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A Sensitive GLC-Method for Component Sugars and *O*-Glycosidic Linkage Monosaccharides of Cartilage Proteoglycans

ANNA-MARJA SÄÄMÄNEN* and MARKKU TAMMI

Department of Anatomy, University of Kuopio, P.O. Box 6, SF-70211 Kuopio, Finland

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A method is described for the measurement of *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose and xylose present in the different carbohydrate chains of cartilage proteoglycans (PG). Bovine articular cartilage PG samples corresponding to the minimum of 1 nmol of each monosaccharide were reproducibly quantified following hydrolysis with 2 M HCl and derivatization into alditol acetates. An on-column injection mode and an OV-1701 fused silica capillary column were used for chromatography.

Alkaline borohydride treatment of the PG was exploited to reduce the acid labile xylose in the base of the chondroitin sulphate chain into more stable xylitol, allowing the assay of chondroitin sulfate chain length as an *N*-acetylgalactosamine/xylose ratio. A novel procedure is described for the measurement of the galactosaminitol evolving from the protein linkage of oligosaccharides and of keratan sulphate.

Gas-liquid chromatography (GLC) offers high specificity and sensitivity which are important in the assay of protein-linked carbohydrates, which are often available only in minute amounts. Recently presented HPLC methods also exhibit enough resolution to separate the common monosaccharides in glycoconjugates [1-3], but applications suitable for microscale analysis require radioactive labelling [4] or derivatization as for GLC (for review see [2]).

A careful study of the hydrolysis conditions is necessary to optimize the yield of the component monosaccharides from any particular type of complex carbohydrate [4-7]. This report describes efforts in optimizing the measurement of different monosaccharides present in PGs, where the resistance of the glycosaminoglycans against protein-catalyzed hydrolysis presents a special challenge. A novel modification of the alditol acetate derivatization procedure, first reported by Sawardeker *et al.* [8], is described for the measurement of *N*-acetylgalactosamine and xylose involved in the carbohydrate-protein linkages. The alditol acetate method was chosen because of the stability of these derivatives.

*Author for correspondence.

Materials and Methods

Materials

Sodium borohydride and acetic anhydride were from Sigma Chemical Company (St. Louis, MO, USA), and Merck (Darmstadt, W. Germany). Methanol and dichloromethane of Lichrosolv® grade, trifluoroacetic acid of Uvasol® grade, hydrochloric acid and acetic acid (p.a.) were purchased from Merck. Methyl-α-D-mannoside, D-glucoheptose and D-glucosamine were from Sigma; D-mannose, D-galactose, D-xylose, and myoinositol were obtained from Merck. D-Galactosamine was a product of Fluka AG (Buchs, Switzerland). Hydrochloric acid was redistilled for amino acid hydrolysis. All other chemicals were obtained from Merck and were of p.a. grade.

Proteoglycan Preparations

The proteoglycan (PG) preparation used in the hydrolysis time experiments was extracted from bovine articular cartilage of about 1.5 year old animals with 4 M guanidinium chloride and purified by DEAE-Sephacel chromatography (Pharmacia, Sweden) in 8 M urea and 50 mM Tris-HCl, pH 6.8, using a 0-1 M NaCl gradient. In other experiments these PGs were further purified by dissociative CsCl density gradient centrifugation (D1-PGs, [9]).

Hydrolysis

The experiments were done in triplicate and repeated three times. All stages including hydrolysis, reduction, acetylation and purification of the derivatives were performed in the same 10 ml PTFE-capped, acid washed, glass vial. The amount of PG corresponding to 26-103 nmol (5-20 μg) of PG uronic acid, and mixtures containing 11-13 nmol (2 μg) each of the standard monosaccharides, were freeze-dried and hydrolyzed in 500 μl of 2 M HCl at 103°C for 1.5, 4, 8, or 17 h. The acid was evaporated to dryness under a stream of air. Hydrolysis with 500 μl of 2 M trifluoroacetic acid (TFA) was performed under nitrogen at 103°C for 1, 2, 4, 6, 8, or 10 h and evaporated to dryness under a stream of air before reduction and acetylation.

Alkaline Borohydride Cleavage

PG (103 nmol as uronic acid) was dissolved in 200 μl of cold 50 mM NaOH on an ice bath. The pH was checked, and raised to above 10 with 50 mM NaOH, if necessary. An equal volume of 2 M $NaBH_4$ in 50 mM NaOH was added and the samples were incubated for 16 h at 45°C [10]. Excess borohydride was destroyed with 200 μl of 2 M acetic acid, and the samples were passed through PD-10 columns (Pharmacia) to remove salts. The effluent was freeze-dried and hydrolyzed in 2 M HCl as indicated above.

Derivatization Procedures

Method 1; reduction and acetylation. Hydrolyzed monosaccharides were reduced and acetylated essentially as described by Torello *et al.* [6]. The internal standards (5 nmol [1 μg] methyl-α-D-mannoside and 10 nmol [2 μg] D-glucoheptose) were added after

evaporation of HCl. Sodium borohydride (0.5 ml, 2 mg/ml in 1 M NH_4OH) was added and left at room temperature for 40 min. Borohydride was destroyed with acetic acid, and boric acid was removed by repeating five times a procedure in which the samples were dissolved in 1 ml of methanol, heated at 90°C for 5 min, and evaporated under a stream of air. The reduced monosaccharides were acetylated in 0.75 ml of acetic anhydride in capped tubes for 30 min at 100°C. Acetic anhydride was evaporated under a stream of air at 40°C, and the samples were dissolved in 1 ml of dichloromethane. Salts were removed by extracting the organic phase five times with 1 ml of water (Water-I System, Gelman Sciences, Ann Arbor, MI, USA). Dichloromethane was evaporated and the samples were stored in a silica gel desiccator before chromatography.

Method 2; direct acetylation. The PG was treated with alkaline borohydride and hydrolyzed as described above. Myoinositol (22 nmol, 4 μg) and methyl-α-D-mannoside (21 nmol, 4 μg) were added as internal standards, and the tubes were either freeze-dried or evaporated under a stream of air. The samples were acetylated in 500 μl of acetic anhydride/distilled pyridine, 1/1 by vol, at 100°C for 1 h [11]. Excess acetic anhydride was destroyed by adding 1 ml of 0.5 M H_2SO_4. Alditol acetates were extracted into 1.5 ml of dichloromethane and washed twice with 1 ml of 0.5 M H_2SO_4, twice with saturated Na_2CO_3 and twice with 1 ml of H_2O. Dichloromethane was evaporated under a stream of air, and the samples were stored in a desiccator until analyzed.

Gas Chromatography

A Carlo Erba 4160 capillary gas chromatograph, equipped with a flame ionization detector and an on-column injector was used. Samples were dissolved in 50-150 μl of dichloromethane and 0.05 μl of this was injected manually into the column. For automatic injection (Carlo Erba Autosampler AS-550) the sample was transferred with 3 $\times$ 200-250 μl of dichloromethane into a 1.2 ml autosampler vial with a PTFE sealed open-hole screw-cap. In this case a wide bore pre-column (Carlo Erba) was used which allows the injection of larger volumes (up to 3 μl). The samples were separated in a 24 m $\times$ 0.35 mm fused silica capillary column, coated with a 0.15 μm film of OV-1701 (Orion Analytica, Espoo, Finland) with H_2 as a carrier gas. The temperature was programmed from 80-180°C at the rate of 25°C/min and then to 240°C at the rate of 3°C/min and maintained for 6 min at 240°C. The peak areas of monosaccharides were compared to those of internal standards and multiplied by the response factor of each monosaccharide using a Varian 4270 Integrator (Spectra Physics, San Jose, CA, USA) and VAX 11/780 Computer (Digital Equipment Corporation, Maynard, MA, USA).

Amino Acid Analysis

The amino acid composition of the PG, before and after alkaline borohydride treatment, was determined on a 4151 Alpha Plus Amino Acid Analyzer (LKB Biochrom, Cambridge, England) using the standard program of the manufacturer for free amino acids with ninhydrin detection. Norleucine (3 nmol/103 nmol uronic acid) was added as an internal standard, and the samples were hydrolyzed in distilled 6 M HCl under nitrogen for 24 h at 110°C.

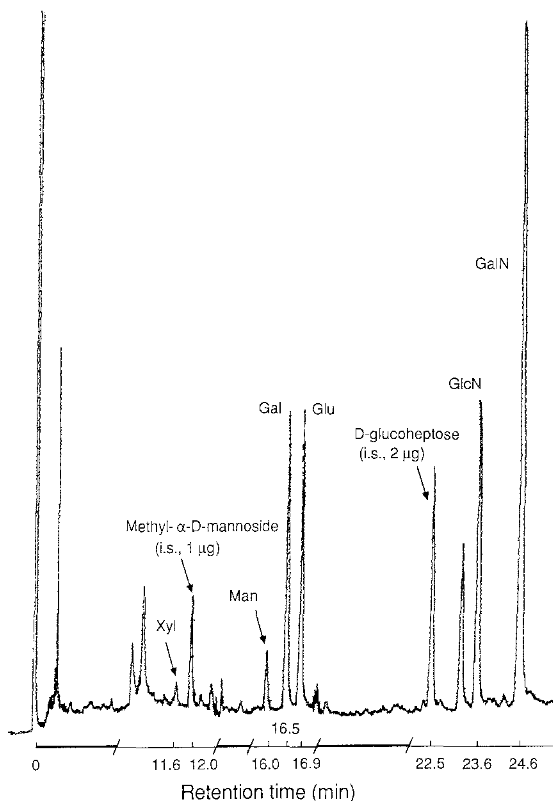

Figure 1. Gas chromatogram of the monosaccharides present in high density PGs purified from canine articular cartilage by associative CsCl gradient centrifugation, hydrolyzed for 8 h in 2 M HCl at 103°C and separated by OV-1701 fused silica capillary column as alditol acetates. A 52 nmol (10 μg) sample (as uronic acid) was derivatized and 1/200-1/1000 of this was injected at 80°C. The temperature was raised to 180°C at a rate of 25°C/min, then to 240°C at a rate of 3°C/min, and kept there for 4 min. The chart speed (in the integrator program) was set to 1.0 cm/min (plain line) or 0.1 cm/min (bold line).

Results and Discussion

Acetylation and Chromatographic Separation

Monosaccharides were routinely peracetylated with acetic anhydride due to the simple washing procedure required. In the linkage sugar analysis, when reduction in the derivatization step was omitted, inclusion of pyridine was necessary for complete acetylation. We also tested methyl imidazole as a catalyst [12] but it resulted in a considerable background and deterioration of the column.

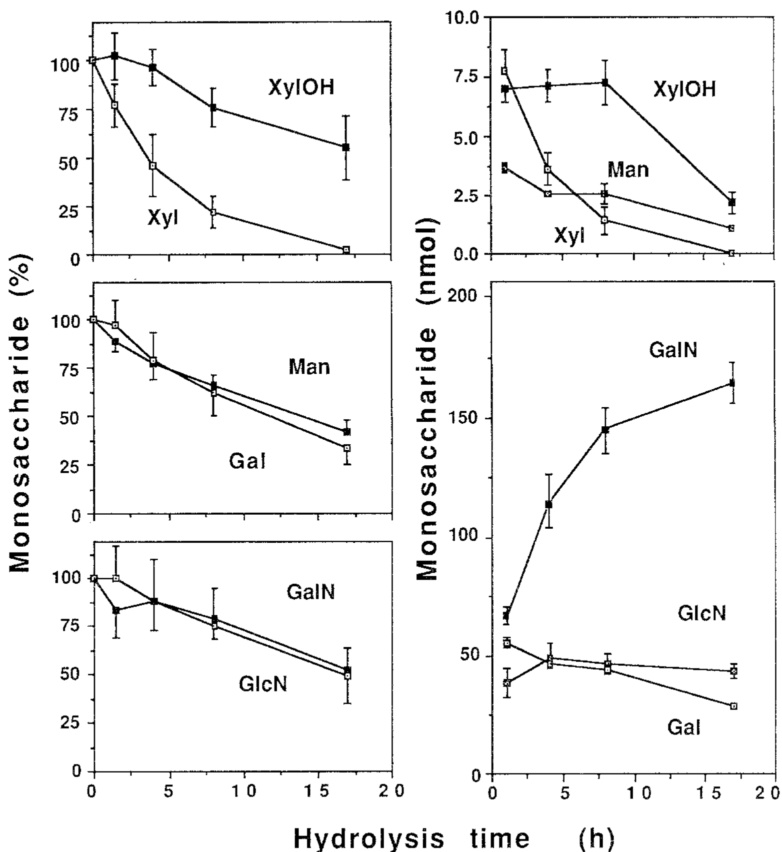

Figure 2. The yield of monosaccharides after hydrolysis in 2 M HCl at 103°C for 1.5-17 h. The left panels show the recovery of free monosaccharides as percentage of the unhydrolyzed samples. The right panels represent the yields (in nmol) from a preparation of bovine articular cartilage PG corresponding to 103 nmol (20 μg) of uronic acid. The values are mean ± SEM, n = 9.

The application of the method for the analysis of a canine articular cartilage PG preparation is shown in Fig. 1. The monosaccharides occurring in cartilage PGs [13] were well separated with the OV-1701 fused silica capillary column (Fig. 1), except sialic acid and uronic acid. These two acidic sugars are not stable to acid hydrolysis nor amenable for GLC as alditol acetates. Alditol acetates of neutral and amino sugars have also been separated on other capillary columns [5, 6, 14-18].

Stability of Monosaccharides on Hydrolysis

The acid resistance of standard monosaccharides was tested in 2 M HCl at 103°C for 1.5-17 h (Fig. 2, left panels). Xylose was decomposed most rapidly; after 4 h only 46% was left, and within 17 h it was completely lost. A significantly better resistance was observed for xylitol (XylOH), the corresponding sugar alcohol, which was stable at least for 4 h. Mannose and galactose had similar types of decomposition profiles on hydrolysis,

Table 1. Determination of the monosaccharides and *O*-glycosidic linkages in bovine femoral cartilage D1-PG.

Compound[a]	Non-treated[b]	Treated[c]	Difference
	(nmol/103 nmol uronic acid)		
Ser	7.2	3.2	-4.0
Thr	4.1	3.1	-1.0
Ser+Thr	11.3	6.3	-5.0
Xyl	2.8	—	
XylOH	—	5.1	
GalN	105.4	—	
GalNOH	—	1.4	
GlcN	30.3	—	
Gal	56.0	—	
Man	5.0	—	

[a] Monosaccharides in the form they occur in before hydrolysis and derivatization.
[b] The PG was hydrolyzed in 6 M HCl for 24 h at 110°C for amino acid analysis. For GLC analysis the monosaccharides were derivatized according to method 1 (see the Materials and Method section) after 1.5 h (neutral sugars) or 8 h (amino sugars) hydrolysis in 2 M HCl.
[c] The PG was treated with alkaline borohydride prior to hydrolysis. Amino acid analysis was done as above; after 1.5 h hydrolysis the monosaccharides were derivatized according to method 2 (acetylation without reduction, see Materials and Methods).

with the recovery of 79% and 77%, respectively, after 4 h. Even hexosamines did not appear completely stable against decomposition. After 17 h 51% of the glucosamine (GlcN) and 48% of the galactosamine (GalN) were destroyed.

Release of Monosaccharides from PGs

The yields of the monosaccharides from a PG preparation were studied in a similar series of hydrolysis periods up to 17 h in HCl (Fig. 2, right panels). Xylose was rapidly released and degraded unless transformed into xylitol by β-elimination before hydrolysis, in analogy with the standard monosaccharides. The amount of xylitol remained stable even up to 8 h (7.3 nmol). Mannose and galactose showed the highest yields after 1.5 h hydrolysis. Glucosamine was released rapidly but its level remained almost constant at all time points during the observation period. Galactosamine was released slowly, with the best yield at 17 h. The fast release of glucosamine obviously reflected the easier hydrolysis of keratan sulphate than chondroitin sulphate.

In general, a good yield of amino sugars was obtained after 8-17 h hydrolysis, while for neutral sugars only 1.5 h were required. For optimum recovery two different hydrolysis times should be used. However, the good yield of both xylitol and galactosamine at 8 h indicates that the galactosamine/xylitol ratio, an indicator of chondroitin sulphate chain length, can be determined from a single hydrolysate of PG pre-treated with alkaline borohydride. In a bovine articular cartilage PG preparation analyzed with this method the mean length of the chondroitin sulphate chains was 20 disaccharide units (GalN/XylOH ratio 20:1, calculated from a 8 h hydrolyzate, Fig. 2, right panels), in agreement with previous studies on this tissue [19].

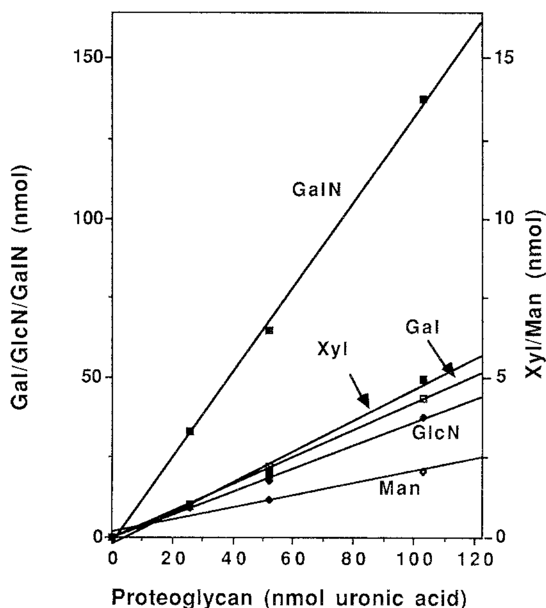

Figure 3. Linearity of the assay. Bovine articular cartilage PG containing 26-103 nmol (5-20 μg) of uronic acid were hydrolyzed for 1.5 h (neutral sugars) or 8 h (amino sugars) and derivatized according to method 1. The correlation coefficients for Xyl, Man, Gal, GlcN and GalN were 0.996, 0.971, 1.000, 1.000 and 1.000, respectively. The values represent the means of three hydrolyzates.

Release of monosaccharides with 2 M trifluoroacetic acid was tested in a series of experiments with different hydrolysis times (1-10 h) at 103°C (data not presented). The recovery of mannose was improved by 29% as compared with HCl (2 M trifluoroacetic acid, 2 h; 2 M HCl, 1.5 h) while no difference was found in the recoveries of xylose and galactose. On the other hand, even after a 10 h hydrolysis in 2 M trifluroacetic acid the recovery of galactosamine was only 50% of that released with 8 h hydrolysis in 2 M HCl. It was concluded that the standard conditions of hydrolysis with trifluoroacetic acid were not effective in releasing galactosamine from chondroitin sulphate chains and did not offer a marked advantage over HCl in the analysis of oligosaccharides and keratan sulphate, either.

Analysis of Linkage Monosaccharides

Chondroitin sulphates are bound to serine and/or threonine residues of the core protein through xylose while cartilage keratan sulphate and *O*-linked oligosaccharides are bound through galactosamine [13]. Treatment of PG with alkaline borohydride detaches chondroitin sulphates and *O*-linked carbohydrates, transforming their linkage sugars into xylitol and galactosaminitol (GalNOH), respectively. These reduced monosaccharides were measured by GLC using direct acetylation, in which the reduction step was omitted from the derivatization procedure, described in the Materials and

Table 2. Reproducibility of the monosaccharide assay of bovine femoral cartilage PG corresponding to 103 nmol uronic acid.

	Coefficient of variation (C.V. %)					
	Xyl[a]	XylOH[a]	Man[a]	Gal[a]	GlcN[b]	GalN[b]
Parallel hydrolysates[c]	22	21	10	17	12	15
Between experiments[d]	56	29	15	15	20	20

[a] Hydrolysis time 1.5 h.
[b] Hydrolysis time 8.0 h.
[c] C.V. between parallel hydrolyzates within an experiment (calculated from three experiments).
[d] Mean C.V. between three separate experiments.

Methods section (Method 2, Table 1). Hence, only those monosaccharides transformed into alditols by alkaline borohydride treatment of intact PG were detected. The result was checked by the analysis of amino acids involved in the binding of the carbohydrate chains to the protein core. Alkaline borohydride treatment destroys serine and threonine residues carrying carbohydrate chains. Serine is recovered as alanine, and threonine mainly as α-aminobutyric acid, but there are also other reaction products [20-21]. A 5.0 nmol decrease in serine+threonine was observed after β-elimination. The ratio of galactosamine (non-treated PG) to XylOH (alkaline borohydride treated PG) was 20.4 (Table 1), suggesting an average chondroitin sulphate chain length of 20 disaccharide units.

Accuracy of the Method

The linearity of the assay was confirmed in 26-103 nmol (5-20 μg) samples of PG uronic acid (Fig. 3). The reproducibility was determined from repeated analyses of the articular cartilage PG preparation (Table 2). The calculated coefficients of variation (C.V.) include all steps from hydrolysis through reduction, acetylation and chromatographic analysis. The reproducibility varied between 10-29% except for xylose (method 1) which was 56% between separate experiments, probably due to the rapid degradation rate in hydrolysis. The pre-hydrolysis reductive cleavage in the procedure reduced the variance of xylose to 20%. The C.V. between separate injections of the same sample was below 5%.

The present assay appears to be the most sensitive among the published applications of the alditol acetate method, when considering the initial amount of monosaccharide required per sample (1 nmol) [6, 12, 16, 22, 23]. A higher sensitivity (0.2 nmol of each monosaccharide per sample), was reported by radioactive labelling with tritiated borohydride and collecting the GLC peaks for scintillation counting [24]. Approximately the same sensitivity as in the present assay was reported by electron capture detection of trifluoroacetic acid derivatives [25].

In conclusion, a sensitive procedure is described for measurement of the alditol acetate derivatives of neutral and amino sugars present in the different glycan chains of PGs, with a specific modification of the assay for their *O*-glycosidic linkages.

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