EDITORIAL

Standard and Method Independent Units for Heparin Anticoagulant Activities

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Summary

It is discussed why the current USP unit of heparin anticoagulant activity necessarily will render inaccurately the anticoagulant activities of low molecular weight heparins. It is shown that the outcome is bound to vary with the method used for comparison of the sample and the standard and with the nature of the standard used. As an alternative we define a unit of heparin in terms of anti-factor Xa- and antithrombin-activity that is independent of the heparin standard and of the assay method, but that is based upon a quantitative description of the catalytic effect of heparin on AT III mediated thrombin- and factor Xa breakdown. Expression of the results of existing anti-factor Xa- and antithrombin tests in terms of these units will allow to express heparin levels in plasma in terms of concentrations of active anticoagulant material. This approach makes it possible to separate heparin pharmacodynamics from heparin pharmacokinetics. Introduction of this unit does not require adaptation of current laboratory practice but changes the way in which the results obtained are expressed.

Introduction

In the early 1920's, Howell (1) defined a unit of heparin as that amount of heparin that causes such retardation of coagulation that a millilitre of cat blood will half-clot when left during one night in the refrigerator. The United States Pharmacopoeia (USP unit), that also figures in many national pharmacopoeias, is similarly defined as that amount of heparin that will cause 1 ml of sheep plasma to half-clot when kept for 1 h at 37°C (see also 2 for a discussion). The International Unit (I. U.) is defined via the International Heparin Standard (I. H. S.) which itself is calibrated in terms of concentrations of active anticoagulant material. That also figures in many national pharmacopoeias. is expressed.

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The direct determination of heparin concentrations in plasma from patients is impossible with the USP method. A large number of other tests is available for this purpose: thrombin time, activated partial thromboplastin time in a multitude of varieties (APTT), and several tests based on the measurement of the decay of added thrombin or factor Xa. In order to express the results of these tests in terms of USP units, they need to be calibrated with a standard preparation, the potency of which has been determined in terms of USP procedure. An important problem in the determination of the heparin concentration by comparison to a standard remains, however, that the outcome will be dependent upon the method used for comparison (see below).

The anticoagulant effect used for the definition of the USP unit is far from being specific; any anticoagulant activity can be expressed in these units. Therefore the USP unit can be used in a meaningful way only as long as fairly similar preparations of heparin are compared. It appears to be a poor tool even to compare low molecular weight heparins to the unfractionated heparin standard (3, 4). Indeed its replacement is being considered (5, 6). Replacement by what, however? In this paper we propose a unit of heparin activity that is not dependent upon the use of a special method and that also is not primarily defined as the activity of a given standard preparation even though in practice standard preparations will remain a necessary means to compare the results in different laboratories.

Biochemical Backgrounds: Heparin

Heparins act on blood coagulation by binding to AT III and enhancing its inhibitory action on thrombin, factor Xa and other blood coagulation enzymes (7–10). Active heparins contain a specific pentasaccharide sequence with which they bind to AT III (see 11 for a review). Only about 30–40% (weight) of UFH contains this sequence. This fraction we call High Affinity Material (HAM). Within HAM we distinguish two classes (Fig. 1): molecules longer than the critical chainlength of 17 monosaccharide units (Above Critical chain Length Material: ACLM, with a Mw >5,400) and molecules below that chainlength (BCLM). ACLM catalyses both factor Xa and thrombin inactivation but BCLM catalyses only factor Xa-inactivation. As discussed in detail in ref. 12 (pp 23–26 and references therein), the cutoff point between ACLM and BCLM heparins can indeed be localised with fair precision around 5,400 MW or 17 monosaccharide units. Our own data also support this conclusion (13, 14).

Different types of heparin may differ as to the HAM content as well as to the partition of the HAM fraction between ACLM and BCLM. In a series of 4 commercially available LMWH preparations the ACLM contents ranged from 10 to 30% and the BCLM contents from 1 to 12%. The HAM content of LMWHs decreases proportionally with the mean molecular weight (14). This seems a logical consequence from the fact that LMWHs are obtained from UFH by chemical or enzymatical splitting of the molecules in a random fashion. A HAM molecule of around 9,000 MW will consist of some 30 monosaccharide units. If such a molecule is
Table 1 Comparison of the activities of different heparins

<table>
<thead>
<tr>
<th></th>
<th>HAM</th>
<th>HAM</th>
<th>International Units</th>
<th>S. I. Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>µM</td>
<td>aXa</td>
<td>aIIa</td>
</tr>
<tr>
<td>A: ICSO of the surface under the thrombin generation curve (intrinsic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFH</td>
<td>0.077</td>
<td>0.0074</td>
<td>0.025</td>
<td>0.373</td>
</tr>
<tr>
<td>LMWH 1</td>
<td>0.153</td>
<td>0.030</td>
<td>0.065</td>
<td>0.357</td>
</tr>
<tr>
<td>LMWH 2</td>
<td>0.189</td>
<td>0.042</td>
<td>0.090</td>
<td>0.454</td>
</tr>
<tr>
<td>PENTX</td>
<td>0.800</td>
<td>0.480</td>
<td>0.520</td>
<td>0.495</td>
</tr>
<tr>
<td>B: Dose doubling the APTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UFH</td>
<td>0.62</td>
<td>0.059</td>
<td>0.192</td>
<td>1.97</td>
</tr>
<tr>
<td>LMWH 1</td>
<td>2.1</td>
<td>0.420</td>
<td>0.9</td>
<td>3.29</td>
</tr>
<tr>
<td>LMWH 2</td>
<td>2.1</td>
<td>0.470</td>
<td>1.0</td>
<td>3.20</td>
</tr>
<tr>
<td>PENTX</td>
<td>50</td>
<td>29.40</td>
<td>32.5</td>
<td>165</td>
</tr>
</tbody>
</table>

Thrombin generation curves were determined as in ref. 13. The APTT was carried out manually by mixing 0.1 ml plasma sample, 0.1 ml Actin FS (Baxter Diagnostics), after 2 min mixing at 37°C, 0.1 ml CaCl₂ (25 mM) was added and the clotting time read by tilting. The dose was 1.7 ml of 10 exponents prolonged the blank of 35 s to between 68 and 72 s. Molar concentrations of HAM were obtained directly via AT III fluorescence titration.

The inactivation of thrombin, factor Xa and other activated clotting enzymes by AT III in plasma is a bimolecular reaction (E + A → I) that according to standard chemical kinetics has a reaction velocity of 

\[ v = k \cdot a \cdot E \]

The concentration of AT III in plasma is about 2.5 pM. In every well designed decay experiment care is taken to keep the initial enzyme concentration below a certain proportion. Therefore, \( k \) can be considered constant and the enzyme decay can be described by pseudo-first order kinetics. The decay of the enzyme is therefore expressed by 

\[ v = k \cdot E' \]

or 

\[ v = k_{dec} \cdot E' \]

where \( k_{dec} = k \cdot A' \cdot E' \). The dimension of \( k_{dec} \) is therefore time, usually it is expressed in min⁻¹. Inhibition of blood clotting, as used in the determination of the USP unit or the APTT, can be obtained both by ACLM and by BCLM, but the activity per unit weight of ACLM is much higher than that of BCLM. The concentrations of different heparins with comparable biological activity on thrombin generation or the APTT differ considerably in weight, molar terms and in anti-Xa activity. Consequently, 1 µg of BCLM contains less USP units of heparin activity than 1 µg of ACLM. In a test based on the anti-factor Xa activity of heparin, BCLM will be about equally active as ACLM is, because the specific anti-Xa activities of ACLM and BCLM, when expressed on a molar basis, are roughly equal (Table 2, see also below). In a test based on the antithrombin action of heparin, however, BCLM will not count at all. The relative importance of ACLM and BCLM for the test outcome is therefore completely dependent upon the test used.

Biochemical Backgrounds: Kinetics of Clotting Factor Decay

The inactivation of thrombin, factor Xa and other activated clotting enzymes by AT III in plasma is a bimolecular reaction (E + A → I) that according to standard chemical kinetics has a reaction velocity of 

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where \( k_{dec} = k \cdot A' \cdot E' \). The dimension of \( k_{dec} \) is therefore time, usually it is expressed in min⁻¹. Heparin catalyses the above reaction. The mechanism of catalysis may be complex (15), its effect is simple: it decreases the half-life time and increases the decay constant \( k_{dec} \). In plasma, the concentration of AT III, the amount of activity declared on the label. For technical reasons current anti-factor Xa activity per unit. We estimated the anti-factor Xa activity of the standard heparin preparation and of the "equipotent" LMWH solution in the presence of a physiological concentration of Ca²⁺ ions and of the "equipotent" LMWH solution. The confusion stems from two sources. In the first place we try to assign a unique potency to a drug that, at the simplest, is a mixture of two distinct active substances: ACLM and BCLM, the proportion of which is not the same in the standard and in the preparation to be tested.

A Proposal for Standard- and Method Independent Units

In the presence of HAM the decay constant of factor Xa increases proportionally to the heparin-concentration and to the concentration of AT III in the plasma. This property we can use to define a standard- and method independent unit (SIU) of HAM activity (16, 17).

**Definition.** One standard independent unit of anti-factor Xa activity (SIU-Xa), of heparin is that amount of heparin that, in

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*In the reaction equation E = enzyme (thrombin, factor Xa etc.), A = AT III, I = inactive product. In the kinetic equations the letters stand for the concentrations of these reactants. The subscripts denote the time, \( E_0 \) = enzyme concentration at zero time etc.

**Because at the half-life time half of the original material is left, and the natural logarithm of \( \log e \) equals \(-0.693\).*
normal plasma, increases the decay constant of factor Xa by 1 min⁻¹ per pM of plasmatic AT III.

The heparin activity thus measured is the activity of all HAM, i.e. both BCLM and ACLM. Varying amounts of ACLM within the same total amount of HAM will not be distinguished by the anti-Xa activity. We therefore also need to define a unit of anti-thrombin activity. The decay velocity of thrombin in plasma increases proportionally with the ACLM concentration and with the AT III concentration (17). We can therefore define a standard- and method independent unit of anti-thrombin activity completely analogous to the anti-factor Xa unit.

**Definition.** One standard independent unit of anti-thrombin activity of heparin (SIU-IIa), is that amount of heparin that, in normal plasma, increases the decay constant of thrombin by 1 min⁻¹ per pM of plasmatic AT III.

The units thus defined are not dependent upon a standard or upon a specific methodology. This does not mean that in practice one or more heparin standards would not be useful or even necessary. The use, in the definition, of the words “in normal plasma” implies that the reaction conditions should be as near as possible to physiological Ca²⁺ concentration, temperature, quality of enzyme, presence of other proteins etc. Reproducible results in different laboratories can only be obtained if the methods are rather strictly defined. If the method is not completely defined then the use of a standard calibrated in the new units can minimise errors due to experimental variations. In principle the deviations from physiological conditions do not count then any more, and any anti-thrombin or anti-factor Xa method that is now in use can be applied to determine heparin contents in terms of the new units, when thus calibrated against well defined standards of anti-factor Xa- and anti-thrombin activity. The only caveat is that the standard and the heparin in question should react in the same way to changes in the reaction conditions. This is not the case e.g. when LMWHs are compared to a UFH standard in mixtures without Ca²⁺ ions (19, see also above). From Table 1 it is seen that heparin concentrations that have similar effects on thrombin generation have comparable activities when expressed in S.I. Units.

**Specific Activities of Heparins, their Use in Heparin Pharmacology**

We can express the specific activity of a heparin in terms of the S.I. units. To do this, we determine how much 1 μg of the heparin under investigation will increase the decay constants of factor Xa and of thrombin in a normal plasma with a known AT III content. If the addition of 1 μg of the heparin to 1 ml of plasma increases the decay constant from 0.490 min⁻¹ (the normal value without heparin) to 4.740 min⁻¹ per μM of AT III, than the specific activity of that heparins is 4.250 SIU-Xa per μM of crude material. In the crude material only the HAM is responsible for this activity, it therefore is logical to express this activity per μg or per nMole of HAM (Table 2).

In a completely analogous way we can determine the specific anti-thrombin activity of a heparin. Again from the specific activity of the crude material one can calculate the specific activity per μg or nMole of active heparin species, i.e. of the ACLM fraction (Table 2).

It should be stressed that these specific activities are inherent properties of a heparin, defined completely by the catalytic potencies of the preparation only and independent of the method with which they are obtained.

Once the specific anti-factor Xa activity of the HAM material of a given heparin is known, we can use the SIU-Xa level of a plasma sample to determine its concentration of HAM by simply dividing that level by the specific anti-factor Xa activity of the HAM that is administered (16). If our conjecture on the identical
specific activity of all HAM would prove to be true, as suggested by the data of Table 2, than the SIU-Xa value would indicate the number of active HAM molecules in the sample independent of the type of heparin injected. Also if it is not true, than the SIU-Xa value still indicates the level of HAM in terms of a mean of the molar activity. Analogously to the HAM level, the ACLM level of a plasma sample can be obtained by dividing its SIU-IIa value by the specific anti-thrombin activity of the ACLM fraction of the material injected.

Thus the definition of SI units and the determination of specific heparin activities enables us to assess separately the levels of ACLM and BCLM (BCLM = HAM - ACLM) in the circulation. This means that we can discuss heparin pharmacokinetics in terms of concentrations of active molecules. Pharmacokinetics can in this way be separated from pharmacodynamics. In order to get an impression of the heparin concentration of a sample it is no longer necessary to compare two pharmacodynamic effects such as the prolongation of the APTT and the thrombotic or bleeding tendency, Fig. 2 gives an example of this approach (from ref. 16).

The TFPI content of the patients sample will as such not significantly influence the half-life time of thrombin or factor Xa in plasma. TFPI has no influence on thrombin. The absolute concentrations of TFPI are maximally 5 nM (20), which is small compared to the amount of factor Xa used in decay experiments (around 100 nM). Also the action of TFPI is practically immediate, so that it will not play a role in the time domain of a decay experiment (min). Any other action of TFPI is dependent upon the presence of tissue thromboplastin, which is absent in anti-factor Xa assays. TFPI therefore will not significantly influence the SIU-Xa determination and not all the SIU-IIa determination.

Practical Consequences for the Clinical Laboratory: Anti-Thrombin and Anti-Factor Xa Tests

Any well-designed anti-thrombin test is dependent upon thrombin breakdown-velocity only. Likewise good anti-factor Xa activity tests will only measure factor Xa breakdown velocity. Therefore these tests already measure k_{dec}, only the results are not expressed in an appropriate way. If standards calibrated in SI units would be available, a conversion coefficient can be found for each method and no important changes in everyday laboratory practice would be required for the adoption of the SI unit.

The results of monospecific tests can than be expressed directly in standard independent units by multiplication with the conversion coefficient. This coefficient will be different for different tests. In any given case it can be determined by calibration of the test against a k_{dec} (t_0) determination or against a heparin preparation of which the potency is known in terms of SI units. This brings back the heparin standard, but it is no longer the standard that defines the unit. The standard is now a tool for calibration that can be abandoned by those who prefer to measure k_{dec} (t_0) directly. A suitable ACLM preparation can be used to calibrate both anti-factor Xa tests in terms of SIU-IIa and anti-thrombin tests in terms of SIU-IIa. In order to see wether the test under observation is indeed monospecific a BCLM standard can be used. A monospecific anti-thrombin test will not react to BCLM at all. A monospecific anti-factor Xa test will give the same result with an ACLM and a BCLM standard.

Monospecific anti-factor Xa tests can then be used to determine HAM (in μg/ml of plasma) and monospecific anti-thrombin tests can be used to determine ACLM (idem).

Practical Consequences for the Characterisation of Heparins

The approach suggested here would make it possible to compare directly the amount of anticoagulant material contained in different preparations of unfractionated as well as low molecular weight heparin. For this it is required to know: a) the content of high affinity material (HAM as % of total); and b) the distribution of the high affinity material around the 5,400 dalton cutoff (ACLM and BCLM as % of HAM). We need also to know: c) the specific anti-factor Xa activity of the HAM fraction and d) the specific anti-thrombin activity of the ACLM fraction.

Optionally the specific anti-factor Xa activity of the ACLM and the BCLM fractions may be given. Again optionally the number average molecular weight of the HAM and the ACLM fractions, or preferably the MW distribution, might complete the characterisation.

With these data (i.e. the heparin dependent constants sub a, b, c and d), it is possible to express the results of well calibrated common anti-thrombin and anti-factor Xa activity tests in terms of circulating concentrations of active (ACLM and BCLM) heparin.

The biochemical characterisation of the heparin is also a necessary prerequisite for describing its pharmacological proper-
ties, were it only because the elimination from the circulation is significantly different for ACLM and BCLM (16).

Of course we cannot exclude the possibility that non-anticoagulant actions of heparin, notably of its LAM fraction, contribute to its antithrombotic effects, either by influencing these effects or by actions that are not related to coagulation. Such actions cannot be rigorously proven to exist unless exact quantitation of the anticoagulant effects is possible.

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REFERENCES


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