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Determination of the Levels of Unfractionated and Low-Molecular-Weight Heparins in Plasma: Their Effect on Thrombin-Mediated Feedback Reactions in vivo

Preliminary Results after Subcutaneous Injection

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Key Words. Thrombin · Low-molecular-weight heparin · Unfractionated heparin · Prothrombinase · Factor Xa

Abstract. We define a standard independent unit (SIU) of heparin as that amount that, in plasma containing 1 μmol of ATIII, raises the (pseudo-)first-order breakdown constant of factor Xa by 1 min^{-1} . These units measure all material with a high affinity for ATIII (HAM); only material above the critical chain length of 17 monosaccharide units (above critical chain length material; ACLM) catalyzes the inactivation of thrombin. An SIU of ACLM is therefore analogously defined as the amount that, in plasma containing 1 μmol of ATIII, will raise the (pseudo-)first-order breakdown constant of thrombin by 1 min^{-1} . Of any given heparin preparation one can determine the specific HAM and ACLM activities in terms of SIU/mg. On the basis of the factor Xa and thrombin breakdown constants found in a plasma sample one can then determine the levels of HAM and ACLM. Preliminary experiments were carried out in plasma samples obtained after subcutaneous injection of unfractionated heparin (UFH) and of two types of low-molecular-weight heparin (LMWH). About three times more of UFH activity than of LMWH activity has to be injected to obtain the same levels of ACLM in the plasma. Only with the LMWHs significant amounts of BCLM are found, which rises higher and persists longer than the ACLM. We determined the course of thrombin generation in platelet-rich plasma (PRP) and in platelet-poor plasma (PPP), as well as in the PPP factor Xa generation curve and the course of prothrombin conversion. The observed inhibitions correlated much better with the levels of ACLM than with those of below critical chain length material. The difference between UFH and LMWHs can therefore not be explained in terms of antithrombin and anti-factor-Xa activity. The essential difference between UFH and LMWH appears in the feedback effect of thrombin in PRP, where thrombin generation is both inhibited and retarded by LMWH, while it is only retarded but hardly inhibited by UFH.

Introduction

In clinical and pharmacological studies anti-factor-Xa activity appears as a sensitive detector of heparins in plasma [1 and references therein]. From fundamental studies such as those reported in this volume, the effect of heparins on the feedback activation of the clotting mechanism by thrombin appears to be the more likely point of attack of heparins [2 and references therein, 3, 4]. Here an apparent paradox arises: *in vitro* heparin seems to act via inhibition of thrombin, but the activity in samples from subjects receiving heparin is adequately rendered by the anti-factor-Xa action. It is the purpose of this article to discuss this paradox. We will suggest a way out by introducing a clear separation between the estimation of the level of two different kinds of active heparin on the one hand and their effects on the different aspects of the biological system of thrombin generation on the other.

All heparins that bind with high affinity to ATIII do so via the specific pentasaccharide region [5, 6] and all heparins that bind to ATIII increase its anti-factor-Xa activity. In principle, this activity is therefore suitable to measure that part of injected heparin that contains this pentasaccharide region (high-affinity material; HAM).

Only high-affinity heparins with a minimal chain length of 18 monosaccharide units, i.e. with an MW > 5,400, will support the antithrombin activity of ATIII [7–13]. The high-affinity material above the critical chain length of 17 units (ACLM) can therefore be specifically measured by the antithrombin activity that it induces. ACLM is a subfraction of HAM. The other subfraction is the below critical chain length material (BCLM) that only enhances factor Xa inacti-

vation and that has no effect on thrombin inactivation. No specific test is available for this material, but it can be calculated from the difference between HAM and ACLM.

Anti-factor-Xa activity is routinely measured in the virtual absence of Ca^{2+} ions, in order to prevent concomitant factor Xa generation. Different types of heparin react differently to the ambient Ca^{2+} concentration [14, 15]. The action of a heparin on factor Xa *in vivo*, at normal Ca^{2+} concentration can therefore not automatically be estimated on the basis of common anti-factor-Xa tests.

Also the assessment of antithrombin activity in samples from *in vivo* experiments is not necessarily ideal. It is often not measured directly but inferred from the prolongation of the activated partial thromboplastin time (APTT) or the thrombin time [see, e.g., ref. 16]. These methods are as convenient as they are imprecise [17] and give only semiquantitative information on the magnitude of the antithrombin effect. The common laboratory tests for heparin in plasma samples are therefore not ideally suited to quantitate the anticoagulant effect of different heparins.

In this article we propose to use the specific antithrombin- and anti-factor-Xa activity of a heparin preparation to determine the concentration of the ACLM and the total HAM fraction in plasma samples. Once these are known, one can determine the effects of the circulating amounts of heparin on biological functions such as thrombin generation in platelet-poor (PPP), and platelet-rich plasma (PRP), prothrombin conversion velocity and factor Xa generation (PPP).

We tested this approach in a pilot experiment in which we injected unfractionated heparin (UFH) and two low-molecular-

weight heparin (LMWH) preparations (A and B) subcutaneously into healthy volunteers. In the plasma samples we determined the increase of the decay constants of thrombin and factor Xa. From these data and the specific activities of the heparins, we calculated the course of the concentrations of ACLM and BCLM. Then we determined the relationship between the levels of circulating heparin found and thrombin generation (in PPP and PRP), factor Xa generation and prothrombin-converting activity.

The Standard-Independent Unit of Heparin

At pharmacologically relevant concentrations, in plasma, the increase of the pseudo-first-order decay constant of thrombin is linearly dependent upon both the concentration of heparin and the concentration of ATIII [18 and discussion]. This property can be used to define a standard-independent unit (SIU) of heparin as that amount of heparin that, when added to 1 ml of plasma containing 1 μ mol of ATII increases the decay constant of a coagulation enzyme by 1 min^{-1} . The unit can be based on factor Xa inactivation to define an SIU of total HAM or it can be based on thrombin inactivation to define an SIU of ACLM.

The Specific Activities of Heparin

The specific activity of a heparin preparation can be expressed in terms of SIU per microgram of material. The factor-Xa-based specific activity is a function of the HAM material. If the HAM content of a heparin is known, it is logical to express this activity

per microgram of HAM. Analogously, the specific activity on factor IIa is a function of ACLM, and therefore it should be expressed in terms of that substance. Inversely, once the factor-Xa-based specific activity of the HAM fraction of a given heparin is known, one can calculate the HAM concentration in an unknown sample from the decay constant of factor Xa. Completely analogously one can calculate the ACLM concentration from the decay constant of thrombin and factor-IIa-based specific activity. One might think of determining specific activities in molar rather than in weight units, but this does not seem possible with the highly polydisperse preparations used.

Although the SIU is independent of a standard, it does not obliterate the use of a good standard preparation because the precise determination, in absolute terms, of specific activities will need a specialized laboratory. Comparative measurements are easier, so that good standard HAM and ACLM heparin preparations remain useful.

Materials and Methods

Materials

Calciparin, LMWH-A and LMWH-B were obtained from the dispensary of the hospital. The figures for HAM and ACLM content were obtained from previous work [11, 12]. Because no conclusions as to pharmacological properties should be drawn from an experiment in which each heparin was administered to 1 subject only, we will not reveal the identity of the heparins.

The chromogenic substrate for thrombin H-D-Phe-Pip-Arg-pNA (S-2238) was obtained from Kabi, Sweden.

Proteins

Reptilase was obtained from Boehringer-Mannheim (Mannheim, FRG) and dissolved according to the instructions of the manufacturer. Human brain

thromboplastin was prepared according to Owren and Aas [19]. Before use it was diluted 1:30 to 1:40 with buffer A containing 0.1 M Ca^{2+} so as to clot a mixture of 30 μl of thromboplastin, 30 μl of buffer A and 120 μl of plasma in 70–80 s. Recombinant hirudin (r-hirudin) was a kind gift of Dr. R.B. Wallis (Ciba-Geigy, Horsham, UK).

Plasmas

The heparins were injected subcutaneously into 2 healthy volunteers. Blood was taken by antecubital venipuncture at 0.75 h (UFH only), at 1.5, 3, 5, 7 h (all three preparations) and at 9 h (LMWHs only). PPP and PRP were obtained according to standard procedures [20].

Blood was collected on 0.13 M trisodium citrate; 9 parts of blood to 1 part of citrate solution. PRP, PPP and defibrinated plasma were obtained as previously published [20]. As shown, reptilase treatment does not significantly alter the concentration of factors II, VII, VIII, IX, X, XI and XII [20].

Activity Measurements

The decay constant of thrombin was determined as described in Béguin et al. [4], i.e. in a system where thrombin generation was inhibited; the half-time ($T_{1/2}$) of thrombin activity was measured and the decay constant was obtained as $\ln 2/t_{1/2}$.

The decay constant of factor Xa was determined as in Schoen et al. [15].

Thrombin generation in plasma was assessed and the constants of inactivation of endogenous thrombin were determined according to Béguin et al. [4]. In short, 240 μl of defibrinated plasma is supplemented with 60 μl of buffer A, containing heparin at the desired concentration and incubated for 5 min at 37°C . At zero time, thrombin generation is started by the addition of 60 μl of a solution containing 100 mM of CaCl_2 , and diluted human brain thromboplastin. At intervals, a 10- μl aliquot of the mixture is sampled into a disposable plastic cuvette containing buffer and chromogenic substrate. The amidolytic activities in the plasma samples are calculated from the optical density and expressed as the equivalent concentration of thrombin (in nM), according to a reference curve obtained with active-site titrated purified human α -thrombin.

The generation of prothrombinase activity is calculated from the amidolytic activity curve of throm-

bin generation, as described previously [20]. Briefly, the observed amidolytic activity is the sum of thrombin activity and the partial activity of α_2 -macroglobulin-thrombin complex. The rate of thrombin generation at any moment is the sum of two processes: (a) The conversion of prothrombin into thrombin by prothrombinase and (b) the decay of thrombin by the action of plasma protease inhibitors. The rate of inhibition of thrombin at any time can be calculated from the concentration of thrombin at that moment and the pseudo-first-order rate constant of thrombin inhibition by ATIII (k_1) and α_2 -macroglobulin (k_2). The rate of prothrombin activation (prothrombinase activity) then can be calculated from the observed rate of generation of amidolytic activity and the rate of thrombin inhibition.

Factor Xa generation curves: plasma (240 μl) was incubated with 60 μl thromboplastin diluted 1:6 in 100 mM CaCl_2 containing $10\text{ }\mu\text{M}$ r-hirudin. At timed intervals, samples were taken and assayed for factor Xa activity using a two-stage bioassay in which factor Xa is the rate-limiting component of prothrombinase (i.e. phospholipids and factor Va are present in excess) and prothrombin is the substrate [21].

Experimental

Specific Activities

In a normal plasma, with a known amount of ATIII and spiked with known amounts of the heparins we determined the specific anti-thrombin and anti-factor-Xa activities for the heparins used. From this and the HAM content we calculated the specific anti-factor-Xa activity of the HAM fraction. In a similar way, the specific anti-thrombin activity of the ACLM subfraction was determined (table 1).

Course of Heparin Concentrations after Subcutaneous Injection

The three heparins were injected subcutaneously in healthy male volunteers in doses as indicated in table 2. In this table, the

amounts of material injected are also recalculated in terms of standard independent units. It is surprising to see that, contrary to what might be gathered from the current literature, injection of LMWHs does not automatically mean administration of large amounts of anti-factor-Xa activity. In fact, the amounts of antithrombin activity as well as that of anti-factor-Xa activity injected with the LMWHs are around 30% of those injected with UFH (table 1).

We determined the increase of the decay constant for thrombin and for factor Xa after injection of the three heparins in amounts indicated in table 1. After normalization for the ATIII levels of the individual plasmas, this led to the course of the plasma levels expressed in SI units shown in figure 1. The ratio of the antithrombin over the anti-factor-Xa activities scatters around unity (fig. 2). Via the specific activities we then calculated the HAM levels

from the increase of the factor Xa decay constants, while the ACLM levels were obtained from the increase of the thrombin decay constants. The difference between HAM and ACLM is the high-affinity material with a molecular weight below the critical limit for thrombin inactivation, i.e. BCLM (fig. 3).

It is evident that the concentrations found in the plasma differ more than can be explained from the difference in the amounts injected. The large amount of antithrombin activity injected with UFH causes lower plasma concentrations of ACLM than the much smaller amounts injected with the LMWHs. This can be attributed to the lower bioavailability and shorter half-time of UFH [22, 23]. Also the roughly twofold difference between the two LMWHs is insufficiently explained by the doses injected and might be due to differences between the heparins and biological variation between the volunteers.

Table 1. Properties of the material injected

		UFH	LMWH-A	LMWH-B
Peak MW, kD		14.5	5.1	4.4
HAM, %		33	24	14
ACLM (HAM > 5.4 kD), %		33	13	8.6
BCLM (HAM < 5.4 kD), %		0	11	5.4
Antithrombin spec. act. ¹	total	10.33	2.36	1.70
	ACLM	31.0	18.2	19.8
Anti-factor-XA spec. act. ¹	total	3.37	0.66	0.55
	HAM	10.21	2.75	3.93
Ratio antithrombin: anti-Xa		3.1	3.6	3.1

¹ In min⁻¹/μmol ATIII/(μg/ml heparin).

After the subcutaneous injection of heparin, the concentration of SIU (Xa)/ml and SIU (thr)/ml in the plasma were measured. The results are shown in Figure 1. The concentration of SIU (Xa)/ml and SIU (thr)/ml in the plasma increased rapidly after the injection of heparin and reached a maximum at 2-4 h. The concentration of SIU (Xa)/ml and SIU (thr)/ml in the plasma then decreased gradually. The concentration of SIU (Xa)/ml and SIU (thr)/ml in the plasma was significantly higher in the LMWH-B group than in the LMWH-A and UFH groups.

Fig. 1. Heparin activities in SIU after subcutaneous injection. **a** Total HAM on the basis of anti-F factor-XA activity **b** ACLM on the basis of anti-thrombin activity. ○ = UFH; ● = LMWH-A, △ = LMWH-B.

Table 2. Doses injected

		UFH	LMWH-A	LMWH-B
Weight of subject, kg		85	89	85
Total antithrombin ¹		228	69	76
Total anti-factor Xa ¹		74.4	19.3	24.6
Dose (calculated), mg	total	22	29	45
	HAM	7.3	7.0	6.3
	ACLM	7.3	3.8	3.8
	BCLM	0	3.2	2.5

¹ Standard independent kilo-units, min⁻¹/μmol ATIII × 1,000.

Fig. 2. Correlation between thrombin based and factor-Xa-based SIU $\circ$ = UFH; $\bullet$ = LMWH-A; $\triangle$ = LMWH-B.

Fig. 3. Levels of active heparin after subcutaneous injection. **a** HAM and BCLM. **b** ACLM. $\circ$ = UFH; $\bullet$ = LMWH-A; $\triangle$ = LMWH-B.

As can be expected, negligible amounts of BCLM are found after injection of UFH, whereas large amounts of this material can be detected after injection of the LMWHs. It is readily seen that the BCLM persists longer in the circulation than the ACLM from the same LMWH doses. Probably, a longer half-time and a higher bioavailability as compared to ACLM accompany its lower molecular weight.

Inhibition of Thrombin- and Factor Xa Generation and of Prothrombin Conversion

In figure 4 we see the effect of heparin injection on the peak activities of the throm-

bin generation curves obtained in each sample. It should be noted that inhibition of thrombin peaks in these experiments reflects the combined effect of increased thrombin breakdown and inhibition of prothrombin conversion [24]. The inhibition of prothrombin conversion necessarily causes a proportional inhibition of the thrombin peak. The amount of inhibition of the thrombin peak above that of prothrombinase consequently is caused by increased thrombin inactivation. The latter in UFH samples in fact contributes all and in LMWH samples more than half of the total inhibition (fig. 4a). It is interesting to see that with the LMWHs an

Fig. 4. Inhibition of thrombin- and prothrombinase peaks. **a** Inhibition of thrombin peaks (—) and prothrombinase peaks (.....). **b** Inhibition of the thrombin peak that cannot be attributed to prothrombinase inhibition. ○ = UFH; ● = LMWH-A; △ = LMWH-B.

Fig. 5. Inhibition of factor Xa and prothrombinase peaks. **a** Inhibition of factor Xa peaks (—) and prothrombinase peaks (.....). **b** Inhibition of prothrombinase as a function of inhibition of factor Xa. $\circ$ = UFH; $\bullet$ = LMWH-A; $\triangle$ = LMWH-B.

inhibition of the thrombin peak, that must be attributed to thrombin breakdown, persists much longer than with UFH (fig. 4b). It seems that there exists ACLM that is sufficiently small to be eliminated with a long half-life.

In UFH as well as in the LMWHs, factor Xa inhibition is higher than the inhibition of thrombin (fig. 4, 5). The ratio of the inhibitions varies with time. The excess inhibition of factor Xa in the later hours can be attributed to the presence of relatively high con-

centrations of BCLM at that moment (fig. 3). The inhibition of prothrombinase is much smaller than the inhibition of factor Xa in all instances (fig. 5a), and the inhibition of factor Xa is not linearly related to the inhibition of prothrombinase (fig. 5b).

Thrombin Inhibition in PRP

An important difference between UFH and LMWHs is to be found when thrombin generation curves are obtained in PRP. Whereas UFH loses its capacity to inhibit

Fig. 6. Inhibition of thrombin peaks in PRP and PPP. **a** Inhibition of thrombin peaks in PRP (—) and in PPP (.....). **b** Inhibition of the thrombin peak in PRP as a function of the inhibition in PPP. $\circ$ = UFH; $\bullet$ = LMWH-A; Δ = LMWH-B.

thrombin generation in the presence of platelets, both LMWHs tested retain their activity under these circumstances (fig. 6).

Discussion

It was the purpose of this article to use recent theoretical insights into the mode of action of heparins as a basis for a rational approach to the interpretation of the data

obtained in plasma samples from persons injected with heparin. The results are given as obtained with three types of heparin, each administered to 1 volunteer only. These are practical examples and illustrations of the proposed approach. Any conclusions that are drawn on the behaviour *in vivo* of UFH and LMWHs are necessarily preliminary and will have to be repeated in larger groups of subjects to allow for the influences of experimental and inter- as well as intraindividual variability.

All heparins in clinical use are highly heterogeneous, both in molecular weight and in ATIII affinity. Only the material with high affinity for ATIII will directly affect the clotting mechanism. The catalytic properties of the HAM vary with MW. The most distinct variation is that with material below the critical chain length of 17 sugar units (MW < 5,400), heparins lose their capability to catalyze thrombin breakdown, whereas they retain their anti-factor-Xa activity down to the size of a pentasaccharide (MW $\geq$ 1,500). There may be variations in specific activity within these groups but as a first approximation the anticoagulant properties of a mixed heparin can be expressed by its content of above- and below critical chain length material (ACLM and BCLM). One can determine the specific activity on factor Xa inactivation of the HAM fraction of a heparin. From the anti-factor-Xa activity in a sample, one can then calculate the amount of HAM present. Completely analogously, one can determine the ACLM concentration in a sample from the antithrombin activity and the specific antithrombin activity of the material injected. Once these concentrations are known, one can try and relate any observed biological effect, such as APTT, inhibition of thrombin generation, performance in a thrombosis model or clinical performance to these concentrations.

We realize that this is only a first step in analyzing the effects of heparin heterogeneity. Complications may arise from different sources:

(a) Within the ACLM and the BCLM class the specific activities may vary with molecular weight. Indeed, in plasma we have found that there is difference between UFH and LMWH in this respect, yet between dif-

ferent MW fractions of one type of LMWH the specific activities were surprisingly similar [11, 12].

(b) Heparins may mobilise endogenous anticoagulants in the circulation [e.g., 24–27], that will obviously not be quantitated via specific activities.

(c) It remains possible that the antithrombotic properties of heparin are not related to their anticoagulant action. For the moment we bypass this hypothesis, which, however, may be readily revived if no satisfying correlation is found between anticoagulant- and antithrombotic effects.

The ultimate consequence of anticoagulant therapy is the diminution of the amount of free thrombin that arises in clotting plasma. The fact that pentasaccharide [29] as well as dermatan sulfate [30] show an antithrombotic effect in animals, whereas pentasaccharide acts on factor Xa only, and hence on prothrombin conversion, while dermatan sulfate only increases thrombin breakdown, strongly suggests that it is immaterial whether thrombin is diminished by inhibition of prothrombin conversion or by enhancement of thrombin breakdown.

The levels of thrombin that are obtained in clotting plasma are the result of the combined action of heparin on prothrombin conversion and on thrombin decay:

An increase in antithrombin activity under the influence of ACLM will lead to a proportional decrease of the amount of thrombin present. A decrease of factor Xa, as caused by both ACLM and BCLM, will cause a decrease of prothrombinase activity,

but that activity, under the conditions in clotting plasma, is *not* linearly proportional to the amount of factor Xa present, so that factor Xa inhibition will *not* lead to a directly proportional decrease of prothrombinase activity [24].

Prothrombinase is a tripartite complex of factors Xa and Va and phospholipid arising from simple chemical equilibria [31]. Pieters et al. [24] found that in clotting plasma, under our conditions, factor Va is the limiting prothrombinase component and that factor Xa is formed at levels of around 10 nmol, i.e. in large excess over the limiting factor Va. The binding constant of factor Xa to phospholipid bound factor Va is 0.1 nmol [32]. This means that at 0.3 nmol factor Va and 10 nmol factor Xa, 99% of factor Va is bound to factor Xa in prothrombinase. With 1 nmol of factor Xa 91% of factor Va will still be bound. This means that the inhibition of factor Xa will have to be very important before any inhibition of prothrombinase is observed. Therefore antithrombin activity of a heparin results in immediate, proportional inhibition of thrombin formation, whereas anti-factor Xa activity does not result in a proportional inhibition of thrombin generation, as can be seen from figure 5.

Ever since Yin et al. [33] suggested that inhibition of factor Xa would be a more efficient means to prevent thrombosis than inhibition of thrombin, it was implicitly understood that the anti-factor-Xa activity induced by a heparin could be an important indicator of its antithrombotic properties. It came therefore as a surprise that heparins, when added to plasma appeared not to cause an important inhibition of prothrombin conversion, with the understandable exception of the ultra-low-molecular-weight- or P-type

varieties, that hardly contain any ACLM and consequently hardly show any antithrombin activity [4, 5, 34, 35]. It appears that the enhanced inhibition of factor Xa that is reputedly observed with LMWHs, does not appear or hardly appears as inhibition of prothrombin conversion, so that the differences between UFH and LMWH cannot be explained on this basis. This was the more confusing because the anti-Xa activities measured in plasma shows a good correlation with antithrombotic efficiency [1 and references therein].

It should be kept in mind that it is entirely possible that the anti-factor-Xa action is a marker of heparin activity while not being itself the instrument of heparin action. Recent observations on the influence of the Ca^{2+} concentration in plasma on the anti-factor-Xa action shed further doubt on the relevance of conventionally measured anti-factor-Xa activities for the anticoagulant effect of a heparin [14, 15].

It appears impossible to explain the action of heparins without referring to the influence of heparins on thrombin-mediated feedback reactions. It becomes increasingly apparent that the positive feedback exerted by thrombin on the procoagulant properties of blood platelets, and inversely the neutralizing effect of platelet factor 4 on heparins, may be important mechanisms to explain the difference between UFH and LMWHs. From figure 6 it is evident that LMWHs retain their activity in the presence of platelets, whereas UFH does not. This behavior of thrombin generation in PRP was to be expected from previous observations in spiked plasmas [36]. The doses of LMWH given, although roughly equivalent to the UFH dose from clinical dose-finding experiments, represent a much

larger number of molecules, due to the much lower specific activities of the LMWH (table 2). Along with the active fractions, an important amount of low-affinity material is injected (fig. 2). These molecules can react with platelet factor 4 and protect active heparin from being neutralized by activated platelets.

Apart from this effect, the main difference between UFH and LMWH probably has to be found in its pharmacological properties. Figure 1 suggests that after the injection of LMWH, not only much more BCLM material reaches the blood stream, but also that the ACLM shows better bioavailability. From table 2 one sees that the LMWH injections contain roughly half as much ACLM as the UFH injection. Yet from figure 2 it is seen that in the circulation the ACLM levels obtained are equal or higher. From figure 4 it is seen that the effect on overall thrombin generation is higher and longer lasting, even (fig. 4b) the anti-thrombin effect lasts longer. This means that the antithrombin material of LMWH has more favorable pharmacological properties than the same type of material from UFH.

BCLM persists in the circulation longer than ACLM does. The persistent inhibition of thrombin generation obtained with LMWHs between 5 and 10 h after injection can be explained by the activity of this material. The possibility of an endogenous anticoagulant released under the influence of the heparin injection must be left open, however. Anyhow, here a difference between the in vivo situation and the situation in spiked plasma becomes apparent. The LMWH injected probably contains material with different half-times so that injection results in an in vivo fractionation of the LMWH injected, and the heparin circulating between 5

and 10 h after injection will become gradually enriched in BCLM.

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