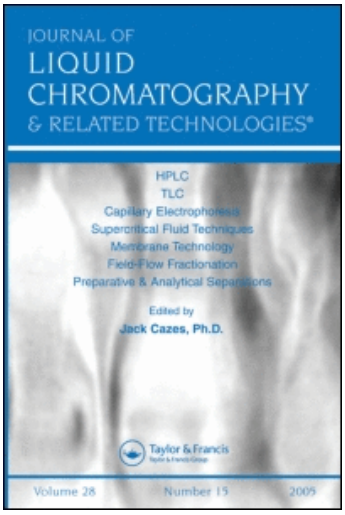

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HPLC AND CE ANALYSIS OF PCR PRODUCTS: A COMPARATIVE STUDY

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ABSTRACT

In this paper we describe the use of high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) as rapid and automated techniques for the analysis of DNA fragments generated by the polymerase chain reaction. Unlike traditional slab gel electrophoresis, these techniques allow a single-step qualitative and quantitative analysis of DNA. Furthermore, both techniques allow the detection of DNA fragments with high sensitivity in the subnanogram to microgram range. The complete automation of the instrumentation permit the unattended analysis of a great number of samples, and the direct entry of the data into a computer facilitates the processing of large numbers of PCR products. Furthermore, the HPLC technique allows the recovery with quantitative yield of pure DNA fragments suitable for other applications.

INTRODUCTION

The polymerase chain reaction (PCR) is a technique for in vitro enzymatic DNA amplification using the simultaneous oligonucleotide primer extension of complementary strands of a target sequence.[1-2] The PCR reaction has rapidly established itself as one of the most widely used techniques in molecular biology; in fact, it permits microgram amounts of target DNA to be obtained from minute quantities (just a few molecules) of nucleic acid template, such as genomic DNA, RNA, cloned DNA, and PCR products themselves, as well as material from archival specimens.[3]

With the continuing development of PCR technology, there is now a growing need for PCR product quantitation in areas such as therapeutic monitoring, disease diagnosis, and the study of the regulation of gene expression. Traditional gel electrophoresis has been routinely used for PCR analysis. However, it typically requires at least 10 ng/μL of DNA for detection and the entire procedure is time-consuming and only semiquantitative.

In the last few years, high performance liquid chromatography has been introduced for the analysis of PCR products using different chromatographic techniques.[4] Among these methods, anion-exchange chromatography, which consists of a diethylaminoethyl group (DEAE) supported by small nonporous particles of silica or polymer-based material, is the most commonly used method for the isolation and purification of oligonucleotides[5] and large molecules of (ds) DNA.[6-9] Using a column packed with 2.5 μm diethyl-aminoethyl-bonded nonporous resin particles (DEAE-NPR), Kato et al.[6] demonstrated the successful separation of fragments in a pBR322 DNA-Hae III digest and in a λ DNA-Hind III digest, suggesting that the HPLC method may be complementary to, or able to, replace traditional electrophoretic techniques.

More recently, an ion-pair reversed-phase HPLC method (IP-RP-HPLC) for separation of (ds) DNA[10] and oligonucleotides[11-12] using nonporous polystyrene-divinylbenzene (PS-DVB) particles as chromatographic support. This method is very efficient and allows a better correlation between retention time and chain length with respect to DEAE-NPR separation.[13]

PCR-generated DNA fragments can also be analyzed by capillary electrophoresis.[14-17] Like HPLC, capillary electrophoresis (CE) offers several advantages compared to the traditional separation techniques,[18] including high resolution, good reproducibility, and high sensitivity. Furthermore, CE analysis can be performed in a short time, with automatic loading of sample and on-line detection, without the need for a staining procedure.[19-26] DNA separation is performed in free solutions of polymers such as hydroxypropyl

methylcellulose (HPMC), hydroxymethylcellulose, or hydroxyethylcellulose, which generate a molecular sieving effect.[23-28] Under these experimental conditions, the separation of DNA fragments in the size range of 20-1200 bp was achieved with high resolution in less than 30 minutes, allowing the separation of fragments differing in 1 to 2 bp.[26]

This paper outlines the different characteristics of the CE and HPLC procedures for the analysis of PCR-generated fragments compared to traditional agarose-gel electrophoresis, with the aim of providing the researcher information useful in choosing the strategy appropriate for his specific needs.

MATERIALS AND METHODS

PCR Amplification

The primers used permitted the amplification of a 414 bp region of the human Met-H gene.[29] Genomic DNA was purified from blood of different subjects.[18] PCR was performed in a total volume of 100 µL containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP), and 10 pmol each of the primers Met-H A (5'-GCCAGTCATGCTTGATTACCTGG) and Met-H B (5'-GGGGG TAAAAGGGTGGGCACC).

The mixture was overlaid with mineral oil (Sigma Chemical, St.Louis, MO, USA), and after 5 minutes of initial denaturation at 94°C, 2.5 U of Taq DNA Polymerase (AmpliTaq; Perkin-Elmer, Norwalk, CT, USA) were added.

35 PCR cycles were then carried out in a thermal cycler Perkin-Elmer 2400 GenAmp PCR System with a 94°C denaturation step for 15 s, a 60°C annealing step for 15 s, and a 72°C step for 30 s. The amplification was completed by maintaining the sample at 72°C for 7 min.

DNA Separation by Agarose-Gel Electrophoresis

DNA separation was performed using a 2% agarose-gel (Bio-Rad, Hercules, CA, USA) in: 45 mM Tris-borate, 1 mM EDTA, pH 8.3, (TBE 0.5X) as electrophoresis buffer. The gel was run at 80 V. DNA bands were detected by staining with ethidium bromide and UV fluorescence.[18]

DNA Separation by HPLC

The HPLC separation of PCR-generated fragments was performed in a Gold System (Beckman Instruments, Fullerton, CA, USA) consisting of a 125 pump system, a 155 variable wave-length detector and a 507 autosampler with a 100 μL loop volume. The system was interfaced with a PC computer utilizing System Gold software for control and data collection. The column used was a Perkin-Elmer TSK DEAE-NPR anion exchange column (35 mm X 4.6 mm i.d.), 2.5 μm particles, protected by a 0.22 μm filter guard column. Separation was obtained performing a gradient between a buffer A, 25 mM, Tris-HCl pH 9.0, and a buffer B, 25 mM Tris-HCl, pH 9.0, containing LiCl 1 M, (Sigma Chemical, St.Louis, MO, USA), operating at a flow-rate of 1 mL/min. Peak detection was performed at 260 nm.

DNA Separation by CE

Capillary electrophoretic analysis was performed using an automated P/ACE 2100 System (Beckman Instruments, Fullerton, CA, USA) in reverse polarity (negative potential at the injection end of the capillary). Detection was accomplished by UV absorption at 254 nm. Post-run analysis of data was performed using the Beckman Gold software. Separations were performed on a (37 cm X 100 μm i.d.) deactivated fused silica capillary (home made silanization). The basic running buffer was: 89 mM Tris-borate, 2 mM EDTA, pH 8.3, (TBE) containing 0.5% of HPMC (Sigma Chemical, St.Louis, MO, USA) as sieving matrix and electrosmotic flow (EOF) suppressor. Ethidium bromide, 10 μM, was included in the electrolyte solution in order to increase the DNA absorption at 254 nm. Separation was performed with constant voltage (0.25 kV/cm) at 24°C. Voltage injection at negative polarity setting of the P/ACE System was performed for 99 seconds at 0.085 kV/cm. In order to increase the sensitivity, without losing efficiency and resolution, the injection time was increased by introduction at low pressure of a small plug of Tris-Acetate buffer, pH 5.2, before sample injection.

RESULTS

HPLC Analysis

Figure 1 shows the HPLC separation, using a TSK DEAE NPR column, of a mixture of DNA-fragments obtained by digestion of pUCBM21 with endonuclease Hpa II and pUCBM21 with endonucleases Dra I and Hind III

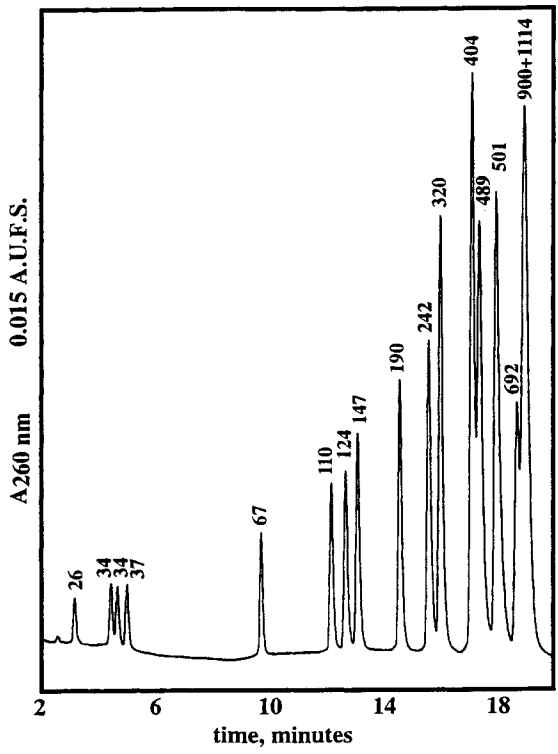

Figure 1. HPLC separation of DNA fragments Marker VIII. The separation was obtained injecting 20 µL of a 60 ng/µL solution of the DNA fragment mixture in a TSK DEAE-NPR column (35 mm x 4.6 mm i.d.) and performing a linear gradient of LiCl from 0.37 M to 0.57 M in 20 minutes. The column was then washed with 1 M LiCl for 3 minutes and returned to its initial condition. Peaks were detected recording the value of absorbance at 260 nm.

(Marker VIII, Boehringer Mannheim, Mannheim, Germany). Under these chromatographic conditions, it is possible to perform the separation of DNA in a range of 20 to 1000 bp. The use of 2.5 µm nonporous particles of silica as packing material allows a wide area of interaction without the diffusion of large molecules through the stationary phase. This characteristic permits a high resolution of molecules such as (ds) DNA with a rapid analysis time. In anion-exchange chromatography, DNA retention is dependent on electrostatic interaction between the phosphate groups of DNA and cationic sites of the chromatographic matrix. DNA is eluted from the column, increasing the ionic strength of the mobile phase by varying the salt concentration (LiCl or NaCl).

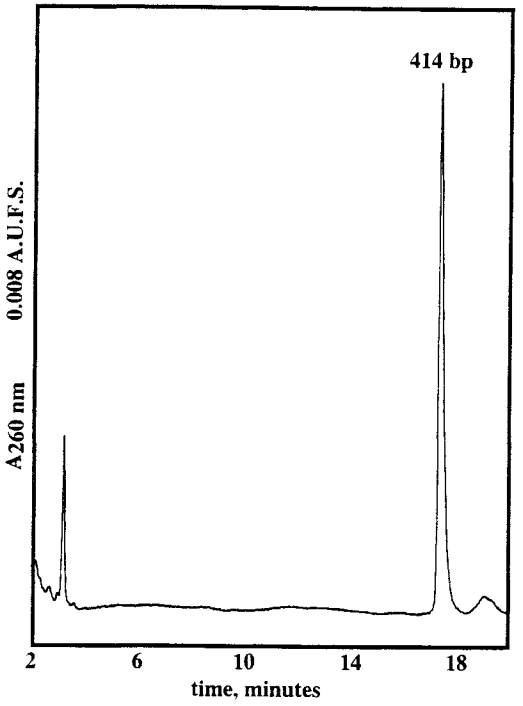

Figure 2. HPLC separation of the 414 bp PCR product. The separation was obtained injecting 20 μL of a 1:10 dilution of PCR mixture in a TSK DEAE-NPR column (35 mm x 4.6 mm i.d.) and performing a linear gradient of LiCl from 0.37 M to 0.57 M in 20 minutes. The column was then washed with 1 M LiCl for 3 minutes and returned to its initial condition. Peaks were detected recording the value of absorbance at 260 nm.

DNA elution is generally a function of the number of negative charges and should thus be directly proportional to chain length. However, this is not always the case because AT base pairs interact with the stationary phase more strongly than GC base pairs.

A recent study reported a better correlation between the retention time and DNA fragment size obtained by replacing sodium chloride with tetramethyl ammonium chloride.[30] The HPLC analysis of the PCR product reported in Figure 2 was performed under the same conditions used for the Marker VIII analysis (Fig. 1). The separation required only about 18 minutes; however, the analysis time was reduced to less than 7 minutes by performing a gradient in the short range of salt concentration required for PCR-fragment elution (Fig.3).

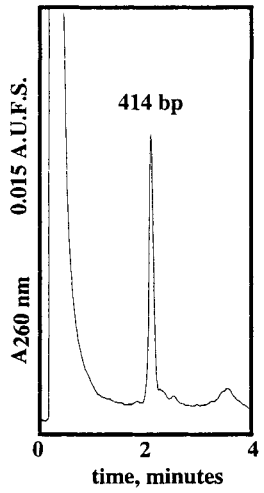

Figure 3. Rapid separation of the 414 bp PCR product by HPLC. The separation was obtained by injecting 20 µL of a 1:10 dilution of PCR mixture in a TSK DEAE-NPR column (35 mm x 4.6 mm i.d.) and performing a linear gradient of LiCl from 0.48 M to 0.56 M in 4 minutes. The column was then washed with 1 M LiCl for 3 minutes and returned to its initial condition. Peaks were detected recording the value of absorbance at 260 nm.

CE Analysis

Many studies reported in the literature have shown the successful use of CE in the separation of DNA fragments and PCR products. Figure 4 shows the electrophoretic separation of Marker VIII DNA fragments obtained with the method described in this paper.

The presence of HPMC as molecular sieve in the buffer allows the separation of DNA restriction fragments from the lowest to the highest molecular weight. Under these experimental conditions, an excellent correlation, in the 26-800 bp range, between migration time and molecular weight is achieved.

This characteristic is very helpful in molecular weight determinations of PCR-generated DNA-fragments. Figure 5 shows the separation of 414 bp PCR product obtained using this method. Furthermore, the CE separation is performed in a short time, is highly reproducible and can be completely automated.

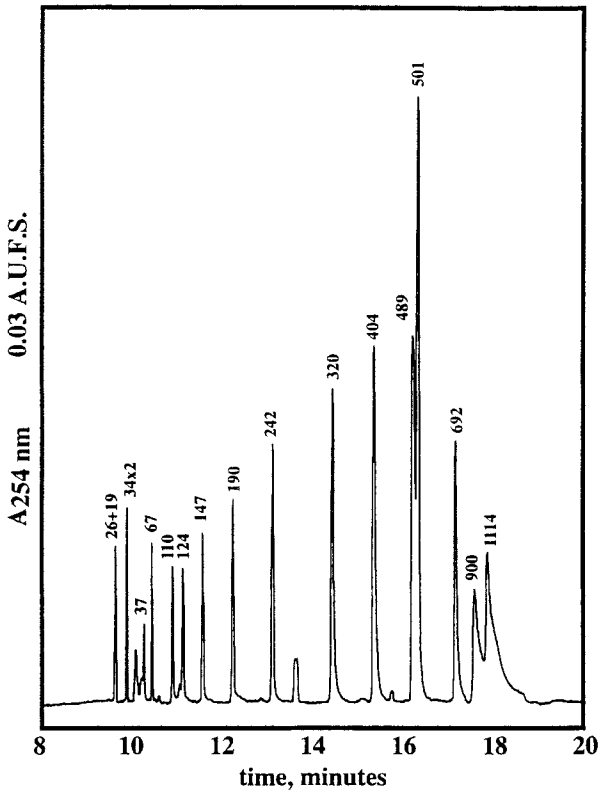

Figure 4. CE separation of DNA fragments of Marker VIII. The separation was performed in reverse polarity on a deactivated fused silica capillary (37 cm x 100 μm i.d.). The running buffer was 89 mM Tris-Borate, 2 mM EDTA, pH 8.3, (TBE) containing 0.5% of HPMC and 10 μM ethidium bromide. A 2.5 μg/mL solution of Marker VIII was voltage-injected at 0.085 kV/cm for 99 seconds. Separation was carried out at a constant voltage of 0.25 kV/cm at 24°C. Detection was accomplished by UV absorption at 254 nm.

The sensitivity of CE is about ten times higher than traditional agarose gel electrophoresis (Fig. 6) when the analysis is performed without sample purification. The removal of salts, primers, and dNTPs from PCR samples by ultrafiltration and the introduction of a stacking buffer in the capillary before injection dramatically increase the sensitivity of this method. In fact, sample purification reduces the competition of primers and dNTPs with the DNA fragments during voltage injection and the stacking buffer plug allows longer injection time without band diffusion.

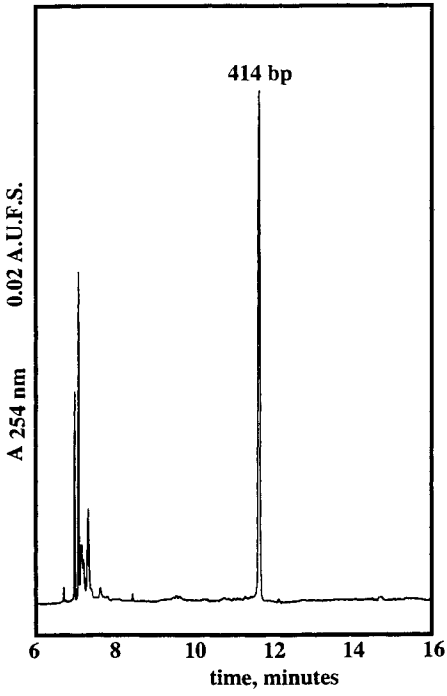

Figure 5. CE separation of the 414 bp PCR product. The separation was obtained by voltage-injecting for 99 seconds, a 1:20 dilution of PCR mixture on a deactivated fused silica capillary (37 cm x 100 μm i.d.). The running buffer was 89 mM Tris-Borate, 2 mM EDTA, pH 8.3, (TBE) containing 0.5% of HPMC and 10 μM ethidium bromide. Separation was carried out at constant voltage, in reverse polarity, of 0.25 kV/cm at 24°C. Detection was accomplished by UV absorption at 254 nm.

DISCUSSION

Unlike traditional agarose gel electrophoresis, modern techniques such as HPLC and CE can be successfully employed in automated single-step separation and quantitation of PCR products.

The reproducibility of DNA elution time with HPLC (CV=1%) and CE (CV=0.4%) surpass the known precision levels of all other DNA analysis techniques (Table 1). Using HPLC analysis, the total PCR product analysis time was 8 - 10 min per sample (Fig. 5), while the identification and

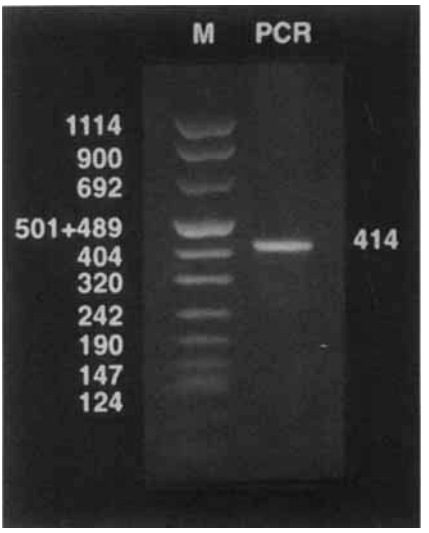

Figure 6. Agarose gel electrophoresis of: lane M, Marker VIII; lane PCR, 414 bp PCR-amplified DNA. Separation was performed using a 2% agarose gel run in 0.5X TBE buffer at constant voltage of 80 V. The gel was stained with ethidium bromide.

Table 1

Reproducibility of Retention Times of PCR Product in Capillary Electrophoresis and High Performance Liquid Chromatography

Run Number	Retention Time CE	HPLC
1	10.13	17.84
2	10.15	17.68
3	10.09	17.34
4	10.11	17.81
5	10.20	17.35
6	10.12	17.58
7	10.13	17.77
8	10.14	17.50
Mean	1013	17.61
SD	±003	±0.20
CV	0.32%	1.13%

quantitation of the unpurified PCR products was obtained within 12 min using CE. Although both of the latter separation techniques are rapid, the throughput with HPLC and CE is about the same as that of conventional agarose gel electrophoresis because they permit only one PCR sample to be run and detected at a time. However, the autosampler enables overnight, unattended analysis of a large number of samples. In addition, the interfacing of the system with a computer facilitates the processing of large amounts of data.

The analysis of amplified DNA with CE, or HPLC utilizing a DEAE-NPR column, is a rapid and easily automated method for qualitative and quantitative analysis over a wide range of PCR product concentrations and allows the detection of subnanogram to microgram amounts of DNA. The sensitivity of CE by UV- detection is 500 ng/mL but, by using a stacking buffer injection, was increased to that of laser-induced fluorescence detection (LIF), 10 ng/mL.[15,17]

In HPLC, the minimum peak detection is instead 1 ng, but this technique permits volume injections ranging from 2 μL to 500 μL, raising the sensitivity to 2 ng/mL. Furthermore, PCR samples are injected without any sample preparation prior to injection.

The DNA separation using HPLC and CE, therefore has major advantages when combined with reverse transcription (RT)-PCR, becoming a reliable and powerful tool for quantitative studies of gene expression.

Compared to CE, HPLC allows quantitative recovery of the pure DNA product from the column. The rapid, high-yield purification of PCR products using high performance anion-exchange chromatography yields sequencing results comparable to those obtained with techniques requiring subcloning of PCR products. The HPLC-purified DNA is of consistently high grade, while the other methods reported in the literature, yield sequencing templates of variable quality. The separated products are also devoid of contaminating material such as agarose, ethidium bromide, or non-specific DNA sequences. Because of the non-destructive nature of the HPLC procedure, the purified DNA is optimally suited for cloning experiments.

This comparative study demonstrates that the CE and HPLC techniques offer the important advantages of greater sensitivity, better reproducibility and higher resolution with respect to traditional gel electrophoresis in the automated separation, purification and quantitation of PCR products. The results reported provide information which should be useful to researchers in choosing the appropriate PCR analysis strategies for specific needs in PCR investigations in the biological field.

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