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DETERMINATION OF SULPHATED DISACCHARIDES FROM CHONDROITIN SULPHATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive method for the determination of chondroitin 4- and 6-sulphate is presented. After chondroitinase digestion of the chondroitin sulphate preparations, the obtained disaccharides are separated on a weak anion-exchange resin in a high-performance liquid chromatography system. The method was used to study 4-sulphate to 6-sulphate ratios in chondroitin sulphates prepared from bovine nasal cartilage and human nucleus pulposus. The results show clearly that these two preparations contain considerable amounts of both isomers.

INTRODUCTION

When studying chondroitin sulphates (CSs) and CS-containing proteoglycans, the ratio of chondroitin 4-sulphate (CS-4) to chondroitin-6-sulfate (CS-6) is an important factor in heterogeneity and possibly also in the biological function of the polysaccharides[1]. The relative amounts of these CS isomers have earlier been estimated using IR spectral analysis[2], chromatography on cetylpyridinium-cellulose[3] or ratios of colorimetric reactions following chondroitinase digestion[4]. A common feature of these methods is that the analytical results were related to substances that were assumed to be pure CS-4 and CS-6. However, the methods do not give identical results.

In another approach to the study of the distribution of 4- and 6-sulphates, paper chromatography of chondroitinase digests was used[5], whereby the disaccharide products, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (Δ-di-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (Δ-di-6S), can be separated. This method, however, has an unsatisfactory sensitivity with consequential difficulties in quantifying the results.

The aim of this study was to develop a method accurate and sensitive enough to determine the amounts of 4- and 6-sulphate in CS preparations. To this end, we tried to separate the Δ-disaccharides obtained after chondroitinase digestion using a high-performance liquid chromatography (HPLC) system.

EXPERIMENTAL

Materials

The following CS preparations were used: (a) commercially available CS-4 and CS-6 (Sigma, St. Louis, Mo., U.S.A., grade I, prepared from "whale cartilage" and "shark cartilage", respectively); (b) a mixed isomer preparation (Sigma grade II); and (c) CS-4 and CS-6 prepared from bovine nasal cartilage and human nucleus pulposus according to Antonopoulos *et al.*[6]. In order to remove possible peptide residues from the linkage region, all CS preparations were treated with alkali according to Heinegård[7].

The Δ-disaccharides were prepared for chromatography from the CSs by enzymatic digestion according to Yamagata *et al.*[8] using chondroitinase ABC (Sigma). Chondro-4-sulphatase and chondro-6-sulphatase were obtained from Sigma. Sephadex G-10 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical-reagent grade or better.

Analytical methods

Uronic acid was analysed by the carbazole reaction (Bitter and Muir) modified for the AutoAnalyzer II system[9]. Hexosamines were determined by the Elson and Morgan procedure modified by Antonopoulos *et al.*[6]. N-Acetylhexosamine was assayed by a modification[10] of the Morgan and Elson procedure. UV spectra of materials corresponding to the chromatographic peaks were obtained using an Aminco DW-2 spectrophotometer.

HPLC procedures. The HPLC experiments were carried out at ambient temperature on μBondapak Carbohydrate (Waters Assoc., Milford, Mass., U.S.A.). This resin is a fully porous, weak anion exchanger, with an NH_3^+ group as the active site ($pK_a = 6$). The amine is linked via an isopropyl structure to the silica backbone, the particle size being 10 μm.

The resin was packed in a 300 × 4 mm I.D. stainless-steel column. The samples were injected via a loop injector (loop volume 100 μl) connected close to the column. To remove particulate material, if present, the samples were filtered through a Millipore filter (pore size 0.5 μm) or centrifuged at 10,000 *g* for 30 min. The column was eluted with sodium acetate or sodium sulphate solution containing 0.01 *M* acetate buffer. When the filtered and deaerated eluents were pumped at 1 ml/min, a pressure of 5 MPa was obtained. The elution patterns were recorded spectrophotometrically at 231 nm, using a Perkin-Elmer LC-55 spectrophotometer with an 8-μl cell, or by connecting the column outlet directly to an AutoAnalyzer II, equipped for uronic acid determination[9]. The recorded peaks were integrated using a Kontron MOP/AMO03 morphometric device (Kontron, Munich, G.F.R.).

Experimental procedure. To find suitable conditions for separating Δ-di-4S and Δ-di-6S, disaccharides were prepared from the mixed CS isomers. The samples were injected into the column and different salt concentrations (sodium acetate or buffered

sodium sulphate) were tried, together with variations of the pH and the elution flow-rate. It was found that 0.2 *M* sodium acetate at pH 5.0 or 0.02 *M* sodium sulphate in 0.01 *M* acetate buffer at pH 5.0 and a flow-rate of 0.5 ml/min gave a complete separation of the main material. The digests of the CS-4, CS-6 and mixed isomer preparations were chromatographed in this manner. Guided by the UV detector, the peak materials were pooled and the pooled materials obtained were designated 1–8 (*cf.*, Fig. 1). The purity of these materials was monitored by re-chromatography under the same conditions.

In order to identify the peaks, the following experiments were undertaken: (1) colour reactions as above; (2) digestions of aliquots with chondro-4-sulphatase and chondro-6-sulphatase[8] and subsequent re-chromatography of these digests; (3) comparison of UV detector recordings with recordings obtained from the Auto-Analyzer II (differences in molar UV absorption); and (4) UV spectral analyzes. Prior to UV spectral analyses, the uronic acid-containing materials were separated from the interfering acetate using gel chromatography in a Sephadex G-10 column. In order to detect even more retarded peaks, if present in the mixed isomer digests, chromatograms were obtained under less retaining conditions (0.05 *M* sulfate solution at pH 5.0) and with the AutoAnalyzer II as the detector.

RESULTS AND DISCUSSION

The digests from the commercial CS preparations were eluted as shown in Fig. 1. Except at pH values close to 6 (pK_a for the resin), the k' values were constant in the

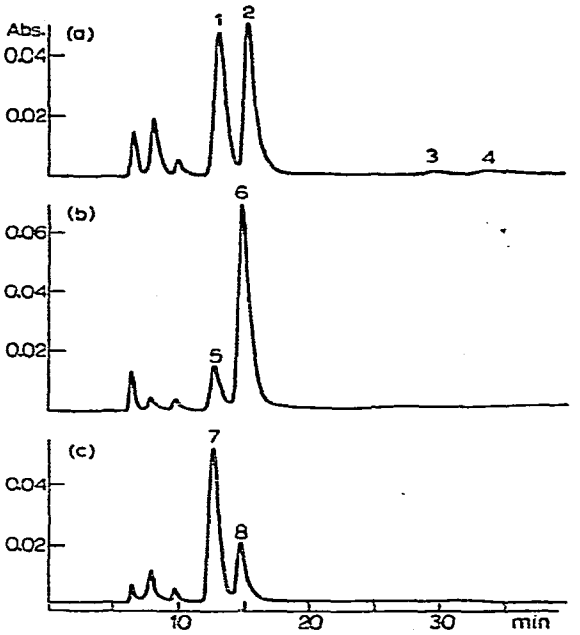


Fig. 1. UV recordings from chromatography of (a) the mixed isomer preparation, (b) CS-4 grade I and (c) CS-6 grade I in 0.02 *M* SO_4^{2-} at pH 5.0. Peak materials were pooled and designated 1–8 as shown. Peaks 1, 5 and 7 were identified as Δ-di-6S and peaks 2, 6 and 8 as Δ-di-4S. Peaks 3 and 4 are tentatively identified as tetrasaccharides. Of the three front peaks, the second contains Δ-di-0S and the third contains acetate (from the digestion mixture).

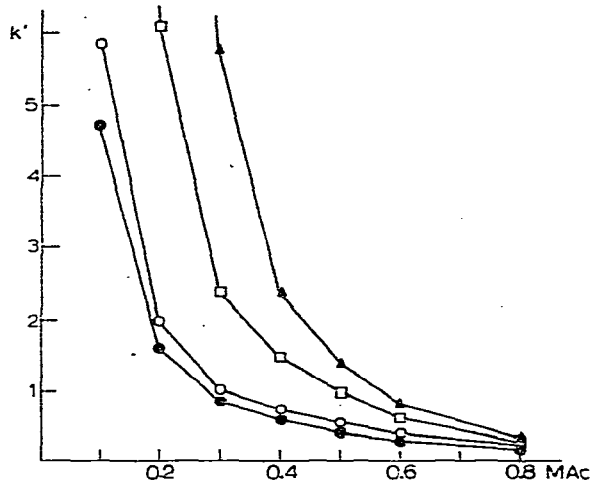
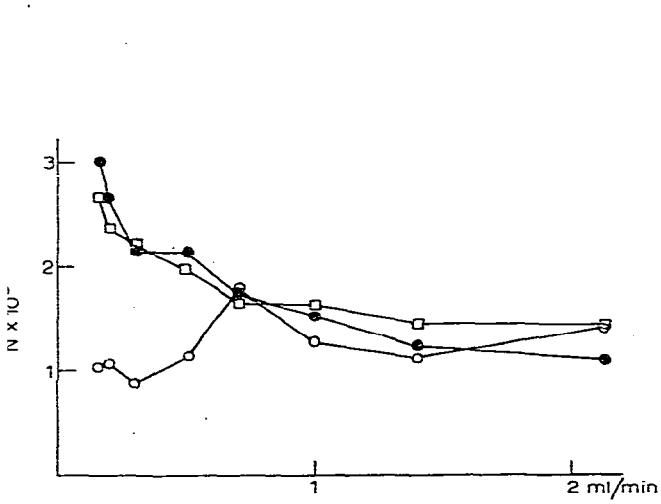


Fig. 2. Retardation of mixed isomer digest material at different ionic strengths. Peak Nos. (*cf.*, Fig. 1a): ●, 1; ○, 2; □, 3; ▲, 4.

pH range tested, giving an α ratio of 1.23 for peaks 1 and 2. As shown in Fig. 2, the material was more retarded as the ionic strength decreased. The average theoretical bottom plate number as calculated from peaks 1 and 2 increased with decreasing elution speed (Fig. 3). Using 0.1 *M* acetate, however, the performance significantly decreased at flow-rates below 0.7 ml/min, which may be due to diffusion effects in the column. The 0.2 *M* acetate solution at a flow-rate of 0.5 ml/min gave a separation of peaks 1 and 2 close to the baseline within 17 min.



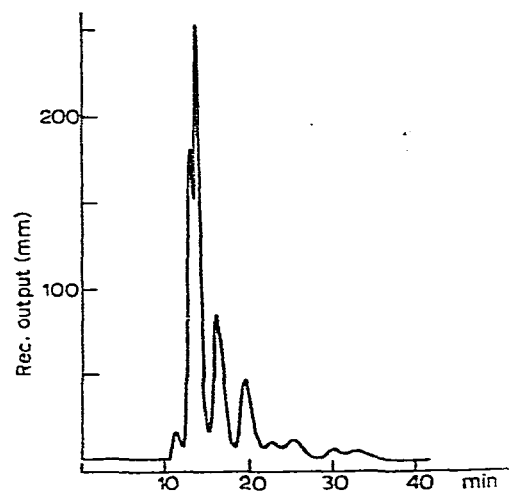


Fig. 3. Theoretical bottom plate number (N) at different ionic strengths and at different elution speeds. Acetate concentration: ●, 0.3 *M*; □, 0.2 *M*; ○, 0.1 *M*.

Fig. 4. AutoAnalyzer recordings (carbazole reaction) from chromatography of an incompletely digested mixed isomer preparation in 0.05 *M* SO_4^{2-}. Close to the front the main disaccharide peaks are eluted together. The following peaks are also eluted in pairs, which may represent isomers of tetra-, hexa- and octasaccharides.

Re-chromatography of the isolated peaks showed a contamination of less than 1% from the adjacent peak, except fraction 8, which showed a 5% admixture. The chemical analysis (Table I) revealed equimolar amounts of hexosamine and uronic acid. The absence of colour development by the Morgan and Elson reaction in fractions 2, 4, 6 and 8 indicates that the hexosamines in these fractions are 4-sulphated. This is also supported by the sensitivity to the different sulphatases as demonstrated in Table I. Partial degradation was, however, found in the less sensitive fractions, probably owing to non-specific enzyme activity. The digested disaccharide material, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (Δ-di-0S), was recovered together with the second of the three front peaks in Fig. 1a. Chemical analysis of material from this peak in the mixed isomer digests also indicated presence of Δ-di-0S in this part of the chromatogram. The results indicate that the materials in farctions 1, 3, 5 and 7 are 6-sulphated whereas the other materials are 4-sulphated.

TABLE I

CHEMICAL ANALYSES OF THE EIGHT POOLED PEAK MATERIALS (*cf.*, FIG. 1)

Parameter	*Peak No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Hexosamine to uronic acid molar ratio	1.01	0.99	1.02	1.01	1.02	0.88	1.02	0.95
Reactivity in the Morgan and Elson reaction	+	−	+	−	+	−	+	−
Sensitivity to chondro-6-sulphatase	+	−	+	−	+	−	+	−
Sensitivity to chondro-4-sulphatase	−	+	−	+	−	+	−	+

UV spectral analyses showed one broad peak with a maximum at 231 nm for the 4-sulphated and at 228 nm for the 6-sulphated materials. The molar absorptivity of the 4-sulphated fractions at 231 nm was only 91% of the corresponding molar absorptivity of the 6-sulphated isomers. Similar results were obtained on comparing the UV detector and AutoAnalyzer results. Digestion with sulphatase changed the absorption maximum to 218 nm, and the molar absorptivity at 231 nm was simultaneously increased by more than 100%.

The molar absorptivities at 231 nm of fractions 3 and 4, however, were approximately half of those obtained for fractions 1 and 2, respectively. This indicates that these fractions are tetrasaccharides with only one of the glucuronic acids being unsaturated. Hence the chondroitinase digestion is incomplete. The relative size of these peaks also varied from digest to digest, but the relative amount was less than 5% after 24 h of digestion. In fraction 3 both hexosamines carry a 6-sulphate and in fraction 4 they are 4-sulphated. The absence of significant amounts of hybrid tetrasaccharides indicates a mixture of low hybridized isomers. Chromatography of the same preparation under less retaining conditions (Fig. 4) showed trace amounts of other oligosaccharides, probably hexa- and octasaccharides.

In order to test the reproducibility of the separation, ten aliquots of the CS grade II preparation were simultaneously digested, and the relative amounts of Δ-disaccharides were determined. The standard deviations were 1.4% of the mean values obtained. These standard deviations did not differ significantly from those obtained when ten attempts were made to integrate the same chromatogram. It therefore seems as if the limiting factor is the precision by which the morphometrical device is handled.

TABLE II

RELATIVE AMOUNTS OF Δ-DI-4S AND Δ-DI-6S IN CS DIGESTS

CS preparation	*Proportion (%)*	
	Δ-di-4S	*Δ-di-6S*
From bovine nasal cartilage	87	13
From human nucleus puposus	28	72
"Whale cartilage" (Sigma CS-4, grade I)	78	22
"Shark cartilage" (Sigma CS-6, grade I)	19	81

The eluate was also analysed for its uronic acid content; 85% of the injected material could be recovered in peaks 1—4 (*cf.*, Fig. 1a).

The CSs from bovine nasal cartilage and human nucleus pulposus have earlier been considered to be almost pure CS-4 and CS-6, respectively. These CSs were studied with the present chromatographic technique and, in contrast to earlier chromatographic investigations[3], both preparations (and also the commercially available "grade I" preparations) were found to contain considerable amounts of both isomers (Table II). The results obtained are similar to those obtained from colorimetric determinations of the Δ-di-4S to Δ-di-6S ratios in CS preparations from corresponding guinea pig tissues[11].

Over-sulphated disaccharides[12] could not be detected with the present method. Other chromatographic systems for the detection of these compounds, involving chromatography on strong anion-exchange resins, have been described earlier[11]. Most CS preparations, however, do not contain significant amounts of over-sulphated disaccharide residues. Therefore, the present method, which also has a satisfactory sensitivity (with detection limits of the order of 10–20 ng of uronic acid for both detectors used), makes it possible to perform reliable determinations of the different disaccharide residues in CS preparations.

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REFERENCES

1 A. Hjerpe, *Dissertation*, Karolinska Institutet, Stockholm, 1976.
2 M. B. Mathews, *Nature (London)*, 181 (1958) 421.
3 C. A. Antonopoulos and S. Gardell, *Acta Chem. Scand.*, 17 (1963) 1474.
4 S. Lohmander, *Eur. J. Biochem.*, 57 (1975) 549.
5 H. Saito, T. Yamagata and S. Suzuki, *J. Biol. Chem.*, 244 (1969) 77.
6 C. A. Antonopoulos, S. Gardell, J. A. Szirmai and E. R. de Tyssonsk, *Biochim. Biophys. Acta*, 83 (1964) 1.
7 D. Heinegård, *Biochim. Biophys. Acta*, 285 (1972) 193.
8 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, *J. Biol. Chem.*, 243 (1968) 1523.
9 A. Hjerpe, C. A. Antonopoulos and B. Engfeldt, *Scand. Clin. Lab. Invest.*, in press.
10 J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, 217 (1955) 959.
11 S. Lohmander, C. A. Antonopoulos and U. Friberg, *Biochim. Biophys. Acta*, 404 (1975) 93.
12 S. Suzuki, H. Saito, T. Yamagata, K. Anno, N. Seno, Y. Kawai and T. Furuhashi, *J. Biol. Chem.*, 243 (1968) 1543.