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Resolution and concentration detection limit in capillary gel electrophoresis

J. MACEK*[a], U. R. TJADEN and J. VAN DER GREEF
Division of Analytical Chemistry, Centre for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden (The Netherlands)
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ABSTRACT

The effects of column length and applied electric field on the column efficiency and the resolution of two oligonucleotides in capillary gel electrophoresis were examined. The best resolution was obtained by using long columns in combination with optimum (and not the highest possible) electric field. Further, the concentration detection limits were optimized; it was found that electrokinetic injection did not cause excessive peak broadening even when applied for 25 s at the operating voltage. Thus sample molecules were concentrated during the injection by isotachophoretic effects resulting in very low concentration detection limits (216 pmol/l for a dodecanucleotide with a relative molecular mass of 3707).

INTRODUCTION

Capillary gel electrophoresis (CGE) is increasing in popularity, especially for the separation of oligo- and polynucleotides [1–3]. Several methods for the preparation of gel-filled capillaries have been described [1,4–6] and the separation of polynucleotides containing up to 431 nucleotide units has been reported [1]. Columns with several gel compositions have been compared [3] and very high plate numbers have been obtained on gels with relatively low monomer contents. However, little attention has been paid to the effects of other separation parameters such as column length, applied electric field and duration of electromigration injection on the resolution and the detection limit. In this work we studied these effects using two oligonucleotides as model compounds.

EXPERIMENTAL

Materials

The oligonucleotides CTT.GTG.GTG.GG (XI, relative molecular mass 3417.6) and CTT.GTG.GTG.GGC (XII, relative molecular mass 3706.6) dissolved in

[a] Present address: Department of Clinical Biochemistry, Faculty Policlinics, Charles University, 12 111 Prague, Czechoslovakia.

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water (0.4 mmol/l) were kindly supplied by CIVO–TNO Institutes (Zeist, The Netherlands) and diluted with water to the desired concentrations. N,N,N′,N′-Tetramethylenediamine (TEMED) was obtained from BDH (Poole, U.K.), ammonium peroxodisulphate, acrylamide, bisacrylamide, urea and polyethylene glycol 6000 from Merck (Darmstadt, Germany), boric acid from J. T. Baker (Deventer, The Netherlands) and tris(hydroxymethyl)aminomethane (Tris) from Aldrich Chemie (Steinheim, Germany).

Apparatus

An Alpha Series II 15-kV high-voltage power supply (Brandenburg, Thornton Heath, U.K.) was used to generate the voltage across the capillary gel column. The compounds were detected by a Spectra 100 UV–VIS detector (Spectra-Physics, San Jose, CA, U.S.A.) operating at 260 nm. Each end of the gel-filled capillary (see below) was dipped in a 15-ml vial filled with 0.1 *M* Tris–0.2 *M* boric acid buffer. Samples were injected electrokinetically into the capillary by immersing the cathodic end of the column in the sample vial and applying a voltage between 0.5 kV and the operating voltage for 1–25 s.

Preparation of gel-filled capillaries

Before filling, a few millimetres of the polyimide coating of the fused-silica capillary (60 cm × 0.075 mm I.D.) were burned off at the distance of 15 cm from the detection end. For some experiments multiple detection windows were created.

The gel-filled capillaries were prepared as described by Yin *et al.* [1]: 4 μl of 5% (w/v) ammonium peroxodisulphate and 4 μl of 5% (v/v) TEMED were added to 0.5 ml of the polymerization solution containing acrylamide–bisacrylamide (T = 6%, C = 5% [5][a]. The capillaries were filled with the polymerization solution by vacuum, then the ends of the capillary were sealed with silicone-rubber septa and the capillary was left in a horizontal position. The gel was formed within 1 h, but capillaries were left overnight for complete polymerization.

In some experiments the capillaries were prepared according to the method of Karger and Cohen [5], in which the gel is bonded to the capillary walls by a bifunctional silane reagent, and contains 20% of polyethylene glycol.

RESULTS AND DISCUSSION

Resolution

The column efficiency, characterized by the number of theoretical plates, N, and the resolution, R_s can be calculated as follows [7]:

$$N = (u_e EL)/(2D) \tag{1}$$

$$R_s = [(u_{e2} - u_{e1})\,(EL)^{1/2}]/[4(2u_e D)^{1/2}] \tag{2}$$

where u_{e1} and u_{e2} are the electrophoretic mobilities of compounds 1 and 2, respec-

[a] C = g N,N′-methylenebisacrylamide (Bis) × 100/(g acrylamide + g Bis); T = (g acrylamide + g Bis)/100 ml of solvent.

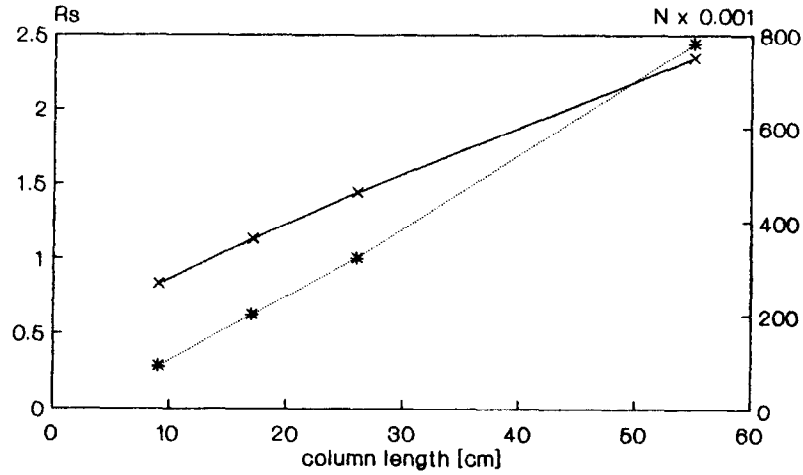

Fig. 1. Separation efficiency (*) and resolution (×) of XI and XII (20 μg/ml) as a function of the effective column length. Capillary, 60 cm × 0.075 mm I.D. with detection windows at 9, 17, 27 and 55 cm, filled with polyacrylamide gel, 6% T, 5% C; buffer, 0.1 *M* Tris–0.2 *M* boric acid; electric field, 270 V/cm; current, 10 μA; electromigration injection, 1 s at 1 kV; detection wavelength, 260 nm; time constant, 0.3 s.

tively, u_e is the average mobility of the two compounds, D is the diffusion coefficient, E is the electric field and L is the column length.

We examined these relationships for two oligonucleotides having 11 and 12 nucleotide units, respectively (Figs. 1 and 2). The dependence of efficiency and resolution on column length follows the theory, but in the case of an applied electric field the above-mentioned equations are not satisfied: the efficiency increases only slowly and the resolution even exhibits a maximum. This behaviour was observed on several columns differing in length and in gel composition. The limited gain in efficiency can be explained by thermally induced zone broadening [8], because at high electric fields

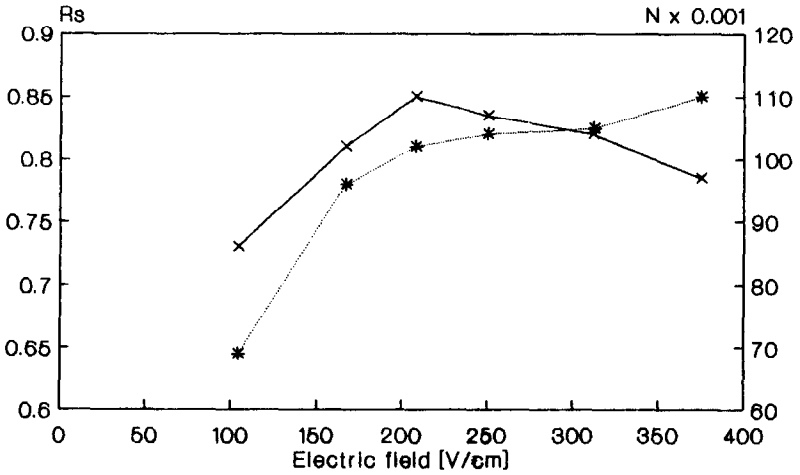

Fig. 2. Separation efficiency (*) and resolution (×) of XI and XII (20 μg/ml) as a function of electric field. Separation conditions as in Fig. 1, except for fixed effective column length, 9 cm.

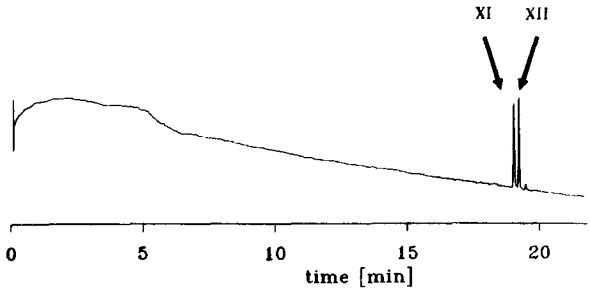

Fig. 3. CGE separation of XI and XII (20 μg/ml). Effective column length, 55 cm; range, 0.01 a.u.f.s.; other conditions as in Fig. 1.

the temperature increases owing to Joule heating. The maximum on the resolution–field curve is caused by the superimposition of the efficiency–field relationship and the increase in electrophoretic mobility with temperature [3] (see eq. 2). It follows that for the best resolution a long column has to be used and that the optimum voltage is not the highest possible (Fig. 3).

Detection limit

One of the main drawbacks of CGE is the high concentration detection limit (*i.e.*, lowest measurable concentration in the sample) usually obtained with conventional UV detection. The reasons are the absorption of the light in the capillary owing to the optical properties of the gel, the short optical pathlength in the (on-capillary) detection cell and the small amount of the sample injected into the column.

We compared the transmittance of capillaries filled with water of CGE buffer with gel-filled capillaries. It is difficult to measure the absorbance of the gel accurately, because some light always passes through only the walls of the capillary. Therefore we evaluated the ratio of the reference and sample beam intensities for different

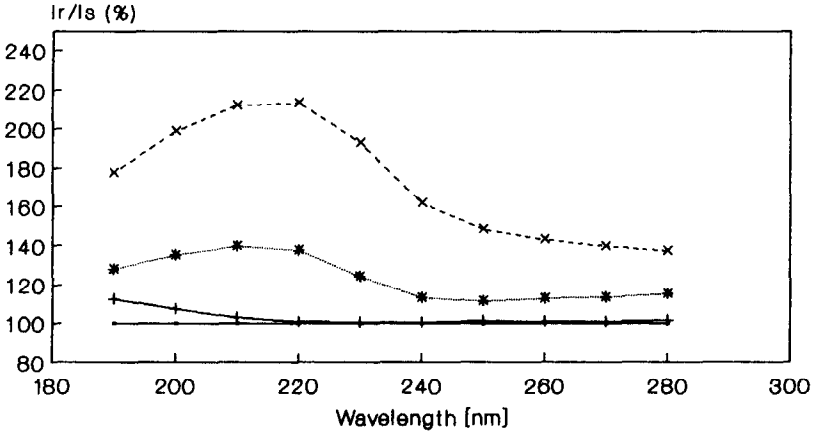

Fig. 4. Optical properties of polyacrylamide gels measured as ratio of intensities of reference and sample beam, and related to water. • = Water; * = gel, T = 6%, C = 5%; + = CGE buffer; × = gel, T = 6%, C = 5%, 20% polyethylene glycol.

materials inside the capillary (Fig. 4). It can be seen that relative to water the intensity of the sample beam at 260 nm decreases by 15% when the column is filled with gel of T = 6%, C = 5%. When the same gel contains 20% of polythelene glycol the intensity is even lowered by 40%. Therefore, we chose the preparation of gel-filled capillaries described by Yin *et al.* [1], because these gels do not contain polyethylene glycol. We did not observe any improvement in the stability as they described for capillaries with walls coated with polyacrylamide. Therefore, we prepared the gel-filled capillaries by the simplest method as described under Experimental. As already described [1,9], the columns are not stable for a long period and occasionally the first few millimetres of the capillary must be cut in order to restore its performance.

The amount of sample that can be injected into the capillary is limited by the requirement not to cause significant zone broadening on sample introduction. This phenomenon can be caused either by distortion of the applied electric field when the concentration of sample ions is too high [10] or simply by injecting too large a zone. However, when the sample is dissolved at low concentration in pure water, a zone-sharpening effect can occur as in isotachophoresis: we used to inject normally at a voltage equal to one tenth of the operating voltage for 1 s (Fig. 3). This is equivalent to an injection for 0.1 s at the operating voltage, *i.e.*, the zone broadening induced by

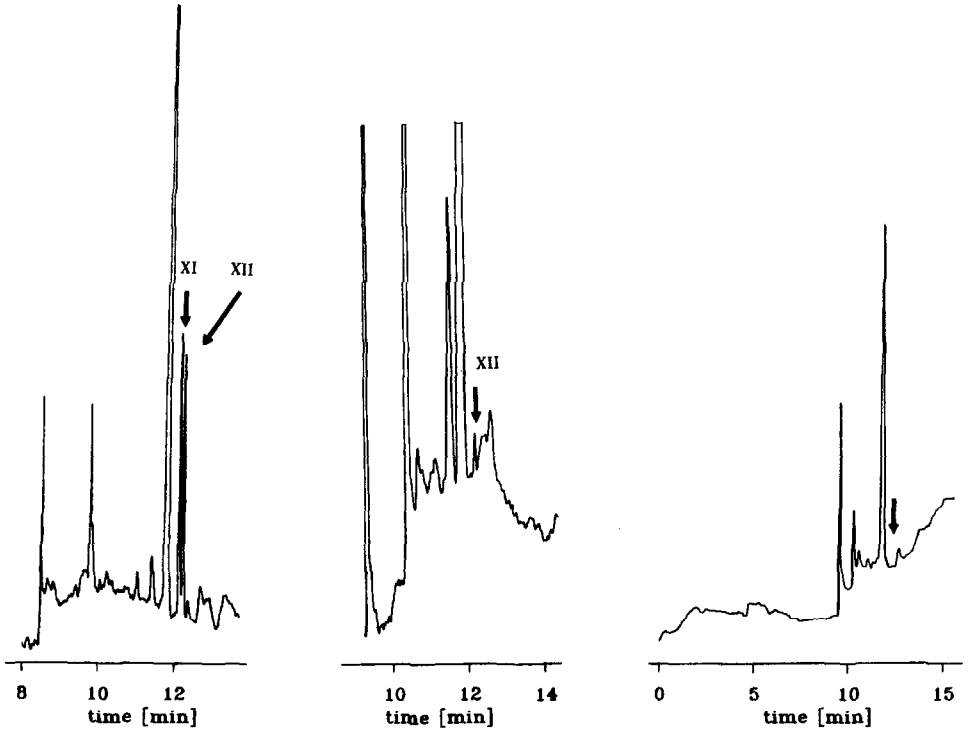

Fig. 5. CGE separation of XI and XII (4 ng/ml). Effective column length, 36 cm; electrokinetic injection, 25 s at 10 kV; range, 0.0005 a.u.f.s.; other conditions as in Fig. 1.

Fig. 6. Electropherogram of XII (800 pg/ml). Conditions as in Fig. 5.

Fig. 7. Electropherogram of pure water. Conditions as in Fig. 5, exception range, 0.002 a.u.f.s.

injection is 0.1 s in time units, which is acceptable for peaks with a width of 2 s at half-height. The concentration detection limit under such conditions is *ca.* 100 ng/ml (based on a signal-to-noise ratio of 3:1). However, on employing injections as long as 25 s at the operating voltage, no excessive peak broadening was introduced and therefore it was possible to lower the detection limit to and even below the ng/ml level without loss of resolution (Figs. 5 and 6). The peak of XII in Fig. 6 corresponds to a concentration of 216 pmol/l, which to our knowledge is the lowest concentration detection limit reported for capillary electrophoresis with conventional UV detection.

It is difficult to calculate the absolute amount injected into the capillary, because the volume fraction occupied by the gel is unknown and the electrophoretic mobilities in buffer and pure water are different [11].

Paulus and Ohms [9] observed severe distortion of oligonucleotide peak shapes after injection for 10 s at 5 kV (operating potential 10 kV), but the concentration of the compounds was 0.2 mg/ml and thus concentration overloading could occur, as discussed above. They assumed that the peak deformation was caused by the high concentration of urea (7 *M*) in the electrolyte solution, but similar effects have not been confirmed by others [2]. We did not observe any gain of efficiency for the tested compounds using 7 *M* urea and therefore we used buffers without urea.

The impurities from water are concentrated in exactly the same way as sample molecules. When only water was injected, the electropherogram showed several peaks (Fig. 7), which can also be found in the sample electropherograms (Figs. 5 and 6). The inherent advantage of CGE is that these peaks belonging to low-molecular-weight compounds are eluted rapidly and therefore they do not interfere with peaks of higher molecular weight, such as oligonucleotides. Some of these peaks might also be due to rapid changes in concentration in the buffer generated during the injection.

CONCLUSION

Samples with concentrations down to 1 ng/ml dissolved in water can be analysed by CGE with conventional UV detection owing to the zone-sharpening effect. The presence of other compounds at high concentrations in the sample could represent a problem, and in such a case a suitable sample pretreatment would be necessary.

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