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Short communication

Detection of glycosaminoglycans as a copper(II) complex in high-performance liquid chromatography

Toshihiko Toida[a], Makiko Shima[a], Saori Azumaya[a], Tomohiro Maruyama[a], Hidenao Toyoda[a], Toshio Imanari[a,*], Robert J. Linhardt[b]

[a]*Faculty of Pharmaceutical Sciences, Chiba University, Yayoi, Inage, Chiba 263, Japan*

[b]*Division of Medicinal and Natural Products Chemistry and Department of Chemical and Biochemical Engineering, PHAR S328, University of Iowa, Iowa City, IA 52242, USA*

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Abstract

Glycosaminoglycans including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronan were analyzed by high-performance gel filtration chromatography. Detection was achieved at 240 nm based on the formation of copper(II) complex in copper sulfate solution at low pH. Detection of the copper(II)–heparin complex is sensitive, permitting the analysis of as little as 10^{-7} g. This method was also successfully applied to the analysis of the chemically derivatized glycosaminoglycans (de-N-acetylated and/or oversulfated chondroitin sulfate) that cannot be depolymerized by enzymes such as heparin and chondroitin lyases. © 1997 Elsevier Science B.V.

Keywords: Detection, LC; Derivatization, LC; Glycosaminoglycans; Glycans; Metal complexes; Copper complexes

1. Introduction

Glycosaminoglycans (GAGs) are a group of sulfated polysaccharides that display a variety of important biological roles [1,2]. There are two major classes of GAGs comprised of disaccharide units of uronic acid and hexosamine: (1) the heparan sulfate family including heparin and heparan sulfate; and (2) the chondroitin sulfate family including chondroitin sulfate, dermatan sulfate and hyaluronan. Keratan sulfate is yet another GAG, containing sulfated disaccharide units of galactose and N-acetylglucosamine. GAGs are polydisperse having average molecular masses ranging from 10 000 to over 100 000 ([3]; see also product literature published by Seikagku, Tokyo, Japan). Except for hyaluronan, their structural complexity is further compounded by sequence heterogeneity, caused primarily by the variation of degree and position of sulfate groups [1,2]. There has been an increased interest in the fractionation and analysis of these complex polymers with the advent of high resolution separation methods. Gel and membrane supported electrophoreses are widely used for GAG analysis [1]. Cellulose acetate membrane electrophoresis provided the earliest method for the resolution of different GAGs and relied on Alcian blue staining for their visualization [4]. While improvements in separations were achieved using high porosity agarose gels that decreased band diffusion [5], polyacrylamide gel electrophoresis (PAGE) offered greatly improved resolution, often resulting in discrete

*Corresponding author.

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banding of structurally simple GAGs, such as hyaluronan [6–8]. Visualization of GAGs on PAGE gels could also be enhanced by silver staining to detect nanogram quantities of GAG [9]. Capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) have also been used in the analysis of oligosaccharides prepared by polysaccharide lyase catalyzed depolymerization of GAGs [10–16]. Chondroitin sulfate/heparin lyases degrade GAGs through an eliminase mechanism that produces unsaturated uronic acid residues at their non-reducing termini [17]. These unsaturated uronic acid residues absorb in the ultraviolet (UV) region at 232 nm, facilitating their detection in CE/HPLC analyses. High-resolution CE has been used to fractionate lyase derived GAG oligosaccharides that could be detected at attomole levels [10].

In general, intact, underivatized GAGs have not been successfully analyzed by CE or HPLC because of the absence of a chromophore. The exception is hyaluronan from eye vitreous humor, which has been analyzed by direct, low wavelength, UV detection [18]. Mukherjee et al. [19] reported that copper(II)–GAG complexes give optical properties that permit their UV detection. Furthermore, Grant et al. showed that these complexes are sufficiently tight to be useful in separations [20]. We recently demonstrated the use of copper(II) complexes to visualize the CE separation of GAGs [21]. These papers suggest that it may be possible to use copper(II) complexes to visualize GAGs separated by HPLC. The current study explores this possibility.

2. Experimental

2.1. Materials

Shark cartilage chondroitin 4-sulfate (average molecular mass=50 000, Seikagaku America, Rockville, MD, USA), porcine skin dermatan sulfate (average molecular mass=16 000, Seikagaku), human umbilical cord hyaluronan (average molecular mass=100 000, Seikagaku), porcine intestinal mucosa heparan sulfate (average molecular mass=14 800, Celsus, Cincinnati, OH, USA), porcine intestinal mucosa heparin (average molecular mass=15 000, Celsus) and low-molecular mass heparin (average molecular mass=4800, Celsus) were obtained from commercial sources. Copper(II) sulfate was from Wako Pure Chemicals, Osaka, Japan. Bovine lung heparin was from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of the highest commercially available grade of purity.

2.2. Derivatization of chondroitin sulfate

The chemical oversulfation of chondroitin sulfates was performed according to the method reported by Nagasawa et al. [22]. N-Deacetylation and re-N-sulfation of chondroitin sulfate procedures followed previously described method [23]. The purity and structure of the products of these derivatization reactions were confirmed by cellulose acetate electrophoresis [4] and by NMR spectroscopy [23].

2.3. HPLC

The HPLC experiments were performed on a Hitachi HPLC LC6000 liquid delivery system (Hitachi Seisakusho, Tokyo, Japan) equipped with a variable-wavelength UV detector, Hitachi Model U3400. The HPLC system was operated in the gel-permeation mode by running using 1.0 m*M* copper(II) sulfate adjusted to pH 4.5 with 0.01 *M* sulfuric acid. The separation column (Toyo-Pearl HW40F, 50 cm×8 mm I.D.) was manually wet-packed in the copper(II) sulfate solution (1 m*M*) and was connected to the guard column (Toyo-Pearl, HW40F, 25 cm×4.6 mm I.D.). Each experiment was performed at a flow-rate 0.5 ml/min. Detection was on-line by UV absorbance at 240 nm.

3. Results and discussion

3.1. Copper(II)–GAG complex formation

All GAGs, except keratan sulfate, which contains a galactose residue, are made up of an uronic acid residue and a hexosamine residue. All GAGs, with the exception of hyaluronan, contain varying degrees of O- and/or N-sulfate groups. Mukherjee et al. [19] demonstrated that all GAGs, except for keratan sulfate, were capable of binding copper(II), affording a complex that had an absorbance maxima near 237

nm. Based on these observations, they suggested that this absorbance was the result of a charge transfer complex between copper(II) ion and the carboxylate group in the GAG. Mukherjee et al. [19] and we also showed that this absorbance was highest at pH 4.5 and considerably reduced at pH 3.0. We have recently demonstrated that it was possible to separate heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate (see Fig. 1) as their copper(II) complexes by CE at pH 4.5 [21]. Based on these data, we undertook to examine whether the copper(II)–GAG complex could be used for detection following separation by HPLC.

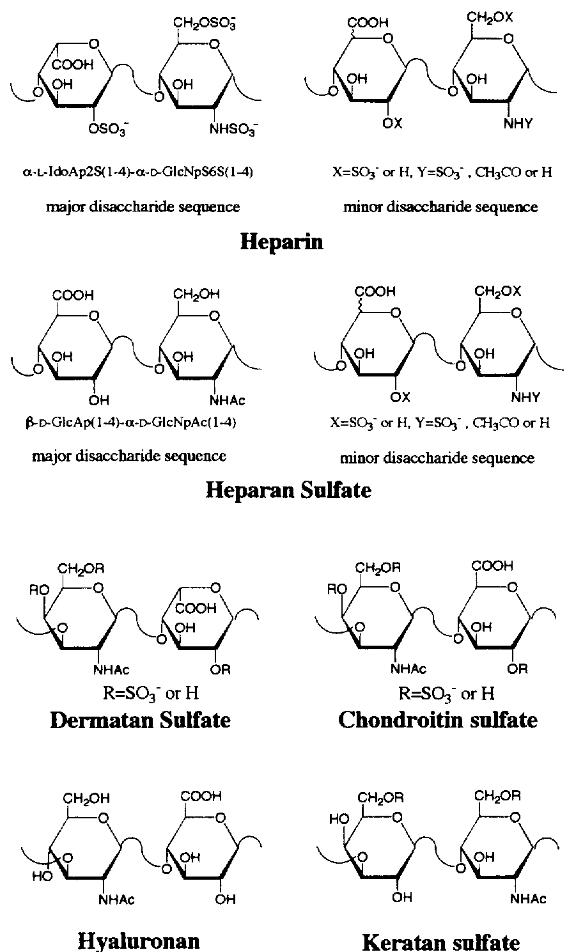

Fig. 1. Structure of the major disaccharide repeating unit found in each GAG. In heparin, heparan sulfate and keratan sulfate, having diverse sequences, $X=SO_3^-$ or H and $Y=SO_3^-$, Ac or H.

Copper(II)–GAG complexes were prepared and their absorbance spectra were obtained at a variety of pH values. As we have previously reported [21], an absorbance maxima of 240 nm was observed at each pH with optimum sensitivity obtained at pH 4.5. The absorbance spectra of copper(II)–GAG complexes of heparin, chondroitin sulfate and hyaluronan are shown in Fig. 2. The response of each copper(II)–GAG complex, however, was different from that previously reported [21] using 5 m*M* copper(II) sulfate as a detection reagent. All the GAGs (Fig. 1), including low-molecular mass (low-M_r) heparin (heparin has an average molecular mass of 15 000 while low-M_r heparin is approximately 5000 [1]), showed similar absorbance maxima at 240 nm. While the detection sensitivity was greatest for the highly sulfated heparin and lowest for unsulfated hyaluronan (Fig. 3), a much higher response was

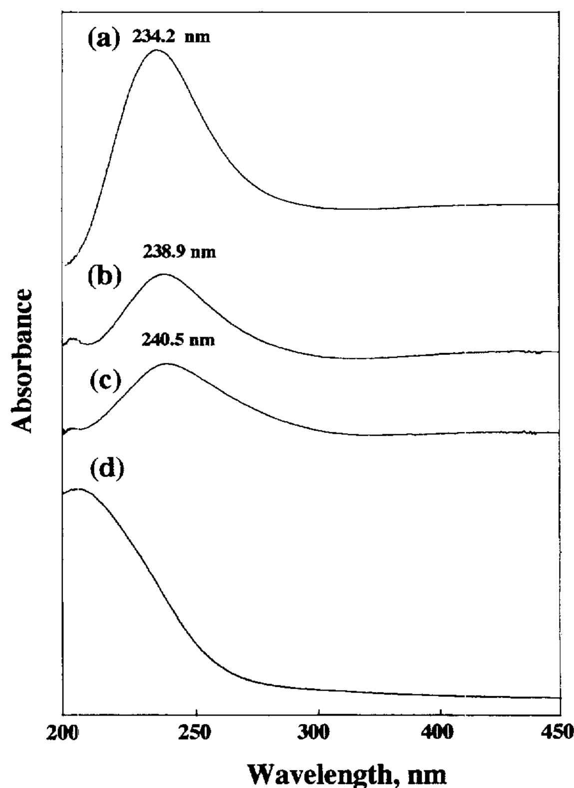

Fig. 2. UV spectra of the copper(II)–GAG complex. (a) Heparin; (b) chondroitin sulfate; (c) hyaluronan; (d) reagent blank. The concentration of each GAG in 1.0 m*M* copper(II) sulfate was 250 μg/ml.

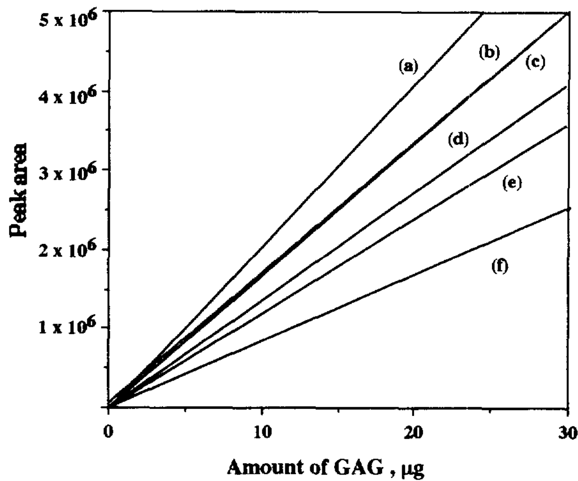

Fig. 3. Absorbance (240 nm) as a function of GAG concentration: (a) heparin; (b) low-M_r heparin; (c) dermatan sulfate;, (d) heparan sulfate; (e) chondroitin 4-O-sulfate and (f) hyaluronan.

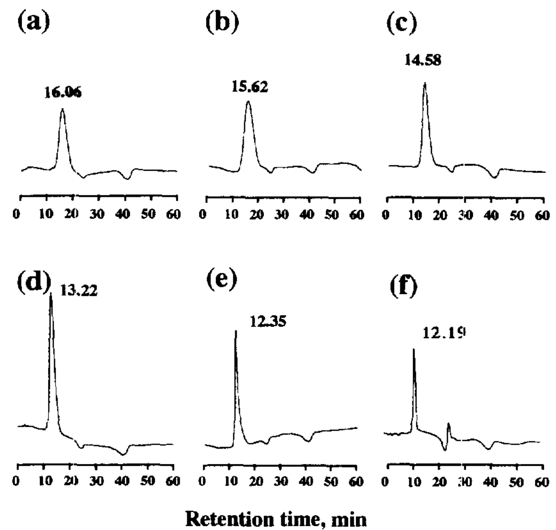

Fig. 4. HPLC analysis of copper(II)–GAG complexes. (a) Heparin, (b) low-M_r heparin, (c) heparan sulfate, (d) dermatan sulfate, (e) chondroitin 4-O-sulfate and (f) hyaluronan. Sample concentration was 1 mg/ml and detection was at 240 nm and separation was in 1.0 mM copper(II) sulfate at pH 4.5.

observed for hyaluronan than previously reported [21]. At 1.0 mM copper(II) sulfate, hyaluronan gave a ten-fold higher response than that obtained in 5.0 mM copper(II) sulfate. This dramatic increase in response for hyaluronan may be attributable in part to a reduction in the absorbances of the reagent blanks.

3.2. HPLC separation of the copper(II)–GAG complexes

Gel-permeation HPLC separation of GAGs was the only separation mode that was examined. While anion-exchange HPLC and reversed-phase ion-pairing separation of GAGs is possible, these methods rely on the use of salt gradients or ion-pairing reagent that can interfere with copper complex formation. Gel-permeation HPLC analysis of the copper(II) complexes of heparin, low-M_r heparin, heparan sulfate, chondroitin 4-O-sulfate, dermatan sulfate and hyaluronan are presented in Fig. 4. Under optimized conditions (see legend of Fig. 4 and Section 2.3), each GAG showed a broad, featureless peak having a distinctive retention time. While the retention times for GAGs were dependent on their molecular masses, the broad peaks that were observed made it impossible to completely separate mixtures of two different GAGs.

A variety of approaches were examined to improve the resolution shown in Fig. 4. The concentration of copper(II) sulfate solution was varied between 0.1 mM and 5 mM. Detection sensitivity increased from 0.1 mM to 1.0 mM where it was optimum. Above 1.0 mM, baseline noise reduced detection sensitivity. The effect of solution pH was examined between 1 and 6. The sensitivity increased with increasing pH reaching a maximum at pH 4.5, after which the sensitivity decreased. Optimal separation and resolution were achieved in 1.0 mM copper(II) sulfate at pH 4.5. A detection sensitivity of heparin of 10^{-7} g was determined under these separation conditions by decreasing the concentration of an analyte until no peak could be detected above baseline noise. The peak breadth observed in Fig. 4 is consistent with the polydispersity and sequence heterogeneity of the GAGs that were analyzed.

3.3. Separation and detection of chemically derivatized GAGs as their copper(II) complexes

Chemically de-N-acetylated re-N-sulfated chondroitin sulfate, and oversulfated chondroitin sulfate were prepared by the methods reported previously

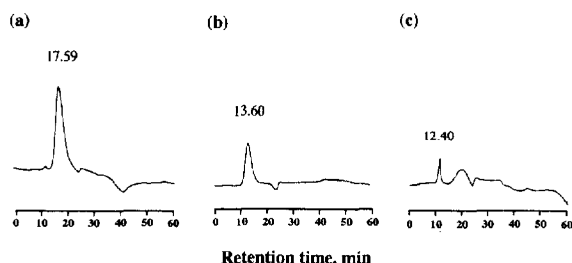

Fig. 5. HPLC analysis of on chemically modified chondroitin sulfates. (a) Oversulfated chondroitin sulfate; (b) de-N-acetylated chondroitin sulfate; (c) de-N-acetylated re-N-sulfated chondroitin sulfate. Sample concentration was 1 mg/ml and detection was at 240 nm and separation was in 1.0 m*M* copper(II) sulfate at pH 4.5.

[22,23]. Because these chondroitin sulfates resists to the degradation enzymes such as chondroitin ACII and ABC lyases (data not shown), their analysis as disaccharides is not possible. Direct analysis of these chemically derivatized GAGs was possible based on the detection of their copper(II) complexes (Fig. 5). Thus, the gel-permeation HPLC method in this paper might be useful in monitoring such structurally modified artificial GAGs that are currently under study as therapeutic medicinal agents [1].

4. Conclusions

In conclusion, HPLC using copper(II) sulfate as the detection reagent can be used for the direct analysis of GAGs and chemically derivatized GAGs that do not possess any additional chromophore. The separations obtained are dependent on GAG molecular mass and the detection sensitivity was remarkably high (as low as 10^{-7} g for heparin).

References

[1] R.J. Linhardt and T. Toida, in Z.B. Witczak and K.A. Nieforth (Editors), Carbohydrates as Drugs, Marcel Dekker, New York, 1997, in press.

[2] D.A. Lane and U. Lindahl (Editors), in Heparin, CRC Press, Boca Raton, FL, 1989, pp. 1–24.

[3] M.B. Mathews and J.A. Cifonelli, in Acid Mucopolysaccharide Reference Standards, Publication of the National Institutes of Health (NIAMD) under contract No. NO1-AM-5-22-5.

[4] N. Seno, K. Anno, K. Kondo, S. Nagase, S. Saito, Anal. Biochem. 37 (1970) 197–202.

[5] C.H.A. van de Lest, E.M.M. Versteeg, J.H. Veekamp, T.H. van Kuppevelt, Anal. Biochem. 221 (1994) 356–361.

[6] D.M. Hittner, M.K. Cowman, J. Chromatogr. 402 (1987) 149–158.

[7] J.E. Turnbull, J.T. Gallagher, Biochem. J. 251 (1988) 597–608.

[8] K.G. Rice, M.K. Rottink, R.J. Linhardt, Biochem. J. 244 (1987) 515–522.

[9] A. Al-Hakim, R.J. Linhardt, Appl. Theor. Electrophoresis 1 (1991) 305–312.

[10] A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 195 (1991) 68–73.

[11] S.L. Carney, D.J. Osborne, Anal. Biochem. 195 (1991) 132–140.

[12] S.A. Ampofo, H.M. Wang, R.J. Linhardt, Anal. Biochem. 199 (1991) 249–255.

[13] U.R. Desai, H.M. Wang, S.A. Ampofo, R.J. Linhardt, Anal. Biochem. 213 (1993) 120–127.

[14] R.J. Linhardt, Methods Enzymol. 230 (1994) 265–280.

[15] A. Pervin, A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 221 (1994) 182–188.

[16] R.J. Linhardt, A. Pervin, J. Chromatogr. 720 (1996) 323–335.

[17] R.J. Linhardt, in A. Varki (Editor), Current Protocols in Molecular Biology, Analysis of Glycoconjugates, Wiley Interscience, Vol. 2, 1994, pp. 17.13.17–17.13.32.

[18] J. Grimshaw, A. Kane, J. Trocha-Grimshaw, A. Douglas, U. Chakravarthy, D. Archer, Electrophoresis 15 (1994) 936–940.

[19] D.C. Mukherjee, J.W. Park, B. Chakrabart, Arch. Biochem. Biophys. 191 (1978) 393–399.

[20] D. Grant, W.F. Long, C.F. Moffat, F.B. Williamson, Biochem. J. 283 (1992) 243–246.

[21] T. Toida, R.J. Linhardt, Electrophoresis 17 (1996) 341–346.

[22] K. Nagasawa, H. Uchiyama, N. Wajima, Carbohydr. Res. 158 (1986) 183–190.

[23] V.D. Nadkarni, T. Toida, C.L. Van Gorp, R.L. Schubert, J.M. Weiler, K.P. Hansen, E.E.O. Caldwell, R.J. Linhardt, Carbohydr. Res. 290 (1996) 87–96.