Characterization of metallothionein isoforms

Comparison of capillary zone electrophoresis with reversed-phase high-performance liquid chromatography

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ABSTRACT

The purpose of this study was to compare and contrast the separation of metallothionein (MT) isoforms by reversed-phase high-performance liquid chromatography (RP-HPLC) with capillary zone electrophoresis (CZE). RP-HPLC was performed on a Vydac C_8 column eluted with a linear acetonitrile gradient. CZE was performed in a 57 cm × 75 μm I.D. fused-silica tube at an operating voltage of 30 kV. Phosphate buffer (10 mM) at pH 2.5, 7.0 and 11.0 was used for both separations. CZE at pH 2.5 resolved three distinct peaks of rabbit liver MT which were incompletely resolved at pH 7.0 or 11.0. RP-HPLC at pH 2.5 gave two peaks and the resolution was not as good as with CZE at the same pH. At pH 7.0 and 11.0, RP-HPLC of rabbit liver MT gave a single predominant peak of unresolved MT-1 and MT-2. Purified rabbit liver MT-1 and MT-2 were used to verify the identity of these peaks. In contrast, MT from horse kidney demonstrated three predominant peaks which were best resolved by CZE at pH 11.0, whereas RP-HPLC resolved only two peaks at pH 11.0 and 7.0. At pH 2.5, RP-HPLC of horse kidney MT gave three peaks, though two of the peaks were incompletely separated. We conclude that pH has a considerable impact on the resolution of MT isoforms by CZE and RP-HPLC and that it is possible to exploit changes in pH to optimize the separation of isoforms for a particular species of MT. When samples of human and sheep liver MT-1, both of which exhibit microheterogeneity, were subjected to CZE, a single predominant peak was observed at each pH value. RP-HPLC of human liver MT-1 at pH 2.5 yielded two peaks that were incompletely resolved. Purified chick liver MT and rat liver MT-1 and MT-2 gave a single predominant peak at all pH values on CZE. In contrast, pig liver MT-1 and MT-2 each exhibited multiple peaks when subjected to CZE, the number of which depended on the pH used to separate the MT. In conclusion, CZE, with its orthogonal selectivity, and RP-HPLC make an excellent combination for the separation and characterization of MT isoforms. Because CZE is rapid (run times typically < 10 min) and requires little sample (< 100 nl), MT samples can readily be analyzed by CZE in conjunction with RP-HPLC or other techniques in order to maximize the information obtained about the individual isoforms.

INTRODUCTION

Because of continuing interest in the unique metal-binding properties of the metallothionein (MT) protein, its putative role in heavy-metal metabolism and numerous investigations into the mechanisms involved in MT gene expression, there is a need for analytical techniques that are capable of rapid separation and sensitive detection of MT isoforms. Although immunological-

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based assays such as radioimmunoassay and enzyme-linked immunosorbent assay have been developed to detect extremely low levels of MTs in tissues and physiological fluids [1–3], in general, they lack sufficient molecular specificity to detect the complete range of isoforms that may be present. In order to study the functional significance of individual MT isoforms, analytical techniques that offer a high degree of resolution are required. Reversed-phase high-performance liquid chromatography (RP-HPLC), which separates compounds based on their hydrophobic character, represents one of the most powerful techniques currently available for the isolation, characterization and quantification of MT isoforms [4]. While the resolution of MT isoforms achieved by RP-HPLC is quite good, this technique can involve relatively lengthy analysis times and requires the use of expensive columns and organic solvents. Although it is possible to separate six isoforms from human liver MT in a single chromatographic step using RP-HPLC at neutral pH [5], this is not always possible for MTs isolated from other eukaryotic species which may require prior purification before they are subjected to RP-HPLC. Recently, capillary zone electrophoresis (CZE) which separates compounds based on their charge-to-mass ratio has shown promise as a rapid, effective and quantitative method for the separation of MT isoforms [6,7]. The objectives of this work were to compare and contrast the separation of MT isoforms achieved by RP-HPLC *vs.* that achieved by CZE using a common phosphate buffer system at acidic, neutral and alkaline pH.

EXPERIMENTAL[a]

Instruments

CZE was performed on a P/ACE System 2100 (Beckman Instruments, Palo Alto, CA, USA). A 57.0 cm (50 cm to detector) × 75 μm I.D. fused-silica capillary column was used. RP-HPLC was performed on a Waters (Waters/Millipore, Milford, MA, USA) chromatography system consisting of dual pumps, an autoinjector, a variable-wavelength UV–Vis monitor and a Vydac C_8 pH-stable reversed-phase column (250 × 4.6 mm). Data were collected and processed from both instruments using System Gold software (Beckman Instruments).

Materials

Standard Cd,Zn-MTs were purchased from a commercial source (rabbit liver and horse kidney, Sigma, St. Louis, MO, USA) or were prepared by sequential gel permeation and ion-exchange column chromatography [8]. Chick liver Zn-MT was further purified by RP-HPLC according to the method of Richards and Steele [9] prior to CZE. Rat and human liver MT isoforms were provided by Dr. Chiharu Tohyama (National Institute of Environmental Studies, Tsukuba, Japan). All buffers and chemicals were reagent grade.

Methods

CZE was performed under the following conditions: Prior to each run, the capillary was flushed for 1.0 min with 0.1 M NaOH followed by water and 10 mM phosphate buffer, pH 2.5, 7.0 or 11.0. Samples of MT were dissolved in deionized water at a final concentration of 1.0 mg/ml and were loaded into the capillary by pressure injection. After a sample was loaded, the run was initiated by applying 30 kV across the capillary. All CZE was conducted at 25°C. RP-HPLC was conducted under the following conditions: samples of MT were dissolved in deionized water at a final concentration of 1.0 mg/ml and loaded onto the column by an autoinjector. Buffer A was 10 mM phosphate adjusted to pH 2.5, 7.0 or 11.0. Buffer B consisted of 60% acetonitrile in buffer A. MT Isoforms were eluted at a flow-rate of 1.0 ml/min using a two-part linear gradient consisting of 0% B by 5 min, 0–25% B by 10 min and 25–50% B by 55 min at ambient temperature. Detection of MT isoforms was accomplished by monitoring absorbance at 200 nm (CZE) or 214 nm (RP-HPLC).

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

RESULTS

Rabbit liver Cd,Zn-MT subjected to CZE at pH 2.5 yielded three resolved peaks, whereas only two peaks were resolved at pH 7.0 or 11.0 (Fig. 1). To verify the identity of the three peaks obtained from the CZE separation conducted at pH 2.5, samples of purified MT-1 and MT-2 from rabbit liver were subjected to CZE individually or as an equal mixture of each (Fig. 2). Under these conditions, the MT-1 isoform yielded two predominant peaks whereas the MT-2 isoform produced a single predominant peak, thus accounting for the three major peaks detected in Fig. 1. Similarly, horse kidney Cd,Zn-MT subjected to CZE at pH 11.0 yielded three resolved peaks, while at pH 2.5 and 7.0 only two were resolved (Fig. 3).

Overall, RP-HPLC gave the best resolution of Cd,Zn-MT isoforms at pH 2.5, but at this pH only two peaks were resolved for rabbit liver and two to three were incompletely resolved for
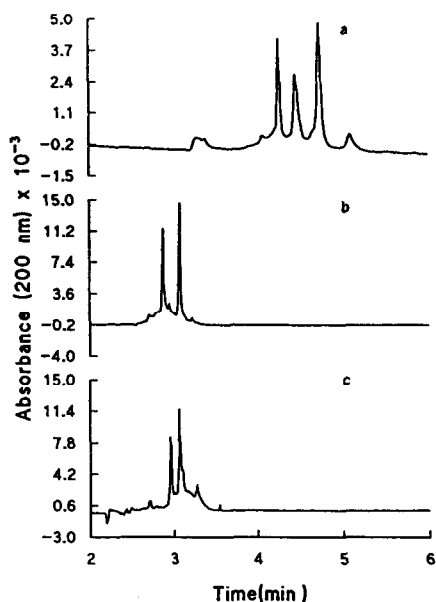
Fig. 1. Capillary zone electropherograms of rabbit liver Cd,Zn-MT conducted at different pH values. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and loaded into the capillary by pressure injection for 1 s. A 57 cm (50 cm to detector) × 75 μm fused-silica, uncoated capillary was used. The running buffer was 10 mM sodium phosphate adjusted to pH 2.5, 7.0 or 11.0 and the running voltage was 30 kV. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.
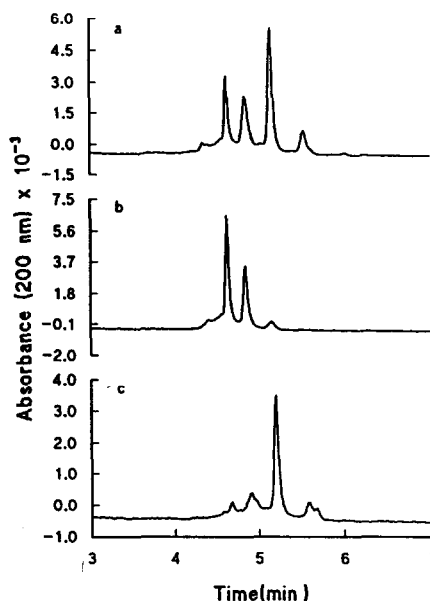
Fig. 2. Capillary zone electropherograms of rabbit liver Cd,Zn-MT isoforms: (a) MT, (b) MT-1, (c) MT-2. Samples of MT isoforms were dissolved at a concentration of 1.0 mg/ml in deionized water and equal volumes of MT-1 and MT-2 solutions were mixed to produce the MT sample. CZE was conducted as described in the caption to Fig. 1 with a running buffer of 10 mM sodium phosphate at pH 2.5.

horse kidney (Figs. 4 and 5). At pH 7.0 or 11.0, rabbit liver MT isoforms coeluted from RP-HPLC, whereas the horse kidney MT-1 and MT-2 isoforms were incompletely resolved.

Human liver MT-1, which is known to be heterogeneous, migrated as a single predominant peak on CZE at each of the three pH values tested (Fig. 6), whereas two predominant peaks were incompletely resolved by RP-HPLC at pH 2.5 (Fig. 7). Sheep liver Zn-MT-1, also characterized by microheterogeneity, migrated as a single peak when subjected to CZE (Fig. 8). The order of migration during CZE for the major MT isoforms (MT-1 and MT-2) from sheep liver reversed as the pH was increased from 2.5 to either 7.0 or 11.0 indicating differences in acidic and basic amino acid composition between these two classes of MT.

Rat liver Cd,Zn-MT-1 and Cd,Zn-MT-2 (Fig. 9) and chicken liver Zn-MT (Fig. 10) migrated as single peaks on CZE at each of the pH values tested. As observed with sheep liver Zn-MT, the
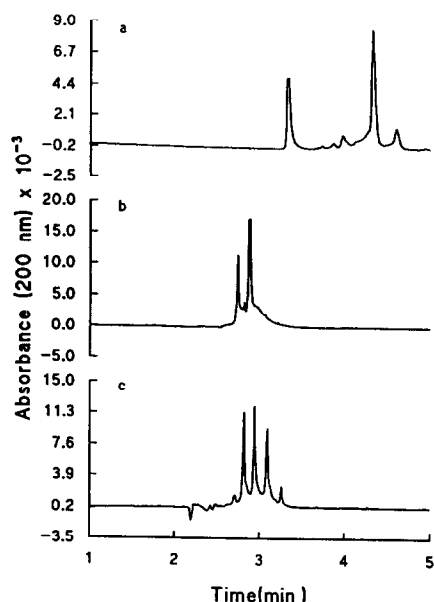
Fig. 3. Capillary zone electropherograms of horse kidney Cd,Zn-MT conducted at different pH values. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.
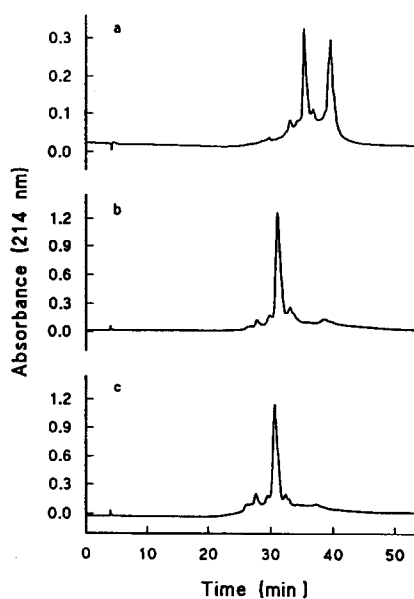
Fig. 4. Rabbit liver Cd,Zn-MT subjected to RP-HPLC at different pH values. MT was dissolved at a concentration of 1.0 mg/ml in deionized water. A 50-μl volume of the MT sample was injected onto a Vydac C_8 pH-stable column (250 × 4.6 mm) and eluted at a flow-rate of 1.0 ml/min with a two-step linear gradient of acetonitrile (CH_3CN) in 10 mM sodium phosphate buffer at (a) pH 2.5, (b) pH 7.0 or (c) pH 11.0 (0–30% CH_3CN over 55 min).

order of migration for rat liver MT-1 vs. MT-2 was reversed when the pH of the buffer was increased from 2.5 to 7.0 or 11.0. In contrast, pig liver Zn-MT-1 (Fig. 11) and Zn-MT-2 (Fig. 12) exhibited multiple peaks on CZE. The number of peaks depended on the pH.

DISCUSSION

MTs isolated from rabbit liver and horse kidney can be separated into three major peaks using CZE. Further analysis of the two major isoform classes from rabbit liver indicated that two of the major peaks originated from the MT-1 isoform group and the third was determined to be MT-2. Our results clearly demonstrate the power of the alternative separation selectivity associated with CZE. Verification of these species as true MT isoforms must await amino acid sequence analysis of the individually collected peaks. However, our findings are consistent with the work of Klauser et al. [10] which demonstrated by RP-HPLC that MT from rabbit liver is comprised of at least four distinct species,

while horse kidney MT is comprised of at least three, each differing in their amino acid composition. In addition, we were able to qualitatively assess the stability of our MT standard solutions prepared in deionized water using CZE. Even after several months of storage at 4°C, there was no evidence of additional peaks nor was there any apparent change in MT isoform peak shape or integrated peak area indicative of MT breakdown [7]. These findings also argue against the possibility that the peaks detected in our study were artifacts arising from MT degradation or modification produced prior to or during CZE. The fact that rabbit liver and horse kidney MTs contain both Cd and Zn could lead to the possibility of MTs differing in their metal content (i.e., metalloforms). Metalloforms could conceivably have different properties related to differences in structure. However, we have found that the type of metal bound to MT does not appear to affect isoform migration time for CZE (unpublished results). Therefore, we do not
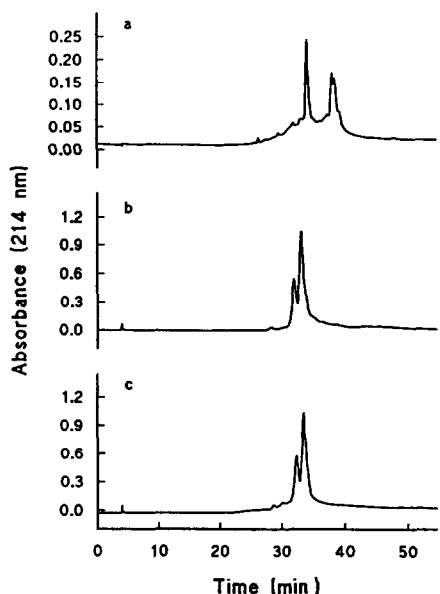
Fig. 5. Horse kidney Cd,Zn-MT subjected to RP-HPLC at different pH values. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and RP-HPLC was conducted as described in the caption to Fig. 4.

Fig. 7. Human liver MT-1 subjected to RP-HPLC at pH 2.5. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and RP-HPLC was conducted as described in the caption to Fig. 4 with an elution buffer of 10 mM sodium phosphate at pH 2.5.
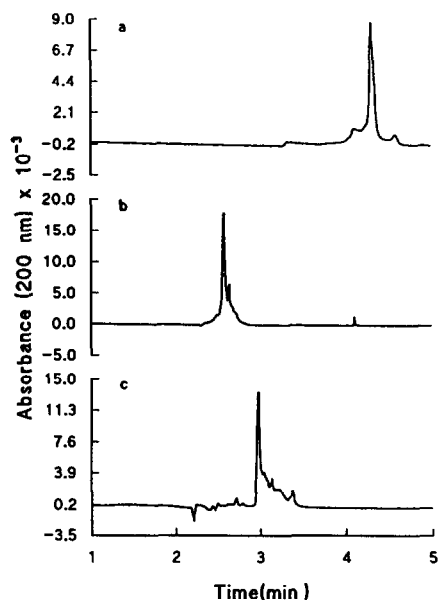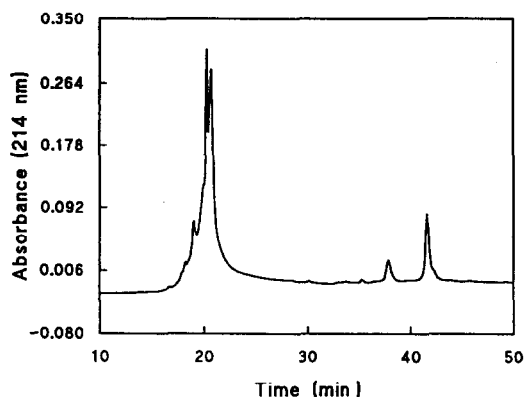
Fig. 6. Capillary zone electropherograms of human liver MT-1 conducted at different pH values. MT-1 was dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.
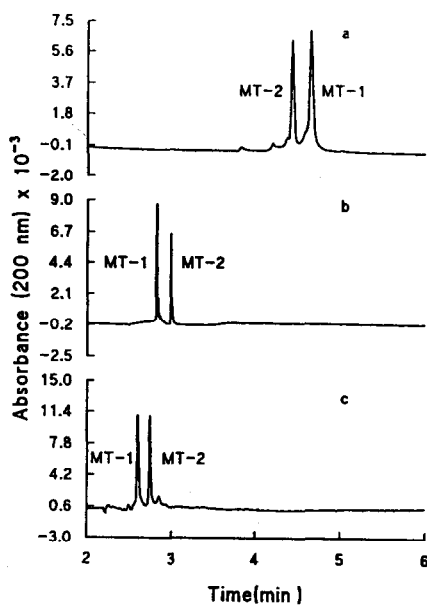
Fig. 8. Capillary zone electropherograms of sheep liver Zn-MT isoforms (MT-1 and MT-2) conducted at different pH values. Samples of MT isoforms were dissolved at a concentration of 1.0 mg/ml in deionized water and equal volumes of MT-1 and MT-2 solutions were mixed to produce the MT sample subjected to CZE. CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.

think that the issue of MT metalloforms represents a complicating factor for interpreting the nature of the individual peaks generated by CZE.

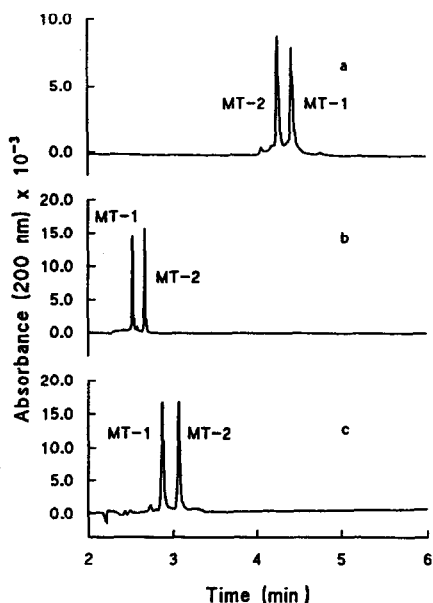The apparent pH dependency for rabbit liver

Fig. 9. Capillary zone electropherograms of rat liver Cd,Zn-MT isoforms conducted at different pH values. Samples of MT isoforms were dissolved at a concentration of 1.0 mg/ml in deionized water and equal volumes of MT-1 and MT-2 solutions were mixed to produce the MT sample subjected to CZE. CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.
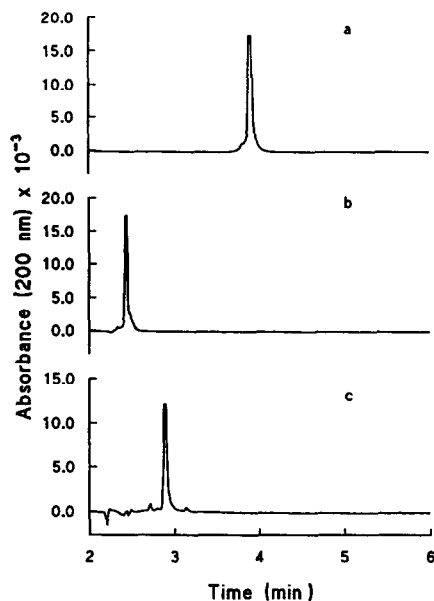
Fig. 10. Capillary zone electropherograms of chicken liver Zn-MT conducted at different pH values. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.

and horse kidney MT isoform separation by CZE is indicative of significant differences in their acidic and basic amino acid contents [11]. Even small changes in pH can produce dramatic shifts in peaks migrating on CZE, especially at or near the pI of the protein because of the contributions of both the basic and acidic amino acids to the net charge on the protein molecule. The metal–thiolate bonds characteristic of MT also contribute negative charge to the molecule at neutral pH and above. This coupled with the contributions of the acidic and basic amino acids results in the overall net negative charge on the MT molecule above the pI (3.9–4.6) of the protein [11]. Since the amino acid substitutions characteristic of MT isoforms do not change the mass of the polypeptide appreciably, the net charge on the molecule undoubtedly determines its behavior on CZE. This most likely explains the separation of three putative MT isoforms from horse kidney at pH 11.0. Moreover, CZE should prove to be an effective means of separat-

Fig. 11. Capillary zone electropherograms of pig liver Zn-MT-1 conducted at different pH values. MT-1 was dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.

Fig. 12. Capillary zone electropherograms of pig liver Zn-MT-2 conducted at different pH values. MT-2 was dissolved at a concentration of 1.0 mg/ml in deionized water and CZE was conducted as described in the caption to Fig. 1. (a) pH 2.5, (b) pH 7.0, (c) pH 11.0.
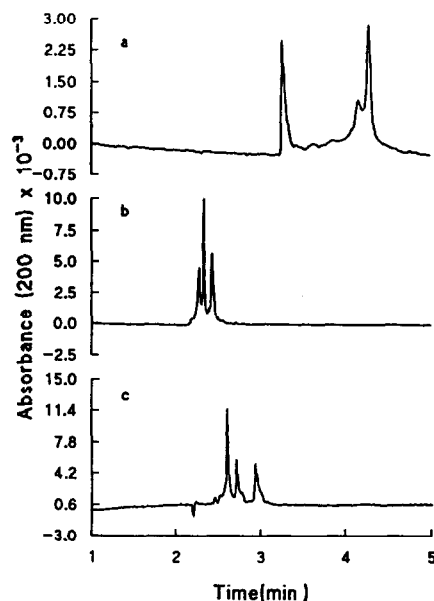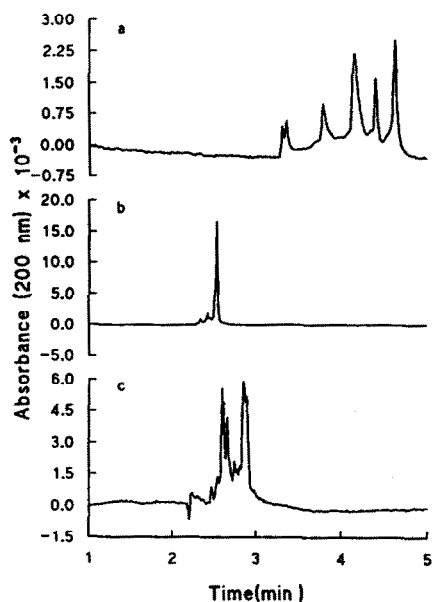
ing a newly discovered isoform class of MT (MT-3) which is expressed only in brain tissue and is characterized by the insertion of three additional glutamic acid residues near the carboxyl terminal portion of the molecule (human MT-3) making it unusually acidic [12,13].

In contrast, at low pH (2.5), where rabbit liver MT showed the best resolution of isoforms, the zinc normally associated with the protein would have dissociated leaving either the metal-free protein (thionein) or a partially metalated species containing cadmium which dissociates from thionein at pH 2.0. The existence of partially metalated MT isoforms (*i.e.*, Cd_4-MT-2a) in a sample of rabbit liver MT-2 has recently been demonstrated by electrospray mass spectrometry [14]. Since metal-binding imparts a distinct tertiary structure to thionein [11], it is likely that at low pH different amino acid residues are exposed to the solvent as a result of metal loss and subsequent unfolding of the polypeptide chain. However, if thionein is first prepared in vitro by exposing MT (rabbit liver Cd,Zn-MT-2) to 0.1 M HCl, a broad peak is

observed when this material is subjected to CZE at pH 2.5 (unpublished results). It would thus appear that differences may exist between subjecting native MT *vs.* thionein to CZE at low pH. This could perhaps reflect differences in structure or stability of the two molecules related to their resultant metal content. It is assumed that metals remain associated with the thionein polypeptide at neutral pH or above. This is apparently true for RP-HPLC conducted at neutral pH [4]; however, it is not currently known what if any loss of metal occurs during CZE at neutral or basic pH. At pH 2.5, even if there is complete dissociation of metal from MT, the quantity present in the buffer and its subsequent dilution would probably result in amounts insufficient to affect the electrophoretic properties of the resulting thionein polypeptide. However, metals such as zinc have been added at mM concentrations directly to the running buffer to improve the separation of peptides subjected to CZE under acidic conditions by differentially decreasing their electrophoretic mobilities [15–17]. This approach seems to be particularly effective for small peptides containing histidine [15]. What effects the addition of metals such as zinc to the running buffer might have on the resolution of MT isoforms by CZE at acidic pH remains to be explored. This study demonstrates the utility of altering pH as a means to improve the separation of MT isoforms by CZE.

From a quantitative standpoint, conducting the analysis of MT isoforms by CZE at acidic pH may offer some advantages over a similar analysis at neutral or alkaline pH. By lowering the pH of the running buffer sufficiently to dissociate all of the metals from the isoforms, the absorbance monitored at 200 nm would reflect the peptide portion of the molecule exclusively. Although both RP-HPLC and CZE conducted at neutral or alkaline pH have been successfully applied to the quantification of MT isoforms [4,6,7], the validity of quantitative estimates relies on the fact that no metal is lost from the protein during the isolation procedure. Under these conditions absorbance originating from the metal–thiolate charge transfer contributes significantly to the overall UV absorbance which is the basis for

quantification of the protein. Trifluoroacetic acid (TFA) has previously been reported to be preferable to phosphate–perchlorate for the RP-HPLC separation of MT isoforms because of its volatile nature [10]. More recently, TFA has been employed in the quantitation of individual MT isoforms by RP-HPLC [18]. These authors reported that because of the low pH of the TFA buffer (ca. 1.8–1.9), zinc and cadmium would completely dissociate from the thionein protein and thus result in a more accurate estimate of actual protein quantity. Thus, the conduct of CZE or RP-HPLC separations of MT isoforms under acidic conditions may yield better estimates of actual protein quantity [18]. However, the resultant reduction in UV absorbance due to the loss of the metal–thiolate charge transfer under these conditions may also reduce the sensitivity of the technique by raising the limits of detection. Since TFA is not commonly used for CZE separations, we chose to use phosphate buffer in this study in order to compare RP-HPLC and CZE on a common basis. However, it is feasible (though perhaps not optimal) to conduct CZE separations of MT isoforms using TFA (unpublished results) so that comparisons of RP-HPLC and CZE using this buffer are possible. Moreover, it is also possible to analyze peaks collected from RP-HPLC in TFA buffer directly using CZE (unpublished results).

Sheep liver MT-1 has been shown to be encoded by a family of three expressed genes [19]. It could be that the amino acid substitutions characteristic of the sheep MT-1 isoforms are such that they do not change the molecule sufficiently, either in its net charge or its hydrophobic character, to obtain complete separation of the individual isoform species under the conditions employed in this study for CZE and RP-HPLC. Less is known about pig liver MTs, since there has been no in-depth analysis of the genes encoding porcine MT. However, from previous studies of the purified isoMTs from porcine liver, there is some indication of heterogeneity beyond the major MT isoform classes [20,21]. It is also possible that incomplete resolution of the MT-1 and MT-2 isoforms during the prior purification by anion-exchange chromatography resulted in some cross-contamination of

the two samples and this occurrence could account for a portion of the observed heterogeneity. The existence of heterogeneity in both the MT-1 and MT-2 isoforms from pig liver detected by CZE in this study is consistent with earlier findings using RP-HPLC [9]. However, as we noted earlier, the ultimate verification of the peaks detected by CZE as distinct MT isoforms depends on subsequent amino acid sequence analysis of the individually collected peaks.

RP-HPLC alone, both as applied in this study and in others [4,10,22] is only capable of resolving the two major MT isoform classes (i.e., MT-1 and MT-2) from rabbit liver and horse kidney. However, if the major MT isoform classes are fractionated by anion-exchange chromatography prior to RP-HPLC, then it is possible to detect additional isoform species for horse kidney and rabbit liver MTs [10]. Without this prior purification step, the different isoform species can coelute on RP-HPLC and thus would not be detected separately. This was clear from our current study which demonstrated coelution or significant overlap of MT-1 and MT-2 isoform peaks from rabbit liver and horse kidney when subjected to RP-HPLC at pH 7.0 or 11.0. In contrast, human liver MT subjected directly to RP-HPLC under neutral conditions has been successfully fractionated into its component isoforms (six altogether) in a single step [5]. It has been reported that human liver MT-1 consists of five subisoform species whereas MT-2 consists of a singular species. In our study, RP-HPLC of pre-purified rabbit liver MT isoforms (MT-1 and MT-2) at pH 2.5 did detect the presence of two major peaks for MT-1 and a singular peak for MT-2 (data not shown), thus confirming our findings with CZE. Our results with human liver MT-1 subjected to RP-HPLC at pH 2.5 indicated some degree of heterogeneity but the two peaks detected were incompletely resolved. Unfortunately, there was insufficient material to fully characterize the separation of human MT-1 on RP-HPLC at pH 7.0 or 11.0. However, CZE of human liver MT-1 at all three pH values studied failed to discriminate significant heterogeneity, yielding instead a single predominant peak. The reasons for these two findings could be due to the nature of the human MT-1 sample used in

the present study since it has been reported that the relative proportions of the five subisoforms for MT-1 can vary significantly from sample to sample [5]. It is also possible that CZE and RP-HPLC as conducted in this study were not optimized and, therefore, lacked the ability to completely resolve the individual human liver MT-1 subisoform species.

Rat liver MT-1 and MT-2 and chicken MT each of which has been found previously to be comprised of a single species on RP-HPLC [10], migrated as a single peak on CZE. Moreover, by changing the pH of the running buffer it was possible to reverse the migration order of MT-1 and MT-2 on CZE which presumably reflects the differences in the net charge of these two classes of molecules. With all of the changes made in buffer pH, at no time were additional peaks indicative of heterogeneity detected for these MT samples. Taken together, the findings of the present study suggest that MTs from different eukaryotic species behave differently when subjected to RP-HPLC and CZE and that both of these modes of separation require optimization for a particular type of MT in order to achieve the best possible separation of the individual isoforms.

In addition to optimizing the conditions under which RP-HPLC or CZE analyses are conducted, some consideration needs to be given to sample preparation. This is particularly true when attempting to analyze for MT isoforms in complex matrices such as liver cytosol. Although this study made use of purified MT samples, both RP-HPLC [4,10] and CZE [6,7] have also been applied to the analysis of MT isoforms in more complex sample matrices such as heat-treated and solvent-fractionated liver cytosols and cell extracts. In fact, it may be possible to combine both of these techniques by performing solid-phase extraction on a reversed-phase support followed by CZE on the fraction eluted to contain MT.

Because it is becoming apparent that no single separation technique possesses the resolving power necessary to completely separate all of the MT isoforms, a multi-dimensional approach applied to the separation of MT isoforms may represent the best approach. Since CZE and

RP-HPLC represent orthogonal separation techniques, a combination of both would give additional capability as opposed to the application of either technique alone. Such a multi-dimensional approach has been successfully applied to the analysis of vasoactive intestinal peptide from rat brain extracts [23] and the separation of tryptic peptides from two species of cytochrome c [24]. Moreover, we have shown that CZE can be successfully applied as a second dimension to gel permeation column chromatography for the separation of MT isoforms from a sheep liver cytosol [6]. We have also been able to directly analyze fractions from RP-HPLC using CZE (unpublished results). In this study, the feasibility of a common buffer system for both CZE and RP-HPLC has been demonstrated. Therefore, a combined approach involving initial sample preparation followed by RP-HPLC and CZE may well offer the most complete separation of MT isoforms to date. Clearly, more work is required to assess the potential of such an approach.

In conclusion, CZE is a new analytical technique for the characterization of MT isoforms which offers rapid analysis times, small sample requirements and alternative separation selectivity as compared to RP-HPLC. CZE affords an excellent means of separating MT isoforms that differ in their net charge. By varying the pH of the buffer used in CZE, it is possible to affect the selectivity of the separation. Since CZE separates proteins according to mass and charge and RP-HPLC separations reflect differences in hydrophobicity, the two techniques are complimentary to each other and, therefore, form a powerful combination for the isolation and characterization of MT isoforms.

REFERENCES

1 R.K. Mehra and I. Bremner, *Biochem. J.*, 213 (1983) 459.

2 J.S. Garvey, R.J. Vander Mallie and C.C. Chang, *Methods Enzymol.*, 84 (1982) 121.

3 A. Grider, K.J. Kao, P.A. Klein and R.J. Cousins, *J. Lab. Clin. Med.*, 113 (1989) 221.

4 M.P. Richards, *Methods Enzymol.*, 205 (1991) 217.

5 P.E. Hunziker and J.H.R. Kägi, *Biochem. J.*, 231 (1985) 375.

6 J.H. Beattie, M.P. Richards and R. Self, *J. Chromatogr.*, 632 (1993) 127.

7 M.P. Richards, J.H. Beattie and R. Self, *J. Liq. Chromatogr.*, 16 (1993) 2113.

8 M. Vasak, *Methods Enzymol.*, 205 (1991) 41.

9 M.P. Richards and N.C. Steele, *J. Chromatogr.*, 402 (1987) 243.

10 S. Klauser, J.H.R. Kägi and K.J. Wilson, *Biochem. J.*, 209 (1983) 71.

11 J.H.R. Kägi and Y. Kojima (Editors), *Metallothionein II*, Birkhäuser Verlag, Basel, 1987, p. 25.

12 Y. Uchida, K. Takio, K. Titani, Y. Ihara and M. Tomonaga, *Neuron*, 7 (1991) 337.

13 R.D. Palmiter, S.D. Findley, T.E. Whitmore and D.M. Durnam, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 6333.

14 X. Yu, M. Wojciechowski and C. Fenselau, *Anal. Chem.*, 65 (1993) 1355.

15 R.A. Mosher, *Electrophoresis*, 11 (1990) 765.

16 H. Kajiwara, *J. Chromatogr.*, 559 (1991) 345.

17 H.J. Issaq, G.M. Janini, I.Z. Atamna, G.M. Muschik and J. Lukszo, *J. Liq. Chromatogr.*, 15 (1992) 1129.

18 M. Wan, J.H.R.H. Kägi and P.E. Hunziker, *Protein Expression Purif.*, 4 (1993) 38.

19 M.G. Peterson, F. Hannan and J.F.B. Mercer, *Eur. J. Biochem.*, 174 (1988) 417.

20 I. Bremner and B.W. Young, *Biochem. J.*, 155 (1976) 631.

21 M. Webb, S.R. Plastow and L. Magos, *Life Sci.*, 24 (1979) 1901.

22 H. van Beek and A.J. Baars, *J. Chromatogr.*, 442 (1988) 345.

23 J. Soucheleau and L. Denoroy, *J. Chromatogr.*, 608 (1992) 181.

24 P.M. Young, N.E. Astephen and T.E. Wheat, *LC·GC*, 10 (1992) 26.