

NEW APPROACHES FOR ANTICOAGULATION IN EXTRACORPOREAL THERAPY

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Abstract

The need to fully heparinize patients undergoing extracorporeal therapy often leads to hemorrhagic complications. Two approaches have been used to solve this problem. The first involves full heparinization of blood entering the extracorporeal device followed by the elimination of heparin from the blood returned to the patient using an immobilized heparinase reactor system. Animal studies have demonstrated the successful elimination of heparin's anticoagulant activity using this reactor. The second approach uses very low molecular weight (VLMW) heparins with improved properties. Although low molecular weight heparins and heparinoids have been successfully used in hemodialysis, these preparations are polydisperse mixtures. New VLMW heparins are described which are pure, monodisperse, structurally defined drugs and show improved pharmacokinetics and greater specificity than heparin. The separation of ATIII and HCII mediated activity against factors IIa and Xa may permit extracorporeal therapy with only partial anticoagulation resulting in increased anti-thrombotic activity with decreased hemorrhagic side-effects. Finally, these VLMW heparins suggest certain desirable structural characteristics

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in the design blood compatible non-thrombotic synthetic polymers for use in extracorporeal devices.

INTRODUCTION

Extracorporeal devices are used in a variety of clinical situations including kidney dialysis, cardiopulmonary bypass, and plasmaphoresis. These devices rely on systemic heparinization to improve blood compatibility. Systemic heparinization often leads to hemorrhagic complications, and chronic exposure has been associated with thrombocytopenia and osteoporosis (1). The high incidence of hemorrhage (1) among the large number of patients undergoing extracorporeal therapy has resulted in heparin being cited as the drug responsible for most deaths in otherwise healthy patients (2).

Heparin is a polydisperse sulfated polysaccharide which acts to inhibit blood coagulation, in the fluid phase, by activating serine protease inhibitors antithrombin III (ATIII) and heparin cofactor II (HCII) (3,4). These activated inhibitors can then irreversibly inhibit one or more of the serine protease coagulation factors (FIIa - XIIIa) (3). Heparin also acts on platelets (5), on proteins on the vessel wall (6) and on other circulating proteins in a less well defined fashion (7).

One approach to avoiding heparin's side-effects would be to eliminate its systemic use in extracorporeal therapy while still preventing thrombosis. Despite the considerable effort devoted to developing blood-compatible materials from which devices could be fabricated (8), systemic heparinization continues to be routinely practiced in extracorporeal therapy.

We have proposed an approach, using an immobilized enzyme reactor, which would permit the anticoagulation of the extracorporeal device without relying on systemic anticoagulation of the patient (9). Initial studies of this device in dogs and sheep will be discussed.

The hazards associated with heparin's use have also prompted the search for heparin substitutes with improved properties. Their reduced effect on platelets (10), and reduced anticoagulant activity has resulted in decreased bleeding complications in extracorporeal therapy (11-14). These heparinoids generally act through ATIII and exhibit high anti-Xa to anti-IIa activity ratios (15). Recently heparinoids containing dermatan sulfate (DS) have been studied because of their capacity to inhibit IIa through HCII (14).

Our second approach to reducing the risk of anticoagulation in extracorporeal therapy involves the development of improved heparin-derived anticoagulants. This requires the preparation and structural characterization of monodisperse, homogenous, VLMW heparins. These are then screened in

vitro to obtain compounds which act specifically at defined sites in the coagulation cascade. Subsequent in vivo testing is then required to select the VLMW heparin having appropriate antithrombotic activity but not exhibiting the side-effects commonly associated with the use of heparin. Unlike heparin, and other heparinoids currently being studied, these VLMW heparins are homogenous preparations and should exhibit simpler pharmacokinetics, be easier to monitor, and thus result in improved dose control.

MATERIALS AND METHODS

Heparin, sodium salt, was from porcine mucosa and had a specific activity of from 140-170 U/mg. Heparinase (E.C. 4.2.2.7) was prepared from Flavobacterium heparinum (16) and purified to either catalytic purity (17) or homogeneity (18). Enzymatic activity was determined by the appearance of products (19) or the disappearance of substrate as measured chemically (16,20), enzymatically or by coagulation assays (20,21). Heparinase was immobilized onto cyanogen bromide-activated-Sepharose 4B (17) or agarose (22) beads. The reactors consisted of a Bentley infusion filter (DFT 100) and a Bentley arterial filter (AF 1025) for use in dog and sheep, respectively. In each case the devices were modified by the inclusion of an internal recirculation line (9,23) and the beads were entrapped within the particulate filter. The blood was pumped into the inlet port through a suspension of immobilized enzyme and out the exit port. The flow rates were 50 mL/min in the 10-20 Kg dogs and 250 mL/min in the 40 Kg sheep. Heparin was measured chemically (9,24) and its anticoagulant activities determined (9). Heparinase was also used to prepare substantial quantities of VLMW heparin which was fractionated on the basis of size (25) and charge (26) to obtain monodisperse homogenous VLMW heparins. These homogenous VLMW heparins were structurally characterized by chemical, enzymatic, spectroscopic, and electrophoretic methods (21,27). Their anticoagulant activity (20,21) and their ability to inhibit complement (25) activity were measured in vitro.

RESULTS

Heparinase was prepared from Flavobacterium heparinum (16) and purified by batch ion-exchange chromatography to obtain enzyme with high catalytic purity (17,18).

Heparinase acts on heparin to afford a distribution of VLMW-heparins (Figure 1). The kinetics of heparinase depolymerization of heparin was examined in vitro by a number of chemical, enzymatic and coagulation assays (Figure 2). Heparinase was immobilized onto Sepharose 4B with an activity recovery of 78-91% and onto high density cross-linked agarose.

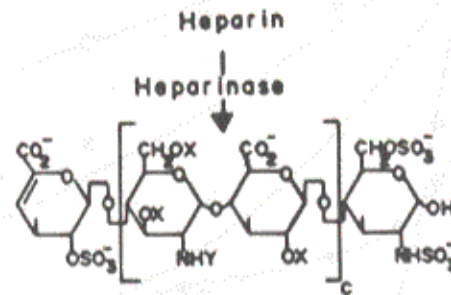


Figure 1. Action of heparinase on heparin. Heparin is cleaved by heparinase at specific sites. The products formed are a disaccharide ($c = 0$), tetrasaccharides ($c = 1$), etc.; X is SO_3^- or H and Y is SO_3^- or CH_3CO .

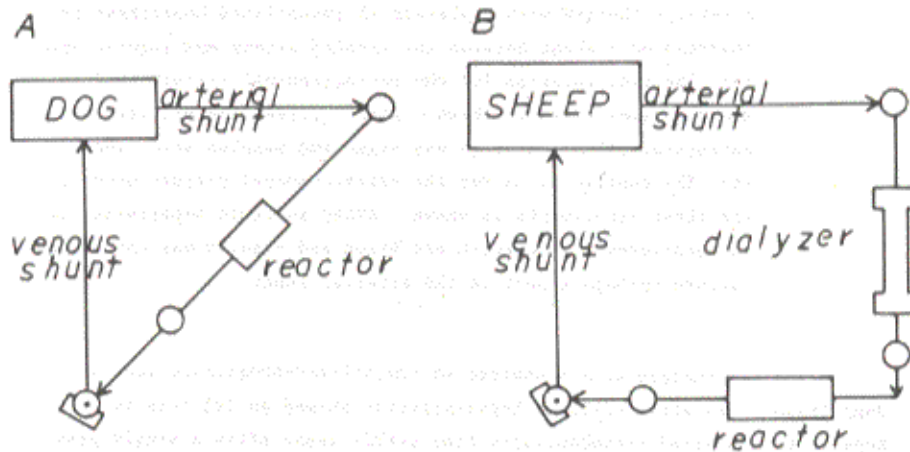


Figure 2. Kinetic profile of heparinase action on heparin. Heparin was treated with heparinase and aliquots were removed at various times and assayed for anticoagulant activity: for ATIII mediated anti IIa (\blacklozenge) and anti Xa (\times) activity; for HCII mediated anti IIa ($+$) activity; by aPTT assay (\diamond); by FXa coagulation assay (\blacksquare); and for metachromatic activity (\square). The heparin measured by these assays are plotted against reaction completion as measured by UV-232 assay.

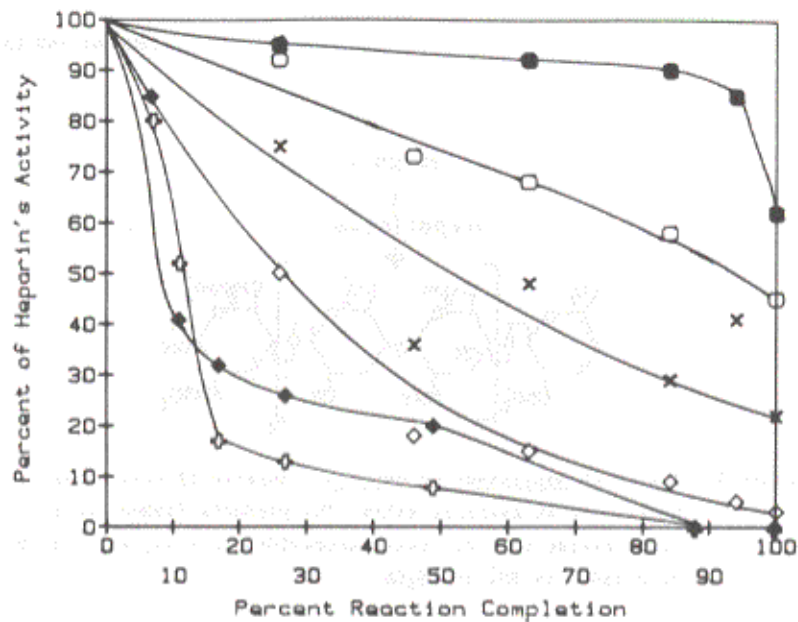


Figure 3. In Vivo deheparinization using an immobilized heparinase reactor. A reactor charged with Sepharose 4B immobilized heparinase is inserted on a shunt between the carotid artery and jugular vein. (a) The configuration for the extracorporeal circuit used in the dog experiments is shown. After systemic heparinization extracorporeal circulation was begun and samples were taken. (b) The configuration for the extracorporeal circuit used in the sheep experiments is shown. After systemic heparinization extracorporeal circulation was begun and heparin was continuously infused through a port in the arterial shunt.

A reactor charged with Sepharose 4B-immobilized-heparinase used in dogs (Figure 3a) after systemic heparinization showed an 80% loss in heparin by activated thromboplastin time (aPTT) assay after a single pass (9). Platelet and white cell counts dropped to 20% of their initial levels but returned to 30 and 70%, respectively, of their initial values by the end of the procedure (28). A reactor charged with agarose-immobilized heparinase used in sheep (Figure 3b) resulted in up to 70% single pass conversion (29). No hemolysis was observed and the red cell count remained at its initial level. The white cell and platelet count dropped to 50% of their initial value with the white cell count returning to normal by the end of the procedure (29). The decrease in formed blood components in these studies are similar to or less than that caused by other extracorporeal systems using these animal models. Toxicology studies showed

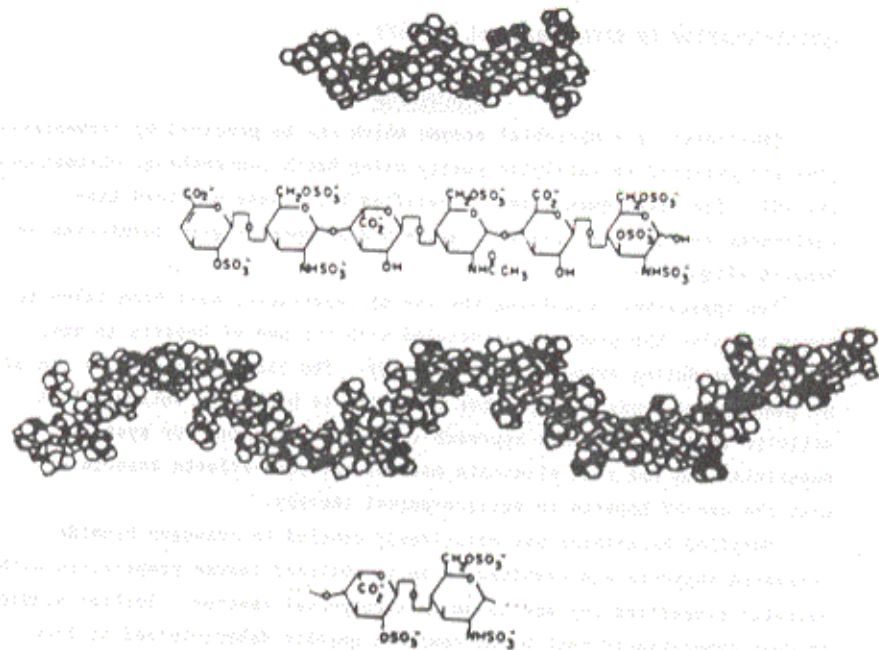


Figure 4. Structure of an anticoagulant hexasaccharide and a heparin chain.

The hexasaccharide (Mr 1833) and a heparin chain (Mr 14,000), comprised of a repeating trisulfated disaccharide, are shown in both spacefilling and Haworth formula representations.

that the products of this reactor were neither cytotoxic nor mutagenic at concentrations in excess of those which would be clinically anticipated (9). These products are also excreted much more rapidly than heparin in both normal and nephrectomized rats (30).

By treating gram quantities of heparin with heparinase in buffer solution and fractionating the products by gel permeation chromatography followed by strong-anion-exchange chromatography, milligram quantities of a variety of VLMW heparins were prepared (21,27). A disaccharide, five tetrasaccharides and a hexasaccharide were structurally defined using chemical, enzymatic, spectroscopic and electrophoretic methods (21,27).

The hexasaccharide (Figure 4) showed only low (5-10 wt% of heparin's activity) ATIII and HCII potentiated anti-IIa activity as measured by amidolytic assay using pure, homogenous plasma proteins. By aPTT assay this hexasaccharide had a specific activity of 68 Units/mg or 40 wt% of heparin's activity and approximately 30 wt% of heparin's capacity to inhibit complement activation (21,25).

DISCUSSION

Heparinase is a microbial enzyme which can be prepared by fermentation (16) and purified to catalytic purity using batch ion-exchange chromatography (17,18). The large quantities of purified heparinase obtained have sufficient catalytic activity to quickly depolymerize gram quantities of heparin (Figure 1).

Two approaches, involving the use of heparinase, have been taken to begin to solve the problems associated with the use of heparin in anticoagulation during extracorporeal therapy. The first involves the use of an immobilized heparinase reactor to eliminate heparin's anticoagulant activity (Figure 2). This approach can reduce the need for systemic heparinization and thus eliminate many of the side-effects associated with the use of heparin in extracorporeal therapy.

Purified heparinase was efficiently coupled to cyanogen bromide activated supports and resulted in an immobilized enzyme preparation with suitable properties for use in an extracorporeal reactor. Initial studies in dogs demonstrated that blood could be quickly deheparinized at flow rates of 50 mL/min with minimal damage to formed blood components (Figure 3a). Subsequent studies turned to sheep as an animal model because they have blood volumes comparable to man, can support higher flow rates during extracorporeal therapy, and have been widely used in artificial heart implantation studies (31). These studies (Figure 3b) have included an artificial kidney in the circuit and continuous infusion of heparin to more closely resemble the ultimate clinical aims for this device. The results showed a high efficiency of blood deheparinization, a modest effect on formed blood components (well within the range of a normal kidney dialysis procedure) and a high level of safety. Initial toxicology studies indicated that the products formed are relatively non-toxic and are cleared rapidly. In addition the use of high density cross-linked agarose beads decreased their susceptibility to rupture reducing the possibility of introducing the microbial enzyme into the animal and eliciting an immune response.

Heparinase acts on heparin to reduce its anticoagulant activity. Our early studies (20) found, however, the rate and amount of activity reduction depends on the assay used to measure that activity (Figure 2). The ATIII and HCII mediated anti-IIa activity of heparin was reduced more rapidly and to a greater extent than was ATIII mediated anti-Xa activity. Additionally, when immobilized heparinase was used in an extracorporeal circuit (Figure 3a) the reactor continued to support unrestricted flow

even after the heparin had been completely depolymerized and showed no activity by aPTT (9). These observations suggested that the products of heparinase depolymerization of heparin might exhibit anti-thrombotic activity *in vivo* while not demonstrating anticoagulant activity by conventional assays performed *in vitro*.

A second approach to reduce the side-effects associated with the heparin anticoagulation of extracorporeal circuits uses heparinase to prepare a mixture of VLMW heparins having reduced anticoagulant activities (Figure 2). These mixtures are then fractionated into homogenous VLMW heparins. While most of these components show no activity at all, some, such as a recently characterized hexasaccharide (Figure 4) have unusually high activities by certain assays. *In vivo* evaluation of the antithrombotic activity, pharmacokinetics, and metabolism of the structurally defined homogenous VLMW heparins is just beginning. The possibility of simultaneously inhibiting complement activation while preventing thrombus formation during hemodialysis by using a VLMW heparin is also being examined.

The results of these studies as well as the establishment of the mechanism by which these VLMW heparins function as antithrombotics may ultimately lead the way to a new class of heparinoid antithrombotics devoid of side-effects, which can be easily monitored and which display predictable pharmacokinetics.

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