Standard and method independent units for heparin anticoagulant activities

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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 via all the major assay methods.

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

Abbreviations used: AT III: antithrombin III; USP: United States pharmacopoeia; IU: International unit; SIU: standard independent unit; UFH: unfractionated heparin; LMWH: low molecular weight heparin; HAM: high affinity material; ACLM: above critical chainlength material; BCLM: below critical chainlength material; APTT: activated partial thromboplastin time.

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Table 1 Comparison of the activities of different heparins

	HAM	HAM	International Units		S.I. Units	
	µg/ml	µM	aXa	aIIa	aXa	aIIa
A: IC50 of the surface under the thrombin generation curve (intrinsic)						
UFH	0.077	0.0074	0.025	0.025	0.373	1.198
LMWH 1	0.153	0.030	0.065	0.022	0.357	1.164
LMWH 2	0.189	0.042	0.090	0.027	0.434	1.047
PENTA	0.800	0.480	0.520	–	0.495	–
B: Dose doubling the APTT						
UFH	0.62	0.059	0.192	0.192	1.97	6.36
LMWH 1	2.1	0.420	0.9	0.3	3.29	10.72
LMWH 2	2.1	0.470	1.0	0.3	3.20	7.72
PENTA	50	29.40	32.5	–	165	–

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 $CaCl_2$ (25 mM) was added and the clotting time read by tilting. The dose was sought that in 9 out of 10 experiments prolonged the blank of 35 s to between 68 and 72 s. Molar concentrations of HAM were obtained directly via AT III fluorescence titration.

split in two random parts, than there is a 5 in 30 chance that the pentasaccharide sequence will be split that is responsible for AT III binding and two LAM molecules will result.

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 ($E + A \xrightarrow{k} I^*$) that according to standard chemical kinetics has a reaction velocity of $v_t = k \cdot A_t \cdot E_t$. The concentration of AT III in plasma is about 2.5 µM. In every well designed decay experiment care is taken to keep the initial enzyme concentration below a certain proportion (e.g. 30 times less) of the AT III concentration. Therefore A_t can be considered constant and the enzyme decay can be described by pseudo-first order kinetics. The decay velocity of the enzyme is therefore expressed by $v_t = k \cdot A_0 \cdot E_t$, or $v = k_{dec} \cdot E_t$, where $k_{dec} = k \cdot A_0$. In this situation the time-course of E is given by $E_t = E_0 \cdot e^{-k_{dec} \cdot t}$; i.e. the enzyme decays semilogarithmically as e.g. a radioactive element. The decay constant is inversely proportional to the half-life time of the enzyme: $k_{dec} = 0.693/t_{½}$**. The dimension of k_{dec} therefore is inverse 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, at heparin concentrations that may be encountered in actual prophylactic and therapeutic practice, the increase of the decay constant is proportional to the AT III concentration and to the concentration of heparin: $k_{dec} = k \cdot A \cdot H$. This proportionality, that was found for all the different heparins that we tested (13, 14, 16–18), we will use as a basis for the definition of a heparin unit. It is important to notice that in this formula H stands for the concentration of *active* heparin. For the decay of factor Xa only HAM molecules count, for the decay of thrombin only the ACLM fraction.

The Confusion Inherent to the Old Units

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 (13, 14). In Table 1 it is seen that concentrations of different heparins with comparable biological activity on thrombin generation or the APT differ considerably in weight volume or molar terms and in anti-X_a 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.

Obviously only a standard with an identical ratio of ACLM:BCLM as the sample to be tested will not be influenced by the type of test used. This is the rationale behind the usefulness of the a LMWH standard (4). This standard is a mixture of ACLM and BCLM, the composition of which approaches the composition of other LMWHs better than the UFH standard does. But the units of a LMWH standard, nor of any other LMWH can be compared to units of UFH, in which hardly any BCLM is present.

A second complication is that the UFH standard, like all other UFHs but unlike LMWHs, has a low anti-factor Xa activity in the absence of Ca^{2+}, i.e. in all current anti-Xa tests. Any LMWH in these tests is measured against a "handicapped" standard and hence is overestimated (19).

The following may serve as an illustration. We took 0.01 USP unit of standard heparin and determined the amount of a LMWH that in a commercial, amidolytic anti-factor Xa test showed the same activity, so that by definition it contained also 0.01 USP unit of anti-factor Xa activity. This, in fact is the way in which the manufacturers arrive at the amount of activity declared on the label. For technical reasons current anti-factor Xa tests are carried out in the absence of Ca^{2+} ions. 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 Ca^{2+} ions and found the UFH preparation to be 1.37 times as potent as the LMWH. In the body heparin acts in the presence of Ca^{2+} ions, so the label of the LMWH must be judged to overestimate the contents by 1.86 times (see also 19).

So the label on the LMWH bottle overestimates the potency of its contents nearly two times. Similar results were obtained with other LMWHs, including the international standard for LMWH.

The confusion stems from two sources. In the first place the test conditions may differ considerably from the conditions in the body. In the second 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*

* 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 ½ equals −0.693.

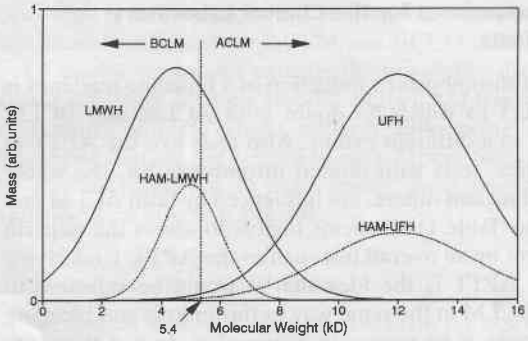

Fig. 1 Schematic representation of the molecular weight distribution in unfractionated and in low molecular weight heparin (see text)

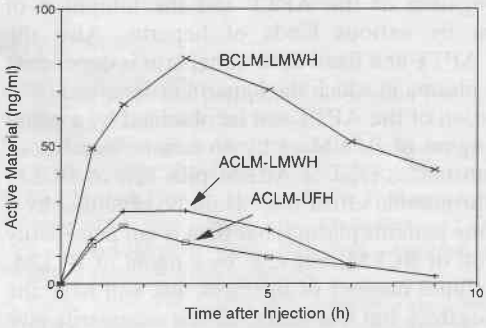

Fig. 2 The course of ACLM and BCLM levels after injection of UFH and LMWH. At zero time a subcutaneous injection was given of UFH (22 mg, containing 7.3 mg of ACLM and no detectable BCLM) and of LMWH (30 mg, containing 3.8 mg ACLM and 3.2 mg BCLM). No significant levels of BCLM were found when UFH was injected. (From ref. 16, to which we refer for details on materials and methods)

normal plasma, increases the decay constant of factor Xa by 1 min^{-1} per µM 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* µM 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 Ca^{2+} 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 Ca^{2+} 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 SI 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 µg 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

Table 2 Specific activities of a LMW heparin and its subfractions

Heparin	MW (dalton)	Specific anti-IIa act. per: µg crude	nMole HAM	nMole ACLM	Spec. anti-Xa act. per: µg crude	nMole HAM
Fraxiparin	5,100	2.36	56	>106	0.72	17.1
HAM, total	5,400	5.96	42	79	2.57	18.0
HAM, medium MW	7,740	16.88	136	140	1.77	14.3
HAM, low MW	4,620	0.62	3	7	3.66	17.8
HAM, ultralow MW	3,400	0.00	0	0	3.46	21.0

The specific activities are given in min^{-1} per unit weight as indicated, determined as described in ref. 16 and 17. Essentially similar data were obtained with Enoxaparin and Logiparin fractions (Béguin et al. unpublished).

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, then 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-aXa determination and not at all the SIU-aIIa 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} ($t_{1/2}$) 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} ($t_{1/2}$) directly. A suitable ACLM preparation can be used to calibrate both anti-factor Xa tests in terms of SIU-Xa 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 Clinical Laboratory: The Over-All Tests

Reduction of thrombosis or induction of a bleeding tendency is an over-all effect to which no doubt both ACLM and BCLM contribute, but to a different extent. Also tests like the APTT in its many varieties, tests with diluted thromboplastin, the whole blood clotting time and others, are influenced by both ACLM and BCLM (see also Table 1). It seems logical to assess the heparin effect in a patient by an overall test such as the APTT. Underlying the use of the APTT is the idea that it would be sensitive to ACLM and to BCLM in the same way as thrombosis and bleeding are. This, however, is an unproven assumption. In fact there are substances such as pentasaccharide or dermatan sulfate that hardly influence the APTT in doses that have a significant antithrombotic effect (21, 22). This is again seen in Table 1, from which it also can be deduced that there is a poor correlation between the prolongation of the APTT and the inhibition of thrombin-generation by various kinds of heparin. Also the prolongation of the APTT at a fixed dose of heparin is dependent upon the individual plasma in which the heparin is dissolved (23).

A fixed prolongation of the APTT can be obtained by *a* ng/ml of ACLM or by *b* ng/ml of BCLM or by an infinite number of mixtures of both substances (½*a* of ACLM plus ½*b* of BCLM etc.). A given antithrombotic effect may again be obtained by *a* ng/ml of heparin in the patients plasma, but than is not necessarily also obtained *b* ng/ml of BCLM, but e. g. by *c* ng/ml of BCLM. There is again an infinite number of mixtures that will have the same antithrombotic effect, but this series will *not* necessarily give an identical prolongation of the APTT. This shows that the APTT can be used without problems if we deal with ACLM only (i. e. in UFH) but looses tether as soon as mixtures with BCLM appear, i. e. in LMW heparins. The only way to understand what is indicated by a given prolongation of the APTT is to determine *a* and *b* as in the example above. They will probably differ even between different types of APTT.

One can imagine an ideal test that reacts to ACLM, BCLM and all other anticoagulants in the same way as the thrombotic tendency does. For the moment we can only prove that the current over-all tests are not suitable candidates. It is our guess that the thrombin potential, i. e. the surface under the thrombin generation curve might be more suitable for this purpose (24).

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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