Figure 6. A chromatogram comparing porous vs non porous HPLC columns for the separation of pBR 322/Hae III digest. Other experimental conditions as in Figure 3.

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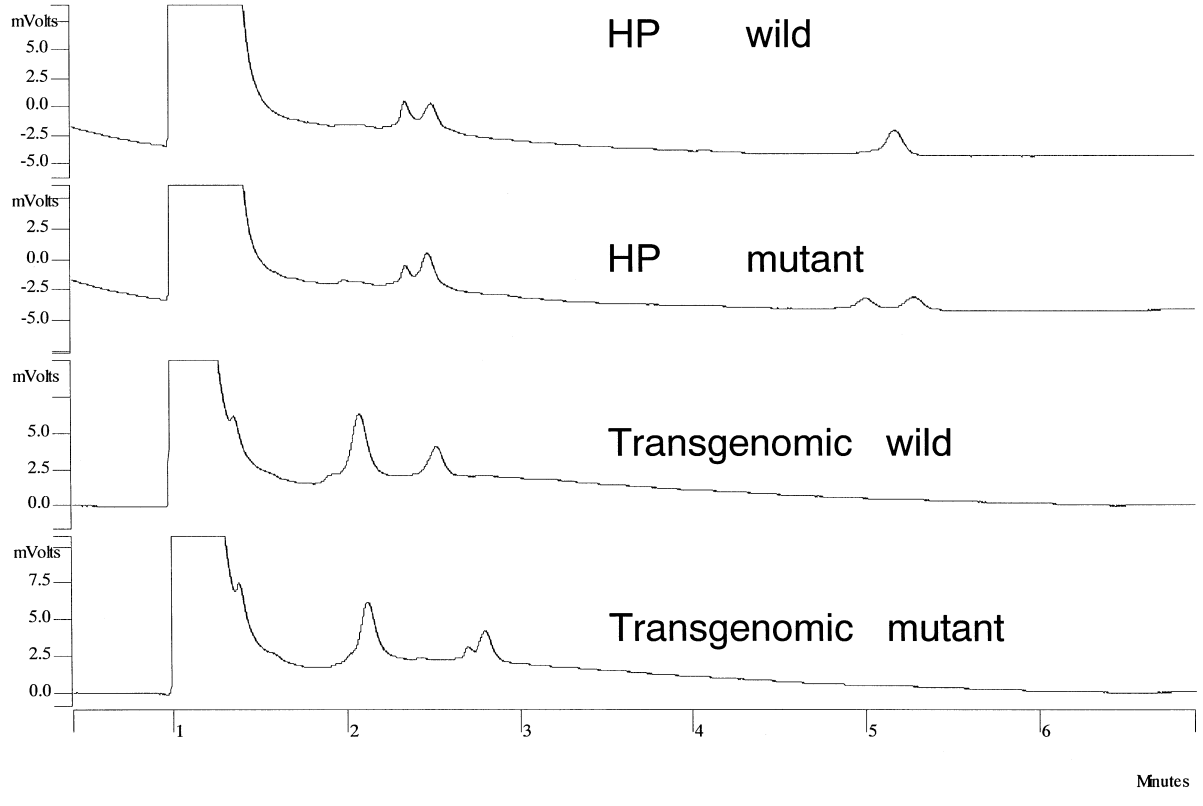

Figure 7. Comparison of the DHPLC separation of mutant and wild type DNA fragments using porous silica based column (upper two traces) and nonporous PS/DVB based column (lower two traces), at 58°C. Other experimental conditions as in Figure 3.

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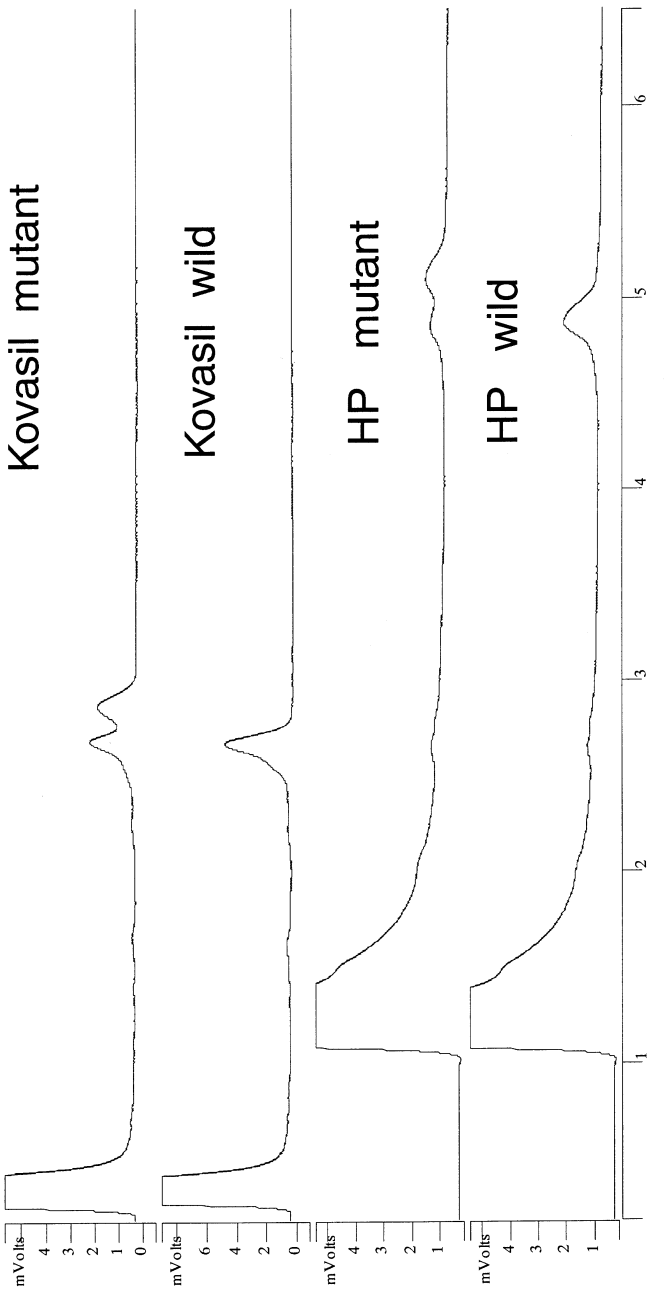

Figure 8. Comparison of HP and Kovasil columns for the DHPLC separation of heteroduplexes. Experimental conditions as in Figure 7.

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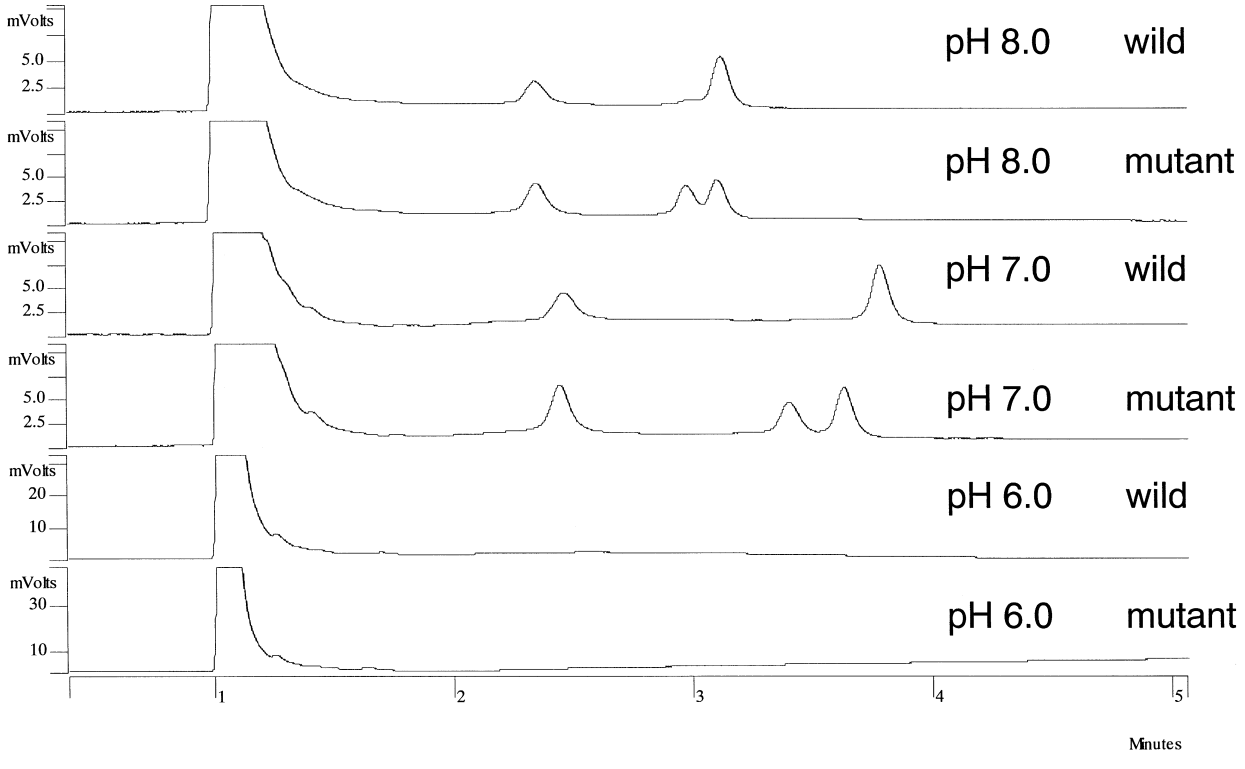

Figure 9. A chromatogram showing the effect of pH on the DHPLC separation of wild and mutant DNA fragments. Other experimental conditions as in Figure 3.

easier, and faster than CE (4 min/run versus about 20 min/run). CE also required a good control of Joule heating. Gao and Yeung(12) used array capillary electrophoresis (96 capillaries) with gradient temperature (10°C) to detect the mutation of 96 samples simultaneously in under 25 minutes. In this case, CE rather than HPLC, becomes the method of choice due to its high throughput, sensitivity, small sample size requirements, and solvent consumption.

Two commercially coated fused silica capillaries were evaluated for DNA fragment separation. Fluorocarbon coated capillary was found to be more stable than the DB-17 capillary. However, both capillaries gave good and reproducible results.

In previously published research,(14) we studied the effect of different entangled polymers, namely hydroxy cellulose and polyacryl amide, on the CE separation of DNA fragments. Both polymers gave good and reproducible results.

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CONCLUSION

The results of this study show that both HPLC and CE are useful techniques for the separation of DNA fragments and for the detection of DNA mutants. Not all HPLC columns tested resolved the DNA fragments or detected the mutation. The packing material type and physical and chemical properties play a significant role. It was found, that ion-pair DHPLC is easier and faster than single capillary CE for detecting DNA mutants at the melting temperature (5 min. vs 25 min.). However, CE is faster for the separation of DNA fragments (6.5 min. *vs* 20 min.). Most HPLC column packing materials tested resolved small DNA fragments, less than 200 bp, but not large fragments, greater than 200 bp. Packing material, silica pore size, and purity affects efficiency and resolution.

DNA mutants at their melting temperature, and DNA fragments, 21-587 bp, at 40-50°C, but not at room temperature, are resolved by DHPLC using special columns; ultra pure, large pore, high coverage C-18-silica, nonporous C-14-silica, or nonporous PS/DVB. Ion-pair DHPLC gradient elution, as described in the Experimental section, is the optimum gradient for resolving DNA mutants and fragments. Efforts to resolve DNA fragments and mutants using isocratic elution were not successful. HPLC using gradient elution temperature for the separation of DNA mutants were also not successful due to the inefficient transfer of heat to the wide internal diameter column. CE can resolve the 21-587 bp fragments at room temperature, and at 30 to 50°C. CE migration times, efficiency, and resolution decreased with increasing column temperature from 25 to 50°C. Gradient temperature was successfully used for the CE detection of mutation.(12) It was also found that fluorocarbon coated capillary was more stable

than DB-17, however, both capillaries gave good and reproducible CE results. The effect of other CE parameters, such as applied voltage, gel type, concentration, and capillary length on the separation of DNA fragments has been previously studied.

The interested reader should consult the following references for details. (14, 15).

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