APPLICATION OF BIOLOGICAL SAMPLES TREATED BY WET-DIGESTED MINERALIZATION TO CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN REACTION BY 4-(2-PYRIDYLAZO)-RESORCINOL

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Abstract.....To quantify metals in biological samples, we tried to find good conditions for wet-digested mineralization of the samples and for separation of metal ions in chromatography. A 500 μ l volume of aliquot was transferred to a glass tube, and was evaporated at 100°C for 2 hours. A 5.5 ml volume of a mixture of concentrated nitric acid-70 % perchloric acid (10 : 1, v/v) was added and heated, consecutively, at 80°C for 12 hours, at 140°C for 2 hours, at 180°C for 2 hours, and finally at 190°C for 1 hour to evaporate the residual acids. After addition of 500 μ l of 10 mM nitric acid, the metals were extracted by a suspension mixer (32 r.p.m., 1 hour). One hundred μ l of the extracted solution was applied to the chromatographic system : cation-exchange column, TSKgel IC-Cation SW (Tosoh Co.) ; eluent, 0.35 M lactic acid-0.35 M sodium lactate (pH 3.0) ; flow rate, 0.7 ml/minute ; column temperature, 30°C. After adding a color-forming reagent (100 mg/l 4-(2-pyridylazo)-resorcinol in 40 g/l Na₂CO₃ ; flow rate, 0.7 ml/minute) to the effluent, five different metal ions of Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺ and Zn²⁺ were detected at 520 nm. The peaks were separated in approximately 25 minutes, and were quantified even at the 1–10 ppb levels. The present procedures were considered to provide simultaneous detection and accurate quantitation of the above five metals in the biological samples.

Key words : Metals, wet-digested mineralization, cation-exchange chromatography, 4-(2-pyridylazo)-resorcinol, biological samples.

INTRODUCTION

Several metals such as cadmium, mercury, lead, zinc etc. have been recognized as

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toxic trace elements for humans and animals (Smith and Larson, 1946 ; Ganther et al., 1972 ; Goyer, 1989). For biological and clinical researches on these metals, atomic absorption and emission spectrometry (Paulev et al., 1978 ; Tölg, 1977), stable isotope dilution mass spectrometry (Veillon et al., 1979), X-ray fluorescence spectrometry (Tölg, 1977), proton induced X-ray emission spectrometry (Berti et al., 1977), neutron activation analysis (King et al., 1978 ; Greenberg, 1980), inductively coupled plasma emission spectrometry (Uchida et al., 1981), ion-exchange chromatography (Nickless, 1985) and others (Ujiie et al., 1978 ; Matsumoto and Fuwa, 1986 ; Tamari et al., 1986 ; Turanek et al., 1987) have been employed. Among those, an application of high-performance liquid chromatographic (HPLC) technique using ion-exchange resin to separation of metal ions has enabled analysis times to greatly shorten (Seymour et al., 1971 ; Seymour and Fritz, 1973 ; Kawazu and Fritz, 1973). This ion-exchange HPLC method was furthermore developed by combining post-column color-forming reaction with 4-(2-pyridylazo)-resorcinol (Fritz and Story, 1974). This 4-(2-pyridylazo)-resorcinol has been known to react only with the metals including bismuth (Bi^{3+}), cadmium (Cd^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}), iron (Fe^{2+} , Fe^{3+}), hafnium (Hf^{4+}), mercury (Hg^{2+}), lanthanide (La^{3+}), manganese (Mn^{2+}), nickel (Ni^{2+}), lead (Pb^{2+}), thorium (Th^{4+}), vanadium (V^{3+}), zinc (Zn^{2+}) and zirconium (Zr^{4+}) (Fritz and Story, 1974). By these efforts, the analytical sensitivity as well as the selectivity in separation of metals were successfully increased. Though having the advantages of shortening of analytical time and of simultaneity in determination of several metals, the ion-exchange HPLC method in combination with the post-column reaction with 4-(2-pyridylazo)-resorcinol has been scarcely used for determination of the above metals in the biological samples. The followings are raised as the reasons : Samples of tissues and biological fluids generally need wet-digested mineralization with strong acids prior to application to the chromatography, and this strong acidity interferes the separation of metals. Even if neutralization of the acids with alkalis was done, increased salt concentration also interferes the separation. Accordingly, when the mineralized samples are applied to the chromatography, the samples are obliged to be diluted in order to weaken the acidity and/or to lower the salt concentration. As the result, errors will be largely brought forward to the determination.

In this paper, in order to resolve the above-mentioned problems, we tried to find good conditions for wet-digested mineralization of biological samples and for separation of metals in ion-exchange HPLC.

MATERIALS AND METHODS

Chemicals and Materials : 4-(2-Pyridylazo)-resorcinol monosodium salt was purchased from Merck Co. (Darmstadt, Germany). $\text{Bi}(\text{NO}_3)_3$, CdCl_2 , CoCl_2 , CuCl_2 , FeSO_4 , FeCl_3 , HfCl_4 , HgCl_2 , LaCl_3 , MnCl_2 , NiCl_2 , $\text{Pb}(\text{NO}_3)_3$, VCl_3 , $\text{Zn}(\text{NO}_3)_2$ and $\text{Zr}(\text{CH}_2\text{COCH}_2\text{COCH}_3)_4$ were obtained from Wako Pure Chemical Co. (Osaka,

Japan). All other reagents used in this study were commercial products of the highest grade available. A cation-exchange column (TSKgel IC-Cation SW; 5μ ; 4.5 mm i.d. X 5.0 cm length) was obtained from Tosoh Co. (Tokyo, Japan). Male Sprague-Dawley rats weighing nearly 200 g were obtained from Japan Charles River Co. (Atsugi, Kanagawa, Japan).

Preparation of Standard Solutions of Metal Ions : Acidic solutions at concentration of 1,000 ppm were prepared : Bi_3^+ in 0.5 N HNO_3 , Cd^{2+} in 1 N HCl , Co^{2+} in 1 N HCl , Cu^{2+} in 0.1 N HCl , Fe^{2+} in 0.1 N H_2SO_4 , Fe^{3+} in 0.1 N HCl , Hf^{4+} in 1 N HCl , Hg^{2+} in 0.02 N HCl , La^{3+} in 1 N HCl , Mn^{2+} in 0.02 N HCl , Ni^{2+} in 0.1 N HCl , Pb^{2+} in 1 N HNO_3 , V^{3+} in 1 N HCl and Zr^{4+} in 1 N HCl . These solutions were used at given concentrations by dilution with water and/or by mixing each other, when necessary.

Preparation of plasma and Homogenates of Liver, Pancreas and Kidney from Rats : Ten rats were allowed free access to water and standard diet pellet (MF ; Oriental Yeast Co., Chiba, Japan). Five rats received intravenous injection of 1 ml of 0.489 mg/ml CdCl_2 in saline per kg body weight, at 0.3 mg Cd^{2+} per kg body weight. The other 5 rats received intravenous injection of 1 ml of saline per kg body weight. At 30 minutes after the injection, operation to prepare plasma and homogenates of liver, pancreas and kidney were carried out. Under ether-anesthesia, the abdominal cavity was opened through ventral incision. Blood was taken from the abdominal aorta, put into a heparinized tube, and centrifuged ($1,700 \times g$, 10 min, 4°C) to obtain the plasma. After taking the blood, the liver, pancreas and kidney were perfused through the abdominal aorta with ice-cold saline, and the organs were removed. The organs were singly homogenized by a glass-teflon potter homogenizer with 0.25 M sucrose.

Extinction Coefficients of Metal Ions in Reaction with 4-(2-Pyridylazo)-Resorcinol : A 500 μl volume of one of the above metal ion solutions that were properly diluted by water was mixed with a 500 μl volume of color-forming reagent solution (100 mg/l 4-(2-pyridylazo)-resorcinol in 40 g/l Na_2CO_3). The absorbance was determined at 520 nm by a spectrophotometer (model 320, Hitachi Spectrophotometer ; Hitachi Co., Tokyo, Japan), and extinction coefficient of the metal ion was calculated and represented as $\text{cm}^2/\mu\text{g}$ metal.

Determination of Protein Content : Protein content of the homogenates was spectrophotometrically determined by the method of Bensadoun and Weinstein (1976) in which the method of Lowry et al. (1951) was modified so that protein could be assayed in the presence of interfering substances.

Wet-Digested Mineralization of Samples : Wet-digested mineralization of samples were done by modifying the method of Bayfield and Romalis (1985) and of Vezina and Bleau (1988). A 500 μl volume of the standard solutions, plasma and homogenates were singly transferred to 12 mm X 100 mm borosilicate glass tubes, and were heated at 100°C for 2 hours, to evaporate their water constituents, by heating blocks (model HF-41, Heating Block ; Yamato Scientific Co., Tokyo, Japan). After the tubes were

cooled in room temperature, a 5.5 ml volume of a mixture of concentrated nitric acid-70 % perchloric acid (10 : 1, v/v) was added. Then, they were heated by the heating blocks, consecutively, at 80°C for 12 hours, at 140°C for 2 hours, at 180°C 2 hours, and finally at 190°C for 1 hour to completely evaporate the residual acids. Then, the tubes were cooled at room temperature. After addition of 500 μ l of 10 mM nitric acid solution, the tubes were stopped and rotated by a suspension mixer (model RT-50, Rotator ; Taitec Co., Tokyo, Japan) at 32 r. p. m. for 1 hour to effect extraction of metals. These extracted water solutions were applied to the below HPLC system.

Cation-Exchange HPLC with Post-Column Reaction of 4-(2-Pyridylazo)-Resorcinol : The HPLC system used in this study, outlined in Fig. 1, consisted of two solvent delivery pumps (model LC-9A ; Shimadzu Co., Kyoto, Japan) fitted with dumpers for highly sensitive assay (Shimadzu Co.), an injector (model 7125, Syringe Loading Sampler Injector ; Rheodyne Co., Cotati, Cal., U. S. A.), a column heater for maintenance of column temperature (model 655A-52, Column Oven ; Hitachi Co.), a spectrophotometric detector (model L-4200, UV-VIS Detector ; Hitachi Co.). For recording chromatograms, a recorder (model FBR-252A, Flatbed Recorder ; TOA Electronics Co., Tokyo, Japan) was used. In addition, an integrator (model C-R3A, Chromatopak ; Shimadzu Co.) was used to determine peak area. Two solutions for elution and color-forming reagent solution were continuously degassed using a degasser (model 3510, Degasser ; Erma Optical Works Co., Tokyo, Japan). Reaction coil was of teflon tube (0.25 mm i.d. X 1 m length). Conditions of HPLC were as follows : elution solution, a mixture of 0.35 M lactic acid-0.35 M sodium lactate (pH 3.0) ; color-forming reagent solution, 100 mg/l 4-(2-pyridylazo)-

Fig. 1. Schematic diagram of cation-exchange high-performance liquid chromatography with post-column reaction of 4-(2-pyridylazo)-resorcinol.

Abbreviations : M. P., mobile phase for elution ; PAR, color-forming reagent solution containing 4-(2-pyridylazo)-resorcinol. Other details are represented in the section MATERIALS AND METHODS.

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resorcinol in 40 g/l Na_2CO_3 ; flow rates, 0.7 ml/min in both eluant and color-forming reagent solution ; column temperature, 30°C ; injection volume, 100 μl ; detection wave, 520 nm.

Statistics : Results were represented as the mean $\pm$ S.E. Statistical significance was assessed by the use of Student's t-test for unpaired samples, P values of less than 0.05 being considered significant.

RESULTS AND DISCUSSION

Color-Reaction of Metal Ions with 4-(2-pyridylazo)-Resorcinol : Intensity of color-reaction between respective metal ions and 4-(2-pyridylazo)-resorcinol was examined. Solutions containing each metal ion at given concentrations were singly reacted with 4-(2-pyridylazo)-resorcinol in color-forming reagent solution (final concentrations : 4-(2-pyridylazo)-resorcinol, 50 mg/l ; Na_2CO_3 , 20 g/l), and extinction coefficients ($\text{cm}^2/\mu\text{g}$) of the metal ions were spectrophotometrically determined at 520 nm (Table 1). The reaction with 4-(2-pyridylazo)-resorcinol was high in Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Mn^{2+} , Ni^{2+} and Zn^{2+} , moderate in Hg^{2+} and Pb^{2+} , and low in Bi^{3+} , Hf^{4+} , La^{3+} , V^{3+} and Zr^{4+} .

Table 1. Extinction coefficients of metal ions in reaction with 4-(2-pyridylazo)-resorcinol.

Extinction Coefficients ($\text{cm}^2/\mu\text{g}$ at 520 nm)	
Bi^{3+}	0.037
Cd^{2+}	0.572
Co^{2+}	0.889
Cu^{2+}	0.855
Fe^{2+}	0.797
Fe^{3+}	0.451
Hf^{4+}	0.072
Hg^{2+}	0.252
La^{3+}	0.012
Mn^{2+}	0.913
Ni^{2+}	0.867
Pb^{2+}	0.167
V^{3+}	0.003
Zn^{2+}	0.807
Zr^{4+}	0.037

The above metal ions were singly reacted in color-forming reagent solution containing 50 mg/l 4-(2-pyridylazo)-resorcinol and 20 g/l Na_2CO_3 . Their extinction coefficients at 520 nm were represented as $\text{cm}^2/\mu\text{g}$ metal. The values were represented as the mean in 6 experiments.

Retention Times of Metal Ions in HPLC : The metal ions were applied to the HPLC in order to determine their retention times. The retention times (minute) were as follows : 1.6 in Bi^{3+} , 16.2 in Cd^{2+} , 11.6 in Co^{2+} , 3.1 in Cu^{2+} , 14.8 in Fe^{2+} , 1.3 in Fe^{3+} , 20.1 in Mn^{2+} , 8.0 in Ni^{2+} , 64.9 in Pb^{2+} and 6.6 in Zn^{2+} ; peaks of Hf^{4+} , Hg^{2+} , La^{3+} , V^{3+} and Zr^{4+} were not found. As the reasons why the peaks of Hf^{4+} , Hg^{2+} , La^{3+} , V^{3+} and Zr^{4+} were not observed, the following three were raised individually or in combination with each other : the color-formation was quite low (in cases of Hf^{4+} , La^{3+} , V^{3+} and Zr^{4+}) ; the metals were quickly eluted as the void peak (1.30 minutes) ; the metals were strongly adsorbed in the cation-exchange resin and not eluted in the present HPLC system. In case that the other metal ions were referred as to the suitability in this HPLC, Pb^{2+} was observed as a broad peak ranging between 64 and 90 minutes and at more than 5 ppm. In addition, the peaks of Bi^{3+} and Fe^{3+} were near to the void peak and overlapped with one another. Further, it was found that Fe^{2+} was oxidized to Fe^{3+} by the mineralization described below. Thus, Bi^{3+} , Fe^{2+} , Fe^{3+} and Pb^{2+} were considered not to be directly applicable to the present HPLC determination, and the metal ions that were able to be determined in the HPLC were concluded to be restricted in the 6 metal ions of Cd^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} .

Detection Limits of Metal Ions in HPLC : In order to investigate detection limits and practical detection limits, the standard mixtures containing Cd^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} at given concentrations (0.1 ppb to 10 ppm) were applied to the HPLC with injection volume of 100 μl . Detection limit was defined as concentrations of the signal/noise ratio = 1/3, and practical detection limit was defined as the range in which linearity existed. Detection limits and practical detection limits (in parenthesis) were as follows : >7.0 ppb (>10.0 ppb) in Cd^{2+} , >7.0 ppb (>10.0 ppb) in Co^{2+} , >5.0 ppb (>10.0 ppb) in Cu^{2+} , >0.7 ppb (>1.0 ppb) in Mn^{2+} , >0.5 ppb (>1.0 ppb) in Ni^{2+} , >0.3 ppb (>1.0 ppb) in Zn^{2+} . Linearities in the metal ions excluding Mn^{2+} were recognized between each practical detection limit and 10 ppm, whereas in Mn^{2+} between the practical detection limit and 5 ppm.

Recovery Rates of Metal Ions in HPLC : In order to investigate recovery rates following the treatment of wet-digested mineralization, 0.5 ml of a standard mixture containing Cd^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} , Ni^{2+} and Zn^{2+} at 1 ppm each was treated by the same wet-digested mineralization as in the plasma and homogenates, and extracted with 0.5 ml of water or nitric acid solutions (10, 50 and 100 mM) by rotation at 32 r.p.m. for 1 hour (Table 2). The recovery rates of Co^{2+} , Cu^{2+} and Ni^{2+} were low in the water, while they were increased to nearly 100 % in the 10, 50 and 100 mM nitric acid solutions. The recovery rate of Mn^{2+} was low in the water or nitric acid solutions (10, 50, and 100 mM). Even when the extraction time was prolonged up to 24 hours, and when hydrochloric acid and sulfuric acid solutions (10, 50 and 100 mM) were used, the recovery rate of Mn^{2+} remained nearly 20 %. In order to increase the recovery rate of Mn^{2+} , the molar concentrations of the acids for the extraction were considered to be increased. However, the molar concentrations to

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Table 2. Recovery rates of metal ions in extraction dy water and 10, 50 and 100 mM nitric acid solutions.

	Recovery Rates (%)			
	Water	10 mM HNO ₃	50 mM HNO ₃	100 mM HNO ₃
Cd ²⁺	101.5±2.0	98.6±2.3	100.4±1.9	98.9±1.0
Co ²⁺	82.8±4.0	97.5±2.2	98.5±1.2	98.2±0.6
Cu ²⁺	86.9±3.4	104.5±2.4	109.2±4.6	104.6±1.6
Mn ²⁺	20.9±0.9	22.6±5.5	26.7±4.5	17.9±5.5
Ni ²⁺	55.3±5.5	98.4±1.4	97.7±1.2	94.1±0.9
Zn ²⁺	100.6±2.2	102.9±2.1	102.6±2.9	101.1±1.0

A 0.5 ml volume of a standard mixture containing Cd²⁺, Co²⁺, Cu²⁺, Mn²⁺, Ni²⁺ and Zn²⁺ at 1 ppm each was treated by the same wet-digestive mineralization as in the plasma and homogenates, and extracted with 0.5 ml of water or nitric acid solutions (10, 50 and 100 mM HNO₃) by rotation at 32 r. p. m. for 1 hour. Values were represented as the mean ± S. E. in 6 experiments.

be injected to the column were recommended to be as low as 0.1 M for prevention of deterioration the cation-exchange resin. Thus, based on these findings, we adopted 10 mM nitric acid solution as the extraction solution and 1 hour as the time for extraction, and concluded that Mn²⁺ was not able to be determined by the present HPLC method.

Furthermore, in order to investigate recovery rates of the metal ions in samples, a 0.5 ml volume of a standard mixture containing Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺ and Zn²⁺ at 1 ppm each was added to a 0.5 ml volume of 10 % liver homogenate, and the mixture was treated by the wet-digested mineralization. The recovery rates that were calculated by subtraction from the metal ion contents of the mixture by those in the liver homogenate were nearly 100 % in each of metal ions (Table 3).

Reproducibility : Intra-assay reproducibility was evaluated by measuring the contents of Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺ and Zn²⁺ in the identical mixture of the standard solution (1 ppm) and the liver homogenate. The coefficient of variation was calculated from the mean and standard deviation of the values (N=6). We obtained coefficients of variation of 3.9 % in Cd²⁺, 3.1 % in Co²⁺, 4.0 % in Cu²⁺, 3.5 % in Ni²⁺ and 8.7 % in Zn²⁺.

Determinations of Contents (Concentrations) of Metals in Liver, Pancreas and Kidney Homogenates and Plasma : Rats received intravenous injections of either saline or CdCl₂ solution (0.3 mg Cd²⁺ per kg body weight), and liver, pancreas, kidney and blood were taken at 30 minutes after the injection. Metal ion contents (concentrations) in the liver, pancreas and kidney homogenates and the plasma were determined in the present HPLC method (Fig. 2 and Table 4). Cu²⁺ and Zn²⁺ were detected highly in the plasma, liver, pancreas and kidney of the rats that received saline, while Ni²⁺ and Co²⁺ were sporadically detected in a trace in those samples. In comparison to the rats that received saline, the rats that received CdCl₂ solution

Table 3. Recovery rates of metal ions in standard solution when mixed with liver homogenate.

	Recovery Rates (%)
Cd^{2+}	96.6 ± 1.5
Co^{2+}	98.8 ± 1.2
Cu^{2+}	100.5 ± 1.7
Ni^{2+}	96.9 ± 1.4
Zn^{2+}	91.4 ± 3.2

A 0.5 ml volume of a standard mixture containing Cd^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} and Zn^{2+} at 1 ppm was added to a 0.5 ml volume of 10 % liver homogenate, and the mixture was treated by the wet-digestive mineralization. The recovery rates were calculated by subtraction from the metal-ion contents of the mixture by those liver homogenate. Values were represented as the mean $\pm$ S. E. in 6 experiments.

Fig. 2. Representative chromatogram of metal ions in liver homogenate obtained from rat at 30 minutes after intravenous injection of cadmium chloride solution. Liver homogenate was mineralized and extracted as described in the section **MATERIALS AND METHODS**. Injection volume, 100 μl . Peaks ; 1, void peak ; 2, Fe^{3+} ; 3, Cu^{2+} ; 4, Zn^{2+} ; 5, Ni^{2+} ; 6, Co^{2+} ; 7, Cd^{2+} ; 8, Mn^{2+} . Peaks 1 and 2 probably contained Bi^{3+} and others.

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Table 4. Concentrations and contents of metal ions plasma and liver, pancreas and kidney in rats at 30 minutes after intravenous injection of saline or cadmium chloride solution.

	Cu^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}	Cd^{2+}
Plasma ($\mu\text{g/ml}$)					
Saline	0.786 ± 0.167	0.890 ± 0.145	0.001 ± 0.001	0.003 ± 0.003	0.001 ± 0.001
CdCl_2 Sol.	1.187 ± 0.114	$1.487 \pm 0.059^*$	0.001 ± 0.001	0.004 ± 0.003	$0.262 \pm 0.004^*$
Liver ($\mu\text{g/g}$ protein)					
Saline	24.6 ± 1.4	148.5 ± 3.7	5.3 ± 2.4	0.3 ± 0.2	0.1 ± 0.1
CdCl_2 Sol.	33.2 ± 4.0	162.4 ± 9.0	0.1 ± 0.1	0.8 ± 0.3	$15.1 \pm 4.4^{**}$
Pancreas ($\mu\text{g/g}$ protein)					
Saline	34.7 ± 8.6	192.1 ± 23.0	4.4 ± 2.7	0.2 ± 0.2	1.0 ± 1.0
CdCl_2 Sol.	24.2 ± 7.2	147.7 ± 16.4	0.1 ± 0.1	0.7 ± 0.5	3.9 ± 1.0
Kidney ($\mu\text{g/g}$ protein)					
Saline	29.9 ± 4.9	131.1 ± 7.4	4.4 ± 3.0	2.1 ± 0.9	0.1 ± 0.1
CdCl_2 Sol.	$57.7 \pm 8.5^*$	$200.5 \pm 26.1^*$	4.1 ± 3.4	1.7 ± 0.7	$4.7 \pm 0.6^{**}$

Abbreviations : Saline, rats that received saline ; CdCl_2 Sol., rats that received CdCl_2 solution (0.3 mg Cd^{2+} per kg body weight). Values were represented as the mean $\pm$ S. E. in 5 rats. Significant differences from " Saline" : *, $P < 0.05$; **, $P < 0.01$.

detected highly in the plasma, liver, pancreas and kidney of the rats that received saline, while Ni^{2+} and Co^{2+} were sporadically detected in a trace in those samples. In comparison to the rats that received saline, the rats that received CdCl_2 solution showed increments of Cd^{2+} in the plasma, liver and kidney, of Cu^{2+} in the kidney, and of Zn^{2+} in the plasma and kidney. As to why the intravenous administration of CdCl_2 brought the increments of plasma concentration of Zn^{2+} and of Cu^{2+} and Zn^{2+} , a further discussion shall be done in our next papers on the basis of the interaction among the metals. Here, we discuss on the advantages and disadvantages in the present HPLC and wet-digested mineralization procedures.

As described in the section *Introduction*, atomic absorption and emission spectrometry, stable isotope dilution mass spectrometry, X-ray fluorescence spectrometry, proton induced X-ray emission spectrometry, neutron activation analysis, inductively coupled plasma emission spectrometry etc., are, no doubt, elegant and excellent methods. However, these are quite special and not always utilized in general laboratories. In contrast, the HPLC apparatus are popularized in the laboratories for analytic works. Thus, our method will be able to be applied in the general laboratories.

The ion-exchange HPLC method has the advantages of shortening of analytical time (approximately 25 minutes in our HPLC measurement) and of simultaneity in determination of metals. Concerned with separation of metals in ion-exchange

HPLC, Reiffenstuhl and Bonn (1989) succeeded in separation of Na^+ , K^+ , Cu^{2+} , Ni^{2+} , Co^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , Cd^{2+} and Ca^{2+} using a conductivity detector. However, the HPLC measurement with the conductive monitoring did not completely rule out a risk that one peak contained more than two substances. 4-(2-Pyridylazo)-resorcinol reacts only with the metals including Bi^{3+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Hf^{4+} , Hg^{2+} , La^{3+} , Mn^{2+} , Ni^{2+} , Pb^{2+} , Th^{4+} , V^{3+} , Zn^{2+} and Zr^{4+} (Fritz and Story, 1974). So, the peaks that are found in our HPLC are restricted in these metal ions. Accordingly, our method is considered to rule out the risk that each peak contains other metals than it, when the determination is restricted in the 5 metals of Cd, Co, Cu, Ni and Zn. This is an advantage in selectivity for separation, and, at the same time, a disadvantage with respect to a fact that number of metals determined is restricted in 5. In addition, Cd, Co, Cu, Ni and Zn are metals that are important for researches on metal toxicity, in particular, on interaction among those in the body (Smith and Larson, 1946; Ganther et al., 1972; Goyer, 1989). Therefore, the simultaneous determination of the 5 metals is considered to be greatly helpful to this kind of researches. Further, this method has another advantage that the use of 4-(2-Pyridylazo)-resorcinol increased the sensitivity to ppb level in comparison to the level of ppm in conductivity detection (Reiffenstuhl and Bonn, 1989): this sensitivity in our HPLC is near to that in the above-mentioned special methods of the atomic absorption spectrometry and so on. In addition, it should be emphasized that our procedures in mineralization and extraction treatments made possible to chromatographically determine concentrations (contents) of the 5 metals of Cd, Co, Cu, Ni and Zn in the body fluids and organs.

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