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Separation of metallothionein isoforms by capillary zone electrophoresis

John H. Beattie

Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB (UK)

Mark P. Richards

USDA, Agricultural Research Service, Nonruminant Animal Nutrition Laboratory, Beltsville, MD 20705 (USA)

Ron Self

Division Services, Rowett Research Institute. Bucksburn. Aberdeen AB2 9SB (UK)

ABSTRACT

The potential of capillary zone electrophoresis (CZE) for the analysis of metallothionein (MT) isoforms was investigated. CZE was performed using two different systems, (1) a laboratory-constructed instrument with an ISCO UV detector and (2) a Waters Quanta 4000 system. Capillaries were of 75 μm I.D. × *ca.* 1 m in length and loading times were up to 40 s by gravity or 4 s by electrokinetic migration at 30 kV. Samples were dissolved in 10 m*M* Tris–HCl buffer, pH 9.1, and electrophoresis was performed at 30 kV using a 50 m*M* Tris-HCl, pH 9.1 running buffer. Detection was by UV absorbance at 185 or 214 nm. Purified and semipurified MT samples were analysed for qualitative assessment of purity, relative isoform abundance and separation characteristics of MT from different species. As progress towards the development of a quantitative assay, the linearity of calibration curves and simple methods of sample preparation for analysis by CZE were investigated. Complete separation of a mixture of the two major MT isoforms was achieved in less than 5 min and the technique was found to be very useful for qualitative analysis of MT. Using a rabbit liver MT standard (500 $\mu g/ml^{-1}$), a linear relationship was found between the gravity load time and the integrated peak area. Standard calibration curves were also linear and the detection limit for both CZE instruments under our separation conditions was l-10 μg MT ml^{-1}. The successful use of two solvent extraction procedures for tissue samples demonstrated the potential of CZE for routine quantitative analysis of MT.

INTRODUCTION

The unique chemical and physical properties of metallothionein (MT) and its suggested role in heavy metal metabolism and detoxification have been the subject of intense investigation ever since its initial discovery [1]. In most animal species, MT has 2 major isoforms (MT-l and MT-2) which have a p*I* of between 3.9 and 4.6 and differences of charge due to certain amino acid substitutions [2]. Both isoforms contain 33% cysteine residues with a highly conserved sequence. Transition metals such as Zn, Cd and Cu bind to MT through association with the cysteine thiol groups.

Early separation techniques involved lengthy chromatographic procedures in which MT quantification was indirect, based on measurement of the metal content of the protein [3]. Such techniques are insensitive and impracticable for the study of MT metabolism in some tissues including extracellular fluid matrices in which endogenous MT levels are low. Consequently, a number of different assays

Correspondence to: Dr. J. H. Beattie, Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK.

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have been developed which are more rapid and show improved sensitivity.

Immunological assays [radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA)] offer the highest level of detection sensitivity and are particularly useful for analysing MT present in low quantities such as in urine or plasma [4–6]. Complete crossreactivity of MT antibodies with MT from different species or even between the major isoforms of the protein in a single species is, however, questionable. Indeed, an assay has been developed specifically for the measurement of rat MT-I [4].

Various metal-saturation assays have been published which depend on the displacement of metals bound to MT with a radioactive isotope of Hg, Cd or Ag [7–9]. These assays are of limited value since Hg and Cd will not displace Cu from MT [10] and the binding capacity for Ag is variable [11]. A more recent version of this assay uses ammonium tetrathiomolybdate to remove Cu from MT prior to saturation with ^{109}Cd [12].

A number of high-performance liquid chromatography (HPLC) techniques have been developed to isolate and quantify MT in tissue extracts. Suzuki [13] first developed an isolation procedure for MT which coupled size-exclusion HPLC with metal detection by atomic absorption spectrophotometry. Because this technique relies on a combination of size-exclusion and weak cationic exchange properties of the columns used, MT isoforms are not always completely resolved. Later, reversed-phase HPLC (RP-HPLC) techniques were developed which offer reasonably high detection sensitivity combined with the ability to resolve individual MT isoforms [14]. However, RP-HPLC requires the use of organic solvents and expensive columns.

Recently, CZE has been applied to the analysis of proteins and peptides [15,16] and is particularly suitable for the separation of low M_r molecules. MT is well suited to separation by CZE since it is a low M_r protein (cu. 6600 daltons, including metals) with, in most species, 2 major isoforms which differ in charge over a range of pH conditions. Separation of charged molecules is achieved in narrow-bore capillaries, usually less than 100 μm I.D., and up to 1 m in length, filled with an appropriate buffer solution. The molecules separate in an applied electric field according to their charge to mass ratio at the selected pH. The advantages of CZE over other techniques include the speed of analysis (4–8 min for the separation of MT using the conditions described below), small sample requirements (I-100 nl), nanogram sensitivity, high resolution and improved discrimination. Our objectives were to assess the practicability of using CZE to separate MT isoforms for qualitative analysis and to develop a quantitative assay for routine analysis of MT isoforms in tissue and cell samples.

EXPERIMENTAL

Instrumentation and electrophoresis conditions

Two instruments were used to develop a method for the analysis for MT: a system constructed at the Rowett Institute which is described below and a Waters Quanta 4000 system with an autosampler and 6/12 well carousel (Millipore/Waters Chromatography Division, Watford, UK).

The laboratory-constructed capillary electrophoresis system was made by attaching a plastic box (150 mm × 200 mm × 70 mm deep) with a transparent hinged door, containing the anode and a retractable sample/electrolyte holder, to an ISCO CV^4 variable wavelength (190–750 nm) UV-visible detector (ISCO, Lincoln, NE, USA). The cathode was placed outside the box immediately above an electrolyte vial holder on a retractable platform. The electrodes were constructed out of fine platinum tubing (PT007200, Goodfellow Metals, Cambridge, UK) and each end of a fused-silica, polyimide-coated capillary tube (94 cm × 75 μm I.D.; Composite Metal Services, Worcester, UK) was threaded coaxially through an electrode until they were level. An optical window of about 1 cm length positioned about 20 cm from the cathode end was prepared by heating the polyimide tube coating with a flame and removing the charred material with acetone. The prepared tubing was carefully inserted into the flow cell cassette ensuring that the optical window was located in the light path of the detector. Absorbance was monitored at 214 nm.

The capillary tubing was filled with 50 m*M* Tris–HCl buffer, pH 9.1, under positive pressure applied at the appropriate end. A 2-ml bottle containing the same buffer was placed in position in the anode and cathode cell holders to submerge the ends of the capillary in electrolyte. When setting up the instru-

ment each day, the tube was conditioned by purging with 0.1 A4 KOH and then with electrolyte buffer. A potential of 30 kV was then applied across the capillary from a power supply (Brandenburg, alpha III) for a period of not less than 1 h to allow the system to stabilise. Each sample in 10 m*M* Tris–HCl buffer, pH 9.1, was loaded electrokinetically at 30 kV. Load time varied according to the sample concentration of MT but was usually no more than 4 s.

Using the Waters Quanta 4000 instrument and the same capillary dimensions described above, all samples were loaded under gravity for periods of up to 40 s (this being approximately equivalent to 4 s of electrokinetic loading). The capillary was conditioned with 0.1 *M* KOH at the start of each day and at intervals when required. Sample and electrolyte buffers were as described above and the conditions of electrophoresis were similar. The capillary was monitored for UV absorbance at 185 nm and data were collected and integrated by computer using a datalogging program.

Preparation of MT samples for electrophoresis

Purified rat liver Cd, ZnMT-1 and Cd, ZnMT-2 were gifts from Dr. Chiharu Toyama (National Institute for Environmental Studies, Tsukuba, Japan) and rabbit liver Cd, ZnMT isoforms were obtained from Sigma Chemical Company (Poole, UK). The isoform proteins from both species were used to demonstrate the application of CZE to the assesment of MT purity. In addition, rabbit MT-l and MT-2 were used routinely for evaluating capillary condition and for investigating the potential of CZE for quantitative analysis of MT isoforms. In particular, the linearity of gravity loading time and MT concentration against integrated absorbance peak area was investigated using the Waters Quanta 4000 system. An attempt was also made to estimate the detection limit for MT.

Since CZE analysis of MT is very rapid, we investigated the value of measuring sequential fractions from a gel permeation column, thus adding a second analytical dimension to the chromatography profile. Using a Sephadex G-75 column (30 × 1.5 cm) equilibrated with 10 m*M* Tris–HCl, pH 8.6, and cytosol from a 50% tissue homogenate, sheep MT was separated from the liver of a single adult grey-face ewe which had been dosed orally with 500 mg Zn (as $ZnSO_4$ in saline-glycerol) and injected intraperitoneally (i.p.) with 2 mg Zn kg-' and then 4 mg Zn kg^{-1} ($ZnSO_4$ in saline) on the following 2 days. The Zn concentration in each fraction was determined by atomic absorption spectrophotometry and individual fractions corresponding to the elution position of MT were analysed directly by CZE.

Direct analysis of tissue cytosols by CZE was found to be of little value for the quantification of MT (see Discussion below) and the use of gel permeation chromatography as an initial separation procedure was considered impracticable for routine analysis of MT. An attempt was therefore made to adapt and develop sample preparation techniques that would facilitate the rapid analysis of MT isoforms. Two methods were studied: an acetone precipitation technique and a method using acetonitrile to remove high M_r proteins. Liver homogenates (20% (w/v) in 10 m*M* Tris–HCl, pH 8.6, 0.25 A4 sucrose) were prepared from rats which had been injected i.p. with either saline or 3 mg Zn kg^{-1} ($ZnSO_4$ in saline) for 4 consecutive days. $CdCl_2$ (25 μg Cd/ml cytosol) was added to the homogenates to stabilise MT before centrifugation at 105 000 g for 60 min. The addition of too much Cd at this stage can cause precipitation of MT. Aliquots were treated with acetone using the procedure of Hidalgo *et al.* [17]. The precipitated MT was reconstituted in 10 m*M* Tris–HCl buffer, pH 9.1, and subsequently analysed by CZE. Further ice-cold aliquots of cytosol were treated by the slow addition of cold acetonitrile, while vortexing, to a final concentration of 50% (v/v). The samples were left to stand at 4°C for 1 h before centrifugation at 1000 g for 15 min. The supernatants were analysed directly using CZE.

We were interested to investigate whether very small amounts of tissue could be used for analysis and so 8 mg replicate samples of liver from the saline- and Zn-injected rats were homogenised by sonication in 200 μl of 10 m*M* Tris–HCl buffer, pH 8.6. After centrifugation at 1000 g for 30 min (4°C), 150 μl of acetonitrile was slowly added, while vortexing, to an equal volume of the supernatant. The precipitate was removed by centrifugation at 1000 g for 15 min and the supernatant was analysed directly by CZE.

RESULTS

Purity analysis

Due to the high resolution of the CZE technique and the difference in charge between MT-I and MT-2, the two isoforms of rat and rabbit liver MT showed complete separation (Figs. 1 and 2). When corrected for changes in the endo-osmotic front (EOF), there was no difference in the MT-1 and MT-2 separation characteristics between rat and rabbit. As expected from their negative charge at pH 9.1, both isoforms were detected after the EOF. The migration times for replicate electropherograms of purified MT-1 and MT-2 were consistently reproducible although some variation in migration times were noted when analysing tissue extracts. Marked improvements were made by re-conditioning the capillary with 0.1 *M* KOH between analyses, although this routine treatment was not found to be practicable with the laboratory constructed equipment where the capillary had to be purged manually.

In agreement with established knowledge concerning the charge differences between the isoforms, MT-2, which is more highly charged than MT-1, had the longest migration time ***(ca.*** 260 s compared to 230 s for MT-1; Fig. 1). The rat MT isoforms were found to be of high purity (>95%), although a very small component with a migration time intermediate to MT- 1 and MT-2 was detected. However.

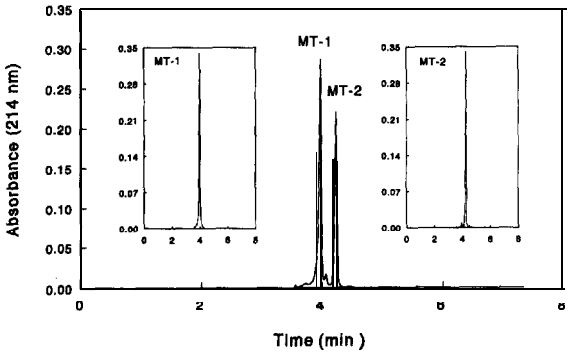

Fig. 1. Capillary zone electropherograms of rat liver MT and the individually purified MT-I and MT-2 isoforms (inserts). Samples of MT (1.14 mg ml^{-1}), MT-1 (0.61 mg ml^{-1}) and MT-2 (0.53 mg ml^{-1}) were dissolved in 10 m*M* Tris–HCl buffer. pH 9.1, and loaded into the capillary by electrokinetic migration at 30 kV for 2 (MT) or 3 (MT-1 and MT-2) s. The running buffer was 50 m*M* Tris–HCl, pH 9.1, and the running voltage was 30 kV.

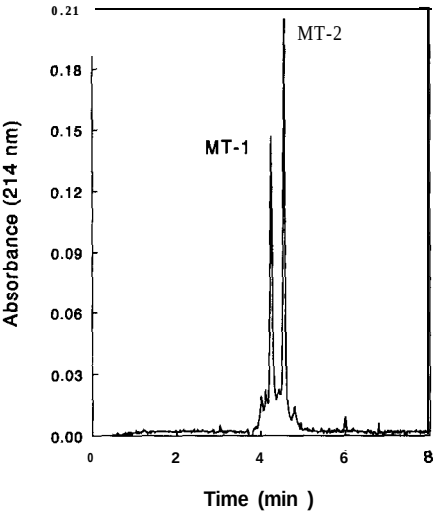

Fig. 2. Capillary zone electropherogram of rabbit liver MT (0.50 mg ml-') loaded by electrokinetic migration for 1 s. The buffers used to dissolve the sample and for running were the same as those described in the legend to Fig. 1.

the combined rabbit MT isoforms contained at least 4 small peaks in addition to the MT isoforms (Fig. 2). It is not clear whether these were contaminants or perhaps variant metalloforms of MT ***(i.e..*** the same MT isoform with different metal composition). From absorbance peak area calculation, the relative proportion of MT- 1 and MT-2 appeared to vary from lot-to-lot of the commercial rabbit protein but replicates from a single lot gave consistent results (data not shown).

Analysis qfgel chromatography fractions

Most of the zinc from the sheep liver cytosol sample subjected to gel permeation column chromatography on Sephadex G-75 eluted, bound to MT, in fractions 12 through 17 inclusive and the CZE analyses of consecutive fractions are shown in Fig. 3. Fractions 13 to 17 show a prominent component which was found to have the same migration time as sheep MT-I purified by ion-exchange chromatography. The migration time of MT-2 was also identified by CZE analysis of the purified isoform and it is clear that its cytosolic concentration was very much lower than that of MT-1. In contrast to MT-1 and MT-2. other contaminants clearly visible in fraction 12, were increasingly less conspicuous in

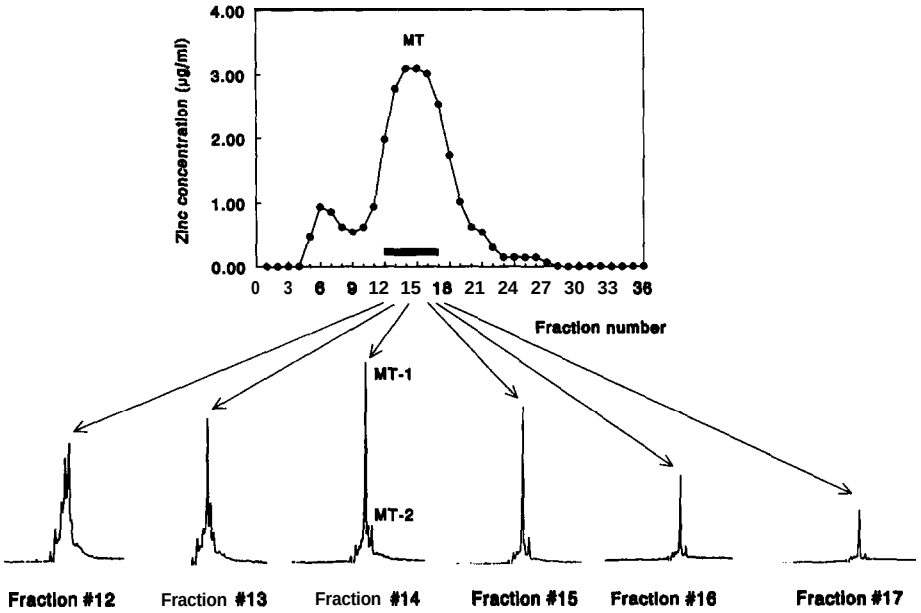

Fig. 3. A two-dimensional analysis of MT from a Zn-treated sheep. The first dimension (top panel) consisted of a gel permeation fractionation of cytosol on a column packed with Sephadex G-75 and eluted with 10 m*M* Tris–HCl, pH 8.6. The MT-containing column fractions (denoted by bar) were identified by an atomic absorption spectrophotometric analysis for Zn. These fractions (12-17) were then subjected directly to the second dimension analysis of CZE with loading by electrokinetic migration for 4 s at 30 kV and run in 50 m*M* Tris–HCl, pH 9. I, at 30 kV.

subsequent fractions. During purification of MT-l and MT-2 on an anion-exchange column (Sephadex DEAE A-25), the appearance of some red coloration was noted in the MT-2 fraction. A sample was therefore desalted and concentrated on a Centricon-3 molecular filtration unit (Amicon, UK) and analysed by CZE. The resulting electropherogram showed 3 components (Fig. 4a), the first of which had the same migration time as the purified sheep MT-l. In order to distinguish the remaining 2 components, the sample was re-analysed by CZE, this time monitoring absorbance at 414 nm. Since the ion-exchange fraction showed a small absorption maximum at 414 nm and MT does not absorb light at this wavelength, the resulting electropherogram clearly demonstrated that the second component (presumably haemoglobin) was the contaminating red protein (Fig. 4b).

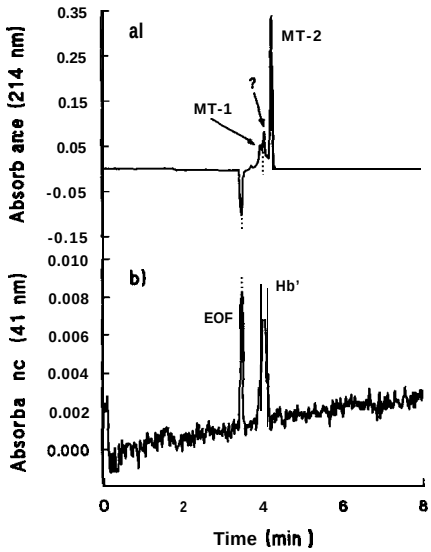

Fig. 4. (a) Capillary zone electropherogram of sheep liver MT-2 previously purified by anion-exchange chromatography and desalted by ultrafiltration. The lower panel (b) represents CZE of the same sample except that absorbance at 414 nm was monitored to detect the presence of a red protein contaminant (presumably hemoglobin, Hb). The endo-osmotic front (EOF) and contaminant MT-l are also indicated.

Standard calibration

The relationship between gravity loading time of MT sample (500 µg ml-') and integrated absorbance peak area was found to be linear over the

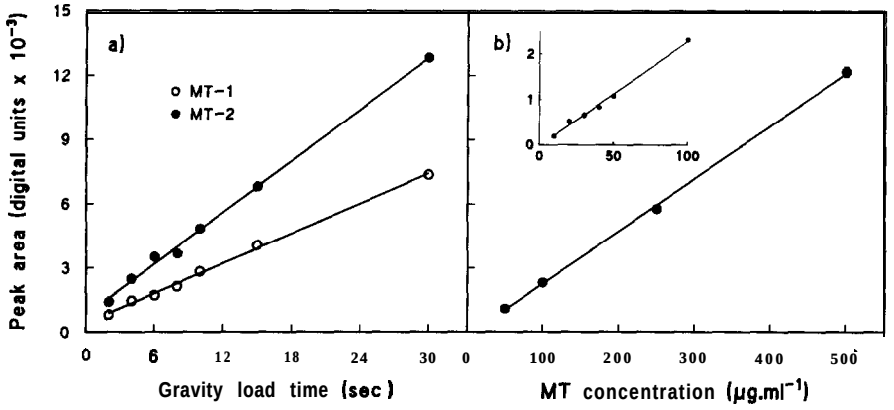

Fig. 5. Integrated peak areas of MT-I and MT-2 isoforms from a rabbit liver MT sample (0.50 mg ml^{-1}) plotted as a function of (a) the gravity loading time or as a function of (b) the concentration of MT loaded into the capillary. A standard curve for the lower concentration range 10–100 $\mu g\ ml^{1}$ is also shown (insert). Each sample was dissolved in 10 m*M* Tris–HCl, pH 9.1, and run at 30 kV in 50 m*M* Tris–HCl, pH 9.1.

range of 2 to 30 s for both MT-I and MT-2 (Fig. 5a). In addition, the ratio of MT-l:MT-2 remained constant over this range indicative of the fact that both isoforms loaded in the same proportion at each time. CZE of standard rabbit MT solutions also gave linear calibration lines across the entire concentration range (10–500 μg ml-' of MT-l + MT-2) as can be seen in Fig. 5b. From linear regression of the data, the absolute limit of detection was estimated to be approximately 1 $\mu g\ ml^{-1}$. Nevertheless, under our separating conditions, it would be appropriate to assume a working detection limit in the range l-10 μg ml-'.

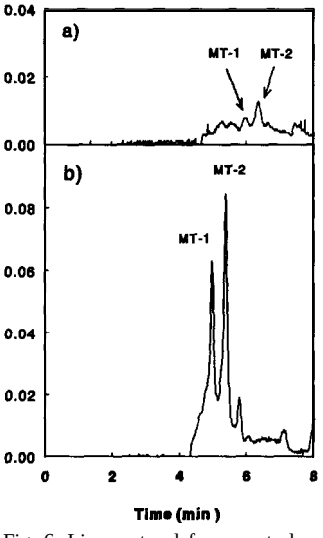

Fig. 6. Liver cytosol from control and zinc-injected rats was fractionated with acetone to enrich for MT and the 80% acetone insoluble pellet was redissolved in *1*.0 ml of 10 m*M* Tris–HCl, pH 9.1, and subjected to CZE as described in the legend to Fig. 1.

Analysis of ***MT*** *in rut liver*

Both MT isoforms were readily detected in the acetone-extracted hepatic cytosol samples from Zn-injected rats (Fig. 6). Relatively little contamination from other components was observed confirming the specificity of this preparation method for MT. MT-2 was clearly identificable in the acetone extract from the livers of saline-injected (control) rats although the level of MT- 1 was considerably lower.

The treatment of cytosol samples with acetonitrile was simple and rapid but could not remove all contaminants, particularly low M_r components. The isoforms were identified by standard additions of MT-l and MT-2 to the acetonitrile-treated samples prior to re-analysis by CZE (Fig. 7). As with the acetone treatment, MT-2 was readily detected in the liver extract from saline-injected rats (Fig. 7a) but MT-I was barely detectable. The presence of both isoforms in the liver extract from Zn-injected rats (Fig. 7d) confirmed the acetone treatment results.

MT was readily detected in the 8-mg liver samples from rats injected with Zn (Fig. 8). In agreement with the results for the 2 previous solvent ex-

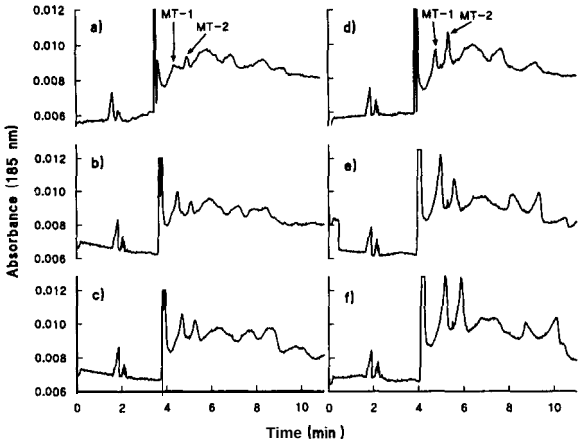

Fig. 7. Liver cytosol as described in the legend to Fig. 6 was treated with acetonitrile (50%, v/v) to remove contaminating proteins and the resulting supernatant after centrifugation was subjected to CZE. MT isoforms were detected in control cytosol (a) and in cytosol from Zn-injected rats (d). The identity of each isoform was confirmed by standard additions of MT-l (b & e) and MT-2 (c & f) to the extract from Control and Zn-injected animals prior to CZE.

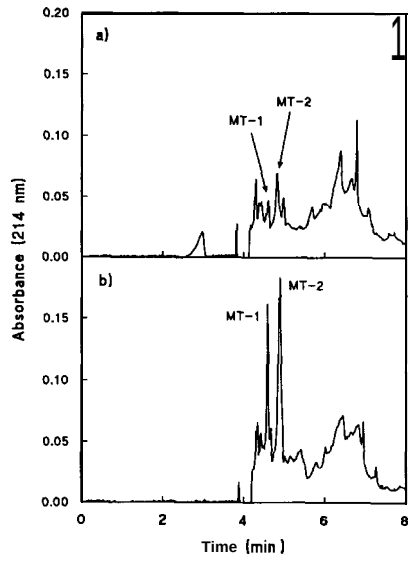

Fig. 8. Capillary electropherograms of acetonitrile-treated cytosol derived from a small (8 mg) sample of liver from (a) control and (b) zinc-injected rats. CZE was conducted as described in the legend to Fig. 1.

traction methods, considerably more MT-2 than MT- 1 was detected in the liver of the saline-injected rats (Fig. 8a). A similar isoform ratio was also evident in the Zn-injected sample (Fig. 8b) and is consistent with co-ordinate induction of MT-l and MT-2 isoforms by zinc.

DISCUSSION

The separation of the major MT isoforms by CZE was found to be simple, rapid and reproducible. In contrast to other electrophoresis techniques, CZE can be automated for batch analysis and would therefore be suitable for the routine analysis of MT-l and MT-2 in large numbers of samples. The high resolution achieved is characteristic of this technique and was not seriously affected by increasing the sample load time up to at least 40 s (gravity) or 4 s (electrokinetic). The estimated detection limit concentration of l-10 μg MT ml^{-1} is high compared to, for example, immunological assays. However, since the volume of sample entering the capillary is usually $<$ 100 nl, it is technically possible to analyse very small sample volumes ($<5\ \mu l$) repeatedly using positive or negative pressure loading techniques e.g. gravity. Thus very small quantities of MT can be extracted into a small volume to achieve an appropriate concentration for analysis by CZE. Consequently both MT-l and MT-2 were readily determined from only 8 mg of liver using the acetonitrile preparation technique (Fig. 8). Further improvements in the sensitivity of analyses by CZE should accompany current advances in detector technology, particularly in the areas of electrochemical, mass spectrometry and laser-induced fluorescence detection.

The application of CZE to the qualitative analysis of MT isoforms has been discussed briefly [18] and the results would indicate that it is an invaluable technique for this purpose. For example, the commercially available rabbit liver MT samples consistently contain at least 4 components in addition to the 2 major MT isoforms. Monitoring the effluent from Sephadex G-75 column chromatography of Zn-induced sheep liver cytosol was valuable in determining the fractions of highest MT content and purity. Without any further chromatographic analysis, it was clear from the CZE data that the liver concentration of MT-l was much higher than

that of MT-2 following injection of sheep with Zn. The preferential induction of MT-l has been noted previously in sheep using traditional chromatographic methods of isoform separation [19]. Since there are 3 functional sheep MT-l genes [20], we were interested to observe if the corresponding proteins could be resolved by CZE. However, only one peak for MT-I was detected and it is unclear whether this contained unresolved components or whether only one MT-l isoform had been induced. Considering the small number of amino acid substitutions [20,21] with relatively little or no change in the molecular weight or charge between MT-l isoforms, it is perhaps not surprising that these proteins could not be resolved, if indeed they were present. It is also possible that the relative abundance of the MT-I isoform compared to the MT-2 isoform reflects the combined contribution of different MT-l isoforms unresolved by CZE. The power of CZE for checking MT purity might be further enhanced using an alternative method of analysis for multi-component identification such as CZE–mass spectrometry since modern mass spectrometers have the required mass resolution and sensitivity for this purpose. Alternatively, a different form of capillary electrophoresis might prove useful in the resolution of MT-l isoforms, one that does not rely solely on differences in charge-to-mass ratio as CZE does. For example, micellar electrokinetic capillary chromatography (MECC) which resolves components partly on the basis of their hydrophobicity could be employed. Since all of the individual MT-l isoforms from human liver samples are resolved with RP-HPLC [22], MECC may likely contribute added resolving capability to a separation of MT isoforms.

The linearity and reproducibility of the calibration curves for MT indicate that CZE is suited to the quantitative analysis of this protein. However, using the above described conditions of electrophoresis analysis of MT in a complex matrix such as liver cytosol was not possible due to the dominating presence of other proteins. Removal of these proteins by precipitation with solvents such as ethanol-chloroform and acetone has frequently been used in the purification procedure for MT [23,24]. Using rat cytosol as starting material, purification of MT for CZE analysis using acetone was successful but the method is relatively time consuming. Acetonitrile is advantageous in that it provides reducing conditions and can be applied directly to the capillary column. Moreover, it has been demonstrated by radioimmunoassay, that the recovery of MT-l from hepatocyte cytosol ($5\ 10^6$ cells) treated with 50% acetonitrile was over 90% (unpublished data). The successful analysis of MT in 8-mg liver samples (Fig. 7) demonstrated the feasibility of using the acetonitrile preparation method with small amounts of tissue such as could be obtained by biopsy or from a single culture dish containing in the order of $5 10^6$ hepatocytes. We therefore believe that acetonitrile extraction shows greatest potential for sample preparation prior to automated CZE analysis.

In conclusion, qualitative analysis of semi-purified or purified MT by CZE is of great value in the assessment of purity and the relative abundance of the two major MT isoforms. CZE also reveals information about differences in the charge to mass ratio of MT isoforms from different animal species. We have not as yet detected any difference in migration time of individual isoforms due to the type of bound metal but this area requires further investigation. Multiple isoforms of MT-l in species such as sheep could not be resolved probably due to the relatively minor changes in charge to mass ratio associated with substitutions of individual amino acids. Standard calibration curves indicated linearity over an acceptable concentration range with an estimated detection limit of 1–10 μg MT ml^{-1}. Prior removal of major protein contaminants by solvent precipitation offers a rapid method of preparing samples for CZE analysis. The use of acetonitrile is recommended for the preparation of small tissue or cell samples prior to quantification of MT by CZE.

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