Biochemical Pharmacology, Vol. 42, No. 8, pp. 1609–1619, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

STRUCTURAL FEATURES OF DERMATAN SULFATES AND THEIR RELATIONSHIP TO ANTICOAGULANT AND ANTITHROMBOTIC ACTIVITIES

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(*Received* 11 *April* 1991; *accepted* 24 *June* 1991)

Abstract—Dermatan sulfate is a polydisperse, microheterogeneous sufated copolymer of *N*-acetyl-D-galactopyranose and idopyranosyluronic acid that is currently under clinical investigation as a new antithrombotic agent. The structure and activity of two pairs of dermatan sulfates, isolated from bovine and porcine mucosa, were studied. One dermatan sulfate from each species demonstrated high *in vivo* antithrombotic activity in the rat vena cava assay. The *in vitro* anticoagulant activity of each dermatan sulfate was determined using activated partial thomboplastin time (APTT), thrombin time (TT) (5 units), calcium thrombin time (CaTT) (5 units), Heptest®, anti-factor Xa and anti-factor IIa antithrombin assays and heparin cofactor II amidolytic assays. The coagulation-based assays gave the best correlation to *in vivo* antithrombotic activity. The physical and chemical properties of each dermatan sulfate were determined using ^{1}H-NMR and ^{13}C-NMR spectroscopy, molecular weight determination, potentiometric titration, chemical degradative analysis, chondroitin lyase degradative analysis and oligosaccharide mapping. These analyses indicated that the major difference between dermatan sulfates from a particular species having high and low *in vivo* antithrombotic activity was their iduronic acid content. The relation between increased iduronic acid content and increased *in vivo* antithrombotic activity may be the result of the conformational flexibility of this residue.

Dermatan sulfate is a polydisperse, microheterogeneous sulfated copolymer of *N*-acetyl-D-galactopyranose (*N*-acetylgalactosamine, D-GalpNAc) and primarily L-idopyranosyluronic acid (iduronic acid, L-IdoAp) that is currently under clinical investigation as a new antithrombotic agent [1]. Dermatan sulfate is found in a wide variety of animal tissues [2] but is prepared commercially from either porcine or bovine mucosa.

The major repeating unit in dermatan sulfate is →3)-β-D-GalpNAc4S(1→4)-α-L-IdoAp(1→ where Ac and S are acetate and sulfate. The amount of →3)-β-D-GalpNAc4S(1→4)-α-L-IdoAp2S(1→ (Fig. 1) in dermatan sulfates of different origins also varies. Other differences in the primary structure of dermatan sulfate include sulfation at the 6-position in *N*-acetyl-D-galactopyranose and the presence of D-glucopyranosyluronic acid (D-GlcAp is the C-5 epimer of L-IdoAp). This sequence variability leads to substantial microheterogeneity in the dermatan sulfate polymer and may play a role in its biological activities.

Heparin, also a glycosaminoglycan, is the most widely used clinical anticoagulant [3, 4]. It blocks the coagulation cascade by inhibiting many of the serine proteases including factor Xa and factor IIa (thrombin). It does this primarily by potentiating the activity of serine protease inhibitors such as antithrombin III [5] and heparin cofactor II [6]. In addition to heparin's anticoagulant activity, measured by its *in vitro* or *ex vivo* plasmatic anticoagulant effects, heparin is also an antithrombotic agent as measured by its *in vivo* blocking of thrombus formation. The antithrombotic effect of heparin also includes its action on platelets and on the endothelium [7].

Although heparin is a major clinical anticoagulant/antithrombotic agent [3, 4], it exhibits significant side-effects, primarily hemorrhagic complications [8], making it the drug cited as the most responsible for death in otherwise healthy patients [9]. Low molecular weight heparins, which act through antithrombin III early in the coagulation cascade, have been introduced in Europe in an effort to circumvent these problems and increase the therapeutic index of heparin [10]. The promise of these drugs has not been fully realized. In clinical studies it appears that their only advantage may be a prolonged half-life and improved bioavailability when administered subcutaneously [10].

Dermatan sulfate is a glycosaminoglycan that is structurally similar to heparin. Unlike heparin, it does not act through antithrombin III on factor IIa and factor Xa. Dermatan sulfate is believed to act primarily on factor IIa through a second serine protease inhibitor, heparin cofactor II [6, 11]. Because of this selectivity, dermatan sulfate has only a weak anticoagulant effect. Interest in dermatan sulfate has centered on its high *in vivo* antithrombotic activity and negligible hemorrhagic complications [12]. These data suggest that dermatan sulfate may possess a higher therapeutic index than heparin, similar to the low molecular weight heparins.

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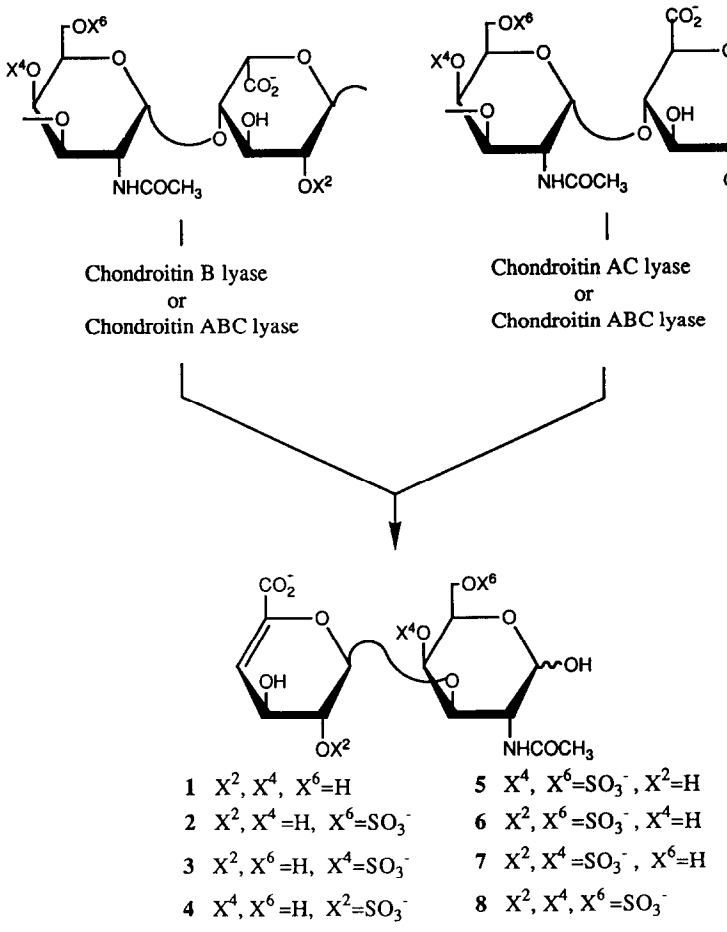

Fig. 1. Treatment of dermatan with chondroitin sulfate lyases. The linkage to L-IdoAp is cut by chondrointin sulfate B and ABC lyases, whereas the linkage to D-GlcAp is cut by chondroitin sulfate AC and ABC lyases. The disaccharide products 1–8 may be formed using any of these lyases.

This paper reports detailed structure and activity studies on two pairs of dermatan sulfates derived from the same tissue that have different *in vivo* antithrombotic activities. Also similarly characterized are a standard dermatan sulfate and two dermatan sulfates currently in clinical trials in Europe as antithrombotic agents.

EXPERIMENTAL PROCEDURES

Materials

Dermatan sulfates DS435, DSOP437 (DSBL), DS7-8HF (DSBH), DS572-2 (DSPH), and DS572-2C (DSPL) were from Opocrin, Corlo, Italy. Dermatan sulfate MF701 was from Mediolanum, Milan, Italy. NIH standard dermatan sulfate was prepared by Mathews and Cifonelli of the University of Chicago from porcine intestinal mucosa [13]. Chondroitin ABC lyase (EC 4.2.2.4, from *Proteus vulgaris*, contains <0.1% and <0.01% chondroitin-4- and 6-sulfatase activity, respectively), chondroitin AC lyase (EC 4.2.2.5, from *Arthrobacter aurescens*), chondroitin B lyase (EC 4.2.2.-, from *Flavobacterium heparinum*) and disaccharide standards (C-kit and D-kit) were from Seikagaku America, Inc., Rockville, MD. Antithrombin III was obtained from KabiVitrum, Molndal, Sweden. Heparin cofactor II was from Diagnostica Stago, Asnieres, France. Human thrombin was from Ortho Diagnostics, Raritan, NJ, and factor Xa from Enzyme Research Laboratories South Bend, IN; chromogenic substrates were from American Diagnostics, Greenwich, CT. All reagents used in electrophoresis were from the Fisher Chemical Co., Fairlawn, NJ. *N,N*-Dimethylformamide was from Merck, Milan, Italy.

A Hoefer (San Francisco, CA) SE600 vertical-slab-gel unit equipped with a Bio-Rad (Richmond, CA) model 1420B power source was used for electrophoresis. Desalting was done with Spectropore dialysis tubing (mol. wt cut-off 1000) from Spectrum Medical, Los Angeles, CA, or with a P-2 gel desalting column from Bio-Rad. For gel permeation HPLC, we used 4.6 × 250 mm Protein Pak 125 and 300 columns (Waters, Milford, MA) in series or a 4.6 × 600 mm TSK G4000 SW gel column from Toso Haas (Lab Service, Bologna, Italy). A Waters 840 liquid chromatography system was used that was equipped with a model 490 multiwavelength UV

detector and a Digital 300 series minicomputer running Waters 840 software specifically designed to determine the molecular weight of polymers. For strong anion exchange high pressure liquid chromatography (SAX-HPLC), we used a Spherisorb analytical column, 4.6 mm × 25 cm, a Spherisorb semipreparative column, 2 × 25 cm, both of 5 μm particle size (Phase Separations, Norwalk, CT), dual LC7A face programmable pumps (Shimadzu Tokyo, Japan) and a variable-wavelength UV detector (LKB, Piscataway, NJ). ^{1}H-NMR spectroscopy was performed at 360 MHz in ^2H_2O (99.996 atom%) at 75° with 3-(trimethylsilyl)propionic-2,2,3-d_4 acid, sodium salt (99 + atom%) as the internal standard (Aldrich Chemical Co., Milwaukee, WI) on a Bruker WM 360 spectrometer equipped with an ASPECT 2000 computer. ^{13}C-NMR was performed at 10–15% (w/v) in ^2H_2O on a Bruker AC300 or CXP300 at 75.5 MHz. The chemical shifts were measured with reference to internal methanol at 51.75 ppm with respect to external tetramethylsilane (Janssen Pharmaceutica, Beerse, Belgium).

Potentiometric titrations were performed on a Titroprocessor automatic potentiometer E636 equipped with an automatic pipette Dosimat E635 (Methrom, Herisau, Switzerland) with a combination monotubular calomel electrode. The anion exchange resin used was amberlite IRA 400 (Rohm & Haas, Milan, Italy) and the cation exchange resin was Relite CF (Sybron Resindion, Milan, Italy).

Methods

Preparation of dermatan sulfates for analysis. Stock solutions of dermatan sulfates were prepared by dissolving bulk drug at 20 mg/mL in distilled water.

Determination of the molecular weight of dermatan sulfates. Prior to their use, each gel permeation column was calibrated with a collection of anionic polymers serving as molecular weight standards [14]. Dermatan sulfate (20 μL, 10 mg/mL) was injected onto the column and eluted with 125 mM sodium sulfate containing 2 mM monosodium phosphate at pH 6.0 at a flow rate of 0.9 mL/min; the eluant was monitored at 205 nm. Values for retention time (t_R) and polymer dispersity (P) were determined and used along with the known molecular weights of the standards to calculate a calibration curve by the third polynomial regression with coefficients D_0, D_1, D_2, and D_3. Molecular weight was calculated using Equation 1:

$$\log \text{mol. wt} = D_0 + D_1(t_R) + D_2(t_R)^2 + D_3(t_R)^3. \quad (1)$$

The total area under the elution curve was determined by integration.

Determination of sulfur, uronic acid content and sulfate/carboxylate ratio. Two chromatographic columns containing amberlite IRA-400 (OH^-, regenerated with 2 N sodium hydroxide) 1 × 10 cm and Relite CF (H^+, regenerated with 2 N hydrochloric acid) 1 × 20 cm were prepared. These columns were washed exhaustively and equilibrated with distilled water (at pH 7.0). The anion exchange column was used to remove contaminating anions (i.e. free sulfate). Dermatan sulfate (200 mg in 2 mL) was then added to the Relite CF column and collected in a distilled, deionized water wash (50 mL). To 10 mL of eluate, 5 mL of *N,N*-dimethylformamide was added and this sample was titrated with 0.1 N sodium hydroxide to obtain two curves, the first corresponding to neutralization of sulfate groups and the second corresponding to the neutralization of carboxyl groups. The percent sulfur is calculated by Equation 2,

$$\%S = \frac{V_1 \times C \times Pa}{Pc} \times 100 \quad (2)$$

where V_1 is the volume of sodium hydroxide used by the titrator to determine the first curve, C is the normality of the sodium hydroxide, Pa is the atomic weight of S (32.06) and Pc is the mg of sample titrated. The percent of uronic acid is calculated by Equation 3,

$$\%UA = \frac{V_2 \times C \times Pm}{Pc} \times 100 \quad (3)$$

where V_2 is the volume of sodium hydroxide used by the titrator to determine the second curve, C is the normality of the sodium hydroxide, Pm is the molecular weight of uronic acid (194.4) and Pc is the mg of sample titrated. The SO_3^-/CO_2^- ratio is obtained from: $(\%S/Pa)/(\%UA/Pm)$.

NMR analysis of dermatan sulfates. The dermatan sulfate samples were exchanged with ^2H_2O before being made up at approximately 1.5–5 wt% and 10–15 wt% for ^{1}H- and ^{13}C-NMR, respectively. The ^{1}H-NMR spectrum of each dermatan sulfate showed the following signals: δ 4.90 (brS, IdoAp 1), δ 4.74 (brS, IdoAp 5), δ 4.66 (brS, GalpNAc-4S 1,4), δ 4.11 (brS, IdoAp 4), δ 4.02 (brS, GalpNAc-4S 2,3), δ 3.91 (brS, IdoAp 3 and GalpNAc-4S 5), δ 3.80 (brS, GalpNAc-4S 6,6′), δ 3.53 (brS, IdoAp 2), δ 2.07 (S, GalpNAc N-CH_3). The ^{13}C-NMR spectrum of each dermatan sulfate showed signals: at δ 177.05 (CO-CH_3), δ 175.79 (CO IdoAp 6), δ 104.52 (IdoAp 1), δ 103.52 (GalpNAc 1), δ 81.69 (IdoAp 4), δ 77.44 (GalpNAc 4), δ 76.87 (GalpNAc 3), δ 76.02 (GalpNAc 5), δ 72.72 (IdoAp 3), δ 70.98 (IdoAp 2,5), δ 62.48 (GalpNAc 6), δ 54.45 (GalpNAc 2), δ 24.05 (CO-CH_3).

Oligosaccharide mapping of dermatan sulfates. Dermatan sulfate (50 μL of 20 mg/mL) was added to 330 μL of 5 mM sodium phosphate and 200 mM sodium chloride buffer, pH 7; chondroitin ABC, AC or B lyase (20 μL, 20 mI.U.) was added to make the total solution volume 400 μL. The reactions were run at 30° for chondroitin B lyase and 37° for chrondroitin AC and ABC lyases to completion in 24 hr and terminated by heating to 100° for 1 min. Depolymerized dermatan sulfate was analyzed by SAX-HPLC by injecting 1.5 μL onto the Spherisorb column equilibrated with 200 mM sodium chloride at pH 3.5. The sample was eluted with the use of a linear salt gradient from 0.05 M to 0.2 M over 1 hr at a flow rate of 1.5 mL/min. The elution profile was monitored at 232 nm, 0.02 absorbance units full scale (AUFS). The column was washed between analyses with 2 M sodium chloride to remove the higher oligosaccharides still bound. Peaks were tentatively identified by their coelution with an authentic sample. Depolymerized dermatan sulfate was analyzed with use of gradient polyacrylamide gel

electrophoresis (PAGE) by adding sample (5 μL of 20 mg/mL) to a stacking gel of 5% (total acrylamide) with a 12–22% linear gradient resolving gel [15]. After 400 V was applied for 16 hr (running a bromophenol blue marker 20 cm into the resolving gel), the gel was fixed and stained in alcian blue and destained [15, 16].

Assays of the anticoagulant and antithrombotic activities of dermatan sulfate. Activated partial thromboplastin time (APTT) was measured in normal human plasma using micronized silica (Organon Technica, Durham, NC) as activator. The routine clotting assays, prothrombin time (PT), APTT, thrombin time (TT) (5 units) and Heptest®, were performed according to manufacturers' specifications. Thromboplastin (Dade, Miami, FL) was used for PT, APTT reagent (Organon Teknika, Morris Plains, NJ) was used for APTT, human thrombin was used for TT (5 units), and the Heptest® kit (Heamachem, St. Louis, MO) was used for the Heptest® assay. Calcium TT (5 units) was measured by reconstituting thrombin with 25 mM calcium chloride (in place of sodium chloride) and determining thrombin time. The anti-factor IIa and anti-factor Xa assays were performed as previously described [17, 18]. Heparin cofactor II mediated anti-factor IIa activity was determined by an amidolytic assay as previously described [15, 19] or using a Stachrom kit [20]. The amidolytic assay data are presented as IC_{50} or the concentration required to inhibit thrombin activity towards chromogenic substrate by 50% as compared to control [15]. Heparin cofactor II activity, determined by a Stachrom kit, is reported as units/mg calculated based on a standard curve using the 4th International WHO heparin standard having 193.4 I.U./mg. The curves obtained were parallel.

In vivo antithrombotic activity was evaluated by the stasis thrombosis model of vena cava ligature in ten rats at each of three different dose levels of dermatan sulfate as previously described [21].

RESULTS

The dermatan sulfates studied included: (1) two experimental samples prepared from beef mucosa according to the method of Rodén *et al.* [22] having high and low *in vivo* antithrombotic activity, DSBH (Dermatan Sulfate, Bovine, High activity) and DSBL; (2) two experimental samples prepared from porcine intestinal mucosa [22]: DSPH (Dermatan Sulfate, Porcine, High activity) having high *in vivo* antithrombotic activity was further fractionated by anion exchange chromatography and the fraction eluting with low salt having low antithrombotic activity was called DSPL; (3) two drug products currently in clinical trials as antithrombotic agents, DS435 (Opocrin) derived from bovine intestinal mucosa and MF701 (Mediolanum) derived from porcine intestinal mucosa; and (4) standard dermatan sulfate (NIH-STD) prepared from porcine mucosa under NIH contract [13].

The molecular weight (mol. wt) and polymer dispersity (P) of the dermatan sulfates determined by gel permeation chromatography ranged from 29,000 to 51,500 and 1.30 to 1.46, respectively (Table

Table 1. Physical, chemical, and enzymatic characterization of dermatan sulfates

Sample	Mol.wt*	P	Opt. rot.†	%S‡	%UA‡	SO_3^-/CO_2^-‡	Sulfuric acid ash§	ABC lyase‖	B lyase‖	AC lyase‖	%IdoAp¶	%IdoAp**
DSBH	51.5	1.46	−60°	5.6	30.6	1.11	23.2	2.09	0.95	0.09	87	91
DSBL	29.0	1.30	−58°	4.9	28.8	1.03	24.2	1.64	0.58	0.11	84	84
DSPH	35.1	1.35	−58°	5.7	31.1	1.11	24.7	2.85	1.37	0.12	93	92
DSPL	33.9	1.36	−66°	5.7	33.5	1.03	23.1	2.29	0.87	0.17	81	84

* Molecular weight (kDa) determination ± 2.
† Degrees at Na_D line in water at 22° and at 30 mg/mL.
‡ Determined by potentiometric titration. Ratio of equivalents of sulfate to carboxylate, indicating the average number of sulfate groups per disaccharide repeat [23].
§ Sulfuric acid ash was determined by the USP method [24]. During the precipitation of the dermatan sulfates, the pH was maintained between 5 and 6.
‖ Exhaustive treatment with chondroitin lyase affords the μmol product/mg of substrate as measured from absorbance at 232 nm using $\varepsilon_M = 5000$ for each disaccharide eluted from the SAX-HPLC column [25].
¶ Determined by ^{1}H-NMR by dividing the intensity of the peak at δ 4.91 by one-third the intensity of the peak at δ 2.08 and multiplying by 100.
** Determined from B lyase absorbance divided by (B lyase + AC lyase) absorbance.

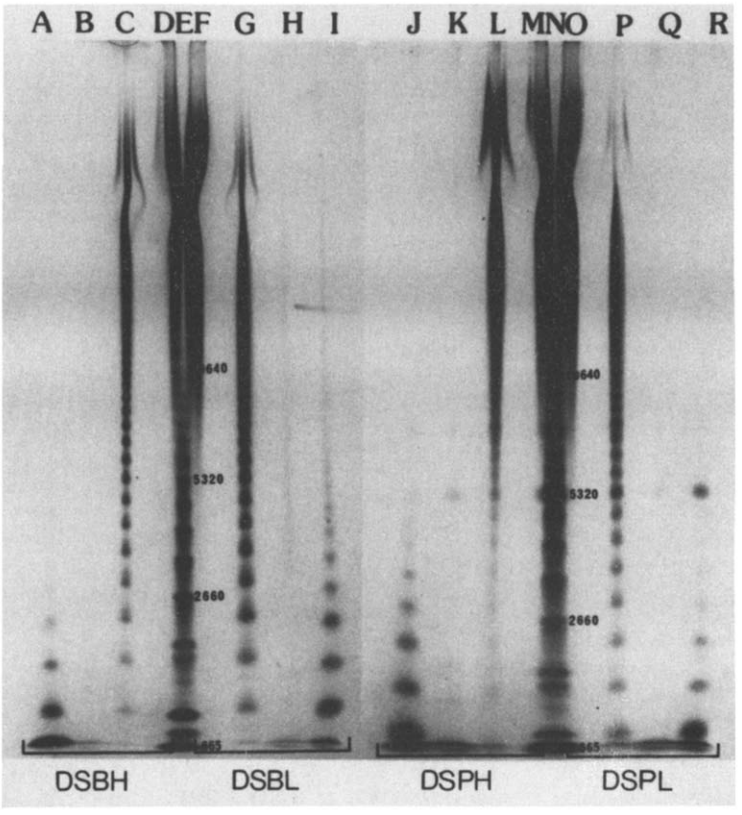

Fig. 2. Gradient PAGE analysis of 100-μg samples of DSBH, DSBL, DSPH and DSPL. DSBH was analyzed in lanes A–D: (A) DSBH treated with chondroitin B lyase; (B) DSBH treated with chondroitin ABC lyase; (C) DSBH treated with chondroitin AC lyase; and (D) untreated DSBH. Lane E shows bovine lung heparin partially depolymerized by heparinase. The molecular weights of the major bands are labeled. DSBL was analyzed in lanes F–I; (F) untreated DSBL; (G) DSBL treated with chondroitin AC lyase; (H) DSBL treated with chondroitin ABC lyase; and (I) DSBL treated with chondroitin B lyase. DSPH was analyzed in lanes J–M: (J) DSPH treated with chondroitin B lyase; (K) DSPH treated with chondroitin ABC lyase; (L) DSPH treated with chondroitin AC lyase; and (M) untreated DSPH. Lane N shows molecular weight standards. DSPL was analyzed in lanes O–R: (O) untreated DSPL; (P) DSPL treated with chondroitin AC lyase; (Q) DSPL treated with chondroitin ABC lyase; and (R) DSPL treated with chondroitin B lyase. All staining was performed using alcian blue.

1). The dermatan sulfates with high and low antithrombotic activity prepared from porcine mucosa had nearly identical molecular weights. Analysis of intact dermatan sulfates DSPH and DSPL, using gradient PAGE, qualitatively confirmed their similar molecular weight profiles (Fig. 2, lanes M and O). The other intact dermatan sulfates showed some differences in molecular weight profiles (Fig. 2, lanes D and F; and Fig. 3, lanes D, I and K).

The ^{1}H-NMR and ^{13}C-NMR spectra of each dermatan sulfate were obtained. Each sample showed an identical number of signals at identical positions; however, the peak intensities varied. From the ^{1}H-NMR spectra of each dermatan sulfate the iduronic acid content as a percentage of total uronic acid was determined (Table 1). The ^{13}C-NMR spectra also confirmed the presence of iduronic acid as a major component (at δ 104.52 ppm) and glucuronic acid as a minor component (at δ 105.8 ppm) in each of the dermatan sulfates. The optical rotation, %S, %UA, SO_3^-/CO_2^-, ash and %IdoAp (by ^{1}H-NMR) were also determined for each dermatan sulfate (Table 1).

Quantitative oligosaccharide maps were prepared for the bovine and porcine dermatan sulfates. Each dermatan sulfate was first depolymerized using three specific chondroitin lyases. SAX-HPLC oligosaccharide maps [26, 27] of DSBL treated with chondroitin ABC, B and AC lyases are shown in Fig. 4. The oligosaccharide maps of the dermatan sulfates DSBH, DSPH, and DSPL were qualitatively similar and the quantitative data derived from these maps are given in Table 2. Each peak was identified by co-injection with disaccharide standards [27]. As is evident from Figs. 2–4 and Table 2, dermatan sulfate was very susceptible to both chondroitin ABC and B lyases but considerably less susceptible to chondroitin AC lyase. From these data the %IdoAp in each dermatan sulfate was calculated (Table 1).

Qualitative oligosaccharide mapping of the chondroitin lyase depolymerized dermatan sulfates was also performed using gradient PAGE [16, 26–

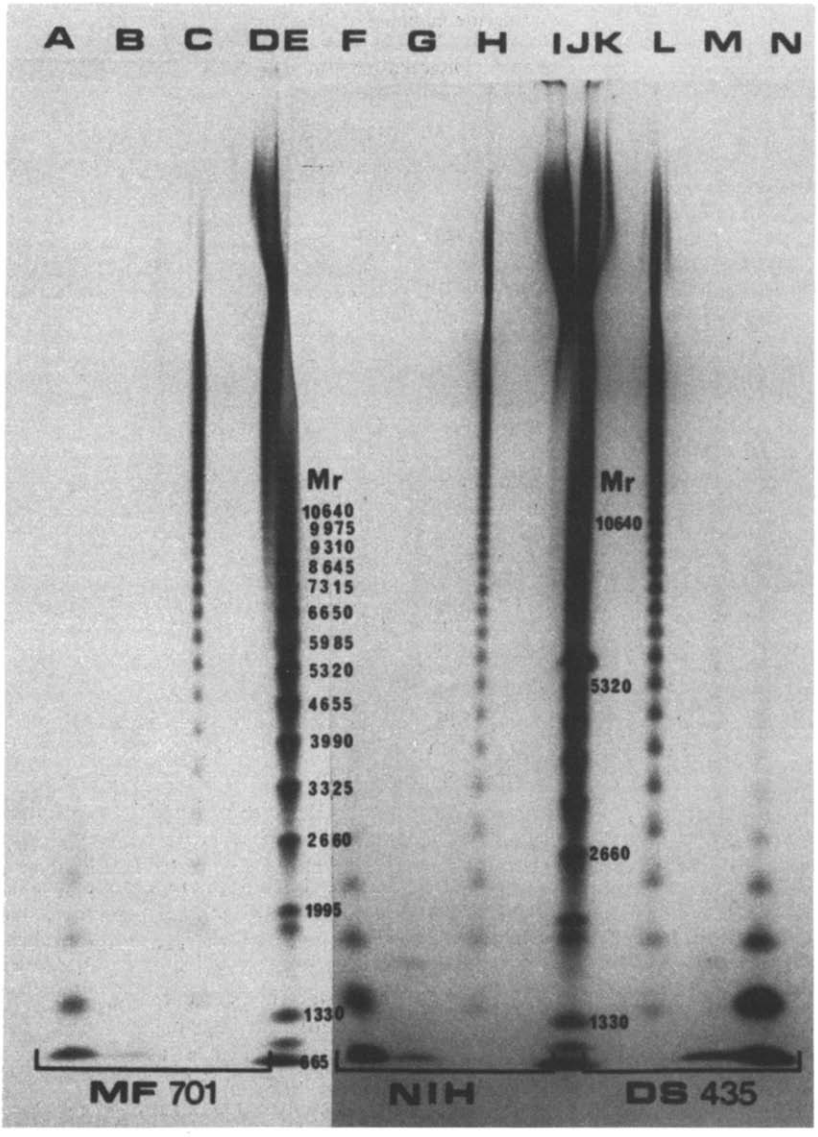

Fig. 3. Gradient PAGE analysis of MF701, NIH standard and DS435. MF701 was analyzed in the first four lanes labeled A–D. Lanes A–D contained 50 μg of: (A) MF701 treated with chondroitin B lyase; (B) MF701 treated with chondroitin ABC lyase; (C) MF701 treated with chondroitin AC lyase; and (D) untreated MF701. Lane E shows bovine lung heparin partially depolymerized by heparinase. The molecular weights of the major bands in the ladder are labeled. NIH reference standard was analyzed in lanes F–I. Lanes F–I contained 100 μg of: (F) NIH treated with chondroitin B lyase; (G) NIH treated with chondroitin ABC lyase; (H) NIH treated with chondroitin AC lyase; and (I) untreated NIH. Lane J again shows the partially depolymerized bovine lung as molecular weight standards. DS435 was analyzed in lanes K–N. Lanes K–N contained 100 μg of: (K) untreated DS435; (L) DS435 treated with chondroitin AC lyase; (M) DS435 treated with chondroitin ABC lyase; and (N) DS435 treated with chondroitin B lyase. All staining was performed using alcian blue.

29] and is shown in Figs. 2 and 3. Gradient PAGE oligosaccharide mapping offers information on higher oligosaccharide products that is difficult to obtain using SAX-HPLC [16, 27]. Heparin that was partially depolymerized using heparinase provided a set of internal standards in the form of a ladder of bands of defined molecular weight [16, 26–29].

The *in vitro* anticoagulant activities of these dermatan sulfate samples were determined using a battery of clotting and amidolytic assays (Fig. 5, A–G). Heparin cofactor II mediated antithrombin activities are presented as both IC_{50} [15, 19] and units/mg [20] (Table 3).

The *in vivo* antithrombotic activity of each dermatan sulfate was determined using the rat vena cava ligature stasis thrombosis model [21]. Both DSBH and DSPH showed high antithrombotic activities having ED_{50} (i.v.) values of 0.75 ± 0.47 and

Table 2. Quantitative oligosaccharide mapping of dermatan sulfates by SAX-HPLC

Dermatan sulfate	Enzyme	Disaccharide* (mol%) 1	2	3	4	5	6	7	8	% of Total peak area corresponding to 1–8†
DSBH	ABC	—	2.83	58.7	0.94	2.55	3.30	4.62	—	72.9
	B	—	—	26.5	—	—	20.8	1.85	—	49.2
	AC	—	—	0.448	0.103	—	3.32	—	—	3.87
DSBL	ABC	—	2.13	78.9	1.73	6.77	—	7.67	—	97.2
	B	—	—	28.2	—	—	25.1	4.46	—	57.8
	AC	—	—	0.322	0.640	—	—	—	—	0.962
DSPH	ABC	—	4.71	61.0	—	7.40	4.92	3.13	—	81.2
	B	—	—	56.4	—	—	5.16	3.08	—	64.6
	AC	—	—	1.51	2.55	—	—	1.74	—	5.8
DSPL	ABC	—	3.78	53.9	—	—	4.83	2.56	—	65.1
	B	—	—	49.3	—	—	5.54	2.44	—	57.3
	AC	2.92	—	—	2.45	—	—	—	—	5.37

* See Fig. 1 for structures of 1–8. The percentage of the total peak area corresponding to each disaccharide peak represents the mol% assuming identical ε_M values. The dashes indicate that no disaccharide was detected. The retention times of 1–8 were: 8.8 ± 0.9, 20.8 ± 0.5, 22.7 ± 0.6, 23.3 ± 0.3, 37.4 ± 0.6, 41.2 ± 1, 42.8 ± 1, and >60 min, respectively.

† Percentage of the total peak area in each chromatogram that was assignable to disaccharides 1–8. One hundred minus this number would represent the mol% of unidentified components corresponding to tetrasaccharides and higher oligosaccharides.

0.75 ± 0.39 mg/kg, respectively. DSBL and DSPL had 3- to 5-fold lower antithrombotic activities with ED_{50} (i.v.) values of 3.48 ± 0.61 and 2.15 ± 0.43 mg/kg, respectively.

DISCUSSION

Although heparin is widely used clinically as an anticoagulant, hemorrhagic complications associated with its use have spurred the search for substitutes [3, 4]. Low molecular weight heparins, originally thought to be a major improvement, have not yet lived up to expectations [30]. Despite lengthened *in vivo* half-life [10] and greater bioavailability [10] (when administered s.c.), clinical studies have been unable to clearly demonstrate a decreased effect on platelets and a decrease in hemorrhagic complications associated with the use of these agents [30].

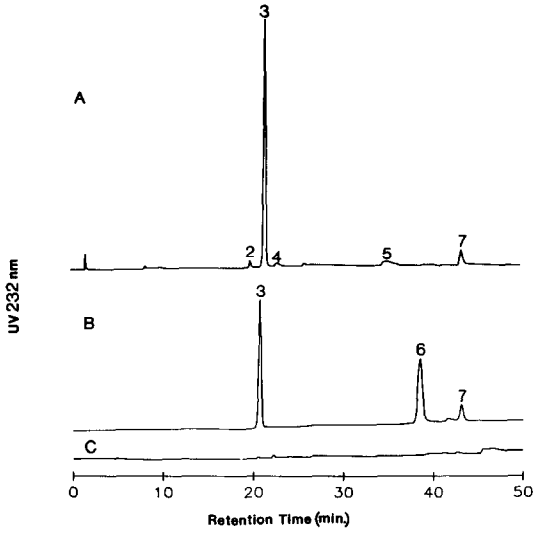

Fig. 4. SAX-HPLC chromatograms of chondroitin lyase-treated DSBL. (A) Products formed using chondroitin ABC lyase; (B) products formed using chondroitin B lyase; and (C) products formed using chondroitin AC lyase. SAX-HPLC analysis was performed without prior size fractionation by gel permeation chromatography. See Fig. 1 for the structure of the disaccharide corresponding to each peak.

Both clinical and *in vivo* and *in vitro* research data suggested that dermatan sulfate might represent an alternative to heparin as an antithrombotic agent [1]. Dermatan sulfate is a major component of a heparinoid drug, ORG 10172, that has performed remarkably well as an antithrombotic agent in clinical studies [31, 32]. Dermatan sulfate has demonstrated high *in vivo* antithrombotic potency in animal studies with very little anticoagulant effect in plasma, suggesting that this drug may demonstrate the successful elimination of anticoagulant activity from the antithrombotic effect [1, 12]. Tollefsen and coworkers [6, 11] provided a biochemical rationale for studying dermatan sulfate when they demonstrated that it could potentiate the HCII-mediated inhibition of thrombin.

As part of an earlier study on dermatan sulfate, we applied oligosaccharide mapping techniques [15] using chondroitin ABC lyase on a variety of commercially prepared dermatan sulfates as well as on fractionated dermatan sulfates [27]. These fractionated porcine mucosal dermatan sulfates demonstrated up to a 200-fold difference in HCII-mediated anti-factor IIa activities. We found that the dermatan sulfate fraction with the highest HCII-mediated activity also gave the most ΔUA(1→3)-β-D-GalpNAc4S6S product (**4**) on treatment with chondroitin ABC lyase. This corresponded to an enrichment of →4)-α-L-IdoAp (or β-D-GlcAp)(1→3)-β-D-GalpNAc4S6S(1→ sequences in dermatan sulfate

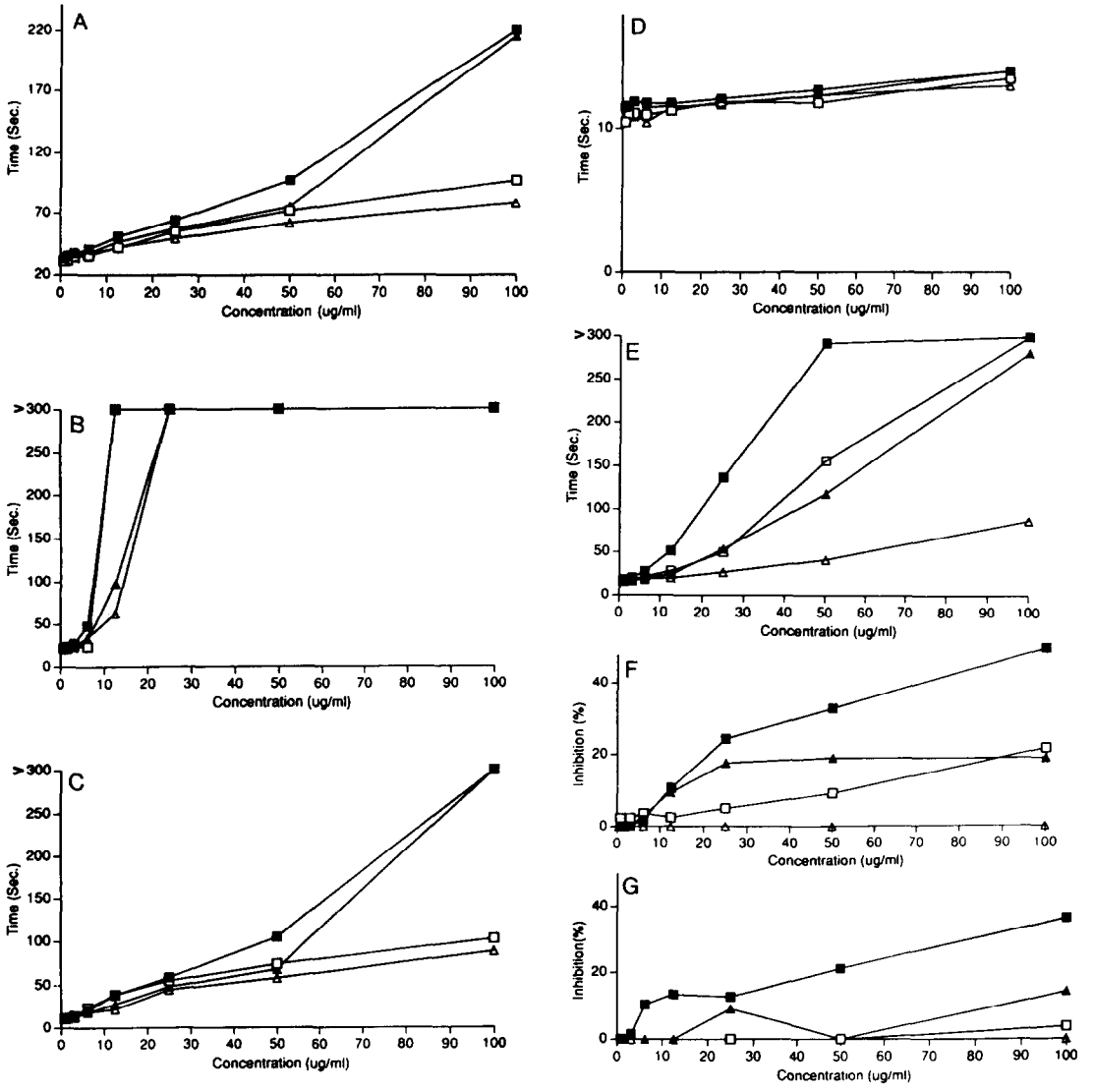

Fig. 5. *In vitro* analysis of dermatan sulfates DSBH, DSBL, DSPH and DSPL. Assays were: (A) APTT, (B) TT (5 units), (C) CaTT (5 units), (D) PT, (E) Heptest®, (F) anti-factor IIa, and (G) anti-factor Xa. Plotted are: DSBH (■), DSBL (□), DSPH (▲), and DSPL (△).

Table 3. *In vivo* heparin cofactor II mediated anti-factor IIa activity of dermatan sulfates

	HCII activity* (IC_{50})	HCII activity† (units/mg)
DSBH	11.5	205.7 ± 11.1
DSBL	6.7	216.8 ± 11.7
DSPH	10	258.6 ± 14.4
DSPL	8.8	238.9 ± 12.4

* HCII-mediated inhibition of factor IIa was measured by amidolytic assay (Chromozym TH®) in duplicate at three concentrations ranging from 5 to 15 μg/mL. The change in absorbance at 405 nm was compared to that obtained with no added dermatan sulfate ($\Delta A_{405} = 0.47$) and the percent inhibition of thrombin calculated. The percent inhibition was plotted against concentration to obtain a linear curve from which the IC_{50} was determined.

† HCII data obtained using a Stachrom kit.

fractions having high HCII-mediated anti-factor IIa activity. It was unclear from this earlier study, however, whether or not the enrichment of this sequence was directly responsible for the increased activity. Recently, Maimone and Tollefsen [33] implicated a second disulfated disaccharide sequence, →4)α-L-IdoAp2S(1 → 3)-β-D-GalpNAc4S(1→, in the interaction of dermatan sulfate and heparin cofactor II.

The current study began with the observation that two pairs of dermatan sulfates (DSBH & DSBL and DSPH & DSPL), which showed only minor differences in their *in vitro* HCII-mediated anti-factor IIa activities (Table 3), had different *in vivo* antithrombotic activity. These data suggested that the *in vitro* HCII assay may not be the best predictor of *in vivo* antithrombotic activity. Thus, we decided to examine the activity of these experimental dermatan sulfates using additional *in vitro* biological assays.

Table 4. *In vitro* activities of commercial and standard dermatan sulfates*

	OP 435	MF701	NIH-STD
APTT at 50 μg/mL†	84.3	55.9	107.7
TT (5 units) at 12 μg/mL†	79.5	26.9	163.5
PT at 50 μg/mL†	12.8	12.4	12.8
Heptest® at 25 μg/mL†	141.7	28.0	>300.0
Anti-factor Xa at 100 μg/mL‡	16	13	42
Anti-thrombin at 100 μg/mL‡	19	0	59
HCII-mediated activity§	<6	8.4	<6

* See Methods for details on assay procedures.
† In seconds.
‡ Percent inhibition.
§ IC_{50} in micrograms per milliliter.

Dermatan sulfates DSBH and DSPH, having the highest *in vivo* antithrombotic activity, also showed the greatest *in vitro* activity by APTT, TT (5 units) and CaTT (5 units) (Fig. 5). The NIH standard dermatan sulfate also showed high APTT activity while the commercial dermatan sulfates and the dermatan sulfates with low *in vivo* antithrombotic activity (DSBL and DSPL) also showed low APTT and CaTT (5 units) activities (Fig. 5 and Table 4). None of the dermatan sulfate samples tested exhibited significant PT activity. Heptest® also demonstrated surprisingly high activity for the NIH standard dermatan sulfate (Table 4). The Heptest® results corresponded to the *in vivo* data for the DSBH & DSBL and DSPH & DSPL pairs. However, using this assay the porcine mucosal dermatan sulfates (DSPH and DSPL) had less activity than did the bovine mucosal dermatan sulfates (DSBH and DSBL), despite the fact that the porcine and bovine dermatan sulfates had comparable *in vivo* antithrombotic activities. The amidolytic assays (Fig. 5) were also able to distinguish between the dermatan sulfates with high and low *in vivo* activity, and again the bovine dermatan sulfates (DSBH and DSBL) showed the greatest activity. Finally, the heparin cofactor II-mediated anti-factor IIa activity of the dermatan sulfates was examined by amidolytic assay and the data were treated in two ways. When HCII activity was expressed as IC_{50}, the samples with the highest *in vitro* activity were those with the lowest *in vivo* antithrombotic activity. The use of the more conventional units/mg expression for HCII-mediated activity gave no significant differences between each pair of samples (i.e. DSBH & DSBL and DSPH & DSPL). These *in vitro* activity data gave few clues as to the origin of the *in vivo* antithrombotic activity and suggested that it was also necessary to examine the chemical structure and physical properties of these dermatan sulfates.

The properties of the intact polymer were first examined to identify structural features that were important for their high *in vivo* antithrombotic activities (Table 1). The molecular weights of the dermatan sulfates DSBL, DSPH and DSPL were very similar and comparable to the standard and commercial dermatan sulfates (Table 1, Figs. 2 and 3). The optical rotations of DSBH, DSBL, DSPH and DSPL were slightly different, although this difference did not correlate to activity differences. Degradative analysis showed no remarkable differences in sulfuric acid ash, %S and %UA, although the SO_3^-/CO_2^- ratio was slightly higher in DSBH and DSPH, the dermatan sulfates having higher *in vivo* antithrombotic activity. The %IdoAp determined by ^{1}H-NMR was higher in the dermatan sulfates having higher *in vivo* activity (DSBH and DSPH). This result was confirmed by depolymerization studies using specific chondroitin lyases (Tables 1 and 2). Chondroitin ABC lyase acts on dermatan sulfate at either the →3)β-D-GalpNAc4X6X(1→4)α-L-IdoAp2X(1→ or the →3)β-D-GalpNAc4X6X(1→4)-β-D-GlcAp2X(1→ (where X may be sulfate) linkages [34], while chondroitin B lyase is specific for the first of these two linkages and chondroitin AC lyase for the second [34]. Some linkages, which are chondroitin lyase cleavable when contained within the polymeric substrate, may become resistant when contained in smaller oligosaccharides, as reported for heparin lyases [29]. The treatment of dermatan sulfate with chondroitin ABC or B lyases forms primarily disaccharides [35], with an $\varepsilon_M = 5000$ [25]. The dermatan sulfates were treated with each lyase, and the micromoles of product formed per milligram substrate was determined (Table 1). Dermatan sulfates DSBH and DSPH, with the highest antithrombotic activity, were depolymerized to a greater extent by chondroitin B lyase, whereas dermatan sulfates DSBL and DSPL, with lower activity, were depolymerized to a greater extent with chondroitin AC lyase. These data confirmed the results of ^{1}H-NMR spectroscopy and again point to the importance of high iduronic acid content for high *in vivo* antithrombotic activity. It is interesting to note that the porcine dermatan sulfates contain considerably more chondroitin lyase resistant linkages than do the bovine dermatan sulfates.

Oligosaccharide mapping of polysaccharides is comparable to the peptide mapping of proteins [26, 36]. Quantitative oligosaccharide mapping of the dermatan sulfates using SAX-HPLC was unable to pinpoint disaccharides that occurred in greatly elevated levels in dermatan sulfates having high *in vivo* antithrombotic activity. Some clear differences were observed in the disaccharide compositions of the four dermatan sulfates studied (Table 2).

Differences in disaccharide composition could even be observed in a single dermatan sulfate when analyzed using chondroitin ABC lyase and chondroitin B lyase (Table 2 and Fig. 4). These results suggest that specificity differences between these two enzymes may result in the enrichment of certain sequences within lyase-resistant tetrasaccharides and higher oligosaccharide products. The major differences between the disaccharide compositions of the dermatan sulfates, however, were associated with the source (i.e. bovine vs porcine) rather than the activity or the preparation method. (The four dermatan sulfates analyzed (Tables 1 and 2, and Fig. 2) were prepared in the same laboratory using a single method [13].)

Qualitative oligosaccharide mapping by gradient PAGE (Figs. 2 and 3) provides an added dimension to the analysis of these dermatan sulfates as the dominant feature observed by this technique is the larger oligosaccharides formed on enzymatic depolymerization. Although these larger oligosaccharides represent the minor sequences present in each dermatan, they stain with greater intensity [28] and are well resolved from the gel front that contains all the disaccharide components. PAGE analysis again confirmed that DSBH and DSPH are highly susceptible towards chondointin B lyase (in Fig. 2 lanes A and J show less high molecular weight products than lanes I and R) and have reduced susceptibility towards chondrointin AC lyase (lanes C and M show more high molecular weight products than lanes G and P). The large number of high molecular weight products, their low concentration and their high affinity for the column prevent their detection by SAX-HPLC. The enzymatic susceptibility results correspond well to iduronic acid content (Tables 1 and 2). Silver staining of these gels [27], carried out after chrondroitin lyase treatment, showed that no heparin was present in any of the dermatan sulfates studied.

The presence of iduronic acid in heparin has been suggested to be important to its anticoagulant activity because it permits greater conformational flexibility of the polymer [37]. Similarly, the content of iduronic acid is also associated with the ability of glycosaminoglycans to self-aggregate [38]. The discovery that dermatan sulfates from two different species with elevated *in vivo* antithrombotic activity also have high iduronic acid content suggests that this flexible sugar residue may also play a role in the antithrombotic activity of the dermatan sulfate.

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