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Separation and quantitation of metallothionein isoforms from liver of untreated rats by ion-exchange high-performance liquid chromatography and atomic absorption spectrometry

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Abstract

A sensitive method for determination of metallothionein (MT) isoform levels in rat liver by ion-exchange high-performance liquid chromatography and atomic absorption spectrometry was developed. Critical steps in sample preparation, like MT extraction, MT saturation with Cd and protein separation, were optimized. This method is capable of measuring levels of 2.0 μg/g liver for metallothionein-1 (MT-1) and 1.3 μg/g liver for metallothionein-2 (MT-2), respectively, with a high recovery of 103% on average. The method described, thus, proved suitable for analyzing metallothionein isoform concentrations even in untreated animals. The ratio of MT-1 to MT-2 was found to be 1:1 on average. MT decomposition during storage was very high in whole livers, but could be reduced by about 80% when extracted liver samples were used.
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1. Introduction

Metallothioneins (MTs) are characterized as metal-binding proteins of low molecular mass with a high cystein content [1]. The biosynthesis of MTs can be induced by a variety of metals, chemical agents, cytokines and stressful conditions. Although the function of MTs is still unclear, it seems that MTs are involved in homeostasis of the essential metal zinc [2] and play a putative role in metabolism and detoxification of heavy metals [3]. Additionally, in most mammalian species two or more MT isoforms are known, which are encoded by different genes. There is also evidence that the expression of some of these genes is under separate control and may serve different biological purposes [4,5]. To investigate the putative role of MT isoforms in physiology, it is of general interest to establish a reliable method for the separation and detection of MT isoforms.

Classical techniques for determination of MTs include the metal saturation assays [6,7], polarography [8,9], radioimmunoassay (RIA) [10,11] and enzyme-linked immunosorbent assays (ELISA) [12,13]. Metal saturation assays use the high affinity of MTs to bind heavy metals (e.g., Cd, Hg, Ag), making MTs easy to detect after saturation. But

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quantitation of MTs is often based on speculative assumptions concerning the stoichiometry of saturation; moreover, this method is incapable of measuring MT isoforms separately. Polarography relies on the high cystein content of MTs for detection, but is very sensitive to other thiol-containing ingredients of a sample, especially cystein-containing proteins. Furthermore, neither polarography nor most of the highly sensitive RIA and ELISA methods can distinguish between MT isoforms in a mixture.

Separation and detection of MT isoforms in several animal tissues can also be performed by high-performance liquid chromatography (HPLC) techniques combined with an ion-exchange column and subsequent measurement by UV or atomic absorption spectrometry (AAS), but only a few MT isoform separation methods based on ion-exchange were available in literature [14–17]. Moreover, some ion-exchange procedures succeeded in separating MT isoforms in tissues but were less sensitive. None of these previously published methods were able to measure the amount of basal MT isoforms in liver or kidney of untreated rodents, only in metal-treated ones where concentrations of MTs are high [14–17].

The purpose of the present study was to devise a fast separation and detection procedure for rat liver MTs with high sensitivity for non-stimulated MT concentrations. Based on the method of Lehman and Klaassen [15] the sample preparation was tested and optimized to reduce possible MT losses. Additionally, the analytical separation conditions and injection volume for separation by HPLC as a whole were adapted to the demand of an improved detection limit. A second topic of this study was to investigate the influence of storage procedures on the measurable MT content, which has not been done before.

2. Materials and methods

2.1. Chemicals

All chemicals used were purchased from Merck, Darmstadt, Germany. Water for HPLC and for all solutions was deionized twice and treated with organic material absorber (Millipore, Bedford, MA, USA).

2.2. Isolation and purification of MT standards from rat liver

For detection and quantitation of MT-1 and MT-2 in rat liver by HPLC–AAS, a MT-1 and MT-2 standard was isolated from cadmium-treated rats. The isolation was based on a procedure described by Garvey et al. [18], which was modified in several steps.

Rat liver MT was induced in female Sprague–Dawley rats by repeated subcutaneous injections (five times) of $CdCl_2$ at increasing concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg Cd/kg bodyweight). The intervals between injections were 48 h and the rats were killed 48 h after the last injection. For the isolation of metallothionein a 40–60 g portion of pooled rat livers was homogenized with three volumes of 0.1 *M* ammonium formate, pH 7.4, 4°C, to which was added 2.0 mg 2-mercaptoethanol per g of liver. All subsequent isolation steps were carried out at 4°C.

The liver homogenate was centrifuged at 48 000 *g* (20 min) and the supernatant filtered through glass wool. Prior to heat treatment 0.4 mg Cd/g liver was added as $CdCl_2$. The supernatant was heated at 82°C to precipitate interfering proteins, immediately cooled in ice and then centrifuged at 48 000 *g* (20 min). The heat soluble material of the resulting supernatant was fractionated with acetone. In a first step pre-chilled acetone was added to 40% (v/v) and after 2 h the pellet was removed by centrifugation at 27 000 *g* (25 min). In a second step the acetone concentration was increased to 80% (v/v) and incubated overnight, after which the precipitated MT was separated by centrifugation at 27 000 *g* (25 min). The pellet was dissolved in 10 ml 5 m*M* Tris–HCl (pH 8.6) and the insoluble particles were removed by centrifugation at 6000 *g* (15 min). The supernatant was applied to a Sephadex G-75 column (98 cm×3.3 cm I.D., Merck) that had been pre-equilibrated with 5 m*M* Tris–HCl (pH 8.6) and the column was eluted with the same buffer at a flow-rate of 17 ml/h. Five-ml fractions were collected and fractions with more than 50% of the maximum UV absorption at 254 nm were pooled for further ion-exchange purification. These MT-containing fractions were applied to a TSK DEAE-650 column (42×4.2 cm I.D., Merck) and eluted with a linear gradient of 20–350

m*M* Tris–HCl (pH 8.6) and a flow-rate of 50 ml/h. Fractions (5 ml) were collected and separated MT-1 and MT-2 were identified by UV absorbance at 254 nm. Fractions from each isoform were pooled, desalinated on a Sephadex G-25 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and then lyophilized. Finally, the lyophilisate was dissolved in triple-distilled water and stored at −80°C.

For quantitation of metallothionein standards the concentrations of the standard solutions were quantified by UV absorbance at 250 nm and the molar extinction coefficient 79 mM^{-1} cm^{-1} [19].

2.3. Optimization of sample preparation

The sample preparation for MT quantitation in rat liver was based on the preparation steps described by Klaassen and co-worker [14,15]; these were tested and modified here in several steps to minimize MT losses and preparation time and to improve the detection limit. Briefly, the preparation procedure involves tissue homogenization and extraction of MT, two centrifugation steps, incubation of the supernatant with cadmium to saturate MT for the subsequent AAS detection, heat treatment to separate protein, followed by centrifugation with membrane filtration and finally HPLC injection. Tissue preparation for MT isoform measurement was improved by varying the liver/buffer ratio for extraction and the incubation period during MT saturation with $CdCl_2$ and by changing the time and temperature of the heat treatment. All livers used in these experiments were obtained from untreated male Sprague–Dawley rats and all preparation steps were carried out at 4°C. Analyses were performed in duplicate.

2.3.1. Extraction of metallothionein

Five extraction buffers were tested to extract maximum amounts of MT from rat liver: 10 and 50 m*M* Tris–HCl, pH 7.4, 10 and 50 m*M* $NaHPO_4$, pH 7.4 and a mixture of 10 m*M* Tris–HCl+250 m*M* sucrose, pH 7.4. Given the small size of a rat liver, the first four buffers were used for extraction of one rat liver while the buffer mixture and a 10 m*M* Tris–HCl were tested on a second liver. The subsequent preparation procedure was the same in each case. Additionally, the optimum liver/buffer ratio was determined. This was done by dividing the liver into five portions which were extracted with decreasing quantities of 10 m*M* Tris–HCl, pH 7.4, to arrive at a final liver concentration of 10%, 20%, 25%, 33% or 50% in the homogenate. The subsequent preparation procedure was the same in each case.

2.3.2. Metallothionein saturation with $CdCl_2$

At a first step, the addition of various quantities of $CdCl_2$ was tested to achieve complete Cd saturation of MT for the subsequent AAS detection. Following liver extraction and centrifugation of the homogenate, 1000 μl of the resulting supernatant were mixed with 50 μl of $CdCl_2$ solution at different concentrations to obtain final cadmium concentrations of 5, 25, 50, 75 or 100 μg/ml. The subsequent preparation procedure was the same in each case.

The second step was to examine different incubation times of $CdCl_2$ with the centrifuged liver extract in order to optimize the Cd-MT yield. Liver was homogenized and centrifuged. To 1000 μl of the supernatant 50 μl $CdCl_2$ was added (50 μg Cd/ml final concentration) and incubated at room temperature for 0, 5, 15 or 45 min to saturate MT prior to heat treatment. The subsequent preparation procedure was the same in each case.

2.3.3. Protein separation by heat treatment

The heat treatment of the samples was performed at different temperatures and for varying periods of time to determine the optimum conditions for maximum protein denaturation with minimum MT losses. Liver was homogenized and centrifuged. The resulting supernatant was mixed with $CdCl_2$ to obtain a cadmium concentration of 50 μg/ml and immediately heat-treated for 1, 2, or 5 min at temperatures of 60, 80 or 100°C. All samples were chilled immediately, centrifuged, membrane-filtered and applied to HPLC–AAS. Additionally, the remaining protein content after the heat treatment was measured in each sample.

2.4. Storage of samples and the influence on the MT content

To investigate the effect of the duration and method of sample storage on the final MT content in liver, two investigations were conducted.

(1) Raw livers from two untreated rats were

divided into equal portions. The liver slices were stored at −20°C and −80°C for 1 or 3 months, while one slice was analyzed for MT immediately.

(2) Prior to storage, a fresh liver was homogenized with three volumes of 10 m*M* Tris–HCl, pH 7.4, followed by centrifugation at 10 000 *g* (20 min, 4°C) and a second centrifugation step at 114 000 *g* (1 h, 4°C). The resulting supernatant was portioned (1 ml), stored at −80°C and analyzed for MT on day 3, 15, 43, 51 and 101, respectively.

2.5. Equipment and method for MT-1 and MT-2 quantitation by HPLC–AAS

A Merck L-6200 high-performance liquid chromatograph consisting of one single L-6200 intelligent pump and a Rheodyne 7125 injector equipped with a 1000-μl sample loop was used for MT analysis. MT separations were achieved on a tentacle anion-exchange column [Fractogel EMD DEAE-650 (S), 20–40 μm; 70 mm×10 mm I.D., Merck] which has a very high protein binding capacity [20,21]. All separation conditions were adapted to the type and capacity of the column and the high injection volume of 500 μl which were used in this study. Gradient elution was performed using 20 m*M* Tris–HCl, pH 7.4 (buffer A) and 200 m*M* Tris–HCl, pH 7.4 (buffer B). MT-1 and MT-2 were eluted with a linear gradient from 0 to 60% B in 18 min at a flow-rate of 1 ml/min. For regeneration the column was rinsed for 7 min with 1 *M* NaCl and then for 25 min with buffer A. All solutions were saturated with nitrogen. According to this elution procedure MT-1 and MT-2 were eluted at concentrations of ~55 and ~90 m*M* Tris–HCl buffer, respectively. The column efflux was collected with a Gilson M 201 fraction collector (Abimed, Langenfeld, Germany) in 1-ml fractions. For MT quantitation the Cd concentration was measured with flame atomic absorption spectrometry (FAAS) at a wavelength of 228.8 nm (Model 5100, Perkin-Elmer, Überlingen, Germany). Prior to the measurements, the FAAS system was calibrated with a Cd^{2+} standard solution of different concentrations (diluted with 20 m*M* Tris–HCl, pH 7.4). The undiluted samples obtained from HPLC were applied to FAAS without dilution and the resulting peak areas were integrated automatically to yield the final cadmium concentration of the sample.

2.6. Quantitation of metallothionein isoforms

Quantitation of the MT isoforms was based on the peak height of Cd detection by AAS. To generate calibration curves cadmium-saturated MT-1 and MT-2 standards were applied to anion-exchange HPLC, eluted, separated and fractionated as described above until each fraction was measured for cadmium content by AAS. The total cadmium content of the fractions corresponding to MT-1 and MT-2 was plotted against the different concentrations of MT injected on the column and linear regression analysis performed to determine the type of relationship. For sample analysis a 500-μl aliquot of the filtered sample was injected into the anion-exchange column, the MT isoforms were eluted in the manner described and collected for cadmium analysis by AAS. The MT concentration of the liver sample results from the final cadmium content of the MT-1 or MT-2 fractions and the linear regression of the standard curves. The levels of MT-1 and MT-2 in liver were calculated using the following equation:

$$\mu\text{g MT-}X\text{/g liver} = \frac{[\mu\text{g MT-}X\text{/0.5 ml injection volume}] \cdot v_H D}{WR_X}$$

where X: 1 or 2 (MT-1 or MT-2); v_H: volume of homogenate (ml); D: dilution of the homogenate (1.05); W: weight of liver used (g); R_X: recovery of MT-X (MT-1: 0.96; MT-2: 1.10).

2.7. Validation of method

The optimized sample preparation and subsequent separation and detection of MT-1 and MT-2 by HPLC–AAS were validated using liver from untreated rats. The method of standard addition was used to determine the recovery and linearity of measurement of MT from rat liver. Five sample preparations were therefore carried out, adding increasing amounts of MT-1 and MT-2 standard to the liver homogenate. The reproducibility of the measured MT concentrations was evaluated by preparing five identical samples and analyzing them for MT-1

Table 1
Concentrations of the metallothionein isoforms and total metallothionein in liver extracted with various buffers[a]

Sample	Buffer	MT-1 (μg/g)[b]	MT-2 (μg/g)[b]	Total MT (μg/g)
Liver 1	10 m*M* Tris–HCl	5.0±0.13	3.7±0.41	8.6
	50 m*M* Tris–HCl	4.6±0.57	2.2±0.48	6.8
	10 m*M* NaH_2PO_4	4.5±1.94	4.0±0.71	8.5
	50 m*M* NaH_2PO_4	4.2±1.09	3.7±0.32	7.9
Liver 2	10 m*M* Tris–HCl	20.4±1.78	19.8±0.61	40.1
	10 m*M* Tris–HCl+0.25 *M* sucrose	16.8±0.12	15.7±1.78	32.6

[a] The first liver was extracted with three volumes of 10 m*M* or 50 m*M* Tris–HCl, pH 7.4, 10 m*M* or 50 m*M* NaH_2PO_4, pH 7.4, and the second liver with 10 m*M* Tris–HCl, pH 7.4 or 10 m*M* Tris–HCl+250 m*M* sucrose in the same manner. The following preparation steps were the same for all preparations with subsequent MT separation and detection by HPLC–AAS.
[b] Values are means±S.E.M.

and MT-2. Finally, the detection limit of this optimized analytical procedure was determined by the method of Kaiser [22].

3. Results

The effect of various extracting reagents on MT yields is shown in Table 1. The amounts of extracted MT from liver were highest for 10 m*M* Tris–HCl or 10 m*M* NaH_2PO_4 buffer. The use of a higher buffer concentration of 50 m*M* gave slightly lower MT yields. A mixture of 10 m*M* Tris–HCl+250 m*M* sucrose provided less MT than extraction with 10 m*M* Tris–HCl buffer. Different buffer volumes also affected the yields of MT (Table 2). The highest MT concentrations were achieved with a liver/buffer ratio of 1:3 (w/v), representing a liver concentration of 25% in the homogenate, followed by the more concentrated 50% and 33% homogenates. The 25% homogenate provided an optimum balance between a high extraction of MT with dilution of the sample (10% and 20% homogenate) and a low extraction of MT with more concentrated extracts (33% and 50% homogenates). The effect of different Cd concentrations and incubation periods for a complete saturation of MT with Cd is presented in Tables 3 and 4. As shown in Table 3, the highest values of total MT were obtained using a Cd concentration of 50 μg/ml, which was in agreement with the recommendation of Lehman and Klaassen [15]. Increasing periods of incubation of the liver extract with cadmium at room temperature resulted in decreasing concentrations of total MT (Table 4). Thus, prolonged incubation at room temperature, rather than enhancing the measurable MT content, reduced it considerably and would therefore appear to be inadvisable. The subsequent heat treatment also had a considerable impact on MT-1 and MT-2 concentrations in the sample (Table 5). The highest MT

Table 2
Concentration of MT-1 and MT-2 in liver extracted with various amounts of buffer[a]

Liver concentration in the homogenate (%)	MT-1 (μg/g)[b]	MT-2 (μg/g)[b]	Total MT (μg/g)
10	7.0±0.06	10.6±2.37	17.6
20	8.1±0.05	10.7±0.90	18.8
25	11.5±1.21	12.8±0.75	24.3
33	9.6±1.89	12.0±1.03	21.6
50	8.9±0.95	14.8±0.28	23.7

[a] Rat liver was divided in equal portions and extracted with decreasing amounts of 10 m*M* Tris–HCl, pH 7.4. The resulting homogenates containing 10%, 20%, 25%, 33% and 50% (w/v) liver were then treated in the same manner and MT separated and detected by HPLC-AAS.
[b] Values are means±S.E.M.

Table 3
Concentration of MT-1 and MT-2 measured in liver after addition of various amounts of $CdCl_2$ during sample preparation[a]

Cadmium content (μg/ml)	MT-1 (μg/g)[b]	MT-2 (μg/g)[b]	Total MT (μg/g)
5	11.5±1.5	13.3±0.5	24.8
25	14.3±0.9	16.2±1.1	30.5
50	14.3±0.8	19.6±0.9	33.9
75	9.9±1.4	15.4±0.2	25.3
100	6.6±1.5	13.8±1.0	20.4

[a] Liver was homogenized with three volumes of 10 m*M* Tris–HCl, pH 7.4, followed by two centrifugation steps at 10 000 *g* (20 min, 4°C) and 114 000 *g* (1 h, 4°C). A portion of 1000 μl of the resulting supernatant was then saturated with 50 μl of $CdCl_2$ at different concentrations to achieve a final cadmium content of 5, 25, 50, 75, 100 μg/ml, respectively. After saturation the samples were treated equally, separated and MT detected by HPLC–AAS.
[b] Values are means±S.E.M.

levels were obtained after 1 or 2 min of incubation in a 80°C water bath while lower temperatures or longer incubation periods decreased the total MT content. For the subsequent column HPLC separation an incubation period of 2 min was favored because of better denaturation of matrix proteins and lower protein concentration in the sample compared with an incubation period of 1 min.

The concentrations of MT in raw liver after storage are shown in Table 6. Storage of tissues led to a reduction in the MT content depending on duration and storage temperature. When livers were stored for 1 month at −80°C total MT losses relative to fresh liver were on average three times greater than after storage at −20°C. After 3 months' storage the reductions of MT in livers stored at −80°C and −20°C respectively were almost identical, the maximum loss being 44% MT.

Table 4
Effect of different incubation times during sample preparation on MT-1 and MT-2 content measured in liver[a]

Incubation time (min)	MT-1 (μg/g)[b]	MT-2 (μg/g)[b]	Total MT (μg/g)
0	17.1±1.63	20.2±0.70	37.3
5	15.2±0.85	19.7±0.70	35.0
15	13.9±2.68	18.1±0.16	32.0
45	9.8±0.66	12.6±3.06	22.4

[a] Liver was homogenized with three volumes of 10 m*M* Tris–HCl, followed by two centrifugation steps at 10 000 *g* (20 min, 4°C) and 114 000 *g* (1 h, 4°C). A portion of 1000 μl of the resulting supernatant was saturated with 50 μl of $CdCl_2$ to achieve a final cadmium concentration of 50 μg/ml and then incubated at room temperature for 0, 5, 15 or 45 min. After incubation the samples were treated equally, separated and MT detected by HPLC–AAS.
[b] Values are means±S.E.M.

Table 7 shows the MT-1 and MT-2 concentrations of the centrifuged MT extract from liver over a 101-day storage period. The MT concentration declined during this time, although losses averaged only 9% after 3 months. With this method of storage the reduction was more marked for MT-1 than for MT-2.

4. Discussion

It is commonly known that the greatest losses of analytical material occurs during sample storage and sample preparation. This is especially true for the quantitation of metallothionein isoforms in animal tissues like rat liver where close attention must be paid to potential losses during sample preparation because metallothionein, whilst being stable to denaturation by short-term heat treatment [23–25], seems to be very labile in tissue extracts [26–28]. We therefore focused on minimizing MT losses during the most critical steps of sample preparation. In addition to the influence of extraction conditions such as buffer concentration and sample/buffer ratio, the cadmium content and the incubation period with the added cadmium also had a marked effect on the measured MT content; in the case of the incubation period this was entirely negative. Incubation of the liver preparation with $CdCl_2$ is essential in order to saturate MT with Cd^{2+} and make it detectable for AAS. High temperatures during incubation should therefore ensure a fully saturated MT for quantitative analysis [15]. But the results show that long incubation periods at room temperature are not necessary and that, on the contrary, degradation of MT-2 and especially MT-1 increases with prolonged incubation. The MT degradation during long-term incubation at room temperature might be caused by the high enzymatic processes which occur in biological samples, so that there predominate proteolytic, oxidative or in general katabolic reactions. Enzymatic katabolic processes may also be responsible for the high MT losses during incubation at 60°C for protein

Table 5
Effect of time and temperature of the heat denaturation during sample preparation on MT-1 and MT-2 content measured in the liver[a]

Temperature (°C)	Incubation time (min)	Protein (μg/ml)[b]	MT-1 (μg/g)[b]	MT-2 (μg/g)[b]	Total MT (μg/g)
60	1	20.2±1.67	10.7±1.79	6.4±0.76	17.0
	2	13.4±0.81	12.0±2.32	11.0±3.21	23.0
	5	11.1±1.41	12.0±3.32	12.9±1.58	24.9
80	1	12.5±0.91	17.1±0.83	15.0±1.11	32.1
	2	7.2±1.96	16.5±1.00	15.7±0.73	32.2
	5	5.1±0.18	13.1±4.43	14.9±4.60	28.1
100	1	6.5±0.51	13.5±2.38	17.1±1.41	30.6
	2	3.5±0.13	11.7±0.70	15.4±0.34	27.2
	5	3.4±0.12	13.0±0.91	11.5±1.41	24.5

[a] Rat liver was homogenized in three volumes of 10 m*M* Tris–HCl buffer, pH 7.4, followed by two centrifugation steps at 10 000 *g* (20 min, 4°C) and 114 000 *g* (1 h, 4°C). The supernatant was mixed with $CdCl_2$ to a final concentration of 50 μg Cd/ml and then heat-denatured at 60, 80 or 100°C for 1, 2 or 5 min without prior incubation. Following heat denaturation the samples were treated equally in the manner described and MT separated and detected by HPLC–AAS. The protein content of the resulting supernatant after heat denaturation was also measured.
[b] Values are means±S.E.M.

Table 6
Effect of storage of raw rat liver samples at −20°C and −80°C on the total metallothionein content

Storage time	Temperature (°C)	Liver 1		Liver 2	
		Total MT (μg/g)	Change[a] (%)	Total MT (μg/g)	Change[a] (%)
Fresh liver	–	15.5	–	16.1	–
1 month	−80	14.5	−6	13.1	−19
	−20	9.9	−36	8.8	−45
3 months	−80	8.7	−44	11.5	−29
	−20	8.7	−44	10.6	−34

[a] Change of MT content in percent compared with fresh liver. Each sample was measured twice for MT-1 and MT-2. For total MT content, MT-1 and MT-2 data were added up.

Table 7
Effect of storage of liver supernatant at −80°C on the metallothionein isoforms[a]

Storage time (days)	MT-1		MT-2	
	μg/g[b]	Change (%)[c]	μg/g[b]	Change (%)[c]
3	16.5±2.4	–	17.4±2.8	–
15	16.0±1.5	−3	19.7±0.7	+13
43	15.8±1.7	−4	15.7±0.7	−10
51	15.1±1.2	−8	15.3±1.7	−12
101	14.4±1.0	−13	16.6±1.2	−5

[a] Prior to storage the liver was extracted and centrifuged and only the supernatant stored at −80°C and measured for MT-1 and MT-2 content over a 101-day period.
[b] Values are means±S.E.M.
[c] Change of MT content in percent compared with fresh liver.

denaturation. Although, MT has been shown to be stable to denaturation by short-term heat treatment [23–25], data from the present study show that temperatures of 80°C and 100°C over a period of 1 or 2 min cause distinct decomposition of MT. Our results show clearly that MT degradation can be largely avoided by short-term heat treatment and an immediate cooling step afterwards, although short-term heat treatment at 80°C and 100°C only constitutes a compromise solution between MT preservation and protein denaturation.

Degradation of MT is also an inevitable reaction that occurs during storage of biological samples. It is evident from the present data that a storage temperature of −80°C and extraction of MT from liver prior to storage can substantially reduce MT losses (Tables 6 and 7). With this method of storage MT losses averaged only about 9% over 101 days, although the reduction of MT-1 was greater than that of MT-2. Compared with the usual way of storing liver slices, MT losses were reduced by over 80%. Interestingly, the MT-2 concentration increased slightly until day 15 of the storage process, before it decreased. Although, the reason for that phenomenon is unclear, we think that the increase in MT concentration is rather based on analytical troubles than on biochemical reactions.

As a result of the analytical improvements described the following optimized method was devised: the fresh rat liver was homogenized with three volumes of 10 m*M* Tris–HCl, pH 7.4 (sparkled with N_2), followed by two centrifugation steps at 10 000 *g* (20 min) and 114 000 *g* (1 h). The resulting supernatant was portioned in 1 ml quantities and mixed with 50 μl of 1000 mg Cd/l $CdCl_2$ solution to achieve complete Cd saturation of metallothionein. The mixture was then heat treated for 2 min in a 80°C water bath and chilled immediately in ice water. Following a further centrifugation at 13 000 *g* (5 min) and membrane filtration (0.22 μm), a 500-μl aliquot was applied to an ion-exchange column and MT-1 and MT-2 were separated by the HPLC elution procedure described. The collected 1 ml fractions of the column efflux were measured for their cadmium content and the MT-1 and MT-2 concentrations calculated by the following regression equations:

$$\text{MT-1 } (\mu g/0.5\ ml) = 0.0413 \times (\text{ng Cd}/0.5\ ml) + 0.210$$

$$\text{MT-2 } (\mu g/0.5\ ml) = 0.0403 \times (\text{ng Cd}/0.5\ ml) + 0.067$$

The validation of this method, which was carried out by the standard addition method, revealed a good linear relationship of MT-1 and MT-2 with regression coefficients of $R^2 = 0.986$ and 1.00, respectively, which are in close agreement with other authors [15,23,24]. The recovery obtained was 96% (MT-1) and 110% (MT-2). Moreover, the reproducibility of five preparations of raw liver provided relative standard deviations of 9.6% for MT-1 and 11.6% for MT-2. Finally, as a result of the optimized sample preparation, together with the described separation and quantitation method for MT isoforms by HPLC–AAS, the detection limit was reduced threefold on average in comparison to the original method of Lehman and Klaassen [15]. In sample solutions a detection limit of 0.46 μg/ml was achieved for MT-1 with this method and an even lower limit for MT-2 of 0.31 μg/ml. This equates to a detection limit of 2.0 μg MT-1/g liver or 1.3 μg MT-2/g liver. In contrast to many other HPLC methods described in the literature, both isoforms of rat liver MT could be separated and detected in the liver of untreated animals [14–17,23,29,30]. Interestingly, Klaassen and co-worker reported that only MT-2 was detectable with their method in untreated rat liver [14,15]. This observation contrasts with the present study, which revealed an isoform ratio of MT-1 to MT-2 of nearly 1:1 on average. There are several possible reasons for this difference. One explanation is that, as MT-1 has been reported to be more sensitive to degradation than MT-2 [28,31], higher MT-1 losses may occur during storage and preparation of the liver sample, resulting in a lower MT-1/MT-2 ratio. This hypothesis was supported by our finding that MT-1 was reduced more than MT-2 during storage of liver preparations (Table 7) and that the incubation period recommended by Lehman and Klaassen [15] of 15 min during sample preparation resulted in distinctly higher MT-1 losses than for MT-2 (Table 4). Moreover, the detection limit of their method of 5 μg/g liver [15] and 3 μg/g liver [14], respectively might have been too high for an accurate analysis of MT-1 in these examined livers. Thus, it can be concluded from this study that modification of some of the critical steps in sample

preparation and HPLC analysis for separation and quantitation of MT from the original method of Lehman and Klaassen [15] resulted in an improved detection limit of MT isoforms and yielded higher MT-1 concentrations, so that the ratio of MT-1 to MT-2 can be set to be about 1.

Compared with other methods based on HPLC separation technology the advantage of this improved procedure is a consistently good separation and detection of both MT isoforms of rat liver even at low MT concentrations in the tissue, as found in the livers of untreated rats (Fig. 1). On account of the low detection limit of this method it may also be possible to analyze tissues other than liver to obtain reliable information about isoform concentrations. Nevertheless, this HPLC–AAS method does not achieve the level of detection sensitivity of immunological techniques like RIA or ELISA [10,32,33]. But these methods, which use mostly polyclonal antibodies, can often detect only one isoform or total MT of rat liver with changing affinity for each isoform. The ability of the method described here to discriminate between individual isoforms and to detect them separately with good reliability therefore makes it a viable alternative to immunological techniques. Our improved method has several other advantages such as ease of handling without the need for a complex and highly sensitive coupling technique between HPLC and AAS [34–37], simple equipment and fast sample preparation and detection.

In summary, improved sample storage and preparation combined with a rapid HPLC–AAS method provides a reliable and sensitive measure of basal hepatic MT-1 and MT-2 levels. The procedure can

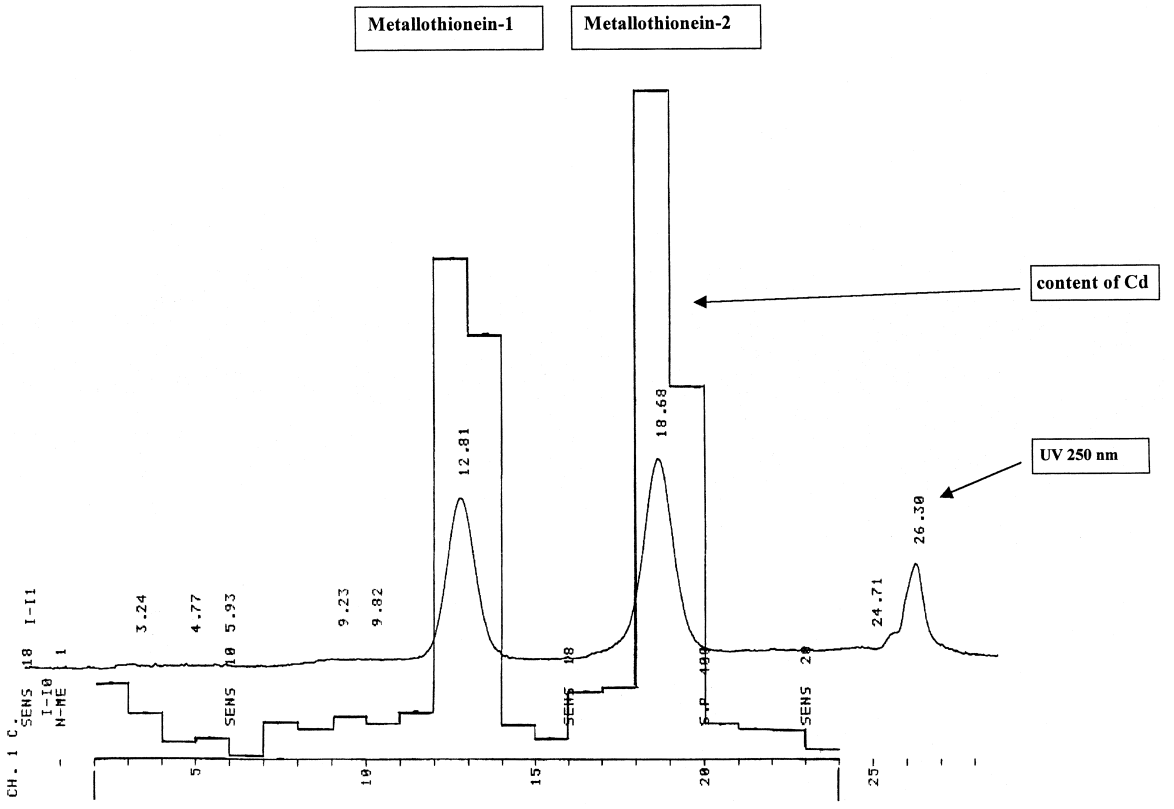

Fig. 1. UV and Cd chromatogram of rat liver. Anion-exchange HPLC separation of hepatic MT isoforms from untreated rat liver. The separations were performed with a Fractogel DEAE column (20–40 μm particle size) using a linear gradient from 20 to 128 m*M* Tris–HCl, pH 7.4 in 18 min at a flow-rate of 1 ml/min. A 500-μl aliquot of the prepared sample was injected. The column efflux was monitored for UV absorbance at 250 nm and 1-ml fractions measured for Cd content.

therefore be considered a helpful tool for investigating the isoform concentrations in a range of tissues and to obtain evidence of their putative roles in physiology.

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