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Note

Chromatography of nucleosides on copper(II)-complexed carbohydrate polymers in an alkaline medium

Hitomi Sasaki *, Masaaki Ohara

Faculty of Pharmaceutical Sciences, Josai University, Keyakidai, Sakado, Saitama 350-02, Japan

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Copper(II) ions have been known to bind to carbohydrate molecules in alkaline solution to form deep blue, stable coordination complexes. The interaction is thought to involve copper(II) ions and dissociated hydroxyl groups [1–4]. We have found that in strongly alkaline medium the principal copper(II)-binding sites in carbohydrate molecules are pairs of vicinal hydroxyl groups, and that the degree of binding is especially influenced by the mutual orientation of the two hydroxyls. Pyranosides possessing the axial–equatorial and/or equatorial–equatorial diol groups were found to complex with copper(II) ions as shown by CD measurements and ESR and NMR spectroscopy [5]. In the case of the furanosides, copper(II) ions bound relatively tightly to ribofuranosides, but complexation with deoxyribofuranosides and arabinofuranosides was not observed to any appreciable extent [6,7].

We now report the application of the copper(II)–carbohydrate interaction to liquid chromatography using copper(II)-complexed carbohydrate polymers as stationary phases. We dealt with two kinds of carbohydrate polymer gels, namely Cellulofine beads, prepared from cellulose, and Sephadex, which is made from dextran cross-linked by epichlorohydrin. On columns of these supports mixtures of nucleosides, such as adenosine and 2′-deoxyadenosine, were separated successfully.

* Corresponding author.

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1. Experimental

Preparation of copper(II)-complexed matrices and packing of chromatographic columns. — Cellulofine GC-100-m was obtained from the Chisso Corporation, Tokyo, as an aqueous slurry. A portion (25 mL settled volume, ~ 20 g) was suspended in ~ 40 mL of 1 N NaOH and allowed to settle. The supernatant was drawn off and replaced with an equal volume of 1 N NaOH. The suspension was then stirred, treated with 0.1 M $CuSO_4$ (50 mL, added dropwise at the rate of ~ 2 mL min^{-1}), and allowed to settle. The pale blue, gelatinous copper hydroxide that formed a layer above the complexed Cellulofine was drawn off by pipet, and the volume was restored with 1 N NaOH. More $CuSO_4$ (50 mL, dropwise, more slowly, with stirring) was added, the suspension was allowed to settle, the turbid supernatant was drawn off, and the volume was restored with 1 N NaOH. The further addition of 0.1 M $CuSO_4$ (50 mL, in portions, with stirring) was necessary to obtain a matrix having a larger amount of complexed copper(II). The copper-loaded matrix was packed into a column and washed with 1 N NaOH.

A 4 g sample of dry Sephadex G-50 medium, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, was swollen overnight in ~ 80 mL of water. The swollen gel was suspended in ~ 100 mL of 1 N NaOH and allowed to settle, and the supernatant was poured off and replaced with a like volume of 1 N NaOH. The gel suspension was then treated with 0.1 M $CuSO_4$ as just described for Cellulofine. In the first treatment ~ 20 mL of the reagent was used, and in the second the addition was continued until copper(II) was seen to precipitate as copper hydroxide, which was drawn off by pipet. A total of 25–30 mL of 0.1 M $CuSO_4$ was used. The copper(II)-complexed Sephadex was packed into a column and washed with 1 N NaOH.

Copper-free control columns were packed with untreated Cellulofine and untreated Sephadex G-50, respectively, suspended in 1 N NaOH.

Chromatography of ribofuranosides and related nucleosides. — In general, 0.5 mL of 1 N NaOH solution containing 0.1 mg of nucleoside sample was placed on a copper(II)-complexed or copper(II)-free carbohydrate polymer column equilibrated with 1 N NaOH. The column was then eluted with 1 N NaOH at room temperature with the aid of a fraction collector. The flow rates were 0.3 mL min^{-1} for Cellulofine columns and 0.5 mL min^{-1} for Sephadex columns.

To determine the amount of nucleoside in each fraction, the absorbance was measured at the relevant ultraviolet absorption maximum. Eluent (1 N NaOH) was employed as a control solution.

Methyl β-D-ribofuranoside was assayed colorimetrically by means of a modified orcinol-pentose reaction [8]. An 0.5 mL aliquot withdrawn from each fraction was neutralized with 0.5 mL of 1 N HCl. To each sample was added 1 mL of 0.3% $FeNH_4(SO_4)_2 \cdot 12\ H_2O$ in concd HCl, and 0.1 mL of 10% orcinol in 95% ethanol. The mixtures were heated for 25 min on a boiling water bath, and cooled. Absorbance was read at 670 nm against a blank of distilled water or 1 N NaOH treated in the same manner as the samples.

In order to examine the effect of flow rate on the chromatographic separations, a series of runs was carried out at flow rates of 0.2 to 0.8 mL min^{-1} for the copper(II)–Cellulofine column, and 0.2 to 0.7 mL min^{-1} for the copper(II)–Sephadex column.

Determination of the amount of Cu(II) bound to the column. — The suction-dried matrix was weighed and suspended in water, and the suspension was adjusted to pH ~ 3 with 1 N HCl. The colorless matrix, to which copper(II) was no longer bound, was removed by decantation. The supernatant and the washings were combined, and neutralized with NaOH. Then the copper(II) content of the solution was determined by the use of EDTA and murexide as titrant and indicator, respectively, after the addition of a few drops of 30% NH_4OH [9].

2. Results and discussion

Chromatography on copper(II)-complexed Cellulofine and Sephadex columns. — Elution chromatograms of ribo- and deoxyribo-nucleosides obtained with a copper(II)-complexed Cellulofine column and a copper(II)-complexed Sephadex column are shown in Figs. 1 and 2, respectively. These results, plus values for methyl β-D-ribofuranoside and two arabinose nucleosides, are summarized in Tables 1 and 2.

With both columns adenosine, guanosine, cytidine, uridine, and methyl β-D-ribofuranoside were found to elute after some delay, which seemed to be attributable to strong interaction with copper(II) ions attached to the column matrices. On the contrary, 2′-deoxyadenosine, 2′-deoxyuridine, thymidine, 9-β-D-arabinofuranosyladenine and 1-β-D-arabinofuranosyluracil were eluted earlier. These nucleosides gave elution volumes less than 26 mL, which were comparable to those observed with copper(II)-free control columns of approximately the same bed depth.

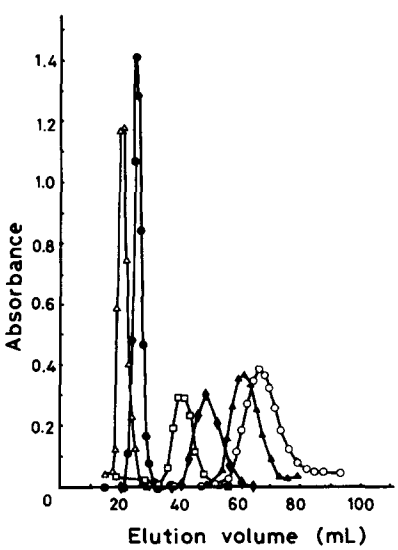

Fig. 1. Chromatography on a copper (II)-complexed Cellulofine column. Dimensions, 12 × 279 mm; copper (II) content, 0.32 mmol g^{-1} wet gel; flow rate, 0.3 mL min^{-1}. Symbols and λ_{max} (nm) used for absorbance measurement: adenosine (○, 261); uridine (□, 266); cytidine (▲, 274); guanosine (◆, 268); 2′-deoxyadenosine (●, 261); 2′-deoxyuridine (△, 262).

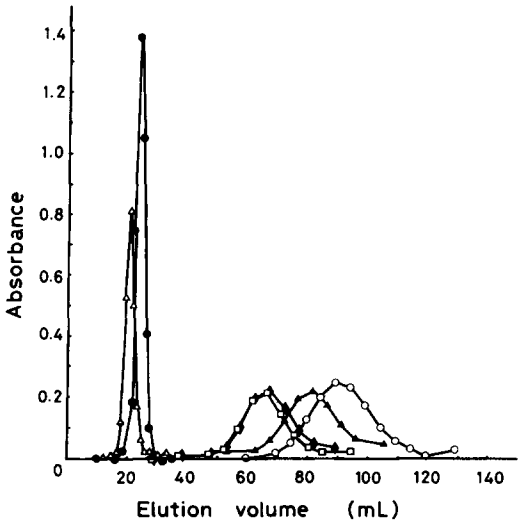

Fig. 2. Chromatography on a Cu(II)-complexed Sephadex column. Dimensions, 12×212 mm; Cu(II) content, 0.042 mmol/g wet gel; flow rate, 0.5 mL/min. Symbols and λ_{max} (nm) used for absorbance measurement: adenosine (○, 261), uridine (□, 264), cytidine (▲, 274), guanosine (◆, 257), 2′-deoxyadenosine (●, 262), 2′-deoxyuridine (△, 264).

Mixtures of two nucleosides (one each of the two types) were chromatographed on columns of the copper(II)-complexed matrices. Well-defined separations were observed for both the adenosine–2′-deoxyadenosine pair and the uridine–2′-deoxyuridine pair, as expected from Figs. 1 and 2.

Table 1
Elution volumes from copper(II)-complexed and copper(II)-free Cellulofine columns

Furanoside	Elution volume (mL)	
	Copper(II)-complexed Cellulofine [a]	Copper(II)-free Cellulofine [b]
Adenosine	66.2, 66.6	23.9, 23.7
Guanosine	47.6	20.3
Cytidine	61.3	21.5
Uridine	39.9, 39.1	19.3
2′-Deoxyadenosine	25.3, 25.2	24.6, 24.4
2′-Deoxyuridine	19.9	19.5
Thymidine	19.9, 19.8	19.1
9-β-D-Arabino-furanosyladenine	22.0	21.9
1-β-D-Arabino-furanosyluracil	19.0	18.6,18.3
Methyl β-D-ribo-furanoside	41.4, 41.6	20.0, 20.1

[a] For column parameters see legend to Fig. 1.
[b] Column size, 12×254 mm; flow rate, 0.3 mL min^{-1}.

Table 2
Elution volumes from copper(II)-complexed and copper(II)-free Sephadex columns

Furanoside	Elution volume (mL)	
	copper(II)-complexed Sephadex [a]	copper(II)-free Sephadex [b]
Adenosine	90.5, 82.3	29.7, 28.5
Guanosine	66.1	26.5
Cytidine	80.6	27.6
Uridine	65.3	26.7
2′-Deoxyadenosine	22.9, 23.4	28.4
2′-Deoxyuridine	20.2	26.1
Thymidine	21.0	25.9
9-β-D-Arabino-furanosyladenine	21.7	26.7
1-β-D-Arabino-furanosyluracil	20.0	25.6
Methyl β-D-ribo-furanoside	60.3, 59.5	26.7

[a] For column parameters see legend to Fig. 2.
[b] Column size, 12×258 mm; flow rate, 0.5 mL min^{-1}.

Differences were found in the capacities of the two column matrices. The elution volume of each retarded furanoside solute on the copper(II)–Sephadex column was greater than that of the corresponding solute on a longer (see Tables 1 and 2) or a comparable copper(II)–Cellulofine column, despite the lower copper(II) content of the Sephadex matrix. In the case of runs using a Cellulofine matrix holding a larger amount of copper [copper(II) content 0.44 mmol g^{-1} wet gel], significant band spreading was observed. Accordingly, the copper(II)–Sephadex columns were superior to the copper(II)–Cellulofine columns for the separation of mixtures of nucleosides.

However, a disadvantage of the copper(II)–Sephadex columns was their greater lability as compared to the copper(II)–Cellulofine columns. Repeated uses of the copper(II)–Sephadex columns caused a shortening of the column length and the decrease in the copper(II) content of the matrix. This decrease is probably due to the release of copper ions from the column. Furthermore, the copper(II)–Sephadex columns exhibited a band of color change from deep blue to dark brown after standing for a long time. This phenomenon presumably arose from changes in the mode of complexation of the copper ions, or in their valence. These serious disadvantages were rarely found with the copper(II)–Cellulofine columns, which were characterized by their high stability.

Elution chromatograms of adenosine and uridine were found to be influenced by flow rate for both the copper(II)–Cellulofine and copper(II)–Sephadex columns. Thus, the peaks in the elution profiles obtained at a low flow rate ($\sim$ 0.2 mL min^{-1} for both columns) were apparently sharper than those seen at faster rates (0.7–0.8 mL min^{-1}), although the elution volumes of the corresponding peaks at different flow rates remained virtually unaltered. On the other hand, the elution profiles obtained at the faster flow rates did not manifest the appreciable, continuous increase in base line absorbance seen in profiles plotted at a lower rate. It appears that fractions taken at the slower flow rate

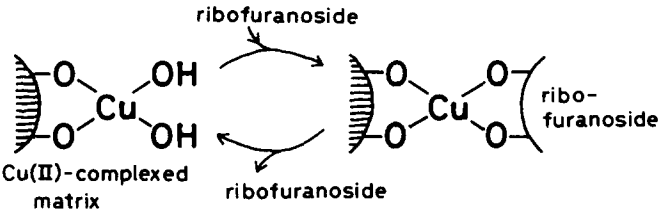

Fig. 3. Probable mode of binding of ribofuranosides to Cu(II)-complexed carbohydrate polymer gels in strongly alkaline medium.

were contaminated by small amounts of copper(II) ions released from the column matrix.

Copper(II)-complexation with carbohydrate molecules during chromatographic processes. — As already mentioned, the fairly slow elution of the ribonucleosides from columns of copper(II)-complexed matrices may be attributed to the strong interaction, in the highly alkaline medium, of the ionized ribose residue with copper(II) ions attached to the carbohydrate polymer gels. Our previous studies [6,7] have demonstrated that the *cis*-1,2-diol grouping on the five-membered ring of ribofuranosides was able to form a quite stable coordination complex with copper(II) ions in strongly alkaline solution. In contrast, with solutes having a *trans*-1,2-diol grouping (arabinose derivatives) or an isolated 3-hydroxy group in their furanose rings, copper(II)-complex formation was hardly discernible [6,7]. These steric and structural requirements for complex formation are further demonstrated by the chromatographic data of the present report.

The liquid chromatographic process presented, employing copper(II)-complexed carbohydrate polymer gels as solid supports in strongly alkaline medium, can be classified as a branch of ligand-exchange chromatography [10]. The basic principle of this type of chromatography is the frequent occurrence of interaction between complex-forming metal ions and the molecules to be separated (Fig. 3). An important feature of the copper(II)-complexed gel structure is the direct binding of copper(II) ions onto the gel matrix, without the intervention of a spacer molecule.

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