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"SUPERSULFATED" HEPARIN FRAGMENTS, A NEW TYPE OF LOW-MOLECULAR WEIGHT HEPARIN

PHYSICO-CHEMICAL AND PHARMACOLOGICAL PROPERTIES

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Abstract—A new type of low-molecular-weight heparin (ss-LMW-H) was prepared (by controlled depolymerization and concurrent sulfation of heparin with a mixture of sulfuric and chlorosulfonic acid), to test the influence of extra-sulfate groups on biological properties of heparin fragments. The fragments had an average molecular weight ranging from 5000 to 10,000, a sulfate-to-carboxyl molar ratio of 2.8–3.1, and electrophoretic mobilities and NMR spectra distinctly different from those of the parent heparins.

Depolymerization with oversulfation reduced the anticoagulant activity of heparin (*ex vivo*, in rats) much more than depolymerization alone, to about 10% of the original APTT and 25–30% of the original a.Xa units. By contrast, the antithrombotic activity (venous stasis model, in rats) was still comparable to that of heparin, and bleeding times were not significantly increased. The lipasemic (lipoprotein-lipase-releasing) activity of ss-LMW-H fragments was more than twice that of heparin. Results are discussed in terms of contribution of charge-density effects to different activities and to different mechanisms for the same activity of heparin.

It is generally accepted that the anticoagulant activity of heparin largely depends on its ability to inhibit thrombin and other activated coagulation factors [1, 2], whereas its antithrombotic activity predominantly involves inhibition of Factor Xa (Xa) [3]. Heparin preparations with low molecular weight (LMW-H), obtained either by fractionation or depolymerization procedures [4], have a lower anticoagulant activity but enhanced anti-Xa activity compared with unfractionated, non-depolymerized heparin (H), and an increased antithrombotic effect in experimental animal models [5]. Indeed, the anticoagulant activity of heparin is mainly associated with its higher molecular weight components [6]. It has also been shown that the degree of sulfation influences most of the biological activities (including the anticoagulant activity) of heparin and heparin-like substances [1]. In order to evaluate the structural factors influencing the biological properties of LMW heparins, we studied the effect of extra sulfate groups on LMW heparin fragments. This paper describes some of the physico-chemical and pharmacological properties of these fragments obtained by a new procedure of depolymerization under conditions of oversulfation.

MATERIALS AND METHODS

Heparins and heparin fractions. Heparins used in this study were pig mucosal preparations, from (A) Proquifin, b.N. 7926; (B) Diosynth, b.N. 57442111; (C) Choay, b.N. 050; (D) Terhormon, b.N. 018; (E) Fournier, b.N. 5094. All heparins had average molecular weight (as determined by gel filtration [7]) in the normal range 14,000–12,000 [7, 8], and showed both "slow-moving" and "fast-moving" components on electrophoresis in barium acetate buffer [8, 9]. A "fast-moving" fraction (H-A_f, MW ~10,000) was obtained by ethanol fractionation [10] of heparin H-A.

"Supersulfated" heparin fragments. Heparins were depolymerized with an appropriate mixture of sulfuric acid and chlorosulfonic acid, under conditions for obtaining structurally homogeneous oversulfated fragments in high yields. In a typical experiment, to 20 ml H_2SO_4 (95%) and 10 ml $HClSO_3$, cooled to −4°, 1 g of heparin (dried at 60° for 24 hr, under vacuum) was added under gentle stirring. The reaction mixture was kept 60 min under stirring at −4°, and a further 60 min at room temperature. The product was precipitated by pouring the reaction mixture into 500 ml cold ethyl ether. The precipitate was filtered, washed with cold ethyl ether, redissolved in H_2O, neutralized with 0.5 N NaOH, and dialyzed through a 3500 D membrane (Thomas 3787-H47) against distilled water. The product was recovered by evaporation (under reduced pressure) of the solution inside the dialysis bag. Yields varied from 85% to 105%.

Determination of degrees of sulfation and molecular weights. The degree of sulfation was determined both by elemental analysis (through the courtesy of Dr G. Cipriani, ANIC) and by conductimetry

[11]. Average molecular weights were determined by gel chromatography on Ultrogel AcA-44 (Biorad) [7] and on Sephadex G-75, using reference standards kindly provided by Dr E. A. Johnson (N.I.B.S., London).

Electrophoretic mobilities. Electrophoreses were performed in both an HCl/KCl buffer [8] and (at low amperage) in a barium acetate buffer [9]. Staining was made with Alcian Blue.

NMR spectra. ^{13}C-NMR spectra of heparins and heparin fragments were obtained either at 20 MHz (with a Varian CFT-20 spectrometer) or at 75 MHz (with a Bruker CXP-300 spectrometer), on about 10% (w/v) solutions in D_2O [12].

Ex-vivo *anticoagulant assays.* Male CD-COBS rats (Charles River, Calco, Italy) weighing 250–300 g were given unfractionated, non-depolymerized heparin (H), supersulfated low-molecular weight heparins (ss-LMW-H) and saline i.v. The animals were anaesthetised with ether and after 15 min blood was collected by intracardiac puncture from open-chested rats into syringes containing 0.125 M trisodium citrate (9 parts blood to 1 part citrate). Platelet-poor plasma (PPP) was prepared utilizing a method previously described [13].

APTT test [14] and anti-Xa assay [15] were performed using the PPP samples. The ratio between both activities was calculated as the conventional anti-Xa/APTT index.

Experimental venous-stasis model. To investigate the possible antithrombotic effect of heparins, we used a venous thrombosis model based on formation of a red thrombus under a ligature applied to the inferior vena cava of rats [16]; this model has repeatedly proved very sensitive to the anti-thrombotic activity of mucopolysaccharides. The venous stasis was induced 15 min after injection of the drugs. Both the incidence of thrombus formation and the red thrombus dry weight were recorded.

Experimental model of bleeding. To evaluate the haemorragic effect of H and ss-LMW-H we used an *in vivo* model of bleeding time in rats [17]. The bleeding time was measured by two techniques [18]: "template" bleeding time, to verify the possible interference of heparins on platelet-vessel wall interaction; "tail transection" bleeding time, which is not only sensitive to vessel wall-platelet interaction, but also to changes in the coagulation/fibrinolysis system.

Platelet count. Platelet was measured using a phase contrast microscope, on samples prepared by using the Unopette diluting system (Becton Dickinson, Novate Mi., Italy).

Ex vivo *fibrinolytic activity.* Plasma fibrinolytic activity was measured as euglobin lysis areas on fibrin plates [19]. Male CD-COBS rats (Charles River, Calco, Italy), weighing 250–300 g, were treated with saline or heparins (0.75 mg/kg b.w. i.v.) and anaesthetized with ether. Blood was collected after 15 min by intracardiac puncture, in syringes containing 0.125 M tri-sodium citrate. Platelet-poor plasma (PPP) was prepared by centrifugation at 4° for 30 min at 2000 g.

To evaluate the influence of H or ss-LMW-H on the fibrinolytic system, we used the conventional euglobulin assay.

Lipoproteinlipase release. Lipoproteinlipase release was measured using enzymatic kinetics based on the ability of the enzyme to hydrolyze ^{14}C-triolein into ^{14}C-oleic acid [20].

RESULTS

Physico-chemical parameters

Depolymerization of heparin under sulfating conditions as described in Materials and Methods, afforded heparin fragments in high yields (up to 105%, as referred to the starting, less sulfated material). As shown by data in Tables 1 and 2, the sulfate-to-carboxyl ratio of the products increased from 1.5–2.0 of the parent heparin (or heparin fraction) to values between 2.5 and 3.1, and the average molecular weight decreased from 13,500 (for the heparin preparation HA to about 6000 for the "prototypes" A-1 and A-2 (Table 1), and to 7500–10,000 for the preparations listed in Table 2, obtained from different heparins. The gel filtration profile of a typical preparation of ssLMW-H (A-5) is compared in Fig. 1 with that of the parent heparin.

The foregoing increase in sulfate content and decrease in molecular weight brought about substantial changes in the electrophoretic profiles of the fragments as compared with the parent heparins. As illustrated in Fig. 2, the "supersulfated" heparin fragments migrate faster than heparin in the acid buffer, and consist of only slow-moving species in barium acetate.

Also the ^{13}C-NMR spectra are dramatically affected by the depolymerization–sulfation reaction. As shown in Fig. 3, most signals of ssLMW-H are displaced as compared with heparin. Also noticeable is the disappearance, in the spectrum of the fragments, of the signal (near 61 ppm) of glucosamine residues nonsulfated at C-6 [8] and the appearance (near 94 ppm) of C-1 signals of reducing end-groups.

Pharmacological properties

The anticoagulant activity of ssLMW-H fragments (as expressed by the APTT and anti-Xa *ex vivo* values) is reported in Tables 1 and 2. Table 1 also reports data for the lipoproteinlipase-releasing activity for prototype preparations of ssLMW-H. Table 3 reports data on the antithrombotic activity, bleeding times and fibrinolytic activity for a typical ssLMW-H and its parent heparin.

The anticoagulant activities of ssLMW-H fragments are consistently low, corresponding to about 10% of the APTT and 25–30% of the anti-Xa units of the reference heparin A. This trend was observed also in a comparison with other unfractionated, non-depolymerized heparins and the corresponding ssLMW-H fragments, in different concentration ranges (data not reported). Anti-Xa/APTT activity ratios observed for the fragments are in the range 2.1–4.4.

Table 3 reports the antithrombotic activity, the bleeding properties in the tail transection and template tests [18], and the fibrinolytic activity of the typical fragment A-5, and the corresponding values for a reference heparin preparation (H-A). The antithrombotic activity of ssLMW-H, as evaluated both

Table 1. Physico-chemical parameters and *ex-vivo* anticoagulant and lipasemic activity of ss-LMW-H "prototypes" and their parent heparins

	Charge density	MW	APTT*	a.Xa*	a.Xa APTT	LPL-releasing activity
			Fragments from heparin			
A-1	3.0	6000	0.06	0.18	3.0	95
A-2	3.0	6000	0.05	0.22	4.4	nd
(H-A)	2.0	13,500	0.67	0.80	1.2	28
			Fragment from a LMW heparin fraction			
A_f1	2.5	4000	0.05	0.22	4.4	nd
($H\text{-}A_f$)	1.5	10,000	0.21	0.32	1.6	nd

* At 0.50 mg/kg.

Table 2. Physico-chemical parameters and *ex-vivo* anticoagulant activity of different preparations of ss-LMW-H

	Charge density	MW	APTT*	a.Xa*	a.Xa APTT
A-3	2.9	7500	0.075	0.30	4.0
A-4	2.8	9000	0.085	0.31	3.6
A-5	3.0	9000	0.10	0.27	2.7
B-1	2.9	10,000	0.10	0.275	2.75
C-1	3.1	10,000	0.14	0.29	2.1
D-1	2.8	8000	0.11	0.28	2.5

* At 0.75 mg/kg.

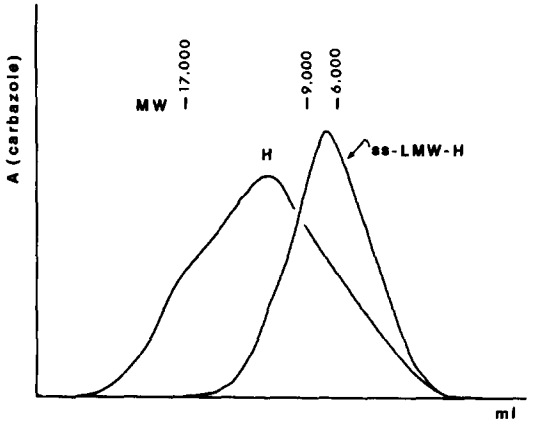


Fig. 1. Gel filtration profiles (Sephadex G-75) of a typical ss-LMW-H preparation (A-5) and its parent heparin (H). Bars indicate the elution volumes of heparin fractions of known molecular weight.

as regards the incidence of thrombus occurrence and the dry thrombus weight, is comparable with that of the unfractionated, nondepolymerized heparin. Also the bleeding times and the fibrinolytic activity of the fragment are not significantly different from those of heparin. (This latter, however, showed a somewhat higher bleeding than the fragment, in the tail transection model.)

By contrast, data in Table 1 and in the stick diagram of Fig. 4 show that the lipoprotein-lipase releasing activity of ssLMW-H fragments is two-to-three times higher than that of heparin.

DISCUSSION

Depolymerization of heparin under conditions of oversulfation afforded in high yield fragments having from one-third to one-half the original size, and degrees of sulfation consistently higher than for the original heparins. Such a substantial increase in sulfate content (from sulfate-to-carboxyl molar ratios of 1.9–2.2 typical of pig mucosal heparins [8], to 2.8–3.1) is reflected in a higher electrophoretic mobility in acid buffer (Fig. 2A), where migration is a function of sulfate content [21]. In barium acetate buffer, where migration decreases with decreasing both charge density and molecular weight [9, 22], the depolymerization–sulfation reaction converted also the "fast-moving" species into "slow-moving" species. ss-LMW-H may indeed be defined as atypical LMW heparin fragments that are slow-moving in cationic buffers [22].

As indicated by the remarkable differences between the ^{13}C-NMR spectrum of ss-LMW-H and that of heparin (Fig. 3), the depolymerization–sulfation reaction affords in fact heparin fragments distinctly different from the original internal segments of heparin. (Other methods of heparin depolymerization, such as those with nitrous acid, heparinase or base, lead to fragments differing from the internal segments of heparin only in the structure of end-residues, and, perhaps, some undersulfation [4].)

Besides sulfation of most hydroxyls at C-6 of the glucosamine residues, the ^{13}C-NMR spectrum of ss-LMW-H clearly indicates complete retention of N-sulfate groups (which, by contrast, are easily cleaved under the usual conditions of acid hydrolysis

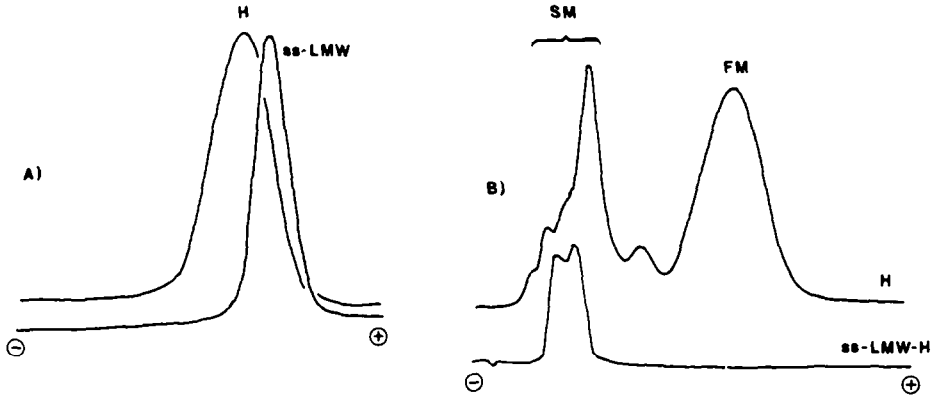


Fig. 2. Electrophoretic patterns of a typical ss-LMW-H (preparation A-5) and its parent heparin (H), in (A) HCl/KCl buffer, and (B) barium acetate buffer. SM = slow-moving species; FM = fast-moving species.

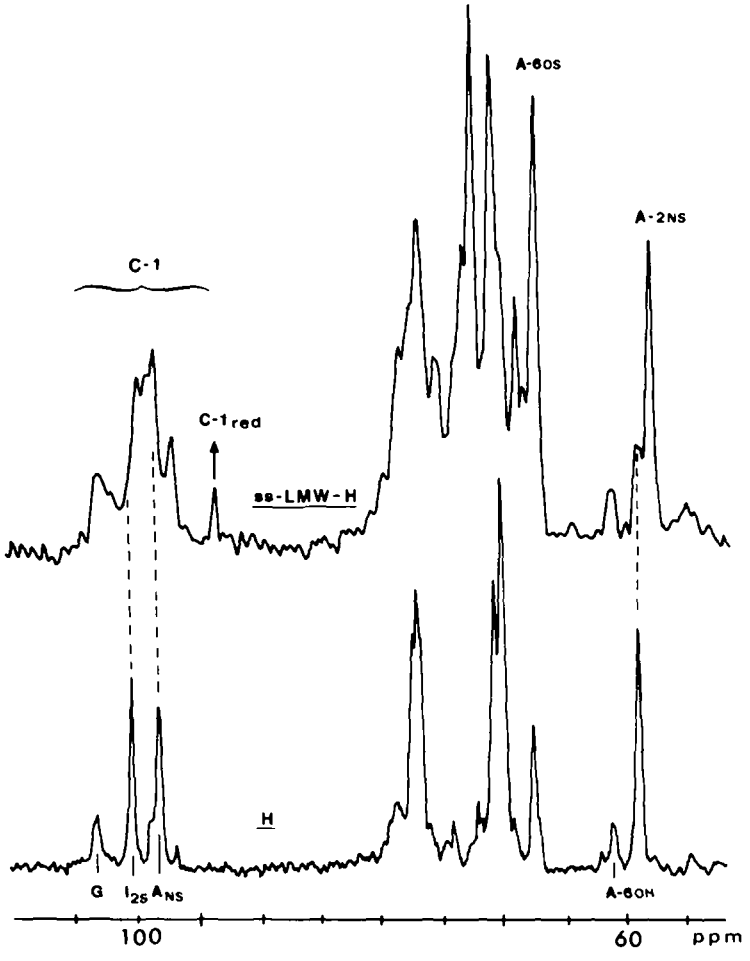


Fig. 3. ^{13}C-NMR spectra (20 MHz, D_2O) of a typical ss-LMW-H (preparation A-5) and its parent heparin (H). (A_{NS} = N-sulfated aminosugar; I_{2S} = iduronic acid 2-sulfate; G = glucuronic acid; A_{NA} = N-acetylated aminosugar.)

Table 3. Biological activities of a typical ss-LMW-H and its parent heparin*

	Antithrombotic activity		Anticoagulant activity			Bleeding time		
	% Occlusion	Thrombus weight (mg)	APTT	a.Xa	a.Xa APPT	Tail trans. (sec)	Template (sec)	Euglobulin lysis area (mm^2)
Saline	70	2.25 ± 0.44	—	—	—	260 ± 27	120 ± 10	163 ± 18
A-5	20	0.20 ± 0.10	0.1	0.27	2.7	295 ± 22	124 ± 11	176 ± 13
H-A	10	0.35 ± 0.10	1.0	1.2	1.2	382 ± 35	123 ± 14	158 ± 14

* At 0.75 mg/kg.

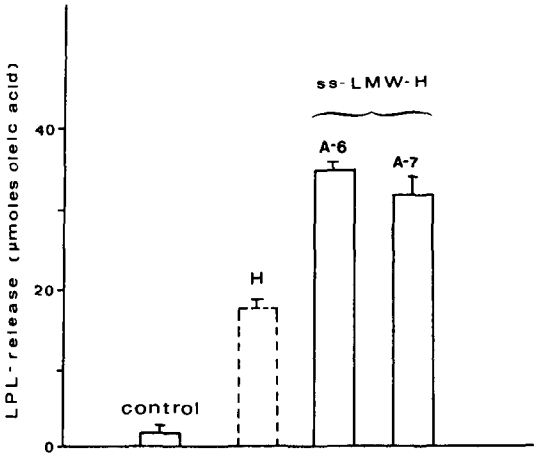
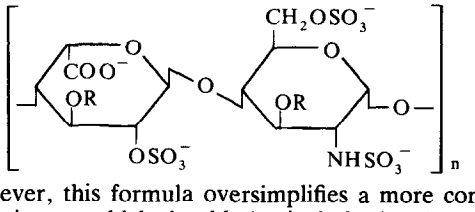


Fig. 4. Lipoprotein-lipase releasing activity of two preparations of ss-LMW-H, and of the reference heparin A-E (H).

[23, 24]). Such a retention, indicated by the relative areas of the C-2 signal of *N*-sulfated aminosugar residues (A_{NS}) to the total area of anomeric (C-1) signals, was further confirmed by a sequence of *N*-desulfation and re-*N*-sulfation reactions (unpublished data). Several features of the spectrum suggest that the extra-sulfate groups are preferentially (but not exclusively) at one of the two possible oxygens, i.e. O–3 of the glucosamine or the iduronic acid residue. The structure of major disaccharide units of ss-LMW-H can be accordingly approximated by the following statistical formula (where R = SO_3^- or H).



However, this formula oversimplifies a more complex picture, which should also include the "irregular" sequences (which account for as much as 35% of the structure of the original heparins [24]). Further studies are in progress to better define the structure of present ss-LMW-H fragments.

The anticoagulant activity of ss-LMW-H is consistently lower than for fragments of the same molecular size obtained by depolymerization of heparin by other methods [25]. However, as observed for other heparin fragments [25], the drop in anti-Xa activity upon depolymerization–sulfation is less than for the APTT activity, leading to a.Xa/APTT activity ratios higher than 2 (up to 4.4).

In spite of low anticoagulant potencies, the ss-LMW-H fragments have an antithrombotic activity comparable to that of heparin (Table 3). This trend was confirmed with other preparations (data not reported). Also the bleeding times and fibrinolytic activity (Table 3) were substantially the same as for heparin. By contrast, the lipoproteinlipase-releasing activity of ss-LMW-H fragments is from two to three times higher than for unfractionated, unmodified heparins (Table 1 and Fig. 4).

Oversulfation of heparin (without depolymerization) was already reported to dramatically reduce the anticoagulant activity of heparin as measured in the USP test [26]. As this activity is largely associated with antithrombin-III-mediated inhibition of thrombin and other coagulation factors [2], it could be implied that extra sulfate groups at the level of the pentasaccharide sequence constituting the binding site of heparin for antithrombin [27, 28] impair the ability of ss-LMW-H fragments to bind to antithrombin (AT-III). Preliminary affinity experiments indicate that ss-LMW-H is bound to AT-III somewhat more than the original heparin [29]. However, further experiments are needed to prove that such an increased adsorption on the AT-III column is not merely due to aspecific interactions with AT-III by the most sulfated fractions of ss-LMW-H.

More relevant to the expression of the biological activities of ss-LMW-H fragments appears to be their very high sulfate content, which implies a high charge density along the polysaccharide chain. Most of the biological properties of heparin and heparin-like substances are in fact due to the anionic polyelectrolyte character of these polysaccharides [1, 24], and charge density was shown to be important also for determining the anticoagulant activity of heparin, at least for the part of this activity mediated by Heparin-Cofactor-2 [30].

Like most of the polyelectrolyte properties, the binding properties of sulfated polysaccharides relevant to their biological interactions are dependent on both charge density and chain length, i.e. they are expected to be stronger for increasing sulfate content and molecular weight [30]. This appears to be confirmed for the APTT activities of the present ss-LMW-H fragments. When the APTT data of Table 2 are plotted against an empirical function (ξ.MW) taken here as the expression of combined

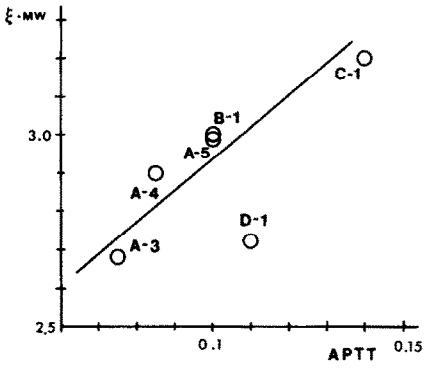


Fig. 5. APTT activities of ss-LMW-H$_s$ prepared from different heparins, as a function of a "charge-density·molecular weight" parameter (ξ.MW) (see text).

influence of charge density (c.d.) and molecular weight,* data for five out of six fragments lie on a straight line (Fig. 5). (Data for the sixth fragment (D-1) could not be re-checked owing to the insufficient amount of sample.)

On an absolute basis, the low anticoagulant activity of ss-LMW-H is thought to be due more to an increased adsorption of these species by the endothelial cell walls than to inactivation of the active site for AT-III. Heparin was shown to be rapidly taken-up by the endothelium [1], and a recent study confirmed that indeed such an adsorption is a function of both molecular weight and charge density [32]. As a result of an increased adsorption induced by extra sulfate groups, the actual concentration in plasma of ss-LMW-H species is thus expected to be lower than for other species having the same molecular weight but lower charge density. Such a low concentration in plasma (leading to low APTT and a.Xa activities) does not significantly impair, however, the antithrombotic activity of ss-LMW-H in the venous stasis model. This raises the question as to whether lining of endothelial wall by the "super-sulfated" polysaccharide fragments is a major contributor to the observed activity.

Of more direct interpretation is the increase in antilipemic (lipoproteinlipase-releasing) activity of ss-LMW-H as compared with the original heparins. In fact, this activity was shown to be related to the sulfate content for a series of heparins and heparan sulfates [8].

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