Measurement of Metallothionein in Rat Tissues by a Chromatographic Method Using the Fluorometry of Thiol Groups

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The estimation of metallothionein (MT) levels in a biological sample is required to study the physiological role or the induction of MT, which is a multifunctional protein. MT contains 61 amino acids with 20 homologous cysteines. The high selectivity, sensitivity and stability of *N*-[4-(dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM) for the determination of SH groups was very useful for the determination of MT. The detection limit of MT by this method was 10 ng/0.5 ml. This method made it possible to measure MT levels in normal tissues in combination with gel filtration chromatography. The age-dependent change in MT levels in normal rat tissues was also investigated by the present method. MT levels in normal rat tissues vary with age. It is thought that this fact is correlated with the biological roles of MT.

Key words metallothionein; fluorometry; age-dependent; rat tissue; *N*-[4-(dimethylamino-2-benzofuranyl)phenyl]maleimide

Metallothionein (MT) is an extremely unique protein, with high metals and thiol groups. MTs occur throughout the animal kingdom and they are induced *in vivo* by metals, hormones, cytotoxic agents, and some kinds of stress. The biological roles of MTs were reported in connection with the regulation of zinc and copper metabolism,¹⁾ detoxification of heavy metals²⁾ and protective function to cells by reducing damage from free radicals.^{3–6)} The assay of MT is required for the investigation of MT, which is a multifunctional protein. Summer and Klein⁷⁾ classified current protocols for MT quantification according to five basic principles, namely chromatography, electrophoresis, metal saturation, immunology and electrochemistry. MT is determined indirectly *via* its thiol or metal content. On the other hand, direct immunological methods such as RIA and ELISA have been used as sensitive methods for MT determination, but these methods are not commonly used. At present, the most commonly used method for MT determination is the metal saturation method, in which saturate thiol groups of MT with Cd, Hg or Ag are used, the unbound metals are removed, then MT level is estimated by determining metals *via* atomic absorption spectrometry or their radioactivity. The SH groups of cysteine residues, which comprise about 30% of the constituent amino acid residues of MT, are capable of being determined by Ellman reagent 5,5'-dithiobis(2-nitrobenzoic acid).^{8,9)} The sensitivity of this method is not satisfactory for the determination of MT in normal tissues; therefore, the method of MT determination using a fluorogenic thiol-selective reagent was investigated as a more sensitive and simple method than the Ellman method. *N*-[4-(Dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM)¹⁰⁾ as a fluorogenic reagent was well suited for this purpose. This method made it possible to measure MT levels in normal tissues in combination with gel filtration chromatography.

In this paper, an age-dependent change in MT levels in normal rat tissues was also investigated.

MATERIALS AND METHODS

Animals Male Wistar rats (3–52 weeks of age) were purchased from Clea Japan, Inc.

Reagents DBPM was purchased from Dojindo Lab., Kumamoto, Japan. Rabbit liver MT as a standard was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Apparatus A Shimadzu model RE-540 spectrofluorophotometer was used for the measurement of fluorescence intensity. A SuperdexTM 75 HR 10/30 column (Pharmacia Biotech) was used for the gel filtration of MT in combination with FPLCTM system (Pharmacia Biotech).

Fluorometric Determination of MT DBPM was used as a fluorogenic reagent for the determination of thiol groups. According to the procedure described by Nakashima *et al.*,^{11–13)} with some modifications, the SH groups of cysteine residues in MT were determined with DBPM, and the amount of MT was calculated with a calibration curve for the MT standard, rabbit liver MT.

To 0.5 ml of a sample solution containing MT were added 0.5 ml of 5 mM EDTA in 0.1 M borate carbonate buffer, pH 8.5, and 1.0 ml of 6 μ M DBPM in acetonitrile. The mixture was incubated at 60 °C for 30 min, then permitted to stand at room temperature for 20 min. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 457 nm with a 4 × 10 mm quartz cell.

Measurement of MT in Rat Tissues The rats, 3, 7, 11, 15, 36 and 52 weeks old, were killed, and ten tissues (kidney, liver, spleen, thymus, heart, lung, small intestine, pancreas, testis, brain) were removed and immediately weighed.

The tissues were each homogenized with 3 volumes of 0.25 M sucrose solution, containing 2 mM dithiothreitol, and centrifuged at 105000 *g* for 60 min at 4 °C. The supernatants, cytosols, were placed in boiling water for 10 min using a glass sphere as a condenser. After cooling, they were centrifuged at 5000 *g* for 30 min at 4 °C, then the obtained supernatant volume was measured. A 200 μ l

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sample of each supernatant was chromatographed on a Superdex 75 HR 10/30 column using 50 mM Tris-acetate buffer, containing 100 mM NaCl and 0.02% NaN_3 , pH 7.4. The flowrate was set at 0.5 ml/min and the eluate was monitored at 254 nm. Each 0.5 ml portion of eluate was collected and the SH groups in each fraction were measured by the method described above. The net intensity of MT fluorescence was calculated by subtracting the background from the integration of MT fractions.

RESULTS

The Determination of MT with DBPM Four fluorogenic reagents, 7-fluoro-4-sulfamoyl-2,1,3-benzoxadiazole (ABD-F), ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), *N*-[4-(2-phthalimidyl)phenyl]maleimide (PPM)¹⁴⁾ and DBPM, were tested for the determination of MT. There was no difference in their sensitivity in terms of MT determination. The fluorescence intensity to MT of PPM or DBPM, bearing a maleimide ring, was more stable with an elapse of time. PPM is not commercially available, while DBPM is easily obtained from

commercially available materials. Therefore, DBPM was employed in the determination of MT.

The fluorescence intensity showed dependence on the concentrations of EDTA and DBPM, as shown in Fig. 1. Optimum concentrations of EDTA and DBPM for the measurement of MT were determined to be 5 mM and 6 μM , respectively, from the concentration that yielded the maximum intensity of each.

The stability of the binding of DBPM to MT is shown in Fig. 2 as a fluorescence intensity after the end of reactions for 20 or 30 min. The fluorescence intensity of both reaction times were stable at room temperature until 60 min after the reaction ended. From this result, the determination condition of MT measurement was decided to be as follows. The reaction was carried out for 30 min and the fluorescence intensity was measured at 20 min after the end of the incubation period.

Standard Calibration and Detection Limit A typical standard curve, the calibration curve, for the fluorometric assay of MT, was obtained by the above method using the MT standard (Fig. 3). A good linear relationship between the amount of MT (0.2–8 μg) and the fluores-

Fig. 1. Effects of Various Concentrations of EDTA and DBPM on Fluorescence Intensity for MT Determination

To 0.5 ml (4 μg MT) of the MT standard solution was added 0.5 ml of various concentrations (0.5–20 mM) of EDTA and 1.0 ml of 8 μM DBPM (left). To 0.5 ml (4 μg MT) of the MT standard solution was added 0.5 ml of 10 mM EDTA and 1.0 ml of various concentrations (1.25–20 μM) of DBPM (right).

Fig. 2. The Stability of the Binding of DBPM to MT

The fluorescence intensity was measured at various times after the end of the incubation period of 20 or 30 min. (MT: 4 μg , EDTA: 5 mM, DBPM: 6 μM).

Fig. 3. Typical Standard Curve for the Fluorometric Assay of MT

Fig. 4. Gel-Filtration Chromatography of 3-Week-Old Rat Liver Cytosol on a Superdex 75 Column

---, absorbance at 254 nm. The symbols A_1 — A_6 represent the fluorescence intensity of each fraction at 27 to 32 min of retention time.

Table 1. MT Concentrations in Normal Rat Tissues of Various Ages

Tissue	MT concentration ($\mu\text{g/g}$ tissue) mean $\pm$ S.D. ($n=3$)					
	3 weeks	7 weeks	11 weeks	15 weeks	36 weeks	52 weeks
Kidney	45.5 ± 2.3	47.0 ± 2.6	39.3 ± 5.0	46.3 ± 7.4	37.7 ± 9.5	40.6 ± 8.0
Liver	47.5 ± 9.9	8.3 ± 1.0	21.4 ± 3.9	26.1 ± 7.9	8.4 ± 3.7	6.5 ± 4.8
Spleen	N.D.	16.1 ± 1.6	13.5 ± 1.6	17.9 ± 7.8	22.8 ± 6.9	27.1 ± 6.2
Thymus	N.D.	15.3 ± 4.1	14.8 ± 0.7	9.3 ± 2.9	25.6 ± 4.0	—
Heart	N.D.	N.D.	N.D.	3.2 ± 1.2	1.7 ± 1.1	2.2 ± 1.2
Lung	N.D.	N.D.	10.4 ± 1.7	6.6 ± 2.1	3.8 ± 1.9	10.7 ± 3.9
Small intestine	9.8 ± 3.3	65.2 ± 4.1	50.2 ± 19.0	31.4 ± 15.3	21.3 ± 3.5	10.6 ± 2.5
Pancreas	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Testis	24.4 ± 3.3	33.9 ± 4.0	48.7 ± 4.4	23.7 ± 6.7	22.1 ± 3.0	54.9 ± 6.4
Brain	19.5 ± 5.8	15.1 ± 3.3	13.4 ± 1.6	7.6 ± 3.4	12.6 ± 2.8	16.9 ± 4.5

cence intensity was obtained. The correlation coefficient was 0.997 and the coefficient of variation was 5.8%. If the limit of detection was defined as three times the standard deviation of ten blank solutions, it was possible to detect as little as 10 ng MT/0.5 ml by this method.

Application to Rat Tissues In the case of the application of this method to a tissue sample, MT fraction must be separated. In addition, the interference of SH groups other than MT must be removed. Chromatographic separation was performed by gel filtration with a SuperdexTM 75 column. Each 0.5 ml portion of eluate was collected and the SH fluorescence was measured (Fig. 4).

The net intensity of MT fluorescence (A_{MT}) was calculated by the following equation:

$$A_{\text{MT}} = \sum_{n=2}^5 A_n - 4/2(A_1 + A_6)$$

where the symbol A_1 — A_6 represents the fluorescence intensity of each fraction at 27 to 32 min of retention time. It was confirmed by the chromatography of standard MT that MT is eluted during a retention time of 28—31 min.

The recovery of added MT by this method was 91.1%.

The MT concentration in normal rat liver obtained by this method was in approximate agreement with published data, $0.8^{(4)}\text{--}20^{(15)} \mu\text{g/g}$.

The Variation of MT Levels in Normal Rat Tissues by Aging MT levels in rat tissues of various ages, 3, 7, 11, 15, 36 and 52 weeks old, were measured by the present method, and these results are shown in Table 1. MT in all pancreas samples was not detected. Renal MT levels in 3- to 52-week-old rats were little changed. The MT level in the brain was at the highest concentration in the 3-week-old, then decreased until the rats were 15 weeks old, then increased again with age. However, the variation in MT levels in the brain as a result of aging was small. Young rat (3-week-old) liver showed a high concentration of MT. Hepatic MT levels in the 7-week-old had decreased to a great extent. It increased until the rats were 15 weeks old, then decreased again with age.

Contrary to hepatic MT levels, the MT level in the small intestine was low in the 3-week-old and it increased greatly at 7 weeks of age, presumably due to the need for

zinc intake, and then it decreased with age. The MT levels in the thymus and spleen, which are the largest lymphoid organs correlated with immunological function, were not detected in the 3-week-old, but they increased with age in the subsequent 7-week-old (the thymus of the 52-week-old rat was not obtained because it had degenerated). The MT level in the testis increased until adulthood, but its concentration in the adult rat fell to a low level, but it increased again at 52 weeks of age. MT in the lung of young rats (younger than 7-week-old) was not detected, but it was measured in lung of adult rats (older than 11 weeks old).

DISCUSSION

The fundamental protocol of the SH groups determined with DBPM, which is fluorogenic thiol-selective and a highly sensitive reagent, is a very simple method; to the sample solution is added EDTA and DBPM, then the mixture is incubated at 60 °C, and the relative fluorescence intensity is measured. Application of this method to MT determination was discussed.

This method is sensitive enough to measure MT in normal rat tissues and is not inferior to the sensitivity of other methods. On the other hand, immunological assays so far allow the measurement of MT in the picogram range and are thus sufficiently sensitive to quantify MT in body fluids. These methods might not be considered to be of general use, particularly for screening MT in various biological systems.⁷⁾

The reason for the decrease in hepatic MT concentration in 7-week-old is not known. The hepatic MT concentration in rat fetus increased from gestation day 16 through day 21, reached a maximum at over 1000 µg/g tissue 1 d after birth, and remained high for the 1st week.¹⁶⁾ Age-dependent changes in the concentrations of MT in human tissues have also been reported.^{17,18)} The analysis of human fetal liver samples showed high concentrations of MT, and hepatic MT levels decreased with age.¹⁷⁾ Ohta *et al.*¹⁸⁾ reported that the high level of MT in human liver was maintained during infancy, and then decreased until the subjects were in their thirties. After that, increases in MT and Zn corresponding to Cd accumulation were observed. Hepatic MT levels decreased markedly during weaning, but MT synthesis could be induced in adult rats on exposure to certain metals.¹⁹⁾ Therefore, our present results are consistent with these published data. A positive linear relationship was observed between Zn and MT levels in liver cytosol (unpublished). One of the major biological roles of MT may be to store Zn in the liver during perinatal mammalian development.¹⁷⁾ Since the rat requires Zn in the growing term, 7-week-old Zn levels in liver decreased and hepatic MT levels also decreased.

It can be seen from the present results that MT levels in normal rat tissues vary with age. It is thought that this fact is correlated with the biological roles of MT. Wormser and Nir¹⁵⁾ have reported that there are age-dependent changes which render the elderly more sensitive to toxic metals and possibly to other hazardous substances. It can therefore be presumed that MT is not required for a tissue when it is growing and functioning smoothly, but when a biological function of a tissue falls off and the tissue reaches an abnormal condition, MT is necessary for the tissue and is thus introduced into the tissue. It was clarified that the present method is not sensitive enough to measure MT concentration in plasma (ng/ml), but it is possible to detect MT in most rat tissues (µg/g). The estimation of MT levels in a biological sample is required for study of the physiological role or induction of MT, which is a multifunctional protein. With this method it is possible to determine MT in normal animal tissues with a fluorophotometer; it is a simplified procedure which does not require an atomic absorption spectrometer or radioisotopes. The parallel use of different methods of MT determination according to the purpose is considered to be most appropriate.

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