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Low Molecular Weight Heparins in Clinical Practice

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Anticoagulant Mechanism of Action of Low Molecular Weight Heparins

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I. INTRODUCTION

In the mid-1960s when the blood coagulation process was formulated in the "cascade" [1] and "waterfall" [2] schemes, only the forward reactions were considered to be of importance. The role of naturally occurring plasma inhibitors in the regulation of thrombin generation was first studied in the years that followed. In a series of papers by Yin and co-workers [3,4] that appeared in 1971, it was postulated that "the efficiency of activated factor X inhibitor as an anticoagulant during normal blood coagulation (an action profoundly enhanced by heparin) may depend more on its preventing any generated activated factor X from activating prothrombin than it may on preventing thrombin from activating fibrinogen." When the same investigators discovered that the activity of the activated factor X inhibitor and that of the earlier described plasma inhibitor of thrombin belong to the same proteinase inhibitor, they suggested that the hemostatic balance was regulated through the neutralization of factor Xa by this inhibitor, presently termed antithrombin III (AT III). Consequently, these authors proposed the new name "activated factor Xa inhibitor" for AT III.

What experimental evidence was then available to arrive at the hypothesis that to date still forms the basis for the antifactor Xa philosophy of the antithrombotic action of heparin? First, it was assumed that because activated factor X is the responsible enzyme in the prothrombin activating complex (prothrombinase), little or no thrombin can be formed when activated factor X activity is rapidly

neutralized. Apparently, it was believed that the coagulation of blood occurs via a sequential series of proenzyme–enzyme conversions (the “cascade” hypothesis). Support for this hypothesis was found in the results of *in vivo* experiments [5,6]. It was observed that on a weight basis, factor Xa was a more potent thrombogenic agent than thrombin in an experimental stasis thrombosis model. Using the same arguments and assumptions, it was calculated that a complete inhibition of thrombin generation via inhibition of factor Xa could be achieved with a 1000-fold smaller amount of AT III than needed for direct inhibition of the generated thrombin [7]. Since heparin dramatically enhanced the rather slow reaction between activated factor X and AT III [8], it was thus logical to assume that the mechanism behind the low-dose heparin therapy of the prevention of postoperative deep vein thrombosis was the heparin-stimulated inhibition of circulating activated factor Xa [7].

Although this hypothesis was never verified experimentally and in fact cannot be tested directly, the very same reasoning provided the rationale behind the development and clinical use of heparin fractions/fragments of low molecular weight [9]. Andersson et al. [10] fractionated heparin by affinity chromatography on matrix-bound AT III and by gel filtration. The heparin fractions thus obtained were determined for their so-called “specific activity” and anticoagulant activity. The specific activity was defined as the ability of the heparin preparation to stimulate the AT III/factor Xa reaction as determined by the assay developed by Yin et al. [11]. The anticoagulant activity was defined as the potency of a heparin preparation to prolong the clotting time of a global test like the activated partial thromboplastin time (APTT). The heparin fractions with low molecular weight were found to have an anti-Xa/APTT ratio of 12, whereas heparin fractions with high molecular weights had an anti-Xa/APTT ratio of less than 1.

Johnson et al. [12] were the first to demonstrate that when given subcutaneously to humans, a low molecular weight heparin (LMWH) resulted in much higher antifactor Xa levels than did unfractionated heparin (UFH). When the LMWH preparations were further tested for their antithrombotic efficacy in an experimental venous stasis thrombosis model developed by Wessler [13], evidence was obtained that the antithrombotic action of heparin could be dissociated from its hemorrhagic one when the anti-Xa/APTT ratio of the heparin was 2 or more [14]. It was then generally believed that LMWHs prevent experimental thrombosis primarily by enhancing the inhibitory effect of the antifactor Xa activity. In addition, these first data also suggested the occurrence of fewer hemorrhagic complications. Because these LMWHs have a diminished effect on overall clotting (less than half that of UFH), the global anticoagulant activity of heparin was thought to cause the bleeding problems during heparin therapy [14].

Studies on the structure–function relationship showed that in addition to a specific pentasaccharide sequence for the interaction with AT III, additional

chain length is required for a stimulating effect of heparin on the thrombin-AT III reaction. Thus, whereas 5 saccharide units are sufficient to stimulate the factor Xa-AT III reaction, at least 18 saccharide units are required for the inhibition of thrombin (see Ref. 15 for an overview). Because LMWH consists of chains with a length of 2 to 30 saccharides (mean molecular weight of 4000 to 6000 D), their lower anticoagulant specific activity, as determined in the global APTT and kaolin cephalin clotting time (KCCT) test and in a thrombin clotting time (TCT) test system, is thought to be caused by lower anti-IIa specific activity in LMWH than in UFH (mean molecular weight 15,000 D; chains of length 10 to 90 saccharide units) [16].

The reason why the APTT and KCCT tests are less sensitive for LMWHs than for UFH is not clear. Although diminished anti-IIa activity might easily explain the lower activity of LMWH in the TCT, the same reasoning cannot be applied on a global test such as APTT and KCCT if one accepts that the underlying principle of the anticoagulant action of LMWH is that direct action on thrombin is much less efficient than inhibition of the prothrombin activator. It has therefore been suggested that there are other mechanisms by which UFH might exert its anticoagulant activity (e.g., an AT III-independent inhibition of the activation of factor IX) [10]. Lane et al. [17] found this explanation unlikely because they could demonstrate a close correspondence between TCT and KCCT results in a study on the anticoagulant activities of heparin fractions.

Since about a third of whole heparin has AT III-binding properties and thus a small fraction of the total heparin preparation accounts for all the anticoagulant activity [18], it might be possible that the lower molecular weight fractions contain less AT III-binding material than do the high molecular weight fractions, and as a result caused diminished anticoagulant activity. However, as pointed out by Barrowcliffe et al. [19], this seemed to be unlikely because they found that there are no major differences between the fractions in their content of AT III-binding material. Thomas et al. [20] suggested that LMWH is unable to inhibit the relatively large amount of factor Xa generated rapidly by optimal surface activation of the intrinsic pathway, but is nevertheless able to neutralize the relatively small amounts produced by other stimuli. However, until now no experimental evidence has been presented that supports that notion. Thus on the basis of the idea that inhibition of factor Xa is the most efficient way to impair thrombin generation, it remains unexplained why LMWH has less anticoagulant activity than UFH in APTT and TCT assays.

In pursuing the anti-Xa concept, ultralow molecular weight heparin (ULMWH) fractions/fragments (mean molecular weight less than 3000 D) were prepared and tested in animal models [21-23]. However, no correlation between ULMWH blood levels as measured by anti-Xa assays and impairment of experimental venous stasis thrombosis could be established. Moreover, it is becoming increasingly apparent that the antithrombotic effects of glycosaminoglycans

might relate solely to their enhancing of the reaction between thrombin and AT III and/or the reaction between thrombin and heparin cofactor II [24,25]. Yet heparin fractions that have antifactor Xa activity alone, such as the synthetic pentasaccharide heparin, have an antithrombotic effect, although it is rather weak [23,26,27]. Thus it seems reasonable to assume that an effective antithrombotic drug must have both antithrombin and antifactor Xa activity. However, it must also be emphasized that the extent to which heparin is able to impair experimental thrombosis depends largely on the type of thrombosis model and type of stimulus used [28]. Unfractionated heparin and a ULMWH produced comparable antithrombotic effects in a model of platelet-dependent thrombus formation when equivalent levels of anti-Xa activity were maintained. However, such a correlation was not found in a model of fibrin-dependent thrombosis formation, nor in a venous stasis model [29].

Clearly, caution should attend the extrapolation of *in vitro* global anticoagulant or specific antiprotease potency figures to antithrombotic efficacy. As a matter of fact, even the anticoagulant mechanism of action, thus the way by which heparin (LMWH in particular) impairs thrombin generation in clotting plasma remains uncertain. This has consequences not only for our understanding of the relationship between anticoagulant and antithrombotic action of heparins, but also causes confusion around the establishment of an international standard for LMWH and the type of assays to be used to determine potency figures for UFH and the various LMWH preparations [30–32].

At present, we have to appreciate that the antifactor Xa philosophy developed beginning in the early 1970s to explain the antithrombotic action of low-dose heparin and later that of LMWHs cannot be maintained in view of our current knowledge of the process of blood coagulation and of how this process is regulated. To view the process of blood coagulation mainly in terms of sequential proenzyme–enzyme conversion reactions is certainly not correct and has to be replaced by a staged cascade model in which the generation of multi-component enzyme complexes are of paramount importance [33]. This chapter therefore contains a section on the mechanism of thrombin generation as it has emerged from biochemical studies.

Oofosu and co-workers [34] were the first to suggest that prevention of prothrombin activating complex formation rather than the inhibition of factor Xa might be the key step for the anticoagulant action of heparin. Uncertainties about the role of antifactor Xa activity in the anticoagulant mechanism of action of LMWH caused renewed interest in the other antiprotease activities of LMWHs and most of all, antithrombin activity. In view of the mean molecular weight of the LMWH preparations (about 4500 D), the assumed homogeneity with respect to the maintained anti-Xa activity might no longer be relevant. Instead of that, an even higher degree of heterogeneity than established for unfractionated heparin could be expected. That is, the biologically active heparin species within a

LMWH preparation are then to be found in those heparin species whose chain length exceeds the mean molecular weight. Because these heparin species must also contain the AT III-binding region, the molecular weight distribution of a LMWH preparation alone is not sufficient to describe the heterogeneity of a LMWH preparation. The distribution of the AT III-binding species must also be taken into account. Surprisingly, despite an extensive search for biological differences among the various LMWH preparations, the latter obvious cause for heterogeneity has not been recognized [35].

Here we wish to present studies and findings from our laboratory which support the hypothesis that the process of blood coagulation is regulated at the level of formation of activated blood clotting factor complexes. Heparins, including LMWHs, impair thrombin generation by inhibition of the generation of the essential cofactors for factor Xa and thrombin generation, either directly via their antithrombin activity or indirectly via their antifactor Xa activity.

II. MECHANISM OF THROMBIN GENERATION

A. Initiation

When blood coagulation is initiated in a natural way (tissue damage, vessel wall lesion) or by artificial means such as kaolin (KCCT), elagic acid (APTT), or thromboplastin (PT), the first traces of factor Xa and thrombin are generated by enzymes lacking their cofactor (Figure 1A). At this (hypothetical) stage of the

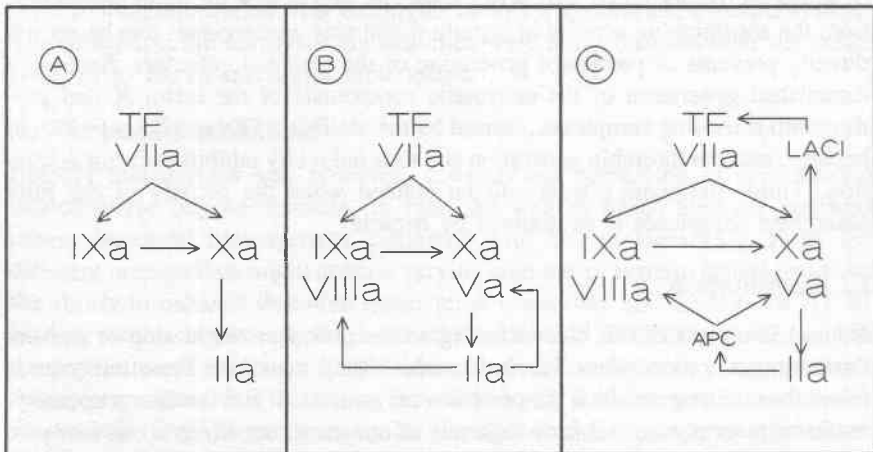


Figure 1 Mechanism of thrombin generation: (A) initiation; (B) propagation; (C) termination.

blood-clotting process, thrombin generation is the result of a series of sequential reactions, and indeed, inhibition of the activator might be much more efficient to achieving an anticoagulant effect than inactivation of the enzyme generated. Under such conditions, antifactor IXa and/or antifactor Xa activity will contribute largely to the anticoagulant activity of heparin. Evidently, when the anti-Xa activity is the common and sole mechanism of action of heparin, LMWH, and ULMWH, all heparin preparations will impair the APTT test equally well. Since the opposite is true, one might expect that the plasma clotting process at this stage is not sensitive for the antifactor Xa activity of heparin.

B. Propagation

The free clotting enzyme factors IXa and Xa are not very efficient activators of factor X and prothrombin, respectively. In the presence of their respective cofactors, activated factors VIII and V, and a procoagulant surface (activated blood platelets), their catalytic efficiency increases four to five orders of magnitude [33]. To date it is not clear which activators of factors V and VIII are physiologically relevant. There is no doubt that thrombin is a very efficient activator of both factors VIII and V [36], but when thrombin activity is neutralized, factor Xa might also directly initiate the generation of both activated factors V and VIII [37,38]. However, trace amounts of thrombin are already sufficient to generate activated factors V and VIII. As a result, thrombin drastically enhances its own generation (Figure 1B).

At this stage of the blood clotting process it becomes very difficult to predict what specific antiprotease activity of heparin could prevail. Since the generation of activated factors V and VIII is of paramount importance for thrombin generation, the antithrombin activity of heparin might play an important role because it directly prevents or postpones generation of the essential cofactors. Second, a diminished generation of the enzymatic compounds of the factor X and prothrombin activating complexes, caused by the antifactor IXa and Xa activities of heparin, reduces thrombin generation and thus indirectly inhibits cofactor activation. Third, the same effects will be obtained when the activity of the fully assembled complexes is neutralized by heparin.

C. Termination

Natural inhibitors of the blood clotting serine proteases might stop or prevent thrombin generation when the rate at which they inactivate these enzymes is faster than the rate at which the proteases are generated. But because a nonenzymatic cofactor is essential for a high rate of enzyme production, it can easily be conceived that destruction of the cofactor would be a very efficient way to turn off thrombin generation. The major negative feedback loops are shown in Figure 1C: the protein C pathway [39] via which activated factors V and VIII are

destroyed and the neutralization of tissue factor by LACI (lipoprotein-associated coagulation inhibitor) [40]. The effects of heparin on the latter reaction system is complex. Heparin alone (in the absence of AT III) accelerated the initial rate of tissue factor inhibition [40]. As yet, no data are available on the differential effects of heparin chain length and the presence of AT III binding sites on the LACI-dependent neutralization of tissue factor.

It has been suggested that activated protein C (APC) by inactivating factor Va might liberate factor Xa. This noncomplexed factor Xa is then rapidly scavenged by AT III-heparin [41]. To what extent heparin is able to prevent the inactivation of thrombin so as to result in a reduced amount of thrombomodulin-thrombin complexes at the endothelial surface, causing diminished protein C activation, is at present not known.

III. SPECIFIC ANTIPROTEASE ACTIVITIES OF LMWHs

Unfractionated heparin, with a mean molecular weight of 12,000 to 15,000 D has, by definition, an antifactor Xa/antithrombin ratio of 1. LMWHs (mean molecular weights between 4000 and 6000 D) have ratios of 2 to 4 or more. Ultralow molecular weight (<3000 D) heparins have only antifactor Xa activity. The theoretical background for this is given by Lane et al. [42], who clearly showed by using monodisperse oligosaccharides with high affinity for AT III in well-defined systems and under well-controlled conditions that an octadecasaccharide is the smallest heparin chain ($M_R = 5400$) that will span the saccharide sequence required to stimulate the AT III-thrombin reaction. Thus they observed an increase in antithrombin activity expressed in units per millimole of heparin from 16 monosaccharide units and higher in both a purified and a plasma system. The molar-specific antifactor Xa activities were fairly constant over the entire range (8 to 18) of saccharide units tested.

A. Thrombin Inhibition

LMWH preparations are, however, certainly not comprised of well-defined monodisperse oligosaccharides. In fact, the way in which they are prepared causes increased heterogeneity compared with that of UFH [35]. First, the different preparations might contain varying amounts of heparin chains that have the ability to enhance thrombin inactivation when they also contain the AT III binding sequence. Second, the distribution of the AT III binding species within the population of the longer (>18 saccharides) chains might vary from preparation to preparation. For this reason it would be very interesting to know how for the various LMWH preparations the functional anti-IIa heparin species are distributed over the total heparin preparation. That is, the interesting question is: How does the mean molecular weight of the entire LMWH preparation compare with that of the anti-IIa species?

1. Exogenous α -Thrombin: Purified System

When human α -thrombin is incubated with an excess of human AT III, thrombin activity decays according to a monoexponential model. The pseudo-first-order rate constant of inhibition of thrombin can be calculated from the slope of a semilogarithmic plot of residual thrombin activity versus time. When heparin is added to this system a linear relationship is found between the amount of heparin added and the rate constant. The kinetic constants of inhibition with the various heparins, expressed in pseudo-first-order rate constants per microgram of heparin per milliliter, are depicted in Figure 2A. The heparin-dependent reaction between thrombin and AT III is affected by calcium ions [43]. It is evident from Figure 2A that Ca^{2+} decreases the rate constant of inhibition of thrombin equally well for all LMWH preparations. When these values are compared with their anti-IIa-specific activities as provided by the manufacturers, a close correlation is found (Table 1).

2. In Situ-Generated Thrombin: Purified System

It might be argued that the kinetics of inhibition of endogenously generated thrombin differ from that of added α -thrombin because of the presence of a number of reaction products, such as prothrombin fragment 2 [44]. We have therefore examined the heparin-dependent neutralization reaction between AT III and the thrombin activity formed during prothrombin activation [45–47]. Much to our surprise we found that part of the thrombin activity generated by the prothrombin converting complex was insensitive for the heparin-stimulated reaction. We could demonstrate that with increasing prothrombin concentration the amounts of meizothrombin (des fragment 1) increased proportionally while the amounts of α -thrombin decreased concomitantly. From another study [48] it became clear that heparin has no affinity for meizothrombin (des fragment 1), and this could be the reason why the reaction between AT III and meizothrombin (des fragment 1) was not stimulated by heparin.

In situ-generated thrombin was inhibited by AT III–heparin more slowly than by purified thrombin. Because the presence of prothrombin activation products was the only difference, we concluded that a noncovalent interaction between thrombin and prothrombin fragment 1.2 or prothrombin fragment 2 caused the rather complex reaction kinetics.

3. In Situ-Generated Thrombin: Plasma System

The plasma situation is expected to be different from that of a purified system because a number of heparin-neutralizing proteins might be present. Competition between these proteins and AT III for heparin are thought to influence the kinetics of the heparin-dependent thrombin inhibition. Therefore, the data

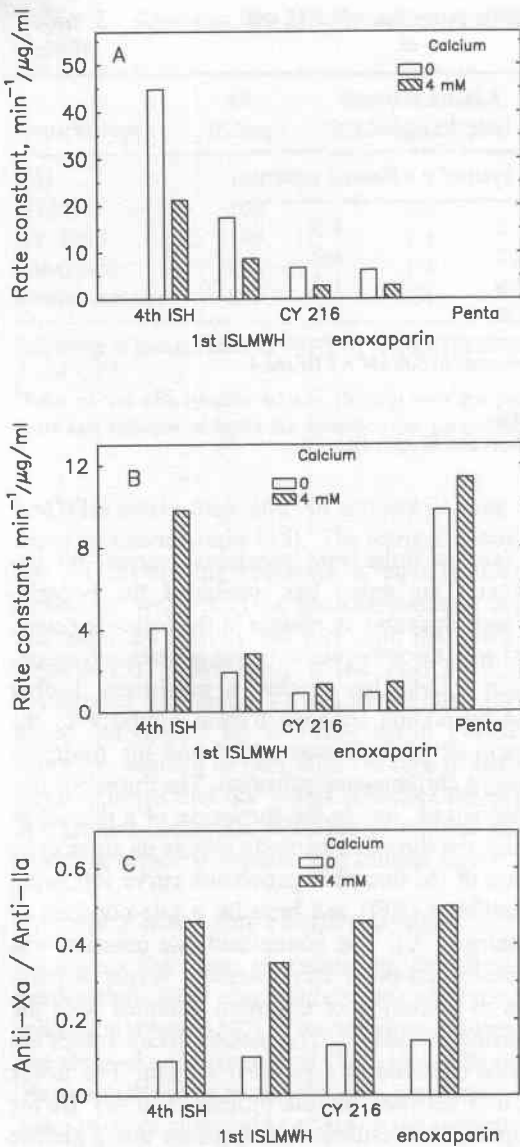


Figure 2 Rate constants for inhibition of human α -thrombin and human factor Xa, with and without Ca^{2+} , by heparins. (A) Human α -thrombin (10 nM) was incubated with human AT III (500 nM) in the presence of varying amounts of heparin. (B) Human factor Xa (10 nM) was incubated with human AT III (500 nM). (C) The ratio of the heparin-dependent rate constants of inhibition of factor Xa over the heparin-dependent rate constants of inhibition of thrombin.

Table 1 Antithrombin (aIIa) Specific Activities of UFH and LMWH

Heparin type	aIIa (IU/mg)	Kinetic constant [min ⁻¹ (μg/mL) ⁻¹]	
		Purified system ^a	Plasma system
ISH ^b	180	21.2	8.5
ISLMWH ^c	67	8.7	nd ^d
CY 216	10–20	2.8	1.8
Enoxaparin	30	2.8	1.2

^aInhibition of human α -thrombin in the presence of 500 nM AT III and 4 mM Ca²⁺.

^bFourth International Standard for Heparin.

^cFirst International Standard for Low Molecular Weight Heparin.

^dnd, not determined.

obtained in a purified system (second-order rate constants) cannot be extrapolated to the plasma situation. Our group has examined the heparin-dependent inactivation of endogenous thrombin in plasma in the following way. Defibrinated plasma was activated with thromboplastin or via contact activation. A few minutes after the thrombin activity has reached a maximum, further thrombin formation was blocked by adding soybean trypsin inhibitor to the plasma. At the same time, heparin or LMWH was added and the thrombin activity was monitored in time using a chromogenic substrate. The thrombin-like activity was never completely neutralized, due to the formation of a thrombin/ α_2 -macroglobulin complex in which the thrombin partially retains its amidolytic activity [49]. From the decay phase of the thrombin generation curve following the addition of soybean trypsin inhibitor (STI) and heparin, a rate constant of inhibition of thrombin was determined [50]. The normalized rate constant was obtained from a plot of the pseudo-first-order rate constant versus heparin concentration. The rate constants of inhibition of thrombin obtained with the various heparin preparations are listed in Table 2. The plasma decay values are surprisingly low compared to those obtained in a purified system. The lower values are explained by competition between plasma proteins and AT III for heparin (Hemker and Béguin, unpublished results). It is apparent that a greater competitive effect is seen with UFH.

B. Factor Xa Inhibition

Heparin chains that contain the specific AT III-binding sequence enhance the reaction between AT III and factor Xa, irrespective of their length. Thus

Table 2 Antifactor Xa Specific Activities of UFH and LMWH

Heparin type	aXa (IU/mg)	Rate constant ^a [min ⁻¹ (μ g/mL) ⁻¹]	Ratio ^b
ISH	180	9.8	18.4
ISLMH	168	2.8	60.0
CY 216	80	1.3	61.5
Enoxaparin	115	1.4	82.1
Pentasaccharide	800	11.4	70.2

^aInhibition of human factor Xa by AT III (500 nM) in the presence of 4 mM Ca²⁺.

^bRatio of the aXa specific activity (IU/mg) over the pseudo-first-order rate constant of factor Xa inhibition per μ g heparin/mL.

LMWHs retain their anti-Xa activity as long as the chain length exceeds 5 or more saccharide units [15]. The intriguing question, however, is whether or not the AT III-binding sequence is equally distributed over the entire range of molecular weights. This question became urgent when it was found that the antifactor Xa activity did vary with the chain length of an AT III high-affinity heparin [51]. Moreover, it has also been reported that when factor Xa is complexed with factor Va at a phospholipid surface, it is partially protected from being inactivated by AT III-heparin [45,52,53]. The extent of protection, however, seemed to vary with the type of heparin used [51]. Another important observation is that Ca²⁺ ions stimulate the heparin-dependent reaction between AT III and factor Xa. Whether or not this stimulating effect was the same for different types of heparin and human factor Xa is not clear [41].

1. Free Factor Xa: Purified System

Following the same procedure as described for thrombin, different LMWH preparations were examined on their catalytic efficiency in the AT III-factor Xa inhibition reaction [47]. The experiments were performed with human proteins in the absence and presence of Ca²⁺ ions. The rate constants of inhibition of factor Xa, normalized as to weight unit, are shown in Figure 2B. It is seen that Ca²⁺ has a twofold stimulating effect on the antifactor Xa activity of UFH. In contrast, the antiXa activity of pentasaccharide is not significantly influenced by calcium. When these data were compared with the anti-Xa specific activities given by the manufacturers, a marked difference became apparent between UFH and the LMWHs. This difference is probably caused by the use of both human factor Xa and Ca²⁺ ions in our assay, where bovine factor Xa and human citrated plasma are used in the conventional anti-Xa assay (either chromogenic or clot-based).

2. Factor Xa Inhibition During Prothrombin Activation

Studies of purified systems have demonstrated that the AT III-dependent rate of inactivation of factor Xa is reduced when factor Xa is bound to a phospholipid surface in the presence of factor V [51] or factor Va [45,52] or when bound to activated platelets [53]. It was also shown that as part of the prothrombinase complex, factor Xa is partially protected from heparin-catalyzed inhibition [45,51,53]. The effect of the molecular size of heparin on the rate constant of inactivation of prothrombinase, and consequently on the extent of protection, has been studied, but the data available are conflicting. In one study it was observed that when factor Xa is bound to activated platelets, the protective effect diminishes with decreasing heparin molecular weight [53], whereas in another study, where factor Xa was bound to a phospholipid surface in the presence of factor V, it appeared that the protective effect became more pronounced with decreasing molecular weight [51]. The latter observation led to the postulation that the lack of correlation between *in vitro* antifactor Xa activity and anti-thrombotic action of LMWH is caused by different inhibitory actions on free factor Xa and on factor Xa in its more physiological form (i.e., as part of the prothrombinase complex) [51].

In an attempt to imitate physiological conditions of factor Xa inactivation as closely as possible, we studied the effect of AT III and heparin on factor Xa *during* human prothrombin activation in the presence of an excess of factor Va and phospholipid, so that all factor Xa was complexed with factor Va at a phospholipid surface. The catalytic specific activities of the heparins increased with increasing molecular size for both the inhibition of prothrombinase and factor Xa. A 10-fold increase over the entire molecular weight range (1700 to 20,000 D) was found [47]. In contrast with the results obtained by others [51,53], all the heparins showed a fivefold higher rate of inhibition of factor Xa compared with the inhibition of prothrombinase (Table 3). This indicates that the factor Va-mediated protection of factor Xa from inhibition by AT III-heparin is independent of the molecular size of the heparin. The discrepancy between our results and that of others is explained largely by the way in which the inactivation of factor Xa was compared with that of prothrombinase. We reasoned that the only relevant comparison is made when the inactivation is studied under exactly the same conditions: during prothrombin activation with factor Va as the only variable component [47].

C. Factor IXa Inhibition

The majority of studies on the regulation of factor IXa by plasma proteinase inhibitors have been done with purified enzyme either in plasma or buffer [54–59]. It has been shown that bovine and human factor IXa react slowly with AT III but do not react with α_2 -macroglobulin and α_1 -proteinase inhibitor

Table 3 Ratio of the Rate Constant of Inhibition of Free Factor Xa to the Rate Constant of Inhibition of Prothrombinase

Heparin	M_r	aXa/aPTase
Pentasaccharide	1,714	8.0
Octasaccharide	2,400	5.7
10–14 Saccharide	3,600	6.7
CY 216	4,500	4.4
18–24 Saccharide	6,300	5.3
Fourth International Standard for Heparin	15,000	4.3
HMW heparin	20,200	4.5

Source: Ref. 47.

[54,56]. It has been proposed that simultaneous binding of the inhibitor and enzyme to the same heparin molecule is required for maximal rates of inactivation of factor IXa [55].

Indeed, we found that pentasaccharide did not stimulate the bovine factor IXa–AT III reaction [59]. Another interesting finding of the same study was that Ca^{2+} ions are absolutely required for the heparin-dependent reaction between bovine factor IXa and AT III. In a recent study from our group [60] on the heparin-stimulated inhibition of factor XIa generation and factor IXa neutralization in human plasma it was found that factor IXa-induced factor IXa activity was not neutralized in recalcified plasma. However, when UFH was present, factor IXa was readily inactivated: a half-life time of 8 s in the presence of 1 μ g UFH/mL (or 0.15 U/mL). The same weight of pentasaccharide also caused a shortening of the half-life time (52 s).

Both the calcium requirement and the fact that pentasaccharide also stimulates the AT III–factor IXa reaction suggest that the reaction is different from that between thrombin and ATIII. In the latter case, calcium reduces the heparin-stimulating effect and the heparin chain must contain more than 18 saccharide units to achieve a so-called “approximation effect,” where binding of both the enzyme and the inhibitor to the same heparin molecule determines the anti-IIa activity of the heparin molecule.

D. The Anti-Xa/Anti-IIa Ratio of Heparin and Heparin Fractions

For different heparin preparations we compared the ratios of the specific catalytic antifactor Xa activity over the specific catalytic anti-IIa activity, as determined

with purified human proteins and in the presence of calcium (Figure 2C). It is seen that ratios of the activities were the same for the Fourth International Standard for UFH and the First International Standard for LMWH, enoxaparin, and CY 216 [61]. Additional findings from our laboratory also suggest that the heparin chains with anti-Xa activity in a LMWH preparation have to be found among the longer heparin chains. Consequently, the mean molecular weight as determined for the total LMWH preparation does not necessarily represent the mean molecular weight of the functional LMW heparin species and might be considered to be a misleading parameter [61].

IV. INHIBITION OF THROMBIN GENERATION IN PLASMA

On the one hand, it is now generally accepted that the anticoagulant potency of a particular heparin preparation cannot be predicted from its specific (antiprotease) activities (i.e., inhibition of factor Xa, thrombin, and factor IXa activity). On the other hand, no consensus has yet been obtained on the question of which of the various possible antiprotease activities are essential for the anticoagulant mechanism of action of heparin and its fragments/fractions. For that reason, a logical extension of the heparin work with purified blood coagulation proteases and inhibitors is a study in plasma. We reasoned that any reaction that can be shown not to play a role in plasma is very unlikely to be important *in vivo*. Although the reverse is not true, studies in plasma are an interesting and necessary step in the process of understanding the *in vivo* action of heparin.

A. Prothrombinase as a Target of Heparin Action in Plasma

From biochemical studies it is expected that significant thrombin generation occurs only when factor Xa is complexed with factor Va in the so-called "prothrombinase complex." The formation of the complex, as well as its activity, is thought to be an important target for heparin action. Unfortunately, no assays for plasma prothrombinase activity are available. To obtain information about the effects of heparin and its fragments/fractions on prothrombinase, we therefore developed a mathematical procedure to calculate the prothrombinase activity from experimentally derived thrombin generation curves.

1. Method of Determination

Thrombin activity as generated in plasma by thromboplastin, for example, can be measured easily and reliably with a chromogenic substrate. Typical examples of thrombin generation curves obtained in the absence and presence of LMWH are shown in Figure 3A. From these curves the effects of heparin on the conversion of prothrombin into thrombin (the generation of prothrombinase activity) can be

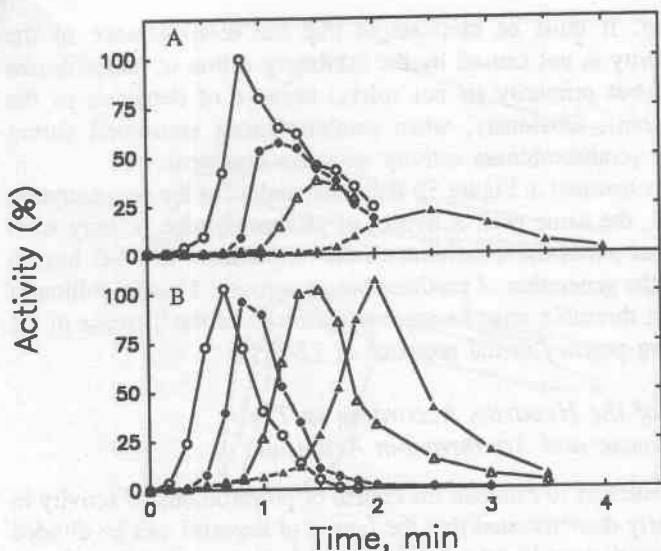


Figure 3 Effect of the low molecular weight heparin CY 216 on the generation of thrombin (A) and prothrombinase activity (B) in thromboplastin-activated plasma. The activities are expressed as a percentage of the maximum amounts in the absence of CY 216 (i.e., 320 nM thrombin and a prothrombinase activity of 700 nM thrombin/min). The concentrations of CY 216 were: \circ , none; \bullet , 2.5 $\mu\text{g/mL}$; \triangle , 10 $\mu\text{g/mL}$; \blacktriangle , 20 $\mu\text{g/mL}$.

estimated as follows. The experimentally observed rate of thrombin formation (the tangent to the thrombin generation curve) is at any moment the sum of two processes (1) the rate of conversion of prothrombin into thrombin by prothrombinase and (2) the rate at which thrombin activity is neutralized by anti-proteases in plasma. The rate of inhibition of thrombin at any moment can be calculated from the concentration of thrombin at that time and the pseudo-first-order rate constant of inhibition of thrombin in plasma (see Section III.A.3). There is one complicating factor—that the thrombin-like amidolytic activity in plasma arises not only from free thrombin but also from a complex formed between thrombin and the protease inhibitor α_2 -macroglobulin. However, with a variable heparin concentration, the contribution of the latter could be eliminated. For more details about the method, the reader is referred to a publication of Hemker et al. [49].

2. Heparin and the Generation of Prothrombinase Activity in Thromboplastin-Activated Plasma

Typical examples of calculated prothrombinase activity generation curves are shown in Figure 3B. The generation of prothrombinase activity is followed by a

disappearance phase. It must be emphasized that the disappearance of the prothrombinase activity is not caused by the inhibitory action of antiproteases (AT III) in plasma but primarily (if not solely) because of depletion of the substrate (prothrombin). Obviously, when prothrombin is consumed during plasma clotting, the prothrombinase activity will also disappear.

It is clearly demonstrated in Figure 3B that independent of the concentration of the LMWH used, the same peak activities of prothrombinase activity were found, indicating that under the conditions of this experiment LMWH has no inhibitory effect on the generation of prothrombinase activity. Thus inhibition of the net generation of thrombin must be entirely the result of the increase in the thrombin-neutralizing potency in the presence of LMWH.

3. Classification of the Heparins According to Their Antiprothrombinase and Antithrombin Activities

The method that enables us to estimate the course of prothrombinase activity in clotting plasma clearly demonstrated that the family of heparins can be divided into two classes: those that act like the smallest heparin fragment with antifactor Xa activity (pentasaccharide) and those that act like standard UFH [62]. The pentasaccharide-like heparins (P-class) had no influence on the decay of endogenous thrombin, but at high dosages they impaired the thrombin generation curve by their antiprothrombinase activity. This antiprothrombinase activity might result from an inhibition of prothrombinase formation (by inhibiting free factor Xa) and/or by a direct inhibition of prothrombinase once it is formed. When a limited series of LMWH preparations and gel filtration fractions of them were tested it was quite surprising to find that despite their increased anti-Xa/anti-IIa ratios the LMWH preparation could be classified as S-type heparins, thus acting on thrombin generation in plasma via their antithrombin activity. Only those fractions that were devoid of anti-IIa activity belonged to the P-class heparins [62-64].

4. Heparin and the Generation of Factors Xa and Va in Thromboplastin-Activated Plasma

From Figure 3B it is also clear that LMWH does dose-dependently prolong the lag phase of the appearance of the prothrombinase activity and thus the onset of thrombin generation. Since factors Xa and Va contribute equally to prothrombinase activity, it remains to be established to what extent its generation is inhibited by LMWH. To this end, generation of factors Xa and Va was monitored in thromboplastin-activated plasma.

Figure 4A shows the effect on factor Xa generation of increasing amounts of a LMWH preparation. As might be expected from its antifactor Xa activity (80 IU/mg), CY 216 did reduce the factor Xa yield. Interestingly, a lag phase in the

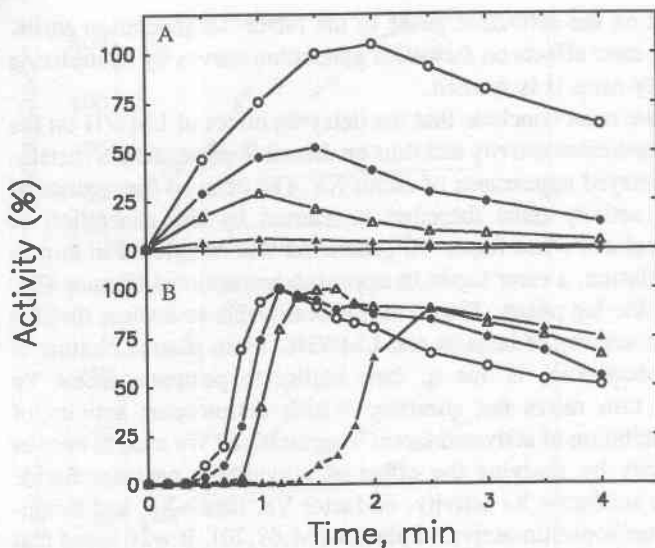


Figure 4 Effect of low molecular weight heparin CY 216 on the generation of factor Xa (A) and factor Va (B) in thromboplastin-activated plasma. The activities are expressed as indicated in Figure 3. The peak value of factor Xa was 12 nM and that of factor Va 25 nM (100% activity). For symbols, see Figure 3.

appearance of factor Xa was not observed, nor was it induced by this or any other heparin. A number of interesting conclusions can be drawn from this observation. First, it indicates that inhibition of factor Xa does not affect its own generation, something that could be expected because the factor Xa-catalyzed activation of factor VII is an important positive feedback reaction [65]. Apparently, when relatively high concentrations of thromboplastin (1:30 dilution) are used to activate plasma, this feedback reaction is probably too fast to be influenced by heparin [66]. Second, the amount of factor Xa generated in the absence of heparin (12 nM) is only about 10% of the amount of factor X present in plasma [67]. From the work of Broze [40] and that of Rappaport's group [68], we now know that the tissue factor-dependent activation of factor X is regulated by an inhibitor of tissue factor, called the extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI).

When rather small amounts of factor Xa (1 nM) are generated in plasma, the tissue-factor VIIa complex becomes rapidly inactivated. In a purified system, the inhibitory action of LACI was abrogated by AT III-heparin, because free activated factor X is rapidly inactivated [40]. In our plasma experiments the tissue factor was destroyed completely within 2 min after the plasma was activated, whether or not heparin was present. Thus heparin and LMWH have a

minor (if any) effect on the activation phase of the factor Xa generation curve. They therefore exert their effects on factor Xa generation curves by neutralizing the factor Xa activity once it is formed.

Consequently, we must conclude that the delaying effect of LMWH on the generation of prothrombinase activity and thus on thrombin generation is certainly not caused by a delayed appearance of factor Xa. The delayed appearance of the prothrombinase activity must therefore be caused by the generation of activated factor V. Indeed, when factor Va generation was monitored in thromboplastin-activated plasma, a clear lag in its appearance was noted (Figure 4B). LMWH did prolong the lag phase. Thus it seems reasonable to assume that the overall anticoagulant activity of heparin and LMWHs, when plasma clotting is initiated with thromboplastin, is due to their ability to postpone factor Va generation. This in turn raises the question: Which antiprotease activity of heparin causes the inhibition of activated factor V generation? We tried to answer this question indirectly by studying the effect of a synthetic pentasaccharide heparin, having only antifactor Xa activity, on factor Va, factor Xa, and thrombin generation in thromboplastin-activated plasma [64,69,70]. It was found that extremely high amounts of pentasaccharide were required to obtain the same anticoagulant effect as that obtained with UFH.

Interestingly, despite the absence of detectable amounts of factor Xa (less than 0.1 nM in clotting plasma), factor Va generation was only slightly delayed and so was thrombin generation. This indicates that inactivation of factor Xa alone is a very inefficient way to prevent the plasma from completely reversing the anticoagulant effect of heparin [69]. Nevertheless, heparins having only antifactor Xa activity do have anticoagulant properties. The question is, however, whether the anticoagulant activity is achieved directly or indirectly. That is, does neutralization of factor Xa result directly in less prothrombinase? Reduced amounts of prothrombinase are also generated when factor Xa is the major activator of factor V [71]. An indirect anticoagulant effect is expected when increased neutralization of factor Xa activity results in a reduced initial thrombin generation, which in turn results in a diminished factor Va and thus prothrombinase formation.

We have obtained strong evidence that thrombin is the only efficient enzyme that activates factor V in clotting plasma [36,72]. Our results suggest that the anticoagulant activity of heparin and LMWH is caused primarily by their ability to postpone the thrombin-catalyzed generation of activated factor V. Figure 5 shows plots of the clotting times as a function of the heparin concentration expressed either as anti-Xa IU/mL or anti-IIa IU/mL. It is clearly seen that the best correlation is found between the anticoagulant effect and the anti-IIa activity for UFH as well as for LMWH. The poor overall anticoagulant activity of the ULMWH pentasaccharide is clearly demonstrated.

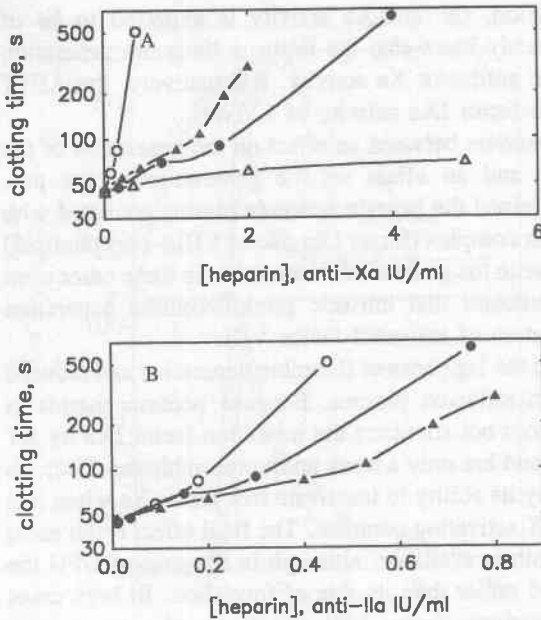


Figure 5 Effect of LMWHs on plasma clotting time triggered with thromboplastin. ○, Fourth International Standard for Heparin; ▲, First International Standard for Low Molecular Weight Heparin; ●, CY 216; △, pentasaccharide.

B. Tenase as Target of LMWH Action

The thrombin generation in plasma activated with kaolin dramatically decreases with increasing amounts of UFH [50]. A much stronger inhibitory effect of heparin on the generation of prothrombinase activity was found in intrinsic activated plasma compared with that of extrinsic prothrombinase generation. Both the peak amount and the time of onset of prothrombinase generation were affected markedly in a dose-dependent manner. As outlined above, intrinsic prothrombinase generation is controlled by the formation of the intrinsic factor X activating complex (tenase), which consists of factor IXa and activated factor VIII. In addition, the generation of activated factor VIII is controlled by thrombin (and to a lesser extent, if at all, by factor Xa). Thus, in contrast to extrinsic prothrombinase generation, where only one component (factor Va) is generated via a feedback mechanism, intrinsic prothrombinase generation depends on two feedback reactions: factor Xa generation and factor Va generation. If the generation of the enzymatic components (factors IXa and Xa) are the rate-limiting

reactions in thrombin generation, the anti-Xa activity is expected to be of importance. However, we already know that the intrinsic thrombin generation (APTT) is not sensitive to the antifactor Xa activity. Alternatively, the APTT could be sensitive for the anti-factor IXa activity of LMWH.

In a first attempt to differentiate between an effect on the generation of the factor X activating complex and an effect on the generation of the prothrombinase complex, we examined the heparin action in plasma activated with the complete factor X activating complex (factor IXa-factor VIIIa-phospholipid) [50]. The absence of a lag phase in the generation of thrombin in these cases even when heparin was present indicates that intrinsic prothrombinase generation depends largely on the generation of activated factor VIII.

Pentasaccharide prolonged the lag phase of thrombin generation and reduced the thrombin yield in contact-activated plasma. Because pentasaccharide is devoid of thrombin activity, does not stimulate the inhibition factor IXa by AT III at the concentrations used, and has only a weak antiprothrombinase effect, its action can only be explained by its ability to inactivate free factor Xa when it is formed by the intrinsic factor X activating complex. The final effect is the same as with UFH: less free thrombin is available, although in the case of UFH the decay of thrombin is enhanced rather than its rate of formation. In both cases reduced rates of feedback reactions (factor VIII and factor V activation by thrombin) will be found.

1. Heparin and the Generation of Factors VIIIa, Va, and Xa and Thrombin in Factor XIa-Activated Plasma

Figure 6A shows that when plasma is activated with factor XIa, factor Xa generation shows the same features as thrombin generation in thromboplastin-activated plasma. That is, despite a linear increase in factor IXa, significant factor Xa generation appears only after a certain time. Thus factor Xa generation is not directly associated with factor IXa generation. The obvious reason is that significant factor Xa generation occurs only when the essential cofactor of factor IXa—activated factor VIII—is also present (Figure 6B). Whether activated factor VIII is generated by factor Xa or thrombin cannot be concluded from the course of factor VIIIa generation. The sudden onset of all these activated factors occurs at about the same time. Such a phenomenon is clearly the result of linked positive feedback reaction systems.

However, if the activation of factor VIII by factor Xa is really important, one would expect that pentasaccharide should effectively postpone factor Xa generation. But the opposite was found. Extremely high amounts of pentasaccharide were required to show a comparable anticoagulant effect with UFH. UFH (0.005 U/mL; 0.25 mg/mL) prolonged the clotting time of factor XIa-activated plasma from 160 s to 260 s (Figure 7A). It required a 10-fold higher

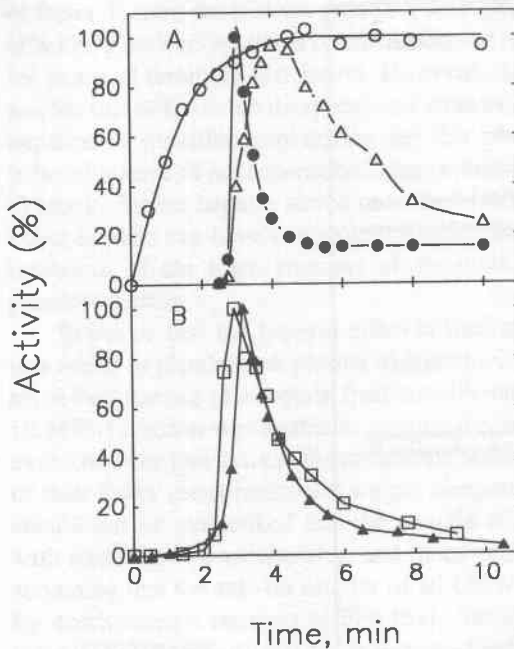


Figure 6 Intrinsic blood coagulation. Plasma was activated with human factor XIa (1.2 nM) in the presence of 10 μ M phospholipid and 4 mM free Ca^{2+} . (A) Generation curves of factor IXa (\circ), factor Xa (Δ), and thrombin (\bullet); (B) generation curves of factor Va (\blacktriangle) and factor VIIIa (\square). The activities of the activated blood coagulation factors are given as a percentage of their peak values.

amount (by weight) of pentasaccharide to obtain the same prolongation of the plasma clotting time (Figure 2C).

Despite the fact that UFH has comparable numbers of anti-IIa units (0.05 U/mL), CY 216 was less effective in prolonging the onset of thrombin generation (Figure 7B). In addition, generation of both factors Xa and IXa was clearly less reduced than in the case of UFH and pentasaccharide. The intriguing question, then, is whether or not the ability of heparin to stimulate the inactivation of factor IXa also contributes significantly to the overall anticoagulant action of heparin and its fragments. Our results also show that, as was suggested for extrinsic prothrombinase formation, extremely low concentrations of the enzymatic component of the factor X activating complex (factor IXa) and that of the intrinsic prothrombin activating complex (factor Xa) are sufficient to produce thrombin at a high rate, provided that the activated cofactors (factors VIIIa and Va) are present.

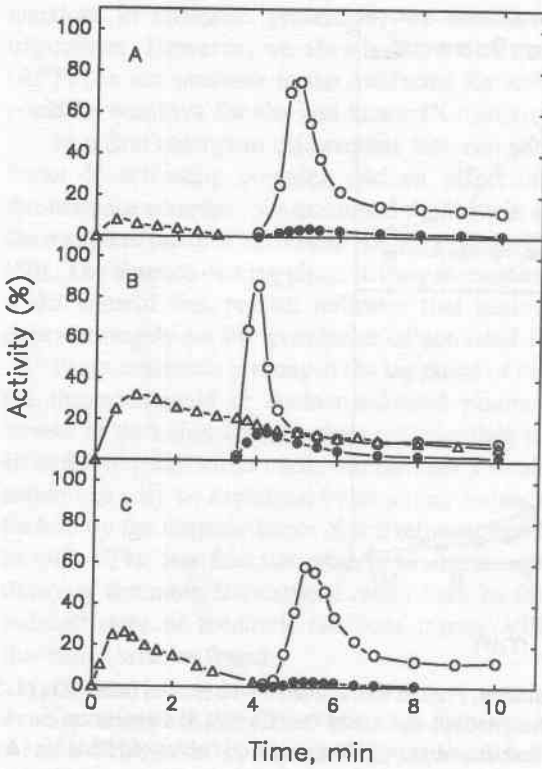


Figure 7 Heparins and intrinsic blood coagulation. Generation of factor IXa (Δ), factor Xa (\bullet), and thrombin (\circ) in factor XIa-activated plasma. (A) Fourth International Standard for Heparin ($0.25 \mu\text{g/mL}$, 0.05 U/mL); (B) LMWH CY 216 ($3.0 \mu\text{g/mL}$, 0.05 aIIa IU/mL , 0.25 aXa IU/mL); (C) synthetic pentasaccharide heparin ($2.0 \mu\text{g/mL}$, no aIIa activity, 1.6 aXa IU/mL).

V. EFFECT OF PLATELETS ON THE ANTICOAGULANT PROPERTIES OF LMWH

Amounts of thromboplastin that do not initiate a rapid onset of thrombin generation in platelet-poor plasma stimulate thrombin formation in platelet-rich plasma [72]. The following explanation can be given. Thromboplastin induces trace amounts of thrombin, which in turn activate the blood platelets. The procoagulant surface so formed serves for assembly of the prothrombin-activating complex. From experiments conducted in the presence of exogenously added factor Va or phospholipids, it was concluded that exposure of procoagulant phospholipids by a platelet membrane translocation reaction [73], not the release

of factor V from the platelet granules, is responsible for most of the stimulating effect of platelets. Addition of unfractionated heparin (0.1 U/mL) prolonged the lag phase of thrombin generation. However, the peak value of thrombin formed and the rate of thrombin disappearance were exactly the same as in the absence of heparin. A plausible explanation for this phenomenon is that the thrombin-induced release of heparin-neutralizing proteins from the platelets (platelet factor 4) blocks further heparin action once the platelets are activated. Hence the only effect heparin can have is a prolongation of the lag phase (clotting time) by the inhibition of the trace amounts of thrombin necessary for the procoagulant platelet reaction.

Evidence that the heparin effect is mediated by the inhibition of thrombin was found in platelet-rich plasma triggered with a small amount of thromboplastin in the presence of a heparin fraction with only antifactor Xa activity [63]. This ULMWH fraction was unable to prolong the lag phase. LMWH preparations are expected to be less susceptible to neutralization by platelet factor 4 (PF4) because of their lower mean molecular weight compared with that of UFH. However, it should not be overlooked that the anti-IIa activity is related to heparin chains with more than 18 saccharides, and these chains do bind to PF4. Thus it is not surprising that the anti-IIa activity of all LMWH preparations can be neutralized by stoichiometric amounts of PF4 [61]. Yet it was found that LMWH preparations (CY 216, PK 10169) did reduce the thrombin peak in platelet-rich plasma, indicating that not all the anti-IIa activity of these LMWHs could be neutralized by PF4 released from activated platelets. This apparent discrepancy is explained by taking into account that on a weight basis more LMWH than UFH had to be used to obtain the same prolongation of the lag phase (the specific anti-IIa activity of LMWH is much less than that of UFH). LMWH also contains non-ATIII binding material and on a molar basis even much more than an UFH preparation. A significant part of these nonanticoagulant heparin species do bind to PF4. Thus, as a minimum, we like to hypothesize that the anti-IIa activity of LMWH is partially protected from the neutralization by PF4 through its own nonactive species.

VI. SUMMARY

Both the anti-factor Xa and anti-IIa activity of LMWH preparations could potentially be of great importance: factor Xa is the enzyme that converts prothrombin into thrombin, and thrombin is necessary for the rapid feedback activation of factors VIII and V, the essential cofactors for the formation of hemostatic amounts of factor Xa and thrombin, respectively.

Our studies have indicated that:

1. The ratio of the factor Xa neutralizing potency to the thrombin neutralizing potency of the LMWH preparations tested does not differ from that of UFH

when examined in purified systems using human proteins and in the presence of Ca^{2+} ions.

2. Heparins with a sole antifactor Xa activity do inhibit formation of the prothrombin converting complex at high dosages. However, when the heparins also have (minimal) anti-IIa activity, the inhibition of formed thrombin becomes more dominant than the reduction observed in prothrombinase activity. That is, at already low UFH concentrations it is seen that the antiprothrombinase activity is completely overshadowed by the direct thrombin inhibition.

3. UFH and LMWH prolong the lag phase of clotting in plasma, activated through either the extrinsic or intrinsic pathway, by inhibition of the formation of the factor X and prothrombin activating complexes. The ultimate targets of LMWH action are the thrombin-dependent feedback reactions (e.g., activation of factors V and VIII).

4. UFH and LMWH inhibit coagulation in platelet-rich plasma activated with low amounts of thromboplastin by eliminating trace amounts of thrombin necessary for triggering the platelet procoagulant reactions.

5. The relatively high amounts of nonanticoagulant species in LMWH preparations might protect the species with anti-IIa activity for neutralization by platelet factor 4.

6. The ideal test for both LMWHs and UFH would be one that shows the effect of heparin on the amount of free thrombin generated during the course of a thrombin generation test and thus independent of the way in which this amount has been diminished.

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