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# ISOLATION AND QUANTITATION OF CADMIUM-, ZINC- AND COPPER-METALLOTHIONEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ATOMIC ABSORPTION SPECTROMETRY

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## SUMMARY

Metallothioneins (MTs) were separated and quantitated by reversed-phase high-performance liquid chromatography (RP-HPLC), in direct combination with atomic absorption spectrometry (AAS) for quantitation of the metal contents in MTs. MTs were eluted from an RP-8 column with a gradient of Tris buffer pH 7.0 and methanol, and were detected by UV absorbance (220 nm). Commercially available purified MTs from horse kidney and rabbit liver were analyzed for purity and metal composition. One lot of horse kidney yielded only 50% of the estimated value. In some cases, the certified metal content differed considerably from the values found. The method was also tested with rat liver and fetal bovine liver. The metal contents found in MTs by use of graphite-furnace atomic absorption spectrometry corresponded well with the values found by RP-HPLC–AAS. Molar ratios of cadmium, zinc and copper were calculated in MT-1, MT-2 and total MT.

## INTRODUCTION

Metallothioneins (MTs) are a group of non-enzymatic, low-molecular-weight proteins (6500 daltons) which bind heavy metals and have a characteristic amino acid composition (high cysteine content, no aromatic amino acids)[1]. MT binds five to seven heavy metal atoms through two clusters of thiolate bonds. The strength of binding can vary by as much as six orders of magnitude depending on the ion involved[2,3]. MTs are thought to play an important rôle in metabolism and detoxification of a number of essential and non-essential trace metals. The basic physiological function of MTs in mammals appears to be the maintenance of zinc and copper homeostasis. They accomplish this by dispensing these metal ions when needed for the biosynthesis of metal-dependent cellular components and also by sequestring the metal ions in a chemically innocuous form when their intracellular concentrations exceed critical values[1,2,4–6].

To study the biochemical toxicology of chronic sublethal cadmium burden in

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living organisms, the isolation of MTs and quantitation of cadmium, copper and zinc in MTs is necessary. A number of methods are known for the quantitation of MTs: radioimmunoassay; mercury-saturation assay; cadmium-saturation assay; thiolate group determination; Sephadex G-75 gel chromatography with subsequent cadmium determination[7]; saturation with cadmium followed by isolation and quantitation of MT isoforms by high-performance liquid chromatography (HPLC) in combination with atomic absorption spectrometry (AAS)[8] and UV detection after reversed-phase (RP)-HPLC[9]. The isolation of MTs by RP-HPLC–AAS has also been described[10,11]. Reversed-phase chromatography seems to be superior to gel permeation and ion-exchange chromatography, because the packing material for reversed-phase chromatography is principally free of ligands for metals[10].

To our knowledge, HPLC–AAS has been used only to isolate MTs and to detect various metals in MTs, but not to quantitate both MTs and metals simultaneously. In this study a method was developed for separating MTs in the two major isoforms, MT-1 and MT-2, and for quantitating MTs and their cadmium, zinc and copper contents by means of HPLC–AAS.

## EXPERIMENTAL

### *Reagents*

Purified rabbit liver MT and horse kidney MT were obtained from Sigma (St. Louis, MO, U.S.A.). Fetal bovine liver was obtained from the Regional Animal Health Service (Boxtel, The Netherlands) by courtesy of Dr. G. H. Wentink. Rat liver from cadmium-exposure experiments was a gift from Dr. A. F. W. Morselt, University of Amsterdam. Methanol was obtained from Rathburn (Walkerburn, U.K.) and all other reagents from Merck (Darmstadt, F.R.G.).

### *Isolation procedure*

A 1-g amount of liver was homogenized, using a glass–PTFE homogenizer, in 5 ml of 50 m*M* Tris–HCl buffer (containing 250 m*M* saccharose and 5 m*M* 2-mercaptoethanol, pH 7.4). Air was removed from the buffer by bubbling with nitrogen. Particle-free cytosol was prepared by centrifugation at 100 000 *g* for 70 min at 4°C. The supernatant was subsequently filtered through a 0.22-$\mu$m Millex Millipore filter and stored under nitrogen at −20°C until analysis.

### *Apparatus*

A Spectra-Physics chromatograph was used, equipped with a solvent-delivery system Model 8700, a Model 8400 UV detector (220 nm) and a Model 4100 integrator. The column (125 mm × 4 mm) was packed with Lichrospher 100 RP-8, particle size 5 $\mu$m (Merck). The sample injection volume was 0.5 ml. The mobile phases were 50 m*M* Tris–HCl, pH 7.0 (A) and methanol (B) distilled from glass before use. MTs were eluted with a linear gradient from 0.5 to 30% mobile phase B in 20 min. After gradient elution, the column was purged with mobile phase B for 5 min. The column was equilibrated with the starting mixture for 10 min before the next injection. The flow-rate was 1.5 ml/min.

The outlet of the UV detector was coupled to the nebulizer of the atomic absorption spectrometer (Varian AA-40) with a PTFE tube. The nebulizer was

adjusted to a flow-rate of 2 ml/min. An air–acetylene flame was used and the absorption line for cadmium was 228.8 nm, for copper 324.8 nm and for zinc 213.8 nm.

Atomic absorption of the metals was measured 12–20 min after injection. Peak areas were determined with a Varian DS-15 data station. For each chromatographic experiment, the total peak areas and peak areas of MT-1 and MT-2 were measured. The total cadmium, copper and zinc contents of livers and purified MTs were determined with a Perkin-Elmer 3030/Zeeman atomic absorption spectrometer according to the method described earlier[12].

## RESULTS AND DISCUSSION

Neutral buffers are necessary for extracting MTs since zinc starts to dissociate from the protein at pH 5[5]. Cadmium and copper are removed from MTs at lower pH values. To prevent formation of dimeric forms of MTs[13,14], 2-mercaptoethanol was added to the homogenization buffer. To prevent oxidation, the supernatant was stored under nitrogen at −20°C till analysis.

Purified MT from horse kidney (Sigma, Lot No. 54F-8590) was used as a reference standard. Total cadmium, zinc and copper contents were determined by Zeeman AAS. Known amounts were injected on the HPLC column, and the peak area units per mg MT, MT-1 and MT-2 were calculated from the UV detector responses. After AAS and integration of the peaks, the peak area units were calculated for 1 μg cadmium and zinc. The copper content of horse kidney MT was below the limit of

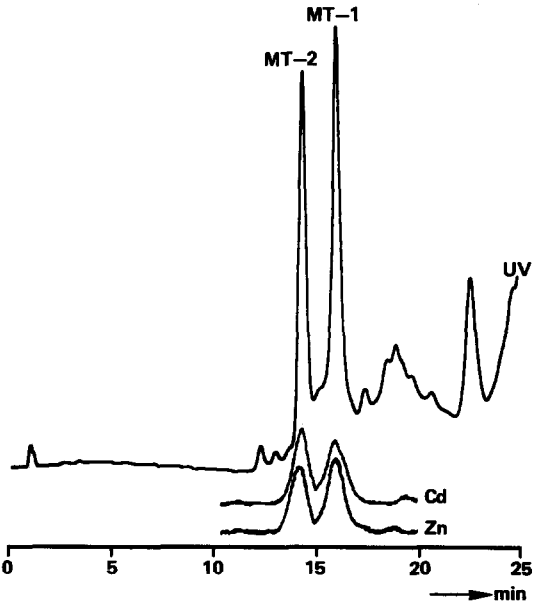


Fig. 1. UV chromatogram with AAS cadmium and zinc signals for purified horse kidney metallothionein (MT; Sigma, Lot No. 54F-8590). The separation was performed with a 100 RP-8 (125 mm × 4 mm) column. MT was eluted with a linear gradient from 0.5 to 30% methanol in 50 m*M* Tris buffer (pH 7.0) and a flow-rate of 1.5 ml/min. Injection volume: 0.5 ml. Metals were determined with an air–acetylene flame.

TABLE I

ANALYSIS OF PURIFIED HORSE KIDNEY METALLOTHIONEIN (SIGMA, LOT NO. 54F-8590) BY ON-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ATOMIC ABSORPTION SPECTROMETRY

| | *MT** | *Cadmium*** | *Zinc*** |
|---|---|---|---|
| MT | 1.35 ± 0.13 | 48.9 ± 4.7 | 24.4 ± 1.8 |
| MT-1 | 0.60 ± 0.02 | 46.7 ± 2.4 | 24.0 ± 0.6 |
| MT-2 | 0.37 ± 0.02 | 46.9 ± 1.5 | 27.7 ± 2.8 |

* MT = Metallothionein, mean ± S.D. (mg) of eight replicates; sample 1.38 mg MT.
** Mean ± S.D. (μg/mg) of four replicates.

detection. A typical chromatogram, representing UV, zinc and cadmium absorption, is shown in Fig. 1.

The day-to-day reproducibility was good (Table I). The ratio between MT-1 and MT-2 is about 2:1, whereas the cadmium and zinc contents per mg MT-1 and MT-2 are equal. The sum of MT-1 and MT-2 is less than the total amount of MT injected; the remaining 0.38 mg are probably other isoforms[9], dimers or polymers of MT.

Fig. 2 shows the simultaneously recorded UV and AAS chromatograms of the

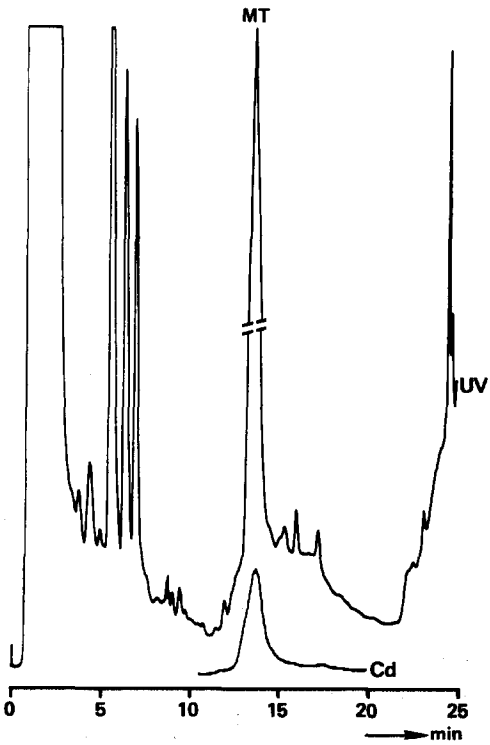


Fig. 2. UV chromatogram and cadmium signal for rat liver subjected to HPLC–AAS, as described in Fig. 1.

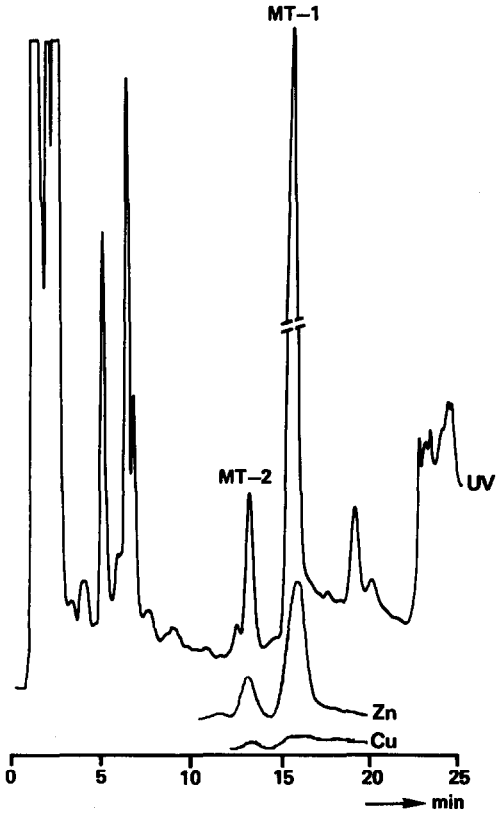


Fig. 3. UV chromatogram with zinc and copper signals for fetal bovine liver subjected to HPLC–AAS, as described in Fig. 1.

TABLE II

QUANTITATION OF CADMIUM, ZINC AND COPPER IN METALLOTHIONEIN

Data are expressed in μg per mg MT, based on the use of horse kidney MT (Sigma, Lot No. 54F-8590) as a reference standard. n.d. = Not detectable.

| | *Certified** | | *ZAAS*** | | | *HPLC–AAS**** | | |
|---|---|---|---|---|---|---|---|---|
| | *Cd* | *Zn* | *Cd* | *Zn* | *Cu* | *Cd* | *Zn* | *Cu* |
| *Horse kidney* | | | | | | | | |
| 54F-8590§ | 63 | 28 | 48.6 | 23.6 | 1.6 | – | – | – |
| 46F-9695§ | 60 | 15 | 49.5 | 16.3 | 3.8 | 49.0 | 27.1 | n.d. |
| 74F-8620§ | 455 | 69 | 50.6 | 11.4 | 6.4 | 44.7 | 18.0 | n.d. |
| *Rabbit liver* | | | | | | | | |
| 105F-9625§ | 94 | 18 | 44.5 | 9.0 | 1.6 | 57.7 | 11.1 | n.d. |
| Rat liver | – | – | 81.5 | 33.2 | 1.3 | 64.0 | 36.7 | n.d. |
| Fetal bovine liver | – | – | n.d. | 51.7 | 0.3 | n.d. | 69.9 | 0.1 |

* Certified value in MT to information from Sigma.
** Determination by graphite-furnace AAS with Zeeman background correction.
*** Determination by present method.
§ Lot. No. of purified MT, obtained from Sigma.

TABLE III

MOLAR RATIOS OF METALS IN METALLOTHIONEIN

n.d. = Not detectable.

| | *ZAAS** | | | | *HPLC–AAS*** | | | |
|---|---|---|---|---|---|---|---|---|
| | *Cd* | *Zn* | *Cu* | *Total* | *Cd* | *Zn* | *Cu* | *Total* |
| *Horse kidney* | | | | | | | | |
| 54F-8590**** | 2.81 | 2.36 | 0.16 | 5.32 | – | – | – | – |
| 46F-9695**** | 2.86 | 1.62 | 0.39 | 4.87 | 2.83 | 2.69 | 0.39 | 5.91 |
| 74F-8620**** | 2.93 | 1.13 | 0.65 | 4.71 | 2.58 | 1.79 | 0.65 | 5.02 |
| *Rabbit liver* | | | | | | | | |
| 105F-9625**** | 2.57 | 0.89 | 0.16 | 3.62 | 3.34 | 1.10 | 0.16 | 4.60 |
| Rat liver | 4.71 | 3.30 | 0.13 | 8.14 | 3.70 | 3.65 | 0.13 | 7.48 |
| Fetal bovine liver | n.d. | 5.14 | 0.03 | 5.17 | n.d. | 6.95 | 0.01 | 6.96 |

* Determination by graphite-furnace AAS with Zeeman background correction.
** Determination by reported method.
*** Lot No. of purified MT, obtained from Sigma.

MT isolate of rat liver and Fig. 3 shows these chromatograms for fetal bovine liver. Rat liver contained 1.29 mg MT per g organ, and fetal bovine liver 1.43 mg MT per g organ (fresh weight).

To test the linearity of the method, different concentrations of horse kidney MT were injected. The UV and AAS responses were linear in the range of 0–200 μg MT/ml, and 0–20 μg metals/ml, respectively. The analytical results for MTs from horse kidney, rabbit liver, rat liver and fetal bovine liver are shown in Table II. These results are calulated on the basis of horse kidney MT (Sigma, Lot No. 54F-8590). The copper content in the purified MTs and rat liver MT was too low to be measured with HPLC–AAS, but could be measured with ZAAS. The certified values of purified MTs

TABLE IV

HPLC–AAS QUANTITATION OF METALLOTHIONEIN ISOFORMS 1 AND 2, AND THEIR CADMIUM AND ZINC CONTENTS

Calculations are based on the use of horse kidney MT (Sigma, Lot No. 54F-8590) as a reference standard.

| | *Found (mg)* | | *Cd (μg/mg)* | | *Zn (μg/mg)* | |
|---|---|---|---|---|---|---|
| | *MT-1* | *MT-2* | *MT-1* | *MT-2* | *MT-1* | *MT-2* |
| *Horse kidney* | | | | | | |
| 54F-8590* | 0.60 | 0.37 | 46.7 | 46.9 | 24.0 | 27.7 |
| 46F-9695* | 0.36 | 0.11 | 44.3 | 34.4 | 30.5 | 37.0 |
| 74F-8620* | 0.51 | 0.31 | 49.2 | 46.9 | 19.2 | 22.5 |
| *Rabbit liver* | | | | | | |
| 105F-9625* | 0.20 | 0.65 | 62.9 | 57.6 | 13.3 | 18.9 |
| Fetal bovine liver | 1.06 | 0.21 | 0.62 | 0.71 | 65.6 | 69.5 |

* Lot No. of purified MT, obtained from Sigma.

TABLE V

MOLAR RATIOS OF METALS IN METALLOTHIONEIN ISOFORMS 1 AND 2

n.d. = Not detectable.

| | *Cd* | | *Zn* | | *Cu* | | *Total* | |
|---|---|---|---|---|---|---|---|---|
| | *MT-1* | *MT-2* | *MT-1* | *MT-2* | *MT-1* | *MT-2* | *MT-1* | *MT-2* |
| *Horse kidney* | | | | | | | | |
| 54F-8590* | 2.70 | 2.71 | 2.39 | 2.75 | n.d. | n.d. | 5.09 | 5.46 |
| 46F-9695* | 2.56 | 1.99 | 3.03 | 3.66 | n.d. | n.d. | 5.59 | 5.65 |
| 74F-8620* | 2.85 | 2.71 | 1.91 | 2.24 | n.d. | n.d. | 4.76 | 4.95 |
| *Rabbit liver* | | | | | | | | |
| 105F-9625* | 3.63 | 3.33 | 1.32 | 1.88 | n.d. | n.d. | 4.95 | 5.21 |
| Fetal bovine liver | n.d. | n.d. | 6.52 | 6.91 | 0.06 | 0.07 | 6.58 | 6.98 |

* Lot No. of purified MT, obtained from Sigma.

sometimes disagreed with the values found by ZAAS and HPLC–AAS. The recoveries for purified MTs from horse kidney and rabbit liver were 90–100%, based on the reference standard horse kidney MT (Sigma, Lot No. 54F-8590). From horse kidney MT (Sigma, Lot No. 46F-9695) only 50% of the injected amount was recovered; apparently this lot was impure.

In Table III are given the molar ratios between cadmium, zinc and copper in 1 mol MT based on a molecular weight of 6500 daltons for MT. One mol MT can bind 5–7 mol zinc and cadmium or both[1,4]. The results for purified MTs from horse kidney, rat liver and fetal bovine liver were in agreement with these values. The lower ratio of mol metal/mol MT in rabbit liver is probably due to the formation of polymers or degradation products of MT during purification[13,14]. The higher amount of metals in rat liver is probably caused by induction of MT via cadmium-exposure experiments to which this rat was subjected.

The two major MT-1 and MT-2 peaks were also integrated separately and quantitated for MT-1, MT-2, cadmium, zinc and copper (Table IV). Although different amounts of MT-1 and MT-2 were found, the cadmium and zinc contents in MT-1 and MT-2 were comparable. MT from rat liver was not separated into MT-1 and MT-2. When methanol was replaced by 1-propanol as the mobile phase B in HPLC–AAS (linear gradient from 0.5 to 7% in 20 min), complete separation of rat liver MT-1 and MT-2 could be accomplished (results not shown). However, the use of 1-propanol considerably decreased the column lifetime. The molar ratios (Table V) of MT-1 and MT-2 agreed with theoretical values[1,4]. Copper could not be measured in the purified forms, because its level was below the detection limit. Fractions can be collected, however, to determine the copper content with graphite-furnace AAS (results not shown).

In conclusion, the method reported allows simultaneous quantitation of MTs and their trace metal composition and requires only 1 g of organ material. The same sample can also be used in Zeeman AAS to determine the total amount of cadmium, zinc and copper. In this way, complex extraction steps are eliminated and consequently the risk of loss of MT and metal is diminished.

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