The Influence of Experimental Conditions and of Organic Solvents as Modifiers on the Separation of Metallothionein Isoforms by Capillary Zone Electrophoresis in an Uncoated Capillary Column

V. Virtanen* / G. Bordin / A.-R. Rodriguez

Commission of the European Communities, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, 2440 Geel, Belgium

Key Words

Capillary zone electrophoresis
Electrophoretic mobilities
Metallothionein isoforms
Organic solvents as modifiers

Summary

Putative metallothionein (MT) isoforms are readily separated by capillary zone electrophoresis (CZE) with a mixture of tris(hydroxymethyl)aminomethane (Tris) and borate buffers in uncoated polyimide-cladded fused-silica capillary tubing. The influence of buffer concentration and composition, pH, running voltage and temperature on the electroosmotic mobility and on the electrophoretic mobilities of model samples (rabbit liver and horse kidney MT) have been investigated. The use of organic solvents as buffer modifiers and their effect on the separation of putative metallothionein isoforms is also described. It is shown that organic solvent modification significantly enhances the resolution – with organic solvents as modifiers it is possible to obtain a good separation of putative isoforms of rabbit liver MT in one run whereas two separate runs were needed at different pHs or at different temperatures when the separation was performed with Tris–borate buffer without organic-solvent modification. The effects of the organic solvents as modifiers were found to be solvent-specific although the trends were in the same direction (i.e. decreasing electroosmotic mobility with increasing percentage of organic solvent). Differences in effect on individual putative MT isoforms were observed, indicative of different chemical and physical characteristics of the isofroms. A difference in polymorphism of horse kidney MT and rabbit liver MT is clearly apparent.

Introduction

Metallothioneins (MTs) constitute a class of low molecular-mass proteins (6–7 kDa) characterized by their unusually high cysteine content (~30 %) which provides the capacity to bind metal ions such as Zn(II) and Cd(II) [1, 2]. Most MTs have two major isoforms (MT-1 and MT-2) [3] which arise from the genetic polymorphism found in many species [4]. Many animal species, furthermore, generate various sub-isoforms of MT-1 and MT-2, leading, therefore, to significant microheterogeneity. Although several functions have been proposed for MTs, including heavy metal detoxification, zinc and copper homeostasis, protection against oxidative damage caused by free radicals and as part of the acute-phase response to inflammation and stress [5], the biological functions of individual isoforms and sub-isoforms remain unknown.

Progress in this field is largely dependent on efficient separation of each isoMT and sub-isoMT and therefore, on the development of high resolution analytical techniques. Anion-exchange chromatography is only capable of resolving the two major MT classes, MT-1 and MT-2 [6]. More recently developed reversed-phase high-performance liquid chromatographic (RPHPLC) techniques are more efficient and can be used to resolve sub-isoforms within each of the two charge classes [7]. Separations of purified mammalian MT by polyacrylamide gel electrophoresis at neutral or alkaline pH also revealed two charge-distinct classes of isoforms [8]. Recently, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) both in uncoated fused-silica capillary tubing [9–15] and in surface-modified, coated, tubing [16–19] has led to promising results, but a good overall method is still required.

Although several publications describe the use of capillary electrophoresis for the separation of MT isoforms, there are, to the best of our knowledge, no data avail-

0009-5893/98/11 637-06 $ 03.00/0

able either on the effect of different conditions on electroosmotic flow and electrophoretic mobilities of MT isoforms or on the use of organic solvents as modifiers in CZE buffers for MT isoform separation.

This work looks at the influence of a range of factors on the separation of MT isoforms by CZE using mixed Tris–borate buffer. In particular, the emphasis is on elucidating the effects of different experimental conditions and organic solvent modifiers on the individual contributions of electroosmotic and electrophoretic mobilities to the overall migration times and hence separation and resolution. A better understanding of the relative effects of conditions and organic solvent modifiers on the electroosmotic and electrophoretic mobilities of structurally very similar MT isoforms is needed. In this text when isoforms are mentioned it should be understood as putative isoforms.

Experimental

Apparatus and Reagents

CZE was performed with P/ACE System 5000 equipment and data were collected and processed with System Gold software (Beckman, Fullerton, CA, USA). Fused-silica capillaries, 50 or 75 µm i.d. and 375 µm o.d. with polyimide cladding, were obtained from Beckman. Overall capillary length was 57 cm with on-line detection at 50 cm. The capillary was housed in a cartridge which enabled liquid cooling to maintain constant capillary temperature at a chosen value during the run. The capillaries were prepared by flushing for 10 min with each of 1 M hydrochloric acid, 0.1 M sodium hydroxide solution and doubly distilled water. The capillaries were then rinsed with buffer solution for 5 min. Between analyses 50 and 75 µm i.d. capillaries were rinsed for 2 or 3 min, respectively, with each of 0.1 M sodium hydroxide, doubly distilled water and buffer. Pressure injection (0.5 psig) of 5 or 10 s, respectively, was used for 75 and 50 µm i.d. capillaries. 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 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 hydrochloric acid to the solutions. The pH of buffers containing organic solvents was adjusted after addition of the organic solvent into the solution. The buffers were filtered through a 0.2 µm filter (Gelman Sciences, Ann Arbor, MI, USA). Cd- and Zn-containing metallothionein samples, rabbit liver MT 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^{-1}.

Measurement of Electroosmotic and Electrophoretic Mobilities

Electroosmotic mobilities, μ_{eo}, were calculated from the migration times of a neutral marker substance, benzyl alcohol, by use of the equation [20]:

$$\mu_{eo} = lL/t_{eo}V \quad (1)$$

where l is the distance between the inlet and the detector, L is the total length of column, t_{eo} is the migration time of the neutral marker, and V is the applied voltage. Benzyl alcohol was diluted in the sample solutions to a concentration of 0.05 % (v/v). Unless stated otherwise measurements of the electroosmotic mobility were conducted at 15 kV, giving a field strength of 263 V cm^{-1}, and at an ambient temperature of 20 °C.

The electrophoretic mobility, μ_{ep}, was calculated by use of the equation [20]:

$$\mu_{ep} = (lL/t_{eo} - lL/t_{app})/V \quad (2)$$

where t_{app} is the migration time of the sample. The negative signs of the electrophoretic mobility values of the MT isoforms are neglected.

Results and Discussion

In our previous study [15] we introduced Tris–borate buffer (110 mM:110 mM, pH 6.9) for the CZE separation of putative metallothionein isoforms. It was found that even when separation efficiency was better than that reported in the literature using an uncoated fused-silica capillary column, complete resolution of putative isoforms of both rabbit liver MT and horse kidney MT was not possible by use of general separation conditions – to achieve the best separation of metallothionein isoforms of different origin it seemed to be necessary to determine a unique set of conditions for optimum resolution of the individual isoforms in each type of metallothionein analysed [15]. In our attempt to overcome this drawback we decided to study the effect of different experimental conditions on the electroosmotic flow and on the electrophoretic mobilities of structurally very similar putative MT isoforms. We also studied the influence of the addition of organic solvents on the electrophoretic behaviour of the MT isoforms to obtain more basic knowledge about the characteristics of the MT isoforms in capillary electrophoretic separations. The metallothionein samples were found to be stable. The relative proportions of the peaks seemed to vary from lot-to-lot of the commercial proteins but replicates from a single lot (as used in this study) gave consistent results, confirming literature reports [9].

Electropherograms of horse kidney MT and rabbit liver MT, obtained using aqueous 110 mM:110 mM Tris–borate buffer (pH 6.90), are shown in Figures 1A and 1B, respectively. The effect of experimental conditions and the use of organic solvents as modifiers on the electrophoretic behaviour of the main peaks (labelled in Figure 1) was studied.

Effect of Experimental Conditions on Electroosmotic Mobility, Current and Migration Time

Increasing the temperature and running voltage were both found to increase electroosmotic mobility. The effect of temperature was not totally linear and the increased electroosmotic flow was a result of the change in the viscosity of the buffer solution. Temperature also influences the chemical equilibrium between the buffer and the inner surface of the capillary [21–23]. Increasing the running voltage directly influences all charged components present in the buffer solution, causing an increase in their velocities and resulting in increased electroosmotic mobility. Increasing buffer concentration resulted in reduced electroosmotic mobility, which seemed to start to level out at higher concentrations, owing to increased ionic strength and viscosity. The effect of buffer composition is shown in Figure 2A; the electroosmotic mobility was nearly constant when Tris was the dominant component of the buffer solution, but as soon as borate became dominant the electroosmotic mobility started to increase with increasing borate dominance. In addition to changes in the ionic strength of the buffer system, this also indicates that the borate ion has higher characteristic mobility than does Tris. The shape of the curve describing the dependence of the electroosmotic flow on buffer pH in a purely aqueous buffer system is already known from the literature [24]. The pH of the buffer affects the extent of ionization of species present in the electrolyte system and differences in the extent of ionization give rise to different electroosmotic mobilities. This is expected because the silanol groups dissociate more at higher pH, causing the zeta potential to increase [25].

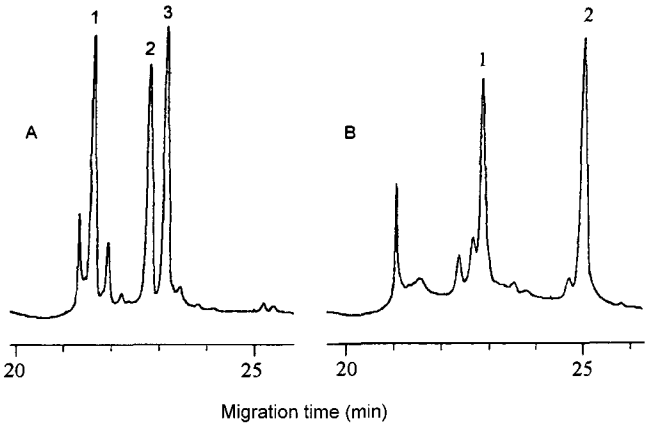

Figure 1
Electropherograms obtained from horse kidney MT (A) and rabbit liver MT (B) by use of 110 mM Tris–110 mM borate buffer (pH 6.90), voltage 11 kV, temperature 20 °C, detection wavelength 200 nm, capillary i.d. 75 μm.

Organic solvents as modifiers increase the viscosity of the buffer and cause changes in the dielectric constant of the buffer. This causes changes in the double-layer of the capillary and results in decreasing electroosmotic mobility (Figure 2B) and in decreasing currents. Similar results with some of the solvents used in this work have previously been obtained by Schwer et al. [26] and Fujiwara and Honda [27]. Changes in the zeta potential must also be considered as one reason for the total effect of organic solvents. The zeta potential decreases with increasing organic solvent content because the points of inflection corresponding to the pK' of the silanol groups shift towards higher pH on addition of the solvents [26]. Recent results from Dittmann et al. [28] confirm that the zeta potential changes on addition of organic solvents. The general trend is the same for all organic solvents, but the rate of the effect is different – a 40 % content of the protic solvents methanol (MeOH), ethanol (EtOH), and 2-propanol (2-PrOH) in the buffer led to a decrease in electroosmotic mobility and current in the ranges 75–90 % and 47–67 %, respectively. The

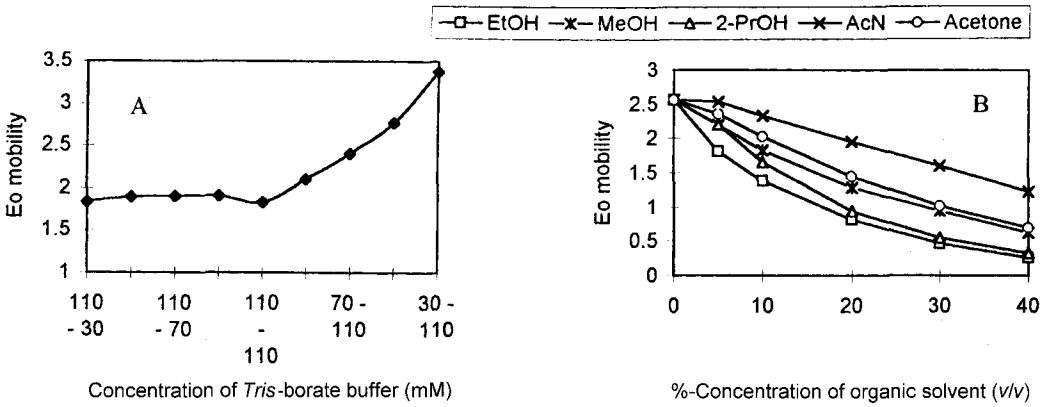

Figure 2
Dependence of electroosmotic mobility (10^8 m^2 V^{-1} s^{-1}) on buffer concentration (A) and content of organic modifier (B). Conditions as for Figure 1 except in B (voltage 15 kV and capillary i.d. 50 μm).

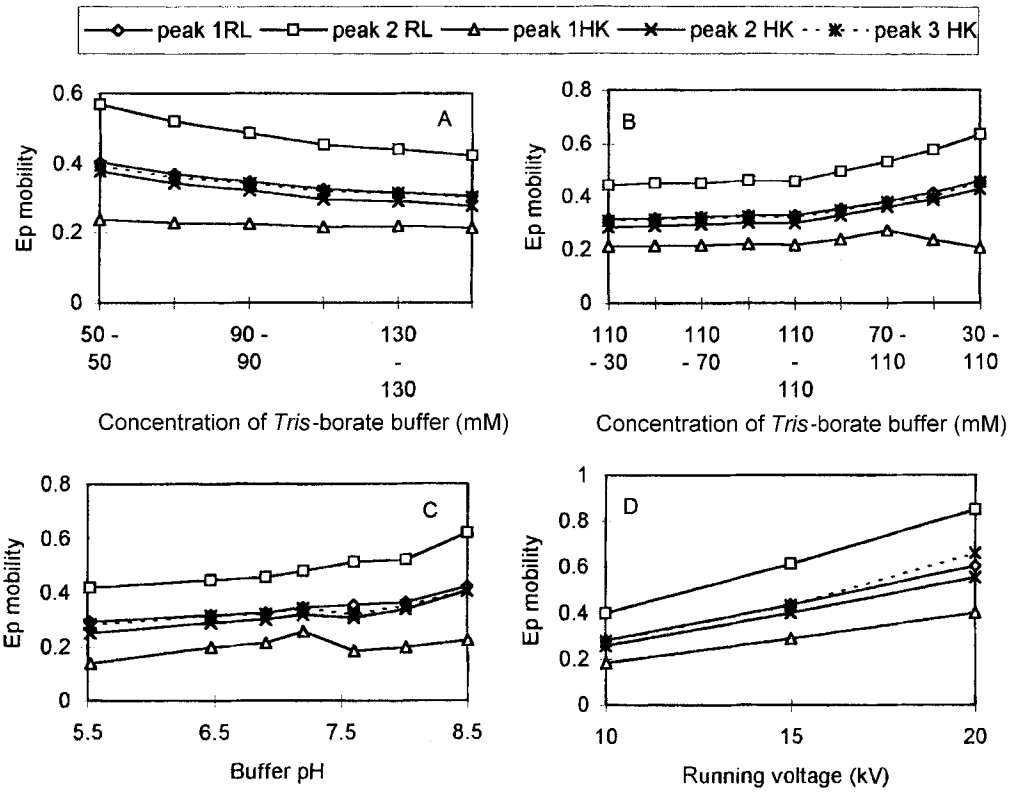

Figure 3

Effect of buffer concentration (A), buffer composition (B), buffer pH (C) and running voltage (D) on the electrophoretic mobilities (10^8 m^2 V^{-1} s^{-1}) of rabbit liver MT and horse kidney MT main peaks. Conditions as for Figure 1.

same concentration of the aprotic solvents acetonitrile and acetone reduced electroosmotic mobility and current by 52–73 % and 17–43 %, respectively. Whereas for acetonitrile and acetone the decrease in electroosmotic mobility is almost linear, for all the protic solvents used the decrease was steeper on addition of the first 15–20 % solvent. As a consequence, 40 %-content of MeOH, EtOH or 2-PrOH in the buffer increased the migration time 4-, 10- or 8-fold, respectively. For the aprotic solvents acetone and acetonitrile the increases were nearly 4- and 2-fold, respectively.

The characteristic electrophoretic mobilities of MT isoforms, and especially sub-isoforms, are likely to be very similar. Reducing the EOF by changing the physical and chemical conditions and changing surface charge-generation by addition of organic solvents are together likely to enhance the separation of putative MT sub-isoforms.

Effect of Experimental Conditions on Electrophoretic Mobility

The electrophoretic mobilities of putative MT isoforms from different species were found to be species-specific. The effect of buffer concentration is shown in Figure 3A. Electrophoretic mobilities were higher for rabbit liver MT isoforms than for isoforms of horse kidney MT, thus revealing the different characteristics of these MTs. The electrophoretic behaviour of horse kidney MT peak 1, which remained nearly constant with changing buffer concentration, was different from that of other horse kidney peaks (other putative isoforms), the electrophoretic mobility of which generally tended to decrease slightly as the buffer concentration was increased. This is indicative of differences even between the isoform groups of horse kidney MT. When Tris was the dominant component of the buffer solution the electrophoretic mobilities remained nearly constant (Figure 3B). As soon as borate became dominant the electrophoretic mobilities of most of the isoforms increased with increasing borate dominance. An interesting exception was the electrophoretic behaviour of horse kidney MT peak 1, which remaining nearly constant irrespective of buffer composition. Compared with the effect of the buffer composition on electroosmotic mobility, the effect is similar but on a smaller scale.

Increasing buffer pH elicited only a slight increase in electrophoretic mobilities, probably because of very minor changes in the chemical equilibrium of the MT isoforms (Figure 3C). The effect of temperature was found to be linear and similar for each MT isoform – electrophoretic mobility increased with increasing temperature. Unlike the behaviour of metalloproteins [22] for which temperature changes led to conformational effects, metallothioneins are known to be heat-stable and no conformational effects have been reported for tem-

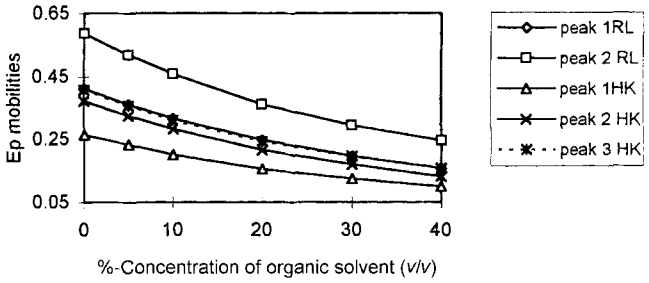

Figure 4

Effect of the MeOH content of the buffer on the electrophoretic mobilities (10^8 m^2 V^{-1} s^{-1}) of rabbit liver MT and horse kidney MT main peaks. Conditions as for Figure 2B.

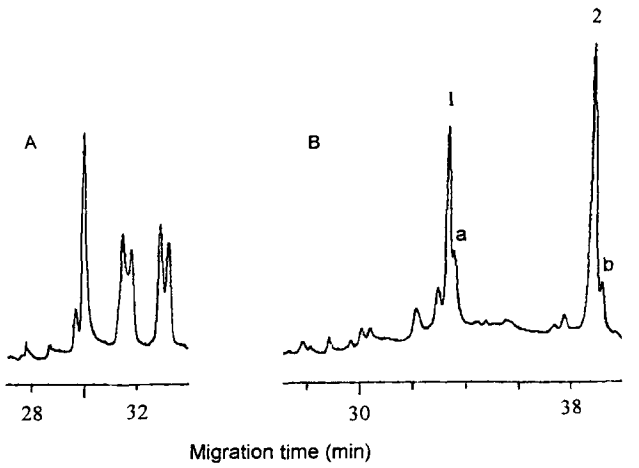

Figure 5

Electropherograms obtained from horse kidney MT (A) and rabbit liver MT (B) by use of Tris–borate–MeOH buffer, 110 mM: 110 mM:40 % (pH 6.70), voltage 22.5 kV, temperature 20 °C, detection wavelength 200 nm, capillary i.d. 50 μm.

peratures up to 85 °C [29]. Differences between the electrophoretic mobilities of MT isoforms were observed when the running voltage was changed. A similar tendency was found for all isoforms, but the slopes of the graphs were different. A higher running voltage caused greater differences in electrophoretic mobilities (Figure 3D). Compared with electroosmotic mobility the effect of running voltage was clearly smaller.

The variation of the electrophoretic mobility of rabbit liver MT and horse kidney MT isoforms with MeOH content is graphically presented in Figure 4. Increasing the amounts of organic solvents as buffer modifiers was found to reduce the electrophoretic mobility of the MT isoforms; solvent-specific effects were also observed.

Separation of Putative Rabbit Liver MT and Horse Kidney MT Isoforms

The electropherograms obtained by use of the optimized aqueous buffer solution, 110 mM : 110 mM Tris–borate buffer (pH 6.90), are shown in Figure 1. Addition of organic solvent as modifier was found to enhance separation, the effect being solvent-specific. Figures 5A and 5B shows electropherograms obtained from horse kidney MT and rabbit liver MT, respectively, by use of MeOH-modified Tris–borate buffer at pH 6.70.

Peaks labelled a and b in the electropherogram of rabbit liver MT (Figure 5B) were previously found to co-migrate with the main peaks labelled 1 and 2 when aqueous buffer was used. Until now two separate runs, under different conditions, were needed to obtain equivalent separation [15]. The faster-migrating small peaks (not labelled) were also better separated by use of an organic modifier. The separation of peak a from the rabbit liver main peak 1 was achieved irrespective of the solvent used. Peak b was clearly resolved by use of acetone, acetonitrile, MeOH, and EtOH but not 2-PrOH. Interestingly, the two main peaks obtained from horse kidney MT, labelled 2 and 3 in Figure 1, each start to split into two peaks when the concentration of organic solvent is increased. This phenomenon is solvent-specific, i.e. when ethanol is used the effect is not observed so clearly, the peaks being broadened but not divided into two peaks. Use of the aprotic solvents acetone and acetonitrile and 2-PrOH results in the splitting of the last two major peaks of horse kidney MT into two peaks, as was observed when MeOH was used. This peak splitting does not necessarily indicate the resolution of additional isoforms – they might, for instance, correspond to metalloforms (i.e. the same MT isoform but different metal composition and stoichiometry) or a conformational change of the isoform. More studies should be performed to investigate the nature of these components.

As is apparent from Figure 6A small amounts (10 %) of organic solvent broaden the later-migrating two major peaks of horse kidney MT – labelled 2 and 3 in Figure 1A. When the organic solvent content is 20 % splitting of both peaks was apparent (Figure 6B). As the MeOH content is increased the splitting of the peaks into two nearly equally sized peaks becomes clearer.

Conclusions

The effects of buffer concentration and composition, pH, temperature and running voltage, and of organic solvents as modifiers, on electroosmotic mobility and on the electrophoretic mobilities of metallothionein model samples have been studied. Different effects on individual putative MT isoforms were observed, indicative of different chemical and physical characteristics. Differences between the behaviour of horse kidney MT and rabbit liver MT were clearly apparent. It was also apparent that organic solvents as modifiers significantly enhance resolution, i.e. with organic solvent as a modifier it is possible to separate putative rabbit liver MT isoforms in one run; this required two separate runs either at different pH or at different temperatures when performed with aqueous Tris–borate buffer without organic-solvent modification. The effects of the organic

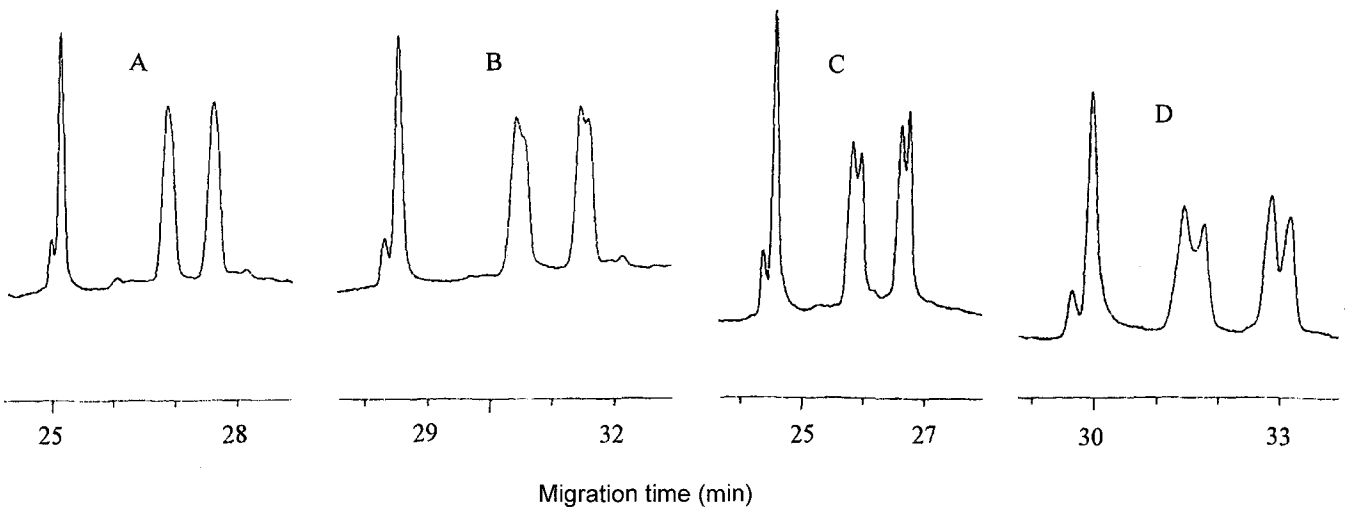

Figure 6

Effect of organic solvent (MeOH) content on horse kidney MT electropherograms. A, 10 %; B, 20 %; C, 30 %; and D, 40 % MeOH (*v/v*). Other conditions: Tris–borate buffer 110 mM:110 mM, temperature 20 °C, pH 6.70, capillary i.d. 50 µm.

solvents were found to be solvent-specific although the trends were in the same direction (decreasing electroosmotic mobility with increasing organic solvent content). Because the peaks separated by CZE represent putative isoforms, and could correspond to oxidized, polymerized, partially degraded artefacts or forms with different metal–thiol stoichiometry, their definite identification as true metallothionein isoforms requires further characterization.

Acknowledgements

This research was carried out within the European Commissions research and development programme in the frame of Human Capital and Mobility.

References

[1] *J. H. R. Kägi, A. Schäffer*, Biochemistry **27**, 8509 (1988).
[2] *M. Vasak, J. H. R. Kägi*, in H. Sigel (Editor), Metal Ions in Biological Systems, Vol. 15, Marcel Dekker, New York, 1983, pp. 213–273.
[3] *J. H. R. Kägi, Y. Kojima*, in J. H. R. Kägi and Y. Kojima (Editors), Metallothionein II, Birkhäuser, Basel, 1987, pp. 25–61.
[4] *R. D. Palmiter*, in J. H. R. Kägi and Y. Kojima (Editors), Metallothionein II, Birkhäuser, Basel, 1987, pp. 63–80.
[5] *I. Bremner, J. H. Beattie*, Ann. Rev. Nutr. **10**, 63 (1990).
[6] *M. Vasak*, Methods Enzymol. **205**, 41 (1991).
[7] *M. P. Richards*, Methods Enzymol. **205**, 217 (1991).
[8] *C. C. McCormick, L. Y. Lin*, Methods Enzymol. **205**, 71 (1991).
[9] *J. H. Beattie, M. P. Richards, R. Self*, J. Chromatogr. **632**, 127 (1993).
[10] *M. P. Richards, J. H. Beattie, R. Self*, J. Liq. Chromatogr. **16**, 2113 (1993).
[11] *M. P. Richards, J. H. Beattie*, J. Chromatogr. **648**, 459 (1993).
[12] *G. Liu, W. Wang, X. Shan*, J. Chromatogr. B **653**, 41(1994).
[13] *J. H. Beattie, M. P. Richards*, J. Chromatogr. A **664**, 129 (1994).
[14] *J. H. Beattie, M. P. Richards*, J. Chromatogr. A **700**, 95(1995).
[15] *V. Virtanen, G. Bordin, A. -R. Rodriguez*, J. Chromatogr. A **734**, 391 (1996).
[16] *M. P. Richards, P. J. Aagaard*, J. Cap. Elec. **1**, 90 (1994).
[17] *M. P. Richards*, J. Chromatogr. A **457**, 345 (1994).
[18] *M. P. Richards, J. H. Beattie*, J. Chromatogr. B **669**, 27 (1995).
[19] *J. H. Beattie, M. P. Richards*, J. Chromatogr. A **700**, 95 (1995).
[20] *R. Kuhn, S. Hoffstetter-Kuhn*, Capillary Electrophoresis: Principles and Practice, Springer, Berlin, 1993, pp. 20–28.
[21] *M. W. F. Nielen*, J. Chromatogr. **542**, 173 (1991).
[22] *R. S. Rush, A. S. Cohen, B. L. Karger*, Anal. Chem. **63**, 1346 (1991).
[23] *P. Oroszian, R. Blanko, X. -M. Lu, B. L. Karger*, J. Chromatogr. **500**, 481 (1990).
[24] *K. D. Lukacs, J. W. Jorgenson*, J. High Resol. Chromatogr. Chromatogr. Commun. **8**, 407 (1985).
[25] *S. F. Y. Li*, Capillary Electrophoresis; Principles, Practice and Applications, Elsevier, Amsterdam, 1992, pp. 215–216.
[26] *C. Schwer, E. Kenndler*, Anal. Chem. **63**, 1801 (1991).
[27] *J. Fujiwara, S. Honda*, Anal. Chem. **59**, 487 (1987).
[28] *M. M. Dittmann, G. P. Rozing*, J. Chromatogr. A **744**, 63 (1996).
[29] *M. Sato, K. T. Suzuki*, Biomed. Res. Trace Elements **6**, 13 (1995).

Received: May 14, 1998
Revised manuscript received: Jun 19, 1998
Accepted: Jul 9, 1998