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Journal of Chromatography A, 700 (1995) 95–103

JOURNAL OF CHROMATOGRAPHY A

# Analysis of metallothionein isoforms by capillary electrophoresis: optimisation of protein separation conditions using micellar electrokinetic capillary chromatography

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**Abstract**

Capillary electrophoresis (CE) techniques have been successfully applied to the separation of metallothionein (MT) isoforms and have proved to be rapid, practical and economical. Study of a variety of different electrolytes and capillaries has shown that electrolyte buffer composition and capillary wall surface modifications can have considerable influence on isoform separation and resolution. Ionic surfactants such as sodium dodecyl sulphate (SDS) form micelles at elevated concentrations and the partitioning of molecules between the hydrophobic micelle phase and the aqueous phase and their resulting migration in an electric field is the basis of the technique known as micellar electrokinetic capillary chromatography (MECC). In the present work, we have used sheep and rabbit MT to optimise MECC conditions for analysis of MT isoforms. Capillaries of 57 cm gave much better separations than shorter columns although analysis times were increased to about 12 min. Changing the buffer and SDS concentration or the pH affected the selectivity of isoform separation and up to 5 isoforms in sheep MT and 6 in rabbit MT were completely or partially resolved. Comparing different diameter capillaries we conclude that 25 $\mu$m I.D. columns give better separations than 50 or 75 $\mu$m I.D. columns although sensitivity is reduced by a factor of about 3 and 5, respectively. Using our MECC conditions, columns coated with $C_1$ or $C_{18}$ hydrophobic material were not found to be useful in improving MT separation or resolution although further evaluation of these columns is in progress. Analysis of sheep liver extracts using optimised conditions showed the expression of at least 4 MT isoforms in response to Zn injection and 3 of these forms were evident in extracts from untreated sheep. We therefore conclude that MECC is a suitable method for MT isoform analysis.

## 1. Introduction

Capillary electrophoresis (CE) has been successfully applied to the study of peptides and proteins [1–5] including metalloproteins [6]. Several different CE techniques have been applied to the separation of metallothionein (MT), a 61 amino acid metalloprotein with 20 conserved cysteine residues binding 7 divalent transition metal atoms such as zinc in a 2-domain structure [7]. In many species, MT is composed of a family of protein isoforms which code from different genes and have distinctive characteristics through substitution of as little as 1 amino acid or up to 15 residues. Separations of purified mammalian MT at neutral/alkaline pH by polyacrylamide gel electrophoresis [8] or capillary zone electrophoresis (CZE) in uncoated silica capillaries [9–11]

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0021-9673/95/$09.50 
*SSDI* 0021-9673(94)01167-2

reveal 2 charge-distinct classes of isoforms, namely MT-1 and MT-2. The differences in charge at this pH range arise largely from substitutions involving acidic residues and MT-2 forms are more negatively charged than MT-1 forms. These proteins are induced by a wide variety of different factors including metals, cytokines and steroids [12,13] but due to limitations in available analytical techniques such as HPLC, routine study of individual isoform expression and tissue concentration has not been practicable.

The binding of metals to MT isoforms is crucial in stabilising the protein secondary structure and so metal dissociation during acidification gives rise to conformational changes. At the same time, the protein charge changes as the pH decreases below the p*I* of MT (3.8–4.4) and isoforms which co-migrate at neutral or alkaline pH can be resolved by CZE at pH 2.5 [14,15]. Unfortunately, decreasing pH also diminishes UV absorbance, which is the usual method of detecting components separated by CE, and hence sensitivity is reduced. We have therefore investigated alternative electrolyte chemistry and capillaries with different surface modifications for use at higher pH.

Capillaries with polyamine coatings reverse the electroosmotic flow (EOF) and their use with phosphate buffers at neutral pH has achieved the complete separation of 4 rabbit MT isoforms [16]. The use of neutral hydrophilic polyacrylamide-coated capillaries, in which the EOF is abolished and separation occurs by electromigration only, also encouraged the separation of different isoforms [15]. The capacity of both the polyamine and neutral coated capillaries to separate MT isoforms was found to be highly dependent on the concentration, pH and chemical nature of the buffer used as the electrolyte.

We have also investigated the application of micellar electrokinetic capillary chromatography (MECC) to the separation of MT isoforms and showed that the anionic surfactant SDS in borate buffer at pH 8.4 was suitable for resolving additional isoforms of MT-1 and MT-2 [17]. MECC does not require specialised capillaries and is a robust CE technique. The principle of MECC involves partitioning of analytes, according to their hydrophobicity, into the micellar phase which is highly charged and therefore migrates rapidly in an electric field [18]. MECC techniques were first developed by Terabe and co-workers [19,20] and a range of anionic and cationic surfactants and modifiers have been used to manipulate the selective partitioning and separation of analytes [21,22] including closely related large peptides [23]. Although the mass limit for MECC is reportedly $M_r < 5000$ [22], MT isoforms, which have metal-saturated mass values of about $M_r$ 6500, are sufficiently influenced by the surfactant to separate during migration.

Our aim in this study was to evaluate the electrolyte and capillary conditions for MECC in order to optimise the separation of MT isoforms in purified proteins and in tissue extracts. Our preliminary investigation of various anionic, zwitterionic and cationic surfactants indicated that SDS was of greatest potential for MT isoform separation (unpublished observations). Additives which modify analyte partitioning into micelles such as methanol and urea were found to be deleterious to isoform separation and borate appeared to be better than phosphate as an MECC electrolyte buffer. Therefore our efforts to optimise the electrolyte have concentrated on modifying borate buffer concentration and pH and also SDS levels. The affinity of some types of protein to bare silica during separation is known to cause poor resolution with uncoated capillaries and the use of hydrophobic coatings in combination with MECC conditions significantly reduces the influence of the column wall [24]. Such coatings also decrease the EOF and both resolution and migration reproducibility of components separated in the capillary are improved [25]. In the case of $C_1$ coated capillaries, a greater proportion of the silanol groups are shielded than with the $C_{18}$ coated capillaries and thus the reduction in EOF as compared to bare silica is reported by the manufacturer to be 47.4 and 34.4%, respectively, for the columns used in this study. Although there is no indication from previous work that MT isoforms interact with bare silica capillary walls, we investigated the utility of hydrophobic capillaries in enhancing

MT separation by EOF manipulation during MECC separations.

## 2. Experimental

### 2.1. Metallothionein samples

Sheep MT-1 and MT-2 were purified from the liver of a Zn-injected grey face ewe using standard chromatographic procedures [26] whereas rabbit Cd,ZnMT (containing MT-1 and MT-2) was obtained from a commercial source (Sigma Chemical, Poole, UK). In order to test the application of the technique to tissue extracts, samples of liver from sheep were processed using a 2-step solvent extraction method based on that of Hidalgo et al. [27] but using ethanol–acetonitrile for protein precipitation. The solvent, (ethanol–acetonitrile, 1:3, v/v) was slowly added, while vortexing, to a 33% (w/v) homogenate of liver in water (usually 1 g liver + 2 ml water) using 15 ml polypropylene tubes and after centrifugation (10 000 *g* for 5 min), the concentration of solvent in the supernatant was increased to 80%. The precipitate was collected by centrifugation at 10 000 *g* for 5 min, re-suspended in 100 μl water, centrifuged in eppendorf tubes, and the supernatant was analysed for MT.

### 2.2. Instrument and separation conditions

Separations were performed using a P/ACE 5000 system (Beckman Instruments, High Wycombe, UK). For studies on the influence of capillary diameter and length and electrolyte modifications, capillary cartridges with 100 × 800 μm windows were fitted with polyimide-coated fused-silica capillaries, 27, 37 and 57 cm in total length (20, 30 and 50 cm from inlet to detector window, respectively) and 25, 50 or 75 μm I.D./375 μm O.D. (Composite Metal Services Ltd., Hallow, UK). For evaluation of surface modified hydrophobic capillaries, $C_1$ (H50) and $C_{18}$ (H250) columns (57 cm total length, 50 μm I.D. and 363 μm O.D.; Supelco, Poole, UK) and uncoated columns of the same length (50 μm I.D., 375 μm O.D.) were fitted in cartridges with 100 × 200 μm windows. The hydrophobic capillaries were conditioned for 2 h prior to use for protein separation, as described in the manufacturers' instructions. Detection with the larger window cartridges was made using a diode array detector monitoring at 200 nm and scanning between 190 and 340 nm whereas a fixed wavelength (200 nm) UV detector was used for cartridges with smaller windows. Separations were performed at 10–25 kV (anode, inlet; cathode, outlet) and the temperature of the capillary was maintained at 25°C by means of circulating coolant. Borate–SDS buffers were made by adjusting the pH of boric acid and SDS using NaOH (all high purity or AristaR reagents, BDH, Poole, UK). Unless otherwise stated, sample introduction by low pressure injection (0.5 p.s.i., $3.45 \cdot 10^3$ Pa) was 1, 3 and 10 sec. for 75, 50 and 25 μm I.D. capillaries respectively.

## 3. Results and discussion

Since previous work indicated the potential application of MECC to MT isoform quantification [17], we have systematically evaluated a range of separation conditions using sheep and rabbit MT isoforms. The separation of sheep MT-1 + MT-2 by MECC using borate buffer concentrations up to 300 m*M* and SDS levels up to 100 m*M* showed 3 main components labelled a, c and e in Figs. 1 and 2. By separation of MT-1 (Fig. 4) and MT-2 (data not shown) individually, components a and c were shown to be MT-1 isoforms whereas component e was an MT-2 form. Additional minor components b and d were also evident and were shown to be MT-1 forms (Fig. 4). Multiple functional MT genes have been identified in sheep [28] and electrospray mass spectra of the proteins to 1 mass accuracy have demonstrated that many, if not all of these genes are translated in response to metal induction [29]. Flow injection mass spectra recorded at acid and neutral pH have shown that all of these components bind up to 7 atoms of Cd or Zn per molecule. As regards sheep MT-1 therefore, we have shown that the components separated by various CE techniques using a wide

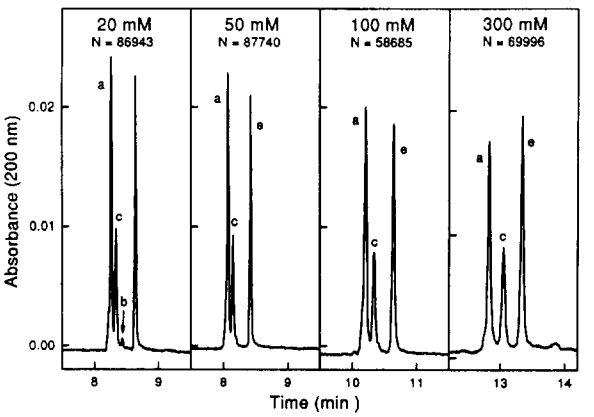


Fig. 1. The effect of increasing sodium borate concentration at pH 8.4 on the separation of sheep MT-1 + MT-2 (1 mg of each isoform/ml water) by MECC at 10 kV. A 57 cm × 75 μm I.D. uncoated capillary was used and SDS concentration was maintained at 75 m*M*. Components a, b and c are MT-1 forms whereas component e is MT-2. The separation efficiency was determined by calculating the number of theoretical plates (*N*).

variety of different CZE and MECC conditions are isoforms of metallothionein rather than contaminants or aggregation/degradation products.

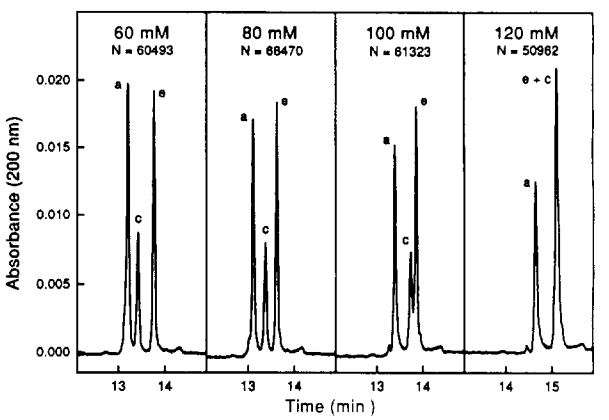


Fig. 2. The effect of increasing SDS concentration on the separation of sheep MT-1 + MT-2 (1 mg of each isoform/ml water) by MECC at 10 kV. A 57 cm × 75 μm I.D. uncoated capillary was used and sodium borate concentration was maintained at 300 m*M* with a pH of 8.4. Components a and c are MT-1 forms whereas component e is MT-2. The separation efficiency was determined by calculating the number of theoretical plates (*N*).

According to the relative abundance of the isoforms detected by mass spectrometry of the purified sheep MT-1 protein, we believe that component a in all MECC separations is MT-1a and that component c is MT-1c, in accordance with the nomenclature designated by Peterson et al. [28] who sequenced the corresponding genes. Component b and d are as yet unidentified but the mass spectra suggest the presence of an unacetylated form of MT-1a and a small amount of MT-1b. The amino acid substitutions which distinguish MT-1a from MT-1c are pro (MT-1a) to ser (MT-1c) at residue 8, gly to ser at residue 11 and val to ile at residue 49. In the case of MT-1b, the difference from MT-1a is even less significant with a single substitution of gly (MT-1a) to ser (MT-1b) at residue 10.

### 3.1. Electrolyte optimisation

When maintaining a constant SDS concentration of 75 m*M* and varying the buffer concentration, separation of c from a was best achieved using a borate level of 300 m*M* although separation efficiency expressed as the number of theoretical plates (*N*) calculated from Eq. (1), where $t$ is the migration time and $\sigma$ is the peak width at half peak height [30], was found to be optimal at 50 m*M* (Fig. 1).

$$N = (t/\sigma)^2 \quad (1)$$

Changing the SDS concentration while maintaining the buffer level at 300 m*M* showed the best separation of a, c and e and the optimum peak resolution at 80 m*M* (Fig. 2). The importance of selecting the correct separation conditions for MT isoforms is illustrated by the tendency of c (MT-1c) to co-migrate with a (MT-1a) under standard CZE conditions [14] and to co-migrate with e (MT-2) under MECC conditions using higher SDS levels (Fig. 2).

The electrolyte pH had a profound effect on rabbit MT isoform separation pattern (Fig. 3) which contrasted with the lack of pH influence on sheep MT isoform migration order (data not shown). The MECC of rabbit MT using 100 m*M* borate and 75 m*M* SDS at pH 8.4 was the same

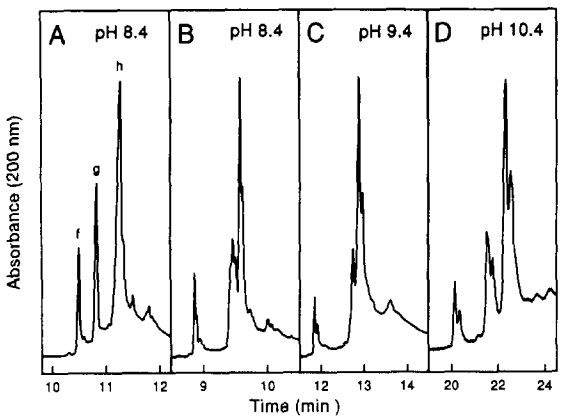


Fig. 3. The effect of pH on the separation of rabbit MT isoforms (1 mg MT/ml water) by MECC at 10 kV and 100 m*M* borate–75 m*M* SDS, pH 8.4 (A) or 20 kV and 500 m*M* borate–100 m*M* SDS at pH 8.4 (B), 9.4 (C) or 10.4 (D). Separations were performed in 57 cm × 25 μm I.D. capillaries and components f and g are MT-1 forms whereas h contains MT-2 forms.

as we have obtained previously [17] and the components labelled f and g are isoforms with a similar net charge to MT-1 whereas component h contains only MT-2 forms. These 3 components were evident at pH 10.4 but 3 further forms were also partially resolved and the separation was a mirror image of that obtained using polyamine coated capillaries and electrolyte buffered at pH 7 [16]. The heterogeneity of rabbit MT confirms other observations using RP-HPLC [31], CZE with uncoated capillaries at low pH [14,15], and CZE with neutral-coated capillaries [15].

The resolution of sheep MT-1 separations in particular, but also that of rabbit MT at 500 m*M* borate–100 m*M* SDS pH 10.4 was reduced, which may have been due to the increased ionic concentration differential between the sample matrix (water) and the electrolyte. We routinely inject samples prepared in water to enhance resolution by stacking and this has proved to be successful for many separation conditions. However, it is thought that the electrolyte:sample concentration differential should be in the order of 10 for optimal stacking effect [32] and it may therefore be possible to improve these separations by increasing the sample ionic strength.

### 3.2. Capillary optimisation

In order to evaluate the separation of sheep MT-1 in different diameter capillaries, buffer conditions (200 m*M* borate, 125 m*M* SDS, pH 8.4) were selected to enhance isoform separation. It should however be noted that the isoform c in Fig. 4 would co-migrate with MT-2 under these conditions. New 57 cm capillaries of all 3 sizes were conditioned by purging with NaOH, water and buffer for exactly the same time, taking into account the different flow rates through each capillary. Injections using low pressure of 1.2, 3 and 10 s duration were made for 75, 50 and 25 μm I.D. capillaries, respectively, so that an equivalent length of plug was introduced. Separations were performed at 10 kV and, as expected, migration time deceased with increasing capillary diameter. These electrophoresis conditions using new capillaries resolved the minor components labelled b and d in all 3 columns although separation was marginally superior with the 25 μm I.D. (Fig. 4). Of greater significance was the considerable difference in sensitivity which was a factor of approximately 5 × higher in the 75 μm I.D. column than the 25 μm I.D. capillary. Thus from the point of view of a potential assay for MT-1a, MT-1c and MT-

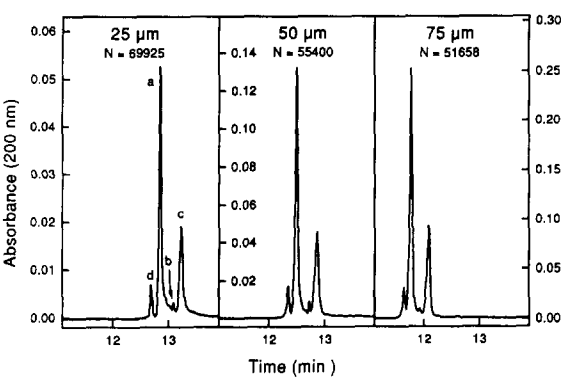


Fig. 4. The influence of capillary diameter on the separation and resolution of sheep MT-1 isoforms (1 mg MT-1/ml water) by MECC at 10 kV in uncoated 57 cm capillaries with 200 m*M* borate–125 m*M* SDS, pH 8.4 electrolyte. The separation efficiency was determined by calculating the number of theoretical plates (*N*).

2, the advantage of enhanced sensitivity using wider columns outweighs the slight loss in resolution. However in compromising on resolution, the assumption that components b and d would not contribute greatly to the peak area of MT-1a under any circumstances may be questionable. In any case, metabolic studies of these components may be of specific interest and the solution to this problem lies in the development or utilisation of more sensitive methods of detection.

One advantage normally associated with the use of smaller diameter capillaries is the ability to use high ionic strength buffers, which enhance resolution, without exceeding the capacity for Joule heat dissipation during electrophoresis [21], which on the P/ACE system is about 0.05 W/cm. However, due to the relatively low conductance of sodium borate and SDS, high concentrations can be used in 57 cm × 75 μm I.D. capillaries without exceeding this limit. The power generated in all separations presented here was $<0.02$ W/cm and usually $<0.01$ W/cm. Nevertheless, small increases in resolution and migration time as found, for example, with capillaries of decreasing I.D. (Fig. 4) may be due to more efficient cooling of the narrower bore columns.

On examining the influence of column length using new 25 μm I.D. capillaries and a 500 m*M* borate–100 m*M* SDS buffer pH 8.4 and 10 kV potential, it was clear that increasing column length was beneficial to isoform separation (Fig. 5). This is to be expected since a longer residence time on the column will enhance separation of closely related compounds. The CZE of MT-1 in 500 m*M* borate buffer without SDS using a 57 cm × 25 μm I.D. capillary at 25 kV partially separated components a and c and this electropherogram was similar in profile to the MECC separation of MT-1 in the 27 cm capillary.

The surface modified hydrophobic capillaries were of no obvious advantage for MT separation using our MECC conditions although we have not made extensive evaluation of these columns with a wide range of electrolyte composition, concentration and pH. The electropherograms

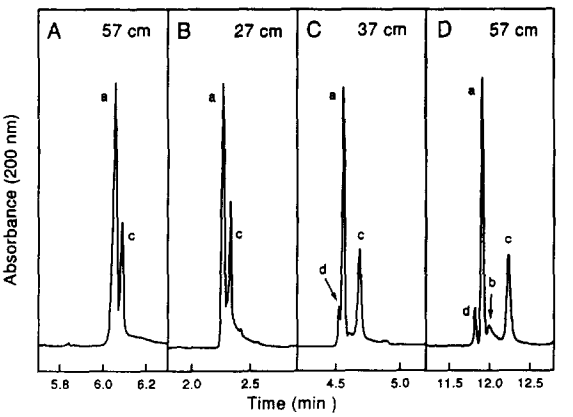


Fig. 5. The influence of capillary length on the separation and resolution of sheep MT-1 isoforms (1 mg MT-1/ml water) by MECC in uncoated 25 μm I.D. capillaries using 500 m*M* borate–100 m*M* SDS, pH 8.4 electrolyte (B–D) and a potential of 10 kV. MECC separations are compared with CZE at 25 kV and 500 m*M* borate buffer, pH 8.4 using a 57 cm × 25 μm I.D. uncoated capillary (A).

for sheep MT-1 using the coated and uncoated capillaries were similar to those in Fig. 4. Separation efficiency ($N$) of the MT-1 in the $C_1$ and $C_{18}$ capillaries was reduced by 0.64 and 0.52 and migration time increased by 1.12 and 1.20, respectively, compared to those in an uncoated column of the same length and I.D. The decrease in EOF observed due to the coating was less than that reported by the manufacturer. However, we used elevated SDS concentrations and the reduction in EOF due to shielding of silanol groups may have been partly counteracted by increased association of the surfactant with the hydrophobic coating, thus exposing more negatively charged detergent sulphate groups to assist in the generation of EOF. Lowering the borate and SDS concentrations to 50 and 10 m*M*, respectively, as used in other protein separation studies [24] resulted in the further deterioration of the MT-1 separation (data not shown). As expected, separations of MT performed in the absence of SDS using a $C_{18}$ capillary gave no absorbance peaks since the protein was entirely adsorbed onto the capillary wall. Separations using electrolytes containing 5% acetonitrile or 10 m*M* SDS were sufficient to prevent adsorption of sheep MT-1 but did not

resolve any of the composite isoforms (data not shown). Attempts to find concentrations of solvent or surfactant which enhanced competitive interaction with the capillary wall but prevented adsorption yielded poorly resolved peaks and better separations may be obtained using capillaries packed with $C_{18}$ material [33].

### 3.3. Tissue extract analysis using optimised MECC conditions

Our experiences with analysing tissue extracts by various CE techniques has been largely favourable although sample preparation is of crucial importance. We have used solvents to precipitate contaminant proteins because MT is relatively soluble at concentrations where many proteins denature or precipitate. Residual solvent in the extract is also compatible with CE analysis and we have injected MT samples containing as much as 50% acetonitrile without significantly affecting the isoform separation (unpublished observations). Because only nanolitres of sample can be injected onto a column, sensitivity is limited by the degree to which extracts can be concentrated into small volumes.

As a compromise between isoform separation, resolution and detection sensitivity, a buffer of 300 m*M* borate–85 m*M* SDS pH 8.4, a 75 $\mu$m I.D. capillary and a potential of 10 kV was used for MECC of these extracts. Isoforms a, c and e in the purified protein separations were identified in extracts from both untreated and Zn-treated sheep although there was considerable induction of all 3 forms in the latter extract (Fig. 6). Isoform d was detected only in the extract from the Zn-injected sheep. Spectral scans of these components in standards and Zn-induced extracts revealed spectra characteristic of MT, with no absorbance at 280 nm due to the absence of aromatic amino acids and a slight shoulder at 230 nm indicating the presence of Zn-mercaptide charge transfer bonds (Fig. 7) [34].

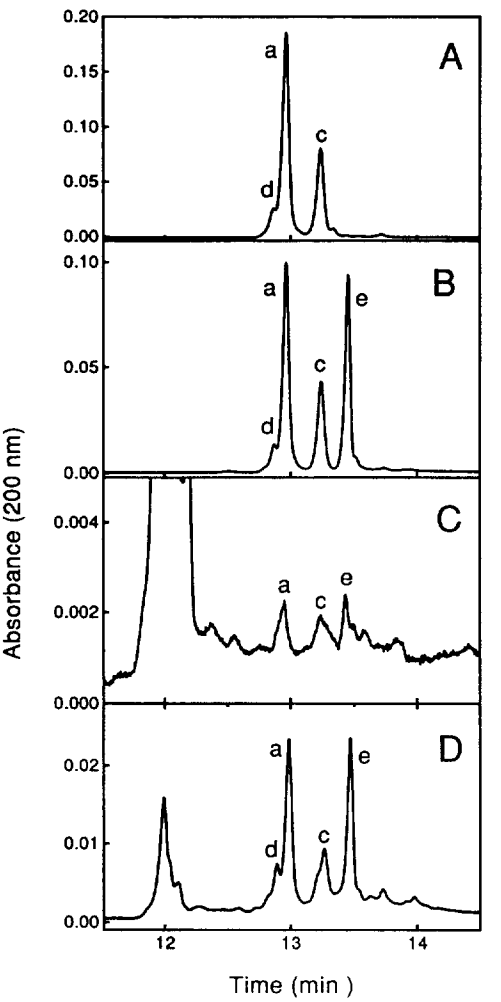


Fig. 6. The separation of MT isoforms in liver extracts from untreated (C) and Zn-injected (D) sheep. Comparison is made with separations of purified MT-1 (A) and MT-1 + MT-2 (B) using identical conditions: 300 m*M* borate–85 m*M* SDS, pH 8.4, at 10 kV using a 57 cm × 75 $\mu$m I.D. uncoated capillary. Components a, c and d are MT-1 forms whereas component e is MT-2.

### 3.4. Conclusions

We have demonstrated the significant influence of capillary column length, pH, buffer and SDS concentration on the separation and resolution of MT isoforms. With the development of more sensitive detectors, it would be appropriate to analyse these isoforms using long, small diameter uncoated capillaries in combination with high ionic strength sodium borate buffers, the pH and SDS level being adjusted from 8.4 to 10.4 and from 70 to 125 m*M*, respectively, to suit the separation of particular MT samples. In the

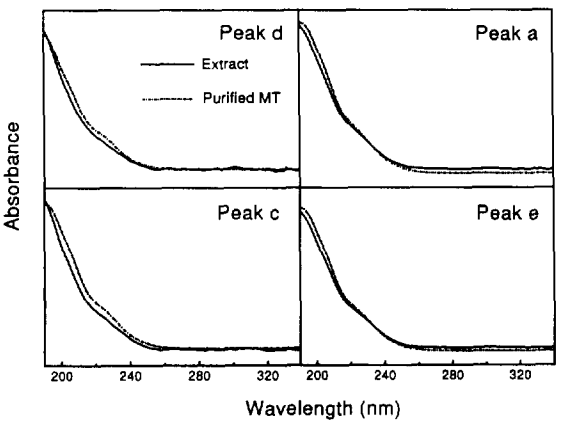


Fig. 7. UV absorbance spectra of the peaks labelled a, c, d and e of the MT-1 + MT-2 purified protein separation and the liver extract from Zn-induced sheep shown in Fig. 6. Scans between 190 and 340 nm were obtained 4 times a second using a diode array detector and the scan at each peak apex is presented.

case of sheep MT, the pH should be around 8.4 and SDS concentration in the range 80–90 m*M*. In contrast, the best separations of rabbit MT were obtained at pH 10.4 and SDS concentrations of 100 m*M*. As regards the electrical potential applied to the capillary, the best separations were obtained when micelle migration rate was low and therefore isoform migration times longer, namely at about 10–20 kV. MT isoforms could be detected even in untreated sheep liver and so using the conditions developed in this study, MECC has practical application to the analysis of MT in tissue extracts.

Comparing MECC with other CE techniques for MT analysis, we conclude that MECC with SDS at alkaline pH and CZE at acid pH, both in untreated fused-silica capillaries, are the most robust, practical and cost-effective methods which have been investigated. The method of choice depends on the intended application since the 2 methods are to some degree complementary. Using a UV detector, MECC gives better sensitivity for MT due to additional absorbance from intact mercaptide bonds and CZE using high concentration phosphate, low pH buffer electrolytes and narrow bore capillaries often yields better resolution. The selection of technique may also depend on the organism from which the MT sample is obtained. Isoforms of, for example, horse kidney MT are better separated by CZE [15] whereas sheep MT isoforms are better separated using MECC conditions. The relative merits of these and other CE methods are further discussed in a separate article [35].

## Acknowledgements

The authors would like to thank Dr. I. Bremner for his critical review of the manuscript. This work was funded by the Scottish Office Agriculture and Fisheries Department, UK.

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