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Simple multi-point detection method for high-performance capillary electrophoresis

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ABSTRACT

A simple device for multi-point detection was designed to record the course of an analysis by high-performance capillary electrophoresis (HPCE). Determinations of mobilities and relative peak areas can be performed with an accuracy higher than that obtained with conventional HPCE equipment. Two principles were utilized (in both instances the polyimide coating is removed at the detection points desired). In one approach, the fused-silica tubing, following a first recording, is moved "backwards" to a new position for a second recording, and so on. The other approach utilizes a piece of tubing which, after a straight stretch, is curved into loops; the first monitoring takes place as the solutes leave the straight stretch, the second when they leave the first loop, the third when they leave the second loop, and so on (the straight stretch can, of course, be replaced with a loop). The technique is illustrated with an electrophoretic analysis in free solution of DNA and peptides. Interestingly, λ DNA is separated into several extremely narrow zones. By using multi-point detection it could be demonstrated that the appearance of the DNA pattern changed in an unexpected, discontinuous way during a run.

INTRODUCTION

Free zone electrophoresis in capillaries with diameters around 1 mm requires rotation of the capillary around its long axis in order to minimize zone distortions caused by convection [1]. The capillaries, which are made of quartz, are ground and polished on both the inside and the outside to make them straighter and to ensure very small variations in the inner diameter and the wall thickness along the length of the capillary. Owing to this high optical quality, the capillaries permit scanning with UV radiation for the detection of the solute zones without giving disturbing fluctuations of the baseline on the recorder chart (when the ratio of the absorption at two wavelengths is recorded, one of them not being absorbed by the solutes, the fluctuations are even smaller). The advantages of scanning these high-quality quartz capillaries are that an electrophoresis experiment can be followed from the start to its termination and that mobilities and peak areas can be determined with high accuracy because the diameter of the capillary varies very little throughout its length and also because several measuring points are obtained.

Fused-silica tubing is not of the same high quality as the above quartz capillaries and therefore does not give a satisfactory baseline on scanning. Further, silica tubing cannot be used with UV scanning, as it is delivered with a non-UV transparent polyimide coating (without the coating it is extremely brittle). In addition, the variations in the diameter of the tubing along its length are too large to permit accurate mobility determinations. Accordingly, in practice it is not straightforward to design a simple scanning device with satisfactory performance. We present here another very simple approach which overcomes partially the problems discussed above: the scanning is replaced with detection at two or more selected migration distances. Only slight modifications of existing monitors for high-performance capillary electrophoresis are required. Multiple

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monitoring has been reported previously, but the equipment was designed such that each detection point required its own detector [2–5]. Our version requires only one detector.

EXPERIMENTAL

Apparatus

The detector was an LKB/Pharmacia (Uppsala, Sweden) HPLC 2141 variable-wavelength monitor, modified for on-tube detection of the solutes. In one version of the equipment the fused-silica tubing was mounted in a V-groove for a fixed position relative to the UV beam, but could still be moved longitudinally in the groove for detection at different points on the tubing (Fig. 1a). The height of the opening in the V-groove was approximately equal to the outer diameter of the tubing and the width was 0.15 mm. In another version, the tubing was bent into loops for detection of the solutes as they entered and left a loop (Fig. 1b). The segments of the tubing that correspond to the beginning and the end of a loop were placed on top of each other in a slit. The height of the slit was slightly larger than the sum of the outer diameters of all the segments. The silica tubing was cooled by means of a fan and the V-groove by flowing water.

For determination of migration times and peak areas we used a Spectra-Physics SP 4270 inegrator. The DNA experiment was monitored with the aid of a recorder (Servograph REC 61) from Radiometer (Copenhagen, Denmark).

Treatment of the fused-silica tubing

By means of an electrically heated tungsten wire [6] the polyimide coating of the tubing was burnt off from places where detection was desired. The non-coated sections must be relatively narrow, otherwise the tubing will break when bent into a loop. The inside of the silica tubing was coated with a monolayer of linear polyacrylamide to eliminate adsorption and electroendosmosis [7]. This coating is stable also at high pH: no changes in migration velocities or the appearance of the electropherograms were observed during a test period of 4 weeks with a coated capillary stored at pH 11.

RESULTS AND DISCUSSION

Four consecutive detections of λ DNA by displacement of the fused-silica tubing (method I, see Fig. 1a)

The electropherograms in Fig. 2a, b, c and d show the patterns obtained when the migration distances were 12, 17, 27 and 37 cm, respectively. The fourth, third and second electropherograms are not simply monotonic extrapolations of the third, second and first electropherograms, respectively. In fact, one reason why we were interested in multiple-point detection was that we wanted to follow the separation of λ DNA, as we had earlier found that λ DNA can be resolved electropheretically into many extremely narrow zones, but not reproducibly [8]. In a forthcoming paper we shall discuss the separation mechanism and the unexpected alterations in the electropherograms. The separations may be dependent on the diameter of the electrophoresis capillary, as the lengths of the λ DNA molecules approach the diameter of the caillary.

In several experiments the unexpected differences between the appearances of consecutive electropherograms were even more pronounced than those in Fig. 2.

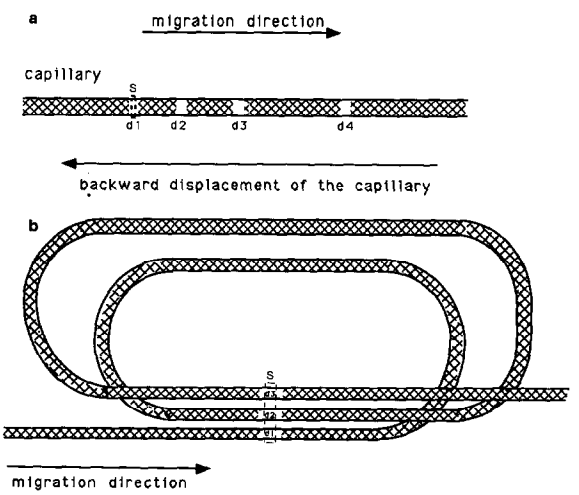

Fig. 1. The principle of multi-point detection in high-performance capillary electrophoresis. S = slit. (a) Method I: following a first detection at d1 the tubing is moved "backwards" for a second recording at d2, and so on. (b) Method II: the tubing is stationary. The first electropherogram is obtained when the solutes pass d1, the second when passing d2, and so on.

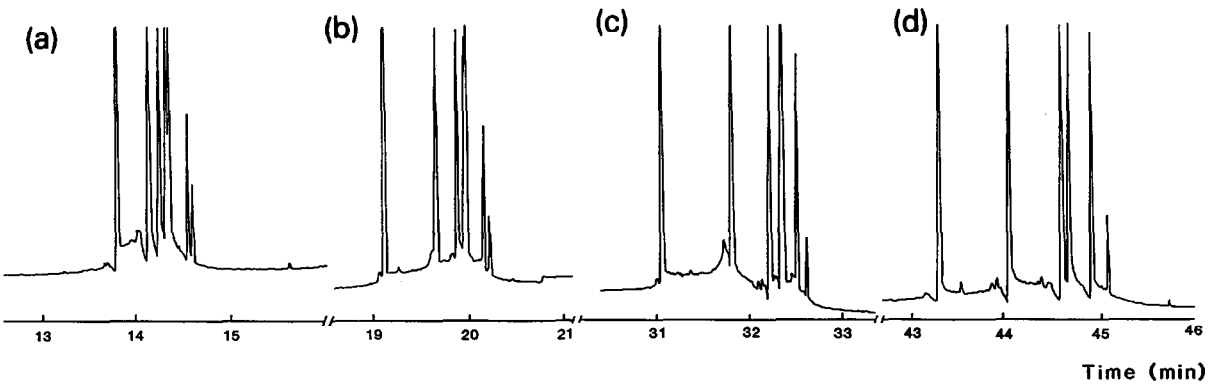

Fig. 2. Electropherograms of λ DNA obtained from four consecutive detections by displacement of the fused-silica tubing (see Fig. 1a). λ DNA was applied electrophoretically at 2000 V for 30 s and analysed at the same voltage in TBE buffer (0.09 *M* Tris–0.09 *M* boric acid–0.002 *M* EDTA) (pH 8.2). When the first detection (a) (migration distance 12 cm) was completed, the fused-silica tubing was drawn backwards to a new position for a second recording (b) (migration distance 17 cm). In (c) and (d) the migration distances were 27 and 37 cm, respectively. Capillary: 540 mm × 0.075 mm I.D. Detection wavelength: 260 nm.

Three consecutive detections of peptides by monitoring the solutes at different points in the stationary fused-silica tubing (method II, see Fig. 1b)

The model peptides were from Bio-Rad Labs. (Richmond, CA, USA). It is striking how an increase in the migration distance increases the resolution (Fig. 3). In contrast to the electropherograms in Fig. 2, those in Fig. 3 exhibit no anomalies.

Determination of relative peak areas and absolute mobilities.

The electropherograms in Fig. 3 show that four, six and all nine peptides were baseline resolved when the migration distances were 4.0, 14.0 and 44.0 cm, respectively. Accurate determinations of peak areas and mobilities could therefore only be obtained for the four peptides bradykinin, luteinizing hormone-

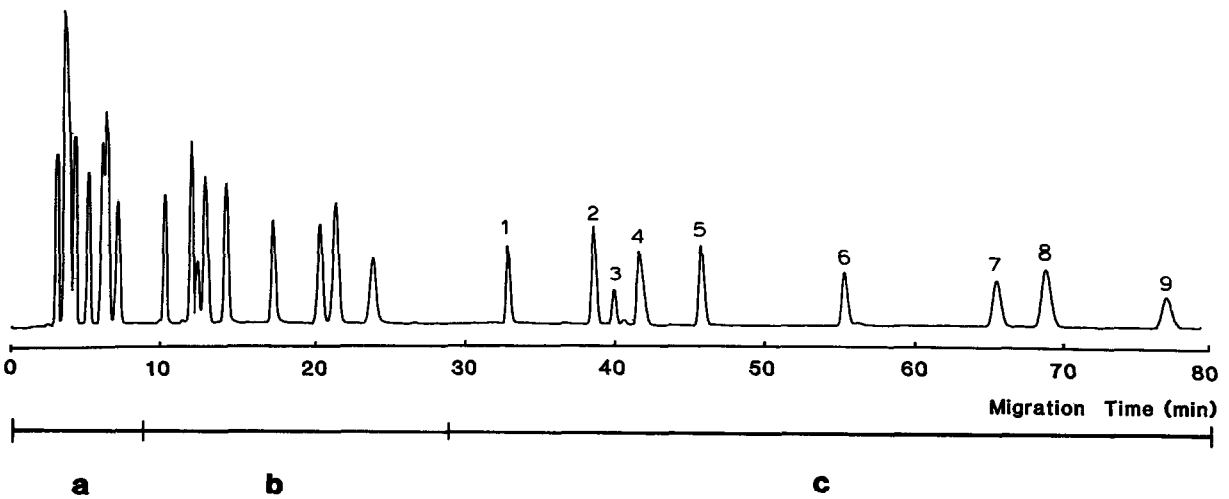

Fig. 3. Electropherograms obtained from three consecutive detections by monitoring the solutes (standard peptides) at different points in the stationary fused-silica tubing (see Fig. 1b). The sample was applied electrophoretically at 8000 V for 10 s. Buffer: 0.1 *M* sodium phosphate (pH 2.5). Running voltage: 8000 V. Dimensions of the capillary: 580 mm × 0.05 mm I.D. Detection wavelength: 200 nm.

TABLE I

DETERMINATION OF RELATIVE PEAK AREAS

Recording	Bradykinin	Luteinizing hormone-releasing hormone	[2–5]Leucine enkephalin	Oxytocin
1st	1.00	1.30	0.91	0.90
2nd	1.00	1.28	0.94	0.91
3rd	1.00	1.33	0.94	0.90

releasing hormone, [2–5]leucine enkephalin and oxytocin (peaks 1, 5, 6 and 9, respectively).

As the light intensity varies over the cross-section of the detecting UV beam and also because the diameters of the capillary are not exactly equal at the different detection points, the area of any particular peak is not the same in the consecutive electropherograms (see Fig. 3). However, this disadvantage should be eliminated if the absolute values of the peak areas are converted into relative units by dividing the areas of all peaks in an electropherogram by that of one of the peaks (*e.g.*, the first). This is confirmed in Table I.

In Fig. 4 the migration distance is plotted against migration time for the four peptides listed in Table I. The points lie surprisingly well on a straight line, indicating that the multi-point detection technique described here gives reliable mobility values (u), provided that the field strength can be determined accurately [$u = v/F$, where v is the migration velocity (cm s^{-1}) (the slope of a line in Fig. 4)].

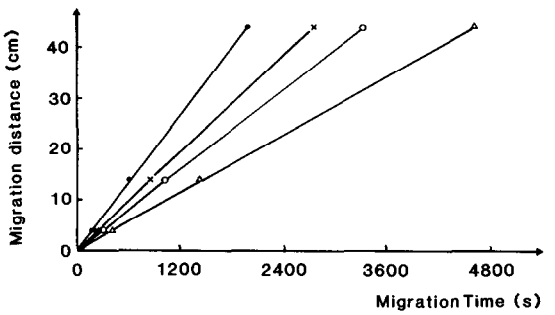

Fig. 4. Plot of migration distance against migration time. The plot refers to peaks 1 (bradykinin), 5 (luteinizing hormone-releasing hormone), 6 ([2–5]leucine enkephalin) and 9 (oxytocin) in Fig. 3. All points fall on straight lines, which indicates good accuracy in the determination of mobilities with the multi-point detection method presented.

Using a field strength obtained by dividing the voltage applied by the length of the capillary, the mobility values for bradykinin, luteinizing hormone-releasing hormone, [2–5]leucine enkephalin and oxytocin were calculated to be $1.61 \cdot 10^{-4}$, $1.17 \cdot 10^{-4}$, $0.96 \cdot 10^{-4}$ and $0.69 \cdot 10^{-4}$ $cm^2\ s^{-1}\ V^{-1}$, respectively.

As the field strength in an electrophoresis tube is determined primarily by the current in the tube and not by the voltage between the electrodes, it is theoretically safer to use the equation $F = I/q\kappa$, where I is the current (A), q the cross-sectional area (cm^2) and κ the electrical conductivity ($\Omega^{-1}\ cm^{-1}$) [9]. It is important that the average cross-sectional area is determined accurately, *e.g.*, by weighing the tubing empty and filled with a heavy liquid of known density.

Sensitivity

A small change in the position relative to the capillary and the photodiode of a light beam with a height as small as that of the inner diameter of the capillary may give rise to a relatively large alteration in sensitivity (deflction of the recorder pen) and in the level of the baseline, as the reflection losses (according to Fresnel's equation) and the light path through the capillary vary with the angle of incidence and also because the photodiode has a different response in different parts of the photosensitive surface. This displacement of the light beam, which can be caused by alterations in the temperature of the detection system, is less pronounced if the light beam is higher than the diameter of the capillary as in Fig. 1. This approach to bathing the capillary in light has been used previously with success [1]. One can therefore expect the detection methods described here to give a relatively small change in the position of the baseline and in

sensitivity if the temperature of the detection system changes during a run. This may explain why the drift in the baseline is extremely small in the electropherogram shown in Fig. 3, despite the long analysis time (80 min).

The slit height is considerably larger in the multipoint detection method II than in method I (see Fig. 1). Accordingly, even when a solute zone absorbs almost all of the light entering the zone, the total light striking the photodiode in method II will be reduced only by about $(1/n) \cdot 100\%$, where n is the number of detection points (most of the light will pass outside the zone). The sensitivity of the multipoint detection method II is, from this point of view, relatively low. However, as a photodiode has more favourable characteristics at high light flux and functions better, the fluctuations in the baseline, including the drift, are smaller in method II than in method I. If one amplifies the photocurrent in method II until the disturbances in the baseline become the same as in method I, one obtains a peak height (for a given solute concentration) which may approach that obtained in method I. Accordingly, the difference in sensitivity between the two methods need not be large. A similar detection technique was utilized in the design of the detector in the first capillary electrophoresis apparatus and worked satisfactorily [1]: the height of the slit was not sufficiently small to restrict the light beam to the centre of the capillary, but was larger than the outside diameter to minimize the variations in the baseline.

Method II works satisfactorily also when the capillaries are placed side-by-side instead of on top of each other as in Fig. 1b.

In any plot of absorption against solute concentration, the curve obtained may deviate considerably from a straight line when the absorption is not zero at infinite concentration, as in the experiments shown in Figs. 2 and 3, where part of the light beam passed outside the solute zone. This case is treated in detail in ref. 1 (pp. 182–190). When a linear relationship is required over a large absorption (concentration) range, only the central part of the tubing should be illuminated. In such experiments, Method II must be used in the version with the capillaries placed side-by-side.

An electrophoresis tube bent into loops gives greater zone broadening than does a straight tube of the same length, as molecules migrating at the outside of the loop lag behind those migrating at the inside. Neglecting the influence of diffusion, it is easy to show that the attendant zone broadening $\Delta X = n2\pi\varnothing$, where n is the number of loops and $\varnothing$ is the inner diameter of the tubing. Method I should, accordingly, be chosen when optimum resolution is required, which was verified experimentally by running DNA fragments in a gel-filled straight tube and in a gel-filled coiled tube (the runs are not shown here). The inner diameter was 0.2 mm. The gel (polyacrylamide) was cast in the coiled tubing. The same result was obtained when the straight gel-filled tube was bent into a loop. By giving the tubing such a shape (*e.g.*, a figure-of-eight or a serpentine path) that the "inner lane" turns into an "outer lane", the zone broadening can be decreased. Another alternative is to cut the end of the tubing where the sample is applied at such an angle that the length of the "inner lane" becomes equal to the "outer lane" at the detection point (suggested by Professor Paul Roos of this Department).

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