A reversed phase HPLC method for the simultaneous determination of all monosaccharides contained in galactosaminoglycan isomers from human aorta proteoglycans

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ABSTRACT

Monosaccharides obtained by reduction and hydrolysis of galactosaminoglycan isomers, are entirely determined as their perbenzoyl derivatives by reversed phase HPLC, without removal of hexosamines prior to benzoylation. The method is suitable for the analysis of arterial proteoglycan constituent galactosaminoglycans, providing specific, precise and reproducible results. Moreover, synthesis and characterization of tri-*O*-benzoyl-1,6-L-anhydroidose and *N*-benzoyl-tetra-*O*-benzoyl-α- and -β-D-galactosamine have been accomplished.

INTRODUCTION

Changes in the distribution of proteoglycans (PGs) seem to play an important role in the pathogenesis of atherosclerosis[1-3].

In a previous study, comparing PGs isolated from normal and atherosclerotic human aorta, we observed an increase of dermatan sulphate-rich PGs (DS-rich PGs) in atherosclerotic areas[4]. Since at least two types of DS-rich PGs differing in iduronic acid content have been found in bovine aorta tissue[5,6], it seems of considerable interest to establish whether the increase of DS-rich PGs associated with atherosclerosis is related to a proportional increase in the degree of DS iduronation. Such data could provide insights into the mechanisms involved in post-translational processes in PG biosynthesis. In fact, the level of iduronation

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depends on the glucuronic acid–iduronic acid epimerase, the activity of which could be different in normal and atherosclerotic areas.

The structural study of arterial PGs requires the identification of the predominating glycosaminoglycan (GAG) on a single core protein. GAGs can display a considerable heterogeneity with respect to their molecular size, disaccharide composition, and sulphate content. Moreover, hexuronate containing GAGs show many differences with respect to the ratio of iduronic to glucuronic acid units[7,8].

Enzymic reactions, electrophoresis, and ion-exchange chromatography have been widely used for the analysis of GAGs[9,10]. The highly heterogeneous structure of copolymeric chains makes it difficult to separate isomers with differing iduronate content by means of conventional electrophoretic or chromatographic procedures. Recently, high performance liquid chromatography (HPLC) has been successfully used for the structural studies of GAGs, both as intact chains[11] and products of chemical and/or enzymic depolymerization[12–23]. Karamanos et al.[24] used a reversed phase HPLC isocratic system for the determination of glucuronic and iduronic acids in standard GAGs after stoichiometric reduction, hydrolysis, hexosamine removal, and per-*O*-benzoylation of neutral sugars derived from hexuronates.

The method described in this paper, which is a modification of the HPLC technique described by Karamanos et al.[24], consists of: (*i*) the reduction of uronic acids in GAGs; (*ii*) the hydrolysis of the reduction products; (*iii*) the direct per-*N*,*O*-benzoylation of the resulting mixture of monosaccharides; (*iv*) the analysis of the perbenzoyl derivatives by reversed phase HPLC with gradient elution.

The aim of the present work is to verify the applicability of our method for the simultaneous determination of iduronic acid, glucuronic acid, and galactosamine in galactosaminoglycans from purified human arterial PGs.

RESULTS AND DISCUSSION

Reduction of galactosaminoglycans was performed in the system 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide–NaBH$_4$ in aqueous solution, according to Karamanos et al.[24]. The crude product so obtained was hydrolyzed with 2 M trifluoroacetic acid to D-glucose, 1,6-anhydro-L-idose, and D-galactosamine. The mixture of the three monosaccharides was treated with benzoic anhydride in pyridine at 37°C, in order to obtain the corresponding perbenzoyl derivatives to be submitted to HPLC analysis.

A set consisting of penta-*O*-benzoyl-D-glucose, anhydro-tri-*O*-benzoyl-1,6-L-idose and penta-*N*,*O*-benzoyl-D-galactosamine, to be used as a standard, was preliminarily prepared by submitting commercial sugars to the previously described procedure, with the exception only of the reduction step *. The structures of the

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* Treatment of the commercial monosaccharides with CF$_3$CO$_2$H allowed us to obtain the same anomeric mixtures as those resulting from the hydrolysis of the biological material (GAGs), as well as to generate the anhydro form of L-idose.

TABLE I

^{1}H NMR signals of perbenzoylated sugars in CDCl$_3$ solution

Com- pound	Chemical shifts (δ) and multiplicities								Aromatic protons
	Sugar protons								
	H-1	H-2	H-3	H-4	H-5	H6a	H-6b	N-H	
1	5.74d	5.29dd	6.09t	5.49ddd	4.93t	4.40d	3.90ddd		8.06–7.30
2	6.86d	5.33m	5.98dd	6.13dd	4.73t	4.62dd	4.42dd	6.57d	8.24–7.28
3	6.23d	5.25q	5.75dd	6.02d	4.50–4.38m ^{a}	4.70–4.58m	4.50–4.38m ^{a}	6.66d	8.24–7.18

^{a} H-5 + H-6b

TABLE II

Vicinal proton–proton coupling constants of perbenzoylated sugars

Compound	Coupling constants (Hz)							
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$	$J_{NH,2}$
1	1.8	8.5	9.0	4.2	< 0.5	5.3	8.3	
2	3.5	10.8	3.2	1.1	6.6	7.0	11.5	8.7
3	9.5	11.6	3.2	n.d. ^{a}	n.d.	n.d.	n.d.	9.9

^{a} n.d., Not determined

derivatized sugars were confirmed by ^{1}H NMR analysis, after a chromatographic separation of the anomeric forms in the case of glucose and galactosamine derivatives.

The compounds 1,6-anhydro-2,3,4-tri-*O*-benzoyl-β-L-idopyranose (**1**) and 2-benzamido-1,3,4,6-tetra-*O*-benzoyl-2-deoxy-α- and -β-D-galactopyranose (**2** and **3**), which were not reported in the literature, were characterized by their melting points, optical rotations, elemental compositions, and ^{1}H NMR spectra at 300 MHz, where an almost complete first-order analysis was possible. The assignments were confirmed by double resonance experiments. The chemical shifts and coupling constants of the three derivatives are listed in Tables I and II.

1   **2** R^1 = OBz, R^2 = H
               3 R^1 = H, R^2 = OBz

The perbenzoylated sugars, both individually and as a mixture, were submitted to reversed phase HPLC analysis with a gradient elution. As shown in Fig. 1, the
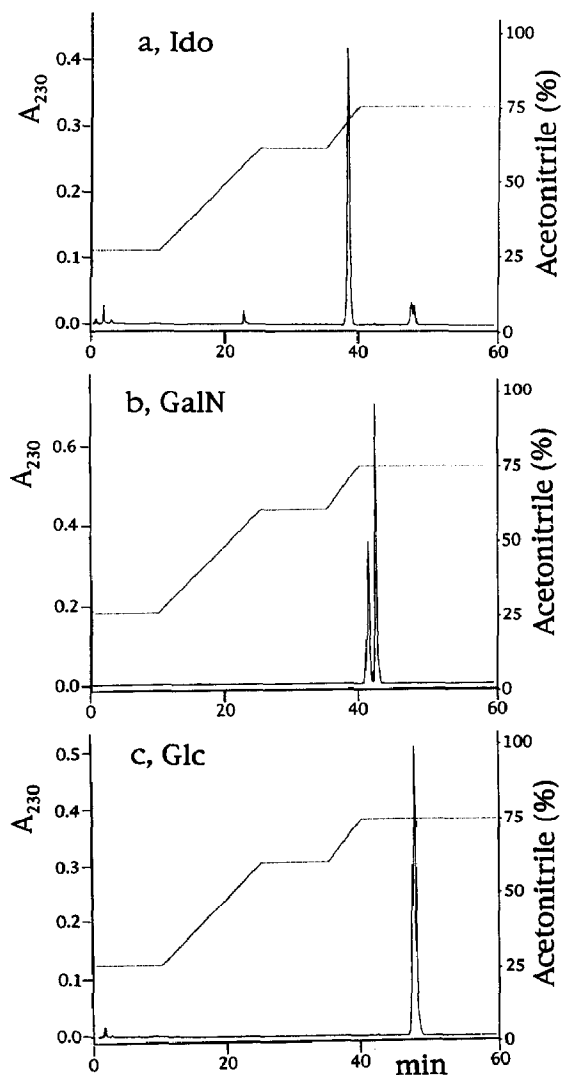
Fig. 1. Reversed phase chromatography of: a, tribenzoyl anhydroidose, b, pentabenzoyl galactosamine, and c, pentabenzoyl glucose. The elution was performed with gradients of acetonitrile in water, as described in the text, flow rate 1 mL/min.

retention times were quite different and in the case of galactosamine derivatives, a satisfactory anomer resolution was also achieved, the major peak corresponding to the α anomer (panel b). The minor peaks at t_R 47.6 and 48.1 which appear in the chromatogram of tribenzoyl anhydroidose (panel a), are probably due to the pentabenzoyl derivatives originating from some unmodified L-idose. As for pentabenzoyl glucose (panel c), the major peak corresponds to the β anomer.

Table III shows the retention times of the perbenzoylated sugars. Our chromatographic conditions allowed us to obtain a good separation of all derivatives, whereas the isocratic elution employed by Karamanos et al.[24] does not separate

TABLE III

Retention times of perbenzoylated monosaccharides on reversed phase HPLC

Sugar	Retention times (min)	
	α anomer	β anomer
1,6-Anhydroidose		37.8
Galactosamine	42.2	41.2
Glucose	47.8	48.2

1,6-anhydroidose from galactosamine, thus making the removal of hexosamines essential. The previous separation of hexosamine by means of ion-exchange chromatography, after GAG hydrolysis, can be omitted in our set-up, thus eliminating an additional step which is time consuming and might cause incomplete recoveries.

Standard calibration curves demonstrating linear relationships between the amounts of benzoylated derivatives and the chromatographic peak areas in UV absorbance units were established (Fig. 2). The different slopes reflect the higher ratio of benzoyl groups to monosaccharide units in glucose and galactosamine with respect to 1,6-anhydroidose (5:5:3).

A linear detector response was obtained for injections up to 4 μg of compound. The reproducibility was 2.9% and the detection limit, calculated as twice the baseline noise, was 10 ng.

To establish the reliability of our modified method in determining the relative proportion of L-iduronic and D-glucuronic acids in tissue galactosaminoglycans, we first tested it with standard solutions of these GAGs.
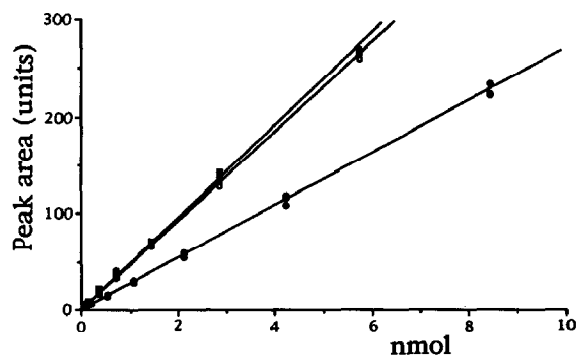
Fig. 2. Calibration curves of perbenzoylated sugars. The detector response at 230 nm was found to be linear in the range tested (0.05–4.0 μg) of perbenzoylated sugar injected. The relative absorbance of glucose, galactosamine and 1,6-anhydroidose derivatives, expressed as peak area per nmol, are in good accordance with the expected ratio of 5:5:3 respectively, based on the number of benzoyl groups bonded to each sugar [95% confidence interval: galactosamine (■) 46.76 ± 0.70 peak area units/nmol; glucose (○) 46.21 ± 0.60; 1,6-anhydroidose (●) 26.76 ± 0.42].
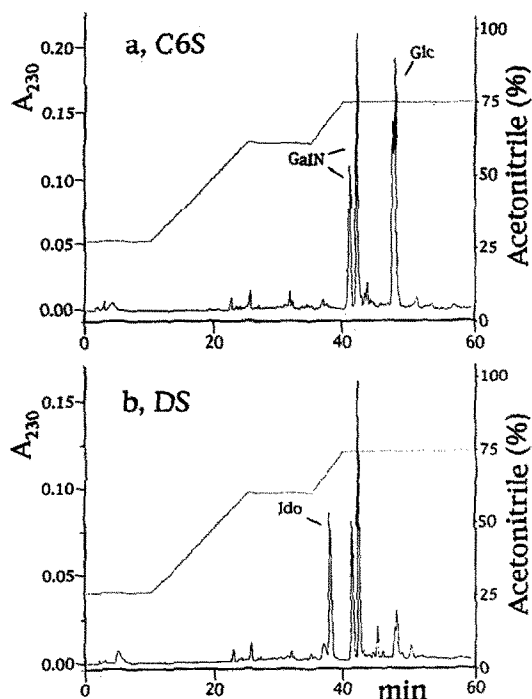
Fig. 3. Reversed phase chromatography of perbenzoylated monosaccharides derived by the reduction and hydrolysis of: a, standard C6S and b, DS.

Fig. 3 shows the separation obtained utilizing dermatan sulphate (DS) and chondroitin 6-sulphate (C6S). The hexosamine–hexuronate molar ratio and the iduronate percentage relative to total hexuronate in the standard galactosaminoglycans are listed in Table IV. The relative percentage of iduronic acid with respect to total hexuronates in standard preparations of commercial C6S, chondroitin 4-sulphate (C4S), and DS were 5, 5, and 85%, respectively. These percentages are only slightly different from those obtained by Karamanos et al.[24] on standard CS and DS. These differences could be due to variability in the commercial preparations used.

TABLE IV

Monosaccharide composition of standard galactosaminoglycans

Standard GAG	Hexuronate : hexosamine molar ratio [a]	Iduronate, per cent of total hexuronate
C4S	0.98	5
C6S	1.02	5
DS	1.03	85

[a] Determined by reversed phase chromatography. The sum of tribenzoyl anhydroidose and pentabenzoyl glucose was compared to pentabenzoyl galactosamine.
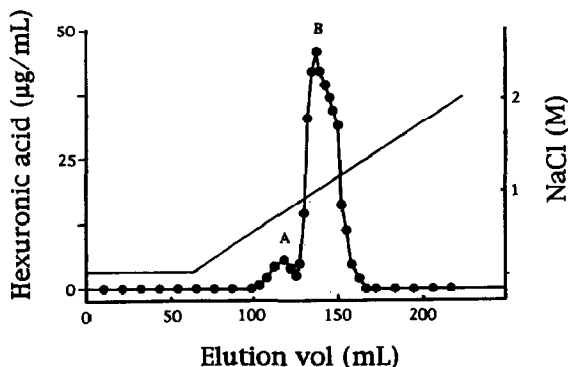
Fig. 4. DEAE-Sephacel chromatography of PGs extracted from human aortas. Elution was performed with a linear salt gradient (0.25–2.0 M NaCl). Two fractions were collected: fraction A contained mostly HS-PGs; fraction B contained a mixture of CS- and DS-PGs.

Control experiments showed that the recovery of the full assay protocol for each standard GAG was ca. 80–90%. However, hydrolysis, perbenzoylation, and C-18 cartridge purification steps gave almost quantitative recoveries, suggesting that ethanol precipitation represents the most critical step.

Moreover, as shown in Table IV, the perbenzoylated products obtained from standard GAG solutions after the whole procedure always gave a molar ratio of galactosamine to hexuronic acid derivatives close to 1, thus indicating similar recoveries for all components.

Experiments performed on galactosaminoglycan mixtures of different relative composition showed that this method gives good results even with samples containing very low concentrations of DS and an excess of CS isomers.

The effective applicability of the modified procedure to the analysis of the composition of PG preparations from human aorta tissue was tested on chondroitin sulphate-rich PGs and dermatan sulphate-rich PGs, purified by means of ion-exchange chromatography on DEAE-Sephacel (Fig. 4) and gel-filtration chromatography on Sepharose CL-4B (Fig. 5), as described in the experimental section. The identity of constituent GAGs was assessed, after papain digestion, by electrophoretic analysis[25,26] associated with the use of specific lyases, as previously described[4].

Ion-exchange chromatography showed two hexuronate containing peaks: the first consisted of heparan sulphate (HS)-PGs, and the second of CS-PGs, DS-PGs, and only traces of HS-PGs and hyaluronic acid (HA). The latter, when submitted to gel-filtration chromatography, produced two peaks: one was eluted with the void volume of the column and consisted almost exclusively of CS-PGs, and the other, roughly separated, eluted in the included volume, was composed mainly of DS-PG. Both fractions were analyzed for their sugar composition.

The galactosaminoglycan analysis of aorta PGs requires a preliminary digestion with papain, in order to obtain polysaccharide chains, followed by the sequential
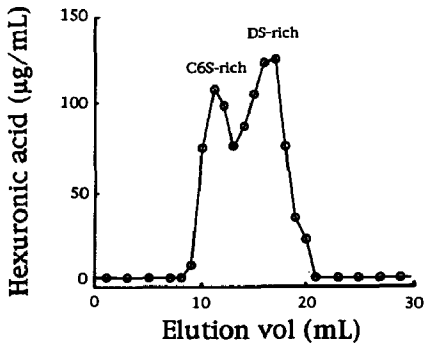
Fig. 5. Sepharose CL-4B gel chromatography of fraction B obtained from the DEAE-Sephacel column. Two fractions were collected, as shown.

use of hyaluronidase, heparitinase, and keratanase to ensure the removal of glycosaminoglycans, which could be derived from hyaluronic acid, heparan sulphate-PGs, and keratan sulphate-PGs, occasionally present as contaminants in purified galactosaminoglycan containing PG preparations. It should be noted that direct analysis of heparan sulphate chains under the experimental conditions used is not possible owing to its resistance to hydrolysis[27].
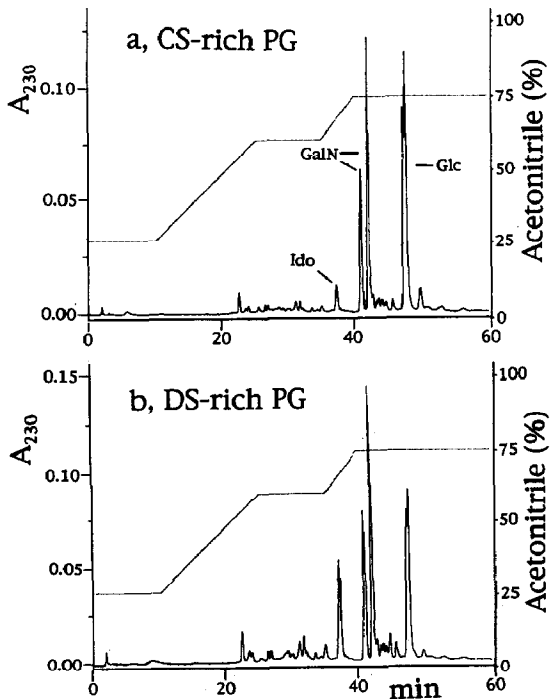
Fig. 6. Reversed phase chromatography of perbenzoyl monosaccharides derived by the reduction and hydrolysis of GAGs from human aorta: a, CS-rich PG; b, DS-rich PG.

TABLE V

Monosaccharide composition of galactosaminoglycans obtained from purified human aorta PGs

Arterial GAG	Hexuronate : hexosamine molar ratio [a]	Iduronate, per cent of total hexuronate
GAGs from CS-rich PGs	1.05	10
GAGs from DS-rich PGs	0.99	45

[a] Determined by reversed phase chromatography. The sum of tribenzoyl anhydroidose and pentabenzoyl glucose was compared to pentabenzoyl galactosamine.

The chromatographic profiles of the perbenzoylated monosaccharides derived from human aorta CS-rich PG and DS-rich PG preparations are shown in Fig. 6. The analytical results obtained for these samples are reported in Table V.

The low iduronate percentage (45%) relative to total hexuronate observed in GAGs from DS-rich PGs with respect to standard DS may be explained by the presence of significant amounts of CS in this fraction, as assessed by electrophoretic analysis (55% DS, 45% CS).

Since arterial PGs could contain neutral sugars other than those derived from the uronic acid containing polysaccharides, we have submitted their constituent GAGs to the full assay protocol after digestion with chondroitinase ABC. The complete absence of peaks at the retention times of 1,6-anhydroidose, galactosamine, and glucose derivatives, as well as a galactosamine–hexuronate ratio close to 1 in the undigested sample, allows us to exclude the risk of interference by contaminants.

In our opinion the method described represents an improvement over other existing methods, because it allows complete analysis of all sugars contained in the disaccharide repeating units of the galactosaminoglycan isomers in a single run, without removal of hexosamines prior to the benzoylation reaction. This is undoubtedly an advantage, not only because it simplifies the procedure and enhances recoveries, but also because hexosamines can themselves be used as an internal standard. In fact, as long as the hexosamine–hexuronate molar ratio is close to 1, the occurrence of coeluting contaminants can reasonably be excluded.

Data obtained on biological samples indicate that the present procedure is suitable for the investigation of the monosaccharide composition of galactosaminoglycan isomers present in arterial PGs.

EXPERIMENTAL

General.—Melting points are uncorrected. ^{1}H NMR 300-MHz Fourier transform spectra were performed on a Varian VXR-300 spectrometer in $CDCl_3$ solutions using Me_4Si as internal standard. Reversed phase chromatography was performed on a Beckman Ultrasphere ODS column (4.6 × 250 mm) protected with a guard-column (4.6 × 30 mm) filled with pellicular ODS material.

Chemicals.—Standard GAGs [CS type A (C4S) from whale cartilage, CS type B (DS) from porcine skin, and CS type C (C6S) from shark cartilage], chondroitinase ABC [CS lyase ABC (EC 4.2.2.4)] from *Proteus vulgaris*, chondroitinase AC [CS lyase AC (EC 4.2.2.5)] from *Arthrobacter aurescens*, hyaluronidase (EC 3.2.1.35) from *Streptomyces hyalurolyticus*, keratanase (EC 3.2.1.103) from *Pseudomonas* species, papain, D-galactosamine hydrochloride, and L-idose were purchased from Sigma (St. Louis, MO, USA). Heparitinase [heparan sulfate lyase (EC 4.2.2.8)] from *Flavobacterium heparinium* was obtained from Seikagaku (Tokyo, Japan). DEAE-Sephacel and Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). Sep-Pak C-18 cartridges were purchased from Waters (Milford, MA, USA) and DEAE MemSep 1000 cartridge from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade.

Synthesis of perbenzoylated sugars.—*General procedure.* Commercial sugars (1.2 mmol) were heated at 100°C for 4 h in 2 M CF_3CO_2H (50 mL). The solvent was removed and the residue treated with benzoic anhydride (10 g, 48 mmol) and 4-dimethylaminopyridine (5 g, 41 mmol) in pyridine (100 mL). After reaction at 37°C for 3 h, water (200 mL) was added, and the mixture was stirred and extracted with diethyl ether (3 × 50 mL). The combined organic extracts were washed with 10% HCl, satd $NaHCO_3$, and water. The ether solution was dried over sodium sulphate and the solvent was evaporated. Crude perbenzoyl derivatives were obtained as crystalline products in yields greater than 95%.

The benzoates were chromatographed on a silica gel column eluted with 1 : 10 EtOAc–benzene, in order to obtain purer materials and/or separate the anomeric mixtures. The following pure products were obtained.

1,6-Anhydro-2,3,4-tri-O-benzoyl-β-L-idopyranose (**1**).—Mp 60–61°C. (plates, from MeOH); $[\alpha]_D^{20}$ + 60.2° (*c* 0.99, $CHCl_3$); ^{1}H NMR, see Tables I and II. Anal. Calcd for $C_{27}H_{22}O_8$: C, 68.35; H, 4.67. Found: C, 68.08; H, 4.54.

2-Benzamido-1,3,4,6-tetra-O-benzoyl-2-deoxy-α-D-galactopyranose (**2**).—Mp 193–194°C (plates, from ether); $[\alpha]_D^{20}$ + 171° (*c* 0.92, $CHCl_3$); ^{1}H NMR, see Tables I and II. Anal. Calcd for $C_{41}H_{33}NO_{10}$: C, 70.38; H, 4.75; N, 2.00. Found: C, 70.11; H, 4.73; N, 1.97.

2-Benzamido-1,3,4,6-tetra-O-benzoyl-2-deoxy-β-D-galactopyranose (**3**).—Mp 149–150°C (needles, from ether); $[\alpha]_D^{20}$ + 46° (*c* 0.86, $CHCl_3$); ^{1}H NMR, see Tables I and II. Anal. Calcd for $C_{41}H_{33}NO_{10}$: C, 70.38; H, 4.75; N, 2.00. Found: C, 70.01; H, 4.62; N, 1.98.

1,2,3,4,6-Penta-O-benzoyl-α- and -β-D-glucopyranose.—These compounds were obtained in pure form according to the general procedure. Their physical and spectroscopic data agreed with those in the literature[28].

Preparation of perbenzoylated sugars from galactosaminoglycans.—*(i) Reduction of uronic acids and hydrolysis*[24]. A 100 mg/mL aqueous solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (10 μL) was added to 40 μL of an aqueous solution containing 1–250 μg of GAG in a screw-capped tube. After 10 min at room temperature, a 5-μL aliquot of 0.04 M HCl was added and the

mixture was regularly stirred in an ultrasonic bath. After 50 min another 5-μL aliquot of 0.04 M HCl was added and the mixture was stirred for a further hour.

To the above solution 2 M aq NaBH$_4$ (100 μL) was added and the mixture was heated at 50°C for 1 h. A second aliquot of 2 M NaBH$_4$ (100 μL) was then added and the heating was continued for another 30 min. After cooling to room temperature, 25 μL of glacial acetic acid was cautiously added, and then 1.3 mL of abs EtOH. The sample was briefly vortexed and kept at 4°C overnight. After centrifugation in a high speed microcentrifuge (9000g), the pellet obtained was dried under a stream of N$_2$.

The sample obtained from the above procedure was dissolved in 2 M CF$_3$CO$_2$H (0.5 mL) and heated at 100°C for 9 h.

(ii) Perbenzoylation of the monosaccharide mixtures. The hydrolyzed mixture was transferred into a 10 mL glass syringe and the solvent removed by lyophilization. The dried product was treated with 0.5 mL of pyridine containing 10% (w/v) benzoic anhydride and 5% (w/v) 4-dimethylaminopyridine at 37°C for 90 min. The reaction was quenched with 4.5 mL of water and the mixture was passed three times through a C-18 cartridge. After washing with 10 mL aq 10% pyridine and 10 mL of water, the benzoyl derivatives were eluted with 15 mL of MeCN, reversing the flow through the cartridge. The solvent was evaporated.

The sample was dissolved in MeCN to a final concentration of 4–40 μg/mL of the original GAG uronic acid content, and then the solution was centrifuged at 9000g to precipitate particulate materials and filtered on a Millipore filter (pore size 0.22 μm) prior to injection into the HPLC system.

Reversed phase HPLC.—The elution was performed with a discontinuous linear gradient of MeCN in water. The chromatographic conditions were the following: 0–10 min 25% MeCN, 10–25 min 25–60% MeCN, 25–35 min 60% MeCN, 35–40 min 60–75% MeCN, and 40–60 min 75% MeCN. The flow rate was 1.0 mL/min at room temperature, and detection was at 230 nm. A 20-μL loop was used for injection. Identity of the peaks was confirmed by coelution with authentic samples. Calibration curves were obtained by injecting known amounts of standard perbenzoylated monosaccharides, ranging from 0.05 to 4 μg.

Preparation of tissue glycosaminoglycans.—PGs were obtained from samples of normal human thoracic aorta intima and media taken at autopsy within 24 h of death. Aortas from two subjects aged 39 and 46 years were used after obtaining appropriate consent from the relatives. After removing the adventitia and outer media, only normal areas were selected. Aorta minces were suspended in 15 times their weight of 6 M urea in 1 M NaCl, containing protease inhibitors (10 mM ethylenediaminetetraacetic acid disodium salt, 5 mM benzamidine hydrochloride, 10 mM phenylmethanesulphonyl fluoride, 10 mM ε-aminocaproic acid, 10 mM *N*-ethylmaleimide), and shaken at 4°C for 24 h. The extract, containing ca. 60% of the total uronic acids, was centrifuged at 100000g for 1 h at 4°C and the pellet discarded. The supernatant was dialysed against 6 M urea, containing protease inhibitors, until a concentration of 0.25 M NaCl was reached.

Samples were submitted to ion-exchange chromatography on a DEAE-Sephacel column (1.5 × 25 cm), equilibrated with a 50 mM sodium acetate buffer, pH 6.2 containing 6 M urea, 0.25 M NaCl, 10 mM $CaCl_2$, and protease inhibitors at a flow rate of 10 mL/h. The column was washed with 3 bed volumes of the same buffer to elute contaminating proteins and the PG elution was carried out with a linear gradient of NaCl in the same buffer, ranging from 0.25 to 2.0 M. The uronic acid profile showed two hexuronate containing peaks. Hexuronate containing material of the second peak was applied onto a Sepharose CL-4B column (1.0 × 50 cm) and eluted with 6 M urea, 1 M NaCl, containing protease inhibitors, at a flow rate of 10 mL/h, producing two PG fractions. Both of these were exhaustively dialyzed and digested sequentially with papain, hyaluronidase, heparitinase, and keratanase before HPLC analysis.

Other analytical methods.—Hexuronic acid content was determined by the method of Bitter and Muir[29], using D-glucuronolactone as a standard. Neutral sugar content in reduced GAGs was measured by the anthrone reaction[30]. Protein concentration was determined by the method of Lowry[31].

Protein digestion was carried out with papain (150 μg/mg protein) in the presence of 5.0 mM cysteine hydrochloride and 5.0 mM ethylenediaminetetraacetic acid, in 0.1 M sodium acetate buffer, pH 5.8 at 60°C for 48 h[32]. The digest was clarified by centrifugation at 8000 g and the residue washed. Washings were combined with the original digest and loaded onto an anion-exchange cartridge to remove oligosaccharides. GAGs were eluted with 1 M NaCl and precipitated by adding 4 vol of EtOH. The mixture was left overnight at 4°C and the precipitate separated by centrifugation, washed twice with EtOH and diethyl ether, and dried.

Electrophoretic separation of GAGs was carried out according to Cappelletti et al.[25,26].

When necessary, aliquots of tissue GAGs (100 μg hexuronic acid/mL) were digested with the following lyases at 37°C for 24 h: hyaluronidase, 10 U/mL in 0.5 M sodium acetate, pH 5.6; keratanase, 0.02 U/mL in 0.05 M tris(hydroxymethyl)-aminomethane hydrochloride, pH 7.4; heparitinase, 0.02 U/mL in 0.05 M sodium acetate, pH 7.0; chondroitinase ABC or AC, 0.1 U/mL in 0.1 M sodium acetate, pH 8.0. Every digestion step was followed by precipitation with EtOH.

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