Journal of Health Science, **45**(4) 222—225 (1999)

Selective and Simple Quantification of Metallothionein III in Mouse Brain

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(Received April 16, 1999; Accepted May 16, 1999)

For the selective quantification of metallothionein (MT)-III, MT samples from mouse brain were converted to Hg-MT, then chromatographed using a double (connected-in-tandem) FPLC-gel permeation column system. The chromatogram showed two peaks with a slight overlap. Hg-MT-III was eluted prior to Hg-MT-I and II, probably due to a slightly higher molecular weight. The amount of each isomer was determined from an analysis of the Hg content in each fraction. The present procedure revealed that MT-III in the mouse brain was comparable in quantity to MT-I and II. Thus, MT-III in mouse brain could be selectively quantified at a sub-picomol level.

Key words —— metallothionein, FPLC, brain, mouse

INTRODUCTION

Since the discovery of a novel metallothionein (MT) family, MT-III, in brain tissue,[1] MT investigators have taken considerable interest in brain MT. Among MT isomers, the distribution of MT-III is limited in the brain.[2] To investigate this brain-specific MT, which coexists with MT-I and II, its selective quantification may be a prerequisite. However, information on the amount of MT-III protein in brain tissue is rare,[3] since most detection has been limited to the messenger level.[3,4–6] Brain MT has proved difficult to induce *in vivo* by various MT inducers such as heavy metals, probably due to their poor permeability.[7–9] We have recently reported an effective induction of rat brain MT by Hg vapor exposure.[10] However, information on the comparative induction rate was limited in mRNA levels.[10]

Here, we showed the selective quantification of MT-III in the mouse brain using a single step of column chromatography following conversion to stable Hg-MT. The present procedure demonstrated that MT-III is present in comparable amounts to MT-I and II in mouse brain.

MATERIALS AND METHOD

Diethylmalate, $CdCl_2$, 5 mM (1000 ppm) $HgCl_2$ standard solution and 100% (w/v) trichloroacetic acid (TCA) aqueous solution were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Ovalbumin was obtained from Sigma Co. (St. Louis, MO, U.S.A.).

MT-null mice, which had null mutation of MT-I and II, and wild-type control mice (OLA129/C57BL6) were kindly provided by Dr. A. Choo.[11] Brain and kidney samples of 3 wild-type control mice and brain samples of 3 MT-null mice were excised, and kept at −80℃ until use. For preparation of the Hg-MT sample, each tissue sample was homogenized (5%, w/v) in N_2-saturated ice-cold 1.15% KCl under N_2 atmosphere to prevent the oxidation of MT. The MTs in each tissue sample were converted to Hg-bound forms according to the procedure reported by Naganuma *et al.*[12] with a slight modification.[10] Briefly, the homogenate (1 ml) was treated successively with diethylmalate (5 μl) and 10 mM $CdCl_2$ (25 μl), and heated at 95℃ for 5 min. Following cooling and centrifugation, the supernatant (0.5 ml) was successively treated with 5 mM $HgCl_2$ (25 μl), 1 mM ovalbumin (225 μl), and 12.5% TCA (250 μl). After centrifugation, the supernatant was filtered through a membrane with a 0.22-μm pore diameter (Ultrafree C3, Millipore). The total MT levels were determined through Hg analysis by an oxygen combustion-gold amalgamation method using an atomic absorption detector, Nihon Instrument MD-1, and were expressed as amounts of Hg bound to the thionein. For gel fractionation, an aliquot of the final Hg-MT sample, containing *ca.* 50 ng Hg, was chromatographed using a Superdex 75 HR column (10 × 300 mm, Pharmacia Biotech, Tokyo) equipped with a Waters 510 pump and U6K injector (Waters Assoc.). The column was

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Table 1. Total MT Levels in Brains of MT-Null Mice and Wild-Type Mice and Kidney of Wild-Type Mice

Tissue	MT-null mice	Wild-type control mice	
	Brain	Brain	Kidney
MT levels (nmol Hg/g tissue)	74.4±2.2	92.9±3.6	12.9±2.8

Each value represents the mean±S.D. obtained from 4 mice.

equilibrated with PBS, and Hg-MTs were eluted with the same buffer at 0.5 ml/min with 1 ml fractions. For accurate separation, the two columns above were connected in tandem, and 0.5 ml fractions were collected. The Hg content in each fraction was determined as above, and was expressed as a percent of the loaded Hg.

RESULTS AND DISCUSSION

HPLC separation of MT isomers (Zn-MT-I and II) has been well performed using an anion-exchange column.[13] In the preliminary experiment, we attempted to separate Hg-MT-I and II using the same HPLC system. If this system was applicable to Hg-MT separation, it was expected to also be useful in separating Hg-MT-III. However, Hg-MT samples prepared from mice kidney showed two broad peaks with considerable overlap on the anion exchange HPLC (data not shown). This might imply that the electric characters of MTs would be different between Zn- and Hg-bound forms.

MT-III has a slightly higher molecular weight than MT-I and II due to an insertion of 7 amino acid residues at the N-and C-terminal domains.[14] This slight deviation in molecular weight may allow a possible separation of MT-III from MT-I and II using gel-permeation chromatography or polyacrylamide-gel electrophoresis. Previously, we found that Hg-MT prepared from rat brain, which should contain MT-I, II and III, was eluted as a single peak at the 13—15 ml fraction on gel chromatography using a commercially available FPLC column (Pharmacia Superdex 75 HR).[10] Here, we used the kidney of wild-type control mice, the brain of MT-null mice, and the brain of wild-type control mice as samples of MT-I/II, MT-III and MT-I/II/III, respectively. Table I shows the total MT levels determined by the Hg-saturation method.[12] The brain MT levels in wild-type control mice

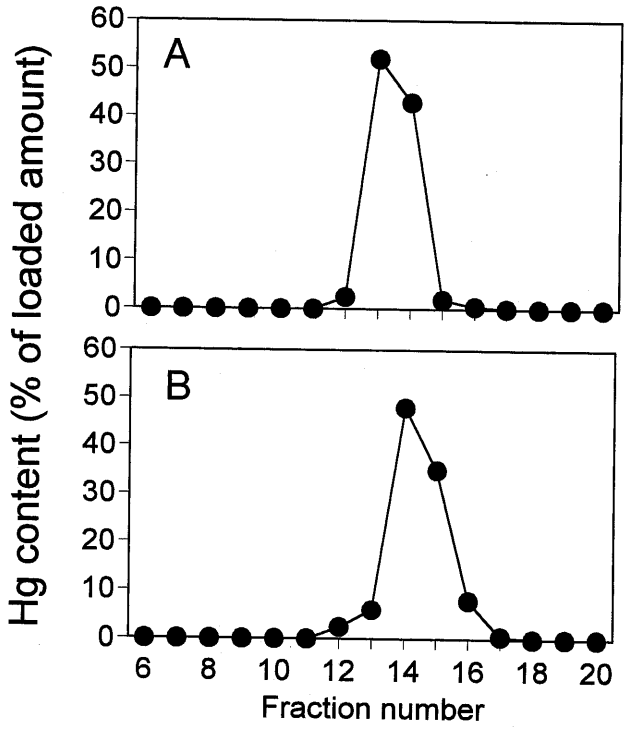

Fig. 1. Typical Gel Chromatograms of Hg-MT Prepared from the Brain of MT-Null Mouse (A) and Kidney of Wild-Type Mouse (B)

Hg-MT samples (containing about 50 ng Hg) were loaded on a Superdex 75 HR column (Pharmacia Biotech), and eluted by PBS. Fractions of 1 ml were collected.

were 7-fold of the kidney MT level, and slightly higher than that in MT-null mice. The Hg-MT samples, which should exclusively contain MT-III or MT-I/II, were eluted using the above-mentioned FPLC column system. The samples prepared from the brain homogenate of MT-null mice and from the kidney homogenate of wild type mice showed a single peak (Fig. 1A, B). No appreciable amount of Hg was detected in other fractions. However, the peak positions of MT-null mice brain (13—14 ml) and wild-type control mice kidney (14—15 ml) seemed to deviate slightly (Fig. 1A, B). This suggested that chromatography using a high-capacity column might provide an effective separation of MT-III from MT-I and II. We examined this possibility using a two-column system connected in tandem with more

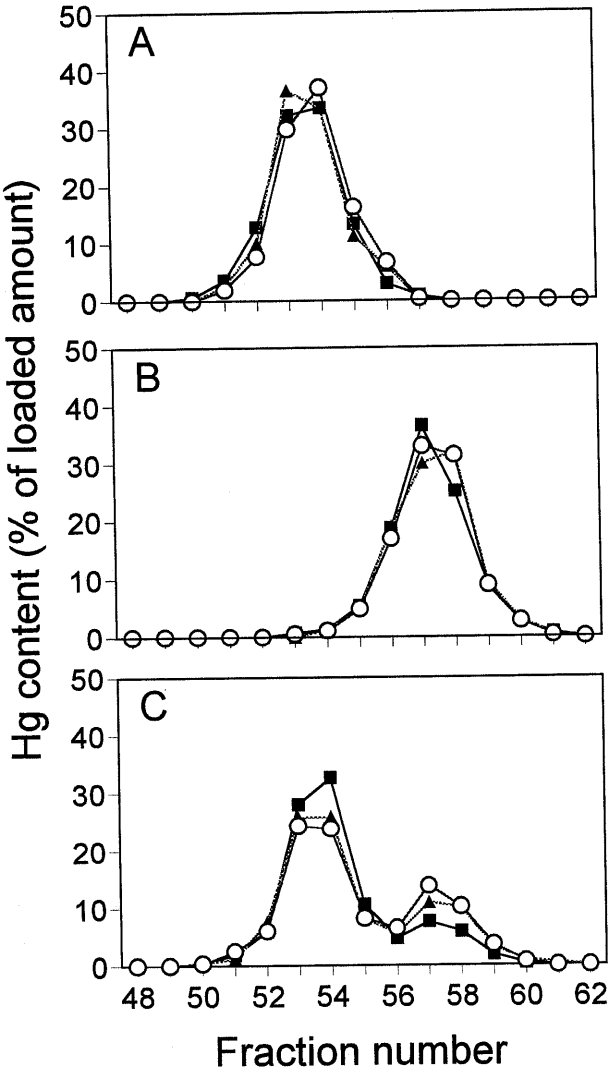

Fig. 2. Gel Chromatograms of Hg-MT Prepared from Brains of MT-Null Mice (A), Kidneys of Wild-Type Mice (B), and Brains of Wild-Type Mice (C)

Hg-MT samples (3 for each chromatogram; containing about 50 ng Hg) were loaded on two connected in tandem Superdex 75 HR columns (Pharmacia Biotech), and eluted by PBS. Fractions of 0.5 ml were collected.

elaborate (0.5 ml) fractionation. Hg-MT samples from the brain of MT-null mice showed a single peak at 26—28 ml (fraction numbers 52—56, Fig. 2A). On the other hand, Hg-MT prepared from the wild-type mice kidney was eluted at 28—30 ml (fraction numbers 56—60, Fig. 2B). The deviation of these two peak positions indicated that Hg-MT-III could be separated from Hg-MT-I and II by a single step of gel chromatography using a double-FPLC column system. The effectiveness of the separation was confirmed by a chromatogram of the Hg-MT samples from the wild type mice brain, which had all three MT isomers. Hg-MT samples from the wild-type mice brain vividly showed two separated peaks at fraction numbers 52—56 and 56—60 (Fig. 2C). As is evident from Fig. 2C, MT-III was the major component in the wild-type mouse brain MTs. Considering that the total MT levels in MT-null mice were slightly (20%) lower than those in wild-type control mice (Table 1), the MT-III levels might be approximately the same between these two strains.

Thus, the amount of MT-III in the mouse brain could be selectively quantified by a single chromatographic procedure following conversion to a stable Hg-MT. Since the Hg analyzing system employed here can accurately detect 0.5 ng (2.5 pmol) of Hg in one measurement, the present procedure will detect Hg-MT on the order of sub-pmol, assuming 10 Hg atoms bound a thionein molecule. This would be much lower than the limit of spectroscopic detection, because the present chromatography gave no appreciable peaks on a UV spectroscopy at the positions where Hg-MTs were detected by Hg analysis.

Acknowledgements The authors thank Ms. T. Onizuka, Ms. Y. Tanaka and Ms. A. Hyodo for technical assistance in Hg analysis.

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