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Note

Use of thin-layer chromatography in the separation of disaccharides resulting from digestion of chondroitin sulphates with chondroitinases*

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An approach to the identification of glycosaminoglycans was developed by Saito *et al.*[1], who used specific enzymes from bacterial origin that degrade chondroitin sulphates. Among these enzymes, chondroitinase ABC degrades chondroitin 4-sulphate (C-4S), dermatan sulphate (DS) and chondroitin 6-sulphate (C-6S). Both enzymes also degrade hyaluronic acid and chondroitin. The degradation is carried out by an elimination reaction yielding disaccharides consisting of 4,5-unsaturated uronic acid and hexosamine at the reducing end carrying a sulphate group at the 4- or 6-position, or no sulphate in the case of non-sulphated glycosaminoglycans[2]. These disaccharides exhibit different mobilities in paper chromatography and can be rendered visible by illumination with UV light. They can be quantified either by measuring their absorbances at 232 nm or by the help of colour reactions for their constituent monosaccharides[2,3].

This method has been widely applied to the identification of glycosaminoglycans in various tissues and in fractions obtained by a variety of procedures. In view of the polydispersity of glycosaminoglycans in biological materials, as shown by their different molecular weights and the variability in the degree of sulphation[4], fractionations generally do not yield homogeneous preparations and glycosaminoglycans of a single type. The determination of disaccharides resulting from digestion with chondroitinases was found to be a very useful means for the identification of the components present.

Paper chromatography is applicable to amounts of disaccharides ranging from 25 to 100 μg of each isomer, which might be higher than desirable when dealing with minor components, and the procedure, including a desalting step, requires about 36 h. The present work was aimed at the development of a more rapid and sensitive method for the qualitative and quantitative determination of these disaccharides. Cellulose thin-layer chromatographic (TLC) plates were found to give satisfactory separations and detection of amounts of disaccharides as low as 2.5 μg. The application of colour reactions with the eluted spots made possible their quantification. An advantage of this method is the short period of time (12 h) required for the development of the thin-layer chromatograms.

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MATERIALS AND METHODS

Chondroitin 4- and 6-sulphate were purchased from Miles Labs. (Kankakee, Ill., U.S.A.). Hyaluronic acid and dermatan sulphate were kindly supplied by Dr. Cifonelli, University of Chicago, Chicago, Ill., U.S.A. Commercial unspecified preparations of chondroitin sulphate were obtained from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.; CS-1) and Delta Chemical Works (New York, N.Y., U.S.A.; CS-2). Chondroitinases ABC and AC and unsaturated disaccharides were obtained from Seikagatu Kogyo (Tokyo, Japan). Pre-coated cellulose F TLC plates, 0.1 mm thick, were supplied by Merck, Darmstadt, G.F.R. All chemicals were of analytical-reagent grade.

Digestion with chondroitinases

The digestion mixture consisted of 10 μl of a solution of glycosaminoglycan in water, 10 μl of enriched Tris buffer (pH 8.0)[1] and 20 μl of an aqueous solution of enzyme containing 10 units/ml. After incubation for $2^1/_2$ h at 37°, another 10-μl portion of enzyme solution was added and incubation continued for another $2^1/_2$ h[5]. The control mixture consisted of the same components (except enzyme), brought to the same final volume. Four volumes of 96% ethanol were then added and the mixtures left overnight at 4°. The clear supernatant obtained by centrifugation was dried in a desiccator and the residue, dissolved in a small volume of water, was applied on the TLC plates.

Thin-layer chromatography

Initially, desalting was carried out using 1-butanol–ethanol–water (52:32:16) for 6 h[5]. After drying in air the plates were developed for 6 h using as solvent 1-butanol–acetic acid–1 *N* ammonia solution (2:3:1)[1]. The unsaturated disaccharides were rendered visible under shortwave UV light as brown spots or after immersion in silver nitrate[6,7] and were identified by comparing their mobilities with those of the reference compounds. For quantitative determinations the areas located by UV illumination representing the various materials as well as clear background areas on the plates were scraped off and eluted with 1 ml water using sonication with a MSE ultrasonicator at maximal intensity for 2 min and subsequent heating at 50° for 1 h. After centrifugation and reduction of the volume of the supernatant by evaporation, the disaccharides were determined either by the method of Hascall *et al.*[8], which determines unsaturated uronic acid, or by the Elson-Morgan reaction for hexosamines as modified by Antonopoulos[9], after hydrolysis with 4 *N* hydrochloric acid for 7 h at 100°.

Uronic acid was determined according to the method of Bitter and Muir[10] and protein by the method of Lowry *et al.*[11].

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of a mixture of 20 μg each of the disaccharides ΔDi-4S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine 4-sulphate), ΔDi-6S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine 6-sulphate) and ΔDi-0S (3-O-Δ^4-glucuronosyl-N-acetylgalactosamine) and the individual compounds separately. Experiments with smaller amounts showed that spots corresponding to 2.5 μg of material could still be detected. It should be pointed out that parallel experiments

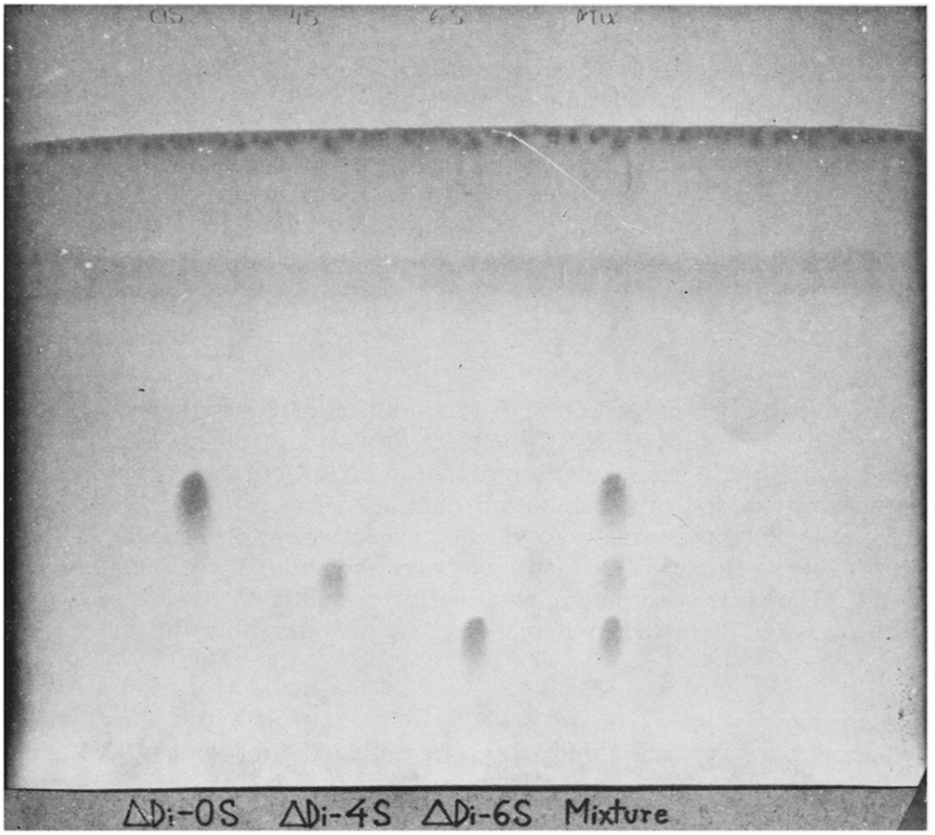

Fig. 1. TLC of standard unsaturated disaccharides. Amounts applied: 20 μg each. Solvents and conditions as described in the text. Spots revealed by UV illumination.

carried out by using paper chromatography indicated that 25 μg was the limit of detection.

Fig. 2 shows the degradation products obtained in the incubation of 50 μg of C-4S and C-6S with chondroitinase ABC and AC. A mixture of the markers developed on the same plate, revealing spots of ΔDi-6S, ΔDi-4S and ΔDi-0S in this order of increasing mobility, permitted the identification of the degradation products. The major spots corresponded to the expected products and the additional minor spots probably represent minor contaminants.

Dermatan sulphate gave, as expected, ΔDi-4S following incubation with chondroitinase ABC and remained undigested by chondroitinase AC (not shown in the figure).

In the experiment illustrated in Fig. 3 we examined the sensitivity of the method by using decreasing amounts of C-6S. The intensity of the spots was proportional to the amount of substrate and the degradation product resulting from 12.5 μg of initial substance is still clearly detectable. In fact, in view of the above-mentioned contamination, the true amount is even lower, indicating the high sensitivity of the method.

The experiment illustrated in Fig. 4 was designed to show the applicability of

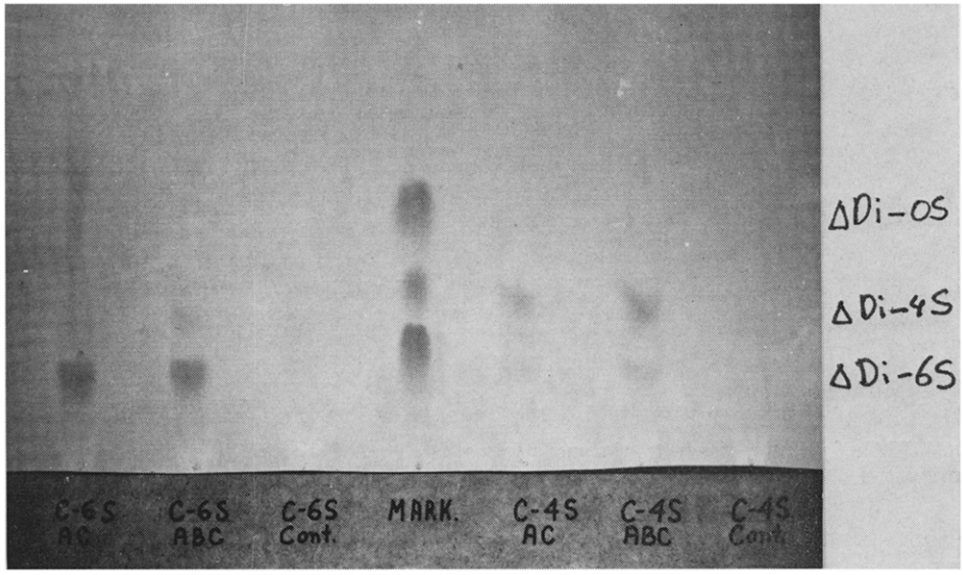

Fig. 2. TLC of digestion products of C-4S and C-6S with chondroitinase ABC and AC. Amounts of substrates used: 50 μg. Controls (cont.) consisted of substrates without enzymes. Mark., reference disaccharide markers.

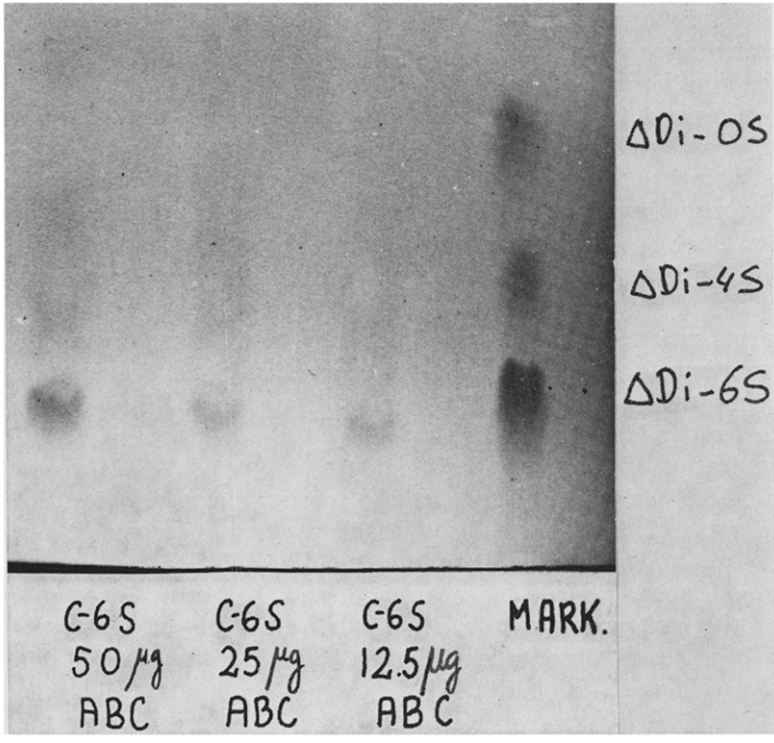

Fig. 3. TLC of digestion products of decreasing amounts of C-6S with chondroitinase ABC.

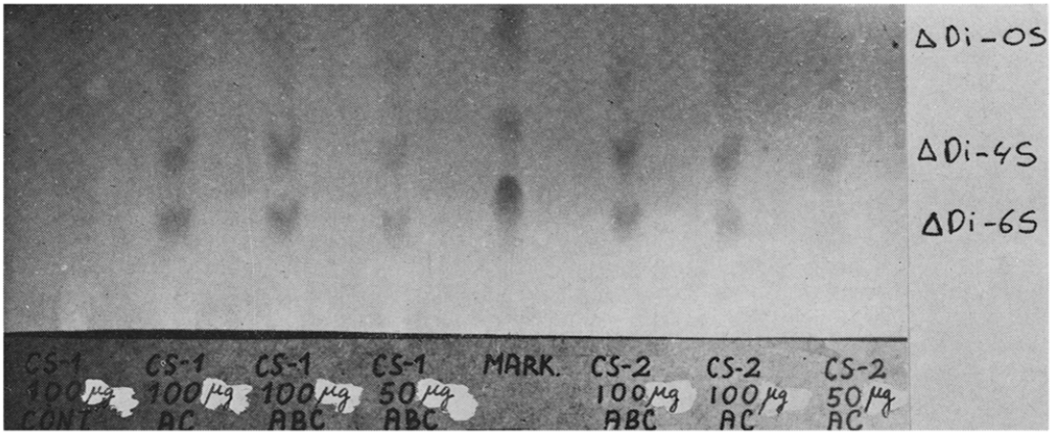

Fig. 4. TLC of digestion products of commercial preparations of chondroitin sulphate with chondroitinase ABC and AC.

TLC in the analysis of impure preparations of mixtures of C-4S and C-6S often encountered in extracts of biological materials. For this purpose, unspecified preparations of chondroitin sulphate from two different commercial sources, found to contain similar concentrations of uronic acid (about 23%) and protein (about 20%), were used. The significant content of protein presumably indicates the presence of proteoglycans. The sensitivity of the method was confirmed by the fact that digestion products from amounts of substrates corresponding to 12 μg of uronic acid were clearly detectable. Other experiments not shown in the figure were carried out with half of this amount and the results were satisfactory.

Experiments not presented in the figures showed that the non-sulphated unsaturated disaccharide derived from the degradation of hyaluronic acid with chondroitinase migrated faster than the reference marker ΔDi-0S supplied by the manufacturer, which is a galactosamine-containing disaccharide, indicating the possibility of resolving mixtures of glucosamine and galactosamine-containing disaccharides. This difference in mobility has also been reported by other workers who used paper chromatography[5].

The experiments reported above included detection of the spots under UV light. It should be mentioned that the spots can also be revealed by the silver nitrate procedure for reducing sugars. The sensitivity of this staining method, however, is lower than that achieved by UV illumination.

For the determination of the various disaccharides separated on the TLC plates two procedures were used. The generally used methods consisting of measurement of absorbance at 232 nm and performance of the carbazole reaction for uronic acid in eluates from paper proved to be inapplicable with the pre-coated TLC plates used in view of the background absorption at this wavelength and the positive carbazole reaction. With two other reactions, however, the results in this respect were satisfactory. Thus, the background absorption with the Elson-Morgan reaction for hexosamines and the periodic acid–thiobarbituric acid reaction for unsaturated uronic acids described by Hascall *et al.*[8] was virtually zero. Experiments with one of the commercial preparations of chondroitin sulphate showed good agreement between

the two colorimetric methods with regard to the relative amounts of the isomeric components. The determination of unsaturated uronic acid has the advantages of being faster and more sensitive than that of hexosamine. It should be noted that with the former method care should be taken to use appropriate standards of disaccharides as the colour intensity of their reaction products is different. Thus, the ratios of the absorbances of equal concentrations were approximately 1:2:3 for ΔDi-4S, ΔDi-0S and ΔDi-6S.

The recovery of the TLC method was examined with the three pure disaccharides. 40 μg of each, individually and in admixture, were applied on the TLC plates. After migration, the recoveries in the eluates of the corresponding spots, using the reaction for unsaturated uronic acid, were 80–105%. Recoveries of this magnitude can be considered satisfactory in view of the possible errors involved in the various steps, and the quantitative procedure can therefore be recommended, especially in experiments in which the relative amounts of isomeric chondroitin sulphates are to be determined in mixtures.

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