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Quantitation of metallothionein and cadmium in metallothionein in mink livers by anion-exchange HPLC-GFAAS method

Shan Xiaoquan, Sun Peng, Zheng Yan,
Jin Longzhu and Xu Weibing

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P. O. Box 2871, Beijing 100085, China

Abstract—Metallothionein (MT) has a great capacity of binding heavy metals showing an interesting connection with metal toxicology, as a biochemical marker for environmental metal pollution. Anino-exchange high per formance liquid chromatography (HPLC) was used to isolate and quantitate MT in livers of minks which were contaminated with heavy metals. MT isoforms (MT-I and MT-II) were eluted at approximately 11.3 and 14.3 min respectively from a DEAE-5 PW anion-exchange column with a Tris-HCl buffer (0.01–0.25 mol/L, pH 8.6) and detected by UV absorbance at 254 nm. The cadmium concentrations in mink liver MT elutions were determined by graphite furnace atomic absorption spectrometry (GFAAS). Obvious increase in liver MT-I concentration rather than liver MT-II was found when the minks were contaminated by feeding contaminated fish captured from the heavy metal-polluted river. The cadmium concentration in mink liver MT-I also increased to some extent as the contaminated level increased.

Keywords: metallothionein; cadmium; anion-exchange HPLC; GFAAS; heavy metal contaminated minks.

INTRODUCTION

Cadmium ranks 62nd in Clarke and its concentration in the earth's crust is extremely low (0.2μg/g; Koizumi, 1989). Nevertheless, this trace element is a very toxic element that has been shown to affect a number of tissues including the liver, kidney, testes, pancreas, bone and nervous system (Klaassen, 1982). Cadmium is widely used in plating certain cooking and baking utensils as well as in the manufacturing of batteries and electronic components. It is well known that cadmium is implicated with many pathological conditions such as persistent hypertension, testicular necrosis, renal diease, liver dysfunction and "Itai-Itai" disease (Kundomal, 1982).

The environmental exposure to cadmium is mainly through the dict. Once absorbed, the cadmium is taken up primarily by livers and kidneys (Shaikh, 1982) and positively accumulated in livers and kidneys (Koizumi, 1989) where it is largely bound to metallothionein (MT). MT is low-molecular weight, heavy metal binding and high cysteine content (approximately 33%) protein (Kissing, 1979) that can be induced by some heavy metals such as Cd and Zn (Whanger, 1983; Ohasaka, 1981). It is well known that MT has some special biological

functions which play important role in heavy metal detoxification (Webb, 1982; Leber, 1976) and metabolism of cadmium, zinc, copper and other metals (Mehra, 1983).

The induction of MT in organisms is often presumed to occur as a result to exposure to environments contaminated with heavy metals. Consequently, such proteins are regarded as potentially specific indicators of heavy metal pollution. It is essential, however, to evaluate their involvement in metabolism, storage and regulation of metals and preferable to quantify levels of MT before the value of these indices can be fully exploited (Langston, 1989). Although many methods have been used to quantitate MT in animal tissues, rapid, specific and sensitive quantitation of MT is difficult. Most methods in use are indirect and complicated in performance. The purpose of the present study is to isolate and quantitate MTs in livers of normal and heavy metal contaminated minks, and determine the cadmium in MTs by a direct and rapid method of anion-exchange HPLC coupled with GFAAS.

MATERIALS AND METHODS

HPLC apparatus

A Shimadzu (LC-3A) high performance liquid chromatograph consisted of two LC-3A pumps, and SIL-1A injector equipped with a 100-μl sample loop, a GRE-3A gradient program controller and a C-RIA data module was used throughout. MT isoforms were isolated by a modification of the method previously described (Lehman, 1986). Chromatography was performed on an anion-exchange column (DEAE-5PW, 7.5 cm × 7.7 mm, HEWLETT PACKARD). The mobile phase consisted of Tris-HCl in concentration of buffer A of 0.01 mol/L and buffer B of 0.25 mol/L (pH 8.6 at room temperature) which was filtered through a 0.45 μm membrane. MT-I and MT-II were eluted with a linear gradient: buffer A retained 100% for 5 minutes, buffer B from 0 to 100% (6%/min) in 16 minutes and then buffer B retained 100% for 10 minutes. The flow rate of mobile phase was 1 ml/min. Proteins were detected at 254 nm with UVD-2 fixed wavelength monitor. All experiments were carried out under the above operating conditions unless otherwise indicated. The mobile phase was prepared by dissolving 2-amino-2 (hydroxymethyl)-1, 3-propanediol (Tris, Shanghai Biochemical Company, Shanghai, China) in quartz twice-distilled water and was adjusted to pH 8.6 using ultrapure hydrochloric acid (Beijin Chemical Company, China).

Preparation of biological samples for HPLC separation and analysis

Mink livers were obtained from Institute of Zoology, Chinese Academy of Sciences (Beijing, China). These minks were divided into three groups. Group one: uncontaminated minks which were fed only with normal fish; group two: half contaminated minks which were fed with half amount of normal fish and half amount of contaminated fish captured from heavy metal polluted water; group three: full contaminated minks which were fed only with contaminated fish. One-g of liver was homogenized in 4.5 ml of Tris-HCl buffer (0.01 mol/L, pH 8.6) at 10000 r/min for 10 minutes. The homogenates were centrifuged at 10000 r/min for 1 hour. The resulting

supernatant was filtered through 0.45 μm membrane and stored at −10°C prior to anion-exchange HPLC analysis.

Quantitation of MT in liver cytosols

Rabbit liver MT isoforms (MT-I and MT-II) were purchased from Life Science Center of Peking University (Beijing, China). Liver MT isoform standard solutions (MT-I 1.544 mg/ml, MT-II 1.087 mg/ml) were prepared by dissolving certain amounts of MTs in 0.01 mol/L Tris-HCl (pH 8.6). MT quantitation was based on peak height of UV absorbance at 254 nm. The liver MT isoform (MT-I and MT-II) standard solutions were used to construct the standard calibration curves. MT standard solution was eluted using the gradient program mentioned above. The peak height was plotted against μg of MT injected onto the column and linear regression analysis was used to determine the relationship. An aliquot of 30 μl of the filtered cytosol sample was injected onto the anion-exchange column, MT was eluted using the above described procedure. Individual MT isoforms were quantitated using peak height and extrapolation from standard curves.

Determination of cadmium in MT

The eluate fractions of MTs were collected. The concentration of Cd in MTs was determined by graphite furnace AAS (P-E 3030 atomic absorption spectrometer equipped with P-E HGA-400 graphite furnace and a Hitachi 056 strip chart recorder).

Operating parameters and programs of anion-exchange HPLC and GFAAS are listed in Table 1.

Table 1 Programs of anion-exchange HPLC and operating parameters of GFAAS

Anion-exchange HPLC		GFAAS	
Column	DEAE-5PW	Wavelength	228.8 nm
Mobile phase	A: 0.01 mol/L Tris-HCl pH at 8.6	Slit width	0.7 nm
		Lamp current	5 mA
	B: 0.25 mol/L Tris-HCl PH at 8.6	D2 background	
Flow rate	1 ml/min	Corrector	on
Wavelength of UV absorbance 254 nm		Program:	
Gradient program:		Dry	110°C, ramp time 1 s hold time 30 s
Buffer A retain 100% for 5 min		Char	300°C, ramp time 1 s hold time 30 s
Buffer B from 0 to 100% at 6% /min		Atomize	2200°C, max. power heating hold time 5 s
Buffer B retain 100% for 10 min		Clean	2650°C, ramp time 1 s hold time 3 s

RESULTS AND DISCUSSION

Currently, high performance liquid chromatography is an important technique to separate proteins. Ion-exchange chromatography seems very useful for both analytical and preparative purpose because it can provide high resolution without denaturation of proteins (Kato, 1985). Some investigators have employed anion-exchange HPLC to separate MT isoforms (Lehman, 1986). Chromatographic properties of purified rabbit liver MT-I and MT-II obtained form our anion-exchange HPLC method mentioned above are shown in Fig. 1 and Fig. 2. Liver MT-I was eluted at approximately 11.3 min and liver MT-II was eluted at 14.3 min (with 0.10 mol/L and 0.14 mol/L Tris-HCl, respectively).

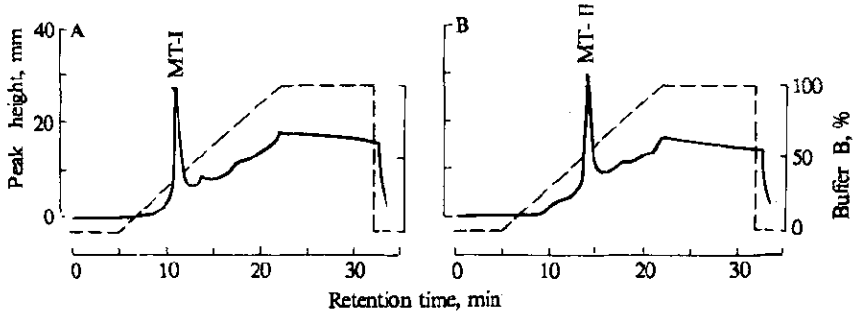

Fig. 1 Chromatograms of standard MTs: (a) 1.0μl of rabbit liver MT-I (b) 1.0 μl of rabbit liver MT-II
The separation of MT isoforms was performed with a DEAE-5PW anion-exchange column (7.5 cm × 7.5 mm) and a linear gradient: 0 to 100% buffer B (6%/ min) in 17 minutes
Mobile phase A was 0.01 mol/L Tris-HCl at pH 8.6, B was 0.25 mol/L Tris-HCl at pH 8.6

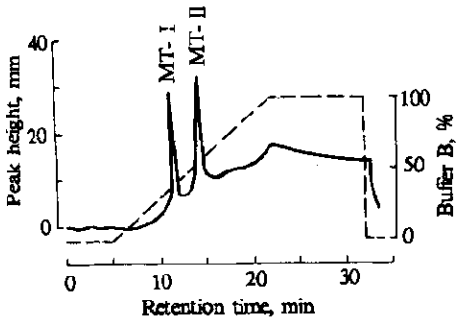

Fig. 2 Chromatogram of 1.0 μl standard MT-I and standard MT-II

In Fig. 3 the chromatograms of liver cytosols from normal mink and heavy metal contaminated mink are shown. There were no MT peaks in Fig. 3a. This means that no MTs were detected in liver cytosol of normal mink which was fed with normal fish. While in Fig. 3b there was a peak of MT-I at 11.34 min and no MT-II was detected.

Fig. 4 demonstrates the linear relationship between weight in μg of MTs and the UV-absorbance peak height. They were used as the typical standard curves in quantitating MTs in mink livers. From these curves it should be

noticed that there was an excellent linear correlation between the UV-absorbance peak height and the quantity of MT injected onto the column.

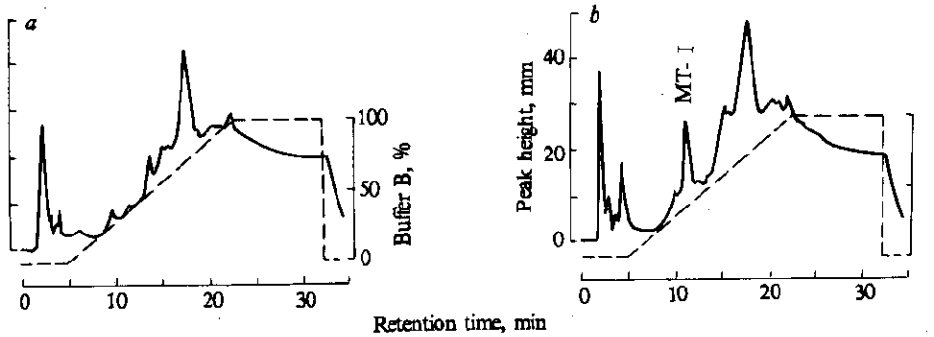

Fig. 3 Chromatograms of mink liver cytosols: (a) 30 μl of normal mink liver cytosol (b) 30 μl of fully contaminated mink liver cytosol

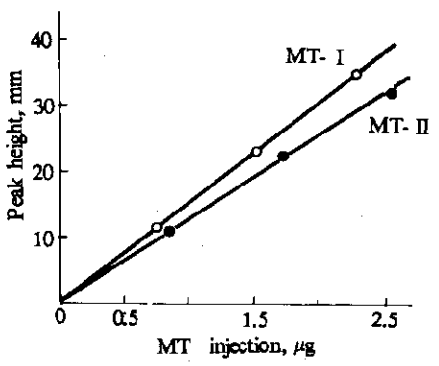

Fig. 4 Calibration curves for MTs determination by anion-exchange HPLC as described for Fig. 1

Concentrations of MTs determined by this HPLC method are listed in Table 2. According to the data we obtained, it is evident that : 1. Concentration of MT-I in mink livers increased with the amount of the heavy metal contaminated fish that the minks were fed with. The more serious the minks were contaminated with heavy metals, the more MT-I was induced in mink livers. 2. No MT-II was detected in normal mink liver cytosol and in heavy metal contaminated mink liver cytosol by our HPLC method. This means that MT-II was not markedly induced in mink livers when minks were contaminated by feeding with heavy metal contaminated fish. The possible reason leading to this difference in synthesis rate of MT-I and MT-II may be that MT-I and MT-II isoforms differ in their isoelectric point, overall net negative charge (Nordberg, 1972; Cherian, 1974) and amino acid sequences (Kagi, 1984).

It is well known that MT synthesis can be highly induced by heavy metals. On the other hand, the induced MTs caused an accumulation of Cd, Zn and Cu in them and retain these elements in tissues as well (Nomiyama, 1982; Deagen, 1980) because MTs contain numerous cysteinyl thiol groups which are responsible for their high-affinity to some heavy metals. The result

Table 2 Concentration of MTs in mink livers determined by anion-exchange HPLC

Sample No.	MTs in liver MT-I, μg/g	MT-II, μg/g	Heavy metal contaminated level
1	ND	ND	Normal
2	ND	ND	Normal
3	93	ND	Half contaminated
4	81	ND	Half contaminated
5	107	ND	Fully contaminated
6	103	ND	Fully contaminated
7	106	ND	Fully contaminated

ND: Not detected

Normal: The minks were fed only normal fish

Half contaminated: The minks were fed half amount of normal fish and half amount of contaminated fish captured from heavy metal polluted water

Fully contaminated: The minks were fed only contaminated fish captured from heavy metal polluted water

of cadmium content in liver MT-I is listed in Table 3. The liver MT-I of heavy metal contaminated mink contained more cadmium (about 1.6 ng/μg) than that of half contaminated mink (about 1.3 ng/μg). This result was an evidence to support the hypothesis that MT played an important role in cadmium detoxification (Leber, 1976). It is believed that MT accomplish the detoxific function by providing high-affinity capacity for cadmium, which is necessary to reduce the excess of free cadmium (Sato, 1982) and to prevent interactions of cadmium with target molecules (Matsubara, 1987). It can be considered that when minks were contaminated with heavy metals, MT synthesis was induced in order to prevent the toxicity of heavy metals such as cadmium.

Table 3 Cadmium concentration in liver MT-I

Sample No.	Cd in MT-I, ng/μg *	Contaminated level
1	ND	Normal
2	ND	Normal
3	1.31	Half contaminated
4	1.25	Half contaminated
5	1.68	Fully contaminated
6	1.55	Fully contaminated
7	1.56	Fully contaminated

* Average values of 2 replicated determinations; ND = not detected

In conclusion, the rapid anion-exchange HPLC-GFAAS method allows simultaneous quantitation of MTs and the trace metals in MTs. Using this method we proved that MT concentration in mink liver cytosols increased when minks were contaminated with heavy metals and the cadmium content in liver MT-I increased with increasing contamination level. This method is also useful to evaluate the heavy metal pollution level of water by determination of MTs and the heavy metal content in MTs in tissues of the animals from the polluted water.

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