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URONIC ACID ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER METHANOLYSIS OF GLYCOSAMINOGLYCANS

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SUMMARY

Two high-performance liquid chromatographic applications for the study of glucuronic and iduronic acid in glycosaminoglycans are presented. In the simplest form these uronic acids are separated on a reversed-phase column as their 1-methylglycoside-6-methyl esters, *i.e.*, the form in which they are recovered after methanolysis. The sensitivity for glucuronic acid is somewhat increased if the polysaccharide is deaminated prior to methanolysis, the subsequent separation being performed on a weak ion-exchange column. To allow this separation, however, an extra preparative step is necessary, consisting of alkaline hydrolysis of the ester bonds. Using these separations, the conditions for methanolysis were studied. Optimal release of uronic acids was achieved after 30–50 h of methanolysis using 1 *M* dry HCl in methanol at 100°C.

INTRODUCTION

The identification of dermatan sulphate in glycosaminoglycan preparations requires the determination of iduronic acid and glucuronic acid. Enzymatic reactions[1], paper chromatography[2], gas chromatography[3] and ion-exchange chromatography[4] have been used to determine the amounts of these uronic acids.

One problem common to all the chromatographic procedures is the depolymerization reactions by which the uronic acids are liberated. One way to perform this disintegration is to hydrolyze the polysaccharides using sulphuric acid[4], formic acid[3] or ion-exchange resins in hydrochloric acid[5]. Since the uronic acids in water are less

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stable at low pH, methanolysis has been used as an alternative[6,7]. By this procedure the uronic acids are obtained as their 1-methylglycoside-6-methyl esters, and problems with mutarotation reactions during the chromatographic procedure are thereby eliminated. Furthermore, it has been claimed that the recovery of glucuronic acid as well as iduronic acid is higher when compared to the hydrolytic procedures.

The ion-exchange systems used to separate the uronic acids, involve chromatography on strong ion-exchange resins, and the times required are in the order of 1.5–6 h. High-performance liquid chromatography (HPLC) on weak ion-exchange resins has proved to be useful for the separation of isomeric anionic disaccharides[8], the separation being completed within 20 min. In order to obtain a simple and more sensitive determination of iduronic acid and glucuronic acid in glycosaminoglycans we tried various HPLC separations of the methanolysate products. Since such a separation by ion-exchange chromatography requires the removal of the 6-methyl esters, the separation of intact esters in reversed-phase systems was primarily studied. Using this determination of uronic acids, we could also study the conditions for the methanolysis.

MATERIALS AND METHODS

Chondroitin sulphate (CS) was prepared from bovine nasal cartilage as described by Antonopoulos *et al.*[9]. Different dermatan sulphate preparations with different iduronic/glucuronic acid ratios, as well as a pure iduronic acid preparation, were gifts from Dr. Anders Malmström, Lund. Commercially available dermatan sulphate, prepared from porcine skin (Sigma), was also used. All other chemicals used were of analytical grade. To evaluate the methanolysis and chromatographic procedures, various chondroitin sulphate–dermatan sulphate mixtures were used. The iduronic/glucuronic acid ratios of these mixtures were estimated enzymatically[1], measuring the amount of 4,5-unsaturated uronic acids[10] liberated by chondroitinase ABC and AC, or by ^{1}H nuclear magnetic resonance (NMR) spectral analysis, comparing the resonance peaks of the anomeric uronic acid protons.

Methanolysis was performed in sealed Pyrex glass tubes using 0.5 *M* or 1 *M* dry HCl in methanol. The reaction was tested at different temperatures (80–120 C) and at different time intervals (6–100 h). The effect of deacetylation and deamination[7,11] prior to methanolysis was also studied. In order to remove irrelevant decomposition products, the dried methanolyzates were dissolved in a small volume of methanol and added to an ion-exchange column (20 × 3 mm I.D.) of Dowex 50-X8, on which 20 × 3 mm I.D. of AG 1-X8 is layered. This column was then washed with 2.0 ml methanol; the pooled eluate containing virtually all uronic acid-6-ester which was subsequently desiccated. This preparative step is, however, not necessary for the subsequent HPLC separation when pure polysaccharide preparations are to be studied. Preparations intended for ion-exchange chromatography were thus prepared and subsequently dissolved in 1 % ammonia in water, thereby hydrolyzing the ester bonds at 100 C for 3 h. After desiccation these preparations were ready for the ion-exchange HPLC procedure.

The materials to be chromatographed were dissolved in eluent and particulate materials were removed by centrifugation at 10,000 *g* for 5 min. The materials were subsequently added to the column via a loop injector. Reversed-phase chromatogra-

phy of non-hydrolyzed methanolyzates was performed using either a ODS-Hypersil column (250 × 4.6 mm I.D.) (Shandon, Southern Products, Great Britain) or a Supelcosil RC 18 column (150 × 4.6 mm I.D.) (Supelco, Switzerland) with a ODS-Hypersil precolumn (50 × 4.6 mm I.D.). These columns were eluted with 5% methanol which was pumped at 0.5 ml/min in an Optilab 931 chromatograph equpped with a high sensitivity refractive index (RI) detector (15-mm cell) at just above room temperature, or by connecting the column to a Technicon AutoAnalyzer II system designed for the carbazole reaction[12]. When using RI detection the dimethyl ester of DL-tartaric acid was found to be a suitable internal standard, eluting shortly after the main glucuronic acid peak.

Ion-exchange chromatography of hydrolyzed methanolyzates was performed on an APS-Hypersil column (250 × 4.6 mm I.D.) (Shandon). The column was eluted with 0.02 *M* sodium acetate, pH adjusted to 3.4 with acetic acid. The eluate was pumped at 0.7 ml/min and monitored with the same high-sensitivity RI detector as above.

RESULTS AND DISCUSSION

As shown in Fig. 1 the material is eluted from the reversed-phase column within seven column volumes, *i.e.*, within 20 min. With shorter methanolysis times a minor peak may be recorded at 23 min. Recovery experiments indicate that no carbazole reactive material is retained on the column after this period. With the RI-detector used, separate peaks containing 0.5 μg can be determined within a 95% confidence interval. When the AutoAnalyzer II was used as detector (Fig. 1d–f), the sensitivity was about the same, but the resolution of the chromatogram was considerably reduced.

The treatment of free glucuronic acid with 1 *M* HCl in methanol gives a product which can be separated into at least four different carbazole reactive peaks (Fig. 1d). The first of these is eluted with the front, similar to free uronic acids. A second peak is eluted at capacity factor, $k' = 1$ (6.6 min). This is the region where glucuronolactone and 1-methylglycosides of hexoses are eluted, while methanol is recovered immediately prior to this peak (a negative peak in Fig. 1a–c) and while the 1-methylglycoside of xylose is eluted slightly later (7.5 min). It may well be that the anhydrous "methanolytic" conditions would also yield a lactone, which is eluted with this second peak. The third and fourth carbazole reactive peaks are eluted at $k' = 3.5$ and 4.5 (13.8 and 16.4 min). From the chromatographic behaviour we suggest that these peaks consist of 1-methylglycoside-6-methyl esters in their β- and α-anomeric configurations respectively.

CS methanolyzates give a pattern similar to "methanolyzed" glucuronic acid (Fig. 1b and e) with two retarded peaks. When dermatan sulphate methanolyzates are chromatographed at least four additional and probably iduronic acid derived peaks occur (Fig. 1c and f). The appearance of several iduronic acid derivatives after methanolysis is in accordance with earlier findings[7]. The major iduronic acid peak is eluted at $k' = 2.9$ (11.4 min). This peak and the main glucuronic acid derivative (16.4 min) are both recorded without interference from other uronic acid derivatives.

A further increased sensitivity of the uronic acid determination could be obtained by ion-exchange chromatography (Fig. 2). This determination is, however,

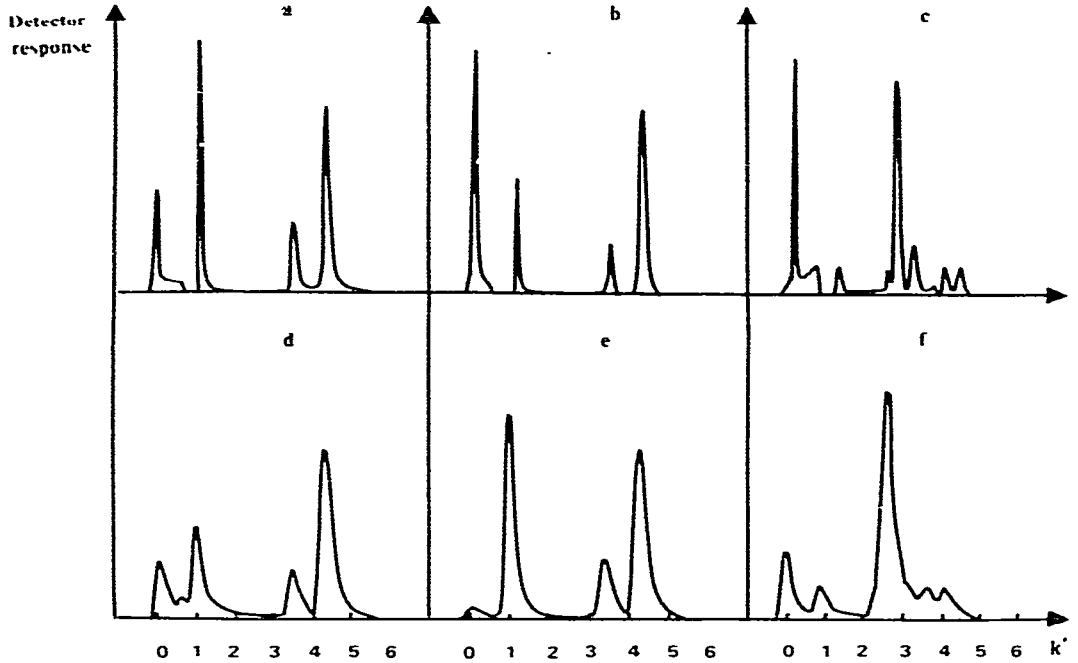

Fig. 1. Chromatography of methanolyzate products on ODS-Hypersil® using RI detection (a–c) or automatic carbazole reaction (d–f) to record the eluate. a, d, Glucuronic acid kept under methanolytic conditions; b, e, chondroitin sulphate and c, f, dermatan sulphate.

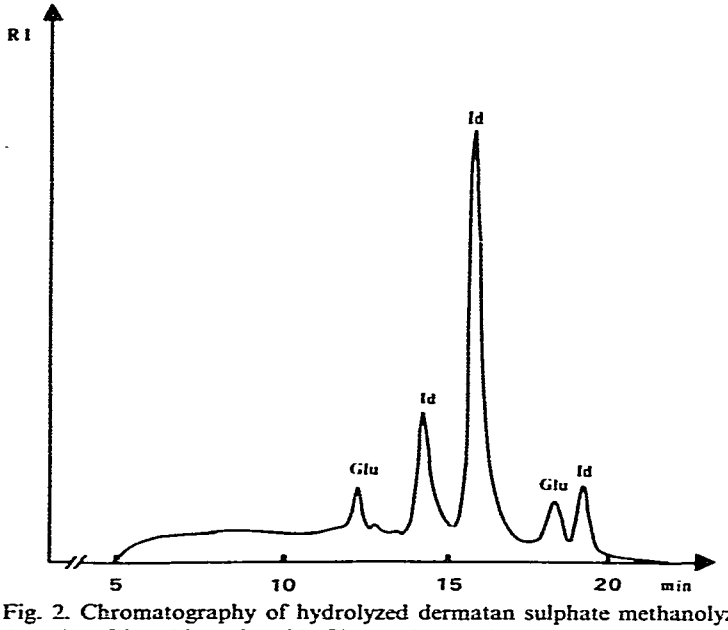

Fig. 2. Chromatography of hydrolyzed dermatan sulphate methanolyzate on APS-Hypersil® using RI detection. Id = Iduronic acid; Glu = glucuronic acid.

somewhat more laborious, since it requires the hydrolysis of the ester bonds. Recovery experiments indicated a virtually complete uronic acid recovery from this hydrolysis, when it was performed with 1% NH_3 as described above. In the ion-exchange chromatogram two glucuronic acid and three iduronic acid derived peaks can be distinguished. Here the main iduronic acid peak is obtained at 16 min and the main glucuronic acid peak at 18.5 min. The pH interval used is just above the p*K* values of the studied uronic acids. This pH was experimentally found to give optimal separation of the peaks, and even minor pH deviations caused confluence of the peak materials.

In order to optimize the release of these main peak materials, the conditions for the methanolysis procedure were studied (Fig. 3). When the lower HCl concentration (0.5 *M* instead of 1 *M*) was used the disintegration was slower and with similar maximal recovery of the uronic acid derivatives. With 1 *M* HCl, maximal iduronic acid recovery was obtained after methanolysis for 6 h, while the glucuronic acid derivative, on the other hand, was not released at the same rate, the maximum being obtained after 18 h. Except for methanolysis at 120°C, longer reaction times did not significantly alter the recovery. The temperatures tested (80°C, 100°C and 120°C) only gave slightly varying results. The iduronic/glucuronic acid ratios obtained from 30–50 h of methanolysis were virtually independent of temperature variations within the interval studied. When the sum of the carbazole reactive peaks was measured, using an external glucuronic acid standard, the total recovery from chondroitin sulphate was 33% and from dermatan sulphate 64% of the expected values. Half of these total recovery figures corresponded to main peak materials.

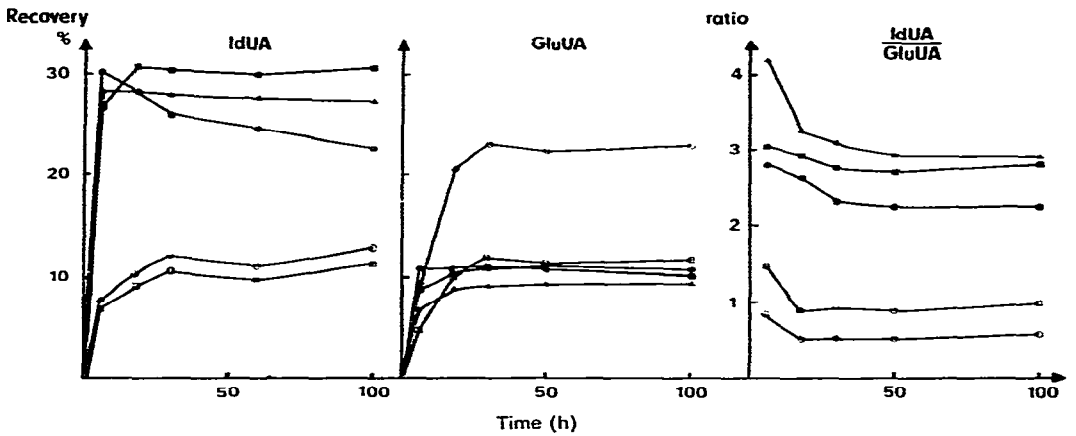

Fig. 3. Recoveries from the methanolysis of iduronic acid (IdUA) main peak material and glucuronic acid (GluUA) main peak material, as well as the obtained ratios between these recoveries. The methanolyses were performed on glycosaminoglycans using 1 *M* HCl in dry methanol at 80°C (▲), 100°C (■) and 120°C (●), as well as on deacetylated deaminated preparations at 80°C (□) and 100°C (○), the time for methanolysis varying from 6 to 100 h.

These recoveries are somewhat lower than those given by Inoue and Miyawaki[7]. The obtained values may, however, be artificially low, as indicated by comparing the carbazole and RI results. The first way of detecting uronic acids gives their molar amounts, while the second is more related to the total mass of the peak

material. The obtained RI/carbazole ratios are higher than can be explained by the molecular weight increase corresponding to two methyl groups. Furthermore, the expected lower carbazole reactivity for iduronic acid derivatives is not observed. One explanation for these findings may be that the derived uronic acids possess lower reactivity in the carbazole reaction, the decrease being somewhat more pronounced with the glucuronic acid derivative. If this is true the reported recoveries are artificially low.

The lower recovery of glucuronic acid after methanolysis alone has been shown by Inoue and Miyawaki[7] to be due to the higher resistance of the glucuronidic linkage compared with the iduronidic linkage. These authors also demonstrated that higher glucuronic acid recovery was obtained after deacetylation and deamination of the glycosaminoglycans prior to methanolysis. After 30–50 h of methanolysis the deacetylated and deaminated sample showed similar recoveries of glucuronic acid main peak material and a considerable decrease in iduronic acid main peak material compared to the non-deacetylated sample (Fig. 3). When the deaminated materials were hydrolyzed and chromatographed on the ion-exchange column, glucuronic acid recoveries considerably increased, which is in accordance with earlier results[7]. The time curve also indicates a pattern of different resistance to methanolysis for these deaminated glycosaminoglycans. While the glucuronic acid derivative shows maximum values after 18 h of methanolysis, the full release of iduronic acid is not obtained until after 50 h. It thus seems as if the deamination procedure somehow decreases the rate by which iduronic acid is released. This effect has not yet been further studied.

Fig. 4 shows the linear recoveries of glucuronic acid and iduronic acid from the different chondroitin sulphate–dermatan sulphate preparations. The deviations from the indicated lines may well be due to errors in the enzymatic determinations rather than in the HPLC procedure. The correct uronic acid ratio in a glycosaminoglycan preparation may therefore be calculated by relating the results to a standard prepara-

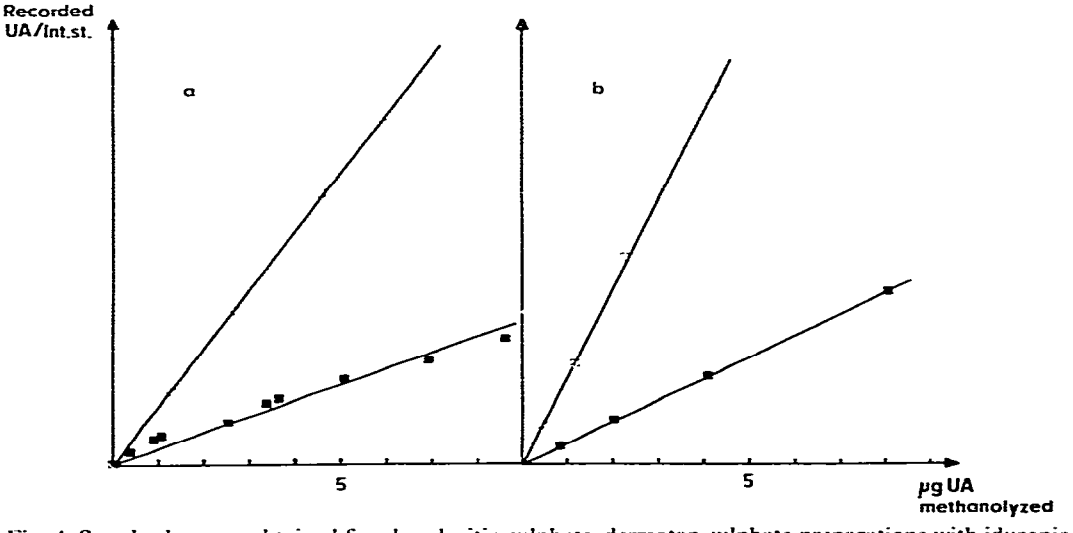

Fig. 4. Standard curves obtained for chondroitin sulphate–dermatan sulphate preparations with iduronic acid/glucuronic acid ratios determined enzymatically (a) and by ^{1}H NMR analysis (b). ■, Glucuronic acid; □, iduronic acid. Note the better correlation in the last case.

tion of known uronic acid composition. Duplicate determinations require 40–80 μg or more of the polysaccharide preparation (corresponding to 10–20 μg uronic acid, *cf.*, Fig. 4). The sensitivity can be increased by the deamination procedure and subsequent ion-exchange HPLC, especially when the glycosaminoglycan in question has a low glucuronic acid content. One reason for the improved sensitivity may be that the alkaline hydrolysis not only splits the methyl ester bonds, but also the lactone bonds formed during methanolysis. A higher recovery within the main peaks would thus ensue.

According to these results the uronic acid composition in chondroitin sulphate–dermatan sulphate preparations can be analyzed according to the following scheme. The dried glycosaminoglycan preparation is methanolyzed in sealed ampoules using 1 *M* dry HCl in methanol for 30–50 h at 100 C. The samples are subsequently dried, and purified with the use of a small Dowex 50-X8/AG 1-X8 mixed ion-exchange column. With the dimethyl ester of DL-tartaric acid as internal standard and comparing the chromatograms with known chondroitin sulphate and dermatan sulphate preparations, the iduronic acid and glucuronic acid contents are directly determined by reversed-phase chromatography. With lower amounts of glycosaminoglycans, it is preferable to deacylate and deaminate the polysaccharides prior to methanolysis. After methanolysis the materials are desiccated and subsequently hydrolyzed with 1 % NH_3 in water. The resulting products are lyophilized again and subjected to ion-exchange chromatography.

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