*Journal of Chromatography*, 572 (1991) 73–84
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6079

# Determination of dietary cadmium-induced metallothioneins in rabbit kidneys and cadmium in metallothioneins by anion-exchange high-performance liquid chromatography coupled with graphite furnace atomic absorption spectrometry

SUN PENG, SHAN XIAO-QUAN*, ZHENG YAN, JIN LONG-ZHU and XU WEI-BING

*Research Center for Eco-Environmental Sciences, Academia Sinica, P.O. Box 934, Beijing 100083 (China)*

(First received May 28th, 1991; revised manuscript received July 12th, 1991)

## ABSTRACT

A rapid method is described for the determination of dietary cadmium-induced metallothioneins (MTs) in rabbit kidneys by anion-exchange high-performance liquid chromatography. Rabbit kidney MT-I and MT-II were eluted at *ca.* 15.0 and 18.8 min, respectively, from a DEAE-5PW anion-exchange column with a Tris–HCl buffer (0.01–0.25 *M*, pH 8.6) and detected by ultraviolet absorbance at 254 nm. A standard calibration curve was constructed using purified standard MT isoforms, which demonstrated an excellent linear correlation between UV absorbance peak heights and the amounts of MT isoforms. Feeding a dose of cadmium for some days resulted in an increase in MT concentrations in rabbit kidneys, but not in the livers. The cadmium concentrations in MT-I and MT-II elutions were determined by graphite furnace atomic absorption spectrometry. MT-I and MT-II showed some differences associated with the oral intake of cadmium. Dietary cadmium also caused zinc to accumulate in kidneys to some extent. The effects of dietary oleic acid on the synthesis of MTs were also studied. Based on the method of standard additions, the recovery of MTs exceeded 93% and replicated injection of samples yielded a relative standard deviation of 2.4% at an MT level of 280 μg/g.

## INTRODUCTION

Metallothioneins (MTs) are proteins of low molecular mass which bind heavy metals and have a high cysteine content (*ca.* 33%) [1]. It is well known that formation of MTs can be induced by some heavy metals, such as cadmium, zinc and mercury [2–4]. Onosaka *et al.* [5] reported that the concentration of MTs in rat livers could also be increased by subcutaneous injection of *n*-heptane, *n*-hexane, oleic acid or linoleic acid. In most tissues MTs have been found to exist in several forms [6]. Because of their physical characteristics they have some special biological functions which play important roles in heavy metal detoxification [7,8] and the metabolism of cadmium, zinc, copper and other metals [9]. The accurate quantitation of MT isoforms and the metals in them is very useful for the study of the biological functions of MTs as well as the toxicity and metabolism of heavy metals.

0378-4347/91/$03.50 

Several methods have been developed to estimate MT concentrations in tissue samples. These include determination of sulphydryl groups of MT by an electrochemical method [10], indirect quantitation based on the metal saturation techniques by radionuclides, such as $^{203}Hg$ [11,12], $^{109}Cd$ [13,14] and $^{110m}Ag$ [15], radioimmunoassay (RIA) [9], determination of MT isoforms using cadmium displacement followed by anion-exchange high-performance liquid chromatography (HPLC) coupled to flame atomic absorption spectrometry (AAS) [16], isolation and quantitation of MT isoforms by reversed-phase HPLC [17] and determination of MTs by inductively coupled plasma atomic emission spectrometry (ICP-AES) of sulphur [18].

Although the RIA method has a higher detection sensitivity than HPLC methods, it is difficult to distinguish various isoforms of MTs by RIA [9]. Currently, HPLC is an important technique for the separation of proteins, and it can separate the distinct isoforms of MTs [19,20]. Indeed, reversed-phase HPLC has been used to isolate MT isoforms easily, but the toxic acetonitrile was used as the mobile phase [17]. Ion-exchange chromatography is very useful for both analytical and preparative purposes because it can provide high resolution without denaturation of MTs [21]. But to date, anion-exchange HPLC has seldom been used as a quantitative method to determine the concentration of MT isoforms in tissues directly based on UV absorbance peak heights.

The purpose of the present study is to use a direct, rapid anion-exchange HPLC method to isolate and quantitate dietary cadmium-induced MT isoforms (MT-I and MT-II) in rabbit kidneys and livers. The relationship between the dietary cadmium and accumulation of zinc or copper in rabbit kidneys and the inducing effect of dietary oleic acid on MTs are also investigated.

## EXPERIMENTAL

### *Chemicals*

2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was purchased from Shanghai Biochemical (Shanghai, China). All chemicals and solvents used in this HPLC system were reagent grade. Water was twice-distilled in quartz.

### *Preparation of MT isoform standard solutions*

Rabbit liver MT isoforms (MT-I and MT-II) were purchased from Sigma (St. Louis, MO, USA). Rabbit kidney MT isoforms were purchased from Life Sciences Center of Peking University (Beijing, China). Kidney and liver MT isoform standard solutions (kidney MT-I 1.071 mg/ml, kidney MT-II 1.066 mg/ml, liver MT-I 1.544 mg/ml, liver MT-II 1.087 mg/ml) were gravimetrically prepared by dissolving certain amounts of MTs in 0.01 *M* Tris–HCl (pH 8.6). These standard solutions were stored at −10°C.

*HPLC apparatus and gradient programme*

A Shimadzu (LC-3A) high-performance liquid chromatograph consisting of two LC-3A pumps, a SIL-1A injector equipped with a 100-$\mu$l sample loop, a GRE-3A gradient programme controller and a C-RIA data module was used throughout. Chromatography was performed on an anion-exchange column (DEAE-5PW, 75 mm × 7.5 mm I.D., Hewlett Packard). Gradient elution was performed using 0.01 *M* Tris–HCl (buffer A) and 0.25 *M* Tris–HCl (buffer B). All the solutions were filtered through a 0.45 $\mu$m membrane. MT-I and MT-II were eluted with linear gradients: buffer A was maintained at 100% for 5 min, then buffer B was increased from 0 to 40% at 6%/min and from 40 to 100% at 8%/min, and finally buffer B was maintained at 100% for 10 min. The flow-rate was 1.0 ml/min. All experiments were carried out under the above operating conditions unless otherwise indicated. MT isoforms were detected at 254 nm with a UVD-2 fixed-wavelength detector. The mobile phase was prepared by dissolving the Tris in the quartz-distilled water and was adjusted to pH 8.6 using ultrapure hydrochloric acid (Beijing Chemical Company, Beijing, China).

*Exposure regime of experimental animals*

Eight New Zeeland male rabbits were used in this experiment. Some of them were fed with a basal diet or this diet plus 30 mg/kg cadmium (as cadmium sulphate) per day for four, seven or fifteen days. One of the rabbits was fed with the basal diet plus 1 ml of oleic acid per day (Beijing Chemical Company) and 30 mg/kg of cadmium per day for four days. Another rabbit was fed with the basal diet plus 1 ml of oleic acid per day and 30 mg/kg of cadmium per day for four days, and the basal diet plus 30 mg/kg of cadmium per day for another three days. After being killed, their kidneys and livers were removed and frozen at − 50°C before analysis.

*Preparation of biological samples for HPLC separation and analysis*

After thawing, 1-g samples of kidneys and livers were homogenized in 4.5 ml of Tris–HCl buffer (0.01 *M*, pH 8.6) at 10 000 rpm for 10 min. The homogenates were heated at 60°C for 5 min and then centrifuged at 17 000 *g* for 1 h. The resulting supernatants (heat-treated cytosols) were filtered through a 0.45-$\mu$m membrane and stored at − 10°C prior to anion-exchange HPLC analysis.

*Quantitation of MT isoforms*

MT quantitation was based on the peak height of UV absorbance at 254 nm. Standard solutions of kidney and liver MT isoforms (MT-I and MT-II) were used to construct calibration curves, following elution as described above. The peak height was plotted against the amount of MT injected into the column, and linear regression analysis was used to determine the relationship. A 30-$\mu$l aliquot of the filtered cytosol sample was injected into the anion-exchange column, and MT-I and MT-II were eluted using the described procedure. Individual MT isoforms

TABLE I

OPERATING PARAMETERS OF GFAAS AND ICP-AES

| GFAAS | | ICP-AES | |
|---|---|---|---|
| Parameter | Value | Parameter | Value |
| Wavelength | 228.8 nm | Incident RF power | 1000 W |
| Slit width | 0.7 nm | Reflected power | < 5 W |
| Lamp current | 5 mA | Viewing height | 12 mm above working coil |
| D2 background corrector | On | Coolant argon flow-rate | 16 l/min |
| *Programme* | | Auxiliary argon flow-rate | 0.5 l/min |
| Dry | 110°C, ramp time 1 s | Carrier argon flow-rate | 1 l/min |
| | hold time 30 s | Sample delivery rate | 1 ml/min |
| Char | 300°C, ramp time 1 s | | |
| | hold time 30 s | | |
| Atomize | 2200°C, max. power heating, | | |
| | hold time 5 s | | |
| Clean | 2650°C, ramp time 1 s | | |
| | hold time 3 s | | |

were quantified using the peak height and extrapolation from the standard curves.

*Determination of cadmium in MT isoforms*

The fractions corresponding to MT-I and MT-II were collected separately according to their retention times detected by UV absorbance. The concentration of cadmium in the MT isoforms was determined by graphite furnace atomic absorption spectrometry (GFAAS) (P-E 3030 atomic absorption spectrometer equipped with P-E HGA-400 graphite furnace and a Hitachi 056 strip-chart recorder). The operating parameters are listed in Table I.

*Determination of cadmium, zinc and copper in animal tissues*

About 0.1 g of kidney and liver tissue was weighed and completely decomposed with 1 ml of ultrapure nitric acid (Beijing Chemical Company) and 0.2 ml of perchloric acid (Beijing Chemical Company) to near dryness. The residue was then redissolved in 10 ml of deionized water with gentle heating. The concentrations of cadmium, zinc and copper were determined by ICP-AES (Jarrell-Ash 1155V inductively coupled plasma atomic emission spectrometer equipped with PDP11/23 computer), under the conditions listed in Table I.

## RESULTS

Chromatograms obtained from [illegible] rabbit kidney and liver by the anion-

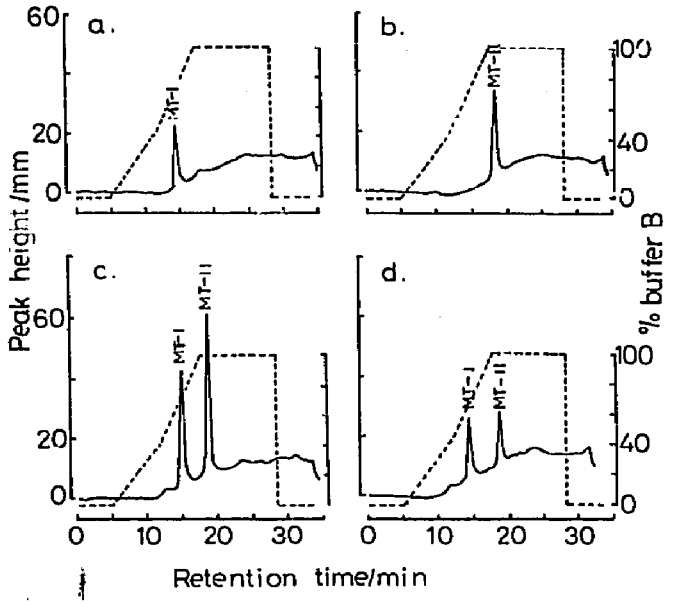


Fig. 1. Chromatograms of standard MTs: (a) 2.5 μl of rabbit kidney MT-I; (b) 2.5 μl of rabbit kidney MT-II; (c) 5 μl of rabbit kidney MT-I and 5 μl of rabbit kidney MT-II; (d) 3 μl of rabbit liver MT-I and 3 μl of rabbit liver MT-II. The separation of the MT isoforms was performed with a DEAE-5PW anion-exchange column (7.5 cm × 7.5 mm I.D.) and a two-step gradient: 0 to 40% B (6%/min) and 40 to 100% B (8%/min) in 15 min. Mobile phases: A, 0.01 *M* Tris–HCl (pH 8.6); B, 0.25 *M* Tris–HCl (pH 8.6).

exchange HPLC method are shown in Fig. 1. Kidney MT-I eluted at 15.0 min and kidney MT-II at 18.8 min (0.17 and 0.24 *M* Tris–HCl, respectively). Liver MT-I and MT-II eluted at 14.9 and 18.8 min, respectively (0.17 and 0.24 *M* Tris–HCl).

MT-I and MT-II isoforms in the controlled rabbit (fed only with a basal diet) kidney cytosols cannot be detected by this HPLC method (Fig. 2).

Fig. 3. shows a chromatogram of kidney cytosol from a rabbit fed with dietary cadmium (30 mg/kg per day for fifteen days). The peaks at 15.03 and 18.71 min

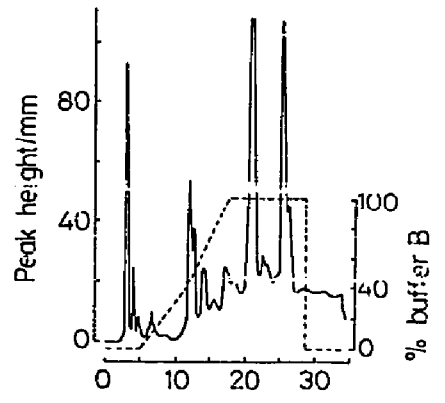


Fig. 2. Chromatogram of 50 μl


Fig. 3. Chromatogram of 30 μl of heat-treated kidney cytosol from a dietary cadmium-treated rabbit. The individual MT isoform peaks are designated as MT-I and MT-II.

Fig. 4. Chromatogram of 30 μl of heat-treated kidney cytosol from a dietary cadmium treated rabbit (same sample as that in Fig. 3), plus 3 μl of standard kidney MT-I and 3 μl of standard kidney MT-II solution.

were identified as MT-I and MT-II, respectively. Fig. 4. depicts a chromatogram of the same sample as in Fig. 3, but with 3 μl of standard kidney MT-I and 3 μl of standard kidney MT-II added. As can be seen, MT-I and MT-II in the kidney cytosol of the rabbit treated with dietary cadmium eluted at the same time as the purified standard kidney MT-I and MT-II. This indicates that the peaks at 15.03 and 18.71 min in Fig. 3 are the peaks of kidney MT-I and MT-II.

Chromatograms of liver cytosols of a controlled rabbit and a rabbit treated with dietary cadmium (30 mg/kg per day for fifteen days) are shown in Figs. 5a and b. There were no MT peaks in either case. Fig. 5c shows a chromatogram of liver cytosol of a dietary cadmium treated rabbit, with 3 μl of standard liver MT-I and 3 μl of standard liver MT-II added. From Fig. 1d and Fig. 5a–c, it is clear

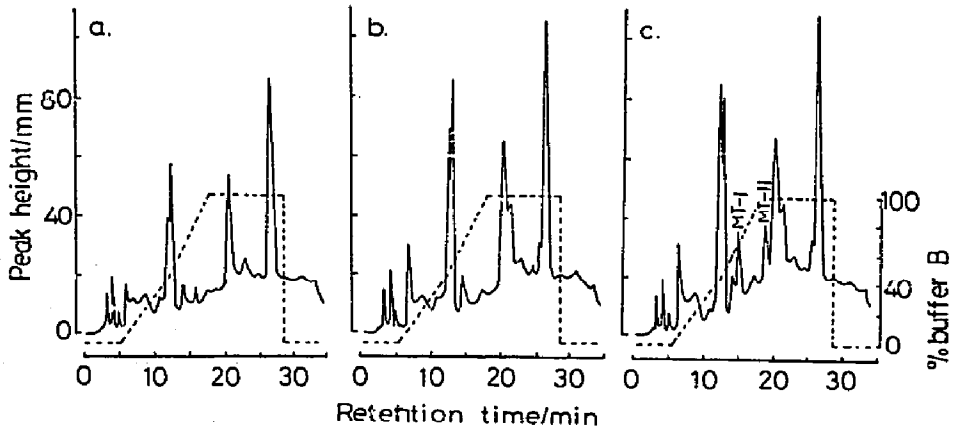


Fig. 5. Chromatograms of heat-treated liver cytosols: (a) 30 μl of control rabbit liver cytosol; (b) 30 μl of dietary cadmium-treated rabbit liver cytosol; (c) 30 μl of dietary cadmium-treated rabbit liver cytosol plus 3 μl of standard liver MT-I and 3 μl of standard liver MT-II solution.

TABLE II

RECOVERY OF THE PROPOSED HPLC METHOD FOR QUANTITATING MT ISOFORMS

| Compound | MTs in cytosol sample (μg) | MTs standard (μg) | Total MTs found (μg) | Recovery (%) |
|---|---|---|---|---|
| Kidney MT-I | 2.9 | 3.21 | 6.25 | 104 |
| Kidney MT-II | 3.3 | 3.20 | 6.35 | 95 |
| Liver MT-I | N.D.[a] | 4.63 | 4.30 | 93 |
| Liver MT-II | N.D. | 3.26 | 3.15 | 97 |

[a] N.D. = not detected.

that no MTs were detected in liver cytosol from the control rabbit or the rabbit treated with dietary cadmium.

There is an excellent linear relationship between the amount of MTs and the UV absorbance peak height. The standard calibration curves constructed using purified standard MT isoforms were used as the typical standard curves to quantitate MT-I and MT-II in animal tissues.

Based on Figs. 1, 3, 4 and 5, the recoveries of kidney and liver MTs were calculated by the method of standard additions (Table II). A satisfactory recovery of 93–104% was achieved.

The detection limit of this method was 10 μg MT per g kidney, and the relative standard deviation (R.S.D.) was 2.4% (six injections of the same sample).

Concentrations of MT isoforms determined by this HPLC method are listed in Table III. According to the data, we obtained the following results: (1) concentra-

TABLE III

CONCENTRATIONS OF METALLOTHIONEINS IN KIDNEYS DETERMINED BY HPLC

| Sample | MT-I (μg/g) | MT-II (μg/g) | Total MT (μg/g) | Dietary levels of cadmium and oleic acid |
|---|---|---|---|---|
| 1 | N.D.[a] | N.D. | | Control |
| 2 | N.D. | N.D. | | Control |
| 3 | 80.8 | 26.9 | 107.7 | 30 mg/kg Cd per day for four days |
| 4 | 138.7 | 38.5 | 177.2 | 30 mg/kg Cd + 1 ml oleic acid per day for four days |
| 5 | 118.8 | 74.2 | 190.0 | 30 mg/kg per day for seven days |
| 6 | 164.1 | 89.5 | 253.6 | 30 mg/kg Cd + 1 ml oleic acid per day for four days and 30 mg/kg Cd per day for another three days |
| 7 | 280.0 | 325.0 | 605.0 | 30 mg/kg Cd per day for fifteen days |
| 8 | 210.0 | 305.0 | 515.0 | 30 mg/kg Cd per day for fifteen days |

[a] N.D. = not detected.

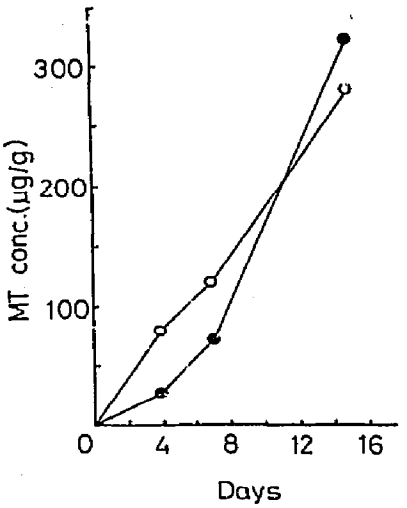


Fig. 6. Relationship between the dietary number of days with cadmium treatment (30 mg/kg per day) and the MT isoform concentration in rabbit kidney (μg/g). (○) MT-I; (●) MT-II.

tions of MT isoforms in rabbit kidneys increased with increasing length of treatment with basal diet plus 30 mg/kg cadmium per day (Fig. 6); (2) concentrations of MT-I and MT-II increased in the kidney of the experimental rabbit at different rates with the same increasing dietary level of cadmium in the kidney of an

TABLE IV

CADMIUM CONCENTRATION IN KIDNEY METALLOTHIONEINS AND TOTAL CADMIUM IN KIDNEYS OF NEW ZEALAND RABBITS

| Sample | Cd in MT-I (μg/μg) | Cd in MT-II (μg/μg) | Total Cd in tissues (μg/g) | MT-Cd/total Cd (%) | Dietary levels of cadmium and and oleic acid |
|---|---|---|---|---|---|
| 1 | N.D.[a] | N.D. | 1.1 | | Control |
| 2 | N.D. | N.D. | 0.77 | | Control |
| 3 | 0.012 | 0.026 | 98.9 | 1.7 | 30 mg/kg Cd per day for four days |
| 4 | 0.015 | 0.024 | 38.1 | 7.9 | 30 mg/kg Cd + 1 ml oleic acid per day for four days |
| 5 | 0.010 | 0.024 | 118.5 | 2.5 | 30 mg/kg Cd per day for seven days |
| 6 | 0.010 | 0.022 | 80.9 | 4.5 | 30 mg/kg Cd + 1 ml oleic acid per day for four days and 30 mg/kg Cd per day for another three days |
| 7 | 0.033 | 0.020 | 137.1 | 11.5 | 30 mg/kg Cd per day for fifteen days |
| 8 | 0.030 | 0.019 | 131.2 | 9.6 | 30 mg/kg Cd per day for fifteen days |

[a] N.D. = not detected.

experimental rabbit (Fig. 6); (3) when rabbits were fed with the same dietary level of cadmium plus an additional dose of oleic acid, a higher content of MTs was found in the kidneys than in rabbits not given oleic acid.

We also found that when rabbits were fed with the basal diet plus 30 mg/kg cadmium per day for less than fifteen days, the formation of MT-I and MT-II in the kidneys was marked, whereas the concentration of MTs in the liver did not increase and was too low to be detected by this method.

The concentrations of cadmium in kidney MT isoform fractions were determined by GFAAS without further treatment. The results are shown in Table IV. The concentration of cadmium in MT-II remained at *ca.* 0.02 $\mu g/\mu g$ during the period of our experiment. The cadmium concentration in kidney MT-I remained at low levels until the seventh day. At the end of the experimental period (fifteen days) the cadmium content in MT-I reached *ca.* 0.03 $\mu g/\mu g$. The ratio of cadmium in MTs to total cadmium in the kidney increased as the content of MTs in the kidney increased.

## DISCUSSION

It is well known that cadmium, zinc, mercury and silver stimulate the synthesis of MTs in animal tissues. A lot of work has been done to investigate the synthesis of MTs in animal tissues after injection of these metals. But to date, very few reports have studied the effects of dietary heavy metals on MTs in animal tissues. Whanger and Deagen [23] studied the effects of dietary mercury on rat tissue MTs. They found that a low dietary level of mercury resulted in its deposition with MTs in the kidney, but not in the liver. Dietary mercury markedly stimulates the uptake of mercury in renal MTs, and increased the zinc content in MTs. Mercury was readily accumulated in the kidney and to a lesser extent in the liver in proportion to induced MT levels. In our studies, we found that dietary cadmium markedly stimulated the synthesis of MTs in rabbit kidneys, but hepatic MTs were not detected by our HPLC method. This means that MT isoforms were first induced and mainly existed in kidneys when rabbits were fed with a dose of cadmium (30 mg/kg per day) for less than fifteen days. This result was different from the effect of cadmium injection. The kidney is a critical organ for dietary cadmium [22]. Many reports suggest that the nephrotoxic effect of cadmium is in fact mediated through MTs [23]. We consider that MTs are constantly resynthesized in the kidney cells in order to combat the toxicity of cadmium. Renal MTs play an important role in binding environmetal cadmium that enters into rabbit bodies through the diet.

In Nomiyama and Nomiyama's experiment [22], two kinds of MT (MT-I and MT-II) appeared in the rabbit renal cortex on the third day after subcutaneous injection of cadmium (0.5 mg/kg per day). Hepatic MTs were also determined on the third day. In this experiment, MT-I and MT-II were detected in the kidney on the fourth day of feeding with a dose of cadmium (30 mg/kg per day), but hepatic

TABLE V

CONCENTRATIONS OF CADMIUM, ZINC AND COPPER IN RABBIT KIDNEYS

| Sample | Concentration of metal (μg/g) | | | Dietary levels of cadmium and oleic acid |
|---|---|---|---|---|
| | Cadmium | Zinc | Copper | |
| 1 | 0.77 | 14.42 | 3.13 | Control |
| 2 | 98.9 | 15.43 | 2.64 | 30 mg/kg Cd per day for four days |
| 3 | 38.1 | 26.74 | 1.88 | 30 mg/kg Cd + 1 ml oleic acid per day for four days |
| 4 | 118.5 | 20.55 | 4.41 | 30 mg/kg Cd per day for seven days |
| 5 | 80.9 | 27.58 | 3.21 | 30 mg/kg Cd + 1 ml oleic acid per day for four days and 30 mg/kg Cd per day for another three days |
| 6 | 147.7 | 61.48 | 6.16 | 30 mg/kg Cd per day for fifteen days |
| 7 | 131.2 | 37.69 | 3.46 | 30 mg/kg Cd per day for fifteen days |

MTs were not detected. The possible reason for this difference is that dietary cadmium has a different effect on MT production and different metabolic mechanism in animal bodies from injected cadmium. Furthermore, this study shows that MT-I and MT-II have a distinct difference in synthesis rate with an increasing dietary level of cadmium and in the associated relationship with cadmium (Fig. 6 and Table III). This may be because MT-I and MT-II isoforms differ in their isoelectric point, overall net negative charge [24,25] and amino acid composition [6].

It has been reported that cadmium enhances the tissue levels of cadmium as well as those of zinc and copper. MTs induced by cadmium might cause an accumulation of zinc and copper in them and retain zinc and copper in tissues as well [22,26]. Koizumi *et al.* [27] investigated the correlations between cadmium and zinc, and between cadmium and copper, in horse tissues. They found that strong correlations existed between cadmium and MTs, between cadmium and zinc, and between cadmium and copper in the renal cortex. But in the liver, the MT concentration was not correlated with the cadmium concentration unless the exposure was to a large amount of cadmium.

We found that, under our experimental conditions, the concentration of MTs in rabbit livers did not increase markedly with the dose of dietary cadmium, but increased in the kidneys with the dose of dietary cadmium. Cadmium in the kidneys reached a level of *ca.* 150 μg/g on the fifteenth day of feeding 30 mg/kg per day (Table V). This was lower than the critical concentration of cadmium in the kidney, which was considered to be *ca.* 200 μg/g [28]. When the rabbits were fed with a dose of cadmium, the zinc concentration in the kidneys also increased compared with that in the basal diet-fed rabbits (Table V). The concentration of copper in the kidneys did not increase noticeably in our experiment. It was postulated that MT concentrations increased with increasing accumulation of dietary

cadmium in the renal cortex, and this resulted in an increase in zinc, which binds to MTs.

The content of MTs in rat tissues were increased by the injection of *n*-heptane, *n*-hexane, oleic acid and linoleic acid. Acute cadmium toxicity can be prevented by preinjection of *n*-heptane [5]. In the present investigation, we studied the inducing effects of dietary oleic acid on MTs in rabbit kidneys. The results indicated that the concentration of MTs in kidneys increased after the rabbits were treated orally with 1 ml of oleic acid for four days. This means that oleic acid has an inducing effect on the synthesis of MTs in animal tissues. Sato and Nagai [29] reported that when cadmium accumulated in renal cells binds to thionein of MTs, renal damage dose not occur. However, renal damage may occur if the concentration of cadmium not bound to MT increases. It can be conjectured that a suitable dose of oleic acid or other non-toxic reagents could be used to induce MTs to eliminate the excess of free cadmium, thus protecting the organism from the toxic effects of cadmium. Further research is required, to investigate this hypothesis.

The anion-exchange HPLC method described was time-saving and easy to use for the isolation and quantitation of proteins. It had the following advantages: (1) the amount of sample required is considerably less than that required for classical chromatographic techniques; (2) the MT-I and MT-II isoforms in heat-treated rabbit kidney cytosols were separated and quantitated directly from the UV absorbance peak heights, without further sample preparation such as application to a Sephadex G-75 gel-permeation column [30] or column-switching manipulation [31]; (3) although anion-exchange HPLC does not achieve the level of detection sensitivity of a typical RIA method, the ability to discriminate between individual isoforms of MTs makes it a useful tool to study the relative rates of synthesis and degradation of MT isoforms; (4) using GFAAS as the method of determination of the concentrations of heavy metals in the elution of MT isoforms can improve the sensitivity for some elements compared with the flame AAS method: MTs were eluted as distinct isoforms by elution with a concentration gradient of Tris–HCl buffer at pH 8.6. Elution with buffers of more basic pH requires higher salt concentration. However, more concentrated buffers easily plug the burner-head of the spectrophotometer when the HPLC system is directly connected to flame AAS system [32]. The GFAAS method can avoid this drawback.

In conclusion, the rapid anion-exchange HPLC–GFAAS method utilized in the present investigation proved to be useful for separating and quantitating MT-I and MT-II induced by heavy metals or other organic reagents. Moreover, this technique is useful for the study of the synthesis, metabolism and biological functions of MTs, and the association of heavy metals with MTs.

ACKNOWLEDGEMENT

This study was supported by National Natural Science Foundation of China.

REFERENCES


1 M. M. Kissing and J. H. R. Kagi, in J. H. R. Kagi and M. Nordberg (Editors), *Metallothionein*, Birkhauser Verlag, Basel, 1979, p. 147.
2 P. D. Whanger and J. T. Deagen, *Environ. Res.*, 30 (1983) 372.
3 S. Onosaka and M. G. Cherian, *Toxicology*, 22 (1981) 91.
4 S. Onosaka and M. G. Cherian, *Toxicology*, 23 (1982) 11.
5 S. Onosaka, Y. Ochi, K. S. Min, Y. Fujita and K. Tanaka, *Eisei Kogaku*, 34(5) (1988) 440 (Japan).
6 M. Nordberg and Y. Kojima, in J. H. R. Kagi and M. Nordberg (Editors), *Metallothionein*, Birkhauser Verlag, Basel, 1979, p. 41.
7 M. Webb and K. Cain, *Biochem. Pharmacol.*, 31 (1982) 137.
8 G. F. Nordberg, *Environ. Physiol. Biochem.*, 1 (1971) 171.
9 R. K. Mehra and I. Bremner, *Biochem. J.*, 213 (1983) 459.
10 R. W. Olafson and R. G. Sim, *Anal. Biochem.*, 100 (1979) 343.
11 J. K. Piotrowski, W. Bolanoskwa and A. Sapota, *Acta Biochim. Pol.*, 20 (1973) 207.
12 A. J. Zelazowski and J. K. Piotrowski, *Acta Biochim. Pol.*, 24 (1977) 97.
13 D. Klein, R. Bartsch and K. H. Summer, *Anal. Biochem.*, 189 (1990) 35.
14 D. L. Eaton and B. F. Toal, *Toxicol. Appl. Pharmacol.*, 66 (1982) 134.
15 A. M. Scheuhammer and M. G. Cherian, *Toxicol. Appl. Pharmacol.*, 82 (1986) 417.
16 M. S. Krug and S. L. Berger, *Anal. Biochem.*, 153 (1986) 315.
17 M. P. Richards and N. C. Steele, *J. Chromatogr.*, 402 (1987) 243.
18 J. Bonger, C. D. Walton and D. E. Richardson, *Anal. Chem.*, 60 (1988) 2033.
19 K. T. Suzuki, *Anal. Biochem.*, 102 (1980) 31.
20 S. Klauser, J. H. R. Kagi and K. J. Wilson, *Biochem. J.*, 209 (1983) 71.
21 Y. Kato, K. Nakamura, Y. Yamazaki and T. Hashimoto, *J. Chromatogr.*, 318 (1985) 358.
22 K. Nomiyama and H. Nomiyama, in E. C. Foulkes (Editor), *Biological Roles of Metallothionein*, Elsevier North Holland, New York, 1982, p. 47.
23 Z. A. Shaikh, in E. C. Foulkes (Editor), *Biological Roles of Metallothionein*, Elsevier North Holland, New York, 1982, p. 69.
24 G. F. Nordberg, M. Nordberg, M. Piscator and O. Vesterberg, *Biochem. J.*, 126 (1972) 491.
25 M. G. Cherian, *Biochem. Biophys. Res. Commun.*, 61 (1974) 920.
26 J. T. Deagen, S. H. Oh and P. D. Whanger, *Biol. Trace Elem. Res.*, 2 (1980) 65.
27 N. Koizumi, Y. Inoue, R. Yinomiya, D. Fujita and T. Tsukamoto, *Environ. Res.*, 49 (1989) 104.
28 K. Nomiyama, *J. Toxicol. Environ. Health*, 3 (1977) 607.
29 M. Sato and Y. Nagai, in E. C. Foulkes (Editor), *Biological Roles of Metallothionein*, Elsevier North Holland, New York, 1982, p. 164.
30 J. H. R. Kagi, M. Vasak, K. Lerch, D. E. O. Gilg, P. Hunziker, W. R. Bernhard and M. Good, *Environ. Health Perspect.*, 54 (1984) 93.
31 K. T. Suzuki, H. Sunaga and T. Yagima, *J. Chromatogr.*, 303 (1984) 131.
32 K. T. Suzuki, H. Sunaga, Y. Aoki and M. Yamamura, *J. Chromatogr.*, 281 (1983) 159.