Carbohydrate Research, 150 (1986) 295–299
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Effect of sulphated derivatives of chitosan on lipoprotein lipase activity of rabbit plasma after their intravenous injection*

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(Received July 9th, 1985; accepted for publication, October 10th, 1985)

Heparin possesses a dual biological function in preventing blood coagulation. It forms complexes with antithrombin III[1] and apolipoprotein C-II[2–6], of which the latter releases lipoprotein lipase (LPL, EC 3.1.1.3) into the circulating blood. LPL-releasing activity has also been observed with some glycosaminoglycans[7–9] (*e.g.*, heparan sulphate and dermatan sulphate) and sulphated polysaccharides[10–13] (*e.g.* sulphated dextran and sulphated xylan). LPL catalyses the hydrolysis of the triacylglycerol moiety of lipoproteins to afford free fatty acids, which generally go into solution as complexes with plasma albumin. However, an abnormally high concentration of free fatty acids in plasma can produce several serious pathological effects[14], especially in hemodialysis with heparin[15]. Therefore, new heparinoids which possess anticoagulant activity but no LPL-releasing activity are desirable.

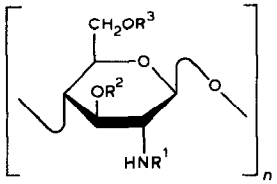
We have described[16] the anticoagulant activity of several sulphated derivatives of chitosan as analysed by activated partial thromboplastin time (APTT), and found that *O*-disulphated *N*-acetylchitosan (mol. wt., 26×10^3) exhibited 1.9–2.2 times the activity of heparin (174 units/mg). We now report on the LPL-releasing activity of some sulphated derivatives of chitosan after their intravenous injection in rabbits.

The following derivatives were prepared, *N,O*-sulphated chitosan (**1**), *O*-sulphated *N*-acetylchitosan (**2** and **3**), *O*-sulphated *N*-hexanoylchitosan (**4**), *O*-sulphated chitosan (**5**), and *N*-sulphated *O*-carboxymethylchitosan (**6**). The molecular weights of these derivatives were in the ranges of $21–26 \times 10^3$ (**1, 2, 4,** and **5**) and $150–245 \times 10^3$ (**3** and **6**). Compounds **3** and **6** were prepared from chitosan I (Flonac-N, commercial chitosan), and **1, 2, 4,** and **5** were prepared from chitosan II (I heated with 45% NaOH in the presence of borohydride for 4 h). Compounds **2** (mol. wt., 25×10^3) and **3** (150×10^3) have similar structures but different molecular weights. Table I summarises the yields, specific rotations, and elemental analyses of **1–6**. Each sulphated derivative had i.r. absorptions at 1240–1250 (S=O) and 800–810 cm^{-1} (equatorial C-O-S and/or C-N-S). The d.s. for *N*-

*Dedicated to Professor N. K. Kochetkov.

0008-6215/86/$ 03.50

acyl was 1.0 in **2–4**, and that for carboxymethyl was 0.61 in **6**. These structures were supported by the elemental analyses, and all the products were soluble in water.



	R^1	R^2	R^3
1	SO_3Na	H	H, SO_3Na
2 and **3**	Ac	SO_3Na	SO_3Na
4	Hexanoyl	H, SO_3Na	SO_3Na
5	H	H	H, SO_3Na
6	SO_3Na	H	H, CH_2CO_2Na

The LPL activity of rabbit plasma after the intravenous injection of **1–6** varied mainly with dose, incubation time, molecular weight, and chemical structure. The maximum activity for a dose of 0.1 mg/kg was 3.4 times that of heparin for **1** at 15 min, and 0.7 and 0.1 times for **6** and **2**, respectively, at 30 min, and **3–5** were inactive (Table II); the maximum activity of heparin occurred at 30 min. Although possessing similar structures, **2** (mol. wt., 26×10^3) had LPL-releasing activity, but **3** (mol wt., 150×10^3) did not, reflecting the difference in molecular weight[5,6]. However, **6** (mol. wt., 245×10^3) had 70% of the activity of heparin in spite of its high molecular weight, because of the presence of carboxymethyl groups at positions 6. The *N*-sulphated derivatives **1** and **6** showed activity, but neither the *N*-hexanoyl product (**4**) nor the NH_2 product (**5**) showed activity. These results indicate that the presence of an acidic group at position 6, an *N*-sulphate[4–6] or *N*-acetyl group[2,8,9], and low molecular weights ($2–3 \times 10^3$)[5,6] confer LPL-releasing activity. The sulphated derivatives of chitosan have low toxicity (LD_{50} 1.25–3.25 g/kg)[17] similar to that of heparin (LD_{50} 1.5–2.0 g/kg)[18].

Both the anticoagulant and LPL-releasing activities can be controlled separately by the chemical modification of sulphated chitosan; thus, **2** (mol. wt., 26×10^3) possesses a high anticoagulant activity (1.9–2.2 times that of heparin)[16] but a low LPL-releasing activity (10% of that of heparin).

EXPERIMENTAL

Chitosan. — Chitosan I was Flonac-N (commercial chitosan of crab shell, Kyōwa Yushi Co.), and had $[\alpha]_D^{26}$ −5° (*c* 0.95, methanesulfonic acid) and d.s. 0.23 for NAc (calculated from the elemental analysis).

Chitosan II was prepared from chitosan I by treatment[19] with aqueous 45% NaOH containing $NaBH_4$ (0.1 g/500 mL) at 115° for 4 h. The product gave no ^{13}C

TABLE I

DATA FOR SULPHATED DERIVATIVES OF CHITOSAN[a]

Compound	*Yield (%)*	$[\alpha]_D$ (c, *temp.*) *(degrees)*	*Formula*	*Calc. (%)*				*Found (%)*			
				C	*H*	*N*	*S*	*C*	*H*	*N*	*S*
1	80	−11 (0.5, 17°)[b]	$[C_6H_8NO_8(SO_3Na)_{1.69} \cdot 3.34\,H_2O]_n$	18.36	3.77	3.55	13.80	18.16	3.91	3.70	14.1
3	75	−16 (0.8, 17°)[b]	$[C_8H_{11}NO_{11}S_2Na_2 \cdot 2.24\,H_2O]_n$	21.04	3.64	3.07	14.04	21.04	3.71	2.93	13.7
4	83	−22 (0.9, 21°)[b]	$[C_{12}H_{19}NO_8SNa(SO_3Na)_{0.77} \cdot (H)_{0.23} \cdot 1.30\,H_2O]_n$	31.11	4.75	3.02	12.25	31.10	4.75	3.00	12.2
5	83	−9 (0.8, 17°)[b]	$[C_6H_{10}NO_4(SO_3Na)_{0.74}(H)_{0.26} \cdot 0.87\,H_2O]_n$	28.56	4.78	5.55	9.40	28.44	4.79	5.85	9.4
6	54	−7 (0.8, 17°)[c]	$[C_6H_9NO_4(SO_3Na)_{1.00}(C_2H_2O_2Na)_{0.61}(H)_{0.39} \cdot 2.56\,H_2O]_n$	24.21	4.43	3.91	8.95	24.22	4.43	3.88	8.9

[a]Chitosan I. *Anal.* Calc. for $[C_6H_{10}NO_4(C_2H_3O)_{0.23}(H)_{0.77} \cdot 0.54\,H_2O]_n$: C, 42.97; H, 7.00; N, 7.76. Found: C, 43.08; N, 6.86; N, 7.64. *N*-Acetyl derivative (d.s. 1.0) of chitosan I was used for the preparation of **3** and **6**. Chitosan II. *Anal.* Calc. for $[C_6H_{11}NO_4 \cdot 0.20\,H_2O]_n$: C, 43.74; H, 6.97; N, 8.50. Found: C, 43.61; H, 7.00; N, 8.34. Chitosan II and its *N*-acyl derivatives (d.s. 1.0) were used for the preparation of **1, 2, 4,** and **5**. [b]In water. [c]In aqueous 0.5% NaOH.

TABLE II

LPL ACTIVITY, MOLECULAR WEIGHT, AND DEGREE OF SUBSTITUTION FOR SULPHATE

Compound	*Mol. wt.*[a] *(× 10³)*	*D.s. for sulfate*[b]	*Dose (mg/kg)*	*LPL activity (meq./L)*[c]		
				15[d]	*30*[d]	*60*[d]
Heparin	21	—	0.1	360	950 (1.0)	500
1	22	1.7	0.1	3200 (3.4)	2600	2000
2[e]	26	2.0	0.1	20	100 (0.1)	i.a.[f]
2[e]	26	2.0	1.0	350	2600 (2.7)	1300
3	150	2.0	0.1	i.a.	i.a.	i.a.
4	27	1.8	0.1	i.a.	i.a.	i.a.
5	22	0.7	0.1	i.a.	i.a.	i.a.
6	245	1.0	0.1	200	700 (0.7)	90

[a]Estimated by gel filtration (see Experimental). An average mol. wt. is shown. [b]Determined from the elemental analyses. [c]The average value of 2–3 experiments. The values relative to that of heparin are shown, in parentheses, at the maximum activity of each sulfated derivative. [d]Incubation time (min). [e]See ref. 16. [f]Inactive.

signals for C=O and CH_3 of NAc at ~174 and ~23 p.p.m., respectively, in the ^{13}C-n.m.r. (c.p./m.a.s.) spectrum, and had $[\alpha]_D^{26}$ −10° (*c* 0.8, methanesulphonic acid) and d.s. <0.05 for NAc.

Sulphated derivatives of chitosan. — Chitosan was *N*-acetylated[19] with acetic anhydride in aqueous 2% acetic acid–methanol (1:2), and *O*-carboxymethylchitosan (d.s. 0.61) was prepared from *O*-carboxymethyl-*N*-acetylchitosan[20]. Sulphation was effected with *N,N*-dimethylformamide–sulphur trioxide[21], and the product was isolated as the sodium salt. *N*-Desulphation was effected[22] with 0.04M HCl at 100° for 2.5 h. Compounds **3** and **6** were prepared from the *N*-acetylated chitosan I, and **1, 2, 4,** and **5** were prepared from chitosan II or its *N*-acylated products.

General methods. — I.r. spectra were recorded with a Hitachi 215 spectrometer, and ^{13}C-n.m.r. spectra with a JEOL FX-100 spectrometer (68.8 MHz). Optical rotations were measured with a JASCO Dip-180 polarimeter. Sulphate content was determined by the barium chloranilate method[23]. Average molecular weights were determined[16] by gel filtration on a column (16 × 46 cm) of Bio-Gel P-300 (fine) for **1, 2, 4,** and **5**, and a column (1.6 × 65 cm) of Toyopearl HW 65 for **3** and **6**. Each calibration curve was obtained by analysis of relationship between V_e/V_o and the log mol. wt. of standard pullulans and dextrans[16]. The eluates were monitored by u.v. absorption at 194 nm.

The other methods have been described previously[16].

LPL activity. — Blood specimens (5 mL), collected from male rabbits (3 kg), were centrifuged at 3,000 r.p.m. at 4° for 20 min to afford serum specimens. A mixture of serum specimen–coconut oil emulsion–0.07M phosphate buffer (pH 8.0)–physiological saline (1.0:0.1:1.0:1.0) was incubated at 37° for 30 min, and used as the substrate[24].

After 14–15-h fasting, a solution of sulphated derivative (0.1 or 1.0 mg/kg) in physiological saline (5 mL) was injected intravenously into each of the three rabbits (2.7–3.0 kg). After 10 min, a blood specimen (5 mL) was collected, mixed with 0.1M sodium oxalate (0.5 mL), and centrifuged at 3,000 r.p.m. at 4° for 20 min. The resulting heparinoid plasma specimen (1.0 mL) was thoroughly mixed with the substrate (1.5 mL) prepared above, and the mixture was incubated at 37° for 15, 30, and 60 min[24]. Free fatty acids were extracted with 2-propanol–heptane–0.5M sulphuric acid (40:10:1), and the heptane layer was titrated with 0.05M NaOH in the presence of Thymol Blue as indicator[25]. The LPL activity was calculated as follows: meq./L = [free fatty acids (mmol/L) after incubation] − [free fatty acids (mmol/L) before incubation]. The results were compared with those for porcine intestinal heparin (lot no. CH-108M, Mitsui Seiyaku Kōgyō Co.) as standard.

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