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Studies of Biologic Properties of a Polysulfated Chondroitin, A73025 (40886)[1]

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Abstract. The chemical composition of a "heparin analog" (A73025) was studied and its biologic properties were compared with those of a heparin preparation from intestinal mucosa. A73025 was characterized as a polysulfated chondroitin with a molecular weight of about 10,000 M_r. Unlike heparin, A73025 showed poor anticoagulant activity *in vitro* by Stypven clotting time, partial thromboplastin time, and thrombin time assays. A73025, like heparin, although to a lesser extent, inhibited thrombin induced platelet aggregation, released lipoprotein lipase when injected into rabbits, and complexed with serum low and very low density lipoproteins.

Although glycosaminoglycans (GAG) of connective tissue have many physiologic functions, these biologic properties are incompletely described. Even though the anticoagulant activity of heparin has been extensively studied, other compounds similar to heparin need to be investigated. Some possess anticoagulant and other biologic activities without the complications of heparin. A "heparin analog" (A73025, Luitpoldwerk, Munchen, Germany), which is a semi-synthetic GAG, has been shown by Thomas *et al.* (1) to have a pronounced effect on *in vivo* blood clotting, but no effect on *in vitro* kaolin–cephalin clotting time. Based on the observation that patients had fewer side effects with A73025 than heparin, Thomas *et al.* (1) suggested a potential use of A73025 to prevent postoperative deep-vein thrombosis. Because the precise chemical composition of A73025 is not known, we studied its chemistry and various biologic properties in comparison with standard heparin.

Materials and methods. A73025 was obtained from the National Institute for Biologic Standards and Control (London, England) through the courtesy of Dr. D. P. Thomas. A heparin preparation from intestinal mucosa, with an anticoagulant activity of 160 units/mg used for comparative purposes was a gift from the Upjohn Company (Kalamazoo, Mich.).

Total uronic acid was determined by the method of Dische (2), and hexosamine by the Boas method (3). Protein was assayed by the Hartree procedure (4). Gas–liquid chromatographic procedures were used for the differential determinations of glucosamine and galactosamine (5), glucuronic acid and iduronic acid (6), and for total sulfate determination (7). *N*-Sulfate was determined by the procedure of Lagunoff and Warren (8). Cellulose acetate electrophoresis was performed in pyridine/formic acid buffer, pH 3.4 (9), and 0.2 *M* cadmium acetate buffer, pH 4.1 (10).

Optical rotation was measured in a Carl Zeiss polarimeter with a 1.0 dm tube at 1.0% concentration of A73025. Fractionation of A73025 was achieved by automated ion exchange chromatography on a Dowex-1 Cl^- column (11) and gel filtration was performed on a Sepharose CL-6B column (12).

The anticoagulant activity of A73025 was assayed in a BBL Fibrometer (Beckton, Dickinson & Co., Cockeysville, Md.) using plasma obtained from laboratory personnel by standard procedures (13) in three different clotting systems, viz. the Stypven clotting time using Russell's viper venom, par-

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tial thromboplastin clotting time (PTT) using kaolin for surface activation, and thrombin time. The effect of A73025 on collagen-, adenosine 5-diphosphate (ADP)-, and thrombin-induced platelet aggregation was studied using a platelet aggregometer (Bio/Data Corp., Norshaw, Pa., Model PAP-2). Platelet-rich plasma was preincubated for 1 min with A73025 or heparin before addition of the aggregating agent. The light transmission registered 8 min after addition of the aggregating agent was taken as the endpoint of aggregation. Lipoprotein lipase releasing activity was determined in postheparin or A73025 plasma as previously described (14). The 1 mg of A73025 or heparin/kg body wt was injected into rabbits through an ear vein and blood was collected between 9 and 12 min over sodium citrate. Lipoprotein lipase activity was assayed in the plasma with Ediol (Calbiochem, La Jolla, Calif.) as a substrate by the method of Korn (15). The ability of A73025 to complex with serum β- and pre-β-lipoproteins was studied in the presence of Ca^{2+} by previously reported procedures (16). The method in brief is as follows: Appropriate volumes of distilled water, 0.1 ml of GAG (0.05–1.0 mg) solution, and 0.5 ml of $CaCl_2$ (7.5–62.5 m*M*), in the order given, were added to pooled human serum (0.1–0.4 ml) in a cuvette, keeping the total volume to 4 ml. A similar mixture omitting GAG was prepared simultaneously as a blank. The contents were mixed and turbidity was measured after 15 min at 24°C against the blank at 600 nm. The reaction mixture was then spun down at 14,000 *g* and cholesterol in the sediment was determined by the method of Pearson *et al.* (17).

Results. The chemical composition and certain physical properties of A73025 are reported in Table I. Total uronic acid and hexosamine were present in almost equimolar proportions. A73025 was devoid of glucosamine and iduronic acid, which are the major constituent monosaccharides of heparin. Four moles of total sulfate per mole of hexosamine were noted in A73025, whereas mucosal heparin contains about 2.5 moles of sulfate per mole of hexosamine. Unlike heparin, A73025 did not contain *N*-sulfate.

TABLE I. PROPERTIES OF A73025[a]

Chemical composition	Chemical properties	
	Percentage[b]	μmole/mg
Uronic acid[c]	17.9	0.92
Hexosamine[d]	24.1	1.14
Total sulfate	38.0	3.96
N-Sulfate	<1.0	—
Protein	0.2	—

[a] Physical properties: molecular weight, 10,000 M_r; optical rotation, $[\alpha]_D^{25}$, − 28°.

[b] The composition reported in the table accounts for only 80%. Although not determined, acetyl groups in equimolar proportion to hexosamine are believed to be present in the material, which accounts for about 8%. Since A73025 was in the form of sodium salt the remainder (12%) could be accounted for as sodium.

[c] Only glucuronic acid was detected by gas–liquid chromatography (GLC).

[d] Only galactosamine was detected by GLC.

The specific rotation $[\alpha]_D^{25}$ −28° observed for A73025 suggests that it is a β-linked polymer, unlike heparin which has a specific rotation of $[\alpha]_D^{25}$ + 49° and contains α-linkages. In cellulose acetate electrophoresis A73025 migrated as a single component with a mobility similar to mucosal heparin. Fractionation of A73025 on Dowex-1 Cl^- column resulted in a minor (<5%) and a major fraction. The minor fraction eluted from the column with 0.5 *M* NaCl, whereas the major fraction eluted with 3.0 *M* $MgCl_2$. A molecular weight of 9,000–10,000 M_r was estimated for the major fraction by gel filtration on Sepharose CL-6B using GAG of known molecular weights to calibrate the column (12). Insufficient amounts were available to determine the molecular weight of the minor fraction.

The effect of A73025 on *in vitro* clotting time in the three different systems is summarized in Table II. Among the three systems *in vitro,* A73025 is most effective in the PTT system and least effective in inhibiting thrombin time. When compared with mucosal heparin it is a poor anticoagulant *in vitro* in all three systems.

A73025 did not influence ADP- or collagen-induced platelet aggregation but inhibited thrombin-induced platelet aggregation (Fig. 1). The inhibition at 5 μg concentration of A73025 in the system was similar to that observed with 0.5 μg of

TABLE II. *In Vitro* Plasma Clotting Assays of A73025 and Mucosal Heparin at Various Concentrations

Clotting assay	Delay in clotting time[a] (sec)
Stypven time[b]	
A73025 (mg uronic acid):	
10.0	10.0
25.0	76.0
50.0	296.3
Mucosal heparin:	
1.0	11.0
2.5	47.5
3.5	No clotting[c]
Partial thromboplastin time[d]	
A73025:	
1.0	120.6
2.5	263.6
3.5	464.1
Mucosal heparin:	
0.2	46.1
0.3	64.1
0.4	84.2
0.5	No clotting
Thrombin time[e]	
A73025	
10.0	3.3
25.0	5.3
50.0	3.8
100.0	2.1
Mucosal heparin:	
0.5	8.7
0.6	9.3
0.8	20.3
1.0	No clotting

[a] Calculated by subtracting the control clotting time from the absolute clotting time, observed in the presence of GAG.
[b] Control clotting time in the system without GAG was 16.4 sec.
[c] Delay in clotting time above 1000 sec was considered as no clotting.
[d] Control clotting time in the system without GAG was 228.3 sec.
[e] Control clotting time in the system without GAG was 8.1 sec.

heparin, suggesting that A73025 is about 10 times less effective than heparin. At higher concentrations of A73025, 25 μg for example, greater inhibition was observed.

When injected into rabbits, A73025 released tissue lipoprotein lipase into the circulation similarly to heparin. Rabbit post-A73025 plasma hydrolyzed Ediol, liberating 0.39 μmoles of glycerol/ml plasma/hr, while post-heparin plasma hydrolyzed Ediol, giving 0.77 μmoles of glycerol/ml plasma/hr, suggesting that A73025 has one-half the lipoprotein lipase releasing activity of heparin.

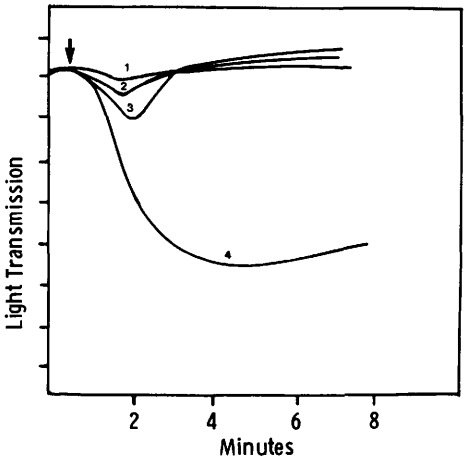

FIG. 1. Inhibition of thrombin induced platelet aggregation by A73025 at 25- (1) and 5.0-μg (3) concentrations and mucosal heparin at a concentration of 0.5 μg (2). Platelet-rich plasma (PRP) was incubated with GAG for 1 min and then thrombin (0.2 U/ml PRP) was added. The light transmission registered for 8 min after the addition of thrombin was noted. The control (4) contained PRP and thrombin only.

The complexing ability of A73025 with serum lipoproteins in comparison with heparin is shown in Fig. 2. A73025 behaved like heparin. The turbidity produced at a concentration of 62.5 μg of GAG/ml in the system is proportional to the volume of serum or β + pre-β-lipoprotein cholesterol in the precipitate. Like mucosal heparin, maximum turbidity was produced at an optimal concentration of 250 μg of GAG and 62.5 mmoles of Ca^{2+} in a total volume of 4.0 ml in the system (16).

Discussion. The chemical analyses and optical rotation of A73025 indicate that it is a polysulfated chondroitin. The sulfate analysis suggests that about four *O*-sulfate groups per disaccharide are present in the compound, but the exact location of these sulfate groups in the disaccharide is not known. Although A73025 has a considerably different chemical structure from heparin, it is probably designated as a "heparin analog" because of its *in vivo* anticoagulant activity.

The anticoagulant activity of heparin is often attributed to its *N*-sulfate ester groups and iduronosulfate esters (18). Recently Lindahl (19) suggested that high-affinity

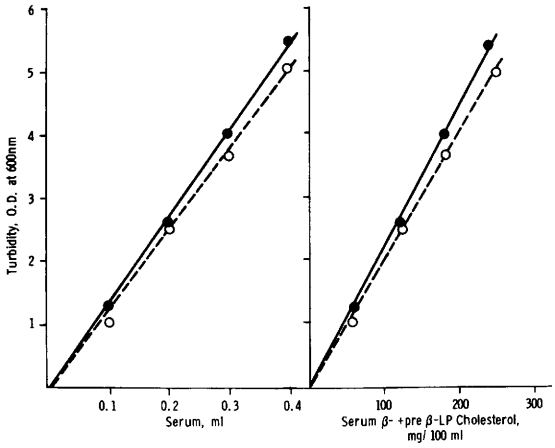

FIG. 2. Interaction of A73025 (○--○) with serum β- and pre-β-lipoproteins. For comparison the interaction between mucosal heparin (●——●) and serum lipoproteins was also studied. Linear relationships between turbidity and volume of serum used in the analysis as well as cholesterol content in the precipitated β- and pre-β-lipoproteins (LP) may be noted. The curve obtained with A73025 is almost identical with that obtained with mucosal heparin. The concentration of GAG in these experiments was 250 μg and that of Ca^{2+} was 62.5 mmoles in a total volume of 4.0 ml of reaction mixture.

binding of heparin to antithrombin III requires a specific sequence of disaccharide units in the GAG. Molecular size of heparin also seems to be important in the anticoagulant activity of heparin (20). Unlike heparin, A73025 lacks *N*-sulfate ester groups and iduronosulfate groups. One or both of these components may at least partly contribute to the *in vitro* anticoagulant activity of GAG. The *in vivo* anticoagulant activity of A73025 might be due to its pronounced effect on antithrombin III (1). When A73025 was subcutaneously administered to patients, Thomas *et al.* (1) observed that it had almost as much effect on antithrombin III as heparin, although it was relatively less effective on the clotting time than heparin. One of the reasons for *in vivo* effect on antithrombin III of A73025, as speculated by Thomas *et al.* (1), could be that the specific action of A73025 on antithrombin III is mediated by a tissue factor rather than a plasma component. The tissue factor might be rapidly converting A73025 into a more active form in the body.

The influence of GAG on platelet aggregation is not well understood. We (21) have previously observed that mucosal heparin inhibits platelet aggregation *in vitro* at least in one system, i.e., thrombin-induced aggregation. The observed inhibition of thrombin-induced platelet aggregation by A73025 suggests that certain constituents of A73025 that are common to mucosal heparin might be at least partly responsible for this inhibition. Mucosal heparin and A73025 differ in the nature of their hexosamines and hexuronic acids, but both have sulfate ester groups. It could be that the anionic nature of these polysulfate ester groups is important for the *in vitro* inhibition of the thrombin-induced platelet aggregation. The polyanionic nature of A73025 might also be responsible for its lipoprotein lipase releasing activity and lipoprotein binding ability. Such properties have implication in the pathogenesis of atherosclerosis.

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