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Separation of acidic proteins by capillary zone electrophoresis and size-exclusion high-performance liquid chromatography: a comparison

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ABSTRACT

Successful separations of alcohol dehydrogenase (pI = 5.4), β-amylase (pI = 5.2) and albumin (pI = 4.7) by capillary electrophoresis in uncoated fused-silica capillaries are reported. Different electrophoretic conditions, consisting in variation of temperature, applied voltage and ionic strength of the buffer used as electrolyte, were tested in order to compare the separation efficiency, resolution and selectivity of the acidic proteins. The results were compared with those obtained by size-exclusion chromatography. Rinsing of the capillary between runs, in order to eliminate adsorbed proteins, can shorten its useful lifetime.

INTRODUCTION

Electrophoresis is the separation of charged molecules based on differential migration in an applied potential field. In capillary zone electrophoresis (CZE), compounds are resolved according to their ability to migrate in an electric field gradient inside a fused-silica capillary of 200 μm I.D. or less. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flows of buffer solutions and ionic species, respectively, within the capillary.

Zone electrophoresis in open capillaries is uniquely simple in the number of factors affecting solute band broadening. Electroosmosis resulting in a flat flow profile and the lack of a stationary phase virtually eliminate any band broadening due to resistance to mass transfer. However, adsorption is a particularly serious problem for proteins because of the multiplicity of polar, charged and hydrophobic sites on the molecular surface [1]. Protein adsorption can change the capillary surface characteristics, affecting results in subsequent runs and consequently the magnitude of electroendosmosis can drift as the capillary surface is modified by adsorption. McCormick [2] reported that phosphate forms complexes with silanols on the silica surface, reducing electroendosmosis and protein–silica interactions. Zhu *et al.* [3] suggested the use of acidic solutions to wash capillaries of 25 μm I.D. between runs. Rinsing with a solution of 0.1 *M* NaOH between protein runs resulted in restoration of the initial conditions of analysis [4]. However, all these procedures can cause capillary damage when using open tubes and examination of the capillary under a light microscope before and between several runs is strongly recommended.

In comparison with HPLC, CE is more limited with respect to the maximum mass load (*i.e.*, the maximum amount of sample that can be loaded on to the capillary without causing intolerable band broadening). In pure CZE, band broadening occurs only due to a single effect: longitudinal diffusion. Therefore, in CZE, the dispersion characteristics are more favourable compared with HPLC.

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The objective of this work was to establish the potential utility of CE with particular emphasis on using open-tube, untreated fused-silica capillary columns for the separation of acidic proteins. Analyses of these proteins by CE were compared with those obtained by size-exclusion HPLC using a conventional TSK G5000 PW XL column.

EXPERIMENTAL

Chemicals

All chemicals used for the preparation of the buffers were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. Deionized water was doubly distilled and filtered through Millipore GS filters (0.22 μm pore diameter).

Albumin from egg (M_r 45 000), β-amylase from sweet potato (M_r 200 000) and alcohol dehydrogenase from yeast (M_r 150 000) were purchased from Sigma (St. Louis, MO, USA), kit No MW MWGF-1000. The neutral marker, benzene, was purchased from Merck (Darmstadt, Germany).

Instrumentation

Zone electrophoresis was performed using a Spectraphoresis 500 system from Spectra-Physics (Fremont, CA, USA). Microbore fused-silica tubing coated with polyimide (Scientific Glass Engineering, Milton Keynes, UK) of 50 and 75 μm I.D. and 190–360 μm O.D. with a total length of 70 cm and a separation length of 63 cm were used. The capillary was enclosed in a cassette for easy handling. On line-detection was performed with a variable-wavelength UV–Vis detector of band width 6 nm (Spectra-Physics). Detection of proteins was monitored at 200 nm and electropherograms were recorded using an SP 4290 integrator (Spectra-Physics).

HPLC separation was performed using a system consisting of an SP 8800 gradient pump module, an SP 8490 UV–Vis detector (both from Spectra-Physics) and a Model 7125 injector (Rheodyne, Cotati, CA, USA). Retention times and peak areas of proteins were measured by using the same SP 4290 integrator after detection at 200 nm.

Capillary photographs were taken with a light Nikon Labophot microscope system (Nippon Kogaku, Tokyo, Japan) by using a 20× acromatic objective, numerical aperture 0.4 (Ref. 160/0.17, Nikon). Prints were made using Kodak PlusX Pan film (125 ASA).

Electrophoresis

A capillary just purchased was first conditioned with 0.5 *M* NaOH for 5 min at 60°C, 0.05 *M* NaOH for 5 min at 60°C and doubly distilled, deionized, filtered water for 5 min at 60°C. Equilibration of the capillary was then performed by washing it with different concentrations (15, 25 and 75 m*M*) of sodium borate–phosphoric acid buffer (pH 7.2) for 10 min at 25°C. After this, the capillary was washed again with the same buffer for 10 min at 25°C under an applied voltage of 20 kV. This buffer system was chosen in order to produce a pH value higher than the isoelectric point (p*I*) of the proteins to be separated. This renders the proteins negatively charged, resulting in repulsion from the charged fused-silica capillary walls and thereby minimizing adsorption. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence: 0.05 *M* NaOH for 3 min, doubly distilled, deionized water for 5 min and the corresponding conditioning buffer for 3 min. The buffer used as electrolyte was 15, 25 or 75 m*M* sodium borate–phosphoric acid (pH 7.2).

Protein solutions of 29.5 μM albumin (pI = 4.7) + 6.6 μM β-amylase (pI = 5.2) + 8.4 μM alcohol dehydrogenase (pI = 5.4), prepared in the electrophoresis buffer diluted threefold, were injected into the capillary by siphoning for a fixed time of 8 s. Benzene at a concentration of 4% (v/v) in the same diluted buffer was used as a neutral marker. Two different voltages, 17 and 22 kV, were applied using positive-to-negative polarity. During electrophoresis, temperature control was employed as indicated.

HPLC separation

Protein solutions of 35 μM albumin + 2.5 μM β-amylase + 5.3 μM alcohol dehydrogenase, prepared in the mobile phase buffer, were injected on to a TSK G5000 PW XL column (30

cm × 7.8 cm I.D.) from Supelco (Progel, Saint Germain-en-Laye, France). The flow-rate of the mobile phase, 75 m*M* Tris–HCl buffer (pH 8.0), was 0.3 ml min^{-1} and the injection volume was 10 μl.

RESULTS AND DISCUSSION

Some aspects of the capillary

The fused-silica capillaries coated with polyimide of 50–75 μm I.D. (Fig. 1) used for free zone electrophoresis received no surface treatment in order to maintain electroosmotic flow. This electroosmotic flow is dependent on the concentration and pH value of the buffer electrolyte, corresponding to the degree of dissociation of the silanol groups. Especially with proteins, interactions with the untreated wall can lead to poor reproducibility of migration times because of changes in the electroosmotic flow. On the other hand, the separation efficiency decreases owing to adsorption of proteins on the wall [4,5]. In that event, in order to achieve a good optimization of the separation parameters, regeneration of the surface by rinsing the capillary with NaOH between runs is recommended [3,6]. However, such rinsing steps, which were done automatically after every run in our analysis and are essential, can shorten the useful lifetime of capillaries. Other phenomenas that can damage the inner wall of the capillary are temperature gradients across it or temperature changes with time due to heat dissipation between runs. Both events are the inevitable result of the application

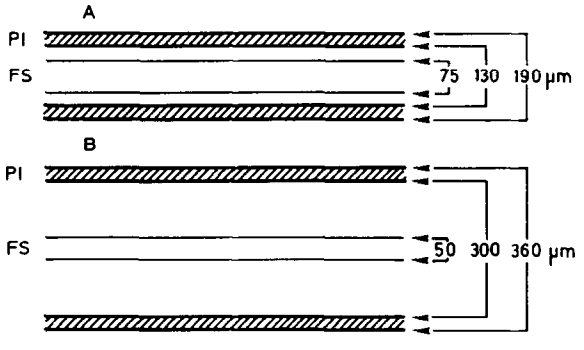

Fig. 1. Schematic representation of (a) 75 μm I.D. and (b) 50 μm I.D. untreated fused-silica capillaries. PI = Polyimide; FS = fused silica.

of high field strengths in capillary electrophoresis. Narrow-diameter capillaries help dissipation but are also more prone to clogging. Mechanical manipulation of the capillary should also be avoided and, once enclosed in the cassette, it should not be removed.

The effect of this damage can be detected by successive current decreases at the same applied voltage between runs. Fig. 2 illustrates some alterations produced in the capillary as a consequence of its utilization, analysed under a light microscope. A defective cutting (c) can lead to a flow interruption (d) by penetration of polyimide inside the capillary. Therefore, when a long capillary is purchased, the user must take care to ensure efficient cutting (b) in order to avoid the production of bubbles. A flow decrease or even interruption can be produced by irregularities in the inner wall (e–k). Damage to this wall caused by irreversible adsorption of proteins, silica cracks, pore formation (l), etc., causes a cavitation phenomenon that impedes the migration of solutes under constant electroosmotic flow. For all these reasons, the routine use of a light microscope is strongly recommended, to obtain good reproducibility of the results, especially in the analysis of proteins, when untreated fused-silica capillaries are used. As damage to the capillary inner wall seems to be an inverse function of the inner diameter, capillaries of 75 μm I.D. were used throughout this work.

Factors affecting mobility of acidic proteins in CZE

Efficiency is a function of the electroosmotic mobility [7], hence controlling the flow will be beneficial in maximizing efficiency and resolution, which could lead to superior separations. Without considering electroosmosis, the migration velocity, ν, in electrophoresis is given by

$$\nu = \mu_e E = \frac{\mu_e V}{L} \quad (1)$$

in cm s^{-1}, where μ_e is the electrophoretic mobility, E is the potential field strength (V/L), V is the voltage applied across the capillary and L is the capillary length [8]. Thus, higher voltages and shorter capillary lengths provide faster elution times. However, in the presence of electro-

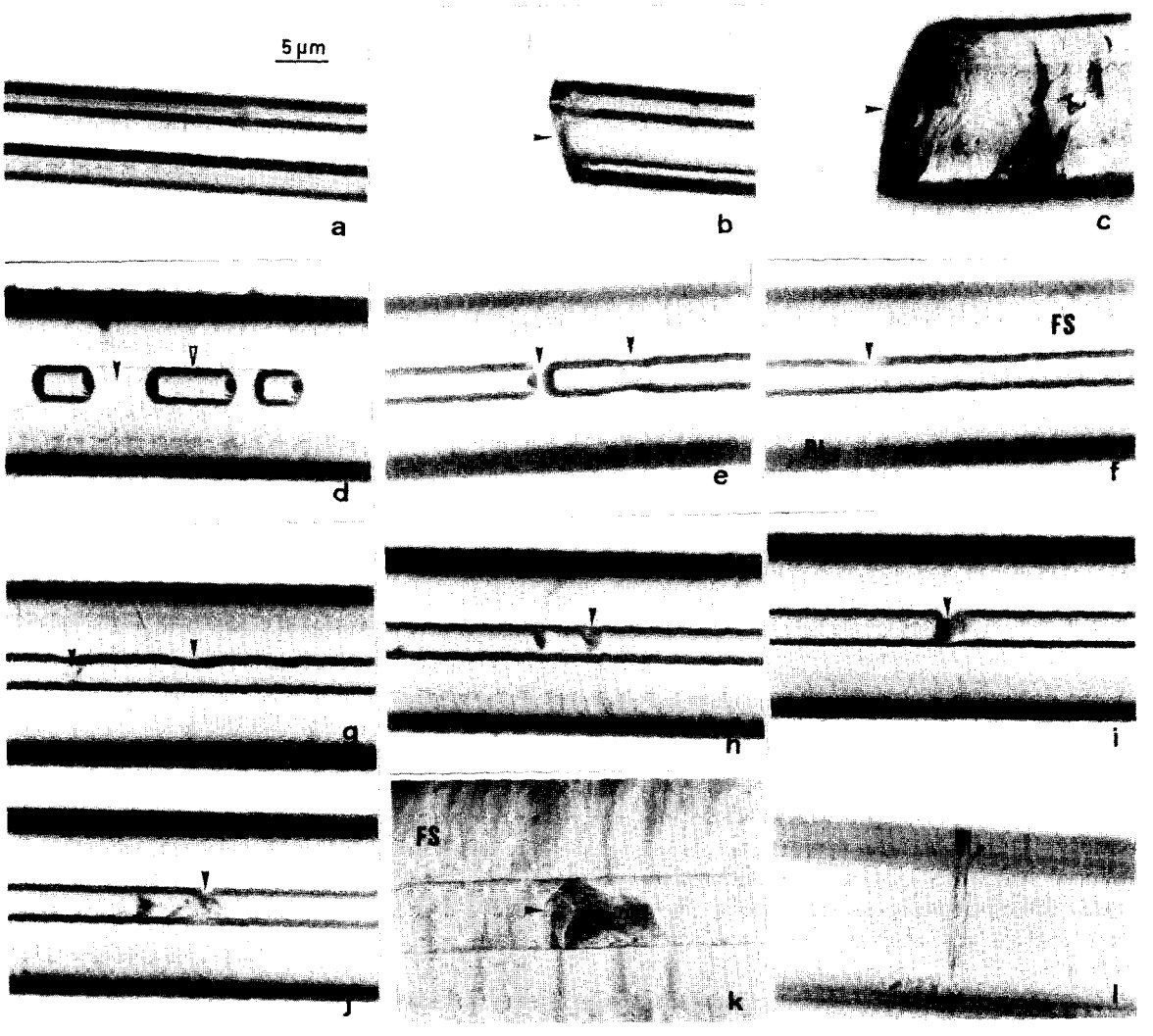

Fig. 2. Internal structure of the capillaries under a light microscope. (a, b) 75 μm I.D. capillary; (c–l) 50 μm I.D. capillary. Magnification procedure is described in the text. (a) Intact empty capillary; (b) good end cutting; (c) defective cutting with disrupted polyimide layer; (d), aspect of empty (open arrow) and full (full arrow) capillary; (e–k), different irregularities in the inner wall of the capillary; (l), pore formation through the wall of the capillary. PI = Polyimide; FS = fused silica.

osmotic flow, the migration velocity can be rewritten as

$$v = \frac{(\mu_e + \mu_{eo})V}{L} \qquad (2)$$

in cm s^{-1}, where μ_{eo} is the coefficient of electroosmotic flow [9]. This is because electroosmotic flow affects the amount of time a solute resides in the capillary and, in this sense, both separation efficiency and resolution are related to the flow-rate. The total velocity was higher for cations and lower for anions (as are the cited proteins at pH 7.2) than their electrophoretic velocity. Neutral molecules (benzene) moved with the same velocity as the electroosmotic flow. The migration sequence was thus cations (if present), benzene (neutral), alcohol dehydrogenase, β-amylase and albumin. The electroosmotic flow was dependent on the concentration and pH of the buffer electrolyte, corresponding to the

degree of dissociation of the silanol groups. We then attempted to use 4% (v/v) benzene as a neutral marker in the corresponding buffer system for each analysis.

Migration velocities calculated for alcohol dehydrogenase ranged from 0.089 to 0.226 cm s^{-1}, β-amylase from 0.082 to 0.213 cm s^{-1} and albumin from 0.073 to 0.193 cm s^{-1} (Table I). It is known that the mobility increases by 2% when the temperature is raised by 1°C [4]. Although temperature differences inside capillaries are small (less than 1°C under realistic conditions), they can be as high as several tens of degrees between the wall of the capillary and the surrounding air [10]. Therefore, an effective thermostating device is important in order to obtain sufficiently reproducible migration times over a long period of use. In our system, control of temperature with a precision of ±0.01°C was achieved with an oven Peltier high-speed fan and resistive thermal device (RTD).

The electrophoretic mobility for anions ($\mu_e + \mu_{eo}$) can be calculated from eqn. (2) and expressed as

$$\mu_e + \mu_{eo} = \frac{\nu L}{V} \quad (3)$$

in $cm^2\ V^{-1}\ s^{-1}$.

It can be seen from the results in Fig. 3 that the electrophoretic mobilities varied as a consequence of the current applied inside the capillary, although this is not a direct relationship. The current is proportional to the resistance, which, in turn, is a function of the temperature, the applied voltage and the ionic strength of the electrolyte. Using the same buffer molarity, 15 m*M* borate–phosphoric acid (Fig. 3A), the electrophoretic mobility of alcohol dehydrogenase, β-amylase and albumin increased as the current increased from 40 to 56 μA, and the differences between each pair were maintained. At 25 m*M* borate–phosphoric acid buffer (Fig. 3B), an increase in the current form 63 to 69 μA produced higher electrophoretic mobilities, but the differences of these mobilities between each pair of proteins were greater than those observed with a 15 m*M* buffer electrolyte. Moreover, the electrophoretic mobilities of alcohol dehydrogenase and β-amylase were similar to those obtained at 63 μA when the current was 99 μA, but the mobility of albumin was much lower than that observed at 63 or 69 μA. This implies a better resolution of the last peak. The resistance of the capillary to the flow current at 75 m*M* borate–phosphoric acid buffer (Fig. 3C) was the highest and the electrophoretic mobility differences between alcohol dehydrogenase and β-amylase and between β-amylase and albumin decreased as the current increased. In this instance, a higher electrolyte concentration resulted in a higher

TABLE I

MIGRATION VELOCITIES OF ALCOHOL DEHYDROGENASE, β-AMYLASE, ALBUMIN AND BENZENE (NEUTRAL MARKER) IN CZE DEPENDING ON TEMPERATURE, VOLTAGE AND IONIC STRENGTH OF THE BUFFER USED AS ELECTROLYTE

Capillary, 70 cm × 75 μm I.D. (63 cm to the detector); separation solutions, (A) 15, (B) 25 and (C) 75 m*M* borate–phosphoric acid (pH 7.2).

Protein	Migration velocity, ν (cm s^{-1})						
	A		B			C	
	30°C, 17 kV	30°C, 22 kV	17°C, 17 kV	25°C, 17 kV	25°C, 22 kV	17°C, 22 kV	25°C, 17 kV
Benzene	0.206	0.278	0.141	0.166	0.237	0.182	0.142
Alcohol dehydrogenase	0.158	0.226	0.089	0.096	0.119	0.128	0.105
β-Amylase	0.149	0.213	0.082	0.088	0.106	0.122	0.096
Albumin	0.134	0.193	0.073	0.076	0.080	0.116	0.088

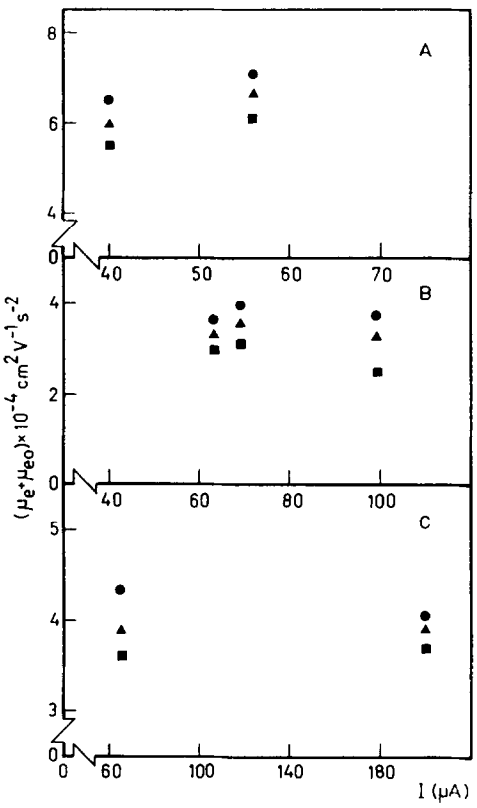

Fig. 3. Plot of electrophoretic mobility ($\mu_e + \mu_{eo}$) as a function of current (I) for (●) alcohol dehydrogenase, (▲) β-amylase and (■) albumin using as buffering electrolyte borate–phosphoric acid (pH 7.2) at (A) 15, (B) 25 and (C) 75 mM concentration. Electrophoresis was performed in a 70 cm × 75 μm I.D. (63 cm to the detector) capillary. Detection was achieved at 200 nm.

current being passed (199 μA) and therefore more heat to be dissipated. However, the electrolyte concentration of a solute in a zone or the change in solution conductivity in that zone will produce a change in the local potential gradient [8].

Factors affecting efficiency, resolution and selectivity of acidic proteins in CZE

Electroosmosis should not, in principle, affect the broadening of solute zones on the capillary for a given period of time, assuming longitudinal diffusion to be the main source of zone broadening [1,11]. However, electroosmotic flow can modify the time a solute resides in the capillary and then the efficiency and resolution are related to the flow-rate.

Fig. 4A–G illustrates this effect, where it can be observed how the band broadening of alcohol dehydrogenase, β-amylase and albumin differed according to the electroosmotic flow, which, in turn, is dependent on temperature, applied voltage and ionic strength of the electrolyte. Calculation of separation efficiency, including electroosmosis, as

$$N = \frac{(\mu_e + \mu_{eo})V}{2D} \quad (4)$$

where D is the diffusion coefficient of the solute, gave similar results for the three proteins, as can be seen from the results in Table II. The lowest separation efficiency was achieved by using 25 mM borate buffer at 25°C and 22 kV applied voltage. Under these electrophoretic conditions, the current was 99 μA and electrophoretic mobility decreased (Fig. 3B) with respect to that obtained at 63 and 69 μA using the same molarity of electrolyte. The highest efficiency was obtained at 199 μA with a temperature of 17°C and 22 kV applied voltage.

It has been demonstrated that high salt buffers reduce protein adsorption. Lysozyme (pI = 10.8) migrated as a sharp peak faster than the electroosmotic flow in a pH 9.0 high-salt buffer, even though the pH of the buffer was 2 units below the pI value of lysozyme [12]. Jorgenson [13] suggested that the protein adsorption may be due to the ion-exchange interaction between cationic sites in the protein and silica moities on the fused-silica surface.

The maximum differences in the number of theoretical plates between electrophoretic conditions that provide the highest (Fig. 4F) and the lowest (Fig. 4E) efficiency are 1409 for alcohol dehydrogenase, 1410 for β-amylase and 1623 for albumin. It is important to note that the separation efficiency, calculated from eqn. 4, is based on applied voltage but not on capillary length for similar diffusion coefficients. Under our experimental conditions, the diffusion coefficient of alcohol dehydrogenase ranged from $1.28 \cdot 10^{-5}$ to $3.22 \cdot 10^{-5}$ cm^2 s^{-1}, that of β-amylase from $1.17 \cdot 10^{-5}$ to $3.04 \cdot 10^{-5}$ cm^2 s^{-1} and that of albumin from $1.04 \cdot 10^{-5}$ to $2.76 \cdot 10^{-5}$ cm^{-2} s^{-1}

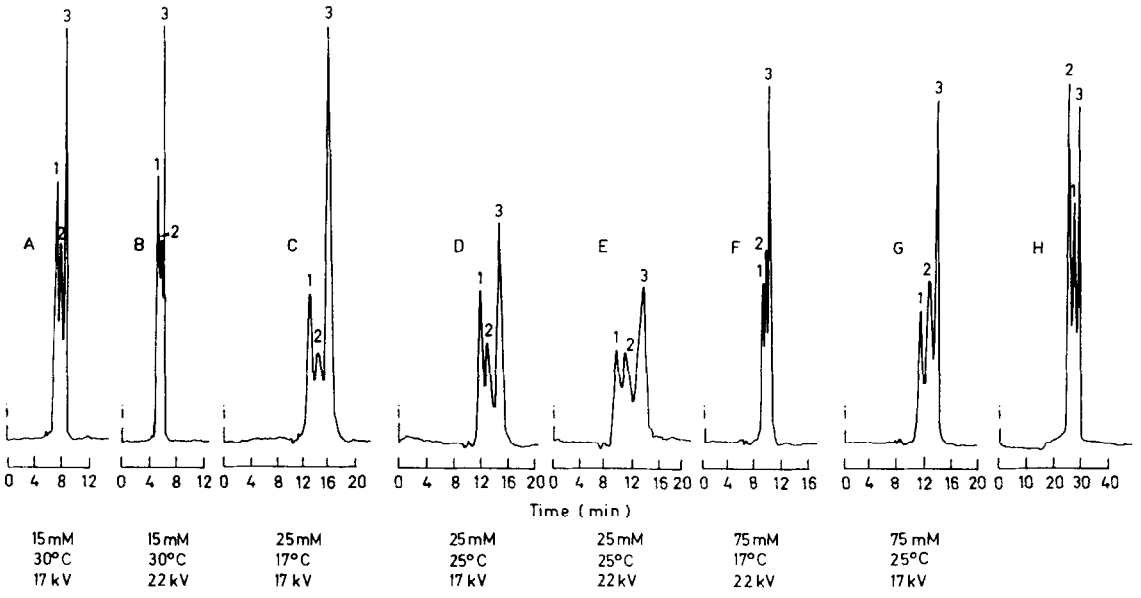

Fig. 4. (A–G) Electropherograms and (H) chromatogram of a mixture of (1) alcohol dehydrogenase, (2) β-amylase and (3) albumin. Proteins were prepared in a 1:5 dilution of the electrophoresis buffer. Electrophoresis was performed in a 70 cm × 70 μm I.D. (63 cm to the detector) capillary under the conditions specified in Table II. Detection was achieved at 200 nm. HPLC separation was performed in a 30 cm × 7.8 mm I.D. column packed with TSK G5000 PW XL. Mobile phase, 75 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 0.3 ml min^{-1}. Detection was achieved at 200 nm. i = Injection.

(data not shown). The values of this coefficient were calculated from Fick's first law:

$$J = -D \cdot \frac{\delta c}{\delta x} \quad (5)$$

where J is the flux density in mol cm^{-2} s^{-1} and $\delta c/\delta x$ is the gradient concentration in mol cm^{-4}. Given the limitation of efficient heat dissipation, maximum separation efficiency will be obtained at high voltages and low diffusion coefficients with the highest value of the sum of the coefficients for electrophoretic mobility (μ_e) and electroosmosis flow (μ_{eo}).

The resolution (R) was also influenced by the buffer electrolyte molarity, temperature and applied voltage (Table II). The resolution between alcohol dehydrogenase and β-amylase and between β-amylase and albumin was improved with an increase in the buffer concentration at the same applied voltage. At an applied voltage of 17 kV, the resolution between alcohol dehydrogenase and β-amylase ranged from 0.39 at 15 m*M* buffer to 0.56 at 25 m*M* buffer and to 0.71 at 75 m*M* buffer. However, the resolution between β-amylase and albumin was not so linear at either 17 or 22 kV.

The best resolution is observed when $\mu_{eo} = \mu_e$; however, the trade-off is an increased analysis time. Electroosmosis towards the cathode resulted in a better resolution of anions, as were the cited proteins at pH 7.2, which migrated against the electroosmotic flow and were carried back toward the cathode [8]. Isaaq *et al.* [14] also observed a higher resolution of dansylamino acids with 200 m*M* than with 100 m*M* phosphate buffer. Resolution can be improved by increasing the size of the buffer cation [15]. A limited means of improving resolution is to increase the voltage. To double the resolution, the voltage must be quadrupled. The key to high resolution is to increase $\Delta\nu$. The control of the migration velocity is best accomplished through selection of the proper mode of capillary electrophoresis coupled with selection of the appropriate buffer [16].

Differences in selectivity (α) between alcohol dehydrogenase and β-amylase and between β-amylase and albumin are listed in Table II. The

TABLE II

EFFICIENCY (THEORETICAL PLATES N), RESOLUTION (R) AND SELECTIVITY (α) OF ALCOHOL DEHYDROGENASE (AD), β-AMYLASE (βA) AND ALBUMIN (A) IN CZE DEPENDING ON TEMPERATURE, VOLTAGE AND IONIC STRENGTH OF THE BUFFER USED AS ELECTROLYTE

Capillary, 70 cm × 75 μm I.D. (63 cm to the detector).

Electropherogram[a]	Electrophoretic conditions				Protein	N^b (per cm)	R	α
	Temperature (°C)	Voltage (kV)	Buffer concentration (mM)	Current (μA)				
A	30	17	15	40	AD	245 221	0.39	1.06
					β-A	244 800	0.97	1.11
					A	243 932		
B	30	22	15	56	AD	244 899	0.31	1.06
					β-A	244 967	0.74	1.10
					A	244 267		
C	17	17	25	63	AD	244 375	0.56	1.09
					β-A	244 307	0.89	1.13
					A	244 749		
D	25	17	25	69	AD	244 873	0.59	1.09
					β-A	244 909	1.35	1.16
					A	244 372		
E	25	22	25	99	AD	243 924	0.47	1.13
					β-A	243 875	1.05	1.32
					A	243 126		
F	17	22	75	199	AD	245 333	0.42	1.05
					β-A	245 285	0.84	1.05
					A	244 340		
G	25	17	75	67	AD	244 896	0.71	1.09
					β-A	244 594	0.71	1.09
					A	244 207		

[a] Electropherograms in Fig. 4.
[b] Separation efficiency calculated as $N = (\mu_e + \mu_{eo})V/2D$.

results show that the optimum separation for each pair was obtained at 99 μA and 199 μA gave the worst α values. An increase in current was accompanied by higher selectivity with 15 and 25 mM borate buffer, whereas with 75 mM buffer this did not occur. This indicates that differences in selectivity exist, which means that the buffer anion concentration affects the selectivity under the experimental conditions used. Similar results were obtained by Atamna *et al.* [15] working with dansylated amino acids.

Separation of acidic proteins by size-exclusion HPLC

Fig. 4H illustrates the separation of alcohol dehydrogenase, β-amylase and albumin by size-exclusion chromatography with an isocratic mobile phase composition using 75 mM Tris–HCl buffer (pH 8.0). These proteins eluted at 27.3, 29.11 and 33.4 min, respectively. As separation in size-exclusion chromatography is based on a physical sieving process and not on chemical attraction and interactions [17], variation of

TABLE III

EFFICIENCY (THEORETICAL PLATES, N), RESOLUTION (R) AND SELECTIVITY (α) OF β-AMYLASE, ALCOHOL DEHYDROGENASE AND ALBUMIN IN SIZE-EXCLUSION HPLC

The isocratic conditions of the mobile phase and the column used are given in the text.

Protein	N^a (per cm)	R	α
β-Amylase	49 770	0.74	1.07
Alcohol dehydrogenase	57 300	0.80	1.06
Albumin	101 670		

[a] Efficiency calculated as $N = 16(t_r/W_b)$, where t_r is the retention time (min) and W_b is the peak width at half-height (cm).

either mobile phase ionic strength or flow-rate did not produce any improvement of selectivity. The chromatogram observed in Fig. 4H was the best obtained by using a TSK G5000 PW XL column.

Results for the efficiency, resolution and selectivity are given in Table III. The separation efficiencies for alcohol dehydrogenase (N = 49 770), β-amylase (N = 57 300) and albumin (N = 101 670) were lower than those obtained in CZE for the same proteins. The resolution and selectivity of each pair were similar to those achieved by capillary electrophoresis.

CONCLUSION

We have demonstrated the potential applicability of CZE where acidic proteins can be separated within 18 min or less by using unmodified fused-silica capillaries. The experimental conditions used in this work show advantages over surface-modified capillaries where the instability and limited tolerable pH range prevent their routine utilization. Size-exclusion HPLC is an alternative for the separation of proteins although the efficiency is much lower than in CZE. The other great disadvantage of size-exclusion HPLC is the high cost of HPLC columns compared with capillaries for electrophoresis.

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