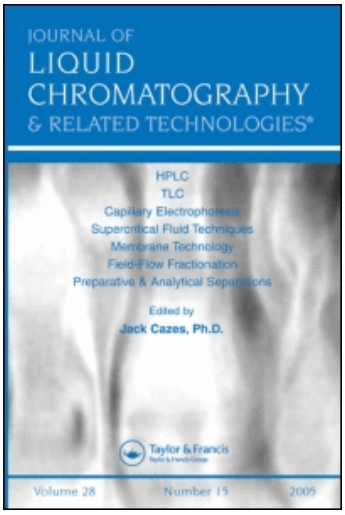

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HIGH RESOLUTION CAPILLARY ELECTROPHORESIS OF FORENSIC DNA USING A NON-GEL SIEVING BUFFER

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ABSTRACT

For the routine application of capillary electrophoresis to forensic DNA analysis, a number of requirements must be met. In the analysis of DNA amplified by the polymerase chain reaction, these requirements include high resolution and consistent and reproducible runs. Genetic markers of interest in this study consist of DNA fragments 200 or more base pairs long with repeat units as small as 2 base pairs. Normally gel-filled columns are necessary to resolve such small differences in size, however these columns suffer from a limited lifetime and a buildup of impurities over time. This paper reports an alternative method using a non-gel sieving buffer prepared from hydroxyethyl cellulose. Application of this method illustrates that a refillable, coated, open tubular column can achieve the necessary separation efficiency. Examples are given showing application of the technique to a number of genetic markers of interest in forensic DNA analysis.

INTRODUCTION

The polymerase chain reaction, PCR, allows the amplification of fragments of DNA present in trace amounts of biological material [1]. Certain specific regions of DNA are important in genetic

typing and are targeted. Such loci are known as variable number tandem repeats, (VNTRs) and when the size of the DNA fragment is relatively small, VNTRs have the potential to allow analysis of DNA that is highly degraded [2]. One particular locus, SE33, has alleles that range from 234 base pairs up to approximately 320 base pairs in size and contain repeat units of 2-4 base pairs [3]. The separation of large DNA molecules with differences in mass this small can be difficult by agarose or acrylamide slab gel electrophoresis.

Capillary electrophoresis (CE), with its high resolving power and automated sample and data manipulation, has great potential for application to typing VNTR alleles. To date, most CE separations of DNA fragments have been performed using gel-filled columns [4-7]. These columns are necessary because the mass/charge ratio of DNA is independent of size, requiring some type of sieving process in order to achieve an effective separation. Typically separations are performed using a CE capillary filled with a crosslinked polyacrylamide gel.

Unfortunately, the gel-filled columns currently being produced have a severely limited lifetime due to gel degradation caused by localized heating effects resulting from the high voltages used in the CE separations [5]. The decomposition of additives such as urea in the buffer can also restrict the shelf life. In addition to short lifetimes, a second problem is caused by the potential for gel-filled columns to irreversibly bind high molecular weight DNA resulting in a reduction of performance over time [8]. Other problems with these columns include high cost and difficulty in installation.

An alternative method for CE separation of DNA fragments has been developed using non-gel sieving media [8-12]. In this

technique an open tubular silica capillary is filled with a buffer containing a water soluble linear polymer such as methyl cellulose. DNA fragments are separated due to entanglement with the polymer network inside the capillary [10]. Unlike gel-filled capillary columns, non-gel sieving offers a clean and stable environment to be used each time a sample is run. Fresh sieving buffer is pumped into the column prior to each analysis and the old separation media is pumped out at the conclusion of the run. Thus, the same capillary column can be used for several weeks before replacement is necessary, and the time spent in the difficult procedure of replacing and aligning the thin, hairlike capillaries is minimized. Another advantage of this technique is that there are no problems with sample carryover from prior analyses. Material which has not eluted by the end of the analysis time is pumped out as the column is refilled.

Generally, this non-gel sieving systems do not have the resolving power of gel-filled columns. Recently, however, the polymer hydroxyethyl cellulose has been shown to achieve separation efficiencies nearly equivalent to those obtained with gel-filled columns [12].

The intent of this study was to investigate these soluble polymer buffers for application to the analysis of PCR amplified DNA of forensic interest, and to determine if the resolution obtained would permit the analysis of the small differences in size of DNA fragments used in genetic typing. The performance goal for the procedure was to achieve better than four base pair resolution with consistent and reproducible runs. In order to achieve this goal, methodology for appropriate sample preparation, injection, and analysis had to be developed. This methodology uses phenylmethyl columns with a tris-borate buffer containing HEC

polymer and ethidium bromide. A special column wash cycle permits consistent and reproducible runs. The resolution obtained (up to 4 million theoretical plates/meter, and less than 3 base pairs) shows great potential for use in DNA typing by PCR based techniques.

MATERIALS AND METHODS

Capillary Electrophoresis

A Spectraphoresis 1000 capillary electrophoresis instrument (Spectra-Physics) was used in the constant current mode at 37.8 uA (approximately -12 Kilovolts) and 25 degrees C. The methodology used was a modification of a procedure published by S. Nathakarnkitkool, et al [12]. The column was a 70cm, 0.1mm ID J&W DB-17 with a 0.05um phase thickness. The buffer was prepared in the following manner: 0.1 mM EDTA was added to 100 mM Trisma base and 100 mM boric acid pH adjusted to 8.7 with cesium hydroxide. Hydroxyethyl cellulose (Aldrich) was dissolved in this buffer at a concentration of 0.5% (w/v), and the solution was filtered through a 0.45um cellulose acetate disposable filter (Corning 25943). Prior to analysis the ethidium bromide was added to a concentration of 1.27 uM.

When fresh capillaries were prepared, the new columns were hand-flushed by attaching a syringe to the end of the capillary column and forcing buffer through the length of the capillary. This syringe was prepared by taking a 5cc plastic luer lock syringe and gluing a glass capillary press fit union (J&W #705-0725) to its tip. Prior to analysis each day, the capillary column was rinsed using a vacuum rinse for 20 minutes with HPLC grade methanol and

then for 20 minutes with buffer. Prior to each individual run, the columns were rinsed for 2 minutes with methanol and 6 minutes with buffer. The methanol rinses helped to keep the column bubble free by wetting the phenylmethyl coating, and also reduced the buildup of impurities on the column that result in loss of efficiency. Following the methanol and buffer wash, the samples were injected electrodynamically at 5 kV for 10 seconds. Typical run times were 30 minutes.

Sample Preparation

The pBR 322 HAE III digested DNA (Sigma) was diluted to 50 ug/ml with triply distilled water. Biological samples were prepared by extracting the DNA and amplified via the PCR [1,13]. The amplified DNA was diluted to 2ml with deionized water and desalted using dialysis via 1 pass through a Centricon 100 ultrafiltration device (Amicon). Following ultrafiltration, the sample was dialyzed further by pipeting it onto a 0.025uM membrane filter (Millipore VSWP) that was floating on a petri dish of deionized water. Float dialysis was carried out for 30 minutes after which time the sample was ready for CE analysis [14]. Salt-free samples were essential as the presence of residual salt from the PCR process degrades the performance of the electrodynamic injection onto the CE column.

RESULTS AND DISCUSSION

The focus of this work has been to improve the separation efficiency of capillary electrophoresis using refillable non-gel sieving buffers and to demonstrate its application to the analysis

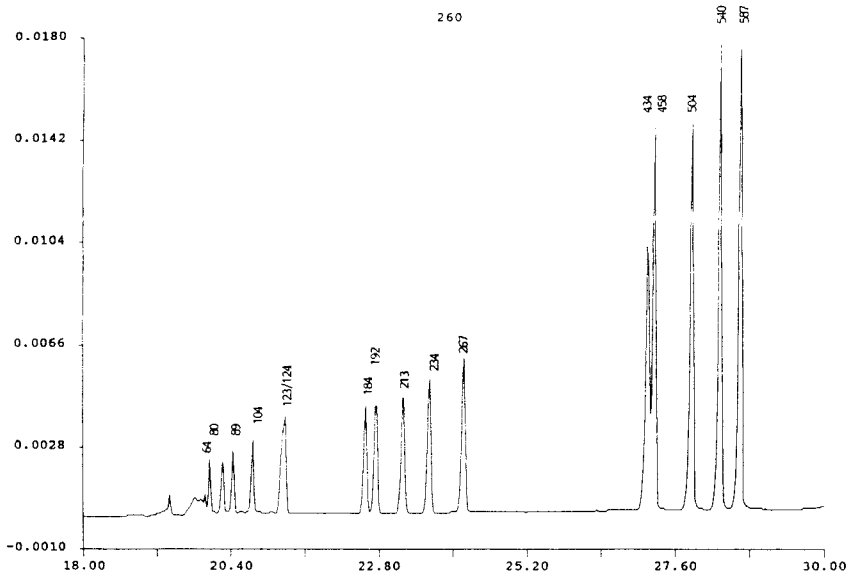

Figure 1. PCR amplified D1S80 standard mixture. Sample dialyzed using float dialysis with a Millipore 0.025 uM filter. The buffer contained 100mM tris-borate, pH adjusted to 8.7 with CsOH, 0.5% HEC and 0.635uM ethidium bromide.

of PCR-amplified loci of forensic interest. Initial development of this methodology focused on obtaining reproducible and sufficient sample injections. The injection of the CE samples is performed electrokineticly by applying a potential difference of 5 kilovolts for 5 seconds to the sample vial. The presence of chloride ions in the PCR mixture has a detrimental effect on the injection of the DNA molecules as the smaller ions preferentially inject due to their lower mass/charge ratio. Thus the lower the concentration of the small ions in the sample, the more efficient the injection of the PCR products.

Because of the importance of obtaining salt-free samples in the analysis of PCR products by CE, three different approaches were

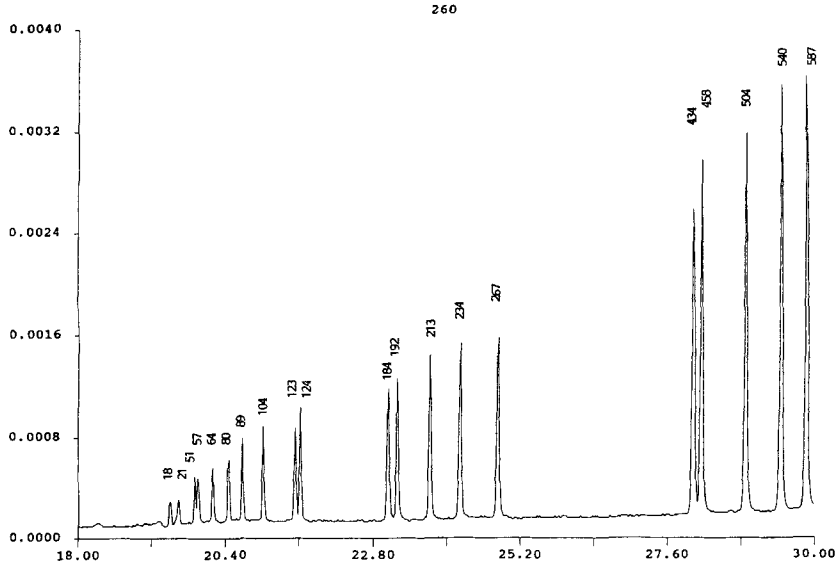

Figure 2. PCR amplified D1S80 standard mixture. Sample dialyzed using 1 pass through a Centricon 0.100 uM filter followed by float dialysis with a Millipore 0.025 uM filter. Run conditions as in Figure 1.

tested for efficacy in dialyzing samples. These are: ultrafiltration, solid phase extraction, and float dialysis. Float dialysis was the only procedure which consistently produced samples which yielded adequate signal on the CE.

Since certain PCR products also contained large quantities of excess primers and other low molecular weight material, a single wash through a Centricon 100 ultrafiltration apparatus was performed prior to float dialysis to help remove these larger molecules. Figures 1 and 2 show the effect of this two step dialysis process. In Figure 1 a PCR amplified mixture of D1S80 alleles is analyzed after being treated solely by float dialysis. Figure 2 shows the same sample analyzed using the two step dialysis process. With the ultrafiltration step, the low molecular weight

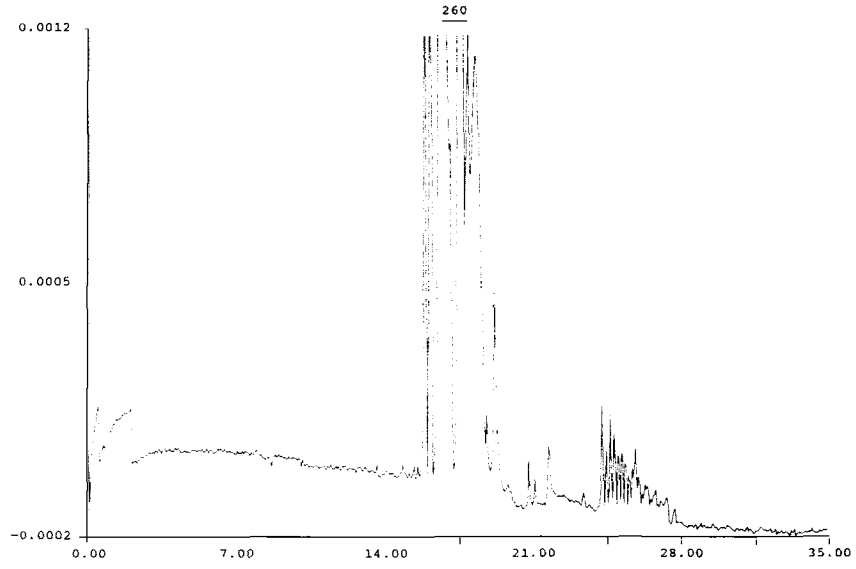

Figure 3. pBR 322 HAE III digested DNA. Experimental conditions as in Figure 1.

primer peaks are at a much lower concentration, increasing the electrokinetic injection efficiency for the higher molecular weight PCR products.

Method Optimization

A number of buffer system variables were evaluated to optimize the separation efficiency. The major variables included polymer concentration, ethidium bromide content, and the type of counter ion. Previous results had shown that these components can strongly influence resolution [10,12]. Experiments were carried out to examine the role of each of these variables in an effort to maximize separation efficiency and ensure consistent and reproducible results.

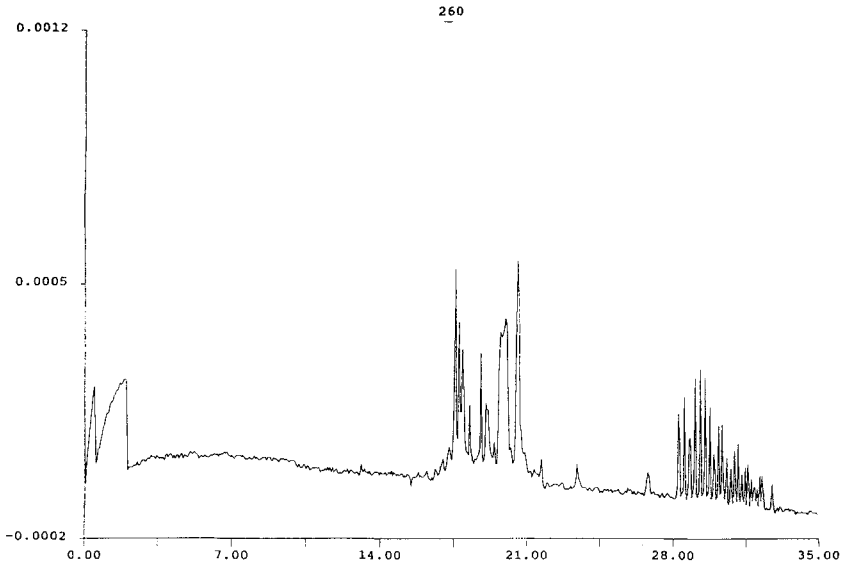

Figure 4. pBR 322 HAE III digested DNA showing improved resolution and with over 4 million theoretical plates per meter. Experimental conditions as in Figure 1 except 6.35uM ethidium bromide.

Increasing the polymer concentration from 0.5% to 0.75% had a positive effect on resolution of smaller fragments. This is presumably due to the reduction in effective pore size although there may be other interactions taking place [10]. For DNA fragments in the range of 150 to 400 base-pairs even higher polymer concentrations may be necessary, although separation of higher molecular weight fragments would suffer. The maximum polymer concentration used is limited only by viscosity, as the CE instrument may not be able to effectively flush the column with an extremely viscous buffer.

Increasing the concentration of ethidium bromide from 0.635 uM to 6.35 uM also improved resolution, particularly for smaller fragments. Figures 3 and 4 illustrate the exceptional resolution

that can be obtained upon increasing the ethidium bromide concentration from 0.635 uM to 6.35 uM with 0.5% HEC in the buffer. In these figures a HAE III digested pBR322 DNA is analyzed via CE. Note the complete resolution of the 123 and 124 base-pair peaks at the higher concentration of ethidium bromide. However, higher concentrations of ethidium bromide appeared to have a deleterious effect on column performance if left for extended periods in the column. For this reason ethidium bromide containing buffers were flushed out of any column which remained unused for an extended period of time, and the columns were stored in non ethidium bromide containing buffer. For most applications, a concentration of 1.27 uM ethidium bromide provided good resolution with a minimum of column deterioration problems.

Adjusting the pH of the tris-borate buffer with CsOH also increases resolution due to the ion-pairing of cesium ions with the DNA oligomers, slowing down their migration rate and improving separation efficiency. Addition of cesium may also help to reduce any remaining electroosmotic flow, although this flow is already quite low due to the presence of the polymer network and the coated capillaries [12,15]. There also are some interesting selectivity differences that occur when the cation is changed from sodium to cesium. In general, with the cesium ions, smaller DNA fragments were better resolved, but the effect on the larger fragments appeared to be sequence or confirmation dependent. For example in the analysis of the pPBR 322 DNA the smaller peaks were better resolved with cesium vs sodium, but the 434 bp and 458 bp fragments moved closer together.

The phenylmethyl coated silica columns typically lasted two to three weeks or up to 300 individual runs before replacement was necessary. Eventually loss of resolution occurred, presumably due

to irreversible chemisorption of residual DNA fragments and buffer components to active sites on the column, as well as degradation of the wall coated capillary column by the relatively high (8.7) pH of the buffer. Frequent flushing of the column with a water soluble organic solvent such as methanol or acetonitrile appeared to aid in reducing wall effects which resulted in increasing peak-broadening as the number of injections on column increases.

Analysis of Samples

Genetic typing using PCR-amplified DNA is carried out on loci which contain a variable number of tandemly repeated sequences (VNTRs). The length of the repeat unit can be as short as 2 base pairs. At a given genetic locus, each individual has a pair of such sequences, known as alleles. In this laboratory we are currently exploring a number of such genetic markers for application to forensic analysis. By combining all the major alleles present in a sample population into a single reference standard, the ability of the CE method to resolve any given pair of alleles can be experimentally verified. Two genetic markers, D1S80 and SE33, were selected for this study to determine whether or not CE with non-gel sieving buffers could be a viable technique for the analysis of PCR-amplified VNTRs. Mixtures of alleles for D1S80 and SE33 were examined using CE in order to gauge what is required for successful application to the technique.

D1S80 is a marker located on human chromosome 1 locus 80 [16,17]. It has a basic repeat unit of 16 base pairs. This marker should be relatively easy to analyze by CE compared to SE33 since it contains a much larger repeat unit, and thus such high resolving power is not required. A standard mixture of alleles from D1S80

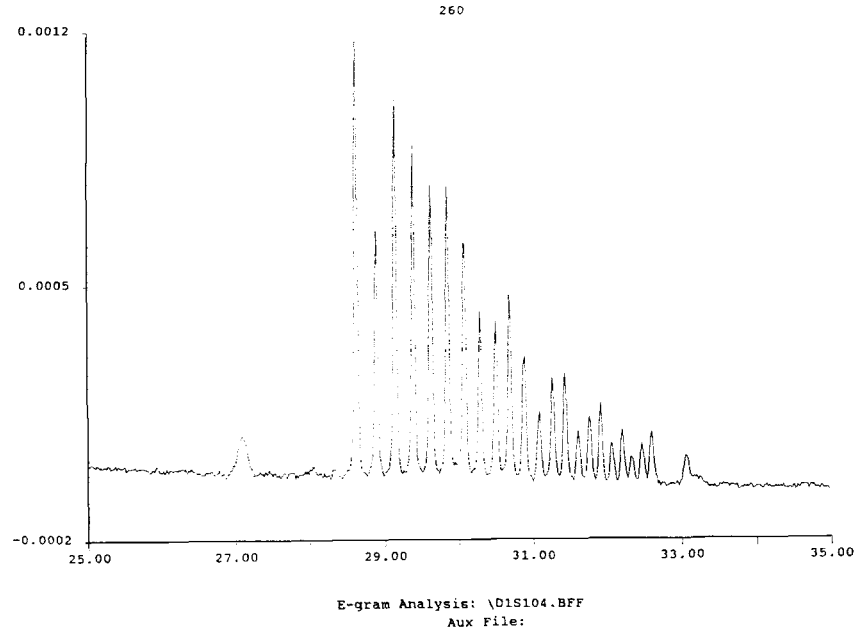

Figure 5. PCR amplified D1S80 allelic mixture. Experimental conditions as in Figure 1.

was prepared which included DNA fragments from 403 to 1069 base pairs, with each allele approximately 16 base pairs apart. Figure 5 shows a mixture of D1S80 alleles which were reamplified by the PCR and analyzed using CE with 0.635 uM ethidium bromide in the buffer and 0.5% HEC. The figure shows clean separation of all the alleles with a gradual loss in intensity as molecular weight increases. It is important in running such samples in gel media that all peaks are clearly resolved. The figure also illustrates the capability of CE to provide quantitative results which at times can be difficult to obtain with slab gel electrophoresis.

The SE33 genetic marker also is potentially useful in forensic analysis [3]. The alleles of this marker are believed to be four

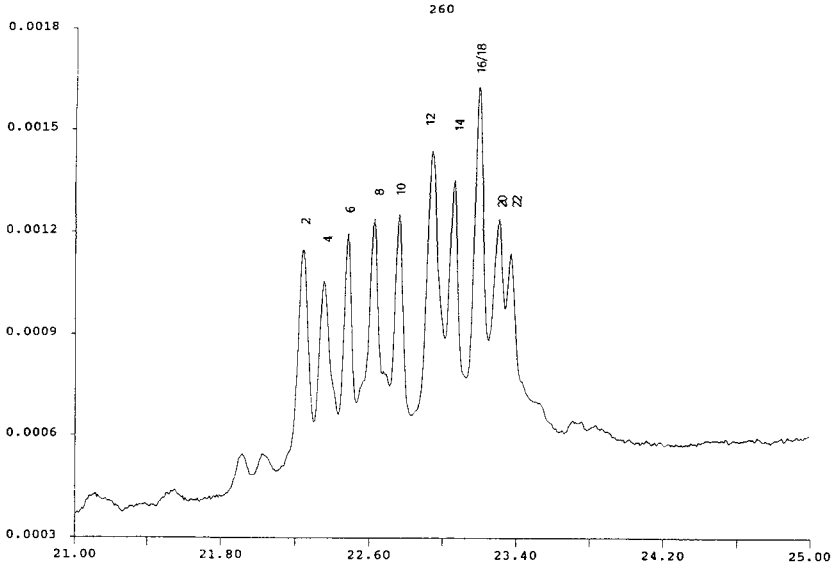

Figure 6. Mixture of SE33 alleles. Experimental conditions as in Figure 1 except 1.27uM ethidium bromide.

base pairs apart although there is also a 2 base pair repeat in the region between the primers. The smallest allele identified is 234 base pairs long, and the other alleles range in size from one to 22 repeats based on a four base-pair sequence. A standard was prepared by eluting individual alleles that were separated by slab gel electrophoresis. Then the alleles were reamplified and mixed together in proper concentrations to achieve similitude in their relative heights. For simplicity, every other allele from 2 to 22 repeat units was included in the mixture.

Figure 6 shows the electropherogram of this standard. The analysis was carried out using 1.27 uM ethidium bromide and 0.5% HEC. While most of the peaks were resolved in this analysis, there was some question about the identity of the peak labeled 16/18 and concern about the resolution between peaks 20 and 22.

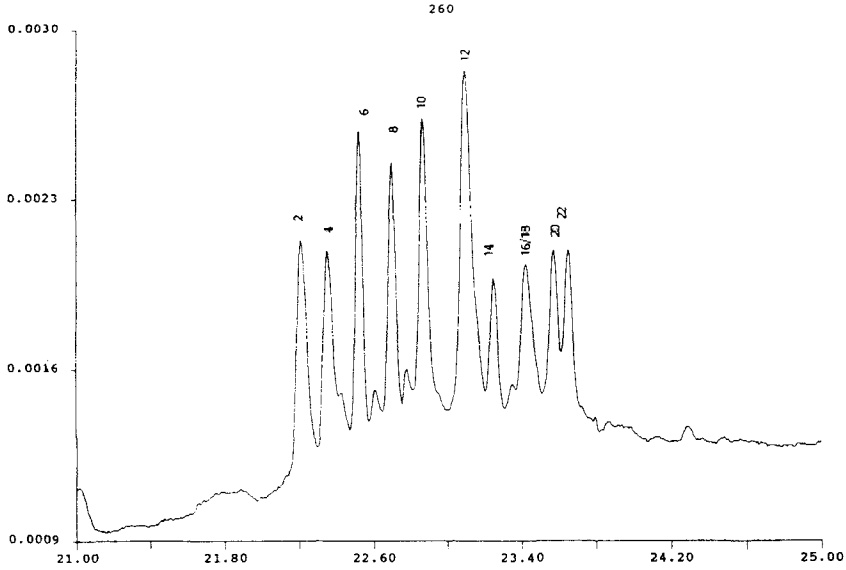

Figure 7. Mixture of SE33 alleles. Experimental conditions as in Figure 1 except 1.27uM ethidium bromide and 0.75% HEC.

To help improve resolution, polymer concentration was increased to 0.75%. Figure 7 shows the results under these conditions. Resolution of the peaks improved greatly, and small shoulder peaks appeared between many of the peaks. Examination of the individual alleles prior to mixture revealed these shoulder peaks. These small peaks are found minus and/or plus one repeat unit from the major allele when analyzed by gel electrophoresis. While indicating that amplification conditions have not yet been optimized for this particular marker, these bands are still useful in illustrating that we have now achieved four base pair resolution. There still is some question regarding the identity of peaks 16/18 and 20/22. The answer to this problem can be found by examining a plot of repeat number vs retention time. Figure 8 shows this plot.

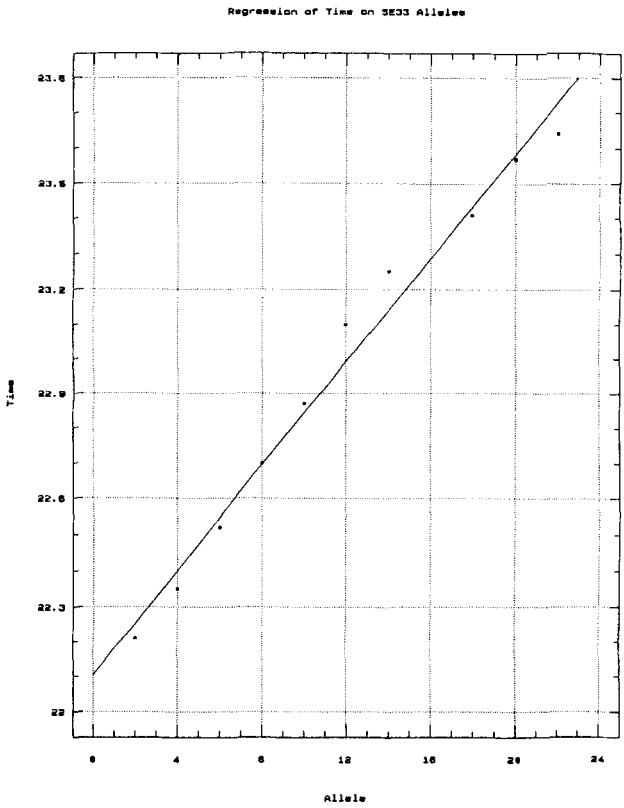

Figure 8. Plot of retention time vs predicted number of base pairs for SE33 alleles. Plot reveals a discrepancy between the analysis of alleles 12, 14, and 22 using CE.

The plot reveals a regular spacing between alleles 2-10 while alleles 10, 12, and 22 are off axis, indicating that the size of these peaks were not assigned correctly. It should be noted that the alleles originally were identified on the basis of their performance on slab gels and not on their size. The repeat numbers were assigned by determining which pair of alleles analyzed on a slab gel had a sufficient gap between them to accommodate a third allele. The presence of a two base pair repeat in this system in

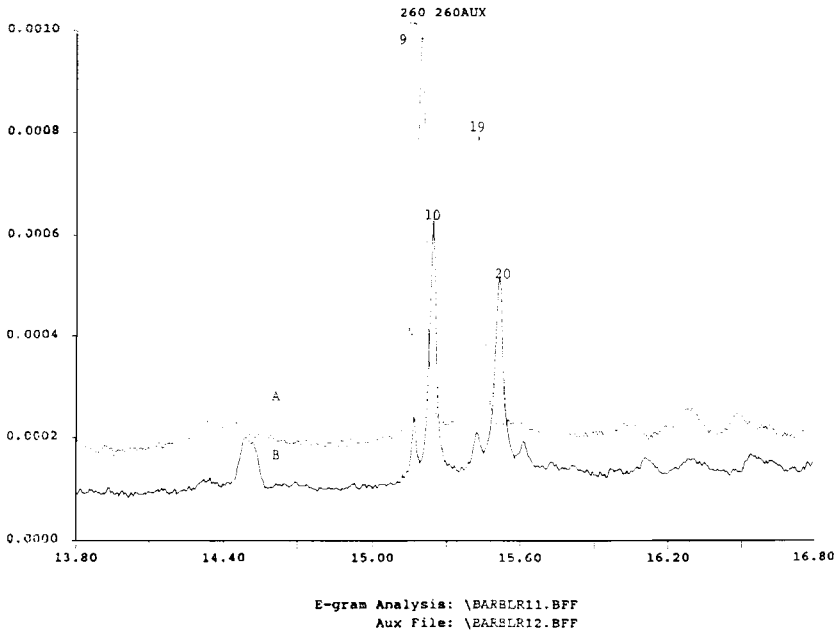

Figure 9. Two sets of SE33 alleles amplified via PCR from liquid blood. Experimental conditions as in Figure 6.

addition to the four base-pair repeat resulted in the initial assignment of these three peaks as 12, 14, and 22. For example if a two base-pair repeat fell between peaks 20 and 21, it would appear that the distance between them was larger than 4 base pairs. If, based on the CE results, these peaks are relabeled 13, 15, and 21 respectively, the data points in figure 8 all fall on the line.

The results given above clearly show the utility of CE in resolving questions of this nature. The ability to precisely determine the retention time of each fragment allows the analyst to better predict the fragment length. It should also be noted that due to the dynamic nature of the sieving buffer and the higher field strengths used, effects resulting from conformational

differences may be less prevalent than those in stable cross-linked gels.

Figure 9 shows two different samples of SE 33 alleles prepared by PCR amplification of extracted genomic material. The two peaks in plot A consist of alleles 9 and 19 while those in plot B show alleles 10 and 20. Note particularly the small shoulder peaks revealed in plot B. These peaks are 4 base pairs removed from the major peak. These results clearly show that we have achieved the necessary resolution to perform this analysis. For greater confidence in identifying these alleles however it would be useful to reference these peaks to an internal time standard. Efforts are presently underway to develop such a standard in order to allow population studies to begin on these and other markers.

CONCLUSIONS

The above results show that CE has great potential in the analysis of PCR amplified DNA of forensic interest. Its advantages over slab gel electrophoresis include speed, resolution and minimal sample preparation. The entire system is computer controlled, allowing unattended operation and minimizing transcription errors. In using sieving buffers, a fresh separation medium is prepared prior to each run, reducing the possibility of contamination or carryover from previous runs. By adding appropriate concentrations of ethidium bromide, polymer, and counter ion, the buffer can be customized to achieve the desired results. Finally, the analysis of real samples shows that CE can be used as a diagnostic tool in the analysis of PCR amplified DNA.

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