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Application of a polyamine-coated capillary to the separation of metallothionein isoforms by capillary zone electrophoresis

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Abstract

In this study a fused-silica capillary treated internally with a polyamine coating which reverses electroosmotic flow in the direction of the anode was evaluated for its ability to resolve metallothionein (MT) isoforms. Analysis of different MTs purified from liver and kidney tissue revealed the following numbers of putative isoform peaks resolved: rabbit (3–6); horse (3–5); rat (2–3), chicken (1); human MT-1 (5–6); sheep (4–5) and pig (4–5). The greater degree of MT isoform heterogeneity detected in this study using the polyamine-coated capillary suggested a higher resolving capacity for capillary zone electrophoresis conducted with this capillary compared to an uncoated one. Using the single isoform of chicken MT (cMT) as a reference standard, relative standard deviations of 2.53, 1.85 and 2.21% for peak migration time, area and height, respectively, were observed for eight consecutive runs. A standard curve for cMT established linearity ($r^2 = 0.99$) for integrated peak area over three log units of cMT concentration with a lower limit of detection estimated to be ≤ 5 μg/ml. Acetonitrile extracts of chick liver tissue homogenates were successfully analyzed for the presence of MT isoforms from both control and zinc-injected animals. Based on our initial evaluation, capillary zone electrophoresis using the polyamine-coated capillary appears to be a very useful analytical method for the separation and quantification of individual MT isoforms.

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1. Introduction

Metallothioneins (MTs) are a family of low-molecular-mass, heavy metal-binding proteins that are thought to play a fundamental role in intracellular metal metabolism [1]. 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 [2]. MT isoforms arise from genetic polymorphism which is found in many species [3]. Specific amino acid substitutions which, in some cases, cause differences in net charge characterize individual isoforms. Both the number and the position of the cysteine residues, however, are highly conserved in MTs across a wide variety of species [2,3]. Anion-exchange chromatography is capable of resolving the two major charge classes of MTs, designated MT-1 and MT-2 based on their order of elution from the column [4]. Reversed-phase HPLC is capable of further resolution of MT isoforms, especially isoforms (subisoforms) within each of the two charge classes [5]. We have recently been investigating capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) separations of MT isoforms conducted in uncoated fused-silica capillaries [6–9]. However, for many MTs a complete separation of all of the isoforms by either of these two methods has yet to be achieved.

There have been a number of developments in

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capillary wall modification aimed at improving separation efficiencies for proteins and peptides [10]. In general, these modifications are concerned with decreasing the interaction between the protein or peptide and the wall. Typical approaches include coating the capillary wall with amphipathic polymers [11], inclusion of zwitterionic reagents in the running buffer [12,13] and partial deactivation of the silanol groups by derivatization with hydrocarbons and neutral hydrophilic compounds [10]. The choice of buffer, as well as the concentration and pH of the buffer are also effective means of altering the protein–wall interaction. Collectively, these approaches have resulted in improved separation efficiency and/or selectivity. We have recently shown that selectivity in the separation of MT isoforms is dependent on pH when using a phosphate buffer in conjunction with an uncoated fused-silica capillary [8].

Another consideration in the separation of proteins by CZE concerns the effects of capillary wall modification and changes in buffer concentration and pH on electroosmotic flow (EOF). Depending on the modification, EOF can either increase, decrease or reverse its direction compared to an unmodified fused-silica capillary. The regulation of EOF is an important aspect in achieving maximum resolution during CZE separations of proteins [14]. If the mixture of proteins to be separated contains individual components that differ significantly in net charge, then a rapid EOF can be used to shorten the time of analysis. However, if the mixture contains proteins with similar or identical net charge, then minimizing the effects of EOF may be necessary to permit resolution of the components based on subtle differences in their electrophoretic mobilities. Towns and Regnier [15] reported that polyamine coatings adsorbed onto fused-silica capillaries can be cross-linked to form a stable, positively charged surface. The advantage of this particular modification was shown to be the ability to alter pH over a wide range (pH 2–12) in order to maximize selectivity for the separation of a mixture of proteins without significantly affecting EOF. This approach was found to be particularly useful for the resolution of a mixture of basic proteins with differing p*I* values [15]. Moreover, the positively charged wall was effective in decreasing the protein–wall interactions due to Coulombic attraction that occur in uncoated fused-silica capillaries.

The two charge classes of MTs (MT-1 and MT-2) differ by a single negative charge at neutral pH [2] and are readily separated by CZE in uncoated fused-silica capillaries [6–9]. Separations of MT-1 and MT-2, conducted at neutral or alkaline pH, can be quite rapid (*i.e.*, < 10 min) because a relatively fast EOF is used to move the negatively charged MTs past the detector. However, for many animal species additional MT isoforms (subisoforms) exist within each of the major charge classes that are not resolved because they possess a similar or identical net charge. In order to study their role in cellular metal metabolism, a technique is required that can both isolate and quantify each of the MT isoforms. Currently the best methods available for this purpose are reversed-phase HPLC or a combination of gel-filtration, anion-exchange and reversed-phase chromatography [5,16]. The latter method involves multiple steps and reversed-phase HPLC is a lengthy procedure requiring the use of expensive columns and organic solvents. Therefore, the objective of this work was to develop a rapid and sensitive technique employing capillary electrophoresis to separate all of the individual MT isoforms in a single step for subsequent quantification. A fused-silica capillary treated with a polyamine coating which reverses EOF in the direction of the anode was evaluated for this purpose.

2. Experimental[a]

2.1. Instrumentation

CZE was performed on a P/ACE System 2100 (Beckman, Fullerton, CA, USA). A 57 cm (50 cm to the detector) × 50 μm I.D. fused-silica

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[a] Mention of a trade name, proprietary product or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other suitable products.

capillary column was used. The capillary, treated internally with a proprietary polyamine coating (Beckman), was housed in a cartridge which permitted liquid cooling to maintain capillary temperature at 25°C during the run. Data were collected and processed with System Gold software (Beckman).

2.2. *Materials*

Standard Cd,Zn-MTs were purchased from a commercial source (rabbit liver and horse kidney, Sigma, St. Louis, MO, USA). An experimentally prepared [17] rabbit liver Zn-MT was a gift from Dr. F.A. Liberatore (DuPont Medical Products, N. Billerica, MA, USA). Pig liver Zn-MT was prepared by sequential gel-permeation and ion-exchange column chromatography [18]. Chick liver Zn-MT was further purified by reversed-phase HPLC prior to CZE [18]. Rat (Cd-induced) and human liver MT isoforms were provided by Dr. C. Tohyama (National Institute of Environmental Studies, Tsukuba, Japan). Rat and sheep liver Zn-MTs were gifts from Dr. J.H. Beattie (Rowett Research Institute, Aberdeen, UK). All buffers and chemicals were reagent grade. The running buffer (0.1 *M* sodium phosphate, pH 7.0) was prepared by adjusting the pH of a solution of monobasic sodium phosphate with 1 *M* NaOH or by mixing, in the correct proportions, solutions of mono- and dibasic sodium phosphate so as to achieve a final pH of 7.0. Both methods of preparation produced a 0.1 *M* sodium phosphate running buffer that performed identically.

2.3. *Methods*

CZE was performed under the following conditions: prior to each run, the capillary was flushed with a wash solution and a regenerator solution (Beckman) followed by buffer (0.1 *M* sodium phosphate, pH 7.0) for 2.0 min each. Samples of MT were dissolved in deionized water at a final concentration of 1.0 mg/ml or at the specified concentration and were loaded into the capillary by pressure injection. After a sample was loaded, the run was initiated by applying 20 kV across the capillary. All CZE was conducted at 25°C. Detection of MT isoforms was accomplished by monitoring UV absorbance at 200 nm. Preparation of chick liver tissue extracts from zinc- and saline-injected chicks by acetonitrile fractionation was performed as described previously [6], except that the 50% acetonitrile fraction was further adjusted to 80% acetonitrile. The resulting precipitate was collected by centrifugation and resuspended in 0.1 ml of deionized water prior to analysis by CZE.

3. Results and discussion

Fig. 1 depicts a typical separation of rabbit liver Zn-MT subjected to CZE using a polyamine-coated capillary with 0.1 *M* sodium phosphate buffer, pH 7.0 at 20 kV. From the inset showing an expanded time scale, it is clear that four predominant peaks (putative isoforms) are resolved with the presence of several more minor ones. MT-2 is characterized by a single predominant species which presumably is the MT-2a isoform [2,16] and a much less abundant species. MT-1, on the other hand is characterized by three major peaks and a number of minor ones. The major peaks most likely include MT-1a and MT-2d and MT-2e, the latter two of which exhibit charge characteristics more like MT-1 than MT-2, which is more negatively charged and thus migrates faster [2,16]. This is in contrast to CZE conducted in uncoated capillaries where at pH 7.0 only two peaks are resolved, those being MT-1 and MT-2 [8]. Since rabbit liver MT has been reported to be comprised of up to six isoforms (*i.e.*, three distinct isoforms within both the MT-1 and MT-2 fractions), the detection of four predominant peaks plus additional minor ones is consistent with this finding [19]. Moreover, recent findings suggest that in response to zinc induction, rabbit kidney cells in culture synthesize four MT isoforms that can be resolved by gel-permeation chromatography followed by consecutive anion-exchange and reversed-phase HPLC [16]. These authors further reported that the individual MT isoforms were differentially stimulated by exposure to zinc and thus the relative levels of each isoform vary according to prior metal exposure.

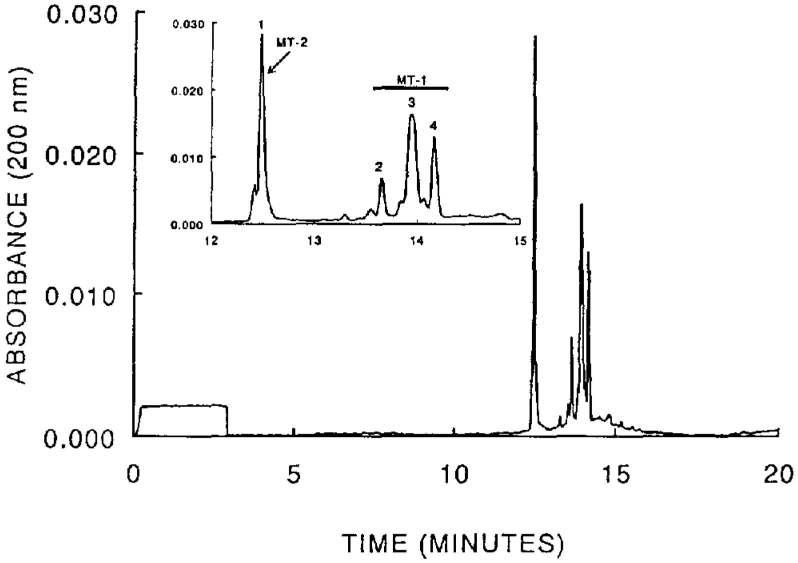

Fig. 1. Capillary zone electropherogram of rabbit liver Zn-MT. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and loaded into the capillary by pressure injection for 10 s. A 57 cm (50 cm to detector) × 50 μm fused-silica, polyamine-coated capillary was used. The running buffer was 0.1 *M* sodium phosphate adjusted to pH 7.0 and the running voltage was 20 kV. Inset: an expanded time scale shows more clearly the separated isoform peaks which are numbered according to their order of migration. Those peaks belonging to the MT-1 and MT-2 charge classes are also indicated.

In order to determine the optimal conditions to perform the separation of MT isoforms, the concentration and the pH of the sodium phosphate running buffer were varied while using the rabbit liver Zn-MT sample depicted in Fig. 1 (Figs. 2 and 3). At low ionic strength (10 m*M*) the resolution and peak shapes are poor, undoubtedly due to significant interaction between the negatively charged MT isoforms and the positively charged wall (Fig. 2). Increasing the concentration to 50 m*M* improved both peak shape and resolution which was further improved by increasing the buffer concentration to 100 m*M*. This concentration of phosphate does generate a rather high current of about 90 μA. A concentration of 200 m*M* gave even further improvements, mainly in peak shape (data not shown); however, because of the high current generated it was necessary to decrease the running voltage from 20 to 15 kV. Thus a concentration of 100 m*M* was chosen as optimal for a routine separation of MT isoforms.

Fig 3. depicts the effect of pH on the separation of MT isoforms in 0.1 *M* sodium phosphate buffer. At pH 6.0, there is incomplete resolution of the MT-1 subisoforms and the migration times of the MT-2 and MT-1 isoform groups are shorter than at higher pH values. The latter finding may reflect the fact that EOF is increased as pH decreases due to an increase in the number of positively charged amine groups on the surface of the capillary [15]. As the pH is increased to 7.0 or 8.0, the peak migration times also increased and the resolution of the isoform peaks is improved, although the difference between pH 7.0 and 8.0 is less dramatic than that observed between pH 6.0 and 7.0. Fig. 4 depicts a separation in 0.1 *M* phosphate buffer at pH 2.5. Below a pH value of 5.0, metals begin to dissociate from MTs [2] and at pH 2.5, all of the zinc bound to the MT protein (thionein) would be removed leaving the metal-free proteins (apothioneins). Three predominant peaks were resolved at pH 2.5 with the presence of a number of minor peaks. Although the separation appears to be quite good, it has been our experience with the polyamine-coated capillary that there is significant peak broadening, prolonged migration times and somewhat erratic behavior among repetitive runs all of which

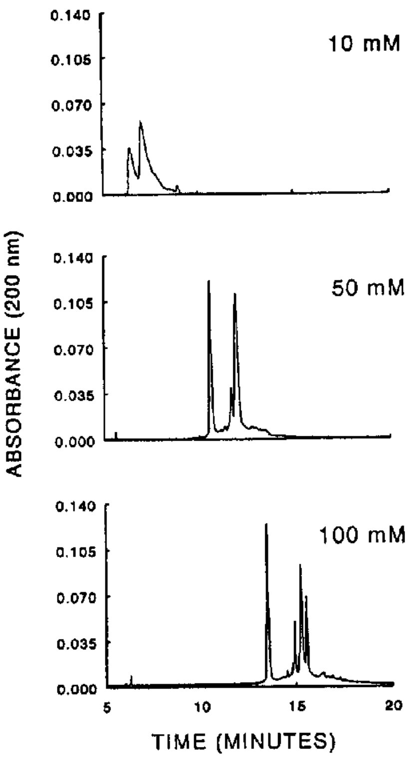

Fig. 2. Capillary zone electropherograms of rabbit liver Zn-MT separated using a running buffer of sodium phosphate, pH 7.0 at a concentration of 10, 50 or 100 m*M*. With the exception of changes to the concentration of phosphate in the running buffer, CZE was conducted as in Fig. 1.

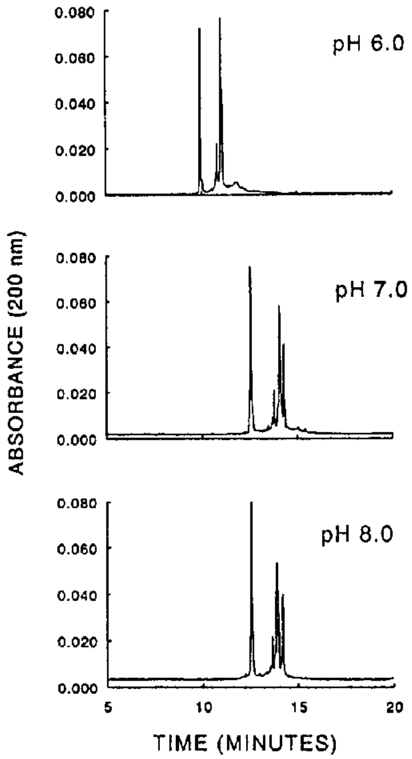

Fig. 3. Capillary zone electropherograms of rabbit liver Zn-MT separated using a running buffer of 0.1 *M* sodium phosphate at pH 6.0, 7.0 or 8.0. With the exception of changes to the running buffer pH, CZE was conducted as in Fig. 1.

suggest that this pH is not optimal for routine separation of MT isoforms. On the contrary, using uncoated fused-silica capillaries, we have previously found that significant improvement in the resolution of rabbit liver MT isoforms can be achieved at pH 2.5 using a phosphate buffer [8]. In fact, the separation achieved with an uncoated fused-silica capillary [8] is the exact "mirror image" of the one achieved in this study using the polyamine-coated capillary (Fig. 4), indicative of the charge reversal on the surface of the capillary and the resulting difference in the direction of EOF. There may be merit in separating MT isoforms at low pH where metals are dissociated from the protein because quantitative determinations are then only dependent on the detection of UV absorbance due to the peptide bonds and not that coming from the

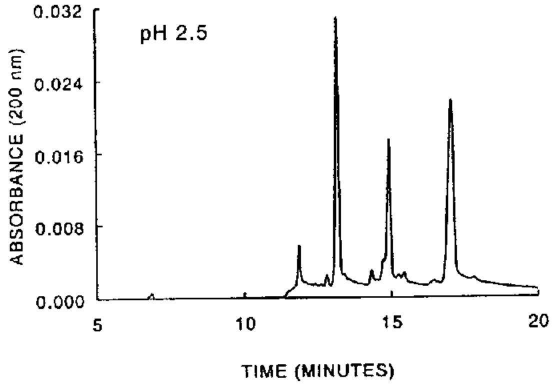

Fig. 4. Capillary zone electropherogram of rabbit liver Zn-MT separated using a running buffer of 0.1 *M* sodium phosphate at pH 2.5. CZE was conducted as in Fig. 1, except that the running buffer pH was adjusted to 2.5 and the voltage was increased to 30 kV.

metal–thiolate charge transfers which can contribute significantly to the overall absorbance at 200 nm. This would tend to improve the reproducibility of quantitative assays since the nature and amount of bound metal would not be a factor [8,16]. However, at pH 7.0, the native conformation of the MT isoforms which depends on the bound metal would be preserved [2]. This conformation may prove to be an essential component in the separation of all of the MT isoforms. More work is required to establish the optimal pH at which to isolate MT isoforms for subsequent quantification.

Fig. 5. depicts the separation of rabbit liver MT derived from two sources. Both MT preparations were the result of cadmium induction *in vivo*. The commercial preparation contains both cadmium and zinc with the former metal being the predominant one. The experimental preparation underwent additional modification during the purification procedure in that the zinc and cadmium bound to the native protein were removed by acid extraction and replaced exclusively with zinc [17]. Fig. 5A and B depict the separations of the experimental and commercial preparations, respectively. Both electropherograms look remarkably similar with the exception of a single peak which is much more pronounced in the experimental compared to the commercial preparation. Thus, the commercial preparation exhibits three predominant peaks, whereas the experimental preparation exhibits four, as was found in Fig. 1. It is not presently known if this difference constitutes a difference in the isoform pattern of the two preparations of MT or if it originated from differences in the preparation of the two samples. Klauser *et al.* [19] reported that rabbit liver MT-1 subjected to reversed-phase HPLC at neutral pH exhibited two predominant peaks, while MT-2 exhibited a single predominant peak and a less abundant one. Together these findings are consistent with our observation of three predominant peaks for the commercial preparation. If in fact differences in isoforms exist, then it could reflect a difference in the strain of animals used or in the induction procedure. It is also possible that this peak arises from the metal-exchange procedure applied to the experimental MT preparation and the fact that the commercial preparation is a mixed Cd,Zn-MT, whereas the experimental preparation is a Zn-MT. We have previously found that the metal composition of MT isoforms apparently has little or no impact on their separation by CZE [6,7,9]. In any event, the comparison of both MT preparations clearly demonstrates the resolving power of this technique which is capable of detecting subtle differences in these two MT preparations that CZE and MECC techniques conducted using uncoated fused-silica capillaries could not (unpublished results).

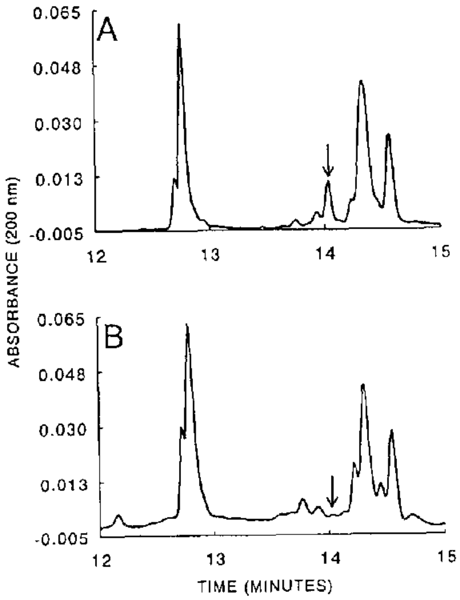

Fig. 5. A comparison of electropherograms of experimental Zn-MT (A) or commercial Cd,Zn-MT (B) prepared from cadmium-injected rabbit liver. CZE was conducted as in Fig. 1. The presence of an isoform peak that differs in amount between the two MT preparations is noted with an arrow.

Fig. 6 depicts the separations of MTs derived from liver or kidney tissue from a number of animal species, including humans. Horse kidney MT exhibited three predominant peaks with the presence of two additional minor peaks detected (Fig. 6A). Klauser *et al.* [19] previously found that horse kidney MT could be resolved into five or six separate isoproteins using reversed-phase HPLC. The results of this study represent the first independent verification of that finding.

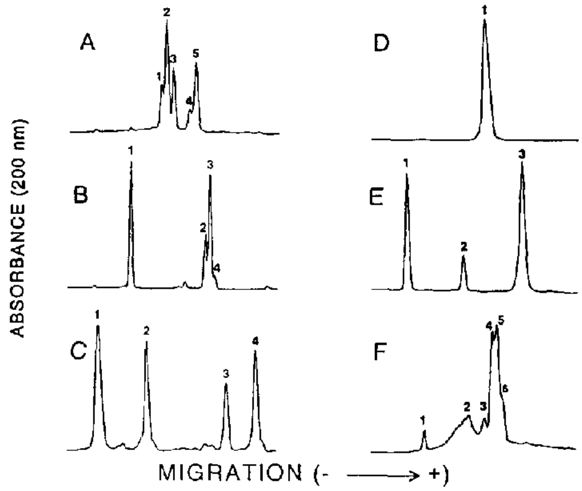

Fig. 6. A comparison of electropherograms of MTs isolated from horse kidney (A), sheep liver (B), pig liver (C), chicken liver (D), rat liver (E), or human liver MT-1 (F). MT samples were dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as in Fig. 1. Each electropherogram depicted represents a 3-min "window" that contains all of the isoform peaks for each MT sample separated. The major isoform peaks are numbered according to their order of migration.

Since, it has been reported that equine MT isoforms all share a common net negative charge at pH 7.5 [2], it is clear that some factor other than net charge differences is responsible for effecting their separation by CZE. This fact might also explain the migration behavior of horse kidney MT isoforms which tend to migrate more closely together than those from other species. Sheep MT has previously been found to be coded for by four genes (one MT-2 and three functional MT-1 genes), thus giving rise to four isoforms [20,21]; however, these have yet to be isolated and completely characterized. It is clear from this study that sheep MT separated on the polyamine-coated capillary exhibited four discernible peaks (Fig. 6B). Moreover, MECC does resolve the same number of peaks from this sample and thus confirms the existence of four sheep MT isoforms [9]. We have previously reported on the heterogeneity characterizing pig liver MT isoforms isolated by reversed-phase HPLC [5,18]. In this study, pig liver MT was characterized by four major peaks (Fig. 6C). This is also consistent with CZE analysis done in uncoated capillaries at several different pH values [7,8] as well as with previously published findings using low-pressure chromatographic separations [22,23] both of which detected heterogeneity for porcine MT. Most avian MTs, including chicken, are characterized by the presence of a single isoform encoded by a single gene [24]. When subjected to CZE [7–9] or reversed-phase HPLC [5,18], a single predominant peak is observed, indicating homogeneity of the preparation. Similarly the results of this study indicated a single peak for chick liver MT (Fig. 6D). Rat liver MT has been previously found to be encoded by two genes, one each for the MT-1 and MT-2 isoforms [2,3]. Clearly when subjected to CZE under the conditions used in this study, two predominant peaks are detected (Fig. 6E). However, a third minor peak migrating between the MT-2 and MT-1 peaks is observed, indicating the existence of further heterogeneity for rat liver MT. The existence of a third rodent MT isoform has also been observed for rat liver MT subjected to MECC analysis [9] as well as anion-exchange HPLC of rat and mouse liver MTs [25,26]. The level of this putative isoform has been suggested to depend on whether the MT was induced by zinc or by cadmium, since zinc has been suggested to be a more potent inducer than cadmium [25,26]. The rat liver MT in Fig. 6E was zinc-induced and this could explain the existence of the third isoform peak. When a Cd-induced rat liver MT sample is separated, much less of the intermediate peak was observed (data not shown). Human liver MT is characterized by extensive heterogeneity with the MT-1 charge class exhibiting five subisoforms, all of which can be isolated by reversed-phase HPLC [27]. Fig. 6F indicates considerable heterogeneity with a major peak apparently comprised of two to three closely migrating components (Fig. 6F, peaks 4–6) as well as the existence of three other lesser abundant peaks with at least one of them (Fig. 6F, peak 2) exhibiting significant microheterogeneity. Thus the isoform pattern for human liver MT-1 is quite complex compared to the other MTs analyzed. Together these data emphasize the utility of the polyamine-coated capillary in the analysis of MT isoform heterogeneity which can vary considerably among different animal species.

The reproducibility of repetitive runs was tested using chick liver MT because this particular MT is comprised of a single isoform (Fig. 6D). Table 1 summarizes the findings for eight consecutive runs. Peak migration time, integrated peak area and peak height gave relative standard deviations of 2.53, 1.85 and 2.21%, respectively. These data confirm the reliability of the technique for MT isoform analysis and was somewhat better than that previously achieved for CZE in uncoated capillaries [7]. A trend toward decreasing peak migration time is apparent in the data (Table 1). This effect seemed to depend on the degree of prior conditioning or use the capillary had received. Also, changes in peak migration times were observed after the running buffer was replenished. Peak migration times appeared to stabilize after several repetitive runs. Despite this variation, the resolution of individual MT isoform peaks appeared to be unaffected since the decline in migration time was uniform for all isoform peaks. Thus, the prior conditioning of the capillary and the age of the running buffer are both variables to be considered when evaluating reproducibility of a separation using the polyamine-coated capillary.

To test the quantitative nature of this separation technique, chick liver MT (cMT) was again employed to establish standard curves (Fig. 7). Integrated peak area showed excellent linearity over three orders of magnitude of MT concentration (1.0 μg/ml to 1.0 mg/ml). It was possible to detect as little as 1.0 μg/ml of cMT, although it was necessary to increase the time of injection two- to three-fold above that required for a 5.0 μg/ml standard in order to get sufficient mass into the capillary for accurate peak detection and integration. Using a 1.0 μg/ml cMT standard, it was also found that integrated peak area was linear over a range of loading times (10–60 s, data not shown). This detection limit (*ca.* 1.0 μg/ml) is equal to or better than that achieved for CZE conducted in uncoated capillaries [6,7] and probably reflects the higher peak efficiencies obtained using the polyamine-coated capillary.

In view of the fact that this technique was determined to be reproducible and quantitative, more complex sample matrices containing MT were investigated (Fig. 8) Liver tissue obtained from chicks injected with saline (control) or zinc (+Zn) were first fractionated using acetonitrile treatment in order to remove as much extraneous protein as possible prior to electrophoresis [6]. The control liver extract was found to have a small amount of MT, but it was too low to be accurately quantified. To verify that this was

Table 1
Reproducibility of chicken liver MT isoform migration time, peak area and peak height for eight consecutive runs

Run No.	Time (min)	Area (V s)	Height (V)
1	16.06	7.62	0.076
2	15.98	7.59	0.077
3	15.50	7.39	0.077
4	15.31	7.49	0.077
5	15.18	7.40	0.079
6	15.11	7.72	0.080
7	15.15	7.47	0.078
8	15.09	7.77	0.081
Mean	15.42	7.56	0.078
S.D.	0.39	0.14	0.002
R.S.D. (%)	2.53	1.85	2.210

S.D. = Standard deviation of the mean; R.S.D. = relative standard deviation. Chicken MT was dissolved in deionized water at a concentration of 1.0 mg/ml. Injection time was 5 s.

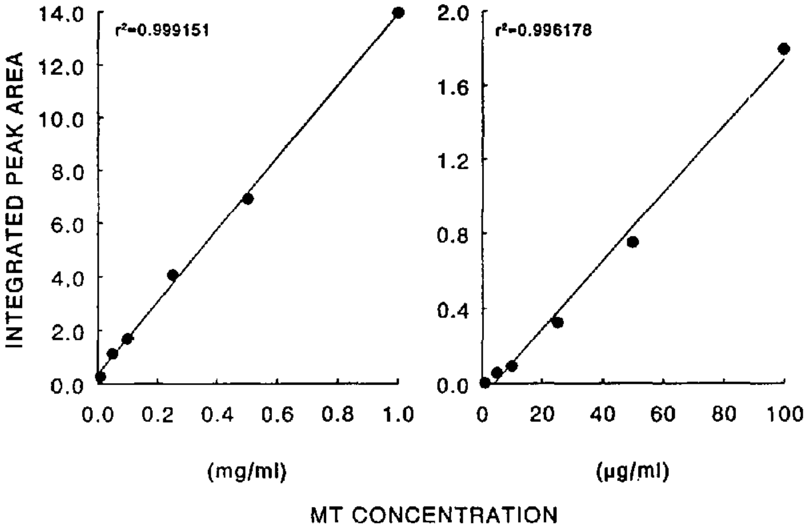

Fig. 7. Integrated peak area of chicken liver MT plotted as a function of the concentration of the MT sample loaded into the capillary. MT samples were diluted to the appropriate concentration from a 1.0 mg/ml stock using deionized water and were then loaded into the capillary by pressure injection for 10 s. CZE was conducted as in Fig. 1. Standard curves were constructed for the ranges of 0.05–1.0 mg MT/ml and 1–100 µg MT/ml. The curves were derived by linear regression analysis of the data and the correlation coefficient (r^2) for each curve is listed.

indeed the MT peak, 100 µg of cMT standard was added to the control extract prior to separation (Fig. 8, inset). The MT peak was thus verified. Fig. 9 depicts the separation of liver tissue extract from zinc-injected chickens. Since zinc is known to be a primary inducer of the MT

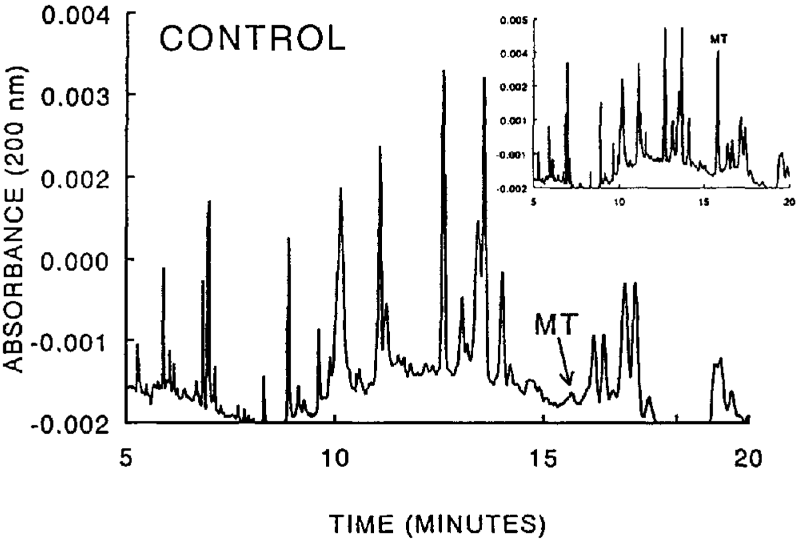

Fig. 8. Capillary zone electropherogram of an acetonitrile-fractionated liver homogenate extract from a saline-injected chicken (control). The extract, dissolved in deionized water, was loaded into the capillary by pressure injection for 10 s. CZE was conducted as in Fig. 1. The inset shows the same sample rerun after it was adjusted to 100 µg MT/ml using standard chicken liver MT. The MT isoform peak is so designated.

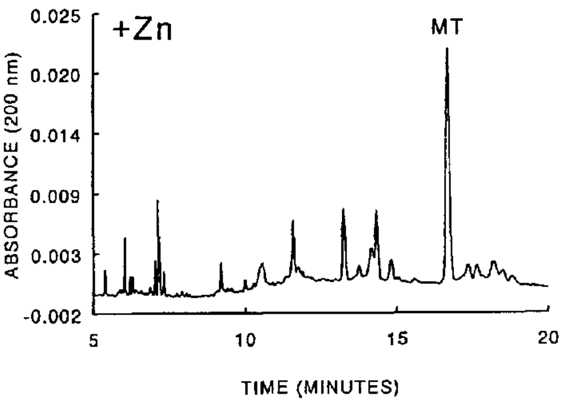

Fig. 9. Capillary zone electropherogram of an acetonitrile-fractionated liver homogenate extract from a zinc-injected chicken (+Zn). The extract, dissolved in deionized water, was loaded into the capillary by pressure injection for 10 s. CZE was conducted as in Fig. 1. The MT isoform peak is so designated.

gene in birds [28], it was interesting to note that the MT peak was the most prominent one observed in the extract. Furthermore, it was estimated, based on the standard curve previously derived (Fig. 7), that the zinc-injected sample contained in excess of 1.0 mg MT/g of liver tissue. Thus, it appears that CZE using the polyamine-coated capillary is applicable to the analysis of MT in complex matrices such as tissue extracts. Clearly, more work is required to confirm the utility of this technique for the routine analysis of MT in tissue and cell samples.

Although the polyamine-coated capillary was developed to resolve basic (positively charged) substances, the results of this study suggest that the utility of this capillary can be extended to the analysis of MT isoforms (and presumably other proteins) which exhibit acidic p*I* values. At pH 7.0, MTs have a net negative charge and therefore the ionic strength of the running buffer must be sufficiently high to suppress Coulombic interactions between MTs and the polyamine coating of the wall of the capillary. A 0.1 *M* sodium phosphate buffer pH 7.0 was found to be adequate for this purpose. Phosphate as a buffer choice appears to be key to the separation of MTs, since equimolar (0.1 *M*) solutions of other buffers such as 3-(N-morpholino)propanesulfonic acid (MOPS) and Tris at pH 7.0 or sodium borate buffer at pH 8.4 produced incomplete resolution of MT isoforms compared to phosphate. These buffers were only capable of resolving the MT-1 and MT-2 charge classes (data not shown). The use of 0.1 *M* sodium phosphate buffer at pH 7.0 significantly reduced EOF in this column. This may result from an interaction between phosphate ions in the buffer and the positively charged surface amine groups which effectively screens the surface of the capillary, thereby diminishing EOF. Differences in elctrophoretic mobility may then become the more dominant factor in the separation of individual MT isoforms [14]. McCormick [29] previously reported that phosphate was capable of modifying the surface of uncoated fused-silica capillaries. This had a significant impact on the resolution of a mixture of peptides and proteins, especially at low pH, by greatly reducing EOF and permitting separations based predominantly on differences in electrophoretic mobility. He suggested that phosphate bound to exposed silanol groups on the capillary surface. Because of the surface charge reversal characteristic of the polyamine-coated capillary, EOF and electrophoretic mobility of MT isoforms are aligned in the same direction (toward the anode). This differs from the situation in uncoated silica columns where, at neutral pH, EOF and electrophoretic mobility are opposed to one another. Under those conditions, EOF is required to move the MT isoforms past the detector toward the cathode. The fact that alignment of a weak EOF and electrophoretic mobility may be an important aspect of the selectivity of the polyamine-coated capillary is strengthened by our previous finding that at pH 2.5 in an uncoated fused-silica capillary, rabbit liver MT isoforms were better resolved [8]. Under the latter conditions, the surface silanol groups would be more highly protonated thereby reducing flow toward the cathode while the MT isoforms, which undergo a charge reversal at this pH to a net positive charge, migrate along with EOF toward the cathode. Thus, control of EOF and allowing electrophoretic mobility to become a more prominent factor in the separation may be a useful mechanism to achieve a complete resolution of MT isoforms, especially the subisoforms which share a similar or identical net charge.

4. Conclusions

A new method for the separation and quantification of MT isoforms has been developed using a polyamine-coated capillary and phosphate buffer at relatively high ionic strength. Since the pH of the running buffer is 7.0, the MT isoforms retain their native structure and their metal content is preserved. With this method, it was possible to achieve better separations of MT isoforms, especially the subisoforms, than was previously possible by CZE or MECC techniques using uncoated fused-silica capillaries. The reason for this may involve a specific interaction between the buffer and the capillary surface coating which slows EOF toward the anode yet permits the alignment of electrophoretic mobility of the individual MT isoforms in the same direction as EOF. This unique combination of parameters is postulated to increase the selectivity of the resolution of MT isoforms.

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6. References

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