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# Factors influencing the separation of metallothioneins by capillary zone electrophoresis

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**Abstract**

The factors influencing the separation of metallothioneins (MTs) by capillary zone electrophoresis (CZE) were studied using untreated fused-silica capillaries. A comparison was made between the various buffers of 20 m*M* $Na_2B_4O_7$, 10 m*M* $Na_2B_4O_7$–10 m*M* $Na_2HPO_4$ and 20 m*M* $Na_2B_4O_7$–10 m*M* Tris for the separation of MTs in terms of migration time and UV-absorbance under the various applied voltages. The migration times for MT-I and MT-II decreased with increase in the applied voltage and column temperature. However, no significant change in UV absorbance was observed. When $Na_2B_4O_7$–Tris buffer was chosen for the separation of MTs, the migration time, UV absorbance, theoretical plates and resolutions also increased with increasing buffer concentrations. The effect of buffer pH on migration time was relatively complicated. Under the optimal operating conditions the standard MTs were well separated. Determination of MTs in the real samples failed due to the adsorption of other proteins onto the capillary walls.

## 1. Introduction

Capillary zone electrophoresis (CZE) has become a major separation technique for complex mixture of peptides and proteins [1]. McCormick [2] reported the separation of peptides and proteins using low-pH buffers in modified silica capillaries. Separation of proteins with a molecular mass ranging from 12 to 77 kDa and isoelectric points of 4.5–11 has been obtained in less than 25 min. Strong interaction between the proteins and the capillary walls was apparent. CZE was applied for the separation of Ca- and Zn-binding proteins [3]. Ca- and Zn-binding proteins and internal standard proteins were separated completely.

Adsorption of analytes onto fused-silica capillaries is a common phenomenon. In order to overcome this problem various measures, such as changing the separation conditions [4] and derivatizing the silica surface [5], have been taken. Towns and Regnier [6] examined the mechanism of polycation adsorption to capillary walls. They concluded that polycation adsorption caused the mean transport velocity of neutral species to be discontinuous across the length of the capillaries and the separation efficiency to be reduced, and thus the axial distribution of the zeta potential was not uniform across the length of the capillary.

Metallothioneins (MTs) are proteins of low-

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molecular-weight which bind heavy metals and have a high cysteine content (*ca.* 33%) [7]. It is well recognized that formation of MTs can be induced by some heavy metals, such as cadmium, zinc and mercury [8–10]. There are several methods reported in the literature for the separation of MTs from animal tissues. These include determination of the sulphydryl groups of MTs by an electrochemical method [11], indirect quantitation based on the metal saturation technique by radionuclides [12,13], and radioimmunoassay [14]. In recent years high-performance liquid chromatography–flame atomic absorption spectrometry (HPLC–FAAS) or graphite-furnace atomic absorption spectrometry (HPLC–GFAAS) or inductively coupled plasma atomic emission spectrometry (HPLC–ICP-AES) [15–17] have been satisfactorily applied for the separation of MTs.

Although the past two years have seen an explosion of papers utilizing CZE for the separation of complex mixture of peptides and proteins, there has been only one report on the separation of metallothioneins in rat liver by CZE during the revision period of the manuscript. However, prior removal of major protein contaminants was still required for CZE analysis [18]. The purpose of the present study is to investigate the factors which influence the separation of MTs by CZE using untreated fused-silica capillaries. After this has been done the separation and determination of MTs in real samples can be exploited in the future.

## 2. Experimental

### 2.1. Apparatus

CZE was performed with a Spectra Phoresis 1000 high-performance capillary electrophoretic instrument (Spectra-Physics, Fremont CA, USA) equipped with a scanning focus UV-Vis detector and an IBM PS/2 computer with state-of-the-art software, by which the peak area can be divided by migration time, designed specifically for application in capillary electrophoresis. The capillary electrophoresis system was equipped with a 80-position autosampler, 2 automated washes and a temperature controller allowing the capillary to cool down to 15°C and to heat up to 60°C ± 0.01°C. The UV absorbance of MTs was measured at 250 nm. In order to achieve a more linear calibration curve the hydrodynamic injection method was used throughout. An untreated fused-silica capillary (70 cm × 50 μm I.D., Optical Fibre Factory, Hebei Province, China) was used in the present study. After each run the capillary was washed with 0.1 *M* sodium hydroxide solution for 2 min followed by electrophoresis buffer for an additional 2 min. After every four runs the capillary was rinsed with running buffer.

### 2.2. Materials

Rabbit kidney MT isoforms were purchased from Peking University. The stock solution of the MTs (0.901 mg/ml of MT-I and 1.054 mg/ml of MT-II) was gravimetrically prepared by dissolving adequate amounts of MTs in 10 m*M* Tris-HCl (pH 8.6) and stored at 4°C. The working standards of MTs were prepared by diluting the stock MTs solution with 10 m*M* Tris-HCl (pH 8.6) before use. The operating buffer was composed of 20 m*M* sodium tetraborate–10 m*M* Tris. The buffers were filtered through a 0.45-μm membrane prior to use. All other chemicals used in this study were of analytical reagent grade.

## 3. Results and discussion

### 3.1. Choice of operating buffer

In order to achieve a good separation and high sensitivity for the determinations of MTs a series of operating buffers was compared in terms of migration time and UV absorbance values under the various applied voltages. The results are shown in Table 1.

As can be seen the migration time for MT-I and MT-II was reduced with increasing applied voltage no matter what operating buffers were used for capillary electrophoresis. The reason for the shorter migration time for MTs at higher

Table 1
Effect of applied voltages on migration time and UV absorbance of MT isoforms with various operating buffers.

| Applied voltage (kV) | MT-I | | MT-II | |
|---|---|---|---|---|
| | Migration time (min) | UV absorbance (peak area) | Migration time(min) | UV absorbance (peak area) |
| *20 mM $Na_2B_4O_7$ (pH 9.0)* | | | | |
| 10 | 11.67 | 3545 | 12.56 | 4951 |
| 15 | 7.74 | 3569 | 8.33 | 5079 |
| 20 | 5.59 | 3667 | 6.02 | 4744 |
| 25 | 4.67 | 3462 | 4.99 | 4877 |
| *10 mM $Na_2B_4O_7$–10 mM $Na_2HPO_4$(pH 9.0)* | | | | |
| 10 | 11.19 | 3407 | 11.98 | 4680 |
| 15 | 7.41 | 3870 | 7.92 | 4311 |
| 20 | 5.34 | 3611 | 5.71 | 4908 |
| 25 | 4.64 | 3239 | 4.73 | 4573 |
| *20 mM $Na_2B_4O_7$–10 mM Tris (pH 9.0)* | | | | |
| 10 | 11.31 | 4239 | 12.03 | 5993 |
| 15 | 7.45 | 4120 | 7.91 | 6107 |
| 20 | 5.55 | 4204 | 5.90 | 5874 |
| 25 | 4.45 | 3995 | 4.73 | 6294 |

Conditions: hydrodynamic injection of 50 μg/ml of MT-I and MT-II for 8 s, untreated fused-silica capillary 70 cm × 50 μm I.D., length to detector 62 cm, column temperature 30°C.

applied voltage is that in capillary zone electrophoresis the migration time for a solute is reversely proportional to the applied voltage ($t \propto 1/V$) [19]. It should also be pointed out that no significant change in the UV absorbance values for MTs was observed when the applied voltage increased from 10 to 25 kV. When a relatively low voltage of 10–15 kV was used unstable baselines, asymmetrical UV absorption peaks and longer migration times were observed. Therefore, the applied voltage of 20 kV was used throughout the study.

Based on a comparison of the above operating buffers in terms of stable baseline availability, separation efficiency and determination sensitivity for MTs the conclusion can be drawn that $Na_2B_4O_7$–Tris was suitable for this study. A further discussion on $Na_2B_4O_7$–Tris was given below.

The effect of various concentrations of $Na_2B_4O_7$–10 m*M* Tris on the migration time of MTs was studied. The results are shown in Fig. 1. The electropherograms showed that the migration time for both MT-I and MT-II increased with increase in the buffer concentrations. This phenomenon was previously demonstrated by Fujiwara and Honda [20] as well as Bruin *et al.* [21] and is mainly due to strong reduction of the electroosmotic flow. Additionally, the UV absorbance values also increased with increasing buffer concentrations over the range of 10 m*M* $Na_2B_4O_7$–10 m*M* Tris to 30 m*M* $Na_2B_4O_7$–10 m*M* Tris. However, the baseline became unstable at a buffer concentration of 30 m*M* $Na_2B_4O_7$–10 m*M* Tris. Therefore, 20 m*M* $Na_2B_4O_7$–10 m*M* Tris was considered as the optimal buffer concentration.

### 3.2. Effect of buffer concentration on the number of theoretical plates and resolution

The effect of the buffer concentration on the resolution and number of theoretical plates was studied, and the results are shown in Figs. 2 and 3, respectively. Both the number of theoretical plates and the resolution increased with increase in the buffer concentration. This may be due to an increase in the efficiency with increasing

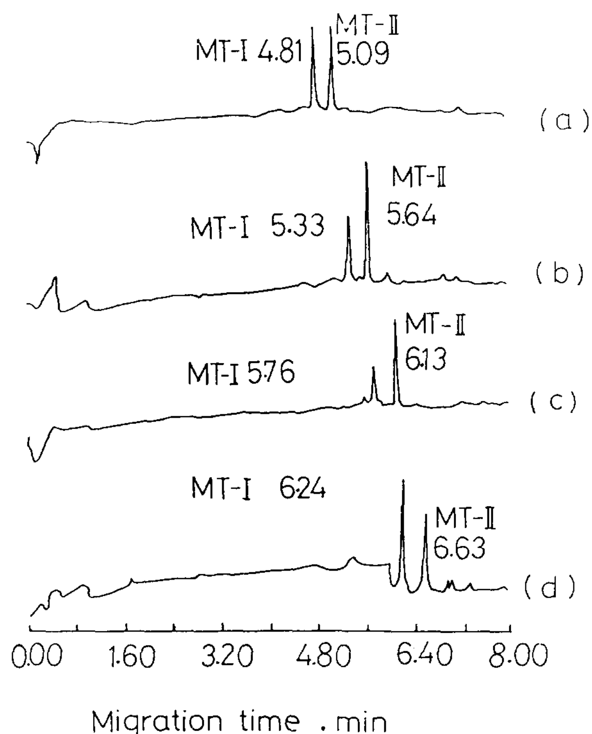


Fig. 1. Electropherogram of MT isoforms with various buffer concentrations under the following operating conditions: hydrodynamic injection of 50 $\mu$/ml MT-I and MT-II for 8 s, untreated fused-silica capillary 70 cm × 50 $\mu$m I.D, length to detection 62 cm, column temperature 30°C, applied voltage 20 kV. (a) 10 m*M* $Na_2B_4O_7$–10 m*M* Tris; (b) 15 m*M* $Na_2B_4O_7$–10 m*M* Tris; (c) 20 m*M* $Na_2B_4O_7$–10 m*M* Tris; (d) 25 m*M* $Na_2B_4O_7$–10 m*M* Tris.

current [22]. The resolution increased rapidly with increasing buffer concentration from 10 m*M* $Na_2B_4O_7$–10 m*M* Tris to 20 m*M* $Na_2B_4O_7$–10 m*M* Tris, and then levelled off with further increase in buffer concentration. For the relationship between the number of theoretical plates and buffer concentration a quite similar trend was observed. This experiment further demonstrated that 20 m*M* $Na_2B_4O_7$–10 m*M* Tris is the optimal buffer concentration.

### *3.3. Influence of buffer pH on the migration time of MTs*

The effect of buffer pH on the migration time of MTs was investigated with a buffer concentration of 20 m*M* $Na_2B_4O_7$–10 m*M* Tris and an applied voltage of 20 kV. The results are given in Fig. 4. It indicated that the migration time for both MT-I and MT-II decreased gradually with an increase in pH from 7 to 9. There was a minimum at pH 9, and then the migration time increased with further increase in pH from 9 to 11. Thus buffer pH 9.0 was used in the following experiments.

In order to explain the reason for the increase

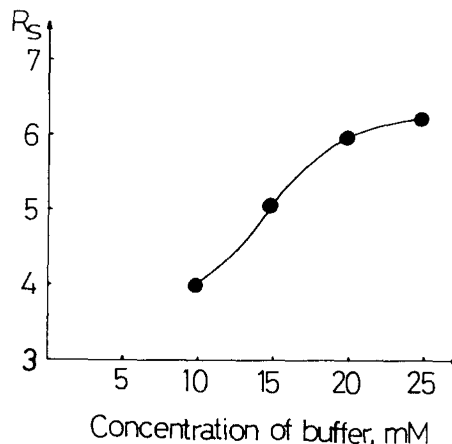


Fig. 2. Effect of buffer concentrations on resolution. Conditions as in Fig. 1.

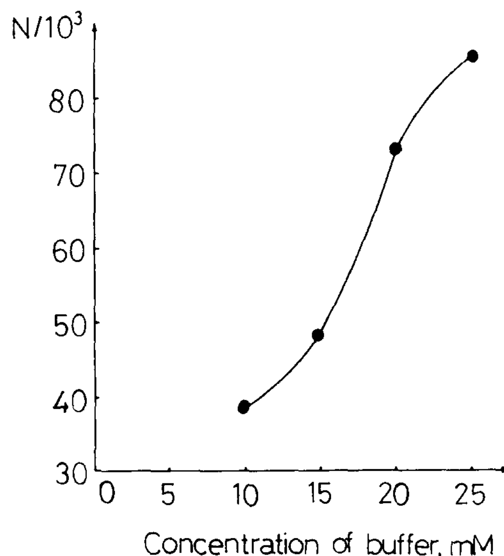


Fig. 3. Effect of buffer concentrations on the number of theoretical plates. Conditions as in Fig. 1.

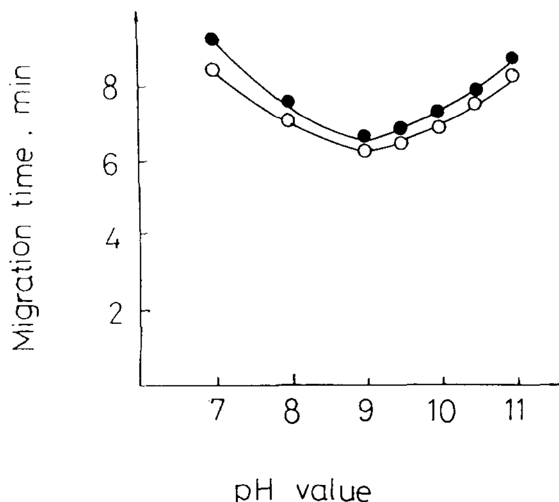


Fig. 4. Effect of buffer pH on migration time of MTs. (○) MT-I; (●) MT-II. Conditions: operating buffer: 20 m*M* $Na_2B_4O_7$–10 m*M* Tris; other conditions were the same as in Fig. 1.

in the migration time of the MTs at a buffer pH of 9–11 we should bear in mind that the electroosmotic mobilities, or equivalent linear velocities and migration times are related to the buffer concentrations [20,21] and ionic strength [23]. In addition, the ionic strength is more important than the concentration. According to Vindevogel and Sandra [24] boric acid and borax are functionally similar. They pointed out that the buffer of Tris-HCl and boric acid-NaOH have the following pH dependence. When Tris-HCl was used a decrease in pH over the range of 7–9 was accompanied by an increase in the amount of ions, and thus an increase in the ionic strength. Both effects cooperatively produced a relatively strong dependence of the electroosmotic mobility on the pH. However, when boric acid-NaOH was used as a buffer, increasing the pH over the range 8–10 would increase the ionic strength. However, the ionic strength was stronger than the pH effect, and thus the reversed dependence of the electroosmotic mobility on pH was observed. Finally, they concluded that the electroosmotic mobility of Tris-HCl increased with increase in pH from 7 to 9 and the electroosmotic mobility of boric acid-NaOH decreased with increasing pH from 8 to 10. The pH-dependence curves of Tris-HCl and boric acid-NaOH intersected at pH 9. In the present study a buffer of $Na_2B_4O_7$–Tris was used and the pH was adjusted with HCl or NaOH. We expect that the relationship between the electroosmotic mobility and pH should be the same as that for Tris-HCl and boric acid-NaOH reported in ref. 24. If that is correct the migration time should decrease with an increase in pH from 7 to 9, and then increase with further increasing pH values from 9 to 11. Thus, there is a minimum in the relationship between the migration time and buffer pH.

### *3.4. Effect of capillary temperature on the migration time of MTs*

The effect of capillary temperature on the migration time of MT-I and MT-II was studied at a fixed applied voltage by using the temperature controller. The results are schematically shown in Fig. 5. It is clearly shown that the migration time for MT-I and MT-II decreased with increasing temperature due to the decrease of the buffer viscosity, and thus an increase in the metallothioneins mobility. Since the ambient temperature was close to 30°C and no denaturation of MTs

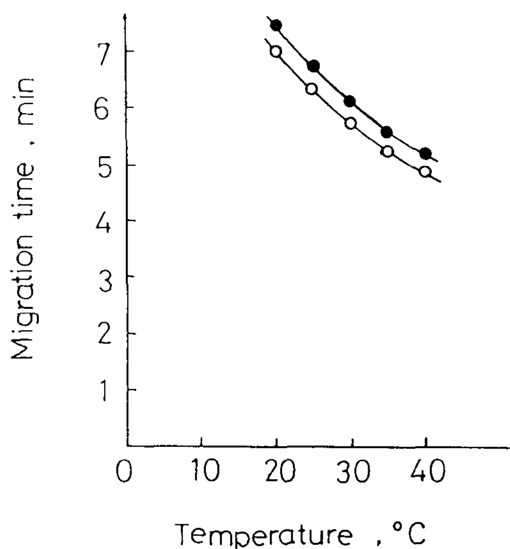


Fig. 5. Effect of temperature on migration time of MTs. (○) MT-I; (●) MT-II.

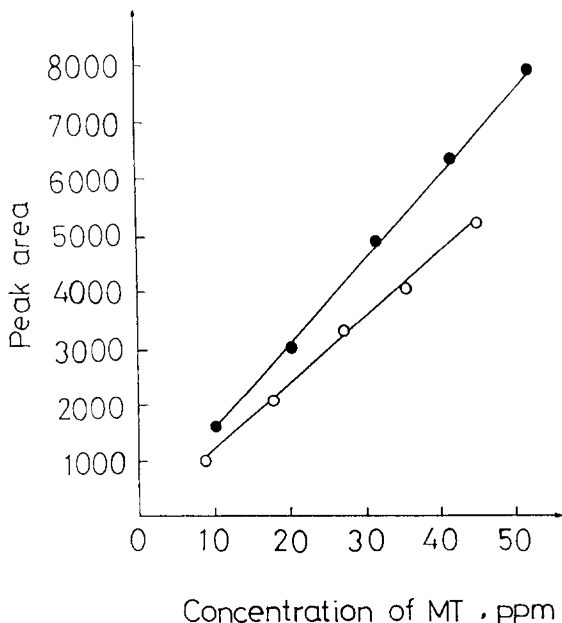


Fig. 6. Standard calibration curves for the electrophoretic analysis of MT isoforms. (○) MT-I; (●) MT-II. MT-I: $r = 0.995$; $y = 111.56x + 78.8$; MT-II: $r = 0.998$; $y = 154.4x - 104.6$.

was observed at this temperature 30°C was chosen for capillary electrophoresis.

### *3.5. Calibration curve for standard MTs*

Under the optimal operating conditions the calibration curves for MT-I and MT-II were obtained from a concentration range of 10–50 $\mu$g/ml (Fig. 6). However, no MTs were detected by CZE in the rabbit kidney samples in which both MT-I and MT-II were determined by high-performance liquid chromatography using a DEAE-5PW anion-exchange column with a Tris-HCl buffer [16]. The migration times for the standard MTs were delayed by *ca.* 1 min after each run with real samples. After several runs of real samples no standard MTs could be detected. Presumably, a build-up of proteins other than the MTs in the real samples on the capillary walls is responsible for the gradual increase in migration time and for the failure of quantitative determination of MTs in the real samples. This adsorption phenomenon was also always observed in CZE by other workers [2,19].

## 4. Acknowledgement

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## 5. References

[1] W.G. Kuhr and C.A. Monning, *Anal. Chem.*, 64 (1992) 389R.
[2] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
[3] H. Kajiwara, *J. Chromatogr.*, 559 (1991) 345.
[4] M.J. Gordon, K.J. Lee, A.A. Arias and R.N. Zare, *Anal. Chem.*, 63 (1991) 69.
[5] J.K. Towns and F.E. Regnier, *Anal. Chem.*, 63 (1991) 1126.
[6] J.K. Towns and F.E. Regnier, *Anal. Chem.*, 64 (1992) 2473.
[7] M.M. Kissing and J.H. Kagi, in J.H.R. Kagi and M. Nordberg (Editors), *Metallothionein*, Birkhauser Verlag, Basel, 1979, p.147.
[8] P.D. Whanger and J.T. Deagen, *Environ. Res.*, 30 (1983) 372.
[9] S. Onosaka and M.G. Cherian, *Toxicology*, 22 (1981) 91.
[10] S. Onosaka and M.G. Cherian, *Toxicology*, 23 (1982) 11.
[11] R.W. Olafson and R.G. Sim, *Anal. Biochem.*, 100 (1979) 343.
[12] J.K. Piotrowski, W. Bolanoskwa and A. Sapota, *Acta Biochim. Pol.*, 20 (1973) 207.
[13] D. Klein, R. Bartsch and K.H. Summer, *Anal. Biochem.*, 189 (1990) 35.
[14] R.K. Mehra and I. Bremner, *Biochem. J.*, 213 (1983) 459.
[15] M.S. Krug and S.L. Berger, *Anal. Biochem.*, 153 (1986) 315.
[16] Sun Peng, Shan Xiao-quan, Zheng Yan, Jin Long-Zhu and Xu Wei-bing, *J. Chromatogr.*, 572 (1991) 73.
[17] J. Bonger, C.D. Walton and D.E. Richardson, *Anal. Chem.*, 60 (1988) 2683.
[18] J.H. Beattie, M.P. Richards and R. Self, *J. Chromatogr.*, 632 (1993) 127.
[19] J.W. Jorgenson and K.D. Lukacs, *Science*, 222 (1983) 266.
[20] S. Fujiwara and S. Honda, *Anal. Chem.*, 58 (1986) 1811.
[21] G.J.M. Bruin, J.P. Chang, R.H. Kuhlman, K. Zegers, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 471 (1989) 429.
[22] A.B. Timerbaev, W. Buchberger, O.P. Semenova and G.V. Konn, *J. Chromatogr.*, 630 (1993) 379.
[23] V. Dolnik, J. Liu, J.F. Banks, M.V. Novotny and P. Bocek, *J. Chromatogr.*, 480 (1989) 321.
[24] J. Vindevogel and P. Sandra, *J. Chromatogr.*, 541 (1991) 483.