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EFFECT OF POLYANETHOLESULPHONIC ACID AND XYLAN SULPHATE ON ANTITHROMBIN III ACTIVITY

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SUMMARY

Both polyanetholesulphonic acid and xylan sulphate prolonged the partial thromboplastin clotting time of plasma. The anticoagulant effect of both compounds was reduced following pre-incubation of plasma with antiserum specific for antithrombin III. Polyanetholesulphonic acid was more effective than xylan sulphate in inhibiting thrombin-initiated clotting of plasma, and potentiated antithrombin III inhibition of both thrombin and Xa. Xylan sulphate was more effective in potentiating antithrombin III inhibition of Xa than of thrombin. These differential effects of xylan sulphate on different blood serine proteases are discussed in terms of the antithrombin III-mediated anticoagulant activity of heparin.

INTRODUCTION

The sulphated glycosaminoglycan heparin exerts an anticoagulant effect by potentiating antithrombin III-induced inhibition of a number of blood serine proteases, particularly thrombin and activated coagulation factor X (Xa)(1). Several other sulphated polymers exhibit anticoagulant activity which, at least in some cases, appears to be mediated through antithrombin III (2,3,4,5,6,7). The chemical parameters associated with highly active heparin preparations are still to be defined (8), and the molecular prerequisites for the interaction of heparin (and of other sulphated polymers) with antithrombin III are unknown. However, heparin may exert different effects on antithrombin III inactivation of different serine proteases (9), and it has been suggested that the structural requirements for potentiation of antithrombin III inhibition by heparin-like molecules may be different for thrombin and Xa (10). In particular, limited data suggest that polyanetholesulphonic acid may preferentially potentiate antithrombin III inhibition of thrombin, and that xylan sulphate may preferentially potentiate antithrombin III inhibition of Xa (10,11). Polyanetholesulphonic acid and xylan sulphate, unlike most heparin preparations, are relatively well-

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defined chemically, and such differential effects might eventually provide insight into the molecular bases of heparin activity.

The present report compares the effects of the two polymers on the activities of thrombin and Xa, and on the reaction between antithrombin III and these clotting enzymes.

MATERIALS AND METHODS

Materials: Sodium polyanetholesulphonate was from Sigma Chemical Company Ltd. The compound is reported by the manufacturers to have a molecular weight of 9,000-10,000. Sodium xylanpolysulphate (SP54) was from Benechemie GMBH. The compound is an artificially sulphated β 1→4-linked D-xylan, and is reported by the manufacturer to have a degree of polymerisation of 6-12 monomer units per oligomer, with 1.5-2.0 sulphate residues per monomer unit. Immediately before use, samples were dissolved at 4 mg/ml in buffer (0.015M 5,5-diethylbarbituric acid, 0.01M sodium 5,5-diethylbarbiturate, 0.125M sodium chloride, pH 7.4), and were subsequently diluted in buffer as appropriate.

The sources and methods of preparation of thrombin, Xa, human antithrombin III, polybrene, amidolytic substrates S-2160 and S-2222 (used in the determination of thrombin and Xa respectively), and antiserum specific for human antithrombin III have been previously reported (5,6).

Preparation of human plasma and measurement of partial thromboplastin times: Methods used were as described elsewhere (12). Control clotting times (in absence of added anticoagulant) were in the range 50-70 seconds. If no clot formed in anticoagulant-containing tubes after 400 seconds, the ratio partial thromboplastin time with anticoagulant/without anticoagulant was recorded as infinity.

Measurement of thrombin times: The method of Ratnoff was used (13). Control clotting times (in absence of added anticoagulant) were in the range 15-18 seconds. If no clot formed in anticoagulant-containing tubes after 120 seconds, the ratio thrombin time with anticoagulant/without anticoagulant was recorded as infinity.

Amidolytic experiments: Details of the methods used have been described elsewhere (5). In brief, the procedures exploit the ability of thrombin and Xa to catalyse the amidolysis of synthetic chromogenic substrates. The extent of amidolysis was estimated by spectrophotometric measurement of the quantity of paranitroaniline released from substrates during a set incubation period. Direct effects of anticoagulants on enzyme activity were measured by pre-incubating anticoagulants with enzyme before substrate addition. Effects of anticoagulants on inactivation of thrombin and Xa by antithrombin III were measured by pre-incubating anticoagulant and antithrombin before addition of enzyme; substrate and polybrene were then added. The polycation polybrene was included to inhibit any direct inactivation of enzyme by the anticoagulant. Paranitroaniline released in the presence of anticoagulant was expressed as a fraction of that released in the absence of anticoagulant.

RESULTS

Effect on partial thromboplastin times: The effects on clotting times of incubating plasma with polyanetholesulphonic acid or with SP54 over a range

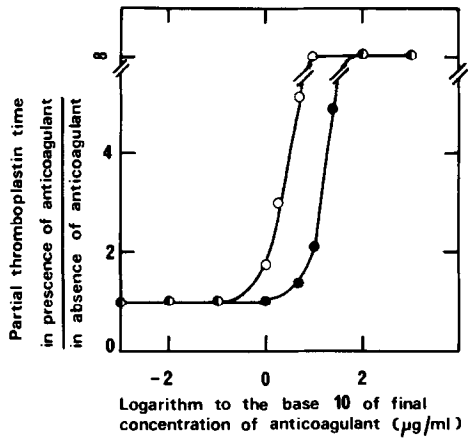

Figure 1. The effect on partial thromboplastin time of polyanetholesulphonic acid (●) and of SP54 (○).

of polymer concentrations are shown in Figure 1. Prolonged clotting times were recorded in the presence of both compounds. On a weight basis, SP54 was the more effective anticoagulant.

Effect of incubation with antiserum specific for human antithrombin III: A concentration of each polymer giving a substantial anticoagulant activity was selected. The partial thromboplastin times were then re-determined following pre-incubation of plasma (100µl) with antiserum specific for human antithrombin (30µl) at 37°C for 10 minutes before addition of polymer. Pre-incubation with antiserum reduced the anticoagulant activity of both compounds (Table 1).

Effect on thrombin clotting times: The effects of polyanetholesulphonic acid and SP54 on thrombin-initiated clotting over a range of polymer concentrations are shown in Figure 2. On a weight basis, polyanetholesulphonic acid was much more effective in prolonging the thrombin clotting time.

Effect on thrombin and Xa activity: Figure 3 shows the effects exerted by polyanetholesulphonic acid and SP54 on thrombin- and Xa-catalysed amidolysis of synthetic chromogenic substrates. Thrombin activity was unaffected, except at the highest concentration of polyanetholesulphonic acid examined (1 mg/ml) (Figure 3a). SP54, at high concentrations, slightly inhibited Xa-catalysed amidolysis; polyanetholesulphonic acid was much more effective than SP54 in inhibiting the action of Xa (Figure 3b).

TABLE 1

Effect of antiserum to antithrombin III on anticoagulant activity

	Anticoagulant concentration (μg/ml)	Partial thromboplastin time (secs)		Clotting ratio*	
		with antiserum	without antiserum	with antiserum	without antiserum
Control	-	45	52	-	-
Polyanetholesulphonic acid	10	90	78	2.0	1.5
SP54	2.5	112	82	2.5	1.6

*Ratio partial thromboplastin time in presence of anticoagulant/in absence of anticoagulant.

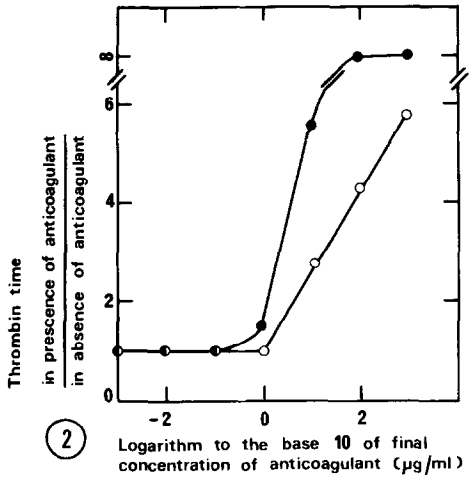

Figure 2. The effect on thrombin time of polyanetholesulphonic acid (●) and of SP54 (○).

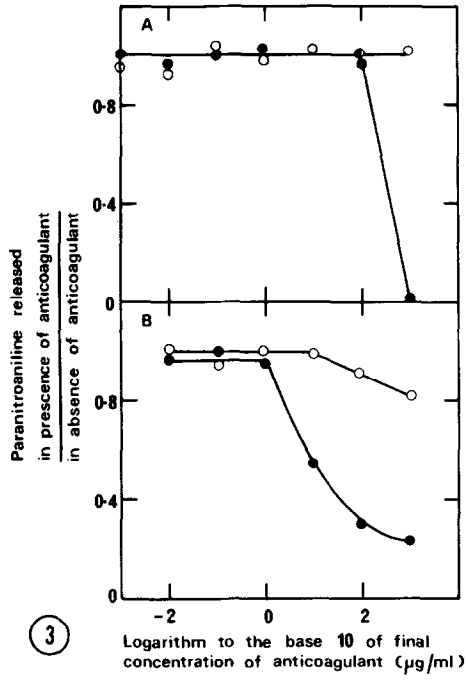

Figure 3. The effect of polyanetholesulphonic acid (●) and of SP54 (○) on amidolytic activity of A: thrombin; B: Xa.

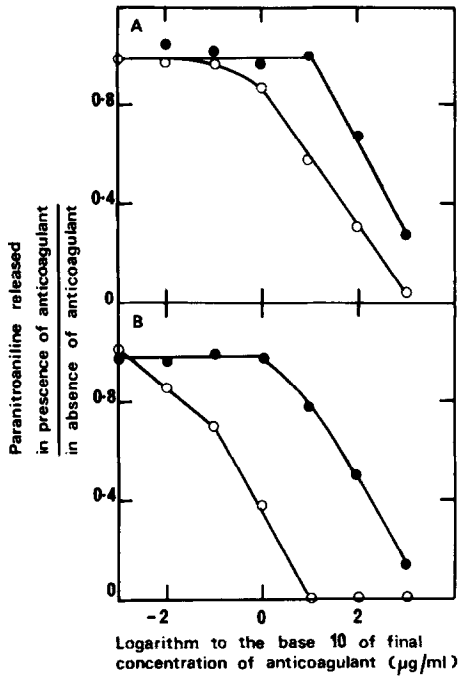

Figure 4. The effect of polyanetholesulphonic acid (●) and of SP54 (○) on antithrombin III-induced inactivation of A: thrombin; B: Xa.

Effect on inactivation of thrombin and Xa by antithrombin III: Both polyanetholesulphonic acid and SP54 potentiated inactivation of thrombin by antithrombin III; on a weight basis, SP54 was the more effective (Figure 4a). Similarly, both compounds potentiated inactivation of Xa by antithrombin III, SP54 being the more effective (Figure 4b).

SP54 was considerably more active in potentiating antithrombin III inhibition of Xa than of thrombin. Polyanetholesulphonic acid was slightly more effective in potentiating antithrombin III inhibition of Xa than of thrombin.

DISCUSSION

The prolongation of partial thromboplastin clotting times by polyanetholesulphonic acid and SP54 suggests that both compounds exert an anticoagulant effect by affecting one or more steps of the intrinsic pathway of coagulation. The reduction in the anticoagulant effects following pre-incubation of plasma with antiserum specific for antithrombin III has been noted for some other

anticoagulant anionic polymers (6), and indicates that the effects are, at least in part, mediated through this protease inhibitor. The different effects shown by the two compounds on thrombin clotting times suggest that, on a weight basis, SP54 is less efficient than polyanetholesulphonic acid in inhibiting the thrombin-catalysed step of the intrinsic pathway. The direct effect shown by high concentrations of polyanetholesulphonic acid on Xa catalysis may not contribute greatly to the anticoagulant effect of the compound, because the concentration of free Xa (and of other activated clotting factors) in plasma is likely to be low.

Our results suggest that, in ex vivo assays using synthetic chromogenic substrates, polyanetholesulphonic acid is approximately as effective in potentiating antithrombin III inhibition of thrombin as in potentiating antithrombin III inhibition of Xa. In contrast, SP54 is more effective in potentiating antithrombin III inhibition of Xa rather than of thrombin. These results are in accordance with a brief previous report describing the anticoagulant activity of SP54 (11), and are also in accordance with our observation that SP54 is relatively inefficient in inhibiting thrombin-initiated clotting.

The molecular basis of this differential action of SP54 is not yet known. Unfractionated heparin itself is reported to potentiate antithrombin III inhibition of thrombin and Xa to different extents, in a mixture containing the two proteases (9). Heparin preparations are known to contain a population of molecules varying in primary chemical structure, molecular size and charge, and some molecules are more potently anticoagulant than others (8). The relative specificity shown by SP54 in our experiments suggests that different antithrombin III-mediated effects exerted by heparin may be properties of discrete, chemically-distinct heparin molecules. At a time when the chemical and anticoagulant properties of fractionated heparins are still being resolved, this is necessarily a tentative speculation. Our results suggest, however, the continued use of anionic polymers of relatively well-defined and potentially variable structure as probes of heparin structure-activity relationships.

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