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Isolation of ^{203}Mercury-Induced Metallothionein in Rat Kidney by Direct Connection of HPLC to a β Radioactivity Detector

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Rat Kidney ^{203}Hg-induced metallothionein (HgMT) was separated on a high performance liquid chromatograph equipped with a gel permeation column and an on-line β radioactivity detector, in order to obtain the simultaneous measurements of renal MT by UV detection and MT-associated ^{203}Hg by a β radioactivity detector. Metallothionein was separated in three major species by both UV detection at 254 nm and ^{203}Hg detection, probably due to the presence of mercury and copper. A standard curve was prepared which demonstrated excellent linear correlation between the integrated HgMT peaks area and the quantity of HgMT injected into the column. In contrast to the results with the gel permeation column above mentioned, rat kidney HgMT was separated in four peaks by reversed-phase hight performance liquid chromatography.

Key words: Metallothionein, Mercury, Rat kidney

Following chronic exposure of animals to inorganic mercury, the highest concentration of mercury was found in the kidney (1, 8). In the soluble cytoplasmatic fraction of this tissue, mercury has been shown to be associated with both high molecular-weight protein (HMWP) and to a higher extent, with metallothionein (MT), a low molecular-weight protein that contains an unusually high concentration of cysteine residues (5). Several functions have been proposed for MT including roles in heavy-metal detoxification, zinc and copper homeostasis (2), as a marker in mercury nephrotoxicity (9) and free radical scavenging (3).

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Gel permeation, ion-exchange and polyacrylamide gel electrophoresis have been used for the isolation of metallothionein in rat tissues (5, 7, 13). However, to our knowledge there are no studies on the renal mercury-metallothionein (HgMT) isolation by high performance liquid chromatography (HPLC).

The purpose of this study was to develop a method for the isolation and quantification of renal ^{203}Hg-induced metallothionein by HPLC (equipped with a gel permeation column) connected to an on-line β radioactivity detector, in order to obtain the simultaneous register of renal MT by UV detection, and MT-associated ^{203}Hg by a β radioactivity detector.

Materials and Methods

Isolation of metallothionein. — Metallothionein was induced in the kidney of male rats of the Sprague-Dawley strain (49 days old when killed) by adding 100 ppm mercury as mercuric chloride to the water, for 4 days. The rats were then injected intraperitoneally with 0.3 mg Hg/kg b.w. (10 μCi $^{203}HgCl_2$) 24 h prior to sacrifice. The kidney was homogenized in 2.5 volume of 0.25 M Sucrose, 0.01 M Tris-HCl pH 8.6 and 5 mM 2-mercaptoethanol, and centrifuged at 105,000 × *g* (90 min, 4 °C). The resulting supernatant was applied to a Sephadex G-75 column (2.6 × 70 cm) and eluted with 10 mM Tris-HCl buffer solution (pH 8.6), and 4.5 ml fractions were collected. Absorbance at 254 nm and the ^{203}Hg content in each fraction were determined. The metallothionein fractions were concentrated on a Diaflo YM-5 membrane and stored at −20 °C prior to analysis by HPLC.

HPLC equipment. — The outlet of a high-performance liquid chromatograph (Watters), equipped with a gel permeation column (Protein Pack 125, 7.8 × 300 mm), was directly connected to the radioactivity monitor (LKB Model 1208 Betacord). Samples were eluted with 20 mM ammonium formiate solution (pH 7.6 at 25 °C) at a flow rate of 0.4 ml/min. The flow rate of scintillation liquid (Atom flow, Dupont) was of 1.2 ml/min. Absorbance at 254 nm and ^{203}Hg were continuously monitored.

Furthermore, the reversed-phase high performance liquid chromatography (RP-HPLC) was also used for the isolation of the MT isoforms, according to the method described by RICHARDS and STEELE (11).

MT quantification. — Rat kidney MT quantification was based on peak area integration of UV absorbance (254 nm). The protein concentration was determined by LOWRY's method (6), using rabbit liver MT (Sigma) as standard. The integrated peak area was plotted versus μg of rat kidney MT applied onto the column; a linear regression analysis was used to determine the relationship.

Results and Discussion

The effect of chronic exposure of animals to inorganic mercury by using ^{203}Hg as radiotracer was studied in our laboratory. The mercury-induced metallothionein was evaluated in rat kidney after the isolation by HPLC equipped with a gel permeation column. This method, allows the isolation of metallothionein as distinct isoprotein peaks by elution with alkaline buffer solution, as in these conditions this column has cation-exchange chromatographic properties (12, 14).

In order to fully characterize the resolution of rat kidney metallothionein isoforms, rat kidney cytosol was fractionated by using classical chromatographic techniques (fig. 1). Gel permeation chromatography (Sephadex G-75) resolved the renal cytosol into four ^{203}Hg-containing peaks. The first peak represented protein complexes of relatively HMW (elution

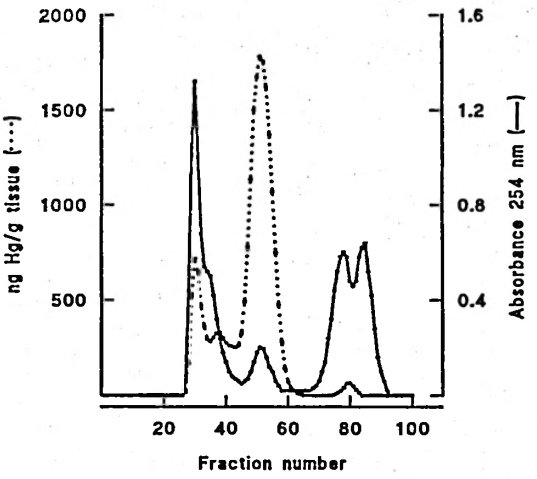

Fig. 1. *Gel filtration chromatography of the soluble supernatant or rat kidney after exposure to inorganic mercury.*
Rat supernatant (6 ml) was applied to a Sephadex G-75 column (2.6 × 70 cm) and eluted with 10 nM Tris-HCl buffer solution (pH 8.6). Fractions of 4.5 ml were collected. Absorbance at 254 nm was continuously monitored. The ^{203}Hg content in each fraction was determined.

volume/void volume, Ve/Vo = 1). The second peak appeared in the eluate in a position similar to that of myoglobine (Ve/Vo = 1.2-1.4). Both peaks represent 27 % of ^{203}Hg present in the renal cytosol. The MT peak, eluted at a position corresponding to a molecular weight of approximately 10.000 daltons (Ve/Vo = 1.7-2.0), represents 71 % of ^{203}Hg present in the renal cytosol. The fourth peak (Ve/Vo = 2.5-2.8), representing 2 % of ^{203}Hg total in renal cytosol, corresponds to complexes of low molecular weight.

The absorption spectrum of MT in the range of 240 to 300 nm results primarily from metal-ligand charge-transfer transitions. The shape of absorption spectrum and extinction coefficients at varied wavelenghts are characteristic of metals bound to the thionein (15). The central fraction of HgMT peak isolated from rat kidney exposed to mercuric inorganic shows an A_{254}/A_{280} ratio of 1.46.

A typical rat kidney metallothionein isolated on a Sephadex G-75 column gives

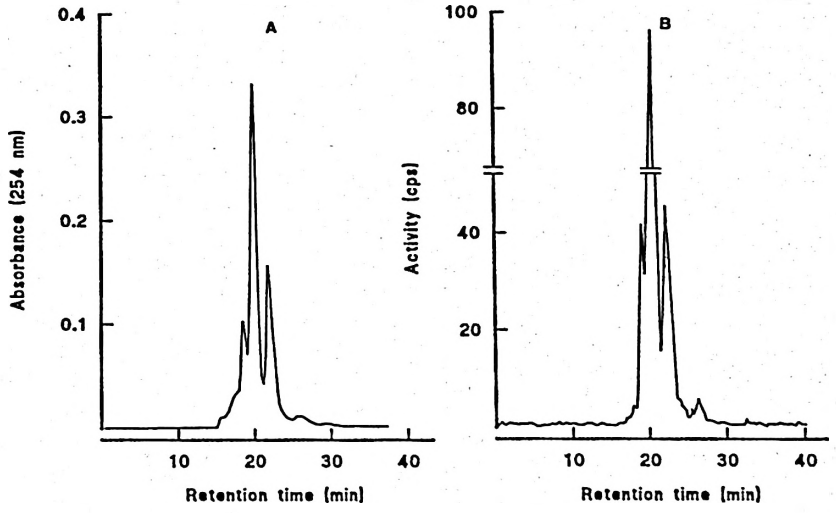

Fig. 2. *Elution profile of rat kidney HgMT on a HPLC equipped with a gel permeation column.*
A 75 µl aliquot of isolated HgMT in 10 mM Tris-HCl buffer, pH 8.6, was applied to a Protein Pack 125 column and the column was eluted with 20 mM ammonium formiate solution (ph 7.6 at 25 °C) at a flow rate of 0.4 ml/min. A liquid scintillation cocktail was added to the HPLC eluent at a flow rate of 1.2 ml/min before the eluent passed through the flow cell of the radioactivity monitor, and the presence of ^{203}Hg was continuously monitored. (A) UV 254 nm and (B) ^{203}Hg.

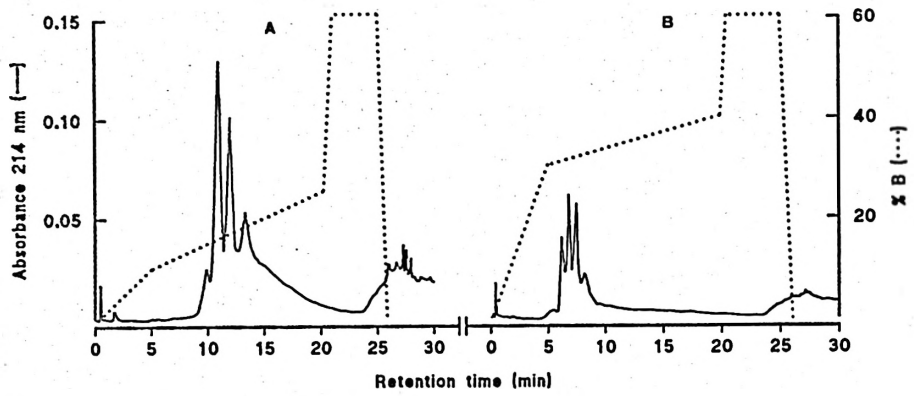

Fig. 3. *RP-HPLC of MT purified from rabbit liver (A) and of HgMT purified from rat kidney (B).* The separation of the isoMTs was performed with a μBondapack C_{18}, radially compressed cartridge column (10 μm particle size) and a two-step linear gradient (dashed line) of acetonitrile [0-6 % and 6-15 % (A); 0-18 % and 18-24 % (B)] in 10 mM sodium phospate, pH 7.0, at room temperature and at a flow-rate of 3 ml/min.

three peaks on a high performance liquid chromatograph equipped with a gel permeation column, due to the presence of copper and intramolecular oxidation (13, 14). By means of atomic absorption spectrophotometry, it was found that following chronic exposure of animals to inorganic mercury, an increase of Cu levels in rat kidney cytosol was observed; this excess of Cu was found to be associated mostly with MT (unpublished results), which is according to the results obtained by NOLAN and SHAIKH (7).

Figure 2 shows the elution profile for the HgMT on a HPLC equipped with a gel permeation column (Protein Pack 125). Three major peaks were resolved by both UV detection at 254 nm (fig. 2a) and ^{203}Hg detection (fig. 2b), probably due to the presence of mercury and copper. The first peak (rt = 18.8 min) represents 16 ± 4 %, the second peak (rt = 19.9 min) 56 ± 3 %, and third peak (rt = 22.0 min) 28 ± 1 % of the total absorption at 254 nm. In the same way, the first peak represents 19 ± 1 %, the second peak a 55 ± 2 %, and the third peak 26 ± 1 % of the total radioactivity associated to metallothionein. Both measurements are, therefore, identical within the experimental error of the technique.

These results are consistent with the data of other authors who reported that rats feeding low levels of stable mercury (100 ppm) in diets caused an increased uptake of labeled mercury in rat kidney MT (16) (this HgMT is separated in three isoforms by DEAE cellulose column) and that three isoforms of Hg, Cu-metallothionein are isolated, by preparative gel electrophoresis, from kidney of rats exposed to mercuric chloride (17) (the rats were administered sc with $^{203}HgCl_2$ at seven doses of 1 mg Hg/kg b.w. every other day).

In order to evaluate the quantification of MT by HPLC, known amounts of MT were injected onto the HPLC column and their peak areas integrated of UV absorbance (λ = 254 nm) were measured. The equation for the rat kidney HgMT applied onto the column was: $y = -2.9 + 0.7x$ ($r^2 = 0.999$). It was possible to detect 5 μg of MT injected into the column. The equations for the three peaks were: Peak I: $y = -4.6 + 0.2x$ ($r^2 = 0.997$), Peak II:

$y = 1.1 + 0.4 \times (r^2 = 0.997)$ and Peak III: $y = -0.7 + 0.2x$ ($r^2 = 0.998$), where y is the integrated peak area, x is μg of rat kidney HgMT injected into the column and r^2 is the correlation coefficient.

On the other hand, we have proceeded to separate the renal HgMT isoforms by RP-HPLC according to RICHARDS and STEELE method (11). One limitation of RP-HPLC has been encountered concerning the detection of copper-induced or copper-enriched MTs (4, 10). Richards founded that MT isoforms containing substantial amounts of copper could not adequately be separated and detected using RP-HPLC at neutral pH. Although the reasons for the inability of RP-HPLC to resolve copper-MTs have not yet been determined, this observation should be taken into account when attempting to analyze MTs containing copper. In contrast to the chromatographic profile after injecting rabbit liver MT into RP-column (fig. 3a), the rat renal HgMT shows a complex chromatographic profile with very poor resolution of several peaks. Nevertheless, with a few chromatographic modifications, we were able to separate the rat renal HgMT in four peaks with good chromatographic resolution (fig. 3b) and all of them contained significant amounts of ^{203}Hg.

Resumen

Se describe la separación de isometalotioneínas de riñón de ratas expuestas a cloruro de mercurio (II) en el agua de bebida. La metalotioneína renal de rata inducida con mercurio (HgMT) puede separarse en tres isoformas mayoritarias mediante cromatografía líquida de alta resolución (HPLC), usando una columna de filtración molecular, la cual, al eluir con una solución tampón débilmente alcalina, muestra una acción cromatográfica de intercambio iónico, además de la propia de filtración molecular. Los resultados sugieren que la separación cromatográfica es debida a la asociación de mercurio y cobre a la metalotioneína.

Palabras clave: Metalotioneína, Mercurio, Riñón de rata.

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