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Journal of Chromatography B, 685 (1996) 353–359

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Identification of metallothionein isoforms with capillary zone electrophoresis using a polyacrylamide-coated tube

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Received 28 December 1995; revised 6 May 1996; accepted 7 May 1996

Abstract

Metallothionein (MT) isoforms from various liver tissues were separated with capillary zone electrophoresis (CZE) using a polyacrylamide-coated tube at neutral pH. The electrophoresis was performed on MT-1 and MT-2 purified from mouse, rat, rabbit and human livers. The retention times of mouse and rat MT-1 coincided, while the retention times of rabbit and human MT-1 were longer. The retention times of MT-2 purified from the four sources were the same. MT-1 and MT-2 separated more definitely with N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)–Tris buffer (25 m*M*, pH 7.4) than with N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid (TAPS)–Tris buffer (25 m*M*, pH 7.7) or with N-(2-acetamido)iminodiacetic acid (ADA)–Tris buffer (25 m*M*, pH 7.4). In addition, liver MT isoforms prepared from Zn- or Cd-administered mice could be separated.

Keywords: Polyacrylamide-coated tube; Metallothionein

1. Introduction

Capillary zone electrophoresis (CZE) is an adequate method for the separation of various substances, and, for some substances, it is better than HPLC [1]. However, many factors such as electroosmotic flow and non-specific adsorption to the inner surface of the tube influence the separation of substances by CZE. Polyacrylamide coating of the tube for the separation of proteins with CZE was introduced by Hjertén [2] and the method was improved by Hjertén and Kubo [3]. The inner surface of the polyacrylamide-coated tube has a neutral pH, which suppresses the interaction between protein and the wall. Thus, the use of a polyacrylamide-coated tube with CZE at neutral pH is a suitable method for the separation of proteins. Indeed, when commercial standard bovine serum albumin was analyzed by this method, three peaks were obtained, whereas with SDS–PAGE, only one peak was obtained (unpublished data).

Metallothionein (MT), which is a low-molecular-

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PII S0378-4347(96)00225-3

mass protein that is induced by various stimuli, has several isoforms. MT isoforms have been separated by several methods, such as HPLC, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [4], as well as by CZE [5–12]. Among the CZE studies, it has been observed that the adsorption of proteins onto an uncoated capillary tube affects the separation of MT isoforms at alkaline pH [7], and that both polyamine-coated and polyacrylamide-coated tubes were superior to an uncoated tube for the separation of MT isoforms [8,11,12]. In addition, good separation depended on the type of capillary, buffer and sample preparation conditions [12]. However, relatively little is known about the separation conditions for MT isoforms by CZE using a polyacrylamide-coated tube at neutral pH. Separation of a molecule's isoforms at neutral pH is of interest in order to study their properties under native conditions. The present study describes the separation of MT isoforms with CZE by using a polyacrylamide-coated tube with a neutral buffer.

2. Experimental

2.1. Materials

For the purification of MTs, female C57 Black mice, male Sprague–Dawley rats and male New Zealand White rabbits were used. Human liver samples were obtained from postmortem examinations and with the permission of the bereaved families. For the crude extract of MT, male ddY mice (6 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and were housed for one week before the experiment. Animals were fed a standard food (Oriental Yeast, Tokyo, Japan) and had free access to tap water.

Acrylamide, ammonium persulfate, N,N,N′,N′-tetramethyl ethylene diamine (TEMED) and tris-(hydroxymethyl)aminomethane (Tris) were purchased from Wako Pure Chemicals (Osaka, Japan). 3-Methacryloxypropyltrimethoxysilane was purchased from Shin-Etsu Chemical (Tokyo, Japan). N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid (TAPS) and N-(2-acetamido)iminodiacetic acid (ADA) were purchased from Dojindo Laboratories (Kumamoto, Japan). Rabbit liver MT-1 and MT-2 (for use as standards) were purchased from Sigma (St. Louis, MO, USA). The other reagents were purchased from Wako Pure Chemicals.

2.2. Coating of the inner surface with polyacrylamide

The polyacrylamide-coated capillary tube was prepared by the method of Hjertén and Kubo [3]. A fused-silica capillary tube (75 μm I.D.) (Otsuka Electronics, Osaka, Japan) was cut to a length of 40 cm. The capillary tube was flamed to remove polyimide and a UV detection window was made about 11 cm from one end. The capillary tube was flushed sequentially with water, 0.1 *M* sodium hydroxide, water, 0.1 *M* hydrochloric acid, water, and acetone, each for 5 min, and then with a 1:1 mixture of acetone and 3-methacryloxypropyltrimethoxysilane. Finally, the tube was filled with the latter to form Si-O-Si-C bonds and was allowed to stand overnight at room temperature. After washing the tube with acetone for 5 min and with water for 5 min, the tube was filled with a mixture of 500 μl of 7% (w/v) acrylamide, 5 μl of 10% (w/v) ammonium persulfate and 5 μl of 10% (v/v) TEMED in 50 m*M* sodium phosphate buffer, pH 7.0, and the mixture was allowed to polymerize overnight. Excess reagents were removed from the ends of the tube by washing thoroughly with 0.5 ml of electrophoresis running buffer containing 2% (w/v) sodium dodecyl sulfate and with water by using an HPLC pump at a flow-rate of 1.0 ml/min before use. The ends of the polyacrylamide-coated capillary tube were cut to give a final column length of 33 cm with a gel length of 25 cm.

2.3. Zone electrophoresis in a polyacrylamide-coated tube

The polyacrylamide-coated capillary tube was installed in a Waters Quanta 4000 (Japan Millipore, Tokyo, Japan) capillary electrophoresis system. Samples of MT were dissolved in distilled water at a final concentration of 1.0 mg/ml. The running buffers used were HEPES–Tris buffer (pH 7.4), ADA–Tris buffer (pH 7.4) and TAPS–Tris buffer (pH 7.7),

each at a concentration of 25 mM. A higher pH was used for the TAPS–Tris buffer because its buffering capacity decreases at pH values below 7.7. The purified MT samples were loaded onto the column by gravity for 3 s, while both the commercial standard rabbit MT-1 and the crude extract of mouse liver MT were loaded for 8 s. The inlet of the capillary was made the cathode. The analysis was performed at 15 kV and 26±1°C and the column eluent was monitored at 214 nm.

2.4. Purification of MT

The purified MTs were prepared from mouse, rat, rabbit and human livers. Mice were intraperitoneally administered 3 mg of Cd/kg of body weight in the form of cadmium chloride three times over a one-week period. Twenty-one days after the last administration, the liver was removed. Rats were also intraperitoneally injected with 3 mg of Cd/kg of body weight in the form of cadmium chloride four times over a one-week period. Seven days after the last injection, the liver was removed. In contrast, rabbits were subcutaneously administered 1 mg of Cd/kg of body weight in the form of cadmium chloride every two days for two months, and the liver was removed two days after the last administration. Livers were stored at −20°C prior to use.

MTs in livers were purified by the method of Kimura et al. [13] as follows: The tissues were homogenized with two volumes of 20 mM Tris–HCl buffer, pH 8.6, containing 5 mM β-mercaptoethanol. Cold ethanol (1.05 volume) and cold chloroform (0.08 volume) were added to the homogenate. After 30 min of stirring, the homogenate was centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was obtained. Three volumes of cold ethanol were added to the supernatant and the mixture was stored at −20°C overnight. A precipitate formed and was separated by centrifugation (6000 g for 10 min). This fraction (crude extract) was dissolved in 20 mM ammonium carbonate solution. The sample was applied to a preparative Sephadex G-75 column, eluted with 20 mM ammonium carbonate solution and the eluent was monitored by both cadmium content and UV absorption (250 and 280 nm). The MT fraction was lyophilized, dissolved in 20 mM ammonium carbonate solution and applied to a DEAE–Sephadex A25 column. MT-1 and MT-2 fractions were eluted with a linear gradient from 50 mM to 2.0 M ammonium carbonate solution. The fractions were lyophilized and redissolved in distilled water prior to analysis by CZE.

2.5. Crude extract of MT from the liver of mice administered zinc or cadmium

Mice were subcutaneously administered 50 mg/kg of zinc or 3 mg/kg of cadmium. Twenty-four hours after the injection, the mice were decapitated and the liver was removed. The crude extract was prepared as described in Section 2.4, re-dissolved in distilled water and analyzed by CZE.

3. Results

MT-1 and MT-2 could be separated with CZE using the polyacrylamide-coated tube and 25 mM HEPES–Tris buffer at pH 7.4. As shown in Fig. 1, purified mouse liver MT-1 showed one peak (Fig. 1a), with a retention time of 11.1 min, and the retention time of rat liver MT-1 coincided with that of mouse MT-1 (Fig. 1b), while rabbit liver MT-1 was delayed with respect to mouse MT-1 (Fig. 1c) (retention time, 11.7 min). Human liver MT-1 also showed a delayed retention time and contained a shoulder peak (Fig. 1d) (retention times, 11.5 and 11.65 min).

Fig. 2 shows the elution patterns of MT-2s obtained from mouse, rat, rabbit and human livers. All four chromatograms showed a single peak with a retention time of 7.6 min.

In contrast, a commercial standard of rabbit liver MT-1 showed eight peaks and MT-2 was also present in the MT-1 sample (Fig. 3a). Using HEPES–Tris buffer (pH 7.4), the MT-1 peak (11.7 min) was found to consist mainly of two incompletely separated peaks. Use of TAPS–Tris buffer (pH 7.7) resulted in a shorter retention time (10.0 min) but did not separate the MT-1 sub-isoforms (Fig. 3b). When ADA–Tris buffer was used (retention time, 12.4 min; Fig. 3c), the baseline was not stable and the separation of the MT-1 isoforms was incomplete.

Fig. 4 shows the separation of MT isoforms in

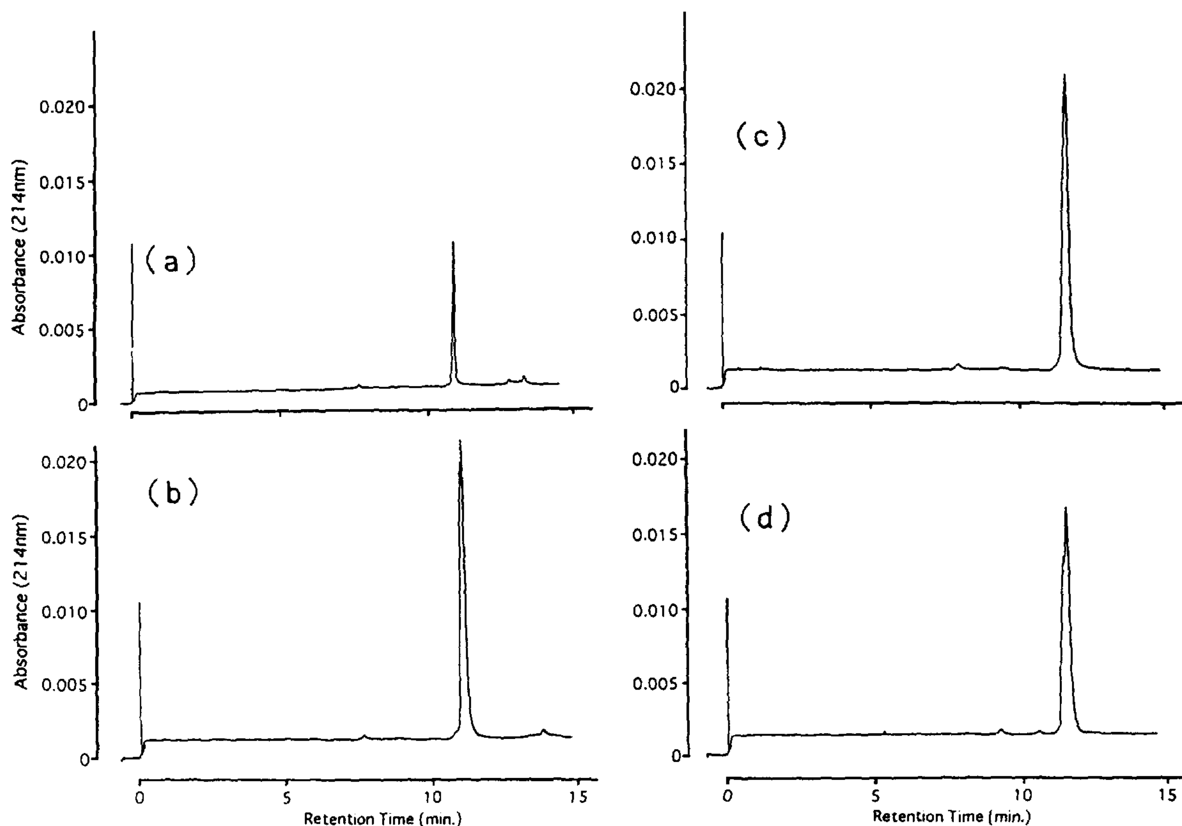

Fig. 1. Typical CZE chromatograms of MT-1 isoforms. Cadmium was administered to mice (3 mg/kg, three times, i.p.), rats (3 mg/kg, four times, i.p.) and rabbit (1 mg/kg, twenty-one times, s.c.). The mouse, rat and rabbit livers were obtained after the administrations and human livers were obtained from postmortem examinations. Liver MT-1 samples were prepared by ethanol extraction and chromatographic techniques are as described in Section 2. The samples were analyzed with a polyacrylamide-coated capillary tube (33 cm) using 25 m*M* HEPES–Tris buffer (pH 7.4). After the samples (1 mg/ml of MT-1) were loaded onto the cathode end of the capillary by hydrostatic injection for 3 s, the separation was performed at 15 kV and 26±1°C, with the separated components being detected at 214 nm. Chromatograms show MT-1 from the livers of (a) mouse, (b) rat, (c) rabbit and (d) human.

mouse liver using the polyacrylamide-coated capillary tube and HEPES–Tris buffer (pH 7.4). In the control group (Fig. 4a), the MT-1 and MT-2 isoforms were detected in slight amounts, with the peak area of MT-1 being larger than that of MT-2. MT-1 had two incompletely separated peaks and the area of the trailing peak was larger than that of the leading one. The MT-1 and MT-2 isoforms from the liver of the zinc-administered mouse could be separated and the ratio of MT-2 to MT-1 was 0.79 (Fig. 4b). The two main MT-1 isoforms could be detected, with the area of the trailing peak being larger than the area of the leading peak. Administration of Cd resulted in an increase in liver MT content. The area of the leading peak of the main MT-1 isoform peaks was larger than that of the trailing peak. The ratio of MT-2 to MT-1 was 0.30 (Fig. 4c).

4. Discussion

MT is a protein induced in various organs by stimuli such as metals, stress and drugs [14]. MT has several isoforms and MT-1 and MT-2 are well known to be induced by metals [4] and to have several sub-isoforms [10]. Recently, MT-3 in the brain and MT-4 in the tongue were also reported [15,16]. MT isoforms have been separated by RIA,

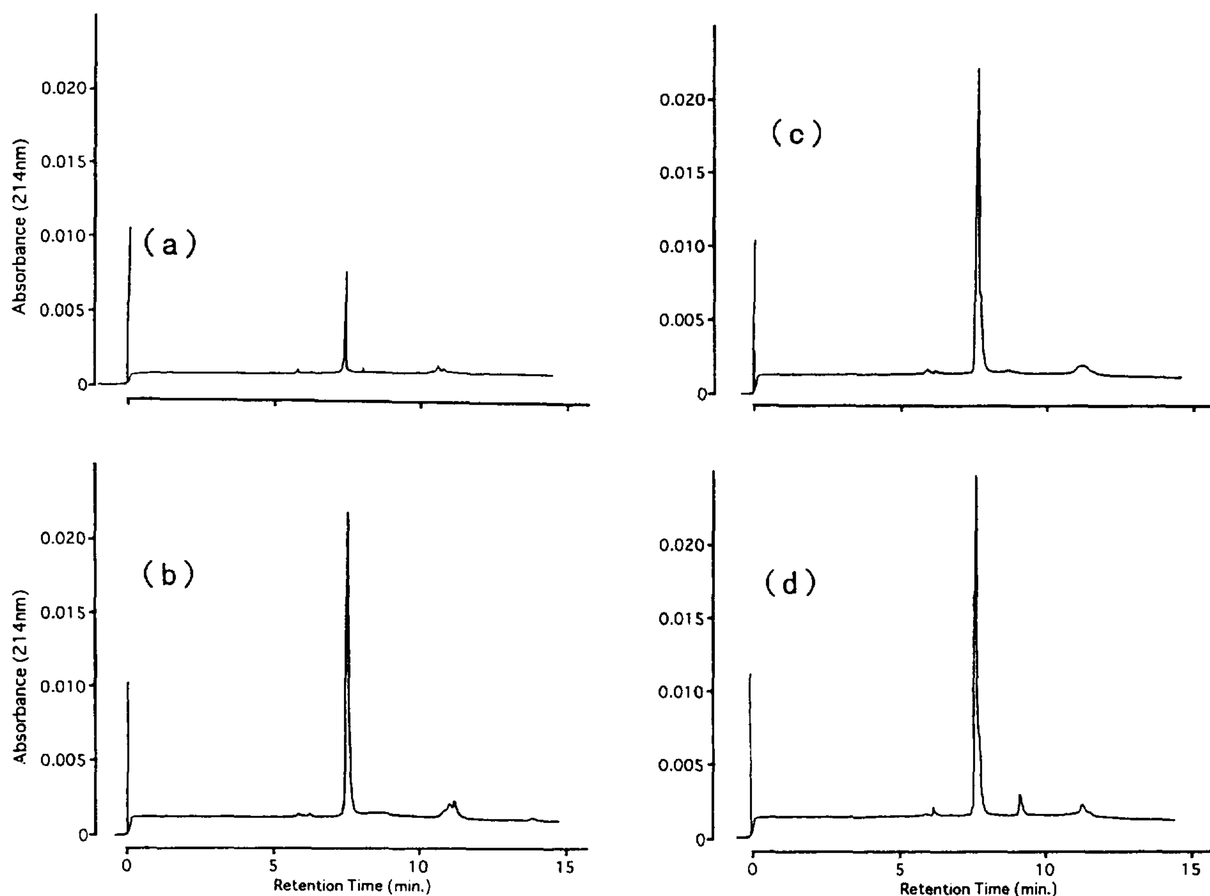

Fig. 2. Typical CZE chromatograms of MT-2 isoforms. Liver MT-2 samples were purified from mouse, rat, rabbit and human by the same method used to prepared MT-1 and were analyzed as described in Fig. 1. Chromatograms show MT-2 from the livers of (a) mouse, (b) rat, (c) rabbit and (d) human.

HPLC, ELISA and CZE [4]. Analysis by CZE has several advantages over the other methods, such as rapid analysis, high resolution and micro-quantity sample requirement. Several types of capillary tubes, such as uncoated, polyacrylamide-coated and polyamine-coated tubes, have been used for the separation of MT isoforms [8,11,12]. The optimal separation of MT isoforms is obtained with the proper combination of capillaries, buffers and sample separation techniques, as suggested by Richards and Beattie [12]. Indeed, when the commercial standard of rabbit liver MT-1, which showed eight peaks when using a polyacrylamide-coated tube (Fig. 3a), was loaded onto an uncoated tube and separated at neutral pH with normal polarity (inlet positive), only three peaks, two of which corresponded to MT-1 and MT-2, appeared (data not presented). Although apothioneins can be separated at pH 2.0 [12], it is important to separate MT isoforms at neutral pH in order to study their properties under native conditions. Coating of the capillary tube with polyacrylamide also reduces interactions between the tube and proteins. We therefore selected a polyacrylamide-coated tube and neutral buffer for the separation of MT isoforms with CZE. As shown in Figs. 1 and 2, MT-1 and MT-2 from mouse, rat, rabbit and human livers could be separated, although the elution of rabbit and human MT-1s was delayed compared with that of mouse and rat MT-1s. As MT samples used in this study were re-purified, subisoforms of MT were not observed. In addition, the MT isoforms purified from the mouse, rat and rabbit

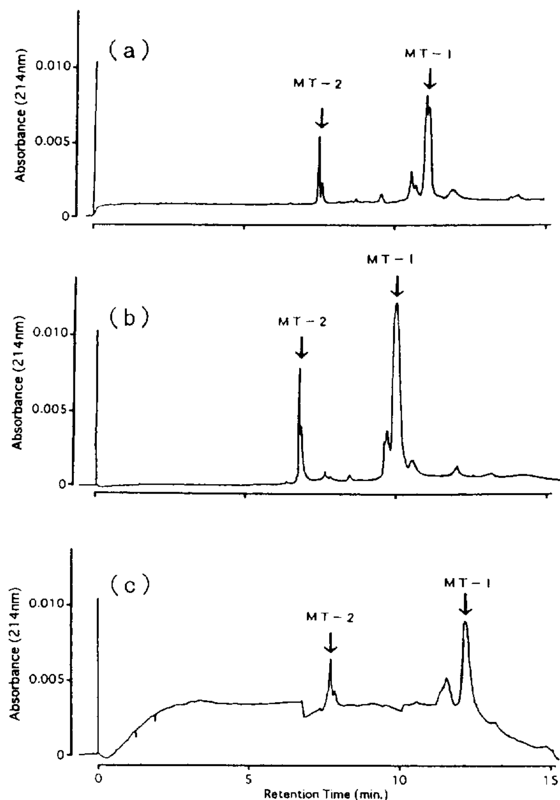

Fig. 3. Effects of different buffers at neutral pH on the separation of MT isoforms. Commercial standard rabbit MT-1 was separated with a polyacrylamide-coated capillary tube using three different buffers. (a) 25 m*M* HEPES–Tris buffer (pH 7.4), (b) 25 m*M* TAPS–Tris buffer (pH 7.7) and (c) 25 m*M* ADA–Tris buffer (pH 7.4). Samples (1 mg/ml) were loaded by hydrostatic injection for 8 s. Column parameters were as described in Fig. 1.

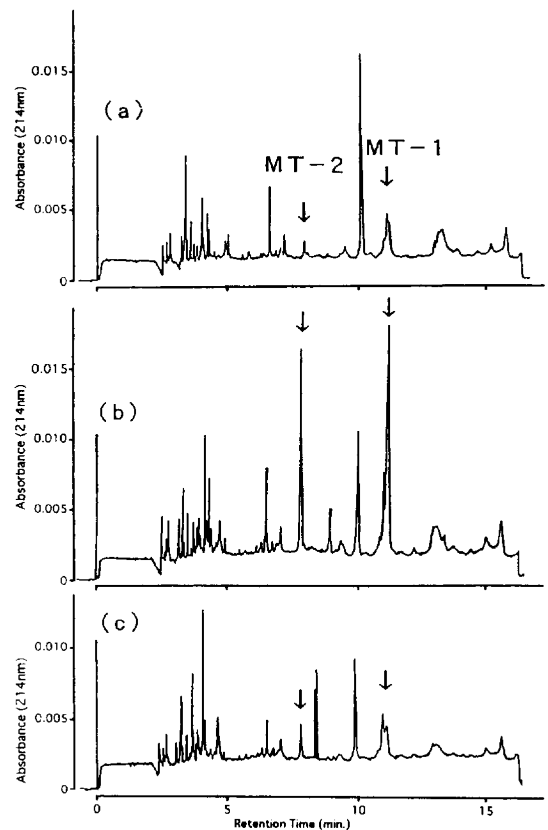

Fig. 4. MT isoforms in mouse liver induced by Zn or Cd. Mice were subcutaneously administered 50 mg/kg of zinc or 3 mg/kg of cadmium. Twenty-four hours after the injection, the mice were decapitated and their livers were removed. Crude extract of mouse liver MT was prepared by ethanol extraction, after which it was dissolved in distilled water and loaded onto the cathode end of the capillary by gravity for 8 s and analyzed as described in Fig. 1. (a) Control mouse liver, (b) Zn-administered mouse liver and (c) Cd-administered mouse liver.

were Cd-thionein, while the MT isoforms from the human were Zn-thionein. In contrast, the commercial standard rabbit liver MT-1 showed eight peaks and the MT-1 isoform mainly consisted of two unseparated peaks (Fig. 3a), while the commercial standard MT-2 showed five peaks (data not presented). Furthermore, it is important to select buffers for CZE analysis, and in this experiment, HEPES–Tris buffer (pH 7.4) was capable of separating the MT isoforms (Fig. 3a). Both MT-1 and MT-2 in mouse liver, induced by either Zn or Cd, could be detected with CZE using a polyacrylamide-coated tube and HEPES–Tris buffer (pH 7.4), although MT-1 had sub-isoforms that were difficult to separate, as was the case with the commercial standard MT-1 isoforms. In addition, the leading peak of the MT-1 isoform coincided with the purified mouse liver MT-1 isoform, but it is unclear whether the relationship between the kind of metal and MT affects the elution time of the MT isoforms.

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