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Separation of basic proteins by capillary electrophoresis using cross-linked polyacrylamide-coated capillaries and cationic buffer additives

A. Cifuentes, M. de Frutos, J.M. Santos and J.C. Diez-Masa*

Institute of Organic Chemistry (CSIC), Juan de la Cierva 3, 28006 Madrid (Spain)

ABSTRACT

A method for the preparation of fused-silica capillaries with a cross-linked polyacrylamide coating bonded to the internal wall is described. When these capillaries are used with acidic separation buffers containing 0.25 *M* morpholine as cationic additive to mask the residual effect of the negative charges on the silica surface, efficiencies between $3 \cdot 10^5$ and $5 \cdot 10^5$ plates/m can be obtained for basic proteins (p*I* 7–11). The reproducibility of migration times for these capillaries is better than 1% run-to-run and 2% capillary-to-capillary. The capillaries can be used for more than 90 h over the pH range 2–10 without any substantial alteration of the migration time or efficiency for the basic proteins assayed. It is also shown that the capillaries can be stored for more than 90 days in air with no appreciable variation in the migration time or the efficiency for several basic proteins.

INTRODUCTION

Capillary electrophoresis (CE) is an analytical and micropreparative technique with great potential, especially in the field of peptide and protein separations [1–3]. Using CE, almost all traditional modes of electrophoresis (free zone, sodium dodecyl sulphate polyacrylamide gel electrophoresis, isoelectric focusing, etc.) can be carried out. However, owing to the capillary format used in CE, shorter analysis times, higher efficiencies and fully automated operation can additionally be obtained with this technique.

Although theory predicts that efficiencies $>10^6$ theoretical plates could be obtained in the separation of proteins by CE, in practice a smaller plate number is obtained in the separation of such biopolymers. Among the several factors controlling band broadening in CE [4], protein adsorption on the internal wall of fused-silica capillaries seems to be the major cause of the low efficiency observed in some separations. Protein adsorption on the capillary can also cause poor migration time reproducibility and low recoveries. Protein adsorption is due to the electrostatic interaction between positively charged residues of the protein and negatively charged silanol groups which are intrinsic to the fused-silica surface. Many approaches have been devised to prevent this interaction. The first is pH manipulation. If the buffer pH is >11–12, most proteins do not have positively charged groups to interact with the capillary. Similarly, if the pH is <2, the capillary wall has no charge. Although working at the extremes of pH has been successful for some proteins [5,6], many of them are not stable or soluble under such conditions. In addition, working at very basic pH can dissolve the silica surface of the capillary, causing irreproducibility of analyte migration times.

The second approach consists in adding a positively charged ion (inorganic or organic) to the separation buffer. The added salt behaves as a competitor of the protein toward the silanol groups of the surface, therefore preventing pro-

* Corresponding author.

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tein adsorption on the wall. This idea was first introduced by Green and Jorgenson [7], who used K_2SO_4 as an additive to the buffer for the separation of some basic ($pI > 7$) proteins. Since then, other salts, such as KCl [8], cetyltrimethylammonium bromide [9,10], tetradecyltrimethylammonium bromide [11] and fluorosurfactant Fluoral FC-134 [12], have also been used. However, the high ionic strength necessary to avoid protein adsorption requires the use of capillaries of small inner diameter and low separation voltages because of the high conductivity of the buffer and the subsequent heat developed by the Joule effect. Zwitterionic additives, which have low electric conductivity, are a good alternative to cations in this approach [13,14]. However, the use of some zwitterions is limited by their low water solubility and the denaturing effect on some proteins.

In the third procedure, capillaries with their internal wall coated with an organic polymer such as the uncharged methylcellulose [15] or a positively charged synthetic polymer [16] are used. As the binding between the adsorbed polymer and the surface is fairly labile, these coatings are very unstable, giving rise to poor migration time reproducibility or requiring frequent capillary recoating.

Finally, a fourth approach has frequently been employed, utilizing capillaries with chemically bonded moieties on the fused-silica surface. Short organic functionalities such as glycero-glycidoxypropyl derivatives [17], arylpentafluoro moieties [18], diol coatings [19], maltose derivatives [19] or other silyl derivatives traditionally used in HPLC have been utilized. These coatings, although successful in certain instances, show poor hydrolytic stability at basic pH owing to the siloxane bond used for immobilization. The use of chemically bonded polymeric coatings has led to high efficiency and reproducible protein separations. Different polymers, including linear polyacrylamide [20,21], polyvinylpyrrolidone [17], poly(ethylene glycol) [22], polyethyleneimine [23], cross-linked polyacrylamide [24], hydroxylated polyethers [25], and even proteins [26], have been used in the preparation of these capillaries. Good separations of basic proteins have also been obtained using capillaries with C_{18} moieties chemically bonded to the capillary surface and surfactants or polymers adsorbed on them [27,28].

This paper describes a method for the preparation of fused-silica capillaries containing a bonded layer of cross-linked polyacrylamide. The associated effect of such a polymeric layer and some cationic additives in the buffer was evaluated as a means of improving the separation of basic proteins ($pI > 7$). Some model proteins such as lysozyme, cytochrome *c*, ribonuclease A and α-chymotrypsinogen were used to test the efficiency of the proposed method to prevent the interaction between basic proteins and the silica wall of the capillaries. These proteins represent a good model for those separations in which the analytes are stable only in a particular pH range (*i.e.*, antigen–antibody complexes, DNA fragment–antibody complexes, pH-sensitive proteins, etc.) and, therefore, CE procedures involving buffer pH control or buffers of high ionic strength cannot be used to prevent protein–capillary interactions.

EXPERIMENTAL

Instrumentation

Separations were carried out using a laboratory-made electrophoresis system. The apparatus included a Glassman (Whitehouse Station, NJ, USA) Model PS/EH50R2 power supply and a Linear Instruments (Reno, NV, USA) Model M-200 variable-wavelength UV–Vis detector with an in-house-modified flow cell operated at 230 nm. The cooling of the capillaries at room temperature was achieved with a fan. Electropherograms were recorded and analysed using an A/D converter (Flytech, Taiwan), an in-house-built amplifier, an Acer 500 PC computer (Multitech, Taiwan) and a Pascal program developed in this laboratory. During electrophoresis, the current through the capillary was measured using a Fluke (Everett, WA, USA) Model 83 multimeter. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 25 μm I.D., 360 μm O.D., 50 cm total length and 25 cm effective length (from the injection point to the detector) were used. Injection was carried out in the anode by electromigration.

The experiments on migration time reproducibility and optimization of morpholine concentration were accomplished in a Beckman (Fullerton, CA, USA) P/ACE 2000 HPCE electrophoresis apparatus controlled by an IBM PS/2 286 computer. The fused-silica capillaries used in this apparatus were similar to those used in the laboratory-made apparatus, but with 27 cm total length and 20 cm effective length. In this instance, the external temperature of the capillaries was maintained at 21°C. Injection was carried out in the anode using nitrogen pressure [0.5 p.s.i. (1 p.s.i. = 6894.76 Pa)]. Detection was effected at 214 nm. All the data were collected and analysed using System Gold software from Beckman running on the IBM PS/2 286 computer. In order to increase the migration time reproducibility, the capillaries were successively rinsed for 30 s each with water, air and buffer between injections.

Samples and chemicals

All the proteins (Table I) were purchased from Sigma (St. Louis, MO, USA) and used as received. The proteins were dissolved at the concentrations indicated in each case (ranging from 0.2 to 1 mg/ml) in water purified with a Milli-Q system (Millipore, Bedford, MA, USA), stored at 0°C and heated to room temperature before use. Phosphoric acid, sodium dihydrogenphosphate, tetramethylammonium bromide (TMAB), hexadecyltrimethylammonium bromide (HTMAB), potassium sulphate (all from Merck, Darmstadt, Germany), morpholine and triethylamine (both from Aldrich, Steinheim, Germany) were used in the different running buffers. Hydrochloric acid, sodium hydroxide (both from Merck), 3-methacryloxypropyl-3-trimethoxysilane (ABCR, Karlsruhe, Germany), acrylamide, N,N′-methylenebisacrylamide, ammonium peroxodisulphate and N,N,N′,N′-tetramethylethylenediamine (TEMED) (all from Schwarz, Cleveland, OH, USA) were used for the preparation of the cross-linked polyacrylamide-coated capillaries.

TABLE I

ISOELECTRIC POINTS OF THE PROTEINS USED

Protein	p*I*
Lysozyme, chicken egg white (Lys)	11.0
Cytochrome *c*, horse heart (Cyt *c*)	10.2
Ribonuclease A, bovine pancreas (Rib A)	9.3
α-Chymotrypsinogen, bovine pancreas (α-Chy)	9.2
Myoglobin, sperm whale skeletal muscle (Myo W)	8.1
Myoglobin, horse skeletal muscle (Myo H)	7.1

Buffers

A stock solution of 20 m*M* phosphate buffer (pH 5.5) was prepared by dissolving a weighed amount of sodium dihydrogenphosphate in Milli-Q-purified water and adding 1 *M* sodium hydroxide to adjust the pH to 5.5. Aliquots of this solution were used to prepare the buffers which contained the cationic additives. The additives (K_2SO_4, TMAB, HTMAB, triethylamine and morpholine) were dissolved in the phosphate buffer at the concentration indicated in each case. When necessary, the pH of these solutions was returned to 5.5 using concentrated phosphoric acid. The other buffers used were prepared by dissolving a weighed amount of sodium dihydrogenphosphate in Milli-Q-purified water and adjusting the pH with 1 *M* sodium hydroxide or 1 *M* hydrochloric acid. The buffers were stored at 4°C and heated to room temperature before use.

Preparation of cross-linked polyacrylamide-coated capillaries

The fused-silica capillaries were successively treated with 0.1 *M* hydrochloric acid for 1 h and 0.1 *M* sodium hydroxide solution for 2 h, then washed with 200 μl of Milli-Q-purified water and 200 μl of methanol. A length of 5 mm of the external polyimide coating was burned off in each capillary to make the detection window. The capillaries were flushed with 100 μl of a solution of 66% (v/v) of methacryloxypropyltrimethoxysilane in methanol and left for 3 h for reaction.

The polyacrylamide gel was prepared by dissolving a mixture acrylamide and bisacrylamide (4.2% C, 10% T) in thoroughly degassed 20 m*M* phosphate buffer (pH 5.5). To carry out polymerization, 3 μl of 10% (v/v) aqueous ammonium peroxodisulphate solution and the same amount

of 10% (w/v) TEMED solution in water were added per ml of buffer. Using such amounts of peroxodisulphate and TEMED, the polyacrylamide–bisacrylamide solution took about 45 min to reach a substantial degree of polymerization. This time interval was long enough to carry out the steps described below that are required for capillary preparation. After adding peroxodisulphate and TEMED, the polymerizing solution was homogenized using first a vortex mixer and then ultrasound for around 30 s each. Next, 50 μl of the polymerizing solution were injected into the capillaries prepared as indicated above. The solution was left for 1 min inside the capillaries before flushing them with nitrogen [0.8 atm (1 atm = 101 325 Pa)]. Immediately afterwards, the capillaries were heated at 140°C for 1 min in a gas chromatograph oven. The process of filling the capillary with polymerizing solution, flushing with nitrogen and heating was repeated three times in order to achieve a homogeneous coating of the inner capillary wall. Finally, in order to check that the gelling reaction had taken place within the capillaries, the unused portion of the polymerizing solution in its vial was heated in the oven for 1 min at 140°C.

RESULTS AND DISCUSSION

To ensure a strong interaction between proteins and silanol groups on the silica surface, which allow the evaluation of the masking efficiency of the different approaches investigated in this work, the buffer pH was fixed at 5.5. At this pH, a substantial proportion of the silanol groups on the silica surface are negatively charged and the proteins used (Table I) have a net positive charge, as can be deduced from their p*I* values.

Separation of basic proteins using uncoated and coated capillaries

The separation of three basic proteins (Cyt *c*, Rib A and α-Chy) using an uncoated fused-silica capillary and a 20 m*M* phosphate buffer (pH 5.5) gave rise to an electropherogram with a unique, tailing peak (result not shown). The poor peak shape is due to the strong electrostatic interactions that take place between the basic proteins and the silica surface at pH 5.5. When the same separation was carried out in the cross-linked polyacrylamide-coated capillaries using the same buffer, the separation of the three proteins was achieved. Reproducibility of the migration time for Cyt *c*, Rib A and α-Chy from run to run was 1.8, 2.8 and 3.4% (R.S.D., $n = 7$), respectively. This reproducibility is poor when compared with the value of less than 1% obtained using fused-silica capillaries with other coatings reported in literature [21,24,27]. The plate number achieved on the coated capillaries (around 10^5 plates/m for the three proteins tested) was almost one order of magnitude smaller than the values predicted by theory and about 3–5 times smaller than that obtained with other silanol-shielding techniques where the capillary–protein interactions have been reduced to a minimum [5,21,24]. This result indicates that some adsorption of the proteins on the capillary still occurs despite the polymeric layer covering the capillary wall. The low efficiency observed could be due to an inhomogeneous coating of the capillary, which could leave some areas of the fused silica uncovered, causing protein adsorption, and/or although the silica is completely covered by the polymeric coating, the small thickness of the layer could permit the electric charges on the silica surface to adsorb the proteins on the top of the polymeric layer; similar results have been observed previously with polyethyleneimine coatings [23].

Separation of basic proteins using uncoated and coated capillaries with cationic additives in the buffer

In order to mask the negative charges that still existed on the surface of the coated capillaries, we thought that an efficient method could be to use cationic additives in the separation buffer. Several additives, such as triethylamine, quaternary ammonium salts and morpholine, have traditionally been used in HPLC [29] to mask the effect of silanol groups. Others have been used for CE [30], particularly phosphate [17] and K^+ [7] ions. We compared the efficiency of an inorganic salt (K_2SO_4), two quaternary ammonium salts (TMAB and HTMAB), and two

amines (triethylamine and morpholine) as silanol maskers. To select the most appropriate additives to be tested with the coated columns, we first tried all of them in non-coated columns, expecting that in such demanding conditions the masking efficiency of each additive would be clearly demonstrated. In all instances, 20 m*M* phosphate buffers (pH 5.5) containing the additives studies at the concentrations indicated in each experiment were used.

When 0.25 *M* K_2SO_4 (a salt whose efficiency in preventing protein adsorption in CE has already been widely demonstrated [5,7,8,30]) was added to the buffer, good efficiency (around $4 \cdot 10^5$ plates/m) was observed for basic proteins. However, owing to the large electric current obtained with these buffers, a low voltage had to be used for the separation, causing a long analysis time (over 30 min). The two ammonium salts used led to electropherograms with broad peaks for the basic proteins, probably due to hydrophobic adsorption between the proteins and the alkylammonium moieties adsorbed on the capillary wall [31]. Finally, buffers containing 0.25 *M* morpholine or 0.25 *M* triethylamine were tried. Using both buffers, good efficiencies (between $1 \cdot 10^5$ and $5 \cdot 10^5$ plates/m) with a short analysis time (*ca.* 10 min) were obtained for the proteins tested. However, a poor reproducibility of the migration time of proteins (6–8.5% R.S.D.) was observed for both amines. It was concluded that morpholine and triethylamine presented some advantages over the other additives studied in terms of efficiency and seem to be better silanol maskers than the other additives. Consequently, we examined the effect of both buffers containing morpholine or triethylamine and coated columns in the separation of basic proteins.

The effect of buffers containing these two additives on the migration time reproducibility and efficiencies achieved on cross-linked polyacrylamide-coated columns is demonstrated in Table II. Better results were obtained in both respects with buffers containing morpholine. According to the pK_a value of these two additives (11.01 for triethylamine and 8.33 for morpholine), the percentage of protonated molecules is higher for triethylamine than for morpholine, and hence a higher shielding effect of the silanol groups would be expected for triethylamine at the working pH. The fact that a more efficient masking effect was observed for morpholine could be interpreted in terms of the stereochemistry of the two additives. Triethylamine, which has bulky ethylene groups surrounding the positively charged nitrogen atom, could have a higher steric hindrance than morpholine for the association with the negatively charged silanol groups on the surface.

In order to optimize the morpholine concen-

TABLE II

MIGRATION TIME REPRODUCIBILITY AND EFFICIENCY OBTAINED WITH CROSS-LINKED POLYACRYLAMIDE-COATED CAPILLARIES AND MORPHOLINE OR TRIETHYLAMINE AS ADDITIVE IN THE SEPARATION BUFFER

Buffer, 20 m*M* phosphate–0.25 *M* morpholine (pH 5.5) or 20 m*M* phosphate–0.25 *M* triethylamine (pH 5.5); capillary, coated 25 μm I.D., 360 μm O.D., 50 cm total length, 25 cm effective length; voltage, 25 kV; injection, 10 kV, 5 s; sample, 0.4 mg ml^{-1} Cyt *c*, 0.58 mg ml^{-1} Rib A and 0.51 mg ml^{-1} α-Chy; detection at 230 nm.

Protein	Morpholine			Triethylamine		
	t_m [a] (min)	R.S.D.$_t$ (%)	N [a] (plates/m)	t_m [a] (min)	R.S.D.$_t$ (%)	N [a] (plates/m)
Cyt *c*	9.6	1.3	322 000	6.8	1.8	256 000
Rib A	17.0	1.7	406 000	10.9	3.2	265 000
α-Chy	20.1	1.9	465 000	12.3	3.5	396 000

[a] t_m and N values are means value of five measurements.

tration, buffers containing 0.1, 0.25 and 0.5 *M* morpholine were compared in terms of the efficiency obtained for three basic proteins. In Table III it can be seen that for all the proteins assayed, the plate number reaches a maximum at 0.25 *M* morpholine. At lower concentrations of morpholine there are still some negative charges on the silica surface able to adsorb the proteins [32]. However, with buffers containing 0.5 *M* morpholine, the high current in the capillary (69 μA at 14 kV) could cause thermal overloading which leads to peak broadening.

In order to check the efficiency of the cross-linked polyacrylamide coating as a masker for the silanol groups of the silica surface, the electroosmotic flows and the electropherograms obtained on coated and uncoated capillaries with the same buffer [20 m*M* phosphate–0.25 *M* morpholine (pH 5.5)] were compared. Under these conditions, the electroosmotic coefficient for the coated capillary ($\mu_{eo} = 1.5 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}) was ten times smaller than that obtained for the uncoated capillaries ($\mu_{eo} = 1.7 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}). The separation of six basic proteins on uncoated and coated capillaries is shown in Fig. 1. As can be seen, peaks with low efficiency and poor resolution are obtained with uncoated capillaries owing to the adsorption of the proteins on the silica surface. The high electroosmotic flow observed for these capillaries also contributes to the low resolution obtained. Conversely, under the same separation conditions, the use of cross-linked polyacrylamide-coated capillaries gives a good separation of the basic proteins with efficiencies close to 500 000 plates (for Fig. 1B, lysozyme 310 000, cytochrome *c* 390 000, ribonuclease A 480 000, α-chymotryp-

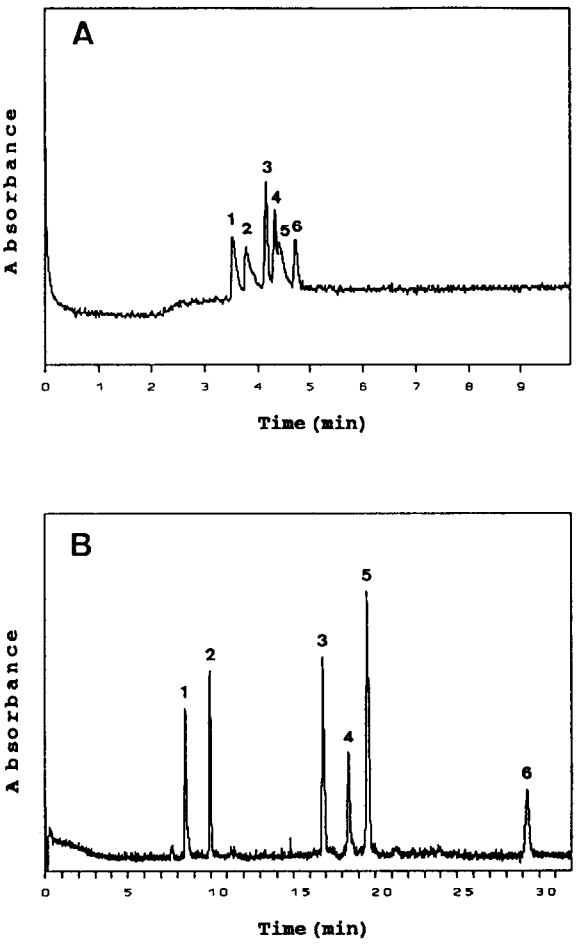

Fig. 1. Separation of basic proteins using (A) uncoated and (B) coated cross-linked polyacrylamide capillaries. Buffer, 20 m*M* phosphate–0.25 *M* morpholine (pH 5.5); capillary, 25 μm I.D., 360 μm O.D., 50 cm total length, 25 cm effective length; separation voltage, 20 kV (30 μA); injection at (A) 2.5 kV for 5s and (B) 4 kV for 5 s; detection at 230 nm. Peaks: 1 = 0.20 mg ml^{-1} lysozyme; 2 = 0.41 mg ml^{-1} cytochrome *c*; 3 = 1.07 mg ml^{-1} ribonuclease A; 4 = 0.21 mg ml^{-1} sperm whale myoglobin; 5 = 0.63 mg ml^{-1} α-chymotrypsinogen; 6 = 0.23 mg ml^{-1} horse myoglobin.

TABLE III

EFFECT OF MORPHOLINE CONCENTRATION ON THE EFFICIENCY FOR BASIC PROTEINS

Buffer, 20 m*M* phosphate–0.25 *M* morpholine (pH 5.5); capillary, coated with cross-linked polyacrylamide, 25 μm I.D., 360 μm O.D., 27 cm total length and 20 cm effective length; voltage, 14 kV; injection using nitrogen pressure (0.5 p.s.i.) for 2 s; sample, 0.25 mg ml^{-1} Cyt *c*, 0.31 mg ml^{-1} Rib A and 0.28 mg ml^{-1} α-Chy; detection at 214 nm.

Protein	Efficiency (plates/m)[a]		
	0.10 *M* morpholine	0.25 *M* morpholine	0.50 *M* morpholine
Cyt *c*	430 000	624 000	400 000
Rib A	300 000	422 000	280 000
α-Chy	300 000	323 000	200 000

[a] Reproducibility: 5% (R.S.D., $n = 5$).

sinogen 370 000, sperm whale myoglobin 430 000 and horse myoglobin 310 000 plates/m).

In order to gain some practical knowledge about the behaviour of the cross-linked polyacrylamide-coated capillaries working with buffers containing morpholine, the coating reproducibility, the stability of the coating at different buffer pHs and the column storage conditions were studied.

Coating reproducibility

The results of the study on the reproducibility of the coating are summarized in Table IV. The run-to-run reproducibility is excellent (R.S.D. 0.57–0.35%), as it would be expected from separations carried out on liquid thermostated (±0.1°C) capillaries. An acceptable day-to-day reproducibility is also obtained. More significant are the results obtained from three different capillaries prepared in the same batch (R.S.D. < 2%). This value is similar to those reported in the literature [21,27] for capillary-to-capillary reproducibility of coated tubes prepared by different methods. This means that a reasonable reproducibility could be expected between capillaries prepared in the same batch.

Coating stability

The stability of the polyacrylamide coatings during continued use with buffers in the pH range 2–12 was tested using a mixture of three basic proteins (Cyt *c*, Rib A and α-Chy) separated in a 20 m*M* phosphate–0.25 *M* morpholine buffer (pH 5.5). Several polyacrylamide-coated capillaries were prepared in the same batch. The test at each pH was run in a different capillary from the batch. Each test was performed in duplicate using different capillaries from the same batch. The capillary was filled with a very acidic (phosphoric acid for pH 2) or very basic (sodium hydroxide for pH 10–12) solution (henceforth called "pH test solution") and left for a certain amount of time. Then, the capillary was successively rinsed with 200 μl of water, air and 200 μl of the buffer used for the separation, electrolysed for 5 min at 20 kV and the migration time and efficiency for the basic proteins on the capillary were measured. The capillary was flushed with 500 μl of the pH test solution and left for a further period. The test lasted up to a total of 90 h. In order to verify if any appreciable modification took place on the polymeric coating by changing the capillary content from the buffer to the pH test solution and back to the buffer during the experiment, the separation of three basic proteins using a 20 m*M* phosphate–0.25 *M* morpholine (pH 5.5) buffer was carried out in a polyacrylamide-coated capillary. The capillary was then flushed with a new buffer consisting of 20 m*M* phosphate (pH 8), electrolysed for 5 min at 20 kV and the separation of acidic proteins (β-lactoglobulin A, β-lactoglobulin B and α-

TABLE IV

MIGRATION TIME REPRODUCIBILITY USING CROSS-LINKED POLYACRYLAMIDE-COATED CAPILLARIES AND MORPHOLINE IN THE SEPARATION BUFFER

Voltage, 12 kV; injection using nitrogen pressure (0.5 p.s.i.) for 4 s; sample: 0.6 mg ml^{-1} Cyt *c*, 0.7 mg ml^{-1} Rib A and 0.5 mg ml^{-1} α-Chy; other conditions as in Table III.

Protein	Run-to-run, ($n = 4$)[a]		Day-to-day, 3 days ($n = 12$)[a]		Capillary-to-capillary, 3 capillaries ($n = 12$)[a]	
	t_m (min)	R.S.D. (%)	t_m (min)	R.S.D. (%)	t_m (min)	R.S.D. (%)
Cyt *c*	9.33	0.35	9.36	0.94	9.31	1.27
Rib A	13.45	0.54	15.43	1.31	15.36	1.81
α-Chy	17.75	0.57	17.73	1.48	17.69	1.96

[a] *n* represents the total number of measurements carried out.

lactalbumin) was run several times. Next, the capillary was flushed with 200 μl of water, air and 200 μl of the initial buffer (morpholine buffer) and the separation of the basic proteins was run again under the above conditions. We observed that it was also necessary to electrolyse the capillary (20 kV for 5 min) before obtaining good reproducibility for the separation of the basic proteins. By using this cleaning routine, the reproducibility of the migration time before and after the replacement of buffer was within 1–1.5% R.S.D. ($n = 10$). This result indicates that it is possible to change from one buffer that contains morpholine to another one that does not and then back to the morpholine-containing buffer without modifying the migration time of the proteins in the capillaries.

A similar result, but with poor reproducibility [10–15% R.S.D. ($n = 10$)], was observed for the efficiency with basic proteins. This result also shows that with our polyacrylamide coating, a cleaning routine consisting in flushing the capillary with 200 μl of water, air and 200 μl of buffer and electrolysing it for 5 min at 20 kV seems to be very effective.

The results obtained for the pH stability test (Fig. 2) indicate that the stability of the polyacrylamide cross-linked capillaries is fairly good between pH 2 and 10. Within this pH range, the reproducibility of the migration time remains between 1 and 2.5% (R.S.D.) for a period of more than 90 h of continued use of the capillary. At pH 11–12, the stability of the column decreases in a short time, as shown by the continu-

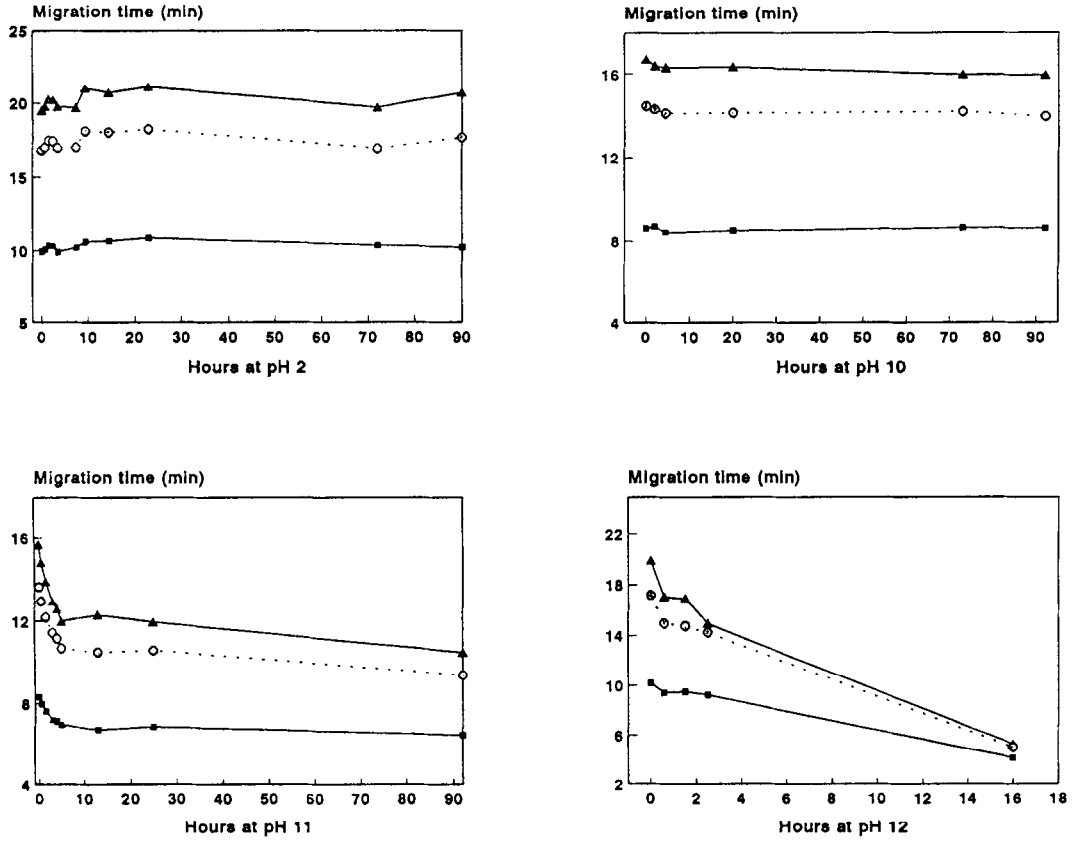

Fig. 2. Stability of the migration time for cross-linked polyacrylamide coatings in the pH range 2–12. Buffer, 20 mM phosphate–0.25 M morpholine (pH 5.5); capillary, 25 μm I.D., 360 μm O.D., 50 cm total length, 25 cm effective length; separation at 25 kV (40 μA); injection at 2.5 kV for 5 s. Sample: ■ = 0.41 mg ml^{-1} cytochrome c; ● = 1.07 mg ml^{-1} ribonuclease A; ▲ = 0.65 mg ml^{-1} α-chymotrypsinogen.

ous decrease of the protein migration time. This could be due to hydrolysis of the bonds that anchor the polymer to the silica [21], leaving an increasing number of free silanol groups uncovered. The increasing electroosmotic flow detected for the coated capillaries subjected to the test solution of pH 11–12 is responsible for the decrease in the migration time observed. On the other hand, a study of the stability of the cross-linked polyacrylamide coating in terms of the efficiency for basic proteins (Fig. 3) shows that the band broadening remains reasonably constant between pH 2 and 10 and quickly decreases at higher pH. As indicated before, degradation of the coating could leave an increasing number of silanol groups uncovered, which are able to adsorb the basic proteins, giving rise to this broadening of the protein peaks.

Column storage

The stability of the cross-linked polyacrylamide coating with respect to the storage time and the substance used to fill the capillary during storage was studied. In a similar fashion to that used for the coating stability test, a mixture of three basic proteins (Cyt *c*, Rib A and α-Chy) separated in a 20 m*M* phosphate–0.25 *M* morpholine buffer (pH 5.5) was used to check the variations of migration time and protein efficiency with the storage conditions. Three different capillaries containing either water, air or buffer [20 m*M* phosphate–0.25 *M* morpholine (pH 5.5)] were kept closed by pinning each end in a small piece of rubber. When the capillary was stored in water, the capillary was flushed with air, rinsed with 200 μl of the separation buffer and electrolysed for 5 min at 20 kV before

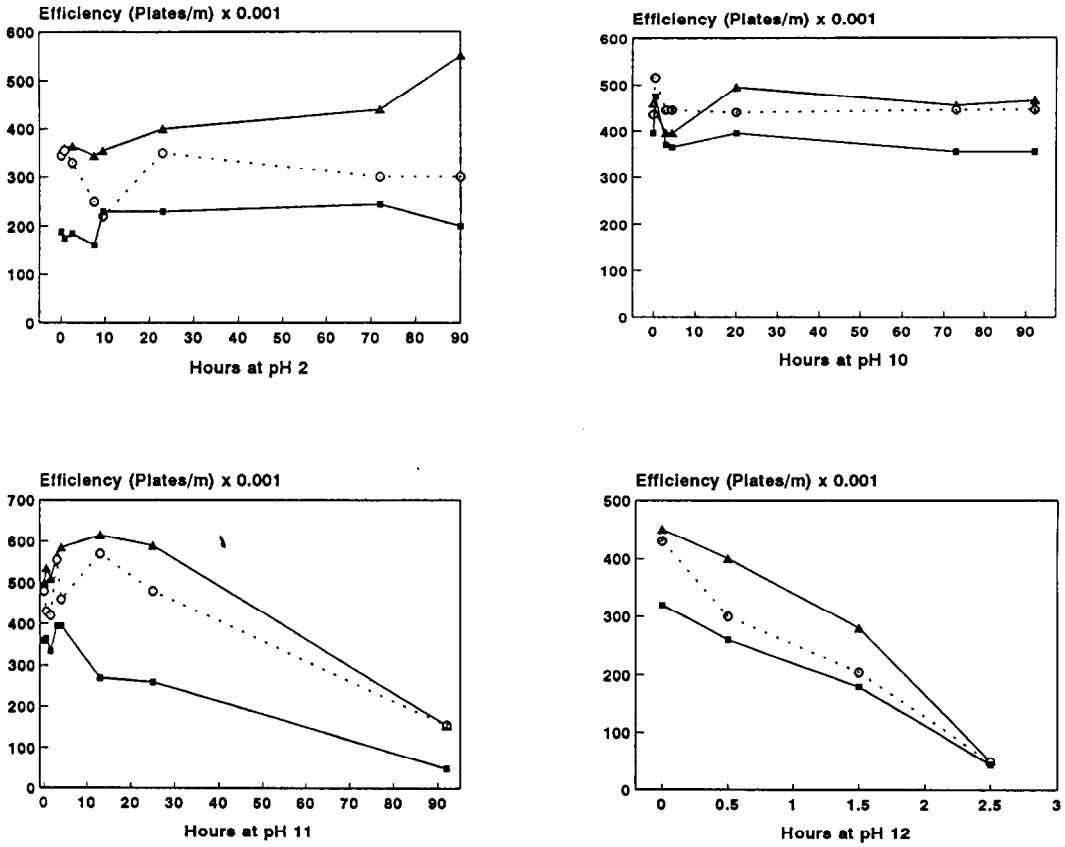

Fig. 3. Stability of the efficiency for cross-linked polyacrylamide coatings in the pH range 2–12. All other conditions and symbols as in Fig. 2.

the test was carried out. Likewise, when the capillary was stored in air, the buffer contained in the capillary was rinsed with 200 μl of water and air after the test.

The best results were obtained when capillaries were stored in air. After 90 days of storage, only a small decrease (*ca.* 1%) of the migration time was observed for the three proteins. However, the capillaries containing water or buffer showed a steady decrease in the migration time of the proteins with storage time (20% and 10%, respectively, in 90 days). Similar results were obtained for the protein efficiency on storage. While the capillaries containing air showed no decrease in efficiency for the three proteins tested, the capillaries kept in water or buffer showed a substantial decrease in efficiency after 12 days of storage. In conclusion, the cross-linked polyacrylamide coatings can be stored for a long period (at least 90 days) in air. After use of the capillaries and before storage, a thorough wash with water is advised in order to flush out the buffer used.

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