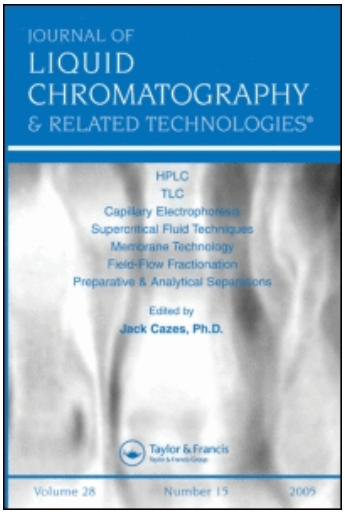

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The Effect of Electric Fields on the Dispersion of Oligonucleotides Using a Multi-Point Detection Method in Capillary Gel Electrophoresis

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THE EFFECT OF ELECTRIC FIELDS ON THE DISPERSION OF OLIGONUCLEOTIDES USING A MULTI-POINT DETECTION METHOD IN CAPILLARY GEL ELECTROPHORESIS

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ABSTRACT

A multi-point detection method is proposed to study the dispersion of oligonucleotides in polyacrylamide gel capillary. In this method a single gel filled capillary is curved into loops, with several detection points aligned with a common detector. In this manner, several electropherograms can be obtained at different migration times during one capillary electrophoresis (CE) run, thus permitting studies of changes in the spatial peak variance σ^2 as a function of time. Investigations of polydeoxyadenylic acids in the range of 40-60 bases indicate that the diffusion coefficient of these compounds increases at higher voltages in the polyacrylamide gel-filled capillaries. Using this simple multi-point detection method one can estimate the band broadening originating from sample injection (σ^2_{inj}), which is otherwise difficult to measure.

INTRODUCTION

Since the successful use of polyacrylamide-filled capillaries for the analysis of small oligonucleotides was first demonstrated (1,2), there has been considerable interest in the application of capillary gel electrophoresis (CGE) to DNA and

oligonucleotide separations. The resolving power and speed of CGE has been shown to be much better than that of slab gel electrophoresis (4-6) and HPLC (7). Compared to HPLC separations of charged large biopolymers, efficiencies in CGE are much higher. Using HPLC in the anion exchange mode for example, the slow diffusion of such large molecules prevents fast mass transfer between the mobile and stationary phases. In capillary gel electrophoresis the slow molecular diffusion of biopolymers is an advantage because the contribution of peak dispersion by axial diffusion decreases with increasing molecular weight of the oligonucleotides. Fast mass transfer in the direction perpendicular to that of the mass transport by the mobile phase as in HPLC is not required. It was presumed that the slow diffusion of large DNA molecules and the number of charges in such molecules, which increase proportionally to the number of base units, could be one of the reason for the high peak capacities which can be achieved with homologous oligonucleotide mixtures. Recently, Yin et al (8) have reported a method to measure the diffusion coefficients of oligonucleotides in gel capillaries without application of an electrical field. A relationship between such diffusion coefficients and molecular size of the oligonucleotides is achieved. However, the slow diffusion of large oligonucleotides in capillaries without high voltage cannot alone explain the high separation efficiencies of CGE. The diffusion coefficients of oligonucleotide molecules and their intermolecular interaction with the polyacrylamide gel may be altered considerably by the application of electrical fields. Thus, it was decided to be important to investigate the dispersive process of oligonucleotide molecules in the presence of strong (>100 V/cm) electrical fields.

Recently, Srichaiyo and Hjerten (9) used a simple multi-point detection method to record the progress of a separation by CZE. In this method a piece of fused-silica tubing coated with a monolayer of linear polyacrylamide was curved

into loops. Using a single detector, detection of the same sample at various points on the capillary could be obtained within a single CZE run. This method is more convenient than the multi-point monitoring method using several detectors ( )which has been reported previously since only one detector is needed.

In this paper, we present a multi-point detection method based on the techniques developed by Hjerten to study the dispersive process occuring to a series of oligonucleotides in gel capillaries in the presence of fields which might typically be applied during capillary electrophoretic separations.

EXPERIMENTAL

Apparatus

A new CZE apparatus which was designed and constructed in our laboratory was used. The modular instrument contains a Spectra 100 UV detector (Spectra Physics, Reno, NV), a CZE 1000 PN 30 high power supply (Spellman, Plainview, NY 11803) and a high power supply local control (Chamonix Industries, Binghamton, NY 13905) for electrokinetic sample introduction as well as adjustment of the actual separation voltage. A special cell fitted to the detector, was designed for multi-point detection (Fig.1). The cell body was constructed by adjoining two pieces of metal. There is a small hole in the center of the lower part of the cell and a ball lens is inserted. The capillary was bent into loops with the detection points inserted on top of each other in a slit on the upper part of the cell. There was a securing arm crossing the slit on each side of the cell that held the looped capillary in position. The top of the cell was covered with two pieces of black electrical tape and a channel (the width of the channel is less than 200 μm) was left between the two pieces of tape for light pass through. During a single CZE run, the solutes in the capillary could be detected several times as they passed the light path at different times. The electropherograms were processed on a SP

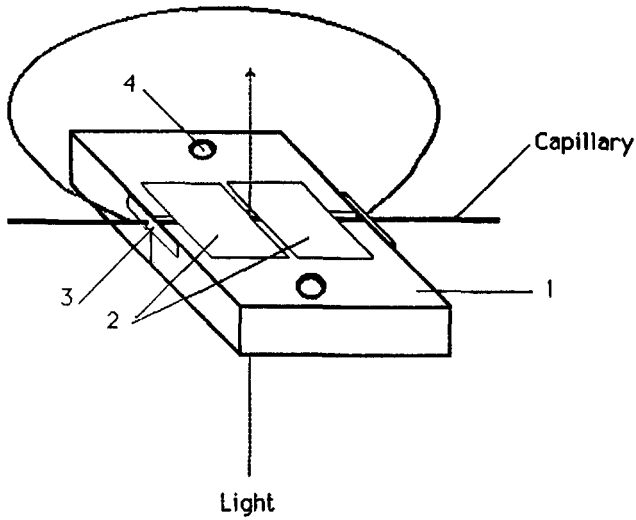

Figure 1. The schematic diagram of the multi-point detection cell. 1. cell body; 2. black tape; 3. securing arm and 4. screw hole.

4400 integrator (Spectra Physics, now a division of Thermo Separations, Fremont, CA).

Chemicals

Tris(Hydroxymethyl aminomethane), Urea ,acrylamide,N,N'-methylenebis(acrylamide)(BIS) and N,N,N',N'tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Richmond, CA). Ammonium persulfate was purchased from Sigma (Sigma,St Louis, MO). All of were of electrophoretic grade. Boric acid (Fisher Scientific Company, Fair Lawn, NJ) was of analytical grade. $PdA_{40\text{-}60}$ was purchased from Pharmacia (Uppsala, Sweden). Adenosine 5'-monophosphate(AMP),adenosine 5'-diphosphate(ADP) and adenosine 5'-triphosphate(ATP) were obtained from Sigma (St Louis, MO). The buffer solution used for electrophoretic runs and gel preparation consisted of 0.10 M Tris, 0.25 M boric acid and 7 M urea (pH 7.6). The solution for the generation of gels contained

acrylamide/bis-acrylamide (6% T, 5% C), 0.10 M Tris, 0.25 M boric acid and 7 M urea. All solutions were filtered through a 0.45 μm membrane.

Methods

Bubble-free polyacrylamide gel filled capillaries without inner surface pretreatment were prepared by the following procedure: the transparent capillary (75 μm i.d. X 356 μm o.d. ,Polymicro Technology, Phoenix,AZ) was rinsed by passing 1 M NaOH for 1 hour and distilled water for 30 min. Five (5) mL of the acrylamide solution (6% T, 5% C) was carefully degassed in an ultrasonic bath for 5 min. Then 10 μL of 10% (w/v) ammonium persulfate and 20 μL of 10%(v/v) TEMED were added to this solution and mixed thoroughly. This final solution was passed through the capillary for 3 min. During the polymerization in the capillary, both ends of the capillary were placed in a buffer solution contained in a septum-topped vial which was pressurized using an injection of air via a 10 mL syringe. The first centimeter at both ends of the capillary was cut before the CZE runs since polymerization was incomplete in those regions. The capillary was equilibriated by running with buffer at 100-150 v/cm for 30 min. The samples were electrophoreticlly injected into the capillary by dipping the cathodic end of the capillary into the sample solution and applying a voltage for a predetermined time. Polynucleotides were detected at 260 nm. Detector rise time was 0.3 sec.

RESULTS AND DISCUSSION

Estimation of Diffusion Coefficients

A number of studies on the various sources of zone broadening in the CZE of molecular ions have recently been published (8, 10-13). The total spatial zone variance in capillary gel electrophoresis is given by:

$$\sigma^2_{total} = \sigma^2_{inj} + \sigma^2_{det} + \sigma^2_{D} + \sigma^2_{T} + \sigma^2_{con} + \sigma^2_{other} \quad (1)$$

where the terms on the right-hand side of the equation represent the variance contributions from injection (σ^2_{inj}), detection (σ^2_{det}), axial diffusion (σ^2_{D}), Joule heating (σ^2_{T}), conductivity changes through the solute zone (σ^2_{con}) and other effetcs (σ^2_{other}). For a carefully designed CGE experiment, the axial diffusion, injection volume and Joule heating terms are the dominant terms contributing to the zone broadening at low velocities (14,15). Thus,

$$\sigma^2_{total} \approx \sigma^2_{inj} + \sigma^2_{D} + \sigma^2_{T} \quad (2)$$

Except for σ^2_{inj}, both the σ^2_{D} and σ^2_{T} terms are directly proportional to the analysis time. Simple diffusion is described using the Einstein equation, as;

$$\sigma^2_{D} = 2Dt \quad (3)$$

The Joule heating (temperature) term can be estimated as (10,16):

$$\sigma^2_{T} = \left(\frac{k^2 B^2 \alpha^6 E^4 \gamma^2}{48 D (8 \lambda_b T_b^2)^2}\right) t \quad (4)$$

where D is the diffusion coefficient of the solute ion, k is the electrical conductivity of the buffer, B is the exponential coefficient relating viscosity to temperature, α is the capillary inner radius, λ_b is the thermal conductivity of the buffer, T_b is the temperature at the inner wall of capillary, and ν is zone velocity. Combining equations yields the relationship:

$$\sigma^2_{total} = \sigma^2_{inj} + 2D't \quad (5)$$

where:

$$D' = D + \frac{k^2 B^2 \alpha^6 E^4 \gamma^2}{96 D(8 \lambda_b T_b^2)^2} \quad (6)$$

D' is defined as the effective dispersive coefficient. In our experiments, the sample solutes pass the detector at several different times within a single CE run. At the first detection point:

$$(\sigma^2_{total})_1 = (\sigma^2_{inj})_1 + 2D't_1 \quad (7)$$

while at the second point:

$$(\sigma^2_{total})_2 = (\sigma^2_{inj})_2 + 2D't_2 \quad (8)$$

Since each detection point was obtained at different times in one run, the band broadening originating from injection was the same for both detection points. Thus, $(\sigma^2_{inj})_1 = (\sigma^2_{inj})_2$. By subtracting Eqn.7 from Eqn.8, we can get:

$$D' = \frac{(\sigma^2_{total})_2 - (\sigma^2_{total})_1}{2(t_2 - t_1)} = \frac{1}{2}k \quad (9)$$

where k is the slope of the line of σ^2_{total} vs. migration time.

Changes in Diffusion Coefficient with Field Strength

Fig. 2 shows two eletropherograms obtained at different field strengths (185 and 233 V cm^{-1} respectively) for four consecutive detections of $pdA_{40\text{-}60}$ by monitoring the solutes at different points in gel filled capillaries. From this figure the relationship between length and resolution is evident. Also, the reduced migration time as a function of field strength is evident.

One of the primary advantages of the multipoint detection technique is that of obtaining information not easily obtained using a conventional single point detection mode. An estimate of D' term of eq. 6 was made by measuring the σ^2 of

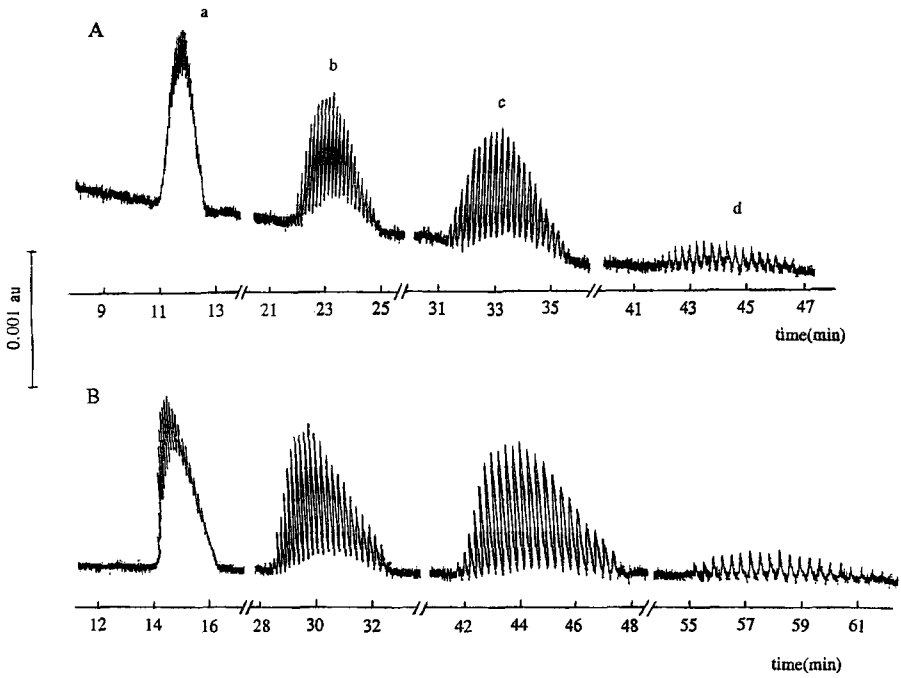

Figure 2. Electropherograms obtained from four consecutive detections of $pdA_{40\text{-}60}$ in gel filled capillary. Capillary: 75 μm i.d. X 356 μm o.d., total length 86 cm. Gel: polyacrylamide, 6% T, 5% C. Buffer: 0.1 M This; 0.25 M boric acid; 7 M urea (pH 7.6). Injection: 5 kv,2s. Sample: $pdA_{40\text{-}60}$ 5 μg/ml in water. Temperature: 24.5 C. **A.** high voltage: 185 v/cm; current:20 μA. **B.** high voltage: 233 v/cm; current: 29 μA. (a) first detection, effective length:18.4 cm; (b) second detection, effective length: 38.5 cm; (c) third detection, effective length: 56 cm; (d) fourth detection, effective length: 73.8 cm.

the peaks of oligomers with 42, 46, 50, and 54 bases at 3 different detection times, as shown in Figure 3. From the slope of the lines, the D' values (D' = 1/2 X slope) for the different oligomers was calculated (see Fig. 4).

There have been several previous studies on the effect of temperature on zone broadening in CZE (17-19). It was concluded that for capillary dimensions of 100 μm i.d. or less, and for a current of less than 30 μA (the same conditions of

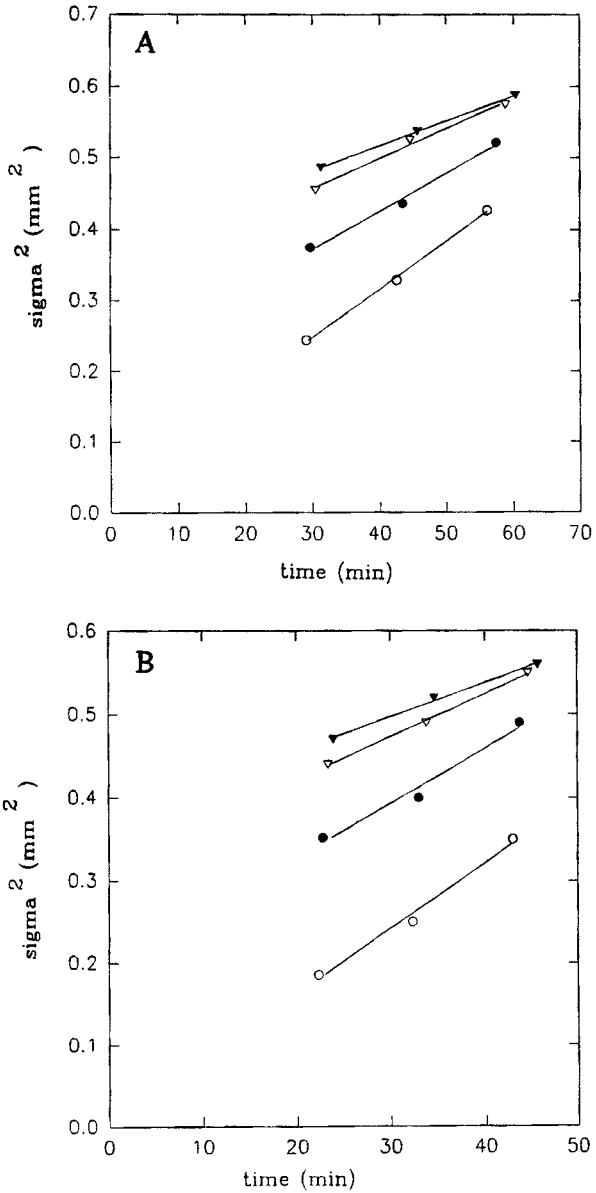

Figure 3. Plot of measured σ^2_{total} against migration time. Separation conditions were showed in Fig.2. Sample: $pdA_{40\text{-}60}$, 5 μg/ml in water. **A.** high voltage: 185 v/cm; **B.** high voltage: 233 v/cm. The four lines correspondent to four oligonucleotides with different number of base units in length: (○) base number: 42; (●) base number: 46; (△) base number: 50 and (▲) base number:54.

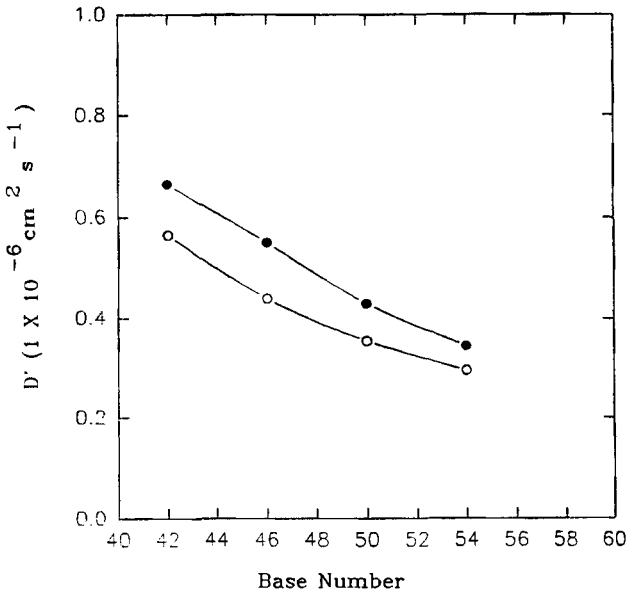

Figure 4. Dependence of dispersive coefficients (D') on the number of base units (i.e. molecular weight)of oligonucleotides(sample: $pdA_{40\text{-}60}$). (○) applied high voltage: 185 v/cm and (•) applied high voltage: 233 v/cm.

our experiments), the effect of heat generated within the capillary tube on the band broadening is much smaller than the effect of axial diffusion (11). Recently, Grossman (15) calculated the contributions of diffusion, injection and heat generated to the band broadening in CE. The σ^2_D value was 9.1 X 10^3 times higher than σ^2_T . The contribution of heat generated to the band broadening was therefore taken as negligible. The values they calculated were comparable to the published experimental values (13). The D' values obtained from our experiments are likewise presumed to be dominated by axial diffusion of the oligonucleotides in the gel capillary, even in the presence of the electrical field. In such a situation, D' approximates the diffusion coefficient (D) under a high electrical field. The diffusion coefficients (see Fig.4) obtained based on this assumption were about 2

to 3 times higher than the results obtained by Yin et al (8). The main reason for this difference might be that the D values they measured were the diffusion coefficients of oligonucleotides in a gel capillary in the absence of an electrical field. Based on theoretical considerations, they concluded that diffusion coefficients of the oligonucleotides might be considerably altered by the application of an electrical field. An electrical field-induced change of conformation of the oligonucleotide molecule may give rise to higher diffusion coefficients (8). The results of our experiments verify this assumption. Recently, Delinger et al (20) reported that the variation of analyte mobility and diffusion coefficients were not constant over a large range of voltages. From the results (Fig. 4), it can be concluded that the diffusion coefficients of the oligonucleotides in the gel capillary decrease nonlinearly with an increase in the number of base units or molecular weight. Yin et al (8) obtained the same results for oligonucleotides in a gel capillary without the application of an electrical field.

Estimation of Injection Variances

Another advantage of the multi-point detection method is that estimates of the σ^2_{inj} value from the plot of σ^2_{total} vs. migration time, which is difficult to measure by other means. In equation 8, it can be seen that $\sigma^2_{total} = \sigma^2_{inj}$ when $t=0$. Thus the value of σ^2_{inj} is given by the intercept of the line of σ^2_{total} vs. time. Fig. 5 shows the electropherograms of a two-point detection of AMP and ADP in a gel capillary with different injection times. The σ^2_{total} and calculated D' values for AMP are listed in Table I. The D' values obtained from the two runs under the same separation conditions with the exception of the injection time are very similar, giving confidence that temperature or other effects are minimal, as assumed in the derivations. Regression of σ^2 versus time yields σ^2_{inj} values for run 1 and 2 are

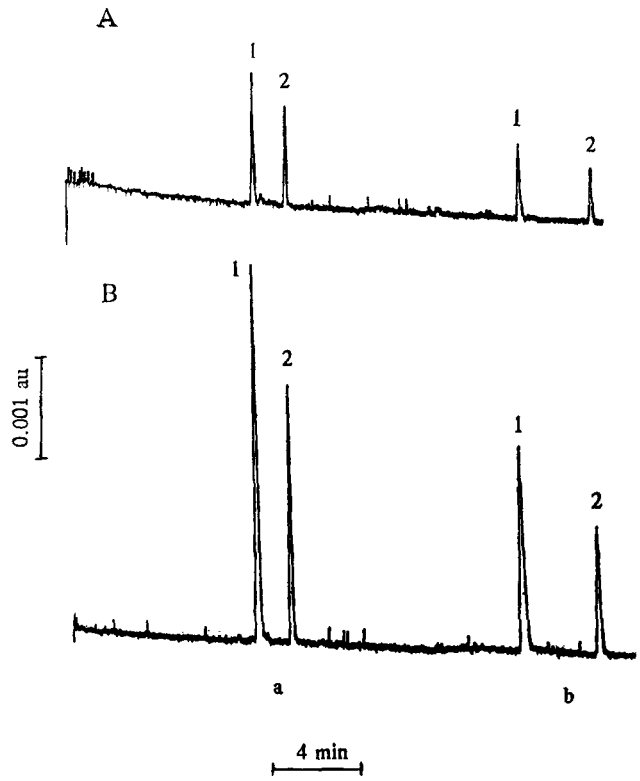

Figure 5. Electropherograms obtained from two consecutive detections of AMP and ADP in gel filled capillary. Capillary: 75 μm i.d. X 356 μm o.d., total length 55 cm, effective length: (a) 16 cm, (b) 40 cm. Gel: polyacrylamide, 6% T, 5% C. Buffer: 0.1 M this; 0.25 M boric acid; 7 M urea (pH 7.6). High voltage: 13.9 kv, 35 μA. Injection: **A.** 3 kv, 3 sec.; **B.** 3 kv, 5 sec.. 1 = ADP, 2 = AMP.

0.25 mm^{2}and 0.55mm^2, respectively. Given that $\sigma^2_{inj} = l^2 / 12$, where l is the width of the analyte plug (11, 14) and that l is proportional to the injection time (t_{inj}) (21), then σ^2_{inj} is proportional to t_{inj}^2. Thus;

$$\frac{(\sigma^2_{inj})_2}{(\sigma^2_{inj})_1} = \frac{(t^2_{inj})_2}{(t^2_{inj})_1} \qquad (10)$$

while taking the square root of both sides yields:

TABLE 1

Measured σ^2 Values for AMP at Two Different Migration Time

Injection time(sec.)	3		5	
Migration time(min)	9.88	23.49	9.71	23.35
σ^2_{total} (mm^2)	0.68	1.26	0.98	1.58
Calculated D' values* (1×10^{-6} cm^2/s)		3.5		3.7

* D' values were calculated based on equation (9).

$$\frac{(\sigma_{inj})_2}{(\sigma_{inj})_1} = \frac{(t_{inj})_2}{(t_{inj})_1} \qquad (11)$$

The ratio of $(t_{inj})_2$ over $(t_{inj})_1$ for the two different injection times in Fig. 5 is 5/3 or 1.67. The ratio of $(\sigma_{inj})_2$ over $(\sigma_{inj})_1$ is $(.55/.25)^{1/2}$ or 1.48. The discrepency of ca. 12% between the two values could be caused by errors in controlling the exact injection times, and to measurement errors of the peak variances.

CONCLUSIONS

In addition to the application of this consecutive multi-point detection method on the study of dispersive processes of analytes during CZE, there are some other advantages of this method which were reported by Srichaiyo and Hjerten recently (9). For example, by using this technique it has been demonstrated that the appearance of the DNA pattern changes in an unexpected, discontinuous way during a CZE run. Also, using this multi-point detection method one can unambiguously study the relationship between the migration distance and

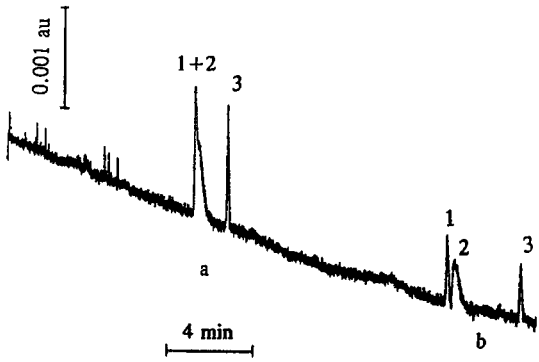

Figure 6. Electropherograms obtained from two consecutive detections of AMP, ADP and ATP in gel filled capillary. Separation conditions are same as those in Fig.5 except that the injection time is 2 sec at 3.5 kv. (a) first detection, effective length: 16 cm; (b) second detection, effective length: 40 cm. 1 = ADP; 2 = ATP and 3 = AMP.

the migration time. Also, it is possible to rapidly choose the optimum effective length for a certain preferred high voltage. This can minimize the time needed to optimize the separation conditions. In Fig. 6, one can see ADP and ATP cannot be separated at the first effective length distance but can be separated at the second detection point. In this way we can obtain good separation conditions in fewer runs than the single point detection method.

In conclusion, the simple multi-point detection method pioneered by Hjerten is very promising for the study of dispersive processes of oligonucleotides in a gel capillary which is subjected to an electric field. The method can help one to investigate the relationship of the diffusion coefficients with the number of base units of oligonucleotides, and to consequently understand the reason for the high resolution and efficiency which can be achieved with homologous oligonucleotide mixtures in gel filled capillaries. Also, one can estimate the σ^2_{inj} value with reasonable accuracy, which is difficult to measure by other methods.

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