Journal of Chromatography, 242 (1982) 193–195
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,768

Note

Determination of disulphated disaccharide residues in chondroitin sulphate, using high-performance liquid chromatography

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(Received January 25th, 1982)

The chondroitin sulphates (CSs) are mainly sulphated at the fourth or sixth carbon of the hexosamine moiety (*cf.* Fig. 1), the position probably being of biological importance[1,2]. The existence of oversulphated disaccharides was first demonstrated in preparations from squid and shark cartilages[3]. The finding of large amounts of oversulphated disaccharide residues in pathologically altered human bone tissue[4,5] indicated that such an oversulphation also might be of biological importance.

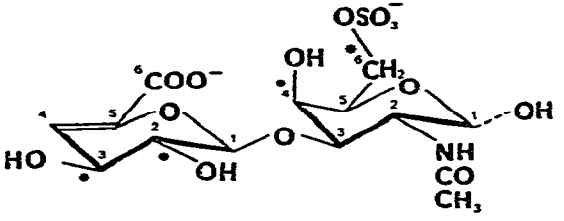

Fig. 1. Structural formula of the 6-sulphated disaccharide derived from 4,5-unsaturated chondroitin sulphate. Asterisks denote the different possibilities for O-sulphation.

Some years ago we described a high-performance liquid chromatographic (HPLC) system, by which non- and monosulphated CS disaccharide residues were analysed in low amounts[6]. The failure then to detect disulphated CS disaccharides may have been due to a sulphatase contamination of the enzyme preparations used. Enzymes now available, however, seem to be pure in this respect, enabling the isolation of such oversulphated disaccharides. The aim of the present paper is to describe briefly the slightly modified conditions by which the oversulphated residue may be studied, also in low amounts, such as in a bone tissue biopsy specimen.

MATERIALS AND METHODS

The CS used was obtained from Sigma (St. Louis, MO, U.S.A.) as their grade II mixed isomer preparation. Because this CS is prepared partly from shark cartilage, oversulphated disaccharide residues might be expected in the chondroitinase digest. Chondroitinase AC, chondroitin-4-sulphatase and chondroitin-6-sulphatase were all obtained from Sigma. All other chemicals were of analytical grade.

Chondroitinase and sulphatase digestions were performed principally according to Suzuki *et al.*[3]. The digests were chromatographed in a Hypersil APS column (250 mm × 4.6 mm I.D.). The column was eluted with 0.1 *M* Na_2SO_4 containing 0.05 *M* sodium acetate at pH 5.0, the eluent being pumped at 0.7 ml/min. The UV absorption at 231 nm was recorded.

Peak materials were pooled and analysed for uronic acid, using an automated carbazole reaction[7,8], as well for 4,5-unsaturated uronic acids, using the periodate–thiobarbituric acid method[9]. In order to detect possible N-sulphation, aliquots were treated[10] with HNO_2 to deaminate N-sulphated hexosamines. After desalting, the materials were hydrolysed with 6 *M* HCl at 100°C for 8 h, and subsequently subjected to hexosamine determination[11]. Attempts to remove possible N-sulphate was also made[10], using 0.04 *M* HCl at 100°C for 1 h. The effect of this treatment was studied with the present chromatographic set-up. Sulphate analyses of pooled peak materials were performed with a scaled-down version of the benzidine method of Antonopoulos[12].

RESULTS AND DISCUSSION

The chondroitinase AC digests separated into five different disaccharide peaks (Fig. 2), which were recovered after 5.2, 6.4, 6.6, 8.2 and 9.2 min, respectively. The chemical analysis indicated that all five peaks consisted of disaccharides and not

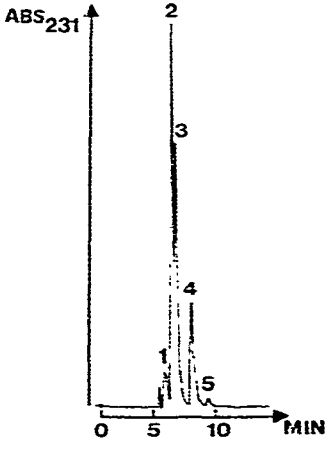

Fig. 2. Chromatography of a chondroitinase AC digest of the Sigma grade II mixed isomer preparation. The material was eluted from the Hypersil APS column with 0.1 *M* Na_2SO_4, also containing 0.05 *M* sodium acetate at pH 5.0. Peaks: 1 = non-sulphated disaccharide; 2 = 6-sulphated disaccharide; 3 = 4-sulphated disaccharide; 4 and 5 = two oversulphated disaccharides.

tetrasaccharides, which also may be found as by-products from chondroitinase digestion[6]. The sulphate analysis showed that the first peak is non-sulphated, the second and third are monosulphated, and the remaining two peaks are oversulphated. The sulphatase experiments also verify this finding. The fourth and fifth peaks are both sensitive to 6-sulphatase digestion, but seem to be unaffected by 4-sulphatase. After 6-sulphatase digestion, materials from peaks 4 and 5 can be recovered as separate peaks eluted later than the 6-sulphated disaccharides, but earlier than the 4-sulphated disaccharides.

These experiments indicate that peaks 4 and 5 represent two different disulphated disaccharides, both with one sulphate at the sixth carbon of the hexosamine, while the fourth carbon carries a hydroxyl group. Because all pooled peak materials were unaffected by HNO_2 and 0.04 *M* HCl, there seems to be no N-sulphation. The only positions thus left for a second sulphate are C-2 and C-3 of the uronic acid (*cf.* Fig. 1). It is tempting to believe that peaks 4 and 5 represent these two alternatives. This interpretation is also supported by the finding of low reactivity in the periodate–thiobarbituric acid reaction. Further attempts to identify the peaks obtained necessitate the collection of larger amounts and have so far not been made.

The molar UV absorption of the different peaks varies considerably. The use of external standards for each peak is therefore advisable. The non- and monosulphated disaccharides are poorly separated with the present set-up, and the previously described[6] lower ionic strength seems more suitable for the determination of these constituents. We have tried gradients to elute all disaccharides in the same chromatogram. This, however, caused a considerable baseline instability and the appearance of ghost peaks, probably originating in the eluent. It thus seems as if the determination of disulphated disaccharides is best performed as a separate experiment.

ACKNOWLEDGEMENTS

This work was supported by grant no B 81-12x-05934-01 from the Swedish Medical Research Council and from the funds of the Karolinska Institute.

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