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Preparation and anticoagulant activity of fully *O*-sulphonated glycosaminoglycans

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Abstract

Glycosaminoglycans including dermatan sulphate, hyaluronan, heparan sulphate and heparin were chemically modified by *O*-sulphonation. By altering the reaction conditions, products having a different degree of *O*-sulphonation could be obtained. Glycosaminoglycan derivatives were prepared having no free hydroxyl groups, with sulphoester group/disaccharide unit ratios of 4.0 for dermatan sulphate and hyaluronan, and sulphoester and sulphamide group/disaccharide unit ratios of 4.22 and 4.88 for heparan sulphate and heparin, respectively. ^{1}H NMR spectroscopy showed that the fully *O*-sulphonated hyaluronan derivative had a glucuronate residue with an altered conformation. Since glycosaminiglycans and their derivatives are often used as anticoagulant/antithrombotic agents, their anti-amidolytic activities were determined. The anti-factor IIa activity of fully *O*-sulphonated dermatan sulphate, hyaluronan and heparan sulphate ranged from 40 to 80 units/mg, while no anti-factor Xa activity of the fully *O*-sulphonated glycosaminoglycans was detected. These values are lower than those reported for low-molecular-weight heparins and are consistent with the requirement of an antithrombin III pentasaccharide binding site for anti-factor Xa activity. Interestingly, the anti-factor Xa of heparin is lost by chemical *O*-sulphonation.

Keywords: Chemical oversulphonation; Glycosaminoglycan; Anticoagulant activity

1. Introduction

Glycosaminoglycans (GAGs) are structurally complex, sulphated, linear polysaccharides comprised of alternating disaccharide units consisting of uronate (glucronate (GlcpA) or iduronate (IdopA)) and hexosamine (glucosamine (GlcpN) or galactosamine (GalpN)) residues [1]. GAGs are divided into four categories based on their composition: the chondroitin sulphate family including chondroitin sulphate, dermatan sulphate: the heparan sulphate family including heparan sulphate and heparin; hyaluronan; and keratan sulphate. Chondroitin sulphate and dermatan sulphate typically contain one sulphate group per disaccharide unit located at either the C-4 or C-6 positions of

Abbreviations: Ac, acetyl; ATIII, antithrombin III; GAG(s), glycosaminoglycan(s); GalpN,D-2-amino,2-deoxy galactopyranoside; GlcpA, D-glucopyranosyluronic acid; GlcpA2S, 2-*O*-sulpho-D-glucopyranosyluronic acid; GlcpA3S, 3-*O*-sulpho-D-glucopyranosyluronic acid; GlcpN, D-2-amino, 2-deoxyglucopyranoside; GPC, gel permeation chromatography; HCII, heparin cofactor II; HexpN, D-2-amino, 2-deoxy hexopyranoside; IdopA, D-idopyranosyluronic acid; IdopA2S, 2-*O*-sulpho-D-glucopyranosyluronic acid; IdopA3S, 3-*O*-sulpho-D-idopyranosyluronic acid; MWCO, molecular weight cut-off; NHP, normal human plasma; 1D, one dimensional; PAGE, polyacrylamide gel electrophoresis; Py·SO_3, Pyridine-sulphur trioxide; TBA, tributylammonium; TBA·SO_3, tributylamine-sulphur trioxide; 2D, two dimensional.

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GalpNAc [1]. Chondroitin and dermatan sulphate are found attached to a protein core forming proteoglycans that are often localized on cell surfaces and in the extracellular matrix, where they are important in cell–cell communication [1–3]. While chondroitin sulphate and hyaluronan lack clinically relevant levels of anticoagulant activity, heparan sulphate and dermatan sulphate appear to be involved in maintaining haemostasis [4]. The anticoagulant activity of heparin is ascribed to specific structural features as well as its high level of sulphation [5]. Heparin has been the drug of choice in clinical, pre-surgical prophylaxis of thrombotic events [6]. However, because of its side-effects, such as bleeding and other disadvantages, developing alternatives to heparin are an important research goal [7]. Hyaluronan lacks sulphation and is present in the extracellular matrix where it is important for the maintenance of tissue architecture [8]. Depolymerization of hyaluronan may facilitate tumor invasion [10].

Recently, modified glycosaminoglycans and polysaccharides have been studies as potential heparin analogues in drug development [6,11]. A fully *O*-sulphonated chondroitin sulphate, synthesized in our laboratory, has been shown to exhibits strong antithrombotic activity [12]. The conformation of glucuronic acid in this chemically modified glycosaminoglycan makes it both structurally and conformationally similar to the 2-*O*-sulpho iduronic acid residues found in heparin [13,14]. In the current study, dermatan sulphate, hyaluronan, heparin and heparan sulphate were completely *O*-sulphonated and the solution conformation of their uronate residues were examined using ^{1}H NMR spectroscopy. The relationship of uronic acid conformation and content of *O*-sulphonate to anticoagulant activity is discussed.

2. Experimental

2.1. Materials

Dermatan sulphate ($M_{w\ av}$, 30000) from porcine skin were kindly donated by Shin-Nippon Yakugyo Co. (Tokyo, Japan). Hyaluronan (($M_{w\ av}$, 100 000) from recombinant *E. coli* was from Kibun Food Chemipha Co. (Tokyo, Japan). Heparin ($M_{w\ av}$, 16 000) and heparan sulphate ($M_{w\ av}$, 14 800) from porcine intestinal mucosa were form Celusus (Cincinnati, OH). Chondroitin lyases ABC (Chase ABC, EC 4.2.2.4) and ACII *arthro* (ChaseACII, EC 4.2.2.5) were form Seikagaku Kogyo Co. (Tokyo, Japan). Heparin lyase I (Hep I, EC 4.2.2.7), heparin lyase II (Hep II, no EC number) and heparin lyase III (EC 4.2.2.8) were prepared from *Flavobacterium heparinum* and purified to homogeneity [15].

2.2. Chemical sulphonation of glycosaminoglycans

Oversulphated glycosaminoglycans were prepared by *O*-sulphonation under mild conditions with adducts of sulphur trioxide (SO_3) in aprotic solvents [16]. Glycosaminoglycans were fully *O*-sulphonated as described previously [12]. In the cases of *O*-sulphonation of heparin and heparan sulphate, partial loss of the sulphate group from GlcpNS residues was observed, requiring re-*N*-sulphonation according to previously described methods [17].

2.3. Determination of molecular weight

Gradient PAGE analysis was used to estimate the weight average molecular weight (M_{wav}) of each sample [18]. A GPC-HPLC column was eluted with 50 mM sodium acetate, pH 7.4 at flow rate of 1 ml/min with detection at 206 nm [19]. The relative molecular weights of each GAG were determined form their elution position.

2.4. Disaccharide and compositional analysis

The determination of unsaturated disaccharides prepared from intact and modified GAGs was performed on the lyase digested samples using HPLC [20]. GAG samples were prepared for the determination of sulphate and hexosamines by exhaustive dialysis (MWCO 3500) against distilled water, lyopholization and drying for 2 days in a desiccator over P_2O_5. Determination of sulphate groups was performed by anion-exchange HPLC after acid hydrolysis of the sample in 6 M HCl at 100°C for 2.5 h using a conductivity detection (Tosoh model CM-8, Tokyo, Japan). Hexosamine was analyzed by the post-column HPLC derivatization method [21] after acid hydrolysis under identical conditions as described for sulphate analysis.

2.5. Optical rotation measurements

The samples, dried for sulphate analysis, were also used to measure optical rotation. Samples were weighed, dissolved in distilled water (5 mg/ml) and their optical rotation was determined at the sodium D-line on a Jasco model DIP-140 spectropolarimeter (Tokyo, Japan).

2.6. IR spectroscopy

IR spectroscopy of solid samples relied on a Jasco model FTIR 230 (Tokyo, Japan). Glycosaminoglycan (100 μg) was mixed with 500 μg of dried KBr and compressed to prepare a salt-disc (3 mm diameter).

2.7. ^1H NMR spectroscopy

^1H NMR spectroscopy was performed under the conditions described previously [21]. 2D spectra were obtained from 512 experiments resulting 1024 data points for spectral width of 2000 Hz and the time domain data were multiplied after zerofilling (data matrix size, 1K × 1K) with shifted sine-bell window functions for 2D double quantum filtered (DQF)-COSY, NOESY and TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D NOESY and TOCSY experiments by using 250 and 500 ms as the mixing time were performed.

2.8. Anti-factor Xa and anti-factor IIa activities

Normal human plasma (NHP) was collected from healthy volunteers. A Coatest LMW heparin/heparin kit (Chromogenix, Mölndal, Sweden) was used to determine anti-factor Xa activity. Anti-factor IIa activity was measured by incubating 50 μl of intact and chemically *O*-sulphonated GAG in 30 μl of NHP with 20 μl of human thrombin (1.2 NIH units/ml) at 37°C for 30 s. Chromogenic TH (ethylmalonly-Pro-Arg-*p*-nitroanilide hydrochloride) substrate 50 μl (1.9 μmol/ml) was added and the amidolytic activity of thrombin was determined at 405 nm. An ACL 300 plus instrument (Lexington, MA) was used and activity was calculated in comparison with USP Heparin Reference Standard (K-3) supplied by US Pharmacopeial Convention (Rockville, MD) [22].

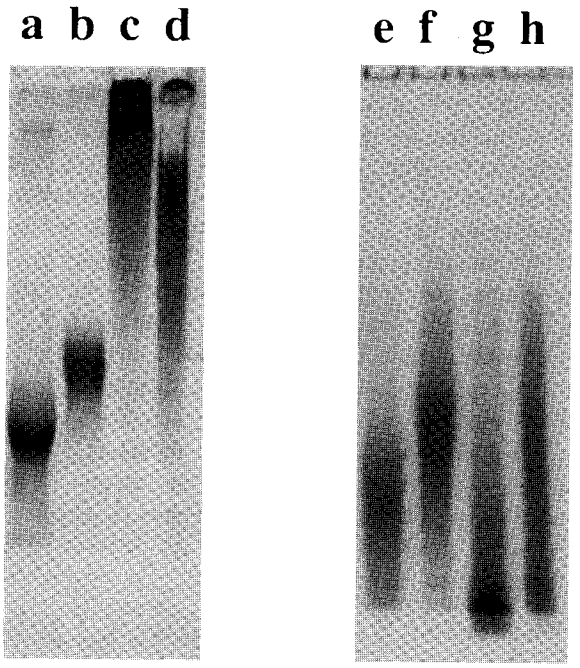

Fig. 1. Gradient PAGE of intact and fully *O*-sulphonated glycosaminoglycans. (a) Dermatan sulphate from porcine skin; (b) Fully *O*-sulphonated dermatan sulphate; (c) Hyaluronan from recombinant *E. Coli*; (d) Fully *O*-sulphonated hyaluronan; (e) Heparin from porcine intestinal mucosa; (f) Fully *O*-sulphonated heparin; (g) Heparan sulphate from porcine intestinal mucosa; and (h) Fully *O*-sulphinated heparan sulphate.

3. Results

3.1. Preparation and characterization of chemically oversulphated GAGs

O-sulphonation of each GAG was performed at different temperatures and resulted in oversulphated GAGs having different levels of *O*-sulphonation. Molecular weights of each sample were determined using gradient polyacrylamide gel electrophoresis (PAGE) analysis. [18] (Fig. 1). A slight increase in molecular weight was observed on *O*-sulphonation. In addition, the microheterogeneity of the partially *O*-sulphonated samples increased, as shown by a reduction in clearly defined banding on gradient PAGE analysis (see Fig. 1). The expected small increase in molecular weight concomitant with increased level of sulphation was demonstrated by gel permeation chromatography (GPC) [19] (data not shown). The results obtained were consistent with the additional mass of the *O*-sulpho groups and the expected stability of the glycosidic linkages in the polysaccharides under the mild reaction conditions.

Exhaustive treatment with the lyases, followed by HPLC analysis was used to determine the composition of the partially and fully *O*-sulphonated GAGs and to confirm that *O*-sulphonation had taken place. The recoveries of the unsaturated disaccharides from partially oversulphated GAGs decreased with increased sulphation. The fully *O*-sulphonated GAGs afforded no unsaturated disaccharide products (data not shown) as expected based on the known resistance of full domains to these enzymes [23].

Changes in the optical rotation of GAGs shown in Table 1 accompany full O-sulphonation. The difference of the optical rotation from the intact GAG was significant in the case of partially oversulphated samples. The magnitude and direction of this change is consistent with either a significant change in the molecular conformation of these derivatives or the dilution effect of newly substituted *O*-sulphonate groups [24]. IR spectroscopy of intact and fully *O*-sulphonated hyaluronan (Fig. 2A and B) strongly suggested the conversion of hydroxy groups to axial/equatorial *O*-sulphonate groups. The intensity of the absorbances at 1240 cm^{-1} and 820–850 cm^{-1} attributed to the stretching of S = O bond and C–O–S bonds, respectively, are dramatically changed by *O*-sulphonation of HA. Similarly, the intensity of the bands at 1440, 1380 and 1100 cm^{-1}, attributed to the stretching and/or deformation vibra-

Table 1
Optical rotation and anti-IIa activity of intact and chemically *O*-sulphonated glycosaminoglycans

GAG[a]	SO_3/disaccharide	Optical rotation	Anti-IIa activity (units/mg)	
			a[b]	b[c]
DS-0	0.01	−41.5	0.8	n.d.
DS-1	2.17	−28.4	2.4	n.d.
Ds-2	2.68	−22.2	8.3	n.d.
DS-3	2.78	−20.3	8.0	n.d.
DS-4	3.95	−10.5	61.9	n.d.
HA-0	0	−66.0	n.d.	n.d.
HA-1	0.90	−32.5	n.d.	n.d.
HA-2	1.82	−30.0	2.2	n.d.
HA-3	2.45	−27.8	8.8	n.d.
HA-4	3.97	−25.0	64.7	n.d.
HS-0	0.92	70.0	2.6	n.d.
HS-1	2.62	39.8	12.6	1.2
HS-2	2.90	37.3	14.2	1.7
HS-3	3.22	35.6	28.7	1.7
HS-4	4.31	31.5	37.8	n.d.
HP-0	2.68	46.5	327.0	258.0
HP-4	4.68	22.3	74.1	n.d.

[a] GAG-0, an intact sample; GAG-1, -2, -3 and 4, derivatives prepared by treating with excess ×4, ×6, ×8 and ×12 molar sulphur trioxide complex of available hydroxy group in each glycosaminoglycans. DS, dermatan sulphate; HA, hyaluronan; HS, heparan sulphate; and HP, heparin.

[b] Human plasma was used for assay.

[c] Purified human antithrombin III was used for assay (Anti-Xa). n.d., not detected.

tion of C–O–H bonds, were decreased in the spectrum of the fully *O*-sulphonated GAGs. Assignment of IR absorption bands at 1240 cm^{-1} [25], and 1430 cm^{-1} in the spectrum of fully *O*-sulphonated HA were based on the reports by Casu et al. [26] and bands in the 820–850 cm^{-1} spectral region were attributed C–O–S stretching based on the results of Orr [27]. Multiple bands at about 800–820 cm^{-1} were tentatively ascribed to sulphate half-ester based on the report of Grant et al. [24] and the band at 800 cm^{-1} was ascribed to C–O–S stretching within predominantly axial/equatorial 2-*O*-, and 3-*O*-sulpho groups of glucuronate residues based on the work of Sanderson and coworkers [28].

^{1}H NMR spectra of intact GAGs and *O*-sulphonated GAGs prepared at 40°C shown in Figs. 3 and 4. With the exception of hyaluronan, the spectra of the parent GAGs showed a substantial level of structural heterogeneity. This heterogeneity results from the presence and/or absence of sulphation at the C-4 and C-6 positions of HexpNAc in chondroitin, dermatan sulphates/hyaluronan, or C-3 and C-6 positions of the GlcpN residues in heparin/heparan sulphate, and the 2 position of the iduronate residue in dermatan sulphate, heparan sulphate and heparin. *O*-sulphonation at 0°C resulted in an expected [29,30] increase in the structural heterogeneity (data not shown). This increased heterogeneity is caused by the introduction of additional sulphate groups at the 4 and/or 6 positions of GalpNAc, or at the 3 and/or 6 positions of uronate residues. Surprisingly, *O*-sulphonation at 40°C resulted in a considerably less complex 1D ^{1}H NMR spectrum, suggesting reduced structural heterogeneity, consistent with full *O*-sulphonation [12]. Two dimensional (2D) ^{1}H NMR experiments, involving DQF-COSY and NOESY spectroscopy, of the fully *O*-sulphonated hyaluronan (Fig. 5), clearly show the down field shifts of ring protons attached to the *O*-sulphonated carbons, such as GlcpA/IdopA H-2, H-3, and GalpNAc H-4, H-6 and GlcpN H-3, H-6, and also affords sequence confirmation. NOESY spectra of the fully *O*-sulphonated GAGs showed crossed-peaks that strongly suggest that the sequence and linkage positions of the GAGs are maintained.

Table 2 summarizes the chemical shifts and coupling constants of ring protons of fully sulphonated dermatan sulphate, hyaluronan and heparin samples. Because of the complexity of the composition of heparan sulphate, which contains GlcpNAc, GlcpNS, IdopA and GlcpA, it was impossible to assign all of the ring protons even after full sulphonation. The coupling constant of each ring proton of glucuronate of hyaluronan at 30°C was used to calculate the dihedral angles of vicinal protons of glucuronate. This dihedral angle was not the 180°, typically observed for a glucuronate residue. These data strongly suggest the glucronate residue had undergone a conformational change from $^4C_1$ to $^1C_4$ resulting from *O*-sulphonation of this residue. The coupling constant between H-1 and H-2 of glucuronate residue of hyaluronan at 60°C dramatically changed from < 1.5 to 4.1 Hz (Table 2). These observations strongly suggest that at 60°C the conformation of the glucuronate residue, in the fully *O*- sulphonated hyaluronan, has changed from $^1C_4$ to $^2S_0$. On the other hand, an unusual coupling constant (< 1.5 Hz) between H-2 and H-3 of GalpNAc residues of dermatan sulphate was observed. The coupling constant $J_{2,3}$, between H-2 and H-3 if GalpNAc residues of intact dermatan sulphate are typically in the range of 8 to 9 Hz at 30°C. This observation suggests that full *O*-sulphonation might have caused the distortion of GalpNAc residues in dermatan sulphate.

3.2. Effects of oversulphated GAGs on the inactivation of factor IIa and factor Xa by human plasma

The correlation between the sulphation level, of chemically *O*-sulphonated chondroitin sulphates, and their inactivation of factor IIa activity was previously demonstrated [12]. In the current study, a clear and dramatic increase in anti-factor IIa activity was ob-

served on the complete *O*-sulphonation of chondroitin sulphate. Since this dramatic activity increase was only observed with full *O*-sulphonation, the increased anti-factor IIa activity probably does not merely result from an increase in overall charge. It is, instead, likely that some other structural change, such as a shift in conformation, is responsible for the increased anti-factor IIa activity of the fully *O*-sulphonated chondroitin sulphate derivative. The increase in anti-factor Xa activity, observed on the incomplete *O*-sulphonation of chondroitin sulphate was considerably smaller. This modest increase may simply result from a non specific effect associated with an increase in the overall molecular charge. The anti-factor IIa or Xa of the fully *O*-sulphonated GAGs are summarized in Table 1. Although each of the fully sulphated GAGs showed the expected enhanced factor IIa activity, expectedly, fully *O*-sulphonated heparin showed no anti-factor Xa activity. Partially *O*-sulphonated heparan sulphate, prepared at 0°C, showed a small amount of anti-IIa activity mediated through ATIII binding. These data strongly suggest that this partial *O*-sulphonation results in a sequence similar to that of ATIII pentasaccharide binding site found in heparin.

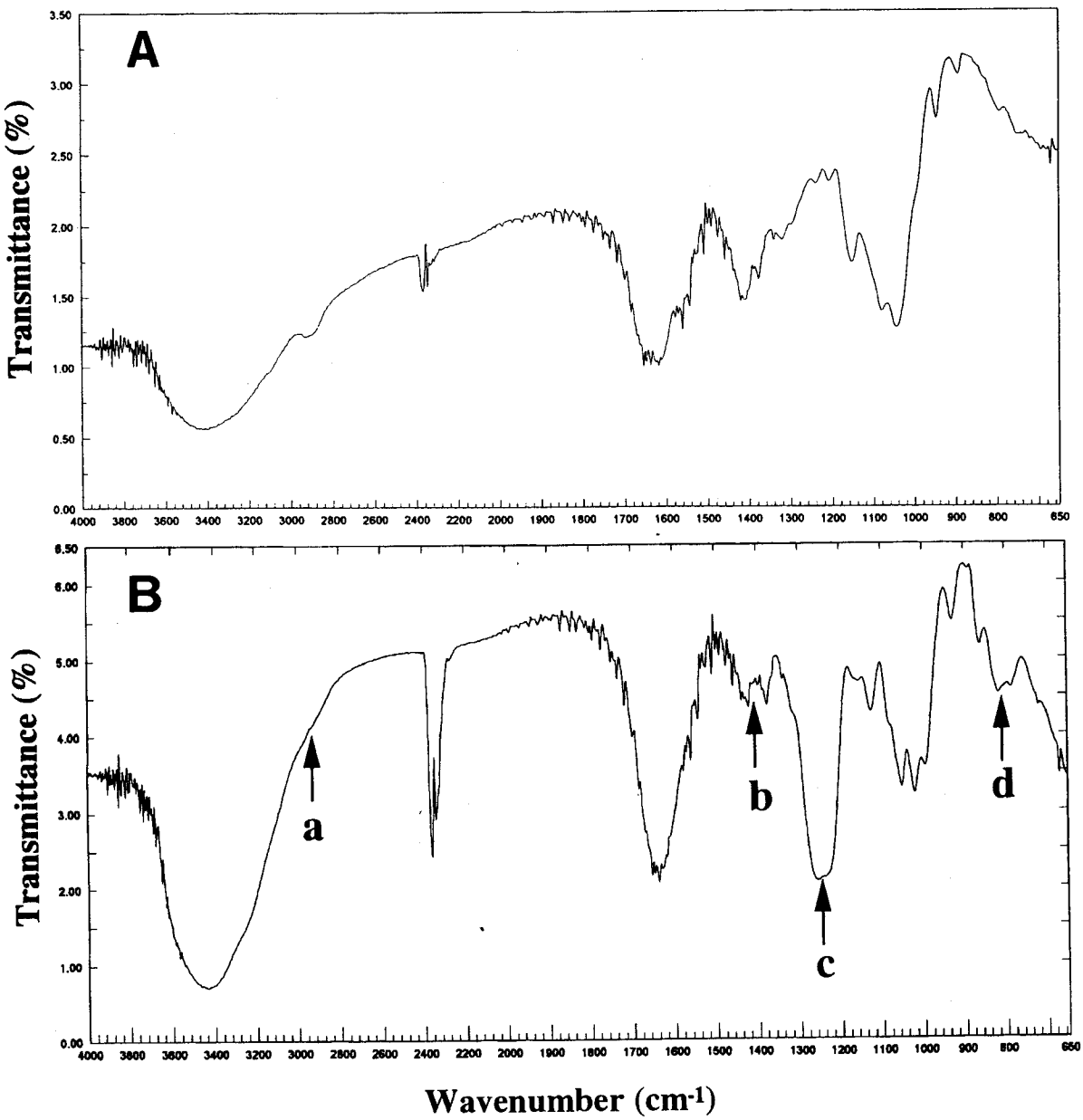

Fig. 2. Infrared spectra of intact hyaluronan and fully *O*-sulphonated hyaluronan. (a) C–H stretching; (b) O–H deformation; (c) S = O stretching; and (d) C–O–S deformation and stretching of C–O–SO_3.

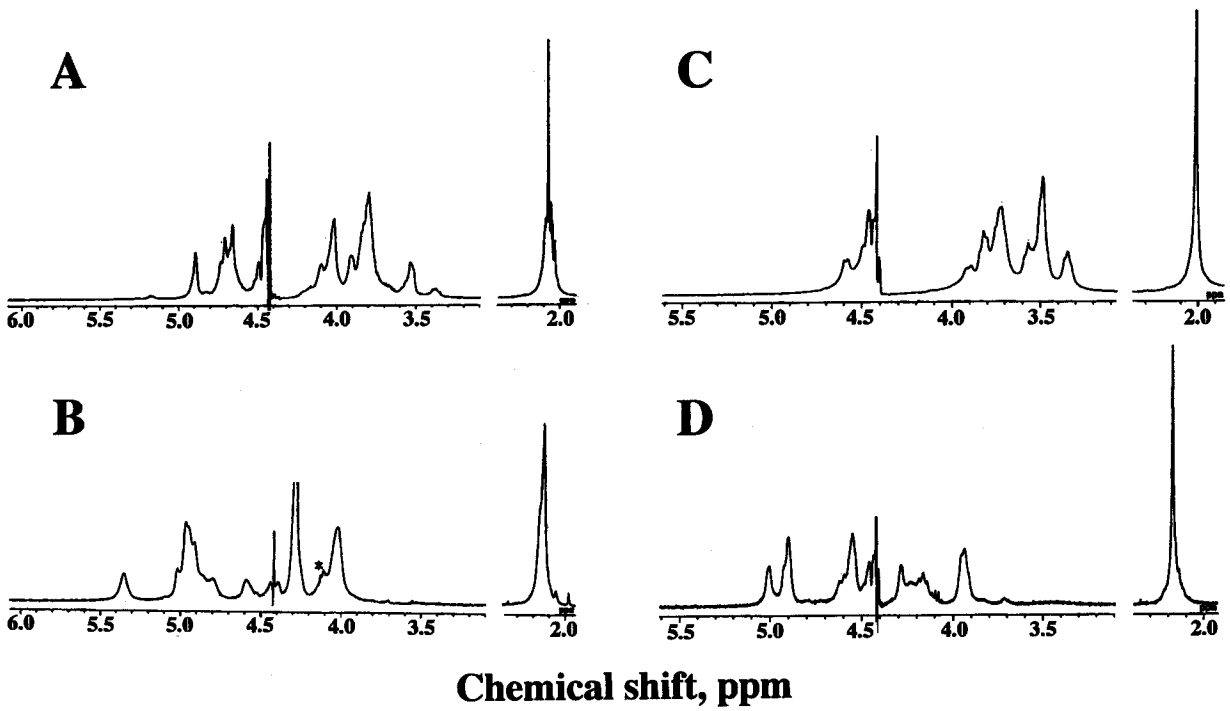

Fig. 3. One dimensional ^{1}H NMR spectra of intact and chemically *O*-sulphonated dermatan sulphate and hyaluronan measured at 333 K. (A) Intact dermatan sulphate; (B) Fully *O*-sulphonated dermatan sulphate; (C) Intact hyaluronan; (D) Fully *O*-sulphonated hyaluronan.

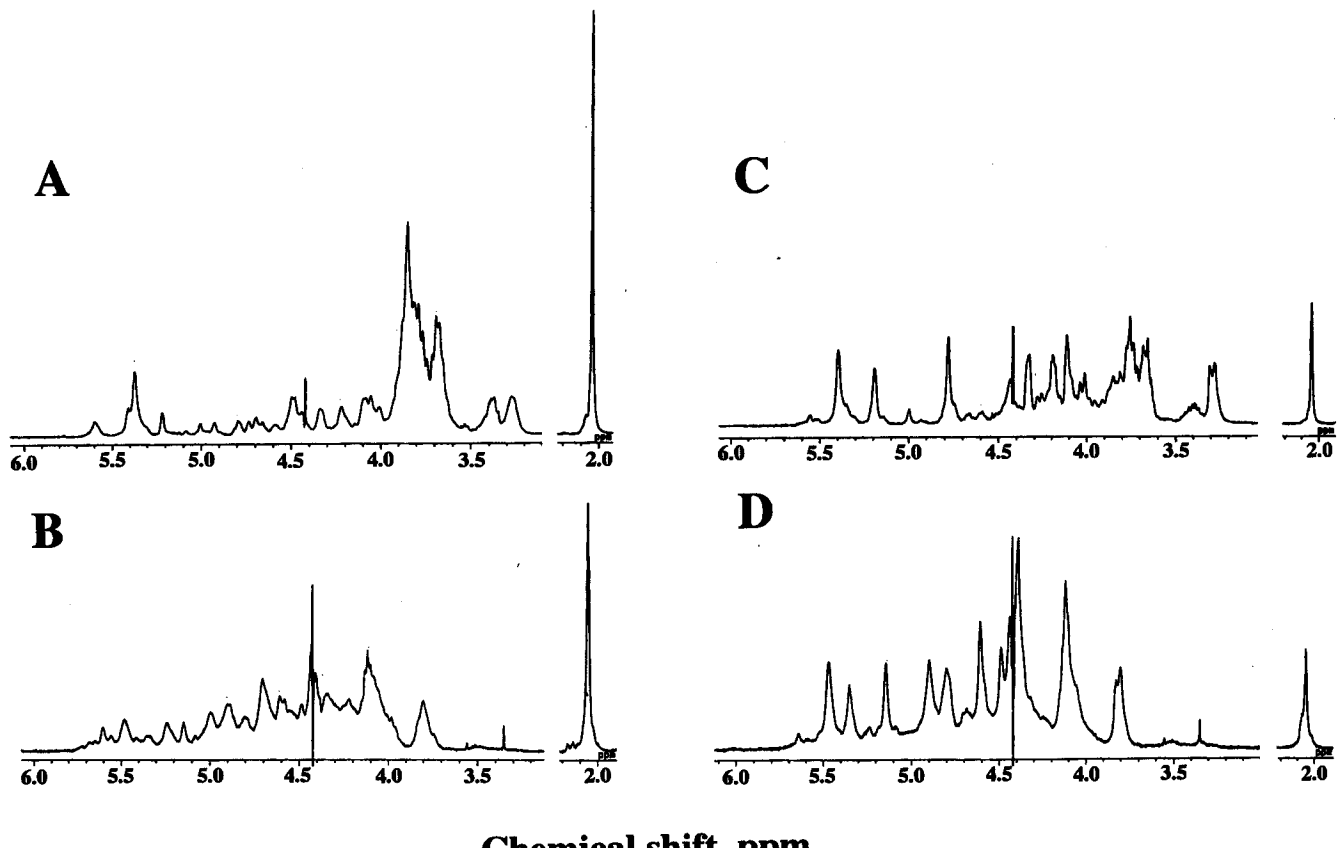

Fig. 4. One dimensional ^{1}H NMR spectra of intact and chemically *O*-sulphonated heparan sulphate and heparin measured at 333 K. (A) Intact heparan sulphate; (B) Fully *O*-sulphonated heparan sulphate; (C) Intact heparin; (D) Fully *O*-sulphonated heparin.

4. Discussion

We previously reported the preparation of a fully *O*-sulphonated chondroitin sulphate that showed strong anticoagulant activity [12]. This activity of this derivative was ascribed to a conformational change of the glucuronate residue. Oversulphated disaccharide sequences account for a minor but important part of the structure of chondroitin sulphate derived from mammalian tissues [31,32]. Dermatan sulphate, hyaluronan, heparin and heparan sulphate were chemically *O*-sulphonated to investigate their physical, chemical biological properties. Optimum conditions for complete *O*-sulphonation of these GAGs were determined to be 40°C for 1 h with 15 equiv. of sulphonation reagent/mol of hydroxy group. Under these conditions, GAG molecular weight increased slightly, consistent with the mass of the added *O*-sulpho and the absence of breakdown of the glycosidic linkages. ^{1}H NMR experiments (Figs. 3, 4 and 5), compositional analysis data, and the failure of polysaccharide lyases to act on these GAG derivatives, demonstrated that they were fully *O*-sulphonated.

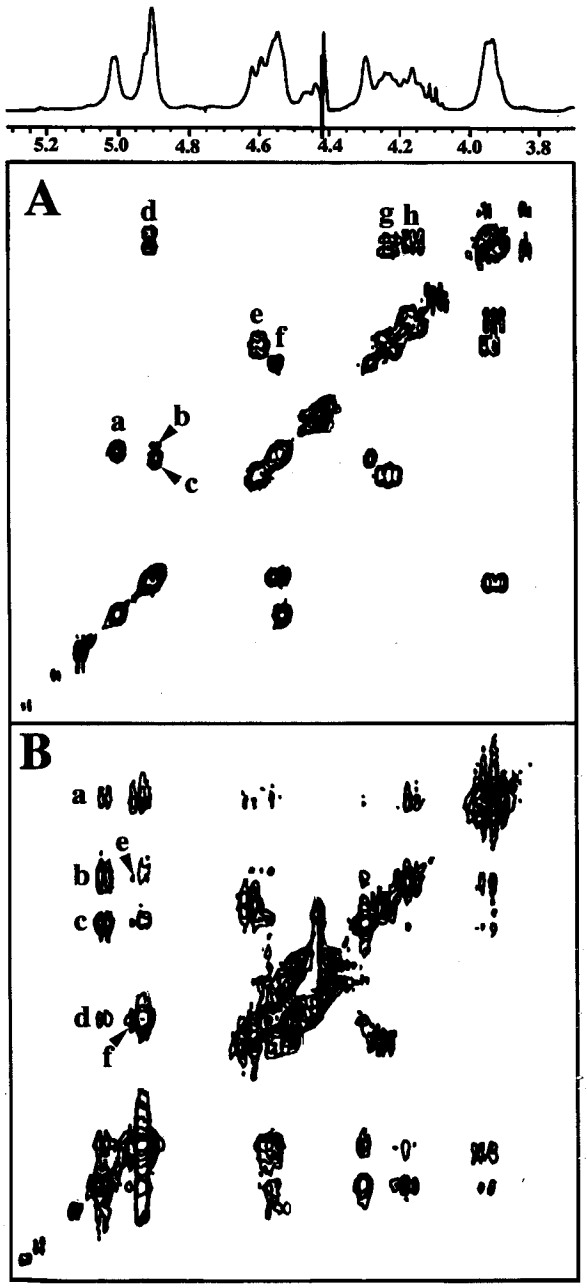

Fig. 5. Two dimensional DQF-COSY and NOESY spectra of fully *O*-sulphonated hyaluronan measured at 333 K. (A) DQF-COSY spectrum and (B) NOESY spectrum of fully *O*-sulphonated hyaluronan. Cross peaks (upper panels): (a) GlcpA H-1/H-2; (b) GlcpA H-2/H-3; (c) GlcpA H-3/H-4; (d) GlcpNAc H-1/H-2; (e) GlcpNAc H-5/H-6; (f) GlcpA H-4//H-5; (g) GlcpNAc H-4/H-5; and (h) GlcpNAc H-2/H-3 and GlcpNAc H-3/H-4: (lower panel): (a) GlcpA H-1/GlcpNAc H-2; (b) GlcpA H-1/GlcpNAc H-3; (c) GlcpA H-1/H-5; (d) GlcpA H-1/H-2; (e) GlcpNAc H-1/H-3; and (f) GlcpNAc H-1/GlcpA H-4.

This study demonstrates that chemically *O*-sulphonated GAGs show anti-factor IIa activities (Table 1) comparable with the activities displayed by previously described heparin analogues [11] and low-molecular-weight heparins [9]. Optical rotation measurements suggests a change in conformation and bands at 800 cm^{-1} in the IR spectra suggests an axial/equatorial disposition of 2- and 3-*O*-sulpho groups in the uronate residue. NMR spectroscopy at 60°C (Figs. 3–5) demonstrates an altered conformation of glucuronate residues from (^{4}C$_1$ to ^{1}C$_4$) in the fully *O*-sulphonated hyaluronan derivative. This conformational change may result from the repulsion of negatively charged sulphate groups, as was observed in that of fully *O*-sulphonated chondroitin sulphate. These conformational changes afford a substantial increase in anti-factor IIa activity (Table 1).

Fully *O*-sulphonated chondroitin sulphate and hyaluronan both shown ^{1}C$_4$ glucuronate, closely resembling the 2-*O*-sulpho-iduronate residue commonly found in heparin. While the anti-factor IIa activities of fully *O*-sulphonated chondroitin sulphate and hyaluronan are comparable to that of low-molecular-weight heparins, the activity of fully *O*-sulphonated dermatan sulphate was much greater than that of fully *O*-sulphonated chondroitin sulphate and hyaluronan.

The same magnitude of increase is not observed in anti-factor Xa activity. The action of different serum protease inhibitors on factor IIa and factor Xa serve as an explanation for this observation. Factor IIa is inhibited by both antithrombin III (ATIII) and heparin cofactor II (HCII) while factor Xa is only inhibited by ATIII. ATIII is known to bind to a specific pentasaccharide sequence found within heparin's structure but, HCII binds with considerably less specificity to oversulphate domains of heparin [33], dermatan and chondroitin sulphates [34]. Therefore, it is likely

Table 2
Chemical shifts (ppm) and coupling constants (Hz) of fully *O*-sulphonated GAGs

GAG[a]	Residue probe temp.	H-1 J1,2	H-2 J2,3	H-3 J3,4	H-4 J4,5	H-5 J5,6	H-6	N-Ac
DS	GalpNAc							
	333 K	4.92 n.d.[b]	4.4 n.d.	4.02 n.d.	4.97 n.d.	4.02 n.d.	4.29	2.14
	303 K	5.00 n.d.	4.39 <1.5	4.06 n.d.	5.00 n.d.	4.06 n.d.	4.31	2.15
	IdopA							
	333 K	5.35 <1.5	4.97 n.d.	4.80 <1.5	4.29 <1.5	4.58	–	–
	303 K	5.35 <1.5	5.00 n.d.	4.9 n.d.	4.31 <1.5	4.55	–	–
HA	GlcpNAc							
	333 K	4.91 n.d.	3.96 n.d.	4.18 8.7	3.96 n.d.	4.22 <1.5	4.62	2.91
	303 K	4.9 n.d.	3.96 n.d.	4.14 n.d.	3.96 n.d.	4.3 n.d.	4.48	2.19
	GlcpA							
	333 K	5.02 4.1	4.56 n.d.	4.91 n.d.	4.56 <1.5	4.29	–	–
	303 K	4.97 <1.5	4.54 n.d.	4.91 n.d.	4.56 <1.5	4.29	–	–
HP	GlcpNS							
	333 K	5.47 2.4	3.81 10.0	4.80 <1.5	4.12 n.d.	4.12 n.d.	4.39	
	303 K	5.48 1.7	3.83 10.5	4.8 n.d.	4.12 n.d.	4.12 n.d.	4.38	
	IdopA							
	333K	5.35 <1.5	4.90 <1.5	4.61 <1.5	4.49 <1.5	5.14	–	–
	303 K	5.36 <1.5	4.90 <1.5	4.62 <1.5	4.50 <1.5	5.20	–	–

[a] DS, dermatan sulphate; HA, hyaluronan; and HP, heparin.
[b] n.d., not determined.

that the large enhancement of anti-factor IIa activity, observed for fully *O*-sulphonated GAGs, is an HCII mediated activity.

The anticoagulant activity might be further increased by refinement of the chemical modification procedure particularly through the *N*-de-acetylation-re-*N*-sulphonation [34] of fully *O*-sulphonated GAGs. Such an approach might represent a new route for the generation of heparin-like compounds with a variety of pharmacological relevant biological activities.

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