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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–INDUCTIVELY COUPLED PLASMA PROFILES OF CADMIUM, ZINC, SULPHUR AND OTHER ELEMENTS IN RAT LIVER SUPERNATANTS AFTER CADMIUM INJECTION

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SUMMARY

The distribution of cadmium, zinc, sulphur and other elements in the liver supernatants of rats and their variation with time following a single intraperitoneal injection of cadmium chloride were characterized by high-performance liquid chromatography (HPLC) coupled with an inductively coupled plasma. Cadmium that was bound to high-molecular-weight-proteins before the induction of metallothionein was analysed more easily using an Asahipak GS-520 column, whereas the distributions of other elements and of cadmium bound to metallothionein were better characterized on a TSK G3000SW column. Cadmium bound primarily to a distinct high-molecular-weight protein before the induction of metallothionein. After the injection its distribution shifted with time to metallothionein. An atomic emission spectrometer, employing an inductively coupled argon plasma and a vacuum UV monochromator was effectively used as a multi-element specific detector for HPLC.

INTRODUCTION

Element-specific detectors are especially useful when the distribution of metals, metalloids, and non-metals that may occur in different chemical forms are to be determined among biological constituents. Distributions of cadmium, zinc, and copper in tissue supernatants and body fluids were effectively determined using an atomic absorption spectrophotometer as a metal-specific detector for high-performance liquid chromatography (HPLC)[1,2].

Metals are known to interact within organisms. Therefore, it is essential to determine in a sample not only the metal in question, but also other biologically active inorganic constituents. Atomic emission spectrometers with an inductively coupled argon plasma (ICP) have the capability to detect many elements simultaneously. With a vacuum monochromator, an ICP can also quantify sulphur compounds, which may serve as ligands for heavy metals. Thus, the ICP is a powerful element-specific detector for HPLC *i.e.* (HPLC–ICP) in the simultaneous multi-element mode[3–6].

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Metals which bind non-selectively to biological molecules usually form complexes of low stability. Such metals are easily redistributed among the biological constituents when the samples are chromatographed. Therefore, the separation of metal-carrying proteins has to be performed with special care to avoid unintended redistributions of metal ions. The column material must not alter the original distribution of metals in the biological samples.

The present study was intended to apply the HPLC–ICP method to analyse metal-binding proteins in biological samples. As biological samples, liver supernatants of rats injected with cadmium were selected. Changes in the distribution profiles of cadmium, zinc, copper, iron, phosphorus, and sulphur with time after the injection were observed. Special attention was paid to metal complexes of low stability.

EXPERIMENTAL

Chemicals

Tris–HCl buffer solutions were prepared by mixing Trizma base and Trizma hydrochloride as indicated by the manufacturer (Sigma, St. Louis, U.S.A.). All other chemicals were purchased from Wako (Osaka, Japan).

Animals

Cadmium chloride was injected intraperitoneally into 45 male SD rats [8 weeks old; body weight (mean ± S.D.), 313 ± 6 g] with a single dose of 1.0 mg Cd/kg body weight (1 ml/rat). The animals were killed by exsanguination after 0.25, 0.5, 1, 3, 6, 12, 24, 48, and 72 h. Rats not treated with cadmium chloride served as controls (0 h). Each liver was homogenized in a Polytron homogenizer under a nitrogen atmosphere after addition of four volumes of 0.1 *M* Tris–HCl buffer solution (pH 7.4) containing 0.25 moles of glucose per l. The homogenates were centrifuged for 1 h at 170 000 *g* at 2°C. A 1.0-ml aliquot of each liver supernatant was digested with 1.0 ml of nitric acid–perchloric acid (5:1, v/v). The total element concentrations in these digests, were determined by ICP spectrometry[7]. Pooled samples for chromatography were prepared by combining 0.5 ml from each of the five supernatants obtained from the five animals in a group. Ten pooled samples (one control, nine cadmium-treated) were thus obtained.

HPLC–ICP

An SW column (TSKgel G3000SW, 600 × 7.5 mm with a guard column of 75 × 7.5 mm; Toyo Soda, Tokyo, Japan) and a GS column (Asahipak GS-520, 500 × 7.6 mm; Asahi, Tokyo, Japan) were used to chromatograph the ten samples. A 0.1-ml portion of the pooled supernatants was applied to the column. A Tris–HCl buffer solution (10 m*M*, pH 8.0 containing 0.1% sodium azide) served as the mobile phase for the SW column, and 0.9% sodium chloride solution containing 0.05% sodium azide was used for the GS column. The flow-rate was maintained at 1.0 ml/min by a Beckman HPLC Instrument Series 340. The mobile phases were degassed with a Shodex Degas degasser (Showa Denko, Tokyo, Japan). Absorbances at 254 and 280 nm were measured with a dual-wavelength ultraviolet detector (Model 152, Altex, Berkeley, U.S.A.). The column exit was connected via a short piece of PTFE tubing to the nebulizer of an ICP (JY48PVH, Seiko, Tokyo, Japan) and the machine

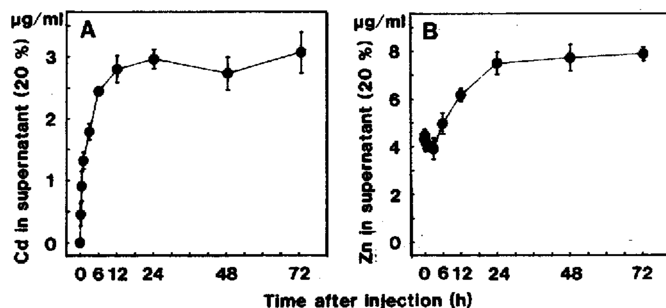

Fig. 1. Changes in the concentrations of (A) cadmium and (B) zinc in the liver supernatants of rats after a single injection of cadmium.

was operated under the conditions reported previously[8]. Emission intensity data were collected every 2 s for 30 min (900 data points) and stored on a floppy disk. Distribution profiles of the respective elements were drawn using our own software developed for a personal computer (PC-9801, NEC, Tokyo, Japan) and an XY-plotter (FP5301R, Graphtec, Tokyo, Japan). The data were expressed as mean ± standard deviation for the five samples in each group.

RESULTS AND DISCUSSION

Cadmium chloride injected intraperitoneally into rats was taken up by the liver. The cadmium concentration in the supernatant rapidly increased with time during the first hour after injection, and then somewhat slower during the next 5 h, until it reached a plateau after 12 h. The zinc concentration in the supernatant appeared to decrease during the first 3 hours, but statistically this was not significant. The zinc concentration then increased during the next 21 h (Fig. 1) at a slower rate

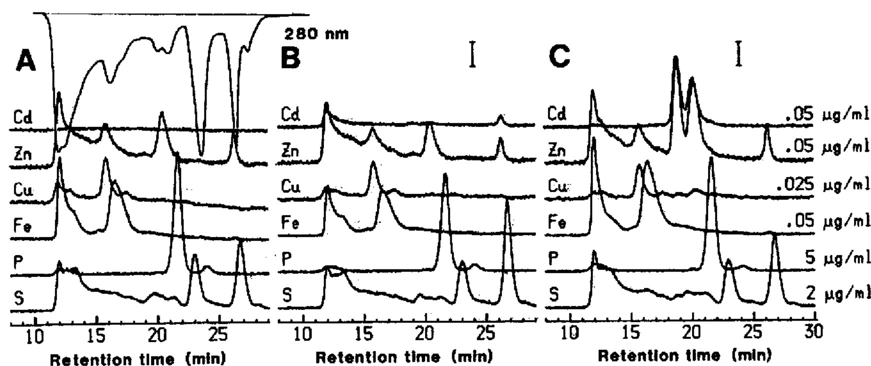

Fig. 2. HPLC–ICP profiles (SW column) of Cd, Cu, Fe, P, S, and Zn in liver supernatants obtained from (A) control rats and cadmium-treated rats, (B) 1 and (C) 72 h after treatment. Vertical bars indicate detection limits for each element. Top left: absorbance at 280 nm for the control profile. Peak assigment: exclusion volume, 11.8 min; superoxide dismutase, 15.7 min; haemoglobin, 16.5 min; metallothionein-II, 18.6 min, and -I, 20.0 min; glutathione, 23.0 min; cysteine, 26.1 min; taurine, 26.7 min.

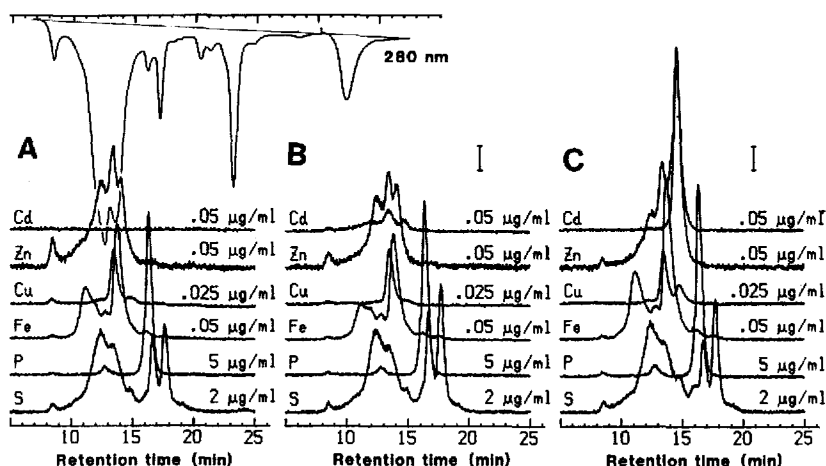

Fig. 3. HPLC–ICP profiles (GS column) for Cd, Cu, Fe, P, S, and Zn in liver supernatants obtained from (A) control rats and cadmium-treated rats, (B) 1 and (C) 72 h after treatment. Vertical bars idicate detection limits for each element. Peak assignment: exclusion volume, 8.2 min; ferritin, 11.0 min; superoxide dismutase, 13.3 min; haemoglobin, 13.5 min; metallothionein, 14.5 min; glutathione, 15.7 min; taurine, 17.6 min.

than that observed for cadmium. The concentrations of copper (0.57 μg/ml), iron (4.4 μg/ml), phosphorus (276 μg/ml), and sulphur (321 μg/ml) in the supernatant did not significantly change with time. The mean concentrations for the control animals are given in parentheses.

The ten pooled supernatant samples were chromatographed on an SW (Fig. 2) and a GS column (Fig. 3). The two columns were used for the following reasons. The GS column material does not contain silanol groups (as does silica gel), and seems to show less interactions with metals during separation procedures. It is suspected that cadmium-binding proteins with low stability constants before the induction of metallothionein could be separated on the GS column. On the other hand, the SW column was used to separate metallothionein into its two isoforms using both size-exclusion effects and cation-exchange properties under the present elution conditions[1,2]. Cadmium, copper, iron, phosphorus, sulphur, and zinc were determined in the eluate and representative profiles are shown in Figs. 2 and 3.

On the SW column with an alkaline buffer eluent metallothionein is separated into its two isoforms, metallothionein-II being eluted faster than metallothionein-I[1,2]. Cadmium was found at the exclusion volume, as well as near the column volume. The former is so-called high-molecular-weight-protein-bound cadmium, while the latter is cysteine-bound cadmium. Small cadmium peaks were also present at the two isoforms of metallothionein before its induction (Fig. 2B) and these two peaks became larger after the induction of metallothionein (Figs. 2C and 4).

A distinct zinc peak was present at a retention time almost identical to metallothionein-I before the induction of metallothionein (Fig. 2A and B). However, this native zinc was not displaced by the cadmium that was distributed over the high-molecular-weight proteins and cysteine (Fig. 2B). Further, the zinc peak at the metallothionein-I showed a slow-eluting shoulder, which was not observed for the cad-

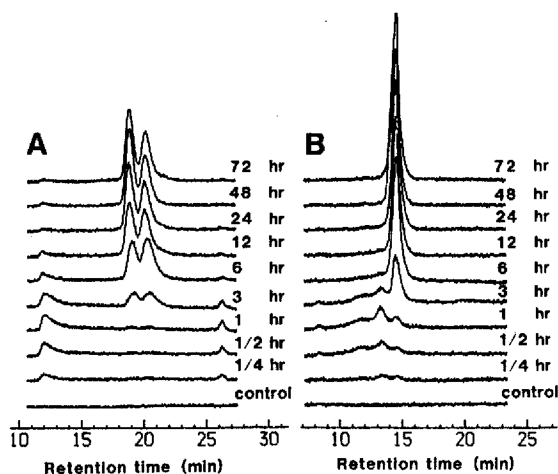

Fig. 4. Changes in the distribution profiles of cadmium in liver supernatants with time on (A) SW and (B) GS columns following treatment with cadmium. Peak assignment on the SW column (A): exclusion volume, 11.8 min; metallothionein-II, 18.6 min, and -I, 20.0 min; cysteine, 26.1 min. Peaks on the GS column (B): exclusion volume, 8.2 min; hepatic cadmium-binding protein-1, 11.9 min, and -2, 13.2 min; metallothionein, 14.5 min.

mium peak in Fig. 2C. This suggests that the zinc peak at the metallothionein-I is a mixture of metallothionein-bound zinc and the native zinc present in the control samples. These results may indicate that the zinc peak eluted near the metallothionein-I peak in the control profile is not metallothionein. The last zinc peak was eluted at the same retention time as the last cadmium peak, just before the big sulphur peak of taurine (Fig. 2).

Copper peaks were observed at 11.7 (the exclusion volume), 15.7 (superoxide dismutase accompanied by zinc), and 17.2 min (unidentified) before the induction of metallothionein. After the induction, copper was also found in the two isoforms of metallothionein (Fig. 2C). The iron peak at the exclusion volume is probably assignable to ferritin.

Sulphur was found throughout the chromatogram since it is a basic constituent of biological molecules. Sulphur peaks were present in the metallothionein fraction of the control supernatant. Oxidized glutathione is eluted around at 21.2 min and the sulphate ions appear as a broad peak just before the oxidized glutathione peak (assigned by co-elution with authentic samples). Sulphur peaks that could be assigned to metallothionein were observed only as tiny peaks (metallothionein-II in Fig. 2C) or were hidden under the peaks present in the control samples.

The same supernatants were also chromatographed on a GS column with 0.9% aqueous sodium chloride as the mobile phase (Fig. 3). Cadmium was found in four peaks on a GS column: a small peak at the exclusion volume, as a broad peak around 11.9 min (tentatively assigned to hepatic cadmium-binding protein-1), as relatively a sharp peak at 13.2 min (tentatively assigned to hepatic cadmium-binding protein-2) and at 14.5 min (metallothionein) (Fig. 3B). Metallothionein was eluted as a single peak on the GS column. The cadmium and zinc that was eluted with cysteine on the

SW column was not detected on the GS column, probably due to the interaction with the gel materials of the GS column.

The changes in the distribution of cadmium with time after the injection are summarized in Fig. 4. Before administration, cadmium peaks appeared at the exclusion volume and at the column volumes on the SW column. Three hours after the cadmium administration two cadmium peaks began to appear in the metallothionein region. The relative ratio of these two cadmium peaks changed with time as observed previously[9]. On the GS column cadmium was found to be mainly associated with hepatic cadmium-binding proteins-2 and -1 before the induction of metallothionein. After the induction it was mainly associated with metallothionein. Before induction hepatic cadmium-binding protein-1 and -2 were detected as distinct cadmium peaks, although it is not known at present why the cadmium bound to the high-molecular-weight-proteins is eluted so differently on the two columns. However, the slow elution of the two hepatic cadmium-binding proteins on the GS column may indicate that these proteins are relatively hydrophilic, because a GS column is known to separate globulins and albumin effectively. Albumin is eluted later than globulins, even when taking the difference in molecular size into consideration[10]. Although the two hepatic cadmium-binding proteins were detected on the GS column before the induction of metallothionein, they are probably related to the toxic form of cadmium after repeated injections of cadmium in the liver and kidney[11-15].

The changes in the distribution of zinc with time after injection are summarized in Fig. 5. Significant changes were observed only in the metallothionein region. A

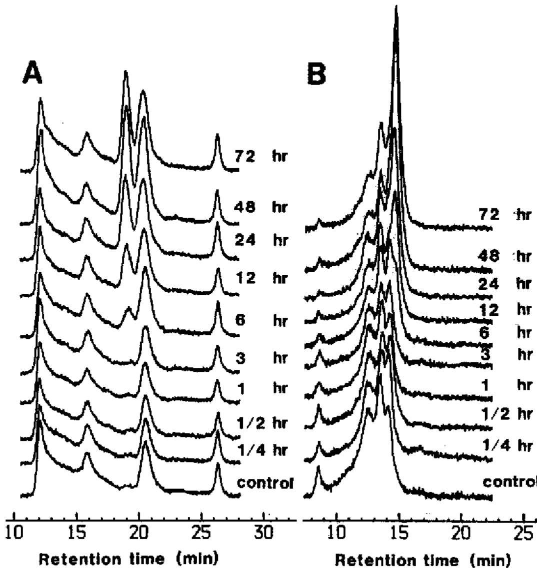

Fig. 5. Changes in the distribution profiles of zinc in liver supernatants with time on (A) SW and (B) GS columns following treatment with cadmium. Peak assigment on the SW column (A): exclusion volume, 11.8 min; superoxide dismutase, 15.7 min; metallothionein-II, 18.6 min, and -I, 20.0 min; cysteine, 26.1 min. Peaks on the GS column (B): exclusion volume, 8.2 min; superoxide dismutase, 13.3 min; metallothionein, 14.5 min.

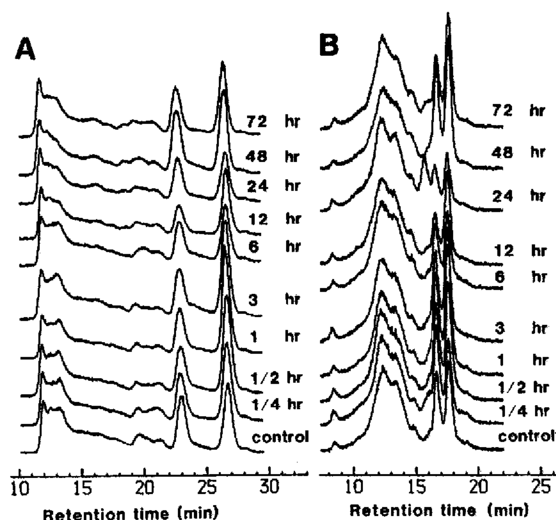

Fig. 6. Changes in the distribution profiles of sulphur in liver supernatants with time on (A) SW and (B) GS columns following treatment with cadmium. Peak assignment on the SW column (A): exclusion volume, 11.8 min; glutathione, 23.0 min; taurine, 26.7 min. Peak assignment on the GS column (B): exclusion volume, 8.2 min; metallothionein, 14.5 min; oxidized glutathione, 15.3 min; glutathione, 15.7 min; taurine, 17.6 min.

zinc containing peak appeared almost at the same retention time as the metallothionein-I peak, even before the induction of metallothionein, and this zinc was not displaced by cadmium in the two hepatic cadmium-binding proteins. Zinc containing peaks in the metallothionein region increased in size during 6 h following the injection, in accordance with the increase in size of the two cadmium peaks in this fraction. However, there was at least a 3-h lag between the start of the increase of zinc compared with that of cadmium in the metallothionein region.

The chromatograms for sulphur hardly changed with time (Fig. 6). A sulphur peak that could be assigned to metallothionein-II was just detectable on the SW column (Fig. 6A) 12 h after injection. However, the sulphur peak that corresponds to metallothionein-I was hidden under other sulphur peaks on the SW column. Oxidation of glutathione occurred accidentally for the 24 h sample (Fig. 6B) during storage between the analyses on the two columns (the same samples applied to the SW column were analysed on the GS column four weeks later because of instrument availability). The chromatograms for sulphur indicate that cadmium does not affect the distribution of endogenous sulphur to a significant extent.

An ICP emission spectrometer was shown to be effective as a multi-element specific detector for HPLC. One of the significant findings in the present work concerns the detection and characterization of cadmium-binding proteins on the GS column before the induction of metallothionein.

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