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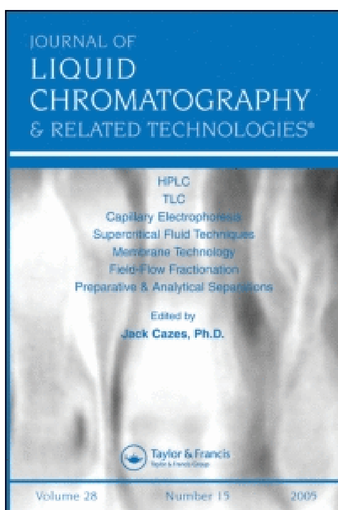

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METHYLATION OF RESIDUAL CARBOXYL GROUPS IN GEL PERMEATION COLUMN AND ITS EFFECT ON ELUTION AND DISTRIBUTION OF METALS AND PROTEINS IN BLOOD SERUM

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ABSTRACT

Residual carboxyl groups in gel permeation column materials (hydrophilic polymer gels) (Asahipak GS-520) were methylated with boron trifluoride methanol complex to minimize the interaction of metals between the ligands in substrates and in gel materials. Although zinc ions were eluted very slowly as an extremely broad peak on the original column, the metal ions were eluted faster as a lesser broad peak on the methylated column. Cadmium ions were eluted faster as a relatively sharp peak on the methylated column than the original column. Alkali earth metal ions were eluted also as sharper peaks on the methylated column. Zinc in rat serum was eluted more with globulins and less with albumin on the methylated column than on the original column. Globulins and albumin in rat and human sera were separated more efficiently on the methylated column. These results suggest that methylation of residual carboxyl groups in gel materials decreased the interaction of metals with gel materials and increased hydrophobicity of the gel materials.

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INTRODUCTION

Metals bound to their specific metal-binding proteins are stable and have less chances to dissociate during usual separation and detection procedures. Superoxide dismutase, for example, belongs to this category of metal-binding proteins. Copper and zinc in this protein bind firmly to the specific ligands forming stable tertiary structures (1).

On the other hand, metals bound non-specifically to proteins and other biological constituents are unstable and easily dissociate or interact with other ligands resulting in the redistribution of metals during analytical procedures. Albumin is probably one of the representative proteins that bind various metals less specifically (2) and may have chances to give distributions of metals different from their original ones due to the interactions of metals with ligands among the substrates, gel materials and buffers. As a trial to minimize the interaction, charged groups in gel materials have been reduced with sodium borohydride (3) and a low molecular weight zinc-binding ligand from human milk was assigned using a modified Sephadex column (4).

Gel materials that can be used for separation of metal-binding proteins have to be carefully examined whether or not they contain functional groups that work as ligands for metals. However, the details of chemical structures of gel materials are usually not known or not open for users. Moreover, manufactures are not interested in the interaction of metals in substrates with column materials.

Comparing with gel materials used for conventional gel chromatography such as Sephadex gels, HPLC gels are made tolerable to high pressure by making microspheres

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of hydrophilic polymers on silica gels. Chemically bonded silica gels such as Proteinpak (Waters Ltd.) and SW columns (Toyo Soda Manuf. Co.) are HPLC columns of this type. Silanol groups in these columns may work as additional ligands for metals when the columns are eluted under alkaline buffer conditions. In fact, an SW column shows both gel filtration and cation exchange properties by elution under an alkaline buffer condition (5,6).

As an HPLC column that interacts lesser extent to metals, we have been interested in an Asahipak column because the column is made of vinyl alcohol copolymers (details are not known) and does not contain additional supporting gels such as a silica gel. Using an Asahipak column we have separated serum proteins to determine cadmium-binding proteins (7). Zinc in serum was also determined simultaneously using an inductively coupled argon plasma-atomic emission spectrometer (ICP) as a detector for HPLC. Although cadmium was believed not to redistribute on an Asahipak column under a neutral condition, we had an impression that zinc might have redistributed on the column (7).

The present study was carried out to examine the distribution of zinc in blood serum on a gel permeation column after methylation of residual carboxyl groups. An Asahipak column was examined as an HPLC column because the column is believed to contain no extra functional groups such as silanol group other than hydroxyl and residual carboxyl groups. Zinc in serum was one of the most unstable metals and we often experienced its easy redistribution in our experiments. Therefore, the distribution of zinc in serum was first examined with the methylated column.

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MATERIALS AND METHODS

Methylation of residual carboxyl groups in Asahipak GS-520 column materials

A 10 g portion of the resin for an Asahipak GS-520 column (Asahi Chem. Ind., Tokyo) was washed twice with 50 ml of methanol (methanol for HPLC, Wako Chem. Ind., Osaka). The resin was allowed to stand in 20 ml of boron trifluoride methanol complex (Wako Chem. Ind.) for 12 hr at room temperature. Then, the resin was washed with methanol to remove boron trifluoride and suspended in doubly distilled water. The resin was packed in a stainless steel column (7.6 x 500 mm). The methylated column was named GST instead of GS for the original column.

HPLC-ICP

Both GS and GST columns were eluted with 10 mM Tris-HCl buffer, pH 7.4, containing NaCl (0.9%) and NaN_3 (0.05%) at a flow rate of 1.0 ml/min on a Beckman HPLC Instrument (Series 340). The mobile phase was degassed with a Shodex Degas degasser (Showa Denko, Tokyo). Absorbances at 254 and 280 nm were measured with a dual-wavelength UV detector (Model 152, Altex, Berkeley). The eluate of columns was introduced directly into the nebulizer of an ICP (JY48PVH, Seiko Instruments & Electronics Ltd., Tokyo) and the machine was operated under the conditions reported previously (8). Emission intensity data were collected every 2 s for 30 min or 4 s for 60 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 microcomputer (PC 9801, NEC, Tokyo) and an XY-plotter (FP5301R, Graphtec, Tokyo).

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Samples

A 0.1 ml solution of metals (Mg, 0.5; Ca, 1; Sr, 0.01; Ba, 0.1; Cd, 10; Zn, 10; and Cu, 10 µg/ml of 0.9% NaCl) or sera of rats (female Wistar rats, 10 weeks old) and human (male, 26 years old) was applied on HPLC columns. Blood sera were obtained by centrifuging at 2,300 g for 10 min after clotting for 20 min at room temperature.

RESULTS AND DISCUSSION

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Residual carboxyl groups in a GS-520 gel material are known to be approximately 0.02 meq/g dry resin (Manufacturer's data) and these in the methylated resin by boron trifluoride methanol complex decreased to a non-detectable level (determined by the Manufacturer).

Seven kinds of divalent metal ions were applied to both GS (Fig. 1A) and GST columns (Fig. 1B). Cupric ions were not detected within 70 min on both columns. However, the metal was washed out easily from the columns with serum proteins added later. The four alkali earth metal ions were eluted faster on the methylated column than on the original column. Although cadmium was eluted at a retention time of 39 min as a broad peak with tailing on the original column (Fig. 1A), it was eluted faster at 24 min as a relatively sharp peak on the methylated column (Fig. 1B). Zinc ions not eluted on the original column were detected at 36 min as a broad peak with tailing on the methylated column. Elution profiles in Fig. 1 indicate that ionic interactions of these metals to column materials, especially to carboxyl groups in the resin were decreased by the methylation procedure.

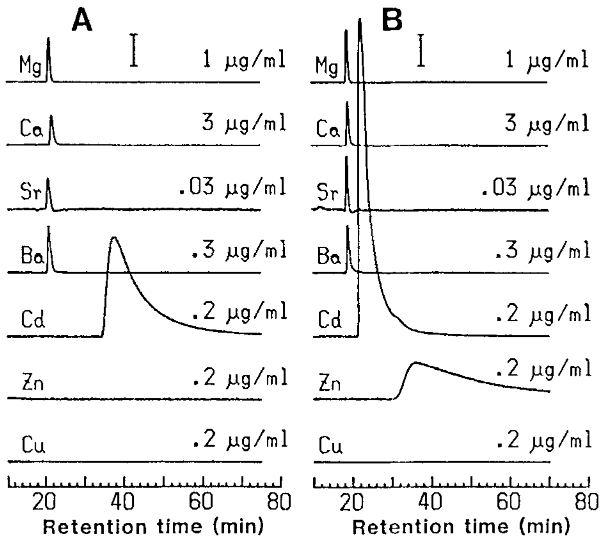

FIGURE 1. Elution of metal ions from GS-520 (A) and GST-520 (B) columns by HPLC-ICP method. A 0.1 ml solution of a mixture of metal ions in aqueous 0.9% NaCl was applied to the original GS-520 (A) or the methylated GST-520 (B) column and the column was eluted with 10 mM Tris/HCl, pH 7.4, containing 0.9% NaCl at a flow rate of 1.0 ml/min. The detector level of each metal was set as indicated in the figures.

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In our previous experiment on separation and detection of cadmium-binding proteins in blood serum (7), we have often experienced a lack of reproducibility in the distribution of zinc among serum proteins; more zinc was eluted at every first application to a GS column than the zinc contained in the applied serum, suggesting that at the first application serum proteins washed out the zinc that had remained in the column. The excess zinc washed out from the column was eluted with globulins. Constant elution and distribution of zinc were usually observed after several applications of the same sample (unpublished observation).

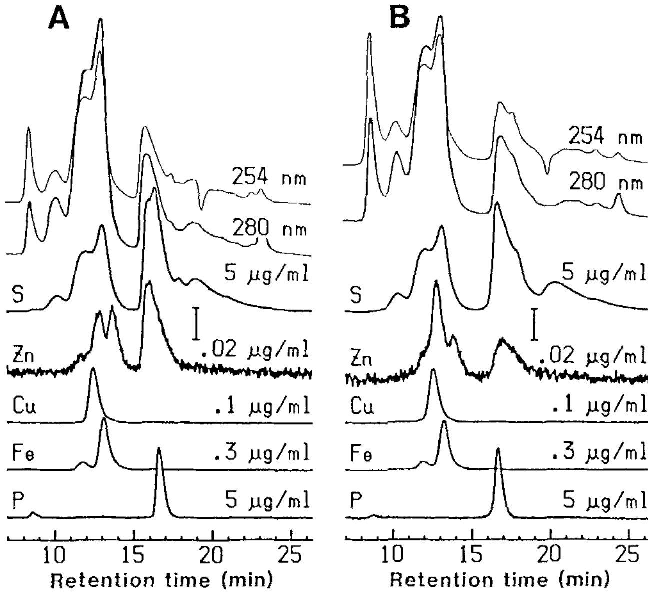

FIGURE 2. Separation of rat serum on GS-520 (A) and GST-520 (B) columns by HPLC-ICP method. A 0.1 ml portion of rat serum was applied to the original GS-520 (A) or the methylated GST-520 (B) column and the column was eluted as described in the legend to Fig. 1.

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Elution profiles in Fig. 2A demonstrate typical distributions of zinc and other elements in rat serum. Serum proteins were divided largely into two fractions on a GS-520 column under the conditions employed in this elution; globulins were eluted faster than a retention time of 15.0 min, while albumin was eluted slower than that retention time (7). Albumin was eluted at 15.8 min as monitored by absorbances at 254 and 280 nm, and sulphur and zinc emission on a GS column (Fig. 2A). This albumin was assigned as mercaptalbumin, whereas albumin eluted at 18.7 min was assigned as non-mercaptalbumin (7). Zinc was eluted only with mercaptalbumin and not with non-mercaptalbumin.

Globulins and albumin were also eluted similarly on the methylated column (GST-520 column) as shown in Fig. 2B. However, separation of globulins and albumin was better on the methylated column than the original one. Zinc in the globulin fraction was eluted as three peaks on both columns. The relative intensity of the three peaks was not only different between the two columns, but also changed when zinc remaining in the columns was eluted out with globulins. The relative distribution of zinc between globulins and albumin fractions was likewise different between the original and methylated columns. More zinc was eluted with albumin on the original column than the methylated column, suggesting that redistribution of zinc occurred in the column during separating procedure. Although zinc remaining in the column was washed out with globulins rather than albumin, zinc ions added into serum distributed more to albumin than to globulins (data not shown). Therefore, the distribution of zinc on the methylated column (GST-520; Fig. 2B) seems to be reflecting the real distribution of zinc among serum proteins.

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An intense peak of copper at 13.2 min can be assigned as ceruloplasmin and only a tiny copper peak was observed at a position of mercaptalbumin. The biggest iron peak at 13.0 min was transferrin and the small iron peak eluted faster than transferrin at 11.8 min was tentatively assigned as haptoglobin-hemoglobin complex. These metal-binding proteins were also eluted similarly on the methylated column (Fig. 2B).

As the distributions of phosphorus and sulphur were determined simultaneously in the present experiment, buffers that contain both elements could not be used. Hence, the best conditions known to separate human globulins and albumin (9) were not applicable,

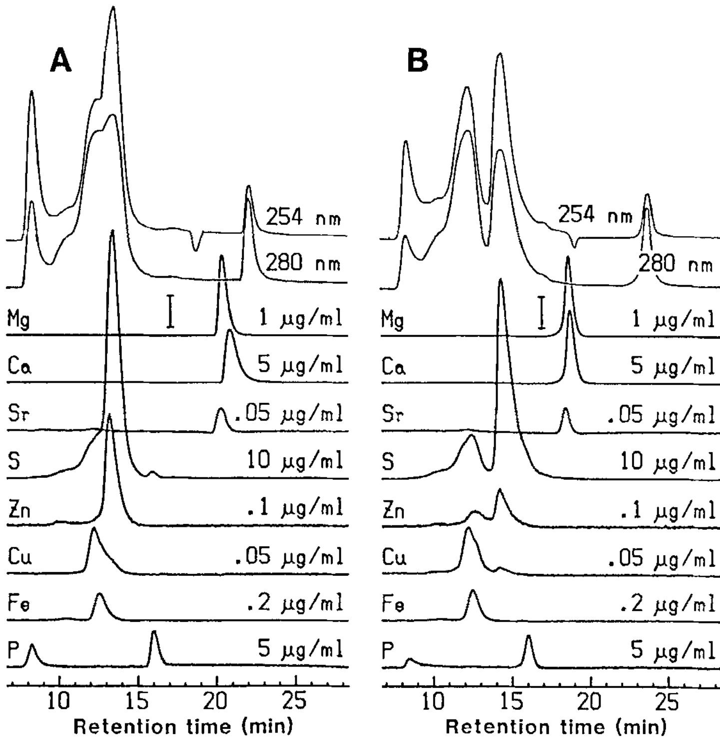

FIGURE 3. Separation of human serum on GS-520 (A) and GST-520 (B) columns by HPLC-ICP method. A 0.1 ml portion of human serum was applied as indicated in the legend to Fig. 2.

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and distributions of proteins and elements in human blood serum were determined under the same conditions employed for rat serum in Fig. 2. On the original GS-520 column, globulins and albumin were not separated as shown in Fig. 3A. However, on the methylated GST-520 column, albumin was separated from globulins as shown in Fig. 3B.

Although methylation of an HPLC column was intended to decrease the ionic interaction of metal ions

with the residual carboxyl groups, the distribution profiles in Fig. 3 indicate that human globulins and albumin can be separated more easily on the methylated column. Human albumin has been separated into mercaptalbumin and non-mercaptalbumin by connecting four GS-520 columns under the sophisticated condition (9). The present result further suggests that the methylated column may have a capacity to separate the two types of albumin more efficiently than the original column.

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