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CHARACTERIZATION OF THE METAL COMPOSITION OF METALLOTHIONEIN ISOFORMS USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ATOMIC ABSORPTION SPECTROPHOTOMETRIC DETECTION

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to separate metallothionein (MT) isoforms and on-line atomic absorption spectrophotometric (AAS) detection was used to quantitatively determine their metal content. With this coupled system (HPLC–AAS), it was possible to determine the zinc, cadmium and copper content of individual horse kidney MT isoforms. When rabbit liver MT and the purified isoforms (MT-1 and MT-2) were subjected to RP-HPLC and the zinc content determined by AAS, it was possible to assign each of the two major zinc-containing peaks of the MT sample to MT-1 or MT-2. HPLC–AAS was used to identify zinc-induced MT in heat-treated cytosol from turkey hen liver, thereby demonstrating its application to the analysis of crude tissue extracts. A standard curve was established using turkey liver MT for the quantitative determination of the zinc content of MT isoforms. There was excellent linear correlation between the μg of zinc bound to MT injected onto the column (ranging from 0.34 to 3.43 μg of MT-bond zinc) and the integrated peak area of the atomic absorbance for zinc. Using this standard curve, it was possible to quantitate the amount of MT-bound zinc in cytosol extracts of cultured turkey embryo hepatocytes exposed to varying levels of supplemental zinc in the culture medium.

INTRODUCTION

Metallothionein (MT) is a low-molecular-weight, heavy-metal-binding protein that is generally characterized by several distinctive features including: (1) a single polypeptide chain comprised of 61–63 amino acids, (2) an abundance of cysteine residues (30%), (3) an absence of aromatic amino acids, (4) a high metal content and (5) a highly conserved structure which, because of the positioning of the cysteine residues, gives rise to two distinct metal-binding domains[1,2]. Another unique feature of MT is that it is a metal-inducible protein such that the metals that bind to the protein (thionein) also induce its synthesis. This observation has been cited as evidence

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indicative of its function in basic cellular metal metabolism[3]. MT has been proposed to play a role in the detoxification of heavy metals such as cadmium and mercury as well as in the homeostasis of essential nutrient metals such as copper and zinc[4–7].

Multiple forms (isoforms) of MT have been isolated and characterized from the tissues of a number of eukaryotic species[1,2]. Generally, these isoforms have been designated as two types, MT-1 and MT-2, based on their order of elution from an anion-exchange column. The MT-1 and MT-2 isoforms differ in their amino acid composition and net negative charge at neutral pH[8]. Sequence analysis of purified MT isoforms revealed the existence of microheterogenity (*i.e.*, the existence of additional isoforms)[1,2] which has subsequently been confirmed and extended using molecular cloning techniques to identify and characterize the individual genes coding for distinct MT isoforms[9,10]. In addition, there is another form of heterogenity characteristic of MT that arises not from genetically determined changes in the primary structure of the thionein polypeptide chain, but from differences in its metal composition[1,2]. MTs that differ in their metal composition but not in their amino acid sequence have been referred to as metalloforms[11].

Several techniques have been developed for the isolation and characterization of MT isoforms from tissue extracts. These include a combination of gel permeation and ion-exchange column chromatography[1,2,4], gel electrophoresis[12] and high-performance liquid chromatography (HPLC)[13–19]. Because of its high metal content, the detection and quantitation of MT in tissues is often performed by measuring the amount of metal bound to the isolated protein. Suzuki[16] first introduced a detection technique for MT based on a gel permeation HPLC separation of MT coupled with atomic absorption spectrophotometric (AAS) detection of the bound metals. The ability of this technique to separate MT isoforms was based on the combination of gel permeation and weak cationic-exchange properties of the column used (TSK gel SW 3000; Toyo Soda, Tokyo, Japan). Since that initial report, the technique of HPLC coupled with AAS (HPLC–AAS) has been modified and extended to include anion-exchange and reversed-phase columns and the combination of gel permeation and ion-exchange columns coupled by means of column switching resulting in a higher degree of resolution in the separation of MT isoforms[17,18]. Lehman and Klaassen[19] have utilized the HPLC–AAS combination with a prior cadmium displacement treatment to detect and quantitate rat liver MT isoforms based on their ability to bind the exogeneous cadmium. Although ion-exchange columns are capable of resolving MT-1 and MT-2, such columns generally do not resolve all of the isoform subspecies. Reversed-phase HPLC (RP-HPLC) has proven to be an efficient method for the complete separation and quantitation of individual MT isoforms[13–15]. However, RP-HPLC may not distinguish between different metalloforms of MT, unless the metal composition affects their elution characteristics. Therefore, the purpose of these studies was to determine the feasibility of coupling RP–HPLC with AAS in order to characterize the metal content of individual MT isoforms and to determine the applicability of such a coupled system to the quantitation of MT-bound metal.

EXPERIMENTAL[a]

Preparation of samples for HPLC–AAS

Rabbit liver MT and the purified isoforms (MT-1 and MT-2) and horse kidney MT were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions (1 mg/ml) were prepared gravimetrically in 10 m*M* sodium phosphate buffer, pH 7.0. Turkey hen liver was obtained 24 h after the second of two intraperitoneal injections of zinc (10 mg/kg body weight, as $ZnSO_4 \cdot 7H_2O$ injected at 24-h intervals) to induce the synthesis and accumulation of MT. A soluble extract was prepared by homogenizing turkey hen liver tissue in one volume of 10 m*M* Tris–HCl, pH 8.6 with a Polytron device set at 3/4 speed for 60 s, followed by heating the homogenate at 60°C for 10 min and centrifugation at 105 000 *g* for 90 min at 4°C. The resulting supernatant (heat-treated cytosol) was filtered through a 0.22-μm membrane and stored at −20°C prior to HPLC–AAS analysis. Purification of the MT-2 isoform (*i.e.*, the predominant MT species present in turkey liver) involved a two-step column chromatographic fractionation of liver cytosol not subjected to prior heat treatment: (1) gel permeation chromatography was performed on a column (60 × 5.0 cm I.D.) of Sephadex G-75, eluted with 10 m*M* Tris–HCl, pH 8.6. Fractions of 10 ml were collected and analyzed for zinc using AAS to detect the MT peak. (2) Those fractions comprising the MT peak from the gel permeation separation were pooled and applied to a column (20 × 2.6 cm I.D.) of DEAE Sephadex A25 and eluted with a linear gradient of 10–300 m*M* Tris–HCl, pH 8.6. Fractions of 5 ml were collected and analyzed using AAS to detect a zinc peak corresponding to the MT-2 isoform. Those fractions containing MT-2 were pooled, dialyzed extensively against deionized water and lyophilized.

HPLC and AAS apparatus

A Waters (Milford, MA, U.S.A.) liquid chromatograph equipped with dual M6000A pumps, a WISP 710 autosampler, a Model 720 system controller and a Model 730 data module (plotter–integrator) was used to perform the separation of MT isoforms. UV absorbance (214 nm) was monitored with a Model 441 fixed-wavelength monitor. The column used was a μBondapak C_{18} cartridge (10 cm × 8 mm I.D., 10-μm particle size) in a Z-module radial compression device. Buffer A consisted of 10 m*M* sodium phosphate, pH 7.0 and buffer B consisted of 60% acetonitrile in buffer A. MT was eluted with a two-step, linear gradient consisting of 0–10% B for 0–5 min followed by 10–25% B from 5–20 min. The column was maintained at ambient temperature and run at a flow-rate of 3.0 ml/min. To determine the metal content of separated MT isoforms, the effluent from the column was fed directly into the nebulizer of a Model 5000 atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, U.S.A.) set to accept a flow-rate of 3.0 ml/min. An air–acetylene flame was used and the spectrophotometer was set to 213.9, 228.8 and 324.8 nm for the determination of zinc, cadmium and copper, respectively. A 1-V output signal from the atomic absorption spectrophotometer was used for peak area integration.

[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.

Quantitation of MT–zinc

MT–zinc quantitation was based on peak area integration of the atomic absorbance for zinc (213.9 nm). Purified turkey hen liver MT-2 was used to construct a standard curve. A stock solution (34.3 μg zinc per ml) was gravimetrically prepared in 10 m*M* sodium phosphate, pH 7.0 (buffer A). The stock solution was diluted with buffer A to yield 100-μl aliquots containing 0.34, 0.86, 1.71, 2.57 and 3.43 μg of MT-bound zinc. Each of the 100-μl aliquots was injected onto the column and eluted as described above. The integrated peak area of the atomic absorbance for zinc was plotted against μg of MT-bound zinc injected onto the column and linear regression analysis was used to establish the relationship.

Quantitation of MT–zinc in cultured hepatocytes

Turkey embryo hepatocytes were prepared as described previously[20]. Briefly, primary monolayer cultures of hepatocytes were prepared from the livers of 16 day-old embryos by digestion with collagenase (0.05%). The isolated cells were plated in medium 199 (M199, Gibco, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum and 0.5 μg/ml bovine insulin. After 16 h, the culture medium was replaced with M199 containing 0–50 μ*M* zinc without serum supplementation. At the end of the exposure period, each plate was washed twice with ice-cold phosphate buffered saline and the monolayer harvested by scraping with a rubber policeman into 2 ml of deionized water. The cell suspensions were sonicated and the protein content determined using a dye-binding procedure (Bio Rad Labs., Richmond, CA, U.S.A.). A soluble fraction of the cell sonicates was prepared by centrifugation (105 000 *g* for 30 min at 4°C) and subjected to HPLC–AAS as described above. MT–zinc was quantified using integrated peak area and extrapolated from the turkey hen liver MT-2 standard curve. Values were expressed as μg MT–zinc per mg cell protein.

RESULTS AND DISCUSSION

Detection of MT-bound metals

RP-HPLC has proven to be an efficient method for the isolation and quantitation of MT isoforms[13–15]. This study confirms the fact that RP-HPLC can be adapted to include AAS detection of the metals bound to the separated MT isoforms. Fig. 1 depicts the separation of horse kidney MT isoforms using RP-HPLC with AAS detection of zinc, cadmium and copper bound to each of the isoform species. Cadmium and zinc, the two most abundant metals bound, gave essentially identical patterns of isoform species, with two predominant peaks (MT-1 and MT-2) and a number of additional less abundant peaks. This pattern of MT isoform peaks is similar to that determined by monitoring UV absorbance at 214 nm[15]. Copper was detected, although at a much reduced level compared to cadmium or zinc. Moreover, the isoform pattern detected by monitoring the atomic absorbance for copper was markedly different from that observed for either cadmium or zinc. The majority of the copper appears to be bound to the MT-2 isoform species with less bound to the MT-1 isoform and the less abundant peaks. The significance of this apparent preferential binding of copper by the MT-2 isoform remains to be explained.

Fig. 2 (MT) depicts the separation of rabbit liver MT on RP-HPLC with AAS detection of the bound zinc. Like horse kidney MT, a complex pattern is demonstrated

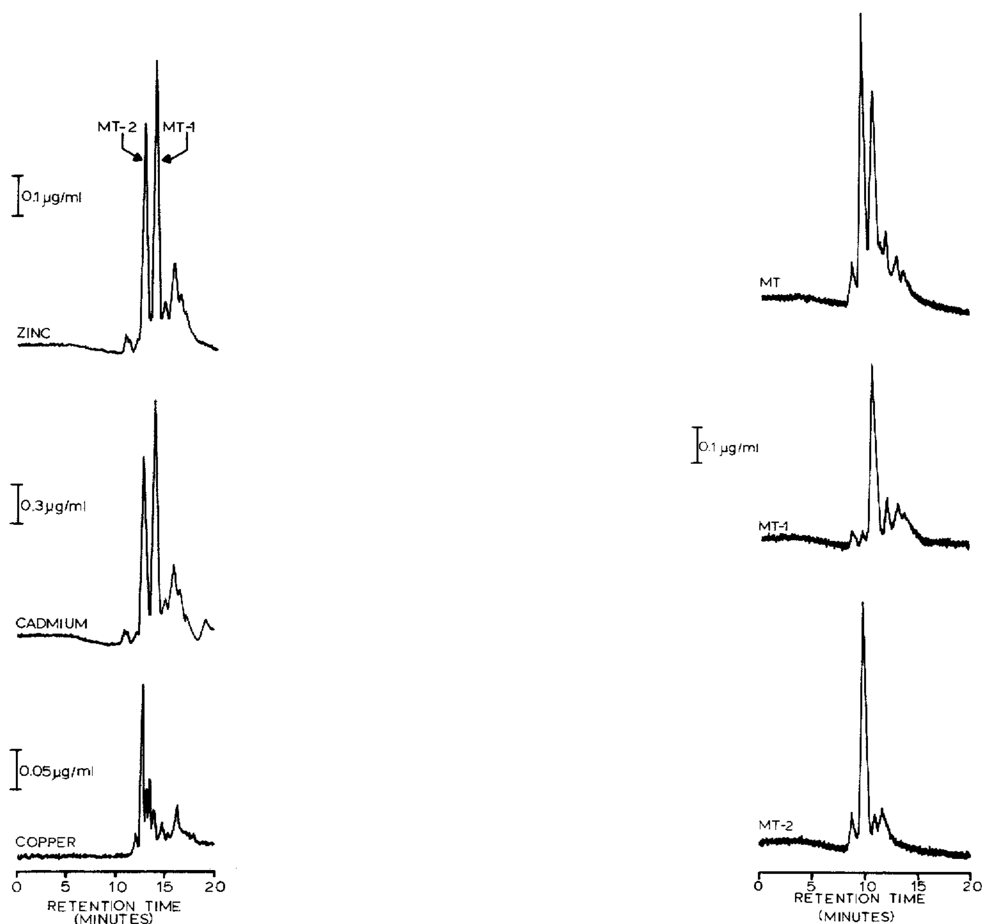

Fig. 1. Horse kidney MT separated using RP-HPLC with on-line monitoring of the atomic absorbances at 213.9 nm (zinc), 228.8 nm (cadmium) and 324.8 nm (copper). The separation of individual MT isoforms was performed with a μBondapak C_{18}, radially-compressed cartridge column eluted with a two-step, linear gradient of 0–6% (0–5 min) and 6–15% (5–20 min) acetonitrile in 10 m*M* sodium phosphate, pH 7.0 at ambient temperature with a flow-rate of 3.0 ml/min. The column effluent was fed directly into the nebulizer of an atomic absorption spectrophotometer for the detection of metals. A separate injection of 100 μg of MT in 100 μl of 10 m*M* sodium phosphate, pH 7.0 was made for the determination of each metal. The vertical bars represent the atomic absorbance level for the indicated concentration of the appropriate aqueous metal standard. The two major MT isoform species are denoted as MT-1 and MT-2.

Fig. 2. Rabbit liver MT and purified MT isoforms (MT-1 and MT-2) subjected to RP-HPLC with on-line monitoring of the atomic absorbance for zinc at 213.9 nm. Separate injections consisted of 100 μg each of MT, MT-1 and MT-2 in 100 μl of 10 m*M* sodium phosphate, pH 7.0. The separation conditions were the same as described in the legend to Fig. 1. The vertical bar represents the level of atomic absorbance for an aqueous standard at a concentration of 0.1 μg zinc per ml.

by the occurrence of 6–7 distinct peaks exhibiting zinc-binding. Furthermore, if purified MT-1 and MT-2 isoform species are subjected to RP-HPLC (Fig. 2, MT-1 and MT-2), it is possible to determine which of the two major zinc-binding peaks observed

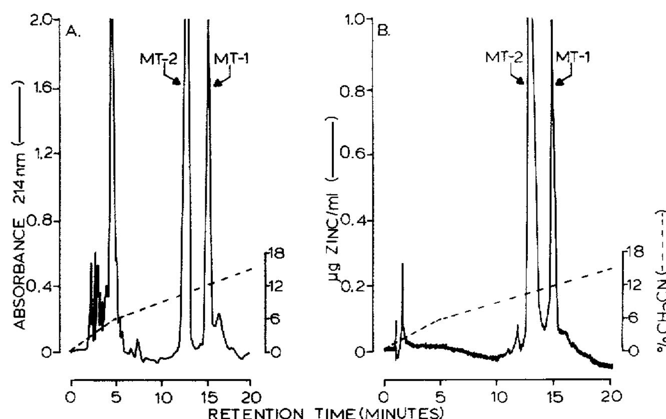

Fig. 3. Turkey hen heat-treated cytosol from birds which had received two consecutive intraperitoneal injections of zinc (10 mg/kg body weight) subjected to RP-HPLC with on-line detection for UV absorbance at 214 nm (A) and atomic absorbance for zinc at 213.9 nm (B). The separation conditions were the same as described in the legend for Fig. 1 with the dashed line denoting the two-step linear acetonitrile gradient used to elute the samples. A separate injection of 100 μl of cytosol was made for monitoring UV and atomic (zinc) absorbances. Two MT species are designated as MT-1 (eluting at 14.83 min) and MT-2 (eluting at 12.93 min).

for MT is classified as MT-1 and which is MT-2. The pattern of peaks for MT and both of the purified isoforms is similar to that observed when UV absorbance was monitored at 214 nm[15].

It is possible to resolve MT isoforms from crude extracts of tissue using RP-HPLC[13-15]. Fig. 3 depicts a comparison of two methods for the detection of MT in heat-treated cytosol from turkey hen liver after its induction by two successive intraperitoneal injections of zinc. The separation of MT from the heat-treated cytosol revealed the presence of two peaks of UV absorbance eluting at 12.93 and 14.83 min which have been designated as MT-2 and MT-1, respectively (Fig. 3A). When AAS detection for zinc was used, a similar pattern of MT peaks was observed indicating that both putative MT species contained zinc (Fig. 3B). It is clear from both chromatograms that the MT species designated as MT-2 is the predominant one which is consistent with what has been reported previously for turkey liver MT[15]. Moreover, the ratio of MT-2 to MT-1 was found to be 4.2 and 4.6 based on the integrated peak areas of UV and atomic absorbances, respectively. The occurrence of two species for turkey liver MT is somewhat curious, since chicken MT has been shown to exist as a singular isoform[21–23]. Although an amino acid analysis of the putative MT-1 and MT-2 species indicated that both were cysteine-rich polypeptides[15], further analysis such as sequencing of the peptides is required to confirm that these species are indeed true MT isoforms. Also it must be emphasized that the turkey liver was obtained from birds that received two large consecutive doses of zinc. It is possible that the additional MT species (MT-1) is present in appreciable quantities only under conditions of maximal induction. Others have reported the existence of at least two forms of MT in quail[18,24,25] and chickens[25] in response to induction by toxic heavy metals or large doses of zinc. Under non-induced conditions, avian MT has been reported to occur as

a singular form[25]. Heterogeneity in chicken MT can arise from differences in the metal composition (*i.e.*, the formation of distinct MT metalloforms), despite the fact that the protein (thionein) portion of the molecule is identical[26]. Moreover, it is possible to separate two different metalloforms of chicken MT using anion-exchange column chromatography[26]. Whether the two forms of turkey liver MT isolated in this study represent distinct isoforms or metalloforms is not known. A similar situation has been reported for rat and mouse MTs. Despite the fact that only two functional MT genes have been identified for rodents[10], a third isoform (a subspecies of MT-2) has been isolated using anion-exchange HPLC[27,28] and RP-HPLC[13]. Suzuki *et al.*[27] have speculated that the third "isoform" may in fact be identical to MT-2 in its amino acid sequence but differ with respect to the state of enzymatically labile functional groups. Clearly, the exact nature of the observed heterogeneity in turkey liver MT remains to be defined. However, RP-HPLC and HPLC–AAS should prove to be useful analytical techniques for the isolation and characterization of heterogeneous MT species.

Quantitation of MT–zinc

In order to investigate the ability of HPLC–AAS to quantitate MT-bound metal, a standard curve was established using zinc-induced MT-2 derived from turkey liver. This particular source of MT affords an excellent standard because it is easily purified and because it is comprised of a singular species[15]. In addition, a standard curve has previously been derived from turkey liver MT-2 for the quantitative determination of MT by monitoring UV absorbance at 214 nm[15]. Varying amounts of MT-2 were

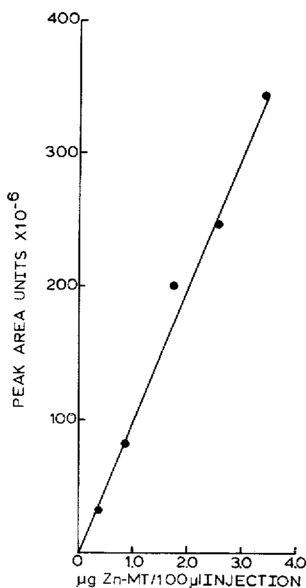

Fig. 4. Turkey hen liver MT-2 standard curve for RP-HPLC with atomic absorbance detecton of zinc at 213.9 nm. Aliquots of MT-2 containing from 0.34 to 3.43 μg zinc in 100 μl of 10 mM sodium phosphate, pH 7.0 were injected onto the column and separated as described in the legend to Fig. 1. The μg of zinc bound to MT-2 (Zn–MT) is plotted against the integrated peak area units of the atomic absorbance for zinc at 213.9 nm. Linear regression analysis was used to fit the line to the data points.

prepared gravimetrically in sodium phosphate buffer (pH 7.0) such that a 100-μl aliquot contained 0.34–3.43 μg of zinc. Fig. 4 demonstrates the linear relationship ($r = 0.991$) between the μg of zinc bound to MT-2 injected onto the column and the integrated peak area of the atomic absorbance for zinc. It is possible to accurately detect as little as 0.1 μg of zinc injected onto the column. This agrees well with a detection limit of 1 μg of MT (assuming that 1 μg of MT fully saturated with zinc would bind about 0.07 μg) determined by monitoring UV absorbance at 214 nm[15]. Furthermore, the recovery of MT-2-bound zinc from RP-HPLC has been estimated to be 97%[15]. Considering the detection limit of HPLC–AAS (1 μg MT), it is clear that the application of this technique is well suited to the detection and quantitation of MT-bound metal in tissues containing considerable amounts of this protein but not to fluids such as serum and urine in which MT levels can be 2–5 orders of magnitude less than typical tissue levels.

Although zinc was the only metal determined quantitatively in this study, it is reasonable to expect that the technique of RP-HPLC coupled with AAS detection could be applied to the quantitation of MT-bound cadmium as well. There have been a number of reports concerning the detection of cadmium following an HPLC separation of MT isoforms[16–19]. Horse kidney MT isoforms were found to contain substantial amounts of this metal (Fig. 1) and, with the appropriate standard, undoubtedly could have been quantitatively determined. Lehman and Klaassen[19] have described a quantitative analytical technique for MT isoforms. This involves an anion-exchange HPLC separation with on-line determination of cadmium by AAS. Previous reports in which HPLC–AAS was employed to detect MT isoforms utilized aqueous metal solutions for quantitation whereas, Lehman and Klaassen[19] were the first to introduce MT isoform standards saturated with cadmium *in vitro*. However, their technique assumes that cadmium completely displaces all of the bound metal on MT which may not always be the case, especially for copper[8]. This study employed a homologous MT standard for which the metal was incorporated into the protein *in vivo*. The use of an MT standard *versus* an aqueous one takes into account any effects that protein-binding might have on the detection and subsequent quantitation of the metal following RP-HPLC. Moreover, it may be possible to use the same MT standard containing more than one metal to quantitate MT-bound zinc, cadmium and perhaps copper. For example, commercially available MTs, such as those from horse kidney or rabbit liver used in this study, contain both zinc and cadmium as well as traces of copper (Fig. 1). Alternatively, since MT can be depleted of its metals *in vitro* by reducing the pH and can be reconstituted with zinc or cadmium upon raising the pH in the presence of either metal, it may be possible to produce MT standards of any desired metal content or combination for subsequent use as standards for the quantitation of MT-bound metal using HPLC–AAS. Moreover, RP-HPLC may be useful for the isolation of singular MT isoform or metalloform species to be used as standards. It has been suggested previously that individual heterologous MT isoforms may be useful as internal standards for the quantitation of MT in tissue extracts[15]. Further studies are required to assess the feasibility of these approaches.

One limitation of HPLC–AAS that has been encountered concerns the detection of copper-induced or copper-enriched MTs. Hunziker and Kagi[29] have found as we[30] have that MT isoforms containing substantial amounts of copper cannot adequately be separated and detected using RP-HPLC at neutral pH. Given the fact that these

species are inherently unstable, readily oxidizable and require anaerobic conditions for their isolation, this finding is not really surprising. Although the reasons for the inability of RP-HPLC to resolve copper–MTs remain to be determined, this observation should be considered when attempting to analyze MTs containing copper. Suzuki *et al.*[18,31] have demonstrated the feasibility of HPLC–AAS for the detection of MT-bound copper using gel permeation and ion-exchange columns to perform the separation of MT isoforms. However, when copper is not the predominant metal bound, as is the case for many naturally occurring MTs isolated from vertebrate animal species, it may be possible to detect as well as quantitate this particular metal using RP-HPLC. Glennas *et al.*[32] have reported that RP-HPLC was well suited for the resolution and detection of cadmium- and zinc-containing MT but was inappropriate for aurofin-induced, gold-containing MT from cultured human epithelial cells. It has

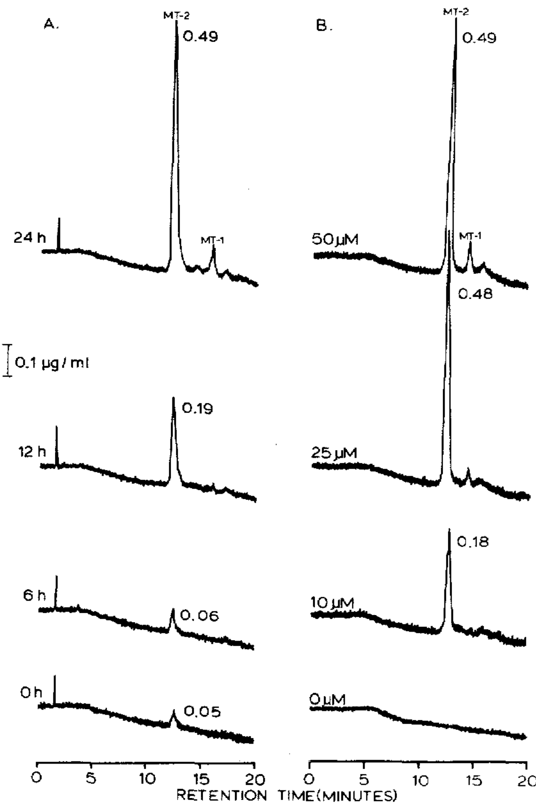

Fig. 5. Cytosol extracts from turkey embryo hepatocytes cultured under serum-free conditions subjected to RP-HPLC with atomic absorbance detection of zinc (213.9 nm). The separation conditions were the same as described in the legend to Fig. 1. The vertical bar represents the level of atomic absorbance for an aqueous standard at a concentration of 0.1 μg zinc per ml. Individual MT species are designated as MT-1 and MT-2. (A) Hepatocytes were cultured in the presence of 50 μM supplemental zinc in the medium and analyzed at 0, 6, 12 and 24 h after exposure. (B) Hepatocytes were cultured in the presence of 0, 10, 25 and 50 μM supplemental zinc in the medium for 24 h. The numbers next to the MT-2 pak represent the amount (μg) of zinc bound per mg of total cellular protein.

been suggested that zinc and cadmium stabilize thionein against degradation[33] and this may, in part, explain our ability to detect copper in the sample of horse kidney MT (Fig. 1). Clearly, more work needs to be done to explore the many possibilities for the application of RP-HPLC with AAS detection to the quantitative determination of MT-bound metals.

Quantitation of MT–zinc in cultured Hepatocytes

One of the advantages of using HPLC to isolate, characterize and quantitate MT and MT-bound metals is that only a small amount of sample is required for such an assay. Therefore, the application of the HPLC–AAS method to the determination of MT-bound zinc in turkey embryo hepatocytes was investigated. Fig. 5A depicts the separation of cytosol extracts from hepatocytes cultured in the presence of 50 μM zinc for up to 24 h using RP-HPLC with detection of zinc by AAS. The amount of zinc bound to the MT-2 species increased from 0.05 to 0.49 μg/mg cell protein by 24 h. Also at 24 h a second zinc-containing peak was detected, although its level was too low to accurately quantitate. This species has been designed MT-1. Similarly, hepatocytes exposed to varying levels of supplemental zinc (0–50 μM) in the culture medium for 24 h exhibited an increase in MT-2-bound zinc from an undetectable level to 0.49 μg/mg (Fig. 5B). In hepatocytes exposed to 25 or 50 μM supplemental zinc, the putative MT-1 species was again detected, although at levels which did not permit an accurate assessment of the amount of bound zinc. The detection of the species designated MT-1 only in the two highest zinc exposure groups (25 and 50 μM) is consistent with its occurrence only under conditions of maximal induction. A similar finding was observed concerning the occurrence of this species in livers from zinc injected chicks[15]. Recently, Klasing and Laurin[34] working with an avian macrophage cell line have reported that two MT species are induced in response to supplemental zinc in the culture medium.

CONCLUSIONS

This study demonstrates that RP-HPLC can be coupled with AAS for the detection and quantitative determination of MT-bound metals. This technique combines the efficiency of RP-HPLC in completely resolving MT isoforms with AAS detection in order to determine the metal content of individual MT isoforms. Moreover, because this technique requires considerably less sample than classical chromatographic techniques, it can readily be applied to pure or partially pure samples of MT or to cytosol extracts from small pieces of tissue or cultured cells. With appropriate standards, it is possible not only to characterize the metals bound to individual MT isoforms, but also to quantitate them. HPLC–AAS represents a useful analytical tool with which to study the metal composition of individual MT isoforms. Furthermore, it may also prove useful in the isolation and characterization of distinct metalloforms of MT.

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