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THE MODE OF ACTION OF HEPARINS IN VITRO AND IN VIVO

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The antithrombotic action of a heparin is not necessarily confined to its effects on the clotting mechanism. Yet we restrict ourselves here to a discussion of the action of heparin on thrombin generation in platelet poor and platelet rich plasma for two reasons. In the first place it is more likely than not that inhibition of the clotting mechanism is at least one of the major working arms of heparin. All medication that inhibits blood clotting has an antithrombotic effect and all dis-orders that are known to impede clotting inhibition are accompanied by a thrombotic syndrome. Also the assumption that heparins act through their interference with blood coagulation is tacitly at the basis of virtually all studies of the pharmacokinetics and pharmacodynamics of heparin action, both clinical and experimental. Finally it is the subject that we studied and the only one that we dare to express ourselves about.

SPECIFIC AND COMPOSITE EFFECTS

The actions of heparin on haemostasis and thrombosis can be devided in specific biochemical effects, that are the effects on those biochemical processes that are at the basis of the action of heparin, and composite biological effects that are the consequence of the specific effects acting in some biological system, either in vivo or in vitro. The specific effects are: Increasing thrombin activation; increasing the activation of factor Xa; increasing the activation of factor IXa (1). There are good grounds to think that inactivation of other clotting factors is of no practical importance. All these activations take place through binding of heparin to antithrombin III (AT III). The action of heparin on thrombin via heparin cofactor II (HC II) occurs at such high concentrations of heparin as are seldomly obtained in clinical practice and will not be subject of discussion (2). Other negatively charged polyelectrolytes such as pentosan polysulfate (3) and lactobionic acid (4) do act via HC II, but they are not subject of this article.

The composite biological effects of heparin on the haemostatic-thrombotic apparatus are many and varied. The main ones are: The antithrombotic effect and the haemorrhagic effect. In fact these two are the only ones that matter if our aim is to find a good antithrombotic drug. They need extensive, laborious clinical experimentation to

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be determined, so it is logical that we resource to model systems in order to find indications on these effects by laboratory shortcuts. They come in two kinds: determination of antithrombotic and haemorrhagic effects in animals and measurements of other composite effects on (human) blood or plasma.

The biological effects observed in the laboratory on isolated blood or plasma, either platelet poor (PPP) or platelet rich (PRP), again come in a great many varieties. Many of them are a variation on the clotting times, such as the activated partial thromboplastin time (APTT) or the Heptest. A clotting time is essentially the time that is necessary for a threshold amount of thrombin (around 15 nM) to be formed. This means that it is especially sensitive to influence on the lag phase of thrombin formation, where thrombin mediated feedback activation of factor VIII takes an especially important place. That is why tests like the APTT, where factor VIIIa formation is a rate limiting step in the lag phase, are sensitive to the action of heparins (fig.1). The prothrombin time (PT), where factor VIIIa does not play a role, is much less, if at all, affected (fig.2).

Our studies since 1985 focussed our attention on an essentially different type of biological effect. We determined the entire course of the thrombin generation curve under the influence of heparin (5,6). These curves yield a wealth of information on the

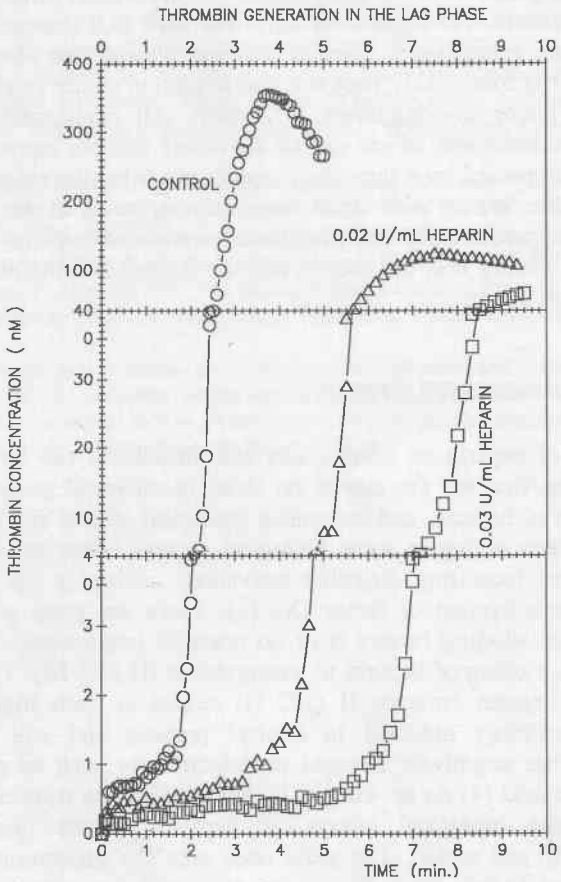

Figure 1. Thrombin generation in plasma (intrinsic system) inhibited by heparin. Methods as in ref. 6 with adaptation of the incubation times of the sample and the chromogenic substrate to the levels of thrombin.

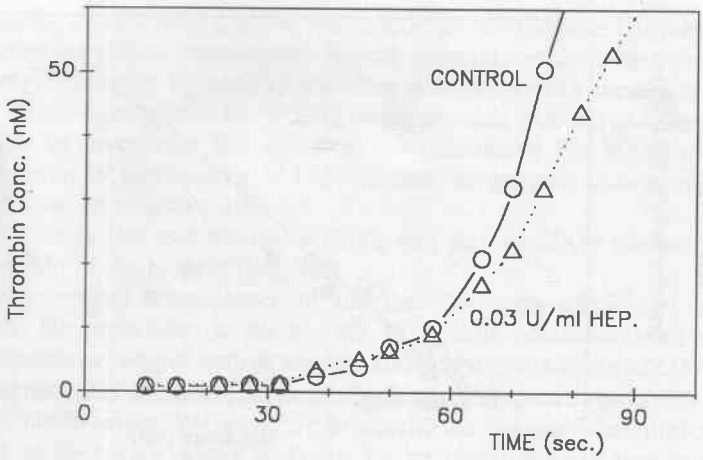

Figure 2. Thrombin generation in Plasma (extrinsic system) inhibited by heparin. Methods as in ref.6 with adaptation of the incubation times of the sample and the chromogenic substrate to the levels of thrombin.

mode of action of heparins, particularly because they allow to separate the effect of heparin on prothrombin conversion (thrombin generation without thrombin decay effects) from that on thrombin inactivation (7). For practical everyday use outside the research laboratory they are much too elaborate however. Reasoning that the action of the enzyme thrombin will be proportional to its concentration and to the time that it is allowed to act we determined the product of time and concentration, i.e. the area under the thrombin generation curve, i.e. the time-concentration integral of thrombin generated in clotting plasma, which we called the *thrombin potential*. We also found a way to determine the thrombin potential in a one-stage spectrophotometric procedure (8).

Any of the composite effects is a complicated function of the ensemble of the specific effects. The example of the APTT and the PT already shows that even in one class of composite effects the sensitivities for the specific effects may differ enormously. There is no *a priori* reason to assume that any one composite or specific effect will be a good yardstick for the antithrombotic- or antihaemostatic action of a heparin. Yet the observation that all anticoagulant drugs invariably are a) antithrombotic, in a dose dependent way, b) antihaemostatic when overdosed, and c) decrease the thrombin potential in a dose dependent way, whereas clotting times do not show such a systematic variation, made us think that the thrombin potential may be a good model for the antithrombotic potency of an anticoagulant drug.

HOW TO CHARACTERISE A HEPARIN

The specific effects are well defined biochemical properties whereas the biological effects are unknown functions of the specific effects. From this consideration it follows that a heparin should be characterised by its specific effects rather than by its biological ones. Unfortunately a biological effect, and a rather bizarre one viz. the prolongation of the prothrombin time of goat plasma, has been chosen to define the pharmacopea unit of heparin activity. Here we will propose a fundamentally different approach that allows to express heparin activity in standard-independent units of activity and to determine the levels of functional heparin in plasma.

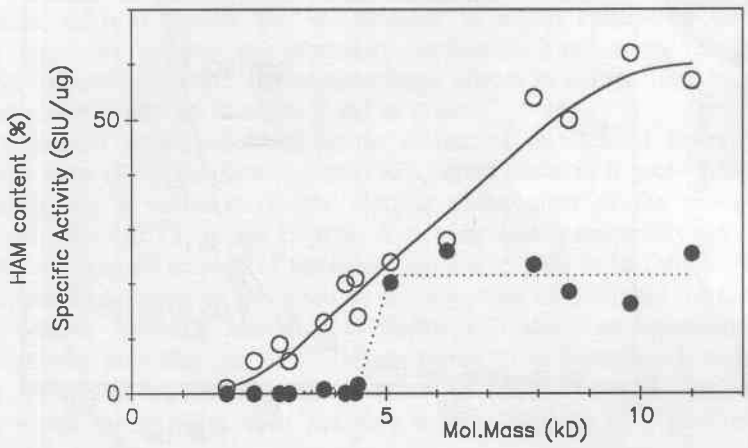

Figure 3. The HAM content and the HAM-specific activity of a series of LMWHs. o——o HAM content; ●·····● specific antithrombin activity.

THE FUNCTIONAL HETEROGENEITY OF HEPARIN

In fact the definition of heparin activity as an arbitrary composite activity did no harm as long as the proportion of the specific activities were similar in the different preparations that were to be compared. This was the case until the low molecular weight heparins (LMWHs) appeared on the scene. In the LMWHs however the antifactor Xa shifts independently from the antithrombin activity. At that moment one can imagine that an infinite variety of different combinations of the antifactor Xa activity and the antithrombin activity can result in the same composite effect on the prothrombin time in goat plasma, so that the unit of heparin activity becomes essentially senseless.

With decreasing molecular weight, glycosaminoglycans loose their capacity to catalyse the AT III dependent inhibition of thrombin (9-15). Barrowcliffe et al (loc.cit.), and Thomas et al (loc.cit.) have shown that heparin fragments with a chainlength of 10-18 monosaccharide units have a high anti-factor Xa activity, and that a length of 20-22 saccharides is necessary for an activity against thrombin. Lane and coworkers (loc.cit.) studied heparins of 8 to >18 monosaccharides and concluded that 18 units is the smallest chainlength that will allow to potentiate the inactivation of thrombin by AT III, whereas the activity against factor Xa was high in all fragments. From this it follows that all the high affinity material (HAM) in a heparin has anti factor Xa activity, whereas antithrombin activity is only expressed in HAM above the critical chainlength of 18 monosaccharides (ACLM)[1]. Below critical chainlength material (BCLM), in order to be active, has to bind to AT III, i.e. it has to contain minimally the AT III binding pentasaccharide. It thus comprises HAM with a length of between 5 and 18 sugar units i.e. a Mr of 1500 - 5400. Classical unfractionated heparin (UFH) hardly contains any BCLM. That explains why, before the advent of low molecular weight heparins, arbitrary units would suffice to indicate the potency of a heparin.

It has often been suggested that the smaller a heparin is, the more it exhibits an anti-factor Xa activity. Apart from the fact that this *per se* is not true (see below) this

[1] In order to prevent long and awkward abbreviations we use ACLM (resp. BCLM) to indicate Above (resp. Below) Critical chainLength *high affinity* Material.

can mean either of two things, given the usually highly disperse LMWH preparations . First, is it possible that in polydisperse heparin preparations the proportion of molecules with uniquely anti-factor Xa activity increases with decreasing mean molecular weight. Second the specific anti-factor Xa activity might increase with the chainlength.

In order to investigate this question, we determined the HAM and the ACLM content in a series of subfractions of LMWH, with the purpose to determine the specific activities per amount of active material.

The first thing that met the eye (16,17), was that the HAM content increases with molecular weight of the heparin (Fig. 3).

This is a natural consequence of the fact that upon scission of a high-affinity heparin chain the probability to hit the AT III binding pentasaccharide increases with decreasing molecular weight until it reaches 100% in a pentasaccharide. We suspect that this phenomenon may be at the basis of much of the reported variability of biological activity with chain length. We therefore expressed the observed activities per amount of HAM as far as they were related to factor Xa inhibition and per amount of ACLM for thrombin related phenomena. As a natural consequence the peak- and mean molecular weights of the active fraction in a LMWH will always be higher than that of the total heparin (fig.4).

THE SPECIFIC ACTIVITY OF A HEPARIN

The catalytic activities of a heparin in the interaction between AT III and thrombin (resp. factor Xa) can be used to quantitate its specific activity.

The reaction:

$$\text{Thrombin} + \text{AT III} \xrightarrow{\text{Heparin}} \text{Inactive product}$$

under conditions occuring in clotting plasma is pseudo first order in thrombin. It is

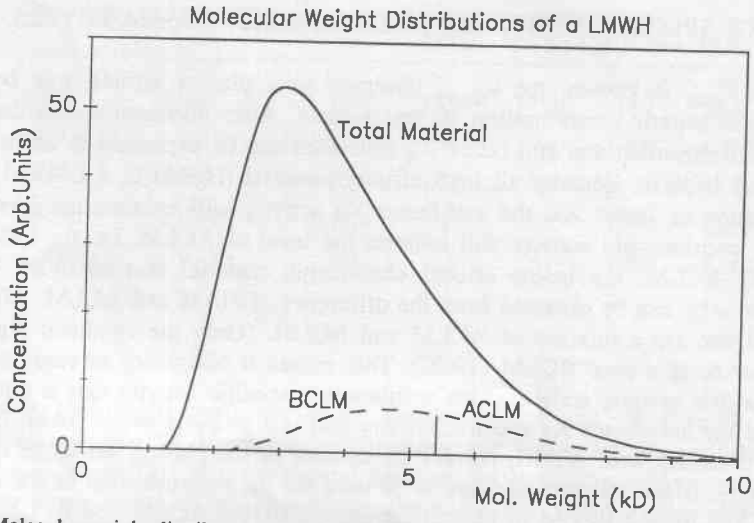

Figure 4. Molecular weight distribution of a LMWH. The area under the hatched line is the high affinity material.

characterised by the half life time of thrombin (resp. F.Xa), or, which is the same thing, by a decay constant, (k_{decay}) that is inversely proportional to the half life time. Upon addition of heparin to plasma, k_{decay} increases from its blank value (k_{blank}), proportional to the concentrations of AT III and heparin in the sample (Béguin et al. in press), hence:

$$k_{decay} = k_{spec}.[AT\ III].[heparin] + k_{blank}$$

Any well designed anti-thrombin or anti-factor Xa activity gives a result that is proportional to k_{decay}. This value thus can be obtained from current laboratory tests if adequate standards are available.

k_{spec} is the *specific activity* of the heparin, i.e. the increase of the decay constant that is brought about by 1µg/ml of heparin in the presence of 1 µM of AT III. It is influenced by a) Ca^{++} ions, b) plasma proteins, c) ionic strength, pH,temperature etc., so it should be determined under well defined, and specified conditions. The decrease of HAM content with Mr in a LMWH preparation is proportional to its molecular weight. This explains most of the variation of specific antifactor Xa activity with molecular weight. If antifactor Xa activity of a number of LMWHs is expressed per µg of ACLM is remarkably constant: (20.4 ± 4.1 SIU/µg, range 15.2 - 26.8, n=11)(fig.3). UFH and the 1st LMW standard are exceptions: 39 ± 0.5 SIU/µg. This may be due to a different affinity to other plasma proteins than that of most other LMWHs.

From the above it will be clear that the simplest possible rational approach to heparin standardisation is somewhat more complicated than accepted practice at this moment. This may be regretted but it may not be overlooked. One can not standardise away the fact that LMWHs have two different activities that cannot be measured with a common yardstick. A good example is the use of antifactor Xa units to compare the potency of UFH and pentasaccharide. One needs much more units of pentasaccharide to achieve the same antithrombotic effect than of UFH (18). This does not mean that pentasaccharide is a "bad" antithrombotic, but merely that it scores high on the antifactor Xa scale and not at all on the antithrombin scale, whereas the antithrombotic effect is a composite effect that responds to both antifactor Xa-and antithrombin specific activity; but in fact better to the latter.

THE USE OF SPECIFIC ACTIVITIES TO DETERMINE PLASMA LEVELS

When k_{spec} is known, the k_{decay} observed in a plasma sample, can be used to determine the heparin concentration in that sample. After adequate standardisation the results of anti-thrombin and anti-factor Xa tests then can be expressed in terms of ng/ml of functional heparin. Because all high affinity material (HAM) in a LMWH catalyses the inactivation of factor Xa, the anti-factor Xa activity will indicate the level of total HAM. The antithrombin activity will indicate the level of ACLM, i.e. the HAM with a $M_r > 5400$. BCLM, the below critical chainlength material that catalyses factor Xa inactivation only, can be obtained from the difference of HAM and ACLM. All LMWHs in practical use are a mixture of ACLM and BCLM. (Only the synthetic high affinity pentasaccharide is a pure BCLM (19,20). This makes it obligatory to characterise each LMWH via two specific activities: the antithrombin specific activity that is a property of ACLM and the anti-factor Xa specific activity that is a property of all HAM. Because of the proportionallity with AT III, the AT III content of the plasma has to be determined independently. If specific activities are to be used for the determination of the levels (in µg/ml) of ACLM and BCLM in plasma samples, different ACLM and BCLM standards should be available.

STANDARD INDEPENDANT UNIT OF HEPARIN ACTIVITY

If one wants to short-circuit the two steps of first determining the specific activity and then the heparin concentration, or when working with insufficiently defined materials, one can always express heparin activity in terms of an unambiguous *Standard Independent Unit (SIU) of heparin activity*, that can be simply defined as that amount of heparin that, when added to 1 ml of normal plasma, containing 1μM of AT III, will raise the pseudo-first order decay constant of a clotting enzyme by one inverse minute. Standard materials can be defined in terms of SIU units. Of course there are two types of SI units, that based on factor Xa inactivation (Xa-SIU) and that based on the inactivation of thrombin (IIa-SIU). Their use is not restricted to heparins, because one can well define a thrombin based SI unit for dermatan sulfate, in that case reported on the heparin cofactor II content. It will be clear that pentasaccharide activities can be expressed in Xa-SI units and dermatan sulfates in IIa-SI units, but that heparins in general will need to be expressed in both. Because of the functional and pharmacological heterogeneity of heparins it can be expected that the course of heparin activity in vivo in terms of the two different units will not be identical (see below).

STANDARD UNITS AND STANDARD INDEPENDENT UNITS, THE EFFECT OF CA^{++}

A classical unit of standard heparin is an amount of heparin, e.g. 5 μg.
It has been automatically assumed to be a unit of antithrombin activity as well as a unit of antifactor Xa activity. In terms of activity one unit is far from being the same thing however. In fact one unit of the 4th international standad unit of heparin is a much more active antithrombin than antifactor Xa agent, as is immediately seen when we express its activity in standard independent units (table 1).

TABLE 1. EQUIVALENCES OF STANDARD UNITS AND STANDARD INDEPENDENT UNITS

STANDARD	AMOUNT	EQUIVALENT AMOUNT				
		μg	IU-IIa	IU-Xa	SIU-IIa	SIU-Xa
UFH	1 mg	1000	193.0	193.0	14.5	4.4
UFH	1 IU.	5.2	1	1	0.075	0.023
LMWH	1 mg	1000	67.0	168.0	7.0	1.7
LMWH	1 IU-IIa	14.9	1	0.4	0.105	0.026
LMWH	1 IU-Xa	6.0	2.5	1	0.042	0.010

There is still more confusion to be cleared. As a rule, in general laboratory practice, it is common to determine antithrombin and antifactor Xa actitvities in the absence of Ca^{++}ions. This is quite natural because the last thing one wants to have in these decay experiments is to complicate them by the simultaneous generation of endogenous factor Xa or thrombin. If one designs experiments that allow te determi-

nation of decay constants in the presence of physiological amounts of Ca^{++}, it appears that the inhibition of factor Xa by standard unfractionated heparin is about twice as efficient as in the absence of Ca^{++} (21). Low molecular weight heparins are hardly affected by these concentrations of Ca^{++}. So in the absence of Ca^{++}, i.e. under the usual laboratory conditions, the antithrombin activity of UFH is significantly underestimated. This makes that the current opinion about a high antifactor Xa over antithrombin ratio of low molecular weight heparins is based on a laboratory artifact.

A SUMMARY OF PREVIOUS IN VITRO RESULTS

At different occasions we have given an overview of our previous results obtained with PPP and PRP in vitro. The main conclusions are therefore only briefly summarised here:

a) The main inhibitory action of UFH in clotting plasma is on thrombin. At concentrations of UFH that inhibit almost completely the appearance of free thrombin, the (extrinsic) conversion of prothrombin into thrombin is inhibited only 20-30 %. This holds for the extrinsic system, where the secundary effects of inhibition of thrombin via feedback mechanisms (see below) is not rate limiting (6).

b) Despite their reputedly high anti-factor Xa action also LMWHs act mainly through inhibition of thrombin, unless they are so small as to have no antithrombin action to speak of, i.e. pentasaccharide and other P-type heparins. In the terms proposed in this article we must conclude that the P-type heparins that we recognised before actually are those heparins that consist almost entirely of BCL material (5,20).

c) The reason for a lack of a direct effect on prothrombin conversion despite a definite anti-factor Xa action is that in clotting plasma, when enough procoagulant phospholipid is added, factor Va is the rate limiting factor and factor Xa is present in excess. Factor Xa levels must be lowered to less than 10 % of their normal values before this diminution shows up as a lack of saturation of phospholipid-adsorbed factor Va, i.e. as an inhibition of prothrombinase (6,22).

d) In the intrinsic system factor VIIIa is rate limiting. Factor VIIIa generates as a result of activation by thrombin. Inhibition of thrombin therefore inhibits the activation of factor VIII and so, indirectly the generation of factor Xa. Therefore, in the intrinsic system, heparins inhibit prothrombin conversion via their antithrombin effect (6,23,24).

e) In platelet rich plasma without added procoagulant phospholipids, phospholipids are rate limiting. The burst of thrombin formation therefore occurs after traces of thrombin have activated the platelets. Heparin will delay the burst because of its antithrombin action. Activated platelets shed heparin-neutralising material (pf4). This neutralises UFH to concentrations of up to 0.4 U/ml. LMWH partly escape this inhibition (25).

AN APPROACH TO IN VIVO EFFECTS

In a preliminary experiment (26) we injected subcutaneously a dose of unfractionated heparin and of two kinds of LMWH (5000 U of UFH, 7500 Choay Units of LMWH-A, 40 mg of LMWH-B). The fact that we only have data on one volunteer per heparin precludes any quantitative conclusion. We present the results here only as a proposal for a rational approach to LMWH pharmacology.

In our volunteers we determined the course of the heparin activity in the blood in terms of SIUs and, via the specific activities, also the course of the blood levels of ACLM and BCLM. Then we determined the main composite biological activities in the

samples, i.e. the thrombin potential, the inhibition of the peak of factor Xa generation, the inhibition of the peak of prothrombin converting activity and the thrombin potential in platelet rich plasma.

The main conclusions are

a) UFH causes no inhibition of prothrombinase and no inhibition in platelet rich plasma. Both LMWHs cause some inhibition of prothrombinase and retain significant activity in PRP.

b) ACLM from LMWHs has a longer half life time than that of UFH. BCLM has a longer half life time than ACLM from the same LMWH. This results in a "fractionation in vivo" of the injected LMWH. Late samples are enriched in BCLM.

c) ACLM in all instances is the most active material. The biological activities correlate much better with the ACLM content than with the BCLM content. Even the inhibition of the factor Xa peak is primarily caused by the ACLM present. Evidently by the antifactor Xa activity of the ACLM, because when expressed in SI-units, i.e. in terms of anti-factor Xa activity, then the inhibition of factor Xa shows a correlation with the Xa-SIU level. This indicates that in practice the anti-factor Xa level will always correlate with any biological effect, even if the anti-factor Xa action itself does not influence the effect. Interestingly the inhibition of overall thrombin generation in PRP, in comparison to that in PPP, is relatively dependent upon BCLM, which possibly reflects the fact that ACLM is more sensitive to neutralisation by activated platelets than BCLM is.

It should once more be stressed that these are preliminary results that may indicate a trend but that are to be substantiated by more data.

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