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Note

Chromatographic method for determination of hexuronic acid in dermatan sulphate

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A number of dermatan sulphate–chondroitin sulphate copolymers (so-called dermatan sulphates) having different hexuronic acid contents, degrees of sulphation and molecular weights, have been reported[1]. The hexuronic acid contents in these polysaccharides were conventionally estimated by the carbazole–orcinol ratio[2], which has been widely used for approximate estimation of the ratio of D-glucuronic acid to L-iduronic acid in glycosaminoglycans containing both hexuronic acids. Later, an enzymatic procedure using chondroitinase AC and ABC[3,4] was frequently utilized for microdetermination of hexuronic acid in the above-mentioned copolymers.

Recently we required a method to check the reliability of the enzymatic procedure. The method described herein was devised to satisfy our requirements, but it turned out to be useful as a general method for the chemical assay of the hexuronic acid content with the aid of a combination of gel filtration and ion-exchange chromatography.

EXPERIMENTAL

Materials and methods

Rooster-comb dermatan sulphates (sodium salts, RC-20 and RC-30 fractions) were as described previously[4]. Pig-skin dermatan sulphate (sodium salt, M_r 20000) was obtained as a 20% ethanol fraction by fractionation with ethanol of the calcium salt according to the procedure of Meyer *et al.*[5]. Derivatives of these dermatan sulphates, which had been labelled with a 2-aminoethylamino group at the reducing end of the polysaccharide chain, were as described previously[6].

Standard N-acetylchondrosine and N-acetyldermosine were as described previously[7]. 1,2-Isopropylidene-L-iduronolactone was obtained from Nakarai Chemicals (Kyoto, Japan), and D-glucurono-6,3-lactone from Sigma (St. Louis, MO, U.S.A.). Chondroitinase AC-II from *Arthrobacter auresens* and chondroitinase ABC from *Proteus vulgaris* were obtained from Seikagaku Kogyo (Tokyo, Japan). AG 1-X4 anion-exchange resin (200–400 mesh) and Sephadex G-25 were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively.

Hexuronic acid was determined by the method of Bitter and Muir[8], modified by increasing the borate concentration to 0.2 *M*, and by using D-glucurono-6,3-lactone and 1,2-isopropylidene-L-iduronolactone as standards[9].

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Enzymatic determination of hexuronic acid in dermatan sulphates and their 2-aminoethylamino derivatives

To a solution of the sample (100 μg in 20 μl of water) were added enriched Tris buffer[10], pH 8.0 (10 μl) and chondroitinase AC-II (0.5 units in 20 μl of water), and the mixture was incubated at 37°C for 5 h. To another sample of the material (100 μg in 20 μl of water) were added enriched Tris buffer (10 μl) and chondroitinase ABC (0.2 units in 20 μl of water), and the mixture was incubated for 5 h at 37°C. The absorbance at 232 nm was measured for each incubation mixture to obtain the ratio of A_{232} (chondroitinase AC-II) to A_{232} (chondroitinase ABC) which gave the proportion of D-glucuronic acid in the total hexuronic acid content (%)[3].

Determination of hexuronic acid in dermatan sulphates and their 2-aminoethylamino derivatives

Hydrolysis of the polysaccharide materials with dimethyl sulphoxide containing 10% water. A solution of the sample (≈ 6 mg per 0.4 ml water) was passed through a column of Dowex 50W-X2 (H^+, 50–100 mesh) at 0–4°C. The eluent and washings were pooled, neutralized (pH 6.0) by the addition of pyridine and lyophilized to give the pyridinium salt as a white powder. A solution of the pyridinium salt (≈ 6 mg) in dimethyl sulphoxide containing 10% of water (1.5 ml) was heated in a Pyrex test-tube (10 cm × 0.7 cm) fitted with a PTFE screw-cap and stirred with PTFE stirrer (diameter 0.5 cm) for 30 h at 108 ± 1°C. After cooling in an ice-batch, the contents of the test-tube were diluted in an equal volume of water and transferred to a distillation flask (volume 20 ml), then neutralized (pH 6.0) with 0.1 *M* NaOH. The solution obtained was evaporated to dryness at 30–35°C under reduced pressure.

Separation on Sephadex G-25 and AG 1-X4 anion-exchange resin, of the hydrolysate into N-acetyldermosine, N-acetylchondrosine, L-iduronic acid and D-glucuronic acid, and determination of the ratio of D-glucuronic acid to total hexuronic acid. The hydrolysate obtained above was dissolved in 0.1 *M* ammonium hydrogencarbonate (0.5 ml) and loaded on a column (85.5 cm × 1.5 cm) of Sephadex G-25 prepared in the same solvent. The column was eluted at 20–25°C with the same solvent at a flow-rate of 34 ml/h. Each fraction (2 ml) was analyzed for hexuronic acid (the elution diagrams are shown in the insets of Fig. 1a, b). The fractions corresponding to the disaccharide and monosaccharide peaks (tube Nos. 46–65 of the elution diagrams in the insets of Fig. 1a, b) were pooled and lyophilized. The residue was dissolved in water (0.5 ml) and loaded on a column (85.5 cm × 1.0 cm) of AG 1-X4 (HCO_2^-, 200–400 mesh) prepared in water. The column was eluted at 40°C with 0.2 *M* formic acid at a flow-rate of 24 ml/h. Each fraction (3.9 ml) was analyzed for hexuronic acid (Fig. 1a, b). The sum of the peak areas due to N-acetylchondrosine and D-glucuronic acid in each elution diagram of Fig. 1 provides an estimate of the content of D-glucuronic acid in the sample, and the sum of the peak areas due to N-acetyldermosine and L-iduronic acid affords that of L-iduronic acid in the sample.

RESULTS AND DISCUSSION

One of us reported previously that the reaction of the pyridinium salts of dermatan sulphates in dimethyl sulphoxide containing 10% of water at 105°C for 30 h afforded higher oligosaccharide (≥ tetrasaccharide, 18.3%), disaccharide (69.2%)

and monosaccharide fractions (12.5%, based on hexuronic acid determination, respectively), and recommended this procedure as an improved method for preparing N-acetyldermosine and L-iduronic acid from dermatan sulphates[7]. The essential feature of the hydrolysis in dimethyl sulphoxide containing a small amount of water is the initial rapid cleavage of the sulphate groups of sulphated mucopolysaccharides under moderately acidic conditions. This probably results from the solvation between protons and dimethyl sulphoxide molecules in the reaction medium: the 2-acetamido-2-deoxy-β-D-hexosyl linkages of the resulting desulphated mucopolysaccharides are

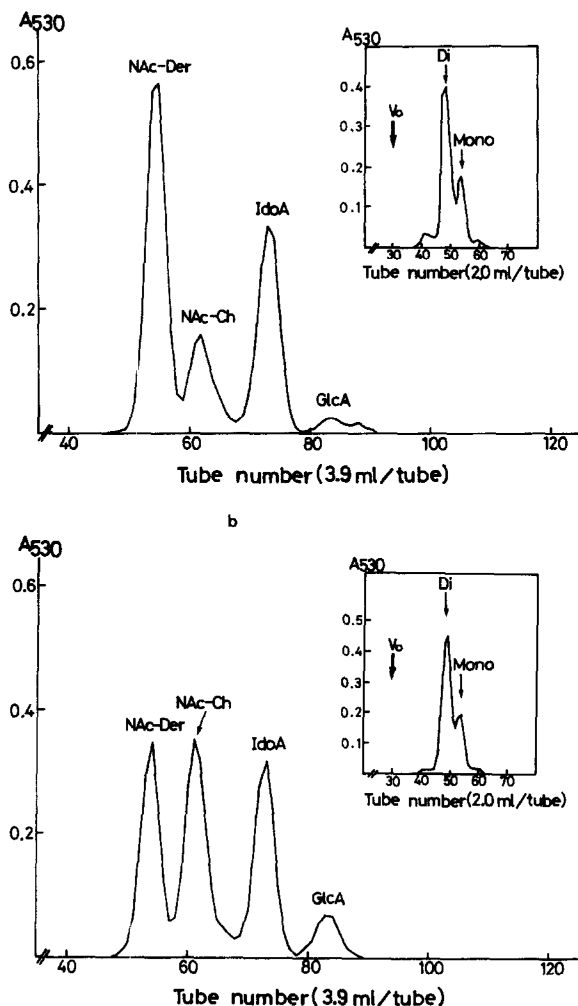

Fig. 1. Anion-exchange chromatography, on AG 1-X4 resin(HCO_2^-), of the mono- and disaccharide reaction products of dermatan sulphates treated with water–dimethyl sulphoxide (1:9, v/v) for 30 h at 108 ± 1°C. (a) Pig-skin dermatan sulphate; (b) rooster-comb dermatan sulphate, RC-30 fraction. Gel filtration diagrams, on Sephadex G-25, of the whole reaction products from dermatan sulphate are shown in each inset. NAc-Der = N-acetyldermosine; NAc-Ch = N-acetylchondrosine; IdoA = L-iduronic acid; GlcA = D-glucuronic acid; Di = disaccharide fraction; Mono = monosaccharide fraction; V_o = void volume.

preferentially cleaved and gradual liberation of the hexuronic acid residues takes place without any marked decomposition of them.

We have investigated an optimum reaction condition to minimize the amount of unreacted oligosaccharide (18.3% of the total material subjected to hydrolysis) without decomposition of hexuronic acid components, especially of L-iduronic acid, and have succeeded in obtaining the disaccharide and monosaccharide fractions almost quantitatively by heating the pyridinium salts of the polysaccharides in water–dimethyl sulphoxide (1:9, v/v) at 108 $\pm$ 1°C for 30 h. As shown in Fig. 1, pig-skin and rooster-comb (RC-30) dermatan sulphates were almost completely hydrolyzed to the constitutional disaccharide and monosaccharide species under the conditions described. Although the elution data are not shown here, rooster-comb (RC-20) dermatan sulphate and 2-aminoethylamino derivatives of these dermatan sulphates all give results similar to those of Fig. 1. A small peak (tube Nos. 35–45) before the disaccharide peak is mainly due to unreacted oligosaccharides, and another small peak (tube Nos. 57–65) after the monosaccharide peak is due to lactones of D-glucuronic acid and L-iduronic acid (the insets of Fig. 1). The carbazole–orcinol ratio of the small peak (tube Nos. 35–45) was assayed to determine the approximate hexuronic acid content, and the value roughly agreed with those obtained by the method proposed herein (experiments and data not shown), indicating no appreciable error due to neglect of this small peak from the whole procedure. All the fractions except the first small peak (tube Nos. 35–45) were subjected to subsequent separation on AG 1-X4 ion-exchange resin. The separation was satisfactory as shown in Fig. 1. HPLC with a Whatman Partisil-10 SAX or Partisil-10 PAC column using a linear gradient of potassium dihydrogenphosphate, or with a reversed stationary phase column (ODS) using an acetonitrile–water system, did not resolve the mono- and disaccharide reaction products from dermatan sulphate (data not shown).

The hexuronic acid contents of the dermatan sulphates and of their 2-amino ethylamino derivatives determined by the above method were in close agreement with those obtained by the enzymatic method as shown in Table I. Our method, which

TABLE I

RATIOS OF D-GLUCURONIC ACID CONTENT TO TOTAL HEXURONIC ACID CONTENT (%) IN DERMATAN SULPHATES AND THEIR 2-AMINOETHYLAMINO DERIVATIVES, ASSAYED BY BOTH ENZYMATIC AND THE PRESENT METHODS

Sample	*Ratio of D-glucuronic acid to total hexuronic acid(%)*	
	Enzymatic method	*Present method*
Pig-skin dermatan sulphate	18.6	19.1
Rooster-comb dermatan sulphate		
RC-20 fraction	21.5	23.7
RC-30 fraction	40.9	42.8
2AEA pig-skin dermatan sulphate[a]	19.1	21.6
2AEA rooster comb dermatan sulphate		
RC-20 fraction	22.6	22.1
RC-30 fraction	41.4	42.8

[a] 2AEA = 2-aminoethylamino.

consists of acid hydrolysis and chromatographic separation, is necessarily accompanied by some errors due to these two processes. One of them would be a deviation from the stoichiometry of the acid hydrolysis, and another would be non-ionic irreversible adsorption on the AG 1 anion-exchange resin. On the other hand, the enzymatic determination using chondroitinase AC and ABC[3] has been demonstrated by us (data not shown) to be susceptible to varous factors specific to the enzymatic reactions, such as variance in the structures of the substrates (the existence of a variety of dermatan sulphate–chondroitin sulphate copolymers) or in the enzyme source (condroitinase AC-I from *Flavobacterium*[3] and AC-II from *Arthrobacter*[11]. Accordingly, we thought that the data on the hexuronic acid content obtained by the enzymatic method need to be supported by some other method differing in principle from the enzymatic method. However, there was only a semiquantitative method based on the carbazole–orcinol ratio[2]. As described above, the method proposed here is very simple both in principle and practice, and the data in Table I show that the values obtained are reliable. Thus, our method is considered to be useful for investigators in the biochemical and medical fields as a complement to the existing methods.

REFERENCES

1 L.-Å. Fransson, in G. O. Aspinall (Editor), *The Polysaccharides*, Vol. 3, Academic Press, New York, 1985, p. 338.
2 P. Hoffman, A. Linker and K. Meyer, *Arch. Biochem. Biophys.*, 69 (1957) 435.
3 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1523.
4 K. Nagasawa, A. Ogamo, H. Ichihara and K. Yoshida, *Carbohydr. Res.*, 131 (1984) 301.
5 K. Meyer, E. Davidson, A. Linker and P. Hoffman, *Biochim. Biophys. Acta*, 21 (1956) 506.
6 H. Uchiyama and K. Nagasawa, *Carbohydr. Res.*, 159 (1985) 263.
7 Y. Inoue and K. Nagasawa, *Carbohydr. Res.*, 97 (1981) 263.
8 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330.
9 M. Kosakai and Z. Yosizawa, *Anal. Biochem.*, 93 (1979) 295.
10 H. Saito, T. Yamagata and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1536.
11 K. Hiyama and S. Okada, *J. Biol. Chem.*, 250 (1975) 1824.