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Investigation of mercury metallothionein complexes in tissues of rat after oral intake of $HgCl_2$

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Mercury, a highly toxic metal found widely throughout the environment, is a potent inducer of metallothionein (MT) expression. The role of MTs in the detoxification of mercury after its oral intake in mammals is studied. After feeding rats with mercuric chloride by gastric gavage, the distribution of heavy metals in rat tissues was investigated by inductively coupled plasma mass spectrometry (ICP-MS). Extensive accumulation of mercury, copper and zinc in kidney and liver is observed. A homemade preparative size-exclusion chromatography (SEC) column (30 cm × 1.9 cm) packed with Sephadex G-75 (40–120 μm particle size) gel (Pharmacia) was used for the purification of MT fractions in rat tissues. Preliminary results from SEC indicate that the mercury-binding MT levels in liver were much lower than in kidney. The MT fractions were collected, desalted, and then separated by reversed-phase high-performance liquid chromatography (HPLC) with UV–Vis spectrometry, ICP-MS and electrospray ionization MS detection. One major and several minor peaks were observed in the HPLC chromatograms of the MT fraction for the kidney sample. UV absorption spectra indicate that MTs were found to bind with mercury. There were no significant mercury-binding MTs detected in the liver sample using UV detection. ICP-MS detection showed that mercury-binding MTs in kidney contained large amounts of mercury and copper but little zinc. Further characterization with ESI-MS showed that the major peak found in kidney contained Hg_6Cu, Hg_5Cu_2-MT-2c and Hg_6-MT-2β, Hg_6Cu-MT-1γ, Hg_7-MT-2α. However, distinction between copper and zinc could not be made based on current mass spectrometric analysis because of instrumental resolution limitations.

KEYWORDS: rat; metallothionein (MT); mercury; separation; UV; ICP-MS; ESI-MS

INTRODUCTION

Metallothionein (MT) is a cysteine-rich, low-molecular-weight intracellular protein with a high affinity for metals such as zinc, copper, cadmium and mercury. It was first isolated 40 years ago from equine renal cortex, and was shown structurally to contain 61 amino acids, of which 20 were cysteine residues.[1] Since then, similar proteins have been found and studied in both prokaryotes and eukaryotes.[2,3] The role of MT has been investigated extensively in the homeostasis regulation of essential metals, including zinc and copper, in the detoxification of potentially toxic heavy metals such as cadmium and mercury, and in the protection against various oxidative stress conditions.[4–6]

Inorganic mercury species existing in the environment are well-established toxicants to human health.[7,8] Severe kidney damage has been found after acute or chronic exposure to these species.[9–11] The kidney damage caused by inorganic

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mercury could be prevented by pre-induction of renal MT, because intracellular mercury in the kidney is firmly trapped by the MT.[12,13] The magnitude of MT synthesis may vary with the route of exposure or administration of metals, and the extent of their accumulation in a particular organ. As oral intake is the most important route for humans, it is quite relevant, therefore, to study the absorption and disposal of an agent after oral intake. The gastro-intestinal absorption following an oral administration of mercury was reported to be poor and the exact mechanism of detoxification of mercury in various organs remains unclear.

This investigation was a follow up of our previous study involving the use of MT as a biomarker to assess mercurial accumulation in rats fed orally with cinnabar, a mineral drug used widely in Chinese medicines.[14] In that study, after the feeding of rats with cinnabar, the distributions of heavy metals and MTs in different organs were measured by inductively coupled plasma mass spectrometry (ICP-MS) and a modified mercury saturation assay respectively. The metals were found to accumulate mostly in the liver and kidney, and a high positive correlation was observed between the MT concentration and metal levels in these organs. In this study, the different MT subisoforms bound to mercury and other metals were separated and characterized in order to gain further insight into the mechanisms involved in metal detoxification by MTs. A set of hyphenated techniques, including high-performance liquid chromatography HPLC–ICP-MS, HPLC–electrospray ionization (ESI)-MS and HPLC–UV, was developed for the characterization study, and details of analytical development work will also be described. $HgCl_2$ was used instead of cinnabar as the mercury source for comparison.

MATERIALS AND METHODS

Chemicals

Methanol was LC grade (Tedia). Nitric acid (Peking Reagent Factory, China) was high purity grade. All other chemicals and reagents used were of A.R. grade. Milli-Q50 (Millipore, Bedford, MA) water was used to prepare all solutions. The water used was sparged with argon gas to remove dissolved oxygen.

MT standard, including MT-1 and MT-2 from rabbit liver, was purchased from Sigma. The MT stock solution was prepared by dissolving 1 mg MT standard in 1 ml water. A working solution was prepared by diluting the stock solution with water or buffer as required.

Apparatus

The ESI-MS instrument used was an HP 1100 MSD pneumatically assisted electrospray octopole–quadrupole mass spectrometer (Agilent, USA). The ICP-MS instrument used was an HP 4500 (Agilent, USA). The HPLC system used was an Agilent 1100 system equipped with a UV diode array detector (DAD; Agilent, USA). Separations were carried out using a Vydac C_8 250 mm × 2.1 mm, 5 μm column with a pore size of 300 Å (Hesperia, USA).

Animal experiments

The animal experiments were conducted according to the protocols described by Huang *et al*.[14] The rats were divided into a control group and an $HgCl_2$ group. Rats were maintained in air-conditioned metabolic boxes in standard conditions of a 12/24 h light/darkness cycle. They were fed with a standard diet and water throughout the experiment. The $HgCl_2$ group rats were fed with $HgCl_2$ at a dosage of 0.01 g per kilogram body weight each week. After 2 weeks, the animals were submitted to total blood extraction by ventral aorta followed by the dissection of the kidney and liver tissues. The dissected tissues were washed with physiological saline solution, cleaned, dried on filter paper, weighed and then stored at −80 °C before chemical analyses.

Metal estimation

For metal quantification, the frozen samples were lyophilized directly for 24 h to obtain the dry weights. The dried tissues were ground in a mortar. About 0.3 g samples were weighed and digested with 5 ml of 65% HNO_3 in a microwave oven. After predigesting overnight, the sample was digested for 5 min and then for another 2 min with 1 ml H_2O_2 in a microwave-assisted oven. Then, the digest was diluted with 4% (v/v) HNO_3 to a final volume of 25 ml and stored at 4 °C for analysis by ICP-MS. Procedural blanks were run for background subtraction. 10 ng ml^{-1} thallium solution was used as an internal standard to correct for the signal fluctuation during long-term measurement. Quality assurance for metal analysis was achieved by the use of standard reference material, such as human hair (GBW07 601).

MT purification and desalting

The rat tissues were thawed, disaggregated and homogenized with a buffer of 0.9% NaCl (pH 7.5) containing 250 mmol l^{-1} glucose (1 : 3, w/v) in a glass homogenizer under an argon atmosphere. The homogenized extract was transferred to 5 ml tubes and centrifuged at 15 000 rpm for 20 min at 4 °C. The supernatant was heated at 85 °C for 10 min, cooled in ice-cold water for 5 min, and then centrifuged again at 15 000 rpm for 10 min at 4 °C. The supernatant was stored at −40 °C for further separation.

A homemade preparative size exclusion chromatography (SEC) column (30 cm × 1.9 cm) used for the purification of MT fractions was prepared by packing Sephadex G-75 (40–120 μm particle size) gel (Pharmacia) in a glass column. A prepared sample of 0.5 ml of MT fraction was injected into the preparative column, which was pre-equilibrated with mobile buffer. The elution was under isocratic conditions of 10 mmol l^{-1} Tris–HCl buffer at pH 8.0 at a rate of 1.0 ml min^{-1}. The eluent was collected every 3 min, giving the equivalent of a 3 ml volume in each fraction. Mercury,

zinc and copper were measured in each fraction by ICP-MS. For MT purification, the MT fractions shaded in the elution profile given in Fig. 1 were pooled and lyophilized using a Model EZ-Dry Kinetics lyophilizer (USA).

The lyophilized residue was dissolved in 1 ml of water and was dialyzed with a floating dialyzer (PD-3-1, Spectrem, USA) with molecular weight less than 1000 Da in fresh stirring water for desaltification at 4 °C for at least 24 h. The desalted MT was lyophilized, and then dissolved in 100 µl water for further characterization.

Separation and detection of MT isoforms and subisoforms

The MT fractions purified with SEC were separated on a narrow-bore Vydac C_8 column. The operating conditions of the separation were optimized with MT standards purchased from Sigma. Buffer A was 5 mmol l^{-1} ammonium acetate in water (pH 6.0) and buffer B was 5 mmol l^{-1} ammonium acetate in a mixture of methanol/water (1 : 1, v/v; pH 6.0). A linear-gradient elution that was performed good separation for MT standard was used by increasing buffer B from 10 to 37.5% in 40 min. The sample injection volume was 20 µl. The flow rate was set at 0.20 ml min^{-1}. The eluent from the C_8 column was detected using UV, ICP-MS and ESI-MS detectors.

ICP-MS spectrometric conditions: the ICP-MS system was optimized by running the tuning solution containing 10 ng ml^{-1} of ^{7}Li, ^{89}Y, ^{140}Ce and ^{205}Tl. A simpler method was developed in this experiment by mixing the eluent from reversed-phase (RP) HPLC on-line with a 20% HNO_3 (v/v) solution pumped with peristaltic pump to adapt to the compositions of RP HPLC into the ICP-MS system. The eluent from the C_8 column was mixed with 20% HNO_3 by a T-joint prior to entering the nebulizer of the ICP system. The metals, including mercury, zinc and copper were monitored in time-resolved analytical mode.

ESI-MS conditions: an m/z range of 1300–1900 was monitored, which allowed the observation of the 4+ and 5+ ionization states of the metallated subisoforms. The mass spectra were acquired with a step size of 0.1 u. The scan time required was 1.46 s per scan.

RESULTS AND DISCUSSION

Metal accumulation

The oral intake of $HgCl_2$ results in an accumulation of mercury, copper and zinc in the rats (Table 1). The concentration of mercury in kidney and liver of treated animals was notably higher than that in other tissues (brain, testicle and heart) in our experiment (data shown in other paper).[14] The concentration of mercury in the liver and kidney of treated animals as significantly higher than that in control animals ($P < 0.01$). The mercury concentration in kidney was increased approximately 570 times that in the control, and in liver it was increased approximately 7900-fold. The total mercury concentration in the kidney of the treated animals

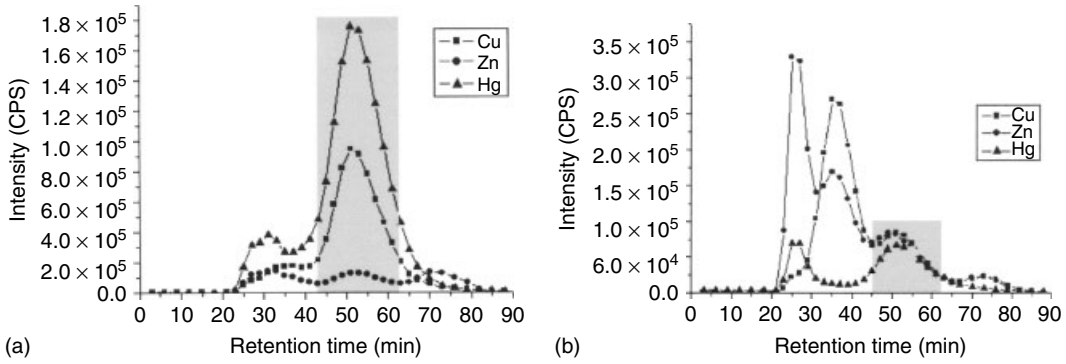

Figure 1. Sephadex G-75 chromatography profile of MT fraction in rat tissues with off-line ICP-MS detection for mercury, copper and zinc: (a) kidney; (b) liver. The elution was under isocratic conditions of 10 mmol l^{-1} Tris–HCl buffer at pH 8.0 at a rate of 1.0 ml min^{-1}. The MT fraction in the range of 45–63 min (shaded) was collected.

Table 1. Distribution of mercury, copper and zinc in liver and kidney following $HgCl_2$ administration in rats

	Liver (µg g^{-1}, fresh tissue)		Kidney (µg g^{-1}, fresh tissue)	
Metal	Control	$HgCl_2$	Control	$HgCl_2$
Hg	0.001 31 ± 0.000 42	10.4 ± 0.3	0.100 ± 0.015	56.7 ± 0.5
Cu	6.84 ± 0.14	7.91 ± 0.05	8.49 ± 0.17	25.9 ± 0.2
Zn	51.2 ± 0.8	75.7 ± 0.8	28.0 ± 0.2	39.6 ± 0.5

was 5.4 times higher than that in the liver. Another interesting result was that the level of mercury increases with increasing copper and zinc levels. For example, the mean concentrations of copper and zinc in the kidney of treated rats were 3.0 and 1.4 times higher respectively than those of the control. The increased levels of these two metals might be related to the detoxicification mechanisms of MTs.

Purification of MT fractions induced in rat kidney and liver

Chromatograms obtained by the preparative SEC for the rat kidney in $HgCl_2$ group are shown in Fig. 1. Mercury, copper and zinc co-eluted with the MT fraction. The eluent between the retention times of 43 to 63 min (shaded) was collected for further characterization. The Hg : Cu : Zn peak intensity ratio at the peak apex of the MT fraction was 13.4 : 7.2 : 1. However, the chromatogram of rat liver obtained by preparative SEC was different from that of the rat kidney. For rat livers, the Hg : Cu : Zn intensity ratios at the apex of the MT fraction was 0.8 : 1 : 1. The mercury-binding MT level in liver was much lower than that in kidney from the SEC chromatogram. The peak area of mercury in the MT fraction of kidney was *ca* 28 times higher than that of liver. The result was somewhat different from what was reported in our previous paper, which showed high MT levels in both the liver and kidney tissues of the treated rats.[14] The difference might have been caused by a deficiency in the mercury saturation assay method used in the study, since low-molecular-weight thiol-containing compounds such as glutathione (GSH) and cysteine in tissues would interfere with MT determination if no preseparation is carried out. However, with concerns about the imprecision that may be brought during the sample preparation, this finding still needs to be carefully checked out.

The MT fractions were preconcentrated by lyophilization and desalted by a dialyzer. Afterwards, the MT was separated by RP HPLC and detected with UV spectrometry, ICP-MS and ESI-MS respectively.

Characterization of MT isoforms and subisoforms in rat kidney and liver

The separation and identification of subisoforms remains a difficult problem because of the lack of standard reference materials and documented reference data. The separation of isoforms can be achieved by various modes of HPLC or electrophoresis techniques. A universally accepted approach to speciation analysis has been the use of hyphenated techniques, which are based on a combination of high-performance separation techniques, such as HPLC and capillary zone electrophoresis, with sensitive and species-selective detectors, such as atomic absorption, plasma emission or mass spectrometers.[15–19]

The MT fractions purified with SEC were separated on a C_8 narrow-bore column and detected on-line with UV spectrometry, ICP-MS and ESI-MS to characterize metal-binding MT isoforms and subisoforms. The separation conditions were optimized with MT-1 and MT-2 reference standards purchased from Sigma.

RP HPLC–UV detection

Figure 2 shows the chromatograms of MT species of rat tissues obtained using RP HPLC with UV detection at 225 nm. For the kidney sample, one major and three minor peaks with retention times of 23.3, 21.5, 22.6 and 29.6 min were found from 20 to 30 min. The peaks were assigned as MT species based on calibrated retention times using cadmium-induced MT standards. Figure 3 shows the UV absorption spectra of the major peak at 23.3 min. Direct evidence for the existence of mercury-binding MT complexes came from the UV absorption spectra, with their most conspicuous feature being characteristic shoulders at 304 nm.[20] On the other hand, the characteristic absorption features of zinc at 220 nm and copper at 270 nm were not obvious for identification purposes under the absorption background. The three minor peaks at 21.5, 22.6 and 29.6 min had the same UV absorption spectra as the major peak at 23.3 min. For the liver sample, no significant peaks were found from 20 to 30 min, suggesting that much less MTs were induced in liver than in kidney. This is consistent with previous SEC results.

RP HPLC–ICP-MS detection

The eluent from the C_8 column was detected on-line with ICP-MS for mercury, copper and zinc. Figure 4 shows the chromatograms of the MT fraction of rat tissues obtained using RP HPLC coupled with ICP-MS. One major and several minor mercury peaks co-eluted with those of copper and zinc were found in the kidney sample, which was consistent

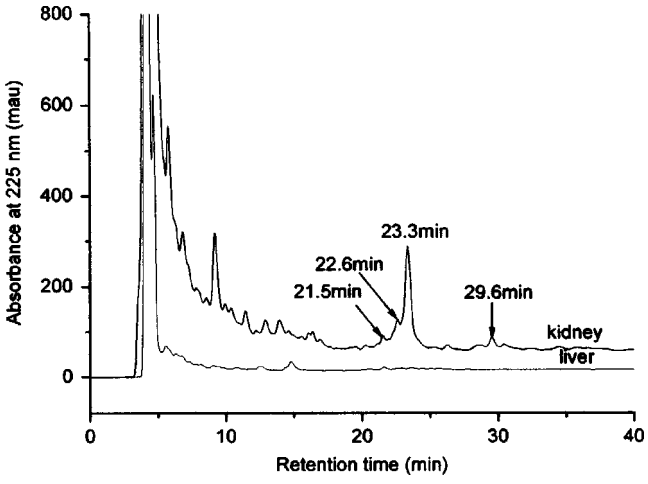

Figure 2. Chromatogram obtained for the separation of MT isoforms and subisoforms of rat tissues on a narrow-bore Vydac C_8 column: 20 µl of MT fractions purified with SEC injected; buffer A, 5 mmol l^{-1} ammonium acetate in water (pH 6.0); buffer B, 5 mmol l^{-1} ammonium acetate in a mixture of methanol/water (1 : 1, v/v; pH 6.0); gradient, 0–40 min, 10–37.5% B, flow rate 0.25 ml min^{-1}.

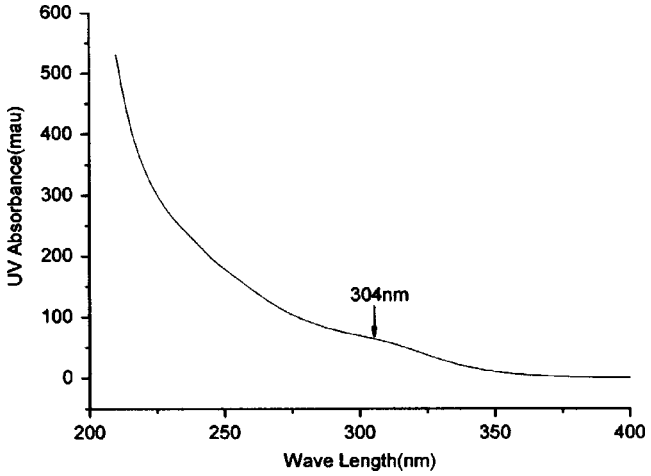

Figure 3. UV absorption spectrum of the peak at 23.3 min in Fig. 2.

with the UV detection result. In the MT fraction region, there was no intense peak for zinc-bound MT, indicating relatively weak binding between apo-MT and zinc in the presence of mercury and copper. The much higher signal intensity of copper compared with mercury may be interpreted that the ICP-MS molar sensitivity for copper exceeds many times that of mercury. The chromatogram of the rat liver sample shown in Fig. 4b is different from that of kidney. Several partially separated small peaks of mercury-binding species in the MT elution region co-eluted with copper and zinc species. The signals of mercury-binding MT species in liver were much lower than that in kidney. The peak intensity of mercury at 23.3 min of the kidney sample was more than 200 times higher than that of the liver sample. The result suggests that the kidney, which induces large amounts of MT, is capable of binding and accumulating mercury more significantly than the liver.[21,22]

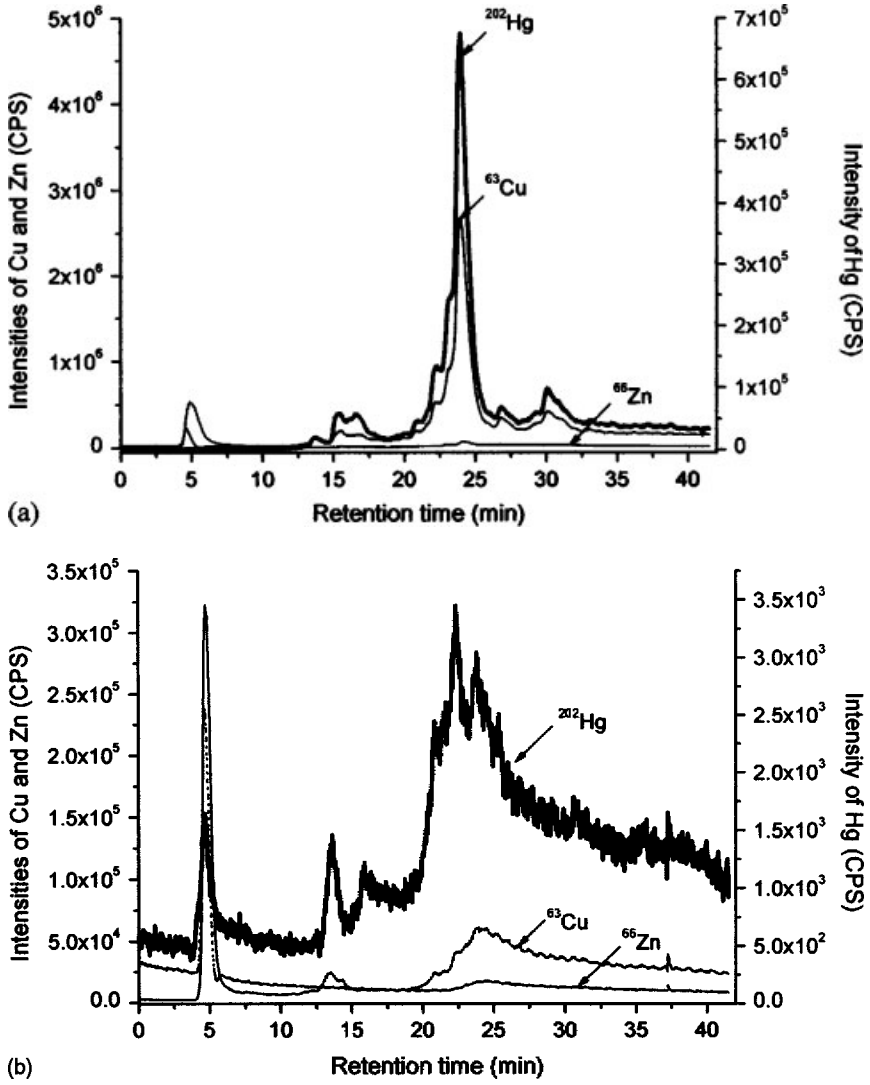

Figure 4. Chromatograms of the MT fraction of rat tissues by RP HPLC coupled with detection by ICP-MS: (a) kidney; (b) liver). The separation conditions are shown in Fig. 3.

The possibility of analytical artifact formation during chromatographic analysis of organometallic species has been assessed. Polec and co-workers, in their research, mentioned the interaction between metal ions (such as mercury(II) and copper(I)) and the stationary phase when using of an RP HPLC column for separation.[23,24] In our experiment, standard MT solution (ZnMT) was used not only to optimize the operation condition, but also to evaluate potential analytical artifact formation problems involving metal–column interactions. One problem of particular concern was residual copper or mercury contamination in the column, which could then cause sample contamination problems through metal exchange, e.g. between zinc, copper and mercury. No such problems were observed based on standards recovery. Furthermore, the copper/zinc and mercury/copper ratios shown in the RP HPLC-ICP-MS chromatogram are consistent with the SEC–ICP-MS result (data not shown), which suggests that the copper atoms assigned to the species found were not formed on the column.

RP HPLC–ESI-MS detection

ESI-MS is one of the most widely used techniques to identify proteins. The eluent from the C_8 column was detected on-line with ESI-MS for the further characterization of the MT isoforms and subisoforms. There were no significant peaks found for the liver sample, which showed very low intensity with UV and ICP-MS detection (not shown). The total ion chromatogram (TIC) of the MT fraction in rat kidney is shown in Fig. 5. One major peak in the TIC of ESI-MS of MT elution region was observed. This major peak, with a retention time of 23.3 min in the RP HPLC–ESI-MS chromatogram, accorded with RP HPLC–UV and RP HPLC–ICP-MS chromatograms at the same retention time. The minor peaks observed with the latter two systems were not observed with RP HPLC–ESI-MS because of the low ESI-MS signal intensity of this sample.

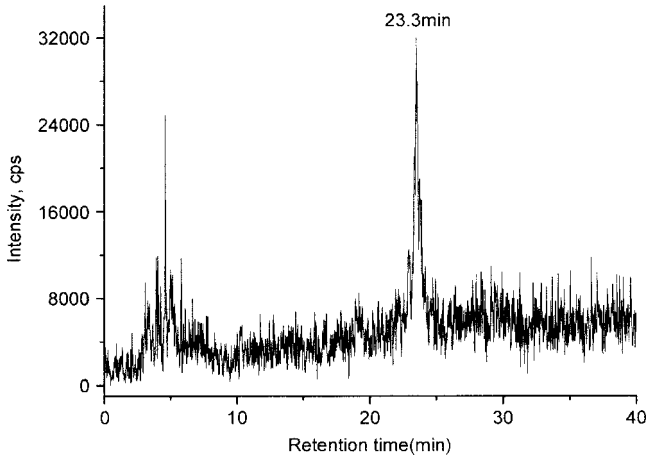

Figure 5. TIC of the MT fraction in rat kidney by RP HPLC coupled with detection by ESI-MS. The separation condition are shown in Fig. 3.

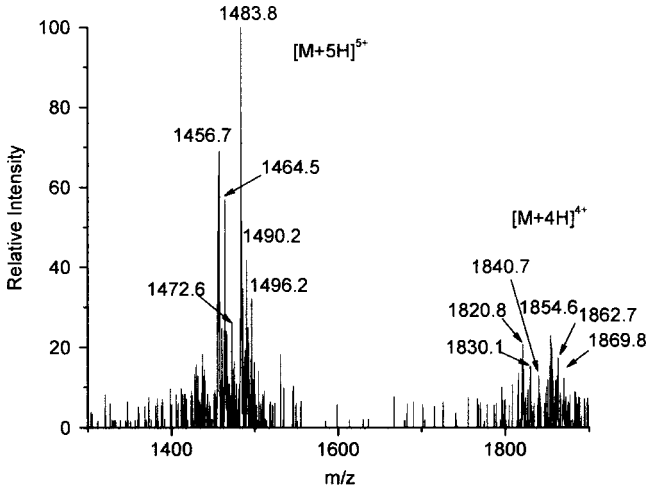

Figure 6. Mass spectra of the peak at 23.3 min in the TIC of the MT fraction (Fig. 5) in rat kidney. For the identification of the masses, see Table 2.

Figure 6 shows the mass spectra of the peak at 23.3 min in the TIC of the MT fraction of rat kidney. Interpretation of the mass spectra data in Fig. 6 is relatively difficult because of the complexity of the clusters of peaks observed (mercury, mercury–copper, and mercury–zinc may co-exist) and the strong binding between mercury, copper and MT which made it difficult to de-metal by acidification to form apo-MT that can be easily identified with ESI-MS.[25] To interpret the MT isoforms and subisoforms of rat kidney from the mass spectra in the vicinity of the TIC, the masses found were compared with the theoretical values calculated by the masses of apo-MT reported in rat tissues and the tentative masses of binding metals in the formula.[18,26] The tentative formulas of the MT isoforms and subisoforms corresponding to the masses found from the mass spectra are listed in Table 2.

Six masses corresponding different metals (mercury, copper or zinc) binding the MT subisoforms were found in the peak at 23.3 min. In this study, it is difficult to distinguish between copper and zinc because their atomic masses are very similar and the resolution of the ESI-MS instrument used in the experiment was not high enough to distinguish between them when binding to MT. As the RP HPLC–ICP-MS result shows only little zinc compared to copper in the peak detected (the amount of copper was more than 30 times than that of zinc in the kidney sample), mercury and copper were considered to be the major metals binding to MT for the tentative molecular formula deduced. The results show that the peak at 23.3 min was a complex that contained different MT subisoforms and different binding metals. The major MT subisoform, i.e. MT-2c, was found to bind to mercury and copper in the Hg_6Cu and Hg_5Cu_2-MT-2c forms. The other MT subisoforms, MT-2β, MT-1γ and MT-2α, bound to mercury and copper in the Hg_6-MT-2β, Hg_6Cu-MT-1γ and Hg_7-MT-2α forms and were co-eluted in the peak. The subisoform of mass 7358.4 was not identified.

Table 2. ESI-MS spectra in the apexes of chromatographic peaks (TIC) of the MT-isoforms of rat kidney(chromatograms given in Figure 6)

Peak	m/z		Mass found (Da)	Tentative formula	Mass calculated[a] (Da)	Reference mass of apo-MT (Da)
	+5 state	+4 state				
23.3 min	1483.8	1854.6	7414.2	Hg_6Cu_1-MT-2c	7416.3	6162.2
	1456.7	1820.8	7278.8	Hg_5Cu_2-MT-2c	7280.2	6162.2
	1464.5	1830.1	7317.0	Hg_6-MT-2β	7316.5	6125.1
	1490.2	1862.7	7446.4	Hg_6Cu_1-MT-1γ	7446.0	6192.0
	1496.2	1869.8	7475.6	Hg_7-MT-2α	7474.6	6084.5
	1472.6	1840.7	7358.4			

[a] Mass of Hg_xCu_y-MT is calculated using the formula: $M_{MT} = M_{apo-MT} + xM_{Hg} + yM_{Cu} - (2x + y)M_H$.

CONCLUSIONS

Mercury, a highly toxic metal found widely throughout the environment, is a potent inducer of MT expression. In this study, significant mercury-binding MTs co-existing with copper and maybe zinc were identified in the kidney of rat after oral intake of $HgCl_2$ using the hyphenated method developed herein. The MTs induced in rat cytosols may promote mercury detoxification by binding with intracellular mercury co-existing with copper, and possibly also zinc, thereby preventing mercury reactions with other cellular targets in rats. Moreover, the combination of HPLC with ICP-MS and ESI-MS detection may be potential tools for the speciation of metals associated with molecules of natural origin.

The mass difference between co-eluted MT complexes with MT isoforms, such as the substitution copper or zinc by mercury, is indicative of the significant role that MT in nephric rat cytosols play in mercury sequestration after exposure to high levels of this toxic metal. Distinction between copper and zinc could not be accomplished in the mass spectra in this experiment. It would require a higher accuracy and sensitivity in the molecular mass measurement by ESI-MS, achieved by using, for example, a time-of-flight mass analyzer.

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