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Note

Determination of hyaluronic acid using high-performance liquid chromatography of chondroitinase digests

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Hyaluronic acid (HA) is a glycosaminoglycan (GAG) made up of alternating D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose. The naturally occurring molecule is usually built up of 500 or more such disaccharide units in an unbranched chain, thereby forming the largest GAG. This polysaccharide is present in most connective tissues, and it has been ascribed a variety of biological functions. Thus HA is supposed to retain water and regulate water flow in the tissues. Furthermore, HA aggregates proteoglycan (PG) monomers, thereby influencing the properties of the tissue, and it is also assumed to participate in the regulation of glycosaminoglycan biosynthesis[1,2].

Many procedures for the determination of HA involve chromatographic isolation and subsequent colorimetric reactions[2]. The separation of HA from other GAGs present in the preparation might, however, offer some difficulties. Thus, for example, chondrotin sulphate (CS) is made up of alternating D-glucuronic acid and 2-acetamido-2-deoxy-D-galactose, the latter most often carrying a sulphate ester group. This GAG may, however, be difficult to separate from HA, especially when the CS molecular weight is low and/or the CS has a low content of sulphate. This problem is considerable when the relative content of CS is very high, and similar problems also appear when HA is to be isolated from tissues with a high content of strongly acidic glycoproteins, *e.g.*, bone.

Other approaches to the problem of HA analysis employ electrophoretic separation[3], enzymatic digestion followed by tritium reduction[4] and specific interaction with the "hook" region of the proteoglycan monomer[5,6]. The latter method is the most sensitive when used in a radioassay test, but it requires access to isotope-labelled "hook" preparations.

The unsaturated disaccharide units obtained from digestion with bacterial hyaluronate lyase or with chondroitinase may also be detected after thin-layer chroma-

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tography[7]. High-performance liquid chromatography (HPLC) of such chondroitinase digests[8,9] has proved to be a very useful tool in the study of GAGs and PGs. In order to measure also the HA contents of such chondroitinase digests we tried to modify the conditions for the separation, so that the nonsulphated galactosamine-containing Δ-disaccharide obtained from CS could be separated from the glucosamine-containing unit derived from HA.

EXPERIMENTAL

Chondroitin sulphate (grade II mixed isomer preparation) was obtained from Sigma (St. Louis, MO, U.S.A.). Hyaluronic acid was prepared from hen aorta according to Antonopoulos and Gardell[10].

The polysaccharide preparations were dissolved in 0.3% Tris buffer at pH 7.5 and digested with condroitinase AC or chondroitinase ABC[11] (Sigma). To reduce the amounts of sulphated CS dissacharides, which might be difficult to wash off the column, choindroitin-4- and -6-sulphatase (Sigma) were also added to the digests. After centrifugation at 10,000 *g* for 5 min to remove particulate matter, aliquots were taken directly for chromatography using a loop injector. In order to avoid problems of mutarotation reactions during the chromatography, the effect of a previous borohydride reduction of aldehyde groups[12] was also tested.

The separation was performed in a 250 × 4.6 mm I.D. Hypersil APS column equipped with a 100 × 4.6 mm I.D. pre-column packed with the same resin. These columns were eluted with 9 m*M* sodium dihydrogen orthophosphate buffer, the pH being adjusted to 2.55 with orthophosphoric acid. The eluent was pumped at 1.1 ml/min, which gave a pressure of 140 bar (2000 p.s.i.). The eluted Δ-disaccharide peaks were recorded spectrophotometrically at 231 nm, the chromatograms shown in this paper being obtained with a LDC Spectromonitor III at 0.05 a.u.f.s. The columns were washed once or twice daily with 25 ml of 2 *M* sodium chloride solution.

RESULTS AND DISCUSSION

With the given procedure the two Δ-disaccharides show baseline separation within 11 min. Optimal separation of the two peaks is obtained at pH 2.5–2.6. At this pH a complete separation was obtained when the concentration was 9 m*M*. The N-acetylglucosamine-containing Δ-disaccharide obtained from the digestion of HA is eluted from the column 7.8–8.8 min after the injection whereas the N-acetylgalactosamine-containing isomer obtained from desulphated CS is eluted at 9.1–10.8 min. The pH used is in the order of 0.5–1.0 pH units below the estimated pK_a values of the Δ-disaccharides. As the separation is dependent on the pH, with an optimal value around pH 2.5–2.6, there may be a minor difference between the pK_a values for the two disaccharides in question, the HA-derived disaccharide being dissociated at a slightly higher pH.

The peaks appear partly divided, with maxima at 8.1 and 8.3 min for the HA preparation and at 9.6 and 10.4 min for the CS preparation (*cf.*, Fig. 1), respectively. This is probably due to mutarotation reactions in the column. This interpretation is supported by three experiments. Firstly, re-chromatography of isolated and probably

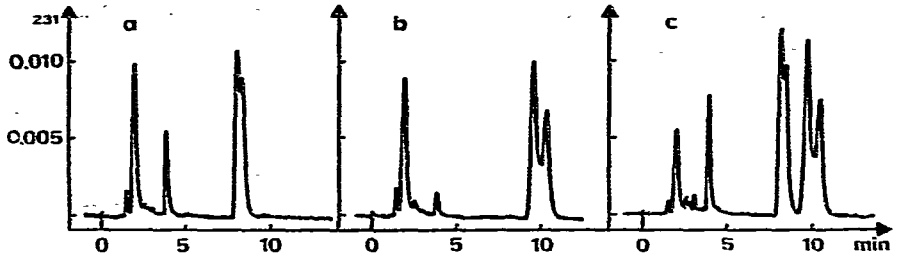

Fig. 1. Chromatography of Δ-disaccharides obtained from chondroitinase AC digestion of (a), hyaluronic acid, (b) desulphated chondroitin sulphate and (c) a mixture of both. The hyaluronic acid-derived peaks each correspond to 1.0 μg of polysaccharide and the CS-derived peaks to 0.75 μg.

anomeric forms resulted in the same original pattern with partly divided, heterogeneous peaks. Secondly, even when attempts were made to separate the components with a low eluent ionic strength and long retardation times, separation was impossible, the form of the peaks indicating that the subfractions were being transformed into each other. Thirdly, the Δ-disaccharides appeared as homogeneous peaks once the aldehyde had been reduced with borohydride (Fig. 2).

Even though the peaks were broadening owing to the existence of anomeric forms, the separation of the HA-derived and CS-derived disaccharides was complete. In the present set-up a detection limit of 10 ng of HA was calculated. When borohydride reduction was used to prevent mutarotation, the products obtained, however, showed a less pronounced separation (Fig. 2) and the reduced disaccharides had to be still more retarded on the column in order to achieve separation. As is also the case with the separation of monosulphated CS-disaccharides[8,12], there was no increase in sensitivity due to this poor separation in comparison with the separation of non-reduced disacchrides.

The described method is thus highly sensitive in its unreduced mode, and the chromatography can be performed using the same digests that are used for the HPLC determination of the CS sulphation pattern. With the baseline separation obtained, this determination can easily be performed in tissues with an unfavourable HA/CS

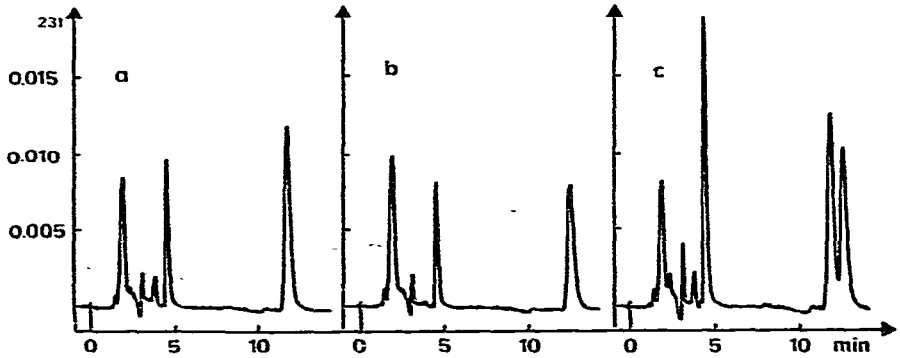

Fig. 2. Chromatography of the same Δ-disaccharide preparations as in Fig. 1, the disaccharides now being reduced with borohydride. Note the homogeneous peaks, which, however, in spite of longer retardation times, show a poor separation (c), while the sensitivity (peak height) is of the same order as in Fig. 1.

ratio, such as in cartilage. The procedure might therefore be a versatile and convenient tool in GAG analysis.

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