Journal of Chromatography, 559 (1991) 43–56
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 455

Factors influencing the resolution and quantitation of oligonucleotides separated by capillary electrophoresis on a gel-filled capillary

DAVID DEMOREST* and ROBERT DUBROW
Applied Biosystems, Inc., 3745 N. First Street, San Jose, CA 95134 (USA)

ABSTRACT

Instrumental and sample matrix factors were examined for their effect on the resolution, sensitivity and detectability of oligodeoxynucleotides separated on 50 μm I.D. gel-filled capillaries. Substantial errors in the calculated percentage purity may result from using peak areas not corrected for peak velocity. Area multiplied times velocity, not area, is used as the quantitative response with units of area counts cm/min. At high oligonucleotide concentrations the response varies linearly with increasing sample oligonucleotide concentration, but at low concentrations the response varies anomalously. This finding indicates the importance of using internal standards for quantitations. Increases in the sample conductivity dramatically decrease the sensitivity, whereas changes in the sample pH have only a minor effect. The mass loading increases linearly with increase in the injection voltage and duration. With injection durations of up to 20 s, the sensitivity is increased in excess of one order of magnitude with only a 15% decrease in resolution. The separation efficiency of larger sized oligonucleotides decreases more readily with increasing on-column mass than smaller species. Increases in the separation field strength and column length increase the resolution consistent with theory only when the detector rise time is reduced. An increase in the column temperature results in a decrease in the migration times by 1.1% per °C with only minor decreases in resolution.

INTRODUCTION

The use of capillary electrophoresis employing gel-filled capillaries for the separation and quantitation of oligodeoxynucleotides is emerging as a powerful tool in molecular biology and biotechnology. This technique, using capillaries of 50–100 μm I.D. and field strengths of 100–450 V/cm, has been used for the single-base resolution of synthetic oligonuclotides of 12–160 bases [1–5] and for the separation of DNA restriction fragments [6], polymerase chain reaction (PCR) amplified products [6] and DNA sequencing products [7–10]. The high resolution achieved on gel-filled capillaries is similar to those of conventional slab-gel separations, but the capillary separations have the additional advantages of short analysis times, low volume–low mass sample requirements and the real-time detection and post-run quantitative data manipulation of traditional chromatographic methods.

As the making of reliable, trouble-free, gel-filled capillaries has been reported to be problematic [4,7], the current availability of consistently performing gel capillaries

0021-9673/91/$03.50

will help dramatically in the acceptance of this technique. With this growth comes the need to understand better the instrumental and chemical factors that influence the performance of gel-filled capillaries for the separation of oligonucleotides. The purpose of this paper is to present, in a quantitative and comprehensive approach, those factors which influence the resolution, detectability and sensitivity of selected synthetic oligonucleotides. With this information, users of gel-filled capillaries should be able to optimize better their separations to suit their individual needs.

EXPERIMENTAL

Materials

Micro-Gel™ 100 capillaries of 50 μm I.D., total length 51 cm and separation length 30 cm, filled with a proprietary gel matrix developed and manufactured by Applied Biosystems (Foster City, CA, USA) were used. The running electrophoresis buffer was 75 m*M* Tris–phosphate (pH 7.6)–methanol (90:10) from Applied Biosystems. Other buffers were made with high-purity Tris (Sigma, St. Louis, MO, USA), phosphoric acid (analytical-reagent grade, J.T. Baker, Phillipsburg, NJ, USA), methanol [high-performance liquid chromatographic (HPLC) grade, Burdick & Jackson, Muskegon, MI, USA], urea (electrophoresis grade, Bio-Rad Labs., Richmond CA, USA), formamide (analytical-reagent grade, Sigma) and ethylene glycol (analytical-reagent grade, Pierce, Rockford, IL, USA). The oligonucleotides $p(dA)_{12,17,18}$ and $d(AGTC)_{3,6,9}$ were synthesized on a Model 380B DNA synthesizer (Applied Biosystems) using standard 5-hydroxyl-protected cyanoethyl phosphoramidite chemistry. The oligonucleotides were purified by HPLC followed by ethanol precipitation. Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and to reconstitute oligonucleotide samples, except were indicated.

Methods and apparatus

Separations were performed on a Model 270A capillary electrophoresis system (Applied Biosystems) under the conditions specified later for total capillary length (*L*), effective separation length (*l*), I.D. (*d*), field strength (*E*), current (*i*), temperature (*T*), electrokinetic injection duration (*t*), voltage (*V*) and oligonucleotide concentration (*C*). The gel concentration (about 8.5% polymer) and running buffer were held constant in order to explore fully the effects of changing the other parameters. On-gel UV detection was applied at 260 nm, range 0–10 ma.u.f.s., with a rise time of 0.1–1.0 s as specified later. The detector signal output was processed through either an HP 3396 integrator (Hewlett-Packard, Avondale, PA, USA) or an SP Chromjet integrator (Spectra-Physics, San Jose, CA, USA) or recorded on a strip-chart recorder (Applied Biosystems). Conductivity measurements were made on a Model 1481-60 conductivity meter (Cole-Parmer Instruments, Chicago, IL, USA) using a micro-conductivity electrode (Microelectrodes, Londonderry, NH, USA). pH measurements were made on a Model 701A digital Ionanalyzer (Orion Research, Cambridge, MA, USA) using micro-pH and reference electrodes (Microelectrodes).

RESULTS AND DISCUSSION

Relationship between peak area and peak velocity

In chromatographic separations using post column detection, the separated analytes pass through a detector flow cell at the same velocity. Analytes separated by electrophoretic methods, using on-column detection, move at different velocities through the detection cell during run-time detection. A given analyte mass moving through a detector cell at a slower rate will be integrated for a longer period of time and, thus, produce a larger area count on a standard laboratory integrator. It would be expected that for the same mass moving at a different rate that area times velocity would be constant according to the following equation:

$$m = kAv \tag{1}$$

where m is the mass moving through the detector cell, A is the area counts, v is the mass velocity and k is a constant dependent on the detector optics and integration sensitivity. According to eqn. 1, the on-gel detection of oligonucleotides during separation by capillary electrophoresis would require, for quantitative accuracy, that area times velocity be used as the quantitative response factor with units of area counts cm/min. For practical purposes, with separations being performed on the same system, we can set $k = 1$ and

$$v = l / t \tag{2}$$

where l is the effective separation length (cm) and t is the peak migration time (min). Combining eqns. 1 and 2, we obtain the following relationship between mass and area:

$$m \propto Al / t \tag{3}$$

Eqn. 3 is valid only if the separating analytes have a uniform velocity during transport through the gel capillary and do not speed up or slow down before they enter the detector cell. The validity of eqn. 3 was demonstrated by the results shown in Fig. 1.

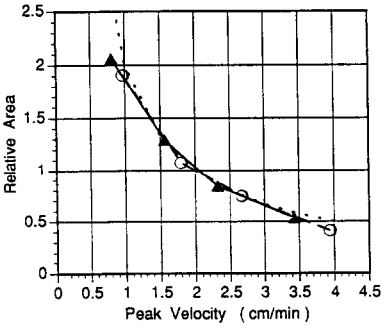

Fig. 1. Effect of oligonucleotide velocity on the area, calculated relative to the area at a velocity of 2.0 cm/min. The data are averages of four separate experiments using gel-filled capillary conditions of L = 39, 43, 44, and 47 cm, l = 20, 24, 25 and 28 cm, T = 50°C, E = 100–400 V/cm, i = 6–24 μA, t = 3 s, V = −5kV and C = 70 ng/ml. ▲———▲ = $p(dA)_{18}$; ○----○ = $p(dA)_{12}$; ····· = theoretical, based on eqn. 3 using a relative area of 1.0 at 2.0 cm/min. See text for definition of abbreviations.

Holding the injection duration and voltage constant, the same concentrations of polydeoxyadenylic acids [$p(dA)_{12}$ and $p(dA)_{18}$] were separated at different field strengths and different separation lengths. The different velocities were achieved by changing the field strengths and the separation lengths were varied to test for on-column time-dependent changes in mass. The average of all data shows a close fit to that expected from eqn. 3 when normalizing to an area of 1.0 at 2 cm/min.

If area instead of area times velocity units are used to calculate the percentage purity of oligonucleotides, errors in the actual mass% may result if there is a significant amount of a major failure sequence or large numbers of minor failure sequences. To put this fact into perspective, consider that equal area counts are obtained for an n-mer and a $(n-1)$-mer oligonucleotide. The calculated purity of the n-mer would be 50%. The migration time of the $n-1$-mer is about 0.95 times that of the n-mer, so that its relative area times velocity would be 1.05 compared with 1.0 for the n-mer. Using area times velocity units to calculate purity would result in 48.8%, a 1.2% difference in the mass purity calculation. In another example, if the n-mer had an area purity of 90% compared with the $(n-1)$-mer, then the calculated area times velocity purity would be 89.6%, only a 0.4% difference in mass purity. Obviously, as the migration time difference between the n-mer and its n-minus (or -plus) failure sequences increase, the greater will be the difference in purity calculations between area and area times velocity units.

The quantitative response units used in this paper are calculated from eqn. 3 and are labelled as area–velocity (counts cm/min).

Effect of oligonucleotide concentration, sample conductivity, pH and organic modifiers on detectability and sensitivity

The results showing the detector response as a function of sample oligonucleotide concentration in water are plotted in Fig. 2. As shown at lower values of concen-

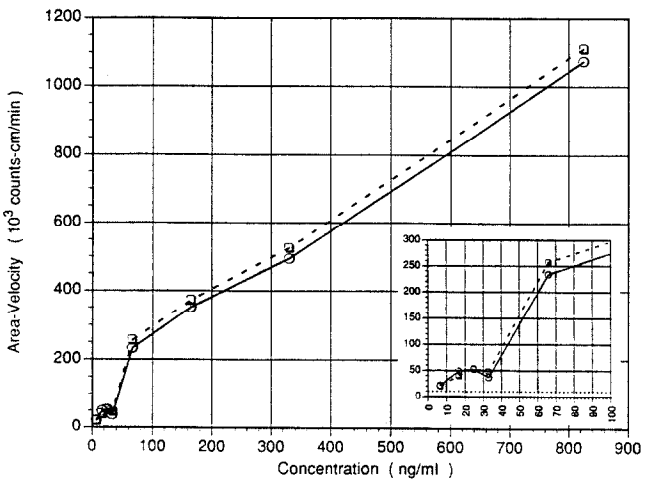

Fig. 2. Effect of sample oligonucleotide concentration on the quantitative response, area times velocity at 260 nm. The gel-filled capillary conditions of analysis were $L = 47$ cm, $l = 28$ cm, $T = 50°C$, $E = 298$ V/cm, $i = 18$ μA, $t = 10$ s and V = −5kV. ○———○ = $p(dA)_{12}$; □-----□ = $d(AGTC)_6$. The dotted line in the inset represents the lower limit of response for $p(dA)_{12}$.

tration, the lower limit of response (r) is the area times velocity for $p(dA)_{12}$ calculated from the equation

$$r = Al / t \tag{4}$$

where r is area times velocity, l is the separation length, t is the migration time and A is the average area of noise peaks plus three standard deviations of the noise. For $p(dA)_{12}$, $A = 1930 + 3(550) = 3580$ counts, $l = 28$ cm and $t = 9.56$ min, then $r =$ 10 485 counts cm/min. Extraprolating the lower curve to zero through this point yields a minimum detectable concentration (MDC) of 3 ng/ml (equivalent to about 0.00009 AU_{260}/ml) for the conditions used. Increases in the injection duration and voltage may be used, as described later, to reduce the MDC further. It should be noted that the MDC will not be a constant for any one system, but according to eqn. 4 will vary with the oligonucleotide velocity. Slower moving oligonucleotides (*i.e.*, larger species with lower mobilities or separations using lower field strengths) will have a lower MDC as the area noise is usually a constant during the run time.

Fig. 2 demonstrates a complex relationship between oligonucleotide concentration and quantitative response. Theoretical factors affecting electrokinetic injections in free solution [11,12] or gels [1] would lead one to expect a linear correlation. A linear correlation is found for relatively high concentrations of the oligonucleotide (>70 ng/ml), but as the concentration falls below 70 ng/ml the response decreases to an unusual plateau region (about 10–40 ng/ml) where the response appears to be independent of concentration. At levels below about 15 ng/ml the response decreases towards the lower limit of response.

It appears that the oligonucleotides are augmenting those factors, such as field strength or possibly electroendosmotic flow at the capillary tip, which influence the rate of migration of the oligonucleotides into the capillary gel during the injection process. The conductivity of the 100 ng/ml sample is equal to the conductivity of the water (5 μS/cm). Therefore, the oligonucleotides do not appear to be affecting the sensitivity by alterations of the sample conductivity. These results stress the importance of using an internal standard for quantification. Fig. 2 demonstrates that equal mass concentrations of $p(dA)_{12}$ and $d(AGTC)_6$ yield similar responses. Over the entire concentration range the ratio of $p(dA)_{12}/d(AGTC)_6$ area times velocity was 0.97 ± 0.13 S.D. This finding indicates that there is no mass discrimination based on size or sequence during the injection cycle. These results demonstrate the feasibility of using a different size and sequence oligonucleotide as an internal standard.

The velocity at which the oligonucleotide migrates from the sample solution into the capillary gel is directly proportional to the field strength between the capillary tip and electrode in the sample solution. It would be expected that if the conductivity of the sample solution is increased (*i.e.*, by the addition of buffer ions), the field strength between the capillary and electrode will decrease, resulting in less oligonucleotide mass migrating into the capillary from solution. The data plotted in Fig. 3 demonstrate exactly this case. The different size and sequence oligonucleotides at different concentrations show a greater than 50% loss in on-column mass at a sample conductivity of 40 μS/cm or greater [equivalent to about a 0.5 m*M* Tris–phosphate (pH 7.2) buffer] when compared with a conductivity of about 15 μS/cm or less. Increasing the conductivity above 500 μS/cm decreases the response to a few percent

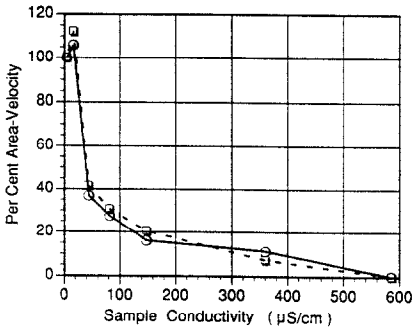

Fig. 3. Effect of sample conductivity on the quantitative response, percentage of area times velocity at 260 nm of the area times velocity at a conductivity of 5 μS/cm. Tris–phosphate buffer (pH 7.2) was used at concentrations from 0 to 10 mM to achieve different sample conductivities at relatively constant pH (7.2–7.5). The gel-filled capillary conditions were L = 47 cm, l = 28 cm, E = 298 V/cm, i = 18 μA, T = 50°C, t = 10 s and V = −5 kV. ○———○ = $p(dA)_{18}$, C = 48 ng/ml; □-----□ = $d(AGTC)_9$, C = 82 ng/ml.

of the lower conductivity value. This higher conductivity, depending on the oligonucleotide concentration, may result in little or no oligonucleotide being detected.

The data in Fig. 3 shows that in order to obtain a sufficient response for the analysis of low levels of oligonucleotides (less than 40 ng/ml), the conductivity must be kept below about 15 μS/cm.

In order to demonstrate the effect of sample pH on response, the oligonucleotides $p(dA)_{18}$ and $d(AGTC)_3$ in dilute sample buffers of nearly equal conductivity (88–102 μS/cm) were analyzed. In the pH range 6.5–9.1 the area times velocity varied by 12.8% (relative standard deviation), demonstrating relatively little or no effect of pH on the migration of oligonucleotides into the gel column from the sample solution. As increasing the pH increases electroendosmotic flow (EOF) [13], these results

TABLE I

EFFECT OF ADDITION OF ORGANIC MODIFIERS TO THE SAMPLE BUFFER ON MASS LOADING

The oligonucleotides were dissolved in a 1.0 mM Tris–phosphate buffer (pH 7.2) (conductivity = 80 μS/cm) at a concentration of 165 ng/ml containing the organic modifiers at the indictated concentrations. The gel-filled capillary conditions were d = 50 μM, L = 47 cm, l = 28 cm, E = 298 V/cm, i = 17 μA, T = 30°C, t = 10 s and V = −5kV. The data are presented as the percentage of area times velocity of that obtained in the absence of any modifier.

Modifier	Oligonucleotide area times velocity (%)			
	$p(dA)_{12}$	$p(dA)_{18}$	$p(AGTC)_6$	Average
Methanol (20%)	89.1	61.4	79.4	76.6
Formamide (10%)	42.1	40.7	35.0	39.2
Urea (2.5 M)	106.7	102.3	101.4	103.4
Ethylene glycol (10%)	109.9	92.1	93.1	98.4

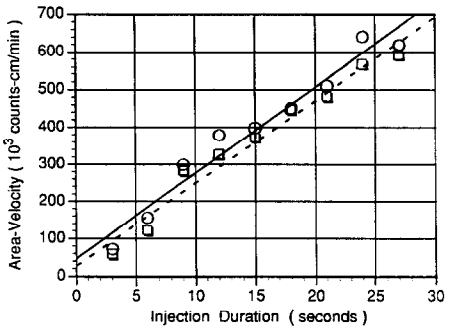

Fig. 4. Effect of injection duration at constant injection voltage on the quantitative response, area times velocity at 260 nm, plotted as linear regressions. The gel-filled capillary conditions were L = 47 cm, l = 27 cm, E = 298 V/cm, i = 17 μA, T = 30°C, V = −5 kV and C = 165 ng/ml. ○———○ = $p(dA)_{12}$ (R^2 = 0.96); □-----□ = $d(AGTC)_6$ (R^2 = 0.97).

suggest that EOF at the capillary tip may not be a factor affecting the migration of oligonucleotides into the gel column.

Various organic compounds were introduced into a sample solution to establish whether or not the decrease in sensitivity due to the increased conductivity of a dilute buffer could be augmented. Samples of $p(dA)_{12}$, $p(dA)_{18}$ and $d(AGTC)_6$ in dilute Tris–phosphate buffer (conductivity *ca.* 80 μS/cm) were analyzed with or without methanol, formamide, urea or ethylene glycol and the results are shown in Table I. Urea and ethylene glycol had no effect on sensitivity, but formamide and, to a lesser extent, methanol caused a reduction in sensitivity.

Effect of injection field strength and duration on resolution and mass loading

The oligonucleotide mass entering the gel capillary from the sample solution would be expected to increase on increasing either or both the injection duration or field strength [1,11,12]. Fig. 4 shows the data from experiments in which the injection voltage is held constant and the injection duration is varied. For the two different oligonucleotides the quantitative response increases linearly with an increase in injection time. The different size and sequence of the oligonucleotides did not appear to influence the amount injected significantly. This lack of discrimination between analytes during injection is in contrast to the discrimination found for electrokinetic injections of differently charged analytes in free solution [12,14]. This lack of discrimination would be expected owing to the equal mobilities of oligonucleotides in the sample solution.

Fig. 4 also shows the feasibility, as discussed previously, of using an internal standard that differs in size and sequence for quantitative analyses. The slopes of the two regression lines differed by only 3.5%. Changing the injection duration will not significantly affect the relative response of different oligonucleotides.

The effect of injection duration on resolution was investigated. Because (as discussed later) the oligonucleotide mass injected affects the resolution, it was necessary to inject from progressively lower concentrations of $p(dA)_{17}$ and $p(dA)_{18}$ as the injection duration was increased in order to maintain the same on-column mass. The

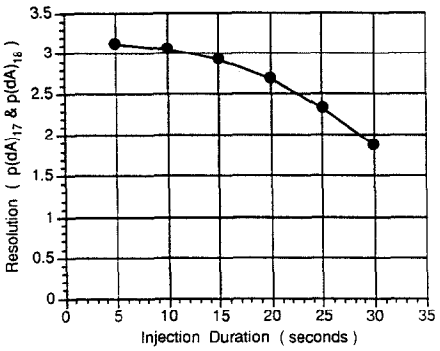

Fig. 5. Effect of injection duration on the resolution of $p(dA)_{17}$ and $p(dA)_{18}$. The gel-filled capillary conditions were L = 45 cm, l = 26 cm, E = 311 V/cm, i = 19 μA, T= 50°C, V = −5 kV and concentration of the oligonucleotides (24–212 ng/ml) adjusted to give a relatively constant on-column mass.

results of this experiment are shown in Fig. 5. The resolution (R_s) was calculated using the equation

$$R_s = \Delta t/w \tag{5}$$

where Δt is the difference in migration times of $p(dA)_{17}$ and $p(dA)_{18}$ and w is the average of the peak widths (as 4σ) of $p(ddA)_{17}$ and $p(dA)_{18}$. Fig. 5 shows that as the injection duration is increased, there is a gradual decrease in resolution. For injection times less than 20s the decrease is less than 15% of the value at 5 s, but above 20 s the resolution decreases more rapidly, approaching a 40% loss at 30 s. For critical separations, it appears necessary to keep the injection time below 20 s in order to maintain resolution.

Increasing the field strength between the capillary tip and electrode is accomplished by increasing the voltage across the entire capillary during the injection cycle. The data in Fig. 6 demonstrate how the quantitative response varies with increasing

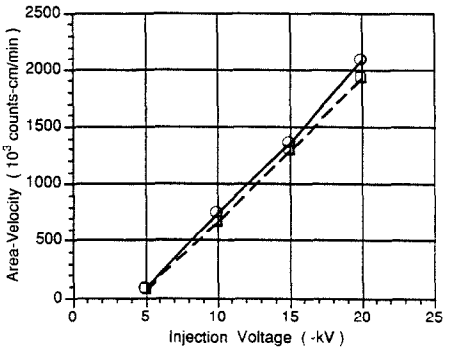

Fig. 6. Effect of injection voltage at constant injection duration on the quantitative response, area times velocity at 260 nm. The gel-filled capillary conditions were L = 48 cm, l = 22 cm, T = 50°C, E = 312 V/cm, i = 19 μA, t = 3 s and C = 20 ng/ml. ○———○ = $p(dA)_{12}$; □-----□ = $p(dA)_{18}$.

injection voltage while the injection duration is held constant. The oligonucleotide mass injected onto the gel capillary increases linearly with increase in injection voltage. There appears to be a slight discrimination such that increasing voltages increase the proportion of mass of the smaller oligonucleotide being injected onto the column. There are other disadvantages to using higher injection voltages: higher injection voltages increase the frequency of column electrical breakdown by the formation of bubbles in the capillary tip, and higher injection voltages have also been observed occasionally to cause severe peak distortion.

The increase in mass loading brought about by the increase in the injection duration and/or injection voltage can exceed one order of magnitude with minimal decrease in resolution. Fig. 7 shows examples of electropherograms that demonstrate the pronounced effect of increasing the injection duration to achieve higher levels of detectability. The electropherogram from the shorter injection duration shows a major $p(dA)_{18}$ peak with marginally detectable minor peaks in a sample from an HPLC-"purified" fraction. When the same sample is analyzed using a longer injection duration, the sensitivity is increased about 17-fold and the electropherogram clearly shows the presence of six or seven minor peaks ranging from 1 to 9% of the total response. This ability of the capillary gels to preconcentrate analytes at the capillary tip during the injections step, referred to as a zone "focusing" [15] or "sharpening" [1] effect, is probably due to the lower mobility of the oligonucleotides in the gel matrix relative to their much higher mobility in the sample solution.

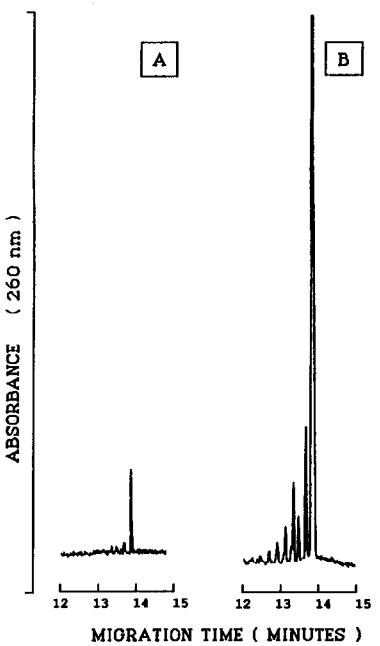

Fig. 7. Effect of sample "focusing" on quantitative response, absorbance at 260 nm, caused by increasing the injection duration. A sample of HPLC-"purified" $p(dA)_{12}$ was separated on the gel-filled capillary using injection times of (A) 3 s and (B) 15 s with conditions $L = 46$ cm, $l = 28$, $E = 304$ V/cm, $i = 19$ μA, $T = 30$°C, $V = -5$ kV and $C = 330$ ng/ml.

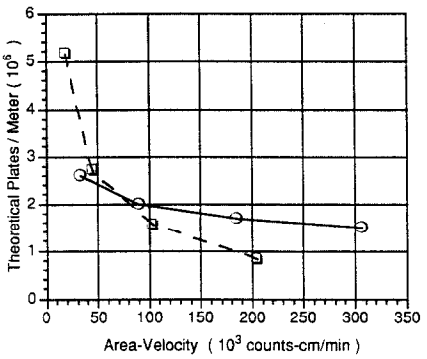

Fig. 8. Effect of on-column mass, as area times velocity at 260 nm, on the separation efficiency of a gel-filled capillary. The analysis conditions were L = 46 cm, l = 24 cm, E = 326 V/cm, i = 19 μA, T = 50°C, t = 3 s, V = −5 kV and C = 20–160 ng/ml. ○———○ = $p(dA)_{12}$; □-----□ = $p(dA)_{18}$.

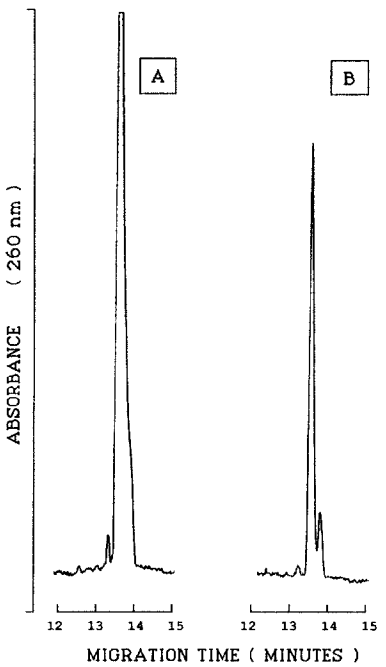

Fig. 9. Effect of on-column mass on the resolution of oligonucleotides of the same size but different sequence. The separation in (A) using a 5-s injection had about a five times greater area times velocity than the separation in (B) using a 2-s injection of the same sample. The large peak at 13.6 min is $p(dA)_{12}$ and the small peak at 13.7 min, resolved in (B) but not (A), is $d(AGTC)_3$. The conditions were L = 47 cm, l = 28 cm, E = 298 V/cm, i = 17 μA, T = 30°C, V = −5 kV and C = 2574 ng/ml for $p(dA)_{12}$ and 396 ng/ml for $d(AGTC)_3$.

Effect of on-column mass on efficiency

It has been shown qualitatively that analyte mass overloading can cause a decrease in resolution and peak distortion in capillary gel separation [1]. Fig. 8 shows the quantitative effect of mass on efficiency in the capillary gel separations of oligonucleotides. The efficiency was calculate using the equation

$$N/L = 5.54\,(t/w)^2/l \tag{6}$$

where N/L is the number of theoretical plates per meter, t is the migration time, w is the peak width at half-height and l is the effective separation length in meters. As the mass injected onto the column increases, there is a corresponding decrease in the efficiency of the separation. The larger oligonucleotide [$p(dA)_{18}$] appears to be more sensitive to decreases in efficiency than the smaller oligonucleotide [$p(dA)_{12}$] on an equivalent mass basis. A five-fold increase in the on-column mass (from $3 \cdot 10^4$ to $15 \cdot 10^4$ counts cm/min) resulted in a 30% efficiency loss for $p(dA)_{12}$ and about a 70% loss for $p(dA)_{18}$. The electropherograms in Fig. 9 illustrate how this increase in on-column mass can significantly compromise the resolution. Fig. 9 shows the separation between $p(dA)_{12}$ and $d(AGTC)_3$ at low and high on-column mass. This critical resolution between oligonucleotides of identical size but different sequence is accomplished in the low-mass but not in the high-mass separation.

It is evident that the needs for resolution may have to be weighed against the needs for detectability in some applications. Separations that require high efficiency, such as sequence dependence, may be limited in their ability to detect trace levels of one component. This problem will be more pronounced in the separation of larger oligonucleotides. For well resolved components, such as single-base size differences, adequate resolution will be maintained over a wider range of on-column mass.

Effect of separation field strength, column length and column temperature on resolution and migration times

In the interests of decreasing analysis times and maintaining resolution, the effects of increasing the separation field strength, decreasing the effective separation length and increasing the column temperature were investigated. Fig. 10 shows the

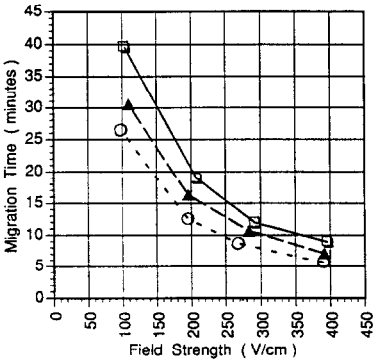

Fig. 10. Effect of separation field strength and effective separation length on the migration time of $p(A)_{18}$. The gel-filled capillary conditions were L = 37, 44 or 47 cm, T = 50°C, i = 6–24 μA, t = 3 s, V = −5 kV and C = 707 ng/ml. Effective separation length: □————□ = 28 cm; ▲-----▲ = 25 cm; ○·····○ = 20 cm.

variation in migration times for $p(dA)_{18}$ as the column field strength and effective separation length are changed. The velocity of charged analytes in capillary electrophoresis are directly proportional to the field strength [15]. It is not suprising to find that the migration time of $p(dA)_{18}$ decreases by about half when the field strength is doubled. Reducing the separation length reduces the distance that the oligonucleotide must be transported from capillary tip to detector. If the oligonucleotide is transported at a uniform velocity, a decrease in this distance would be expected to result in a proportional decrease in the migration time when the field strength is the same. The data show that a proportional decrease does occur. A 29% decrease in the separation length resulted in about a 34% decrease in the migration time of the $p(dA)_{18}$.

The variation in the resolution between $p(dA)_{17}$ and $p(dA)_{18}$ caused by increases in the field strength was examined and the results are plotted in Fig. 11. The data show that the resolution increases by about 40% for every two-fold increase in the field strength, which agrees well with the 41% that would be predicted by theory, *i.e.*, resolution $\propto$ (field strength)$^{1/2}$ [7,15]. The data also demonstrate that if the detector rise time is set to high values, an increase in field strength may actually lead to decreases in resolution. As the difference in migration time, Δt, is inversely proportional to the field strength [7,15], according to eqn. 5 the resolution will decrease if the average peak width, w, does not correspondingly change inversely with the field strength. The detector rise time will add significantly to the peak width if it approaches or exceeds about one third of the peak width at half-height [16]. As the field strength is increased, the peak width decreases and, without any decrease in the detector rise time, may lead to a decrease in resolution. At a field strength of 400 V/cm, the oligonucleotide half-height approaches 1.0 s, necessitating a detector rise time of 0.3 or less to maintain resolution. The signal-to-noise ratio will decrease with decreasing rise time, causing an increase in the MDC. Also, as discussed earlier in relation to eqn. 4, an increased peak velocity caused by increases in the field strength will increase the MDC. Therefore, increasing the field strength as a means of decreasing analysis times is an approach that should weigh in the balance the need for resolution and the needs of detectability.

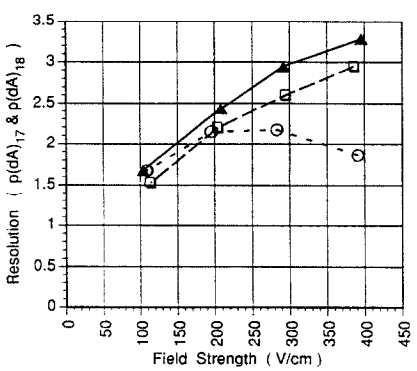

Fig. 11. Effect of separation field strength, effective separation length and detector rise time on the resolution of $p(dA)_{17}$ and $p(dA)_{18}$. The gel-filled capillary conditions were L = 43, 44 or 47 cm, i = 6–24 μA, T = 50°C, t = 3 s, V = −5 kV and C = 707 ng/ml. Separation length and rise time: ▲——▲ = 28 cm, 0.1 s; □-----□ = 24 cm, 0.1 s; ○·····○ = 25 cm, 1.0 s.

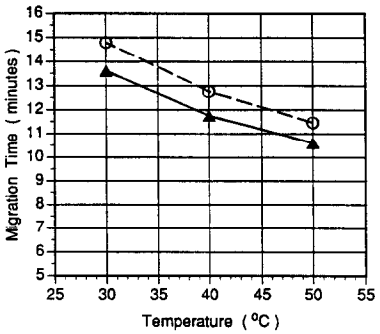

Fig. 12. Effect of separation temperature on the migration time of ▲———▲ = $p(dA)_{12}$ and ○--○ = $p(dA)_{18}$. The gel-filled capillary conditions were L = 48 cm, l = 28 cm, E = 292 V/cm, i = 18 μA, t = 3 s, V = −5 kV and C = 141 ng/ml.

Fig. 11 shows the effect on resolution (R_s) of reducing the effective separation length (l). A 14% reduction in separation length resulted in about a 9% decrease in resolution, which agrees with the 7% that would be predicted from theory, *i.e.*, $R_s \propto l^{1/2}$ [7,15]. Reduction of the separation length is a viable alternative for reducing the analysis time. A 30% reduction in the separation length, with no change in the field strength, should decrease the analysis time by 30% and the resolution by only about 14%. This decrease in resolution will not affect significantly non-critical separations and it avoids the detectability problems associated with increases in field strength. Of course, critical separations may be affected by small reductions in the resolution and necessitate increases in the field strength in order to compensate for the resolution loss.

The variation of migration time and resolution with changes in column temperature were examined. Fig. 12 shows that an increase in column temperature results in corresponding significant decreases in the migration times of $p(dA)_{12}$ and $p(dA)_{18}$. The overall migration time decreases by about 1.1% per °C, which is lower than the 2% quoted in the literature [3]. The migration time of $p(dA)_{18}$ relative to $p(dA)_{12}$ remains the same during temperature changes. Increasing the temperature from 30 to 50°C results in a 22% decrease in the migration times.

The resolution of $p(dA)_{17}$ and $p(dA)_{18}$ was determined at different temperatures. There is about a 15% decrease in resolution when the temperature is increased from 30 to 50°C. Increasing the column temperature is a method that can significantly reduce the analysis time with minimum decreases in resolution. In critical separations, however, this decrease in resolution may have to be offset by a corresponding increase in the field strength.

CONCLUSIONS

The results demonstrate that numerous operating parameters may affect, in a mutually dependent way, the resolution, sensitivity and detectability of oligonucleotides separated on a gel-filled capillary. Analysis times are decreased by increasing the field strength, decreasing the separation length and increasing the column temperature. However, this decrease in migration time may be accompanied by decreases in

detectability (due to increased peak velocity and decreased signal-to-noise ratio) and decreases in resolution (due to shorter separation lengths). A poor response from the injection of an unknown sample may be improved by decreasing the sample conductivity, increasing the injection time or voltage and, in many instances, increasing the oligonucleotide concentration. On the other hand, injection of excessive mass onto the column may decrease the resolution, possibly obscuring the presence of a trace component. Using the information presented, separations may be optimized for particular needs more effectively for speed, resolution and sensitivity.

ACKNOWLEDGEMENTS

We thank Bill Giusti and Tess Adriano, DNA Service Support, Applied Biosystems (Foster City, CA, USA) for the supply of the purified oligonucleotides.

REFERENCES

1 A. Paulus and J. I. Ohms, *J. Chromatogr.*, 507 (1990) 113.
2 A. S. Cohen, D. R. Najarian, A. Paulus, A. Guttman, J. A. Smith and B. L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9660.
3 A. Guttman, A. S. Cohen, D. N. Heiger and B. L. Karger, *Anal. Chem.*, 62 (1990) 137.
4 H.-F. Yin, J. A. Lux and G. Schomburg, *J. High Resolut. Chromatogr.*, 13 (1990) 624.
5 R. Dubrow, presented at the *2nd International Symposium on High Performance Capillary Electrophoresis, January 29–31, 1990*, paper P-208.
6 D. N. Heiger, A. S. Cohen and B. L. Karger, *J. Chromatogr.*, 516 (1990) 33.
7 H. Swerdlow and R. Gesteland, *Nucleic Acids Res.*, 18 (1990) 1415.
8 H. Drossman, J. A. Luckey, A. J. Kostichka, J. D'Cunha and L. M. Smith, *Anal. Chem.*, 62 (1990) 900.
9 A. S. Cohen, D. R. Najarian and B. L. Karger, *J. Chromatogr.*, 516 (1990) 49.
10 H. Swerdlow, S. Wu, H. Harke and N. J. Dovichi, *J. Chromatogr.*, 516 (1990) 61.
11 X.-H. Huang, M. J. Gordon and R. N. Zare, *Anal. Chem.*, 60 (1988) 377.
12 D. J. Rose, Jr., and J. W. Jorgenson, *Anal. Chem.*, 60 (1988) 642.
13 K. D. Altria and C. F. Simpson, *Chromatographia*, 24 (1987) 527.
14 S. E. Moring, J. C. Colburn, P. D. Grossman and H. H. Lauer, *LC · GC*, 8 (1990) 34.
15 B. L. Karger, A. S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585.
16 S. E. Moring, personal communication.