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The Activity of Unfractionated Heparin and Low Molecular Weight Heparins in Rabbit Plasma – The Need for Using Absolute Anti-factor Xa and Antithrombin Activities

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Summary

Rabbit being a common animal model to evaluate the antithrombotic effect of heparins, our purpose was to apply the heparin Standard Independent Unit (SIU) approach to rabbit plasma. To take into account the specificities of the enzymes we have measured the decay constants of factor Xa and of thrombin from autologous and heterologous origins, in presence and in absence of heparin. Different heparins or heparin fractions with a mean molecular weight from 1.7 to 10.5 kDa were used.

We found that: a) the decay constants varied strongly between species and between enzymes; b) the decay constants of thrombin were always higher than those of factor Xa; c) the specific anti-factor Xa activity of heparins increased with the molecular weight and was 1.33 times higher when determined with bovine factor Xa than with rabbit factor Xa; d) the specific antithrombin activity of heparins also increased with the molecular weight but was similar when determined with rabbit and human thrombin; e) the specific anti-factor Xa activity was always lower than the specific antithrombin activity; f) the calibration of the heparins and heparin fractions against the 4th International Standard of Heparin expressed in International Units (IU) lead to a systematic overestimation of the anti-factor Xa activity and to an underestimation of the antithrombin activity.

These observations indicate that it may be very important to use the SIU approach and to know the accurate activities to better understand the mechanism of the antithrombotic activity of heparins in experimental models.

Introduction

Most animal studies on the antithrombotic effect of heparins use rabbit thrombosis models. The antithrombotic action of heparins is mediated by catalysing the inhibition of thrombin, factor Xa and other clotting factors by antithrombin (AT) (1, 2). So, in the rabbit, heparins act through the rabbit coagulation system, which is not necessarily quantitatively identical to that of the human. The results therefore should be interpreted while keeping possible differences between the human and the rabbit in mind.

In order to be active, heparin has to contain the specific pentasaccharide sequence that binds to AT (3) (high affinity material; HAM). HAM molecules with a molecular weight above 5.4 kDa are able to act on both factor Xa and thrombin inactivation, those with a molecular weight below 5.4 kDa are able to catalyse factor Xa inactivation only (4). This distinguishes ACLM (above critical chain length material) from BCLM (below critical chain length material). So the specific anti-factor Xa activity of a heparin is caused by all HAM molecules and the specific antithrombin activity by its subgroup, the ACLM molecules only. Unfractionated heparin hardly contains any BCLM but low molecular weight heparins (LMWHs) do to a varying degree.

A LMWH preparation therefore is always a mixture of ACLM and BCLM. It is impossible to quantify such a mixture by comparing it to a standard that contains ACLM only (like the International Standard Heparin, ISH). When the anti-factor Xa activity is measured, BCLM counts on an equal basis as the much stronger anticoagulant than ACLM. When the antithrombin activity is assessed, BCLM is disregarded. This is not remedied by the introduction of a LMWH-standard, as long as the latter standard is itself characterised by comparison to an UFH standard (5). On top of that, the usual *in vitro* comparison of LMWHs to the ISH, which is carried out in the absence of Ca^{++}, will overestimate the anti-factor Xa activity of LMWH about twice, due to the Ca^{++} dependency of anti-factor Xa activity of large molecular weight heparins contained in the standard (6).

It therefore was proposed to express anti-factor Xa- and antithrombin activity independent of any standard, but by direct quantitation of the catalytic effect of heparin on AT-mediated thrombin and factor Xa breakdown. This led to the definition of a Standard Independent Unit (SIU) of heparin activity (5). One SIU of anti-factor Xa (or antithrombin) activity of heparin is the amount of heparin that, in normal plasma, increases the decay constant of factor Xa (or thrombin) by 1 min^{-1} per μM of plasmatic AT.

Another source of confusion in the heparin field is the expression of heparin concentration. The weight of a heparin preparation is not related to the number of active molecules because: a) there is a large number of inactive heparin molecules present and b) the number average molecular weight of the active fraction is unknown. This however can be circumvented because the molarity of a solution of AT binding heparin can be determined by fluorescent titration (7).

The combination of expressing the activity of a heparin in absolute terms (in SIU) and the concentration in molar terms allows, for the first time, to characterise the specific activity of a heparin, standard- and method-independently, as SIU per mole.

Although expressed in absolute terms, the specific activity of a heparin remains dependent upon the reaction conditions, temperature, pH, ionic strength and the nature of the other reactants: thrombin, factor Xa and antithrombin. The determination should therefore be done under conditions that are as near as possible to *in vivo* circumstances. This not only means that it should be determined in plasma at a normal Ca^{++} concentration but also in plasma of the relevant species.

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The aim of this study was to apply the SIU approach to rabbit plasma. So we determined the specific anti-factor Xa- and antithrombin activities in SIU of a series of heparin preparations under conditions prevailing in the rabbit, i.e. in rabbit plasma with autologous factor Xa and thrombin.

Materials and Methods

Preparation of Plasmas

Blood from 12 New Zealand male rabbits (2-3 kg) was collected on 0.129 M trisodium citrate (9 parts of blood to 1 part of citrate solution) via the left carotid artery. Platelet poor plasma (PPP) was obtained after two centrifugations at 15° C, for 15 min at 3000 × g, and a third centrifugation at 4° C, for 1 h at 20,000 × g. These PPP were pooled and stored at −80° C.

Defibrinated plasma was obtained by mixing an aliquot of plasma with 0.25 IU/ml of ancrod, letting a clot form for 10 min at 37° C. The resultant clot was squeezed and then discarded by winding it on a small plastic spatula.

Human PPP from 10 healthy donors was prepared analogously to rabbit PPP.

Heparins

The 4th International Standard of Heparin (4th ISH) and first International Standard of Low Molecular Weight Heparin (1st ISLMWH) were a kind gift of Dr. T. W. Barrowcliffe from National Institute of Biological Standards and Control (Hertfordshire, UK).

The CY 216 and its ACLM and BCLM fractions previously used in reference (8) (batch XH 716 C, P 2016 XH and P 2017 XH respectively) and the synthetic pentasaccharide (PS) that represents the antithrombin (AT) binding site of heparin (3) were supplied by Sanofi-Recherche (Gentilly, France).

The Logiparin (batch LMW 9101) was obtained from Novo Nordisk (Copenhagen, Denmark).

The Fragmin (batch 45314-51) was obtained from Kabi-Pharmacia (Stockholm, Sweden).

The molar concentrations of the heparin species with high affinity for AT (HAM) in each preparation were determined as described in (7).

The percentage of the heparin species with a molecular weight above 5.4 kDa (ACLM) in each preparation was determined by the manufacturers.

Table 1 summarizes the molecular weight (MW), the HAM and the ACLM in each heparin preparations.

The specific antithrombin and anti-factor Xa activities expressed in IU · mg^{-1} were determined as previously described (9).

Reagents

The chromogenic substrates for thombin (S-2238) and for factor Xa (S-2337) were obtained from Chromogenix (Mölndal, Sweden).

Buffer A: 20 mM Hepes, 150 mM NaCl, 0.5 mg/ml bovine serum albumin, pH 7.35. Buffer B: 20 mM Hepes, 150 mM NaCl, 20 mM EDTA, 0.5 mg/ml bovine serum albumin, pH 7.90. Buffer C: 20 mM Hepes, 220 mM NaCl, 20 mM EDTA, 0.5 mg/ml bovine serum albumin, pH 7.90.

Ancrod was a kind gift of National Institute of Biological Standards and Control (Hertfordshire, UK) and dissolved according to the instructions of the manufacturer.

Recombinant hirudin (r-hirudin) was a kind gift of Dr. R. B. Wallis (Ciba-Geigy, Horsham, UK)

Recombinant tissue factor (rTF) was obtained from Baxter Diagnostics (Switzerland). Before use in thrombin generation test it was diluted with buffer A containing 0.1 M Ca^{++} so as to clot a mixture of pooled human PPP in 34-35 s (30 µl of rTF, 30 µl of buffer A and 120 µl of PPP).

Human thrombin was prepared from euglobulin fraction obtained from defibrinated pooled human PPP by acid precipitation (pH 5.2) at a low ionic strenght as previously described (10). The precipitate was collected by centrifugation and dissolved in half the initial plasma volume in buffer A containing 21.6 mM trisodium citrate. In order to obtain thrombin, thrombin generation was triggered by adding 0.25 vol of diluted rTF to 1 vol of euglobulin fraction.

Human, bovine and rabbit clotting factors were prepared according to techniques described in (11) and (12).

Determination of the Decay Constants of Thrombin

A mixture of 80 µl of defibrinated rabbit plasma and 20 µl of buffer A with or without heparin was prewarmed for 5 min at 37° C. Then 20 µl of human, bovine or rabbit thrombin were added to obtain a final concentration of 70 nM. At timed intervals (3-5 s), a 10 µl aliquot was sampled into a disposable plastic cuvette containing 490 µl of a 200 µM of S-2238 in buffer B. The sampling was done with a pushbutton equipped pipette that recorded the moment of sampling on a personal computer. After about 3 min the amidolytic reaction was stopped by adding 300 µl of 1 M citric acid. The moment of stopping was also recorded automatically. Optical densities were read at 405 nm in a dual wavelength (405-500 nm) dedicated instrument constructed in our workshop. The concentrations of thrombin were read from the ΔOD/min values via a proportionality constant obtained with active site titrated thrombin.

The amidolytic activities (C_t) were fitted to the three parameters curve $C_t = C_\infty + (C_o - C_\infty) \cdot e^{-k\,t}$, where $k = k_{dec}$, C_o is the initial amidolytic activity and C_∞ is the residual, steady end-level activity due to the α_2-macroglobulin-thrombin (α_2M-T) complex. The decay constant (kdec) was the sum of the AT dependent part (k1) and the α_2-macroglobulin dependent part (k2). It was shown that k1 was linearly dependent upon the heparin concentration, whereas k2 was not dependent on heparin (13).

Determination of the Decay Constants of Factor Xa

A mixture of 80 µl of defibrinated rabbit plasma, 7 µl of r-hirudin (35 µM) and 6 µl of buffer A with or without heparin was prewarmed for 270 s at 37° C. Then 7 µl of $CaCl_2$ (0.2 M) were added and then 30 s later 20 µl of human, bovine or rabbit factor Xa so as to obtain a final concentration of 170 nM. At timed intervals (4-6 s), a 10 µl aliquot was sampled into a disposable plastic cuvette containing 490 µl of a 200 µM of S-2337 in buffer C. The disappearance of factor Xa was then measured as described above for thrombin and the decay constants were calculated analogously to those of thrombin.

Calculation of Specific Activities

One SIU of anti-factor Xa activity (SIU Xa) or of antithrombin activity (SIU IIa) is defined as the amount of heparin that, in normal plasma, increases the decay constant of factor Xa (or thrombin) by 1 min^{-1} per µM of plasmatic AT. It therefore is calculated by devision of the increase of the relevant decay constant by AT content of the plasma.

Table 1 Molecular properties of different heparin preparations

Heparins	MW kDa	HAM µM/µg	ACLM %
4th ISH	10.50	0.058	100
Logiparin	6.90	0.040	75
Fragmin	6.20	0.082	55.1
1st ISLMWH	5.39	0.098	61.5
CY 216	5.09	0.038	40
ACLM	6.71	0.052	100
BCLM	3.63	0.024	0
PS	1.70	0.589	0

ACLM and BCLM are the fractions of CY 216 prepared by gel filtration.

Table 2 Half-life times and decay constants of factor Xa and thrombin of different species in rabbit and human plasma

	rabbit plasma		human plasma	
	$t_{1/2}$ (s)	kdec (min^{-1})	$t_{1/2}$ (s)	kdec (min^{-1})
rabbit factor Xa	31.5±1.8	1.32±0.07	30.4±1.1	1.37±0.05
human factor Xa	39.4±2.4	1.06±0.07	79.0±3.3	0.53±0.02
bovine factor Xa	26.0±3.3	1.62±0.18	39.2±2.2	1.06±0.06
rabbit thrombin	9.6±0.5	4.35±0.24	15.9±1.1	2.63±0.17
human thrombin	16.5±1.0	2.53±0.16	16.7±0.4	2.49±0.06
bovine thrombin	14.2±0.5	2.93±0.11	22.2±1.1	1.87±0.09

kdec=ln $2/t_{1/2}$ with $t_{1/2}$ expressed in minutes.
Results are given as mean±SD of at least 3 experiments.

Results

Determination of Functional AT Concentration in Plasma

Because heparin activity is proportional to the AT concentration of the medium, the SIU must be expressed per μM of AT in the plasma (5). To determine the AT content of the plasma we titrated the unknown amount of AT with a known excess amount of thrombin and determined the residual thrombin activity. In a plastic spectrophotometer cuvette 100 μl of a suitable dilution of the plasma (1:100 to 1:400) in buffer B containing 2 IU/ml unfractionated heparin were incubated with 100 μl of a 20 nM thrombin solution at 37° C during 40 min. Then 300 μl of 330 μM S-2238 in buffer B were added, and the amidolytic activity of the residual thrombin was measured at 405 nm in a spectrophotometer at 37° C. This activity was converted into nM thrombin via a reference curve obtained with active site titrated thrombin. The AT concentration was calculated as the difference between the initial thrombin concentration added and the residual thrombin activity found.

Table 3 Specific antithrombin activities of different heparin preparations in rabbit plasma determined with rabbit (R) or human (H) thrombin

Heparins	SIU IIa					
	min^{-1}/μg/ml		min^{-1}/μM HAM		min^{-1}/μM ACL-HAM	
	R	H	R	H	R	H
4th ISH	13.88	15.90	239.31	274.14	239.31	274.14
Logiparin	5.40	5.43	135.00	135.75	180.00	181.00
Fragmin	10.34	9.22	126.10	112.44	228.85	204.06
1st ISLMWH	7.52	7.32	76.73	74.69	127.77	121.45
CY 216	2.48	2.42	65.26	63.68	163.16	159.21
ACLM	10.13	11.46	194.81	220.38	194.81	220.38
BCLM	0.15	0.08	6.25	3.33	----	----
PS	----	----	----	----	----	----

The values reported are the mean of at least 3 determinations with standard deviations of approximately 9%.

In pooled rabbit plasma the functional AT concentration was found to be 2.27 ± 0.14 μM (n = 3) which was similar to that in human plasma (2.24 ± 0.11 μM) (14). Identical results were obtained using rabbit and human thrombin.

AT Specificity for Thrombin and Factor Xa

A marked species specificity was observed in the interaction of clotting factors with AT. Table 2 shows that the decay constants of factor Xa and thrombin differ according to the source of clotting factors and of the plasma used. In rabbit plasma the decay constants of human and bovine factor Xa were respectively 1.25 times lower and 1.23 times higher than that of rabbit factor Xa and the decay constants of human and bovine thrombin were respectively 1.72 and 1.48 times lower than that of rabbit thrombin. In human plasma, the decay constants of human and bovine factor Xa were respectively 2.58 and 1.29 times lower than that of rabbit factor Xa and the decay constants of human and bovine thrombin were respectively 1.06 and 1.41 times lower than that of rabbit thrombin. The decay constants of rabbit factor Xa and human thrombin were similar in rabbit and human plasma but those of the four other preparations were different.

Moreover, the decay constants of thrombin were always higher than those of factor Xa, both in rabbit and human plasma.

The Specific Antithrombin Activities of Different Heparins

The specific activity of a heparin is defined as the increase of the decay constant of thrombin in plasma per amount of heparin and per μM of AT. For a series of different heparins, the pseudo-first order decay constants of exogenous thrombin (rabbit and human) were determined as a function of the concentration of the heparins. The activity, if present, always increased linearly with the concentration of the heparin. Table 3 summarizes the specific antithrombin activities found in rabbit plasma.

We found that the specific antithrombin activity measured in rabbit plasma was independent of the type of thrombin used. The ratio of the specific antithrombin activities determined with human thrombin to the ones determined with rabbit thrombin was 1.02 ± 0.09 (mean ± SD).

The Specific Anti-factor Xa Activities of Different Heparins

Table 4 summarizes the specific anti-factor Xa activities, defined analogously to the specific antithrombin activity above, found in rabbit plasma. For a series of different heparins, the pseudo-first order decay constants of exogenous factor Xa (rabbit and bovine) were determined for increasing concentrations of the heparins as in the previous paragraph. Again the constants always increased linearly with the concentration of the heparin.

We found that for each heparin, except for CY 216, the specific anti-factor Xa activity in rabbit plasma was 1.33 ± 0.11 (mean ± SD) times higher with bovine factor Xa than with rabbit factor Xa.

The specific anti-factor Xa activity of heparins in rabbit plasma increases as a function of the molecular weight, most significantly at molecular weights above 6.2 kDa.

Calculation Coefficients to Shift from IU to SIU; the Ratios of Specific Anti-factor Xa to Antithrombin Activities of Heparins

In Table 5 we give the potency of heparins in IU and SIU and the calculation coefficients to shift between the two types of unit. When

expressed in SIU, the ratios of the specific anti-factor Xa activities to those of thrombin ($min^{-1}/\mu g/ml$) as determined with rabbit factors were calculated for each heparin preparation. The ratio was 0.59 for 4th ISH and the mean ratio was 0.24 (range from 0.17 to 0.36) for the LMWHs, i.e. almost 2.5 times lower; so the specific antithrombin activity was always higher than the specific anti-factor Xa activity and the ratio decreased with molecular weight.

Discussion

In this study, it was our aim to investigate the antithrombin system in the rabbit, the most commonly used animal in thrombosis models. Unfractionated and various low molecular weight heparin preparations were used. Because of species-dependent variation in the behaviour of the enzymes, the relative units have a different meaning in rabbit plasma and in human plasma. Differences can be easily missed if the activities are expressed relative to a standard, if the rabbit system reacts to the standard and to the heparin in a similar manner. E.g. if in rabbit plasma the specific anti-factor Xa activity of a heparin – but not its antithrombin activity – would always be three times higher than in the human, both with the standard and with the heparin under investigation, than this would go unobserved if activities are expressed relative to that standard. Yet the mechanism of action of the drug might be mediated via factor Xa in the rabbit and not in the human. Also the molecular weight dependence of heparin activity could be different in the rabbit and in the human, if their AT molecules would be sufficiently different. Therefore, species differences should be obligatorily expressed in terms of absolute catalytic activity of heparins (5).

For these reasons, we determined the specific anti-factor Xa and antithrombin activities of a series of heparins in a homologous rabbit system in standard independent terms. We also included experiments with heterologous material because bovine factor Xa and human thrombin are more readily available than rabbit products are and therefore are often used in practice.

Table 2 shows that there are important species differences in the absolute decay velocities in the human and in the rabbit, the latter being on the mean about 1.5 times faster.

Human thrombin decays with normal, low velocity in rabbit plasma, which excludes the possibility that the high reactivity of the rabbit thrombin-AT interaction might be due to the presence of endogenous ACLM activity in the rabbit plasma. We must conclude that there are important species differences between the rabbit and the human, and that it seems not justified to extrapolate data obtained in a rabbit system to a human system.

By definition, in international units, the ratio of the anti-factor Xa to antithrombin activities is 1 for the 4th ISH. It is higher than 1 for low molecular weight heparins, which suggests that LMWHs would increase more the AT effect on factor Xa than on thrombin. However, since thrombin is much more sensitive to the heparin-AT complex than factor Xa is, the ratio of absolute anti-factor Xa/antithrombin activities is always smaller than 1 (Table 5), varying from 0.59 for the 4th ISH to 0.24 on average for LMWHs.

Therefore, in rabbit plasma, the 4th ISH shows a slightly higher antithrombin activity than the anti-factor Xa and the LMWHs present a very high antithrombin activity with regard to their anti-factor Xa activity. Consequently, when rabbit thrombosis model experiments were designed on the basis of anti-factor Xa units, a (very) high amount of antithrombin units accompanies this activity, contrary to what we are induced to think when we use international units (15).

Table 4 Specific anti-factor Xa activities of different heparin preparations in rabbit plasma determined with rabbit (R) or bovine (B) factor Xa

Heparins	SIU Xa			
	$min^{-1}/\mu g/ml$		$min^{-1}/\mu M$ HAM	
	R	B	R	B
4th ISH	8.21	10.88	141.55	187.59
Logiparin	1.48	1.93	37.00	48.25
Fragmin	1.88	2.42	22.93	29.51
1st ISLMWH	1.83	2.44	18.67	24.90
CY 216	0.90	0.91	23.68	23.95
ACLM	1.70	2.27	32.69	43.65
BCLM	0.36	0.56	15.00	23.33
PS	7.53	10.06	12.78	17.08

The values reported are the mean of at least 3 determinations with standard deviations of approximately 6%.

Table 5 Calculation coefficients to shift from IU to SIU and anti-factor Xa/antithrombin ratios in rabbit plasma

Heparins	Anti-factor Xa activity			Antithrombin activity			Ratio aXa/aIIa	
	IU	SIU	SIU/IU	IU	SIU	SIU/IU	IU	SIU
4th ISH	194	8.21	0.042	194	13.88	0.072	1.00	0.59
Logiparin	85	1.48	0.017	50	5.40	0.108	1.70	0.27
Fragmin	152	1.88	0.012	66.5	10.34	0.155	2.29	0.18
1st ISLMWH	168	1.83	0.011	66.5	7.52	0.113	2.53	0.24
CY 216	65	0.90	0.014	26	2.48	0.095	2.50	0.36
ACLM	120	1.70	0.014	100	10.13	0.101	1.20	0.17
BCLM	38	0.36	0.009	0.2	0.15	0.75	(190)	(2.4)
PS	350	7.53	0.022	----	----	----	----	----

The activities are expressed in IU/mg for International Unit (IU) and $min^{-1}/\mu g/ml$ for Standard Independent Unit (SIU). Values given in brackets are not relevant, BCLM being almost devoid of anti-thrombin activity.

The specific anti-factor Xa activities of different heparin preparations in rabbit plasma have also been determined with bovine factor Xa and, except for CY 216, all of them have been found about 1.33 times higher than with rabbit factor Xa (Table 4). So if the more convenient commercial bovine factor Xa preparation is used, one has to apply a conversion coefficient of 1.33 to the value found with factor Xa in order to obtain the accurate specific anti-factor Xa activity in rabbit plasma.

With human and rabbit thrombin preparations, the specific activities obtained were similar (Table 3). Therefore it is possible to use directly the values found with human thrombin in rabbit plasma.

Like in human plasma (data not published) we observed in rabbit plasma an increase of the specific activities as a function of the molecular weight of heparin (Tables 3 and 4); for anti-factor Xa activity the effect was more pronounced above 6.2 kDa while the antithrombin activity was increased according to the molecular weight.

This study confirms that the specific anti-factor Xa and antithrombin activities of different heparin preparations may be different according to the species studied, the source of clotting factors used and the definition of units, IU or SIU. Consequently it could be very important to re-evaluate the antithrombotic potencies of heparins using the absolute activities determined in systems of the relevant species.

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