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GEL PERMEATION, ION-EXCHANGE AND REVERSED-PHASE COLUMNS FOR SEPARATION OF METALLOTHIONEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ATOMIC ABSORPTION SPECTROPHOTOMETRY

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SUMMARY

Metallothioneins were separated on three kinds of HPLC column that differ in separation principle: gel permeation, ion exchange and reversed phase. Elution of the columns at neutral to weakly basic buffer conditions prevented dissociation of the metals, and the metals bound to metallothioneins were quantitatively eluted out. The metals in the eluate were continuously determined on an atomic absorption spectrophotometer by introducing the eluate directly to the nebulizer tube. Prerequisites for the on-line separation–detection system are discussed.

INTRODUCTION

Metallothionein is a small protein rich in cysteinyl residues and heavy metals[1]. Although several biological roles have been proposed for the protein[2], no biological activities that can be used as bioassays for the protein have been reported. Hence the protein has been detected and quantitated on the basis of measurement of bound metals or apoprotein. Although simplified methods involving the quantitation of mercury[3] or cadmium[4] after replacement of metals in metallothionein with these metals can be used for known metallothionein samples, more elaborate methods are required to characterize metallothioneins of different biological origins. A similar drawback exists for a sensitive method recently developed as radioimmunoassay[5,6].

Although the conventional method for the separation of metallothionein, a combination of gel filtration and ion-exchange columns, has several disadvantages in sample size and time required for the analysis, it is still the first choice for metallothioneins of unknown species. Recently we have reported a new analytical method for metallothionein which detects the bound metals after separation by a combination of high-performance liquid chromatography (HPLC) on a gel permeation column and atomic absorption spectrophotometry (AAS)[7,8]. The new method has been shown to possess several advantages over the conventional method, and metallothionein can be separated as distinct isoprotein peaks by elution with alkaline buffer solution owing to cation-exchange chromatographic action.

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Metallothionein is a kind of metalloprotein that can be most easily characterized by analysing the metals bound to the protein. Therefore, a specific detector for metals is recommended and seems to be more efficient than general detectors such as UV–visible and refractive index detectors for HPLC. Although a commercially available atomic absorption spectrophotometer is not specifically designed as a detector for an HPLC, it has been used as a sensitive detector for metals[8].

There are several prerequisites for the separation and detection of metalloproteins by HPLC–AAS. Some are common to the separation of metalloproteins: (i) buffers used for the separation should not behave as ligands for the metals and should be neutral or weakly basic to prevent the dissociation of the metals bound to the proteins; (ii) the column materials should not behave as ligands for the metals and should not adsorb the metals. Other conditions relate to the use of AAS: (iii) the concentrations of the buffers and salts in the eluting solutions are recommended to be low so that the burner-head of the spectrophotometer does not become clogged; (iv) the eluting solutions should not contain co-burning materials, such as organic solvents, or at least the composition of the eluting solution should not be changed suddenly during the detection. Any factors that may disturb the baseline of the spectrophotometer give erroneous peaks. As to the interface between the column and the spectrophotometer, we have introduced the eluate into the nebulizer tube through a thin tube, and the difference between the column flow-rate and the spectrophotometer uptake rate has been adjusted by the back-pressure through the thin tube.

The present study was intended to separate metallothioneins on columns of different separation principle and to detect the metals by HPLC–AAS. Gel permeation, ion-exchange and reversed-phase columns were examined to separate metallothioneins of different animal origins, and suitable conditions for the detection of the metals by AAS were selected.

EXPERIMENTAL

Chemicals

The following reagents were purchased from Nakarai (Kyoto, Japan): acetic acid (CHR-101), *n*-propyl alcohol (CHR-176) and trifluoroacetic acid (BIO-74); from Wako (Osaka, Japan): triethylamine; from Kanto (Tokyo, Japan), acetonitrile; from Sigma (St. Louis, U.S.A.), Trizma.

Metallothioneins used in the present experiment were prepared by injecting cadmium chloride into the respective animals and by separating the supernatant fraction of the livers on a Sephadex G-75 column as already reported[9–11].

Apparatus

HPLC for gel permeation chromatography was carried out on equipment consisting of an HLC 803A pump (Toyo Soda, Tokyo, Japan) for solvent delivery, with a column (TSK gel G3000 SW, 600 × 7.5 mm I.D. with a TSK guard column SW 75 × 7.5 mm I.D., Toyo Soda), a dual wavelength ultraviolet detector (Model 152, Altex, Berkeley, U.S.A.) and spectrophotometer with an acetylene flame (Hitachi 170-50A). Anion-exchange chromatography was performed on HPLC equipment (SP-8700, Toyo Soda) with a column (TSK gel DEAE-3SW, 75 × 7.5 mm I.D.), and the eluate was detected with a dual wavelength detector and an atomic absorption

spectrophotometer. Reversed-phase column chromatography was carried out on an HPLC Series 340 (Beckman, Berkeley) with one of three reversed-phase columns (Ultrasphere-Octyl, 250 × 4.6 mm I.D., Beckman; Ultrapore RPSC, 75 × 4.6 mm I.D., Beckman; TSK gel TMS-250, 75 × 4.6 mm I.D., Toyo Soda) and the same detecting system was used.

RESULTS AND DISCUSSION

Gel permeation chromatography

Rat liver metallothionein was separated into the two isometallothioneins (Fig. 1A) and rat kidney metallothionein into the three peaks (Fig. 1B) owing to the presence of copper and intramolecular oxidation, as already reported[12]. Although chicken metallothionein has been reported to consist of a single isoprotein[13], Japanese quail metallothionein was shown to consist of two isoforms[9]; one of them is always a minor constituent as shown in Fig. 1C. Liver metallothionein induced in the frog *Xenopus laevis* is also composed of a single isoprotein (Fig. 1D)[10]. Gibel liver me-

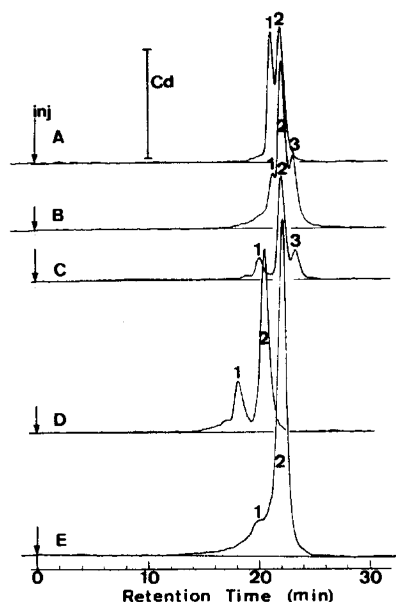


Fig. 1. Elution profiles of metallothioneins on an SW column by HPLC–AAS. A 100-μl aliquot of isolated metallothioneins in 10 m*M* Tris–HCl buffer, pH 8.6, was applied to a TSK gel G3000 SW column and the column was eluted with 50 m*M* Tris–HCl buffer solution (pH 8.0 at 25°C, containing 0.1% NaN_3, dissolved gases were removed at 80°C under reduced pressure before use) at a flow-rate of 1 ml/min on an HLC 803A chromatograph. The eluate was directly introduced into the nebulizer tube and the concentration of cadmium in the eluate was continuously monitored. The void volume of the column corresponds to a retention time of 11.3 min. The vertical bar indicates the detector level of the spectrophotometer (0.1 μg Cd/ml). (A) Rat liver metallothionein: 1 (21.2 min) = MT-II; 2 (22.2 min) = MT-I. (B) Rat kidney metallothionein, 1 (21.3 min) = MT-II; 2 (22.2 min) = MT-I and oxidized MT-II; 3 (23.2 min) = oxidized MT-I). (C) Japanese quail liver metallothionein: 1 (20.1 min) = dimer; 2 (22.1 min) = MT-II; 3 (23.4 min) = MT-I). (D) Frog (*Xenopus laevis*) liver metallothionein: 1 (18.3 min) = dimer; 2 (20.6 min) = MT. (E) Gibel liver metallothionein-I: 1 (20.0 min) = dimer; 2 (22.0 min) = MT-I.

tallothionein consists of two isoforms[11]. The elution profile in Fig. 1E was obtained by applying one isoform (MT-1) separated on a DEAE Sephadex A-25 column.

Gel permeation chromatography of metallothioneins by HPLC–AAS indicates the presence of different chemical species of metallothioneins, or at least the presence of metallothioneins with different electric charges in the respective samples. Metallothioneins with different electric charges can be separated only when the silanol and hydroxyl groups in the silica gel and the polyhydroxylated polymer of the gel material, respectively, dissociate at a basic pH[7,8]. Separation of metallothionein due to the difference of electric charge was not observed by elution at an acidic buffer.

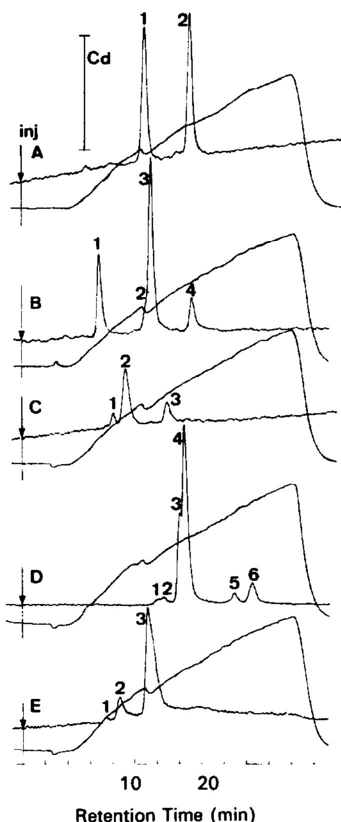
Ion-exchange chromatography

Although metallothionein can be separated into isoforms on an SW column by elution with alkaline buffer as shown in Fig. 1 owing to cation-exchange of the gel permeation column, the protein was not retained on a cation-exchange column for HPLC (TSK gel CM-3SW, 75 × 7.5 mm I.D.) at neutral to weakly basic buffer conditions. On the other hand, conventional conditions for the separation of metallothionein on an anion-exchange column were found to be applicable to separation by HPLC; metallothionein was eluted as distinct isoprotein peaks on an anion-exchange (TSK gel DEAE-3SW) by elution with a concentration gradient of Tris–HCl buffer at pH 8.6 (data not shown). However, as eluting buffers of more basic pH require more concentrated buffers for the elution, and the concentrated buffers choke the burner-head of the spectrophotometer, less basic buffers have to be used for HPLC–AAS. In fact, the burner-head was rapidly choked by 250 m*M* Tris–HCl buffer.

The elution profiles shown in Fig. 2 were obtained by elution with a concentration gradient of Tris-HCl buffer at pH 7.2. The two isoforms of rat liver metallothionein were eluted at concentrations of 42 and 62 m*M* (MT-I and -II, respectively). Intra- and intermolecular oxidation products of metallothioneins were also eluted as distinct peaks under these conditions, and recoveries of cadmium and zinc bound to metallothionein were quantitative (more than 95%).

The order of elution on an anion-exchange column is the reverse of that on an SW column. The two isoforms of rat liver metallothionein were eluted as shown in Fig. 2A, whereas four peaks were observed for rat kidney metallothionein (Fig. 2B), the two peaks corresponding to MT-I and -II being far smaller than the other two. We have explained the different elution profiles of rat kidney metallothionein by the presence of copper and/or oxidative formation of disulphide bonds[12]. The three peaks of rat kidney metallothionein on an SW column (Fig. 1B) have been reconstituted by adding cupric ion *in vitro* to zinc-thionein, and the central peak (peak 2 in Fig. 1B) was suggested to be a mixture of two peaks[12]. The elution profile of Fig. 2B indicates that the central peak in Fig. 1B is a mixture of two peaks: MT-I and oxidized MT-II. The elution profile of rat kidney metallothionein changed with time during storage and analytical procedures. This will be discussed later.

Although *Xenopus* metallothionein was eluted as two peaks (dimer and monomer, the former being formed *in vitro* during storage) on an SW column (Fig. 1D), it was eluted as six peaks on an anion-exchange column (Fig. 2D). The six peaks seem to consist of three pairs of two peaks. We have already pointed out that *Xenopus* metallothionein is easily oxidized to a form more cationic than the original[14]. Further



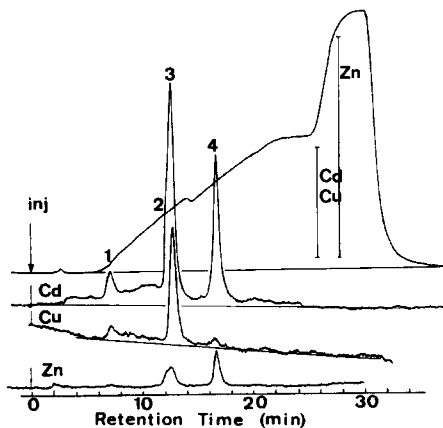


Fig. 2. Elution profiles of metallothioneins on an anion-exchange column by HPLC–AAS. A 100-μl aliquot of isolated metallothioneins in 10 m*M* Tris–HCl buffer, pH 8.6, was applied to a TSK gel DEAE-3SW column and the column was eluted with concentration gradient of Tris–HCl buffer solution (pH 7.2 at 25°C, dissolved gases were removed by bubbling through with helium gas before use) at a flow-rate of 1 ml/min on a SP-8700 chromatograph as follows: Buffer B (limiting buffer, 250 m*M* Tris–HCl solution) was linearly mixed to Buffer A (starting buffer, 10 m*M* Tris–HCl solution) up to 106 m*M* concentration during 20 min and then Buffer A was eluted as shown in the figures. The eluate was directly introduced to the nebulizer tube and the concentration of cadmium in the eluate was continuously monitored. The buffer concentration in the eluate was monitored just before the nebulizer tube in a flow-cell of a conductivity meter (CD-35MII, M & S Instruments, Tokyo). Sodium ion was eluted at a retention time of 2.8 min. The vertical bar indicates the detector level of the spectrophotometer (0.1 μg Cd/ml). (A) Rat liver metallothionein: 1 (11.1 min) = MT-I) 2 (15.2 min) = MT-II. (B) Rat kidney metallothionein, 1 (7.0 min) = oxidized MT-I; 2 (11.0 min) = MT-I; 3 (11.7 min) = oxidized MT-II; 4 (15.4 min) = MT-II. (C) Japanese quail liver metallothionein: 1 (8.2 min) = MT-I; 2 (9.4 min) = MT-II; 3 (13.1 min) = dimer. (D) Frog (*Xenopus laevis*) liver metallothionein, 1 (12.2 min); 2 (12.8 min); 3 (14.1 min) = oxidized MT; 4 (14.7 min) = MT; 5 (19.2 min); 6 (20.8 min). (E) Gibel liver metallothionein-I: 1 (7.5 min); 2 (8.8 min); 3 (11.4 min) = MT-I.

Fig. 3. Elution profile of rat kidney metallothionein on an anion-exchange column by HPLC–AAS. The method was as given in the legend to Fig. 2, except for the buffer gradient. The concentration gradient of Tris–HCl buffer was prepared as follows: Buffer B (limiting buffer, 250 m*M* Tris–HCl solution) was linearly mixed with Buffer A (starting buffer, 10 m*M* Tris–HCl solution) up to 106 m*M* during 16 min, this concentration was maintained for 4 min and then the column was washed by increasing the concentration to 250 m*M* during 1 min and maintaining at 250 m*M* for 4 min. The concentration of cadmium, copper or zinc was continuously monitored. Sodium ion was eluted at 2.7 min. Peaks: 1 (7.1 min) = oxidized MT-I; 2 (12.1 min) = MT-I; 3 (12.6 min) = oxidized MT-II; 4 (16.7 min) = MT-II. The vertical bars indicate that the detector level of the spectrophotometer was set as shown in the figure (0.1 μg Cd, Cu or Zn per ml). The sample used for this figure was same as that for Fig. 2B, but was fresher than that for Fig. 2B.

characterization of the six peaks was not performed in this study. However, peak 3 can be identified tentatively as an oxidized form of peak 4 (original *Xenopus* metallothionein) and the same relationship may be applicable to the other two peak pairs.

The isoform of gibel liver metallothionein separated on a conventional DEAE Sephadex A-25 column was eluted as a broad peak on an anion-exchange column accompanied by two small peaks (peaks 1 and 2, possibly intramolecular oxidation products) as shown in Fig. 2E. The dimer peak was not observed as a sharp peak.

Rat liver metallothionein that contains cadmium and zinc is stable during repeated storage and analytical procedures. On the other hand, rat kidney metallothionein with a high content of copper is unstable and soon gives a different peak ratio among peaks on SW and also on anion-exchange columns. This change is obvious in the two figures, Figs. 2B and 3. The latter was obtained with a fresh sample, whereas the former figure was recorded after repeated storage and analytical procedures of the same sample. Peaks 2 and 4 in Fig. 3 changed to peaks 1 and 3 in Fig. 2B, respectively. This change is probably facilitated by the copper present in rat kidney metallothionein.

This separation–detection procedure thus gives rapid and precise information as to the isoforms and difference in their electric charges. Reproducibility of the results from ion-exchange chromatography is also satisfactory, as shown in Fig. 3 for the repeated analyses.

Reversed-phase column chromatography

Metallothioneins have been demonstrated to be separable on a reversed-phase C_8 column by HPLC[15]. However, metallothioneins were mainly detected using a general detector, a UV detector. An atomic absorption spectrophotometer was also used as a specific detector of the metals, but it was used as an off-line detector by Klauser *et al.*[15].

Our aim is to detect metals bound to less stable complexes, such as metals bound non-selectively to proteins. Reversed-phase columns seem to be superior to gel permeation and ion-exchange columns because the column materials for reversed-phase columns are in principle free of ligands for metals. Therefore, we have examined conditions for the elution of metals in their native forms, and metallothionein was selected as a model of stable complexes. Metallothioneins were separated on reversed-phase columns by HPLC and characterized by detecting the metal bound to the protein on an on-line detector as follows.

Fig. 4 demonstrates the elution profiles of metallothioneins on a reversed-phase C_3 column. The two isoforms of rat liver metallothionein were eluted in the reverse order to an anion-exchange column (Fig. 4A). In contrast to liver metallothionein, rat kidney metallothionein gave a complex profile on a reversed-phase column (Fig. 4B), similar to those on gel permeation and anion-exchange columns. Peaks in Fig. 4B were not correlated to those in Figs. 1B, 2B and 3 in the present study. The avian metallothionein was separated into three peaks (Fig. 4C) as on the other two columns (Figs. 1C and 2C). Although the native frog metallothionein consists of a single isoform, it was separated into complex peaks (Fig. 4D) mainly because of its instability[14]. The complex peaks are probably formed by intra- and intermolecular oxidations and are not due to microheterogeneity of amino acid composition. The fish metallothionein was separated mainly into three peaks, as on an anion-exchange column (Fig. 4E).

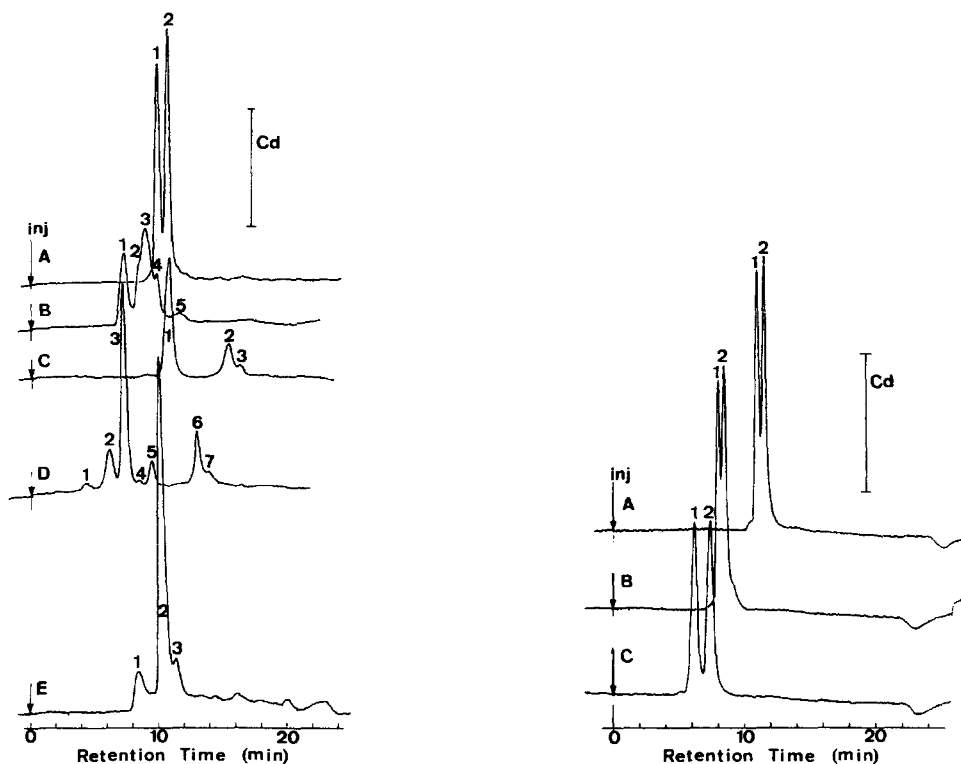


Fig. 4. Elution profiles of metallothioneins on a reversed-phase column by HPLC-AAS. A 100-μl aliquot of isolated metallothioneins in 10 m*M* Tris-HCl buffer, pH 8.6, was applied to an Ultrapore RPSC column and the column was eluted with the following buffer system at a flow-rate of 1 ml/min on an HPLC Series 340 chromatograph: Buffer B (limiting buffer, *n*-propyl alcohol-50 m*M* Tris-HCl, 80:20 v/v pH 7.2) was linearly mixed with Buffer A (starting buffer, 50 m*M* Tris-HCl buffer, pH 7.2) from 2 to 7% concentration (v/v) during 20 min. The eluate was directly introduced to the nebulizer tube and the concentration of cadmium in the eluate was continuously monitored. The vertical bar indicates the detector level (0.1 μg Cd/ml). (A) Rat liver metallotheionein: 1 (9.9 min) = MT-II; 2 (10.8 min) = MT-I. (B) Rat kidney metallothionein: 1 (7.3 min); 2 (8.4 min); 3 (9.0 min); 4 (9.9 min); 5 (11.5 min). (C) Japanese quail liver metallothionein: 1 (10.8 min) = MT-II; 2 (15.4 min) = MT-I; 3 (16.2 min). (D) Frog (*Xenopus laevis*) liver metallothionein: 1 (4.3 min); 2 (6.2 min); 3 (7.2 min) = MT; 4 (8.5 min); 5 (9.4 min); 6 (13.0 min); 7 (13.9 min). (E) Gibel liver metallotheonein-I: 1 (8.5 min); 2 (9.6 min) = MT-I; 3 (9.9 min).

Fig. 5. Comparison of reversed-phase columns for separation of metallothioneins by HPLC-AAS. A 100-μl aliquot of rat liver metallothionein in 10 m*M* Tris-HCl buffer, pH 8.6, was applied to three kinds of reversed-phase HPLC column: Ultrasphere-Octyl (250 × 4.6 mm I.D., Beckman); Ultrapore RPSC (75 × 4.6 mm I.D., Beckman); TSK gel TMS-250 (75 × 4.6 mm I.D., Toyo Soda). The columns were eluted with the following buffer at a flow-rate of 1 ml/min on an HPLC Series 340 chromatograph: Buffer B (limiting buffer, *n*-propyl alcohol-50 m*M* Tris-HCl buffer, 20:80 v/v, pH 7.2) was linearly mixed with buffer A (starting buffer, 50 m*M* Tris-HCl, pH 7.2) from 5 to 25% during 20 min, 25 to 100% during 1 min and then 100 to 5% (v/v) during 1 min. The concentration of cadmium was continuously monitored. The vertical bar indicates the detector level of the spectrophotometer (0.1 μg Cd/ml). (A) C_8 packing (Ultrasphere-Octyl column), MT-II (11.0 min), MT-I (11.6 min). (B) C_3 packing (Ultrapore RPSC column), MT-II (8.0), MT-I (8.5 min). (C) C_1 packing (TSK gel TMS-250 column), MT-II (6.2 min), MT-I (7.4 min).

Metallothioneins were shown to be separable on a reversed-phase C_3 column at a neutral pH (pH 7.2), and the recovery of cadmium in metallothionein was estimated at more than 95% for rat liver metallothionein. Therefore, metallothionein can be separated on two reversed-phase columns, C_8[15] and C_3 columns (present study). In order to separate and detect metals bound to proteins with diverse stability constants, reversed-phase columns of different hydrophobicity were compared by elution with the same condition. Fig. 5 shows the elution profiles of rat liver metallothionein on three reversed-phase columns, C_8(A), C_3(B) and C_1(C). When the column length of C_8 packing is taken into consideration (3.3 times longer than the other two), the separation of the two isoforms of rat liver metallothionein (Fig. 5) indicates that the least hydrophobic column is better. Although metals bound to high-molecular-weight proteins in the liver supernatant fraction were not eluted from C_8 and C_3 columns at neutral pH, a proportion of those metals was eluted from the C_1 column. These results suggest that the C_1 column is better for separation of metallothionein and probably also of proteins at neutral to weakly basic conditions.

CONCLUSION

Separation of metallothioneins on three HPLC columns with different separation principles and detection of the metals by AAS revealed that metallothioneins can be purified and characterized with a small sample size in a short time. The reproducibility is shown to be sufficient to characterize and quantitate the several metals bound to different isoforms by repeating the analytical procedure. The on-line detection of metals by HPLC–AAS at neutral to weakly basic pH was thus demonstrated to be a useful analytical method for metallothioneins. However, separation and detection of metals bound to proteins and other biocomponents with low stability constants must await more elaborate procedures.

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