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Separation of metallothionein isoforms with capillary zone electrophoresis using an uncoated capillary column Effects of pH, temperature, voltage, buffer concentration and buffer composition

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Abstract

Metallothionein (MT) isoforms, sulphydryl-rich proteins with a high affinity for both essential and toxic trace metals, are readily separated by capillary zone electrophoresis (CZE) with an uncoated polyimine-clad fused-silica capillary using Tris–borate buffer. The metallothionein samples investigated were rabbit liver MT, rabbit liver MT-1, rabbit liver MT-2 and horse kidney MT. The effects of temperature, buffer pH, buffer concentration, buffer composition and running voltage on the separation efficiency were investigated. An improved separation efficiency compared with published methods using uncoated fused-silica capillaries was obtained, allowing the separation of peaks which previously co-migrated. The running conditions used were voltage 11 kV, temperature 20°C and buffer 110 m*M* Tris–110 m*M* borate (pH 6.90). Under these conditions, horse kidney MT exhibited five putative isoform peaks and rabbit liver MT nine or ten peaks. Interestingly, the experiments indicated that rabbit liver MT contains one major component which is not found in either isoform MT-1 or MT-2, possibly indicating that the improved separation efficiency made it possible to detect a new group or structure. Based on this initial evaluation, CZE with an uncoated fused-silica capillary using Tris–borate buffer appears to be a very useful method for the separation of metallothionein isoforms.

Keywords: Buffer composition; pH effects; Temperature effects; Metallothioneins

1. Introduction

Continuing interest in the unique chemical and physical properties of metallothionein (MT), a low-molecular-mass sulphydryl-rich protein with a high affinity for both essential and toxic trace metals, and its suggested role in heavy metal metabolism and detoxification [1–7] have led to numerous investigations into the mechanisms involved in MT gene expression. Metals such as zinc, copper and cadmium are bound via thiol linkages from cysteine residues which are clustered in two metal-binding domains within the protein molecule. MT has two major isoforms

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(MT-1 and MT-2) which have p*I* values between 3.9 and 4.6 and differences in charge due to certain amino acid substitutions. These isoforms are rich in cysteine (33%) residues with a highly conserved sequence [3]. MT isoforms arise from genetic polymorphism, which is found in many species [8].

In order to study the functional significance of individual MT isoforms, analytical techniques that offer a high resolution efficiency are required. First, high-performance liquid chromatography (HPLC) coupled with size-exclusion HPLC with metal detection by atomic absorption spectrometry (AAS) was used [9]. The combination of size exclusion and the weak cation-exchange properties of the columns used led to MT isoforms which were not always completely resolved. An anion-exchange chromatographic method resolved the two major charge classes of MTs, designated MT-1 and MT-2 [10]. Later, reversed-phase (RP) HPLC techniques were developed which offered a higher resolution efficiency, being able to resolve subisoforms within each of the two charge classes [11]. However, the requirements for organic solvents and expensive columns restrict the use of RP-HPLC techniques. Separations of purified mammalian MT at either neutral or alkaline pH by polyacrylamide gel electrophoresis also revealed two charge-distinct classes of isoforms [12]. Recently, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) using uncoated fused-silica capillary columns have shown promising results [13–18]. However, for many MTs, complete separation of all isoforms by using either of these two approaches was not achieved. The best results were obtained using acidic buffers, which cause metal dissociation from MTs, resulting in apoprotein forms (apothioneins), not usually present under the neutral conditions occurring in biological samples. The use of surface-modified coated capillaries resulted in improved separations, but still the complete separation of MT isoforms remained to be achieved [19,20]. Surface-modified capillaries also have a shorter lifetime and are more fragile and expensive than untreated capillaries.

The aim of this work was to investigate the possibilities of developing a rapid and sensitive technique to separate individual MT isoforms using uncoated capillary CZE. Several buffers were evaluated for this purpose. Previously, acidic (phosphate, pH 2.5) or basic [borate (pH 8.4) or Tris-HCl (pH 9.1)] buffers were used for the separation of metallothionein isoforms [13–18]. Based on this, other types of proteins, peptides and oligonucleotides have been separated successfully by using a mixed Tris–borate buffer at pH 7.5–8.1 [21–23]. Our preliminary experiments with Tris–borate buffer also showed promising results, and was therefore chosen for a thorough investigation. The effects of voltage, pH, temperature, buffer concentration and buffer composition of the selected Tris–borate buffer system was evaluated for this purpose.

2. Experimental

Capillary zone electrophoresis (CZE) was performed on a P/ACE System 5000 capillary electrophoresis system (Beckman, Fullerton, CA, USA). Data were collected and processed with System Gold software (Beckman). Fused-silica capillaries of 50 or 75 μm I.D. and 375 μm O.D. with polyimide cladding were obtained from Beckman. The overall capillary length was 57 cm with on-line detection at 50 cm. The capillary was housed in a cartridge which allowed liquid cooling to maintain a constant capillary temperature at a chosen value during the run. The capillaries were prepared by flushing the capillary columns for 10 min each with 1 *M* HCl, 1 *M* sodium hydroxide solution and doubly distilled water. The capillaries were then rinsed with buffer solution for 5 min. Between the sample analyses the 75 and 50 μm I.D. capillaries were rinsed for 2 or 3 min with 1 *M* sodium hydroxide, doubly distilled water and buffer successively. Pressure injections (0.5 p.s.i.) for 5 and 10 s were used for 75 and 50 μm I.D. capillaries, respectively. The separated components were detected at 200 nm. All chemicals for buffer solutions were of research grade (Merck, Darmstadt, Germany). All buffers were prepared with doubly

Table 1
Effect of temperature on migration times and observed mobilities (μ_{obs})[a] of horse kidney MT isoforms

Temperature (°C)	Migration time (min)					Mobility ($\times 10^8$ $m^2/V \cdot s$)				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	μ_{obs1}	μ_{obs2}	μ_{obs3}	μ_{obs4}	μ_{obs5}
15	20.99	21.48	21.48	22.57	22.90	2.057	2.010	2.010	1.913	1.886
20	19.18	19.49	19.71	20.53	20.97	2.251	2.216	2.191	2.103	2.059
25	16.98	17.09	17.33	17.93	18.19	2.543	2.527	2.492	2.408	2.374
30	15.11	15.11	15.29	15.75	15.97	2.858	2.858	2.824	2.742	2.704
35	13.40	13.40	13.51	13.84	14.02	3.223	3.223	3.196	3.120	3.080

[a] $\mu_{obs} = lL/t_m U$, where l is the capillary length to the detector, L is the total length of the capillary, t_m is the migration time and U is the running voltage.

distilled water and were degassed in an ultrasonic bath. The pH of the buffers was adjusted by adding either 0.1 *M* sodium hydroxide or 0.1 or 1 *M* HCl to the solutions. The buffers were filtered using a 0.2-μm filter (Gelman Sciences, Ann Arbor, MI, USA). Metallothionein samples, rabbit liver MT (MT-1 + MT-2), rabbit liver MT-1, rabbit liver MT-2 and horse kidney MT were purchased from Sigma (St. Louis, MO, USA). Metallothionein samples were dissolved in doubly distilled water at a final concentration of 1.0 mg/ml in order to enhance the resolution by stacking. Metallothionein samples were prepared weekly and stored at 4°C and an aliquot was taken daily from this stock solution.

3. Results and discussion

3.1. Effect of temperature on separation

The effects of temperature on the migration times and mobilities of horse kidney MT peaks are shown in Table 1. A constant voltage (11 kV), pH (6.90) and buffer concentration (110 m*M* Tris–110 m*M* borate) were used in all measurements. Horse kidney MT exhibited five peaks (labelled 1–5 in Fig. 1A at pH 6.79) under the experimental conditions used. A linear dependence of migration times on temperature was observed throughout the whole temperature range examined (15–35°C). The migration times

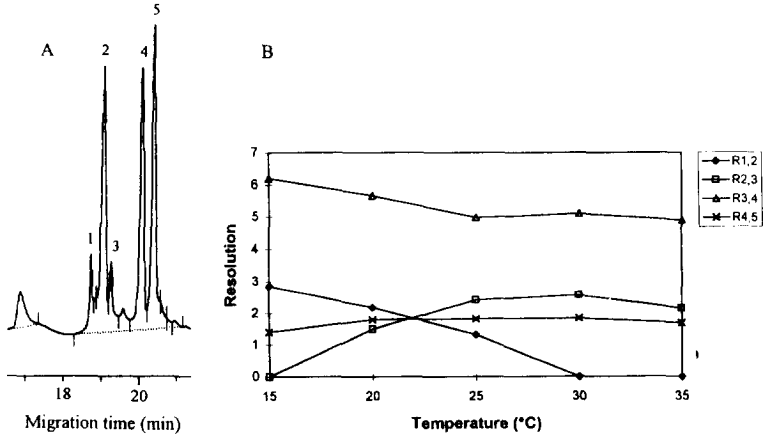

Fig. 1. (A) Electropherogram of horse kidney MT obtained by using 110 m*M* Tris–110 m*M* borate buffer (pH 6.79), temperature 20°C, voltage 11 kV and 57 cm × 75 μm I.D. uncoated fused-silica capillary. (B) Effect of temperature on resolution of horse kidney MT isoforms. Running conditions as in (A) except pH (6.90) and variable temperature (15–35°C).

decreased with increasing temperature. This phenomenon is common in CZE owing to temperature-induced viscosity changes resulting in enhanced electroosmotic flow (EOF) [24]. Small differences in temperature effects on different isoforms were noticed, which is a result of temperature effects on the observed mobilities of the isoforms (μ_{obs}). Neighbouring peaks 1 and 2 co-migrated at 30 and 35°C and peaks 2 and 3 co-migrated at 15°C, but they were separated at other temperatures, as can be seen in Table 1. The best resolution was obtained at 20°C, and that at 25°C was only slightly poorer (Fig. 1B).

With rabbit liver MT, the effect of temperature on both the migration times and resolution of isoforms was clearly observed in particular parts of the electropherogram. At higher temperatures (30 and 35°C) one putative isoform peak (labelled 5) was observed, migrating just behind one major peak 4 (Fig. 2A), but at lower temperatures (<25°C) it migrated together with the major peak (Fig. 2B). This is due to temperature effects on electrophoretic mobilities and on EOF as in the case of horse kidney MT, which could enhance the separation of species migrating in the opposite direction to the EOF. On the other hand, the separation between faster migrating isoforms improved with decreasing temperature. This could be partly a result of the temperature dependence of the chemical equilibrium. Based on the results obtained, 20°C was chosen for subsequent experiments.

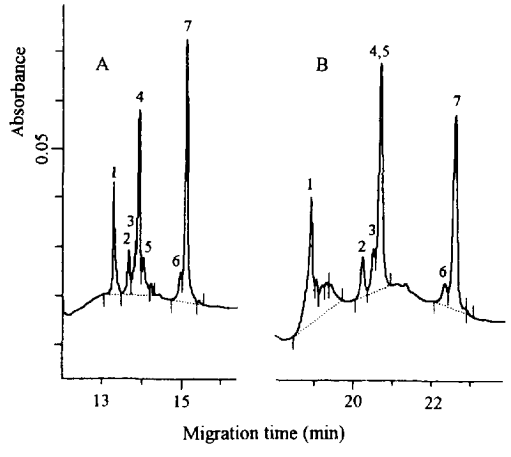

Fig. 2. Electropherograms of rabbit liver MT sample run at (A) 30°C and (B) 20°C. Other conditions as in Fig. 1B.

3.2. Effect of buffer concentration on separation

Another factor which significantly affected the separation efficiency and resolution was the buffer concentration. Its effect on separation was studied. The concentrations used were 50, 70, 90, 110, 130 and 150 m*M* for both Tris and borate. Other parameters (voltage, pH and temperature) were kept constant at 11 kV, 6.90 and 20°C, respectively. As expected, the migration times of putative isoforms of horse kidney MT increased with increasing buffer concentration owing to the increasing viscosity of the buffer. The currents also increased owing to the higher ionic strength. The increase in migration times and the decrease in electrophoretic mobilities versus buffer concentration were linear.

As can be seen from Fig. 3, the resolution between isoform peaks was affected greatly by changes in buffer concentration. At low concentration (50 m*M*), peaks 1 and 2 (labelling of Fig. 1A) migrated together, but using a higher concentration separation was achieved and the resolution increased until it reached a nearly constant level at 90 m*M*. On the other hand, the resolution between peaks 2 and 3 was only slightly affected by the buffer concentration. A critical part of the electropherogram was the separation of isoform peaks 4 and 5. A good

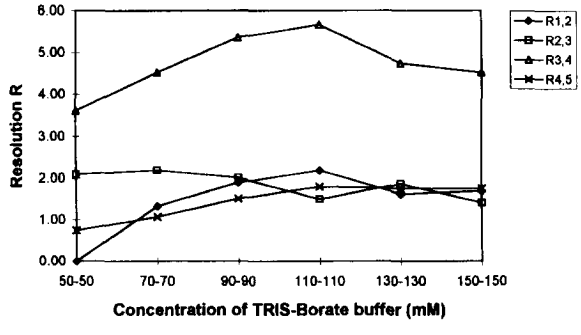

Fig. 3. Dependence of the resolution of horse kidney MT isoforms on the concentration of the Tris–borate buffer. Temperature, 20°C; voltage, 11 kV; buffer pH, 6.90; uncoated fused-silica capillary, 57 cm × 75 μm I.D.

baseline separation was achieved using 110 m*M* or higher buffer concentrations. It must be noted that when using 75 μm I.D. capillaries, the current increased rapidly at higher concentrations. The current also increased during the run, which did not occur using lower concentrations. This is obviously due to Joule heating, indicating also an insufficient cooling capacity. Therefore, 110 m*M* seemed the most suitable concentration for both Tris and borate.

The buffer concentration clearly has an effect on the separation of many putative rabbit liver MT isoforms. Interestingly, using a 70 m*M* buffer concentration, the same putative isoform peak as occurred in temperature dependence experiments at 30°C (labelled 5 in Fig. 2A) could be separated, but the separation of some other peaks was lost. At 90 m*M* a better overall separation was obtained but the isoform peak again migrated together with the main peak (data not shown). The resolution between faster migrating isoform peaks seemed also to diminish at higher concentrations (130 and 150 m*M*).

3.3. Effect of buffer composition

The effect of buffer composition was examined using the buffer concentrations mentioned in Table 2. Tris–HCl buffer (pH 6.52) was also used. Other parameters, voltage (11 kV), temperature (20°C) and buffer pH (6.90), were kept constant. Horse kidney MT exhibited two major peaks and one smaller peak when Tris–HCl buffer was used (Fig. 4A). The second major peak showed severe front tailing, indicating unresolved peaks. The change in buffer composition affected the migration times of the horse kidney MT isoforms only slightly when Tris was the major buffer component. A linear negative dependence was observed when borate was the major buffer component and the amount of Tris decreased. The change in Tris–borate buffer composition affects the separation efficiency and resolution. Isoform peaks 1 and 2 were not resolved using concentration of 110 and 30, 90 and 110, 70 and 110, 50 and 110 or 30 and 110 m*M* Tris and borate, respectively, in the buffer.

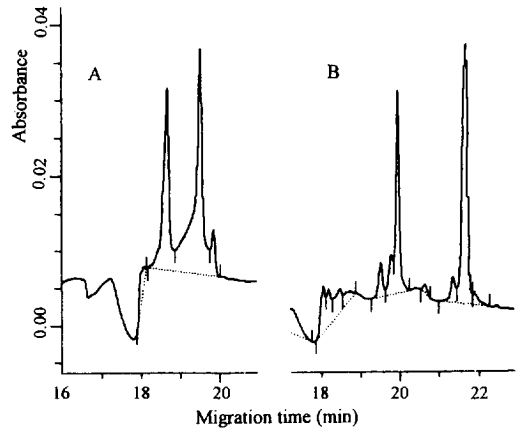

Fig. 4. Electropherograms obtained from (A) horse kidney MT and (B) rabbit liver MT using 110 m*M* Tris–HCl buffer (pH 6.52). Other conditions as in Fig. 3.

Table 2
Effect of composition of Tris–borate buffer on resolution and migration times of horse kidney MT isoforms

Buffer concentration (m*M*)	Resolution $R_{s,r}$				Migration time (min)				
	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,5}$	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
110–30	0.00	1.10	3.77	1.84	19.48	19.48	19.72	20.36	20.72
110–50	2.09	1.18	3.89	1.76	18.29	18.80	19.03	19.66	19.99
110–70	2.37	1.21	4.57	1.77	18.31	18.77	19.01	19.66	19.97
110–90	2.48	1.29	4.69	1.82	18.34	18.70	18.96	19.62	19.92
110–110	2.19	1.49	5.67	1.80	19.26	19.57	19.79	20.60	20.91
90–110	0.00	1.81	4.55	1.60	16.93	16.93	17.16	17.78	17.99
70–110	0.00	1.23	5.24	1.08	14.81	14.81	14.91	15.45	15.59
50–110	0.00	2.41	6.61	0.55	12.49	12.49	12.89	13.29	13.36
30–110	0.00	1.84	1.99	0.82	9.99	9.99	10.29	10.74	10.84

The resolution between peaks 2 and 3 improved slightly at increasing borate concentration when the concentration of Tris was kept constant at 110 m*M*. Also, the resolution between peaks 3 and 4 improved slightly until a drastic loss in resolution was observed at 30 m*M* Tris–110 m*M* borate (Table 2). This was mostly due to the mis-shaped peak 4 (data not shown). Separation of putative isoform peaks 4 and 5 was nearly constant when the Tris concentration was 110 m*M* and borate was either a minor component or at an equal level. When borate became the dominant component, the resolution decreased from 1.82 to 0.82.

Rabbit liver MT was not affected so much by the buffer composition when Tris was the dominant component. Even with Tris–HCl buffer a fairly good separation was achieved (Fig. 4B). A noticeable change was observed when borate became the dominant component in the buffer. The separation of early migrating peaks was immediately lost and the overall separation became poorer with increasing borate dominance (Fig. 5). In order to achieve a good separation for both horse kidney and rabbit liver MT isoforms, a composition of 110 m*M* Tris–110 m*M* borate was chosen.

3.4. Effect of buffer pH

The effect of buffer pH on the separation of metallothionein isoforms was also investigated. The parameters voltage (11 kV), temperature (20°C) and buffer concentration (110 m*M* Tris–110 m*M* borate) were kept constant. Horse kidney MT exhibited only three peaks at buffer pH 5.52 (Fig. 6). The peaks labelled 1, 2 and 3 in Fig. 1A migrated together. When the pH was increased to 6.20 the separation improved so that peaks 1 and 2 were resolved, but peaks 2 and 3 still migrated together. Fig. 7 shows clearly how peaks 2 and 3 were resolved above pH 6.47 and how the resolution improved, reaching a value of 4 when the pH was increased to 8. Large changes were also observed in the resolution between peaks 3 and 4. The resolution slowly decreased when the pH was increased from 5.52 to 6.90, but above pH 6.90 there was a drop in resolution from 5.67 to 1.81. Both of these effects could

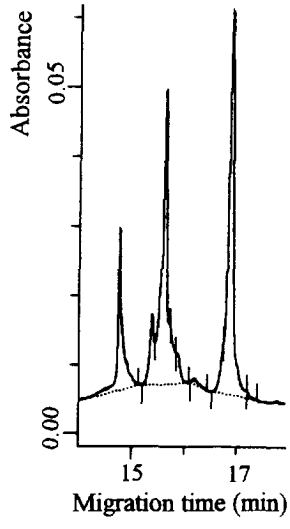

Fig. 5. Separation of rabbit liver MT obtained using 70 m*M* Tris–110 m*M* borate buffer (pH 6.90). Other conditions as in Fig. 3.

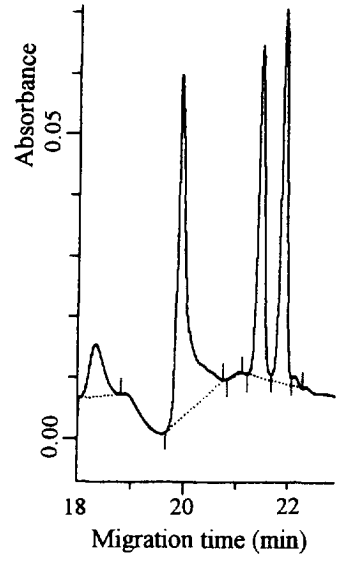

Fig. 6. Electropherograms of horse kidney MT obtained by using 110 m*M* Tris–110 m*M* borate buffer (pH 5.52). Other conditions as in Fig. 3.

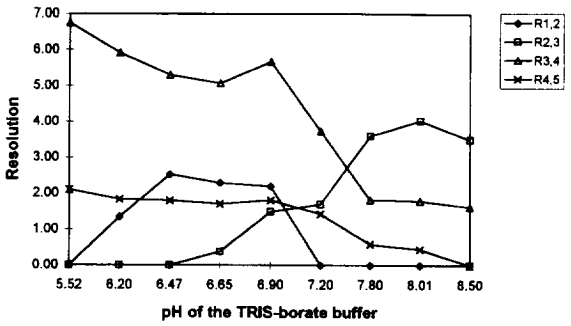

Fig. 7. Dependence of the resolution of horse kidney MT isoform peaks on the pH of the 110 m*M* Tris–110 m*M* borate buffer. Other conditions as in Fig. 3.

Table 3
Effect of buffer pH on migration times of horse kidney MT isoforms

Buffer pH	Migration time (min)				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
5.52	19.93	19.93	19.93	21.46	21.92
6.47	19.39	19.89	19.89	21.07	21.44
6.90	19.21	19.52	19.75	20.62	20.89
7.20	19.45	19.45	19.64	20.22	20.50
7.80	15.93	15.93	16.63	16.97	17.10
8.01	14.46	14.46	15.13	15.45	15.55
8.50	10.99	10.99	11.47	11.72	11.72

indicate changes in isoform structures, maybe in the charge distribution. Differences in the degree of ionization gives rise to differences in electrophoretic and electroosmotic mobilities. Consequently, both the separation efficiency and flow velocities are affected by changes in the buffer pH. Closely migrating horse kidney MT isoform peaks 4 and 5 seemed not to be affected very much when the pH was 6.90 or below, but above pH 6.90 the resolution decreased very rapidly. Obviously, the best separation was obtained at pH 6.90 under the experimental conditions used. A blank solution was used to investigate whether the peaks obtained were totally from metallothionein samples or if they were partly system peaks. The blank solution showed no peaks in the migration zone of MT isoform peaks. The buffer pH affected the migration times of horse kidney MT isoforms only slightly below pH 7.20, but above this pH the migration times decreased very strongly with increasing pH (Table 3). Logically a strong increase in mobilities was also observed above pH 7.20. The same effect was also noticed with migration times and mobilities of rabbit liver MT isoforms.

Rabbit liver MT seemed also to be affected by changes in the pH of the buffer. At low pH (6.20 and below) a total of ten or eleven putative peaks of various intensities but with two main peaks were observed (Fig. 8A). At higher pH the number of peaks decreased to three or four (Fig. 8B). A difference in the electropherograms obtained at higher pH was especially noticed for peaks migrating just after the EOF: four peaks were exhibited at lower pH instead of one or two at higher pH. This could be due to changes in the charge distribution in amino acid substituents. Also the overall separation resolution become poorer with increasing pH, especially above pH 7.2. The separations of rabbit liver MT and horse kidney MT were affected differently by the buffer pH. A buffer pH between 5.50 and 6.20 gave the best separation for rabbit liver MT but

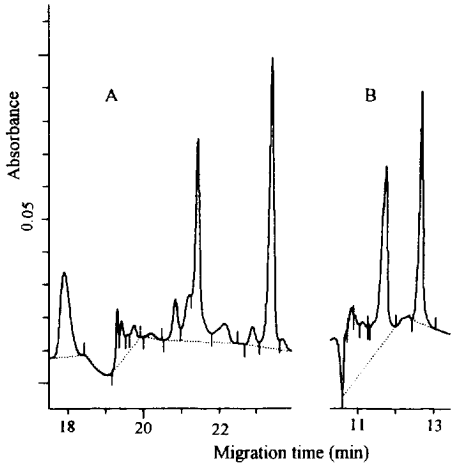

Fig. 8. Electropherograms of rabbit liver MT obtained using 110 m*M* Tris–110 m*M* borate buffer of pH (A) 5.52 and (B) 8.50. Other conditions as in Fig. 3.

pH 6.90 for horse kidney MT. Because of the more dramatic change in the horse kidney MT separation due to changes in buffer pH, the best overall separation for both rabbit liver MT and horse kidney MT was achieved at buffer pH of 6.90.

3.5. Effect of running voltage

Rabbit liver MT and horse kidney MT samples were run at different voltages in order to study the effect of voltage on the separation. As shown in Fig. 9, voltage clearly had an effect on resolution. Both putative rabbit liver MT and horse kidney MT isoforms were better separated at 10 kV. At 20 kV some rabbit liver MT isoform peaks migrated together. It should also be noted that a higher resolution was obtained with the 75 μm I.D. capillary column than with the 50 μm I.D. column. The effect of voltage was studied using the 50 μm I.D. capillary column, which allowed the use of a higher voltage and hence a larger overall range.

3.6. Identification of isoform peaks

Putative MT-1 and MT-2 isoform peaks from rabbit liver MT electropherogram were identified performed by running samples of rabbit liver MT, rabbit liver MT-1 and rabbit liver MT-2 separately (Fig. 10) and also by adding rabbit liver MT-1 and rabbit liver MT-2 to a rabbit liver MT sample solution (Fig. 11). Fig. 10 shows that both rabbit liver MT-1 and MT-2 samples contain residues of the other isoform, indicating that the samples are not totally pure. Rabbit liver MT exhibited ten peaks or bumps at pH 6.0. If rabbit liver MT peaks are labelled a–j, the rabbit liver MT-1 exhibited peaks a, b, c, d, e, i and j and very small residues of f and isoform MT-2 exhibited peaks e, f, i and j. This was also confirmed by the additions of MT-1 and MT-2 to MT (Fig. 11). Interestingly one major peak of MT, g, is not a main peak with MT-1 or MT-2. Adding MT-1 to MT leads to an increase in the main peak e from MT-1 (Fig. 11A), the proportion between peaks g and j remaining similar between MT alone (Fig. 10A) and MT + MT-1 (Fig. 11A), whereas the addition of MT-2 to MT results in a logical increase in peak j (Fig. 11B). Theoretically, all peaks found in the electropherogram of MT should be attributable to either MT-1 or MT-2; however, peak g is only observed with MT. This could indicate that, since rabbit liver MT is a mixture of MT-1 and MT-2, peak g is probably a modified isoform. The

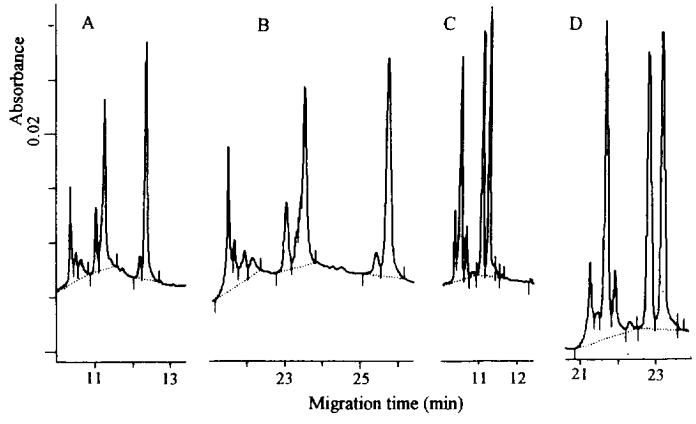

Fig. 9. Effect of running voltage on the separation of (A and B) rabbit liver MT and (C and D) horse kidney MT. Electropherograms obtained using 110 m*M* Tris–110 m*M* borate buffer (pH 6.90) at (A and C) 20 kV and (B and D) 10 kV. Temperature, 20°C; uncoated fused-silica capillary, 57 cm × 50 μm I.D.

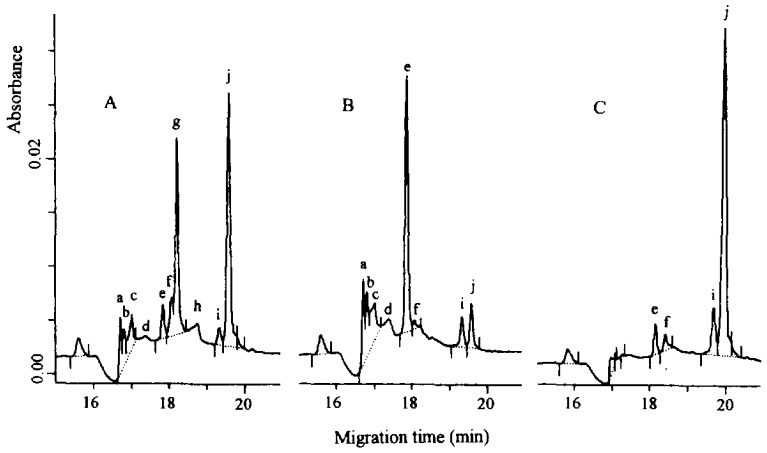

Fig. 10. Electropherograms of (A) rabbit liver MT, (B) rabbit-liver MT-1 and (C) rabbit liver MT-2 obtained using 110 m*M* Tris–110 m*M* borate buffer (pH 6.00). Other conditions as in Fig. 3.

definitive identification of the observed putative MT peaks as true metallothionein isoforms awaits further characterization.

4. Conclusion

A method for the separation of different isomers of biologically active metallothioneins has been developed. Published capillary electrophoretic methods with uncoated fused-silica capillaries use either acidic or basic buffers, thus not giving correct results for the abundances of MT isoforms in tissues. In basic buffers [borate (pH 8.4) or Tris–HCl (pH 9.1)], rabbit liver MT exhibited three or four peaks and horse kidney MT two or three peaks. In the acidic buffer used [phosphate (pH 2.5)], MT isoforms are in the

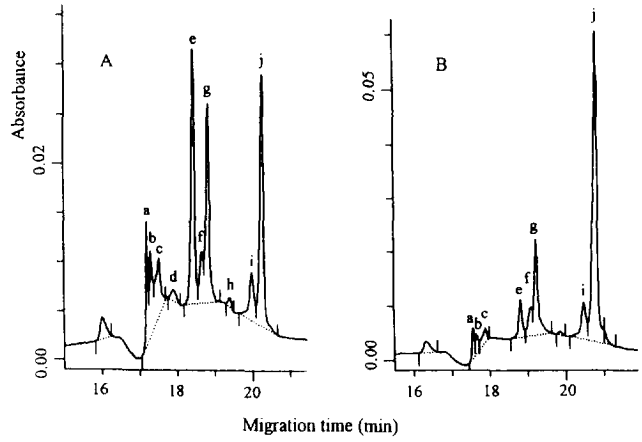

Fig. 11. Electropherograms of samples containing rabbit liver MT and additions of (A) isoform MT-1 and (B) isoform MT-2. The ratio of MT and isoform addition is nearly 1:1. Conditions as in Fig. 10.

apoprotein form not usually present under the neutral conditions that occur in biological samples. Improved separation efficiency and resolution were obtained with the developed method using Tris–borate buffer. Horse kidney MT exhibited five putative peaks and rabbit liver MT nine or ten peaks. Changes in voltage, temperature and buffer pH, concentration and composition affect the resolution. As the experimental conditions are neutral as in tissues, more precise information about the abundance of different isoforms is gained. A difference in polymorphism of horse kidney MT and rabbit liver MT is clearly noticed, horse kidney MT exhibiting three major peaks and rabbit liver MT only two major peaks. The experiments indicate that rabbit liver MT contains one major component which is not found as residues in either MT-1 or MT-2 isoforms. Clearly, more studies should be carried out to investigate the nature of this main component and also its origin. The method used gives a good possibility for comparison of polymorphism of rabbit liver MT and horse kidney MT. However, the achievement of the best separation of metallothionein isoforms may require modification of the conditions to optimize the resolution of individual isoforms for each type of metallothionein analysed.

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