# Low molecular weight heparin-catalyzed inactivation of Factor Xa and thrombin by antithrombin III-effect of Platelet Factor 4

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# Low Molecular Weight Heparin-Catalyzed Inactivation of Factor Xa and Thrombin by Antithrombin III – Effect of Platelet Factor 4

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## Summary

Low molecular weight (LMW) heparin preparations have unknown distributions of ATIII-binding material, so mean molecular weights as such might bear little information on their anti-factor Xa and anti-thrombin activities, and on the neutralization of these activities by platelet factor 4 (PF4). These properties were investigated in pure systems with proteins of human origin. Pseudo-first order rate constants of inactivation of factor Xa and thrombin by antithrombin III were determined as function of heparin concentration, in the presence of 4.0 mM $CaCl_2$. Despite a large variation in the mean molecular weights, the ratios of the anti-factor Xa over the anti-thrombin activities were essentially the same for the 4th International Standard for heparin (0.46), the 1st International Standard for LMW heparin (0.32), CY216 (0.42) and enoxaparin (0.50). The ultra LMW heparin CY222 had only a 2-times higher ratio (0.98). Analysis of CY216 subfractions, obtained by gel filtration, showed that the heparin molecules of the upper region of the molecular weight distribution are responsible for the anti-thrombin, but also to a large extent for the anti-factor Xa activities. The results indicate that depolymerization of unfractionated heparin does not result in an increased anti-factor Xa/anti-thrombin ratio, because in the presence of $Ca^{2+}$-ions the rate constants of inactivation of factor Xa are lowered as compared to those of native heparin. PF4-dependent neutralization of anti-factor Xa and anti-thrombin activities of fixed concentrations of the LMW heparins was studied by measuring rate constants as function of PF4 concentration. All anti-thrombin and 50% of the anti-factor Xa activities were readily neutralized. Excess PF4 was required to neutralize another 35–50% of the anti-factor Xa activities. At PF4 levels obtained at maximal release of the content of platelet α-granules, all anti-thrombin and most (≥85%) of the anti-factor Xa activities can be neutralized.

## Introduction

Unfractionated (UF) and low molecular weight (LMW) heparins inhibit blood coagulation by potentiating the anti-protease activity of antithrombin III (ATIII) (1). Studies on the structure-function relationship of heparin demonstrated that its anti-factor Xa activity depends on the presence of the specific ATIII-binding pentasaccharide domain (see Ref. 2 for a review). The sole binding of heparin to ATIII, however, is not sufficient for catalysis of thrombin inactivation; this process also depends on the simultaneous binding of thrombin (3, 4). The smallest heparin fragment with a noticeable anti-thrombin activity is an octadecasaccharide (5–7). Studies, utilizing heparin fractions with high affinity for ATIII obtained by gel filtration and affinity chromatography on matrix-bound ATIII, have indicated that as the molecular weights of heparin fractions decrease from 10,000 to 2,000 the anti-thrombin activities vanish, while the anti-factor Xa activities diminish much less rapidly.

LMW heparin preparations, as obtained by depolymerization of native heparin, largely consist of molecules with a length of 18 saccharides and less. The lesser ability of these short heparin chains to enhance thrombin inhibition was thought to explain the decreased ability of LMW heparin preparations to prolong the clotting time of global coagulation tests like the aPTT (8, 9). LMW heparins, now clinically used or intended to be used, encompass heparin chains in a vast range of molecular weights (10). In addition to information about the molecular weight distribution of a particular LMW heparin preparation, it is of paramount importance to know the distribution of the heparin chains with affinity for ATIII. This is especially important, because the LMW heparins available at present are prepared using a variety of fragmentation methods, which is an important source for an increase of the inhomogeneity present in UF heparin (10). For instance, certain depolymerization methods (especially the nitrous acid hydrolysis) will also result in cleavage of ATIII-binding sites, which ultimately leads to non-random distribution of ATIII-high affinity saccharides throughout the molecular weight distribution profiles of LMW heparins.

Surprisingly, in spite of an extensive research for biological differences between different LMW heparin preparations, the distribution of the ATIII-binding oligosaccharides has not been evaluated. Moreover, anti-protease specific activities of (LMW) heparin preparations are still determined under rather unphysiological conditions: bovine factor Xa or thrombin in a citrated plasma milieu. At present it has to be realized that the anti-factor Xa and anti-thrombin activities of heparin are influenced by free $Ca^{2+}$-ions. The extent to which the kinetics of inhibition of the proteases are affected by $Ca^{2+}$ depends on the nature (factor Xa or thrombin) and origin (bovine or human) of the protease, and on structural features of the heparin species (chain length) (11–13).

Differences between LMW heparin preparations, regarding their molecular weight profiles and the distribution patterns of their ATIII-high affinity saccharides, might have consequences for their overall anticoagulant activities, and may influence their overall sensitivities towards neutralization by heparin-binding proteins, like platelet factor 4 (PF4). From the work of Lane et al. (3) it is known that the anti-thrombin and anti-factor Xa activities of heparin molecules with 18 or more monosaccharide units can be completely neutralized by PF4. However, as the heparin chain lengths decrease from 18 to 8 saccharides, increasing amounts of

Abbreviations: ATIII, antithrombin III; LMW, low molecular weight; UF, unfractionated; MMW, medium molecular weight; PF4, platelet factor 4; HSA, human serum albumin; S2238, D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride; S2337, benzoyl-L-isoleucyl-L-glutamyl-(γ-piperidyl)-L-glycyl-L-arginine-p-nitroanilide hydrochloride.

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*Table 1* The mean molecular weights, and molecular weight distributions of the UF and LMW heparins used in the study

| Heparin type | Mean $M_r$ | $M_r$ distribution | | | |
|---|---|---|---|---|---|
| | | <2,500 (%) | >7,500 (%) | $M_r$ <95% | 95% < $M_r$ |
| UF standard heparin[1] | 12,500 | 0.5 | 86 | | |
| LMW standard heparin[1] | 6,400 | 4 | 24 | | |
| CY216[2] | 5,100 | 5 | 9 | 2,300 | 9,400 |
| CY216 MMW fraction[2] | 7,900 | 0.5 | 64 | 6,100 | 11,200 |
| CY216 LMW fraction[2] | 4,400 | 0.5 | 0 | 3,300 | 5,500 |
| CY216 ultra LMW fraction[2] | 3,100 | 24 | 0 | 1,800 | 4,500 |
| Enoxaparin (PK10169)[1] | 3,800 | 14 | 23 | | |
| CY222[2] | 3,800 | 24 | 2 | 2,700 | 9,000 |

[1]See Ref. 10.
[2]Weight average, and distribution provided by Sanofi Recherche.

anti-factor Xa activity resist neutralization. The platelet protein PF4, stored in the α-granules, consists of 4 identical subunits, each containing a heparin binding site (14). A strong non-specific electrostatic interaction has been reported between PF4 and heparin molecules with a chain length of 16 saccharides and more (15).

Mean molecular weights as such might actually have no predictive value for the anti-thrombin and anti-factor Xa specific activities of LMW heparins, and their neutralization by heparin-binding proteins like PF4. This provoked us to initiate a reinvestigation of these properties in well-defined buffer systems, utilizing LMW heparin preparations which are at present intended for clinical use and standardization.

## Materials and Methods

*Proteins*

Human PF4 was purified by affinity chromatography on heparin-Sepharose (16). Briefly, outdated platelet concentrates were pooled, and the platelets were lysed by the addition of Triton X100 to a final concentration of 1%. The suspension was heated at 56° C for 45 min and centrifuged at 10,000 × *g* for 30 min at 4° C. Solid ammonium sulfate was added to the continuously stirred supernatant, to a final concentration of 40%. After centrifugation, supernatant was collected and extensively dialysed against 50 nM Tris-HCl (pH 7.5) containing 0.50 M NaCl. The material was applied to a heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer. A linear salt gradient, from 0.50 to 2.0 M NaCl in 50 mM Tris-HCl (pH 7.5), was applied and PF4, assessed by its anti-heparin activity, eluted at about 1.5 M NaCl. The fractions with the highest PF4 activity were pooled, extensively dialysed against 50 mM Tris-HCl (pH 8.0) containing 0.40 M NaCl, and stored at −70° C. The preparation was neither contaminated with heparin nor with ATIII. The molar concentration was assessed utilizing $E_{1\%} = 2.9$ at a wavelength of 280 nm (17), and a molecular weight of 31,200 for the PF4 tetramer.

Human factor Xa and human ATIII were prepared as reported previously (18). Thrombin was isolated, by chromatography of prothrombinase-activated human prothrombin (19) on sulfopropyl-Sephadex (Pharmacia, Uppsala, Sweden) at pH 7.4 (20). The molar concentrations of the factor Xa and thrombin preparations were determined by active site titration with p-nitrophenyl p'-guanidinobenzoate hydrochloride (21, 22). The molar concentration of ATIII was determined by titration with known amounts of factor Xa. The factor Xa, thrombin and ATIII preparations were stored in 50 mM Tris-HCl (pH 7.9) containing 175 mM NaCl, at −70° C.

*Heparins*

The 4th International Standard for heparin (UF standard heparin) and the 1st International Standard for LMW heparin (LMW standard heparin) were a kind gift of Dr. T. W. Barrowcliffe from the National Institute for Biological Standards and Control (London, United Kingdom). The LMW heparin preparations CY216 (batch P 795 XH) and CY222 (batch P 227 WH) were from Sanofi Recherche (Paris, France), and enoxaparin (PK10169) was obtained from Rhône-Poulenc (Gennevilliers, France). At the laboratory of Sanofi Recherche, CY216 was subjected to molecular weight fractionation by gel filtration, and the molecular weight distribution patterns of the obtained fractions were established by (HPLC) gel permeation chromatography (23). Concentrations of ATIII-binding material present in the heparin preparations were determined by stoichiometric titration of ATIII, as monitored by intrinsic fluorescence enhancement (18). The available molecular weight data of all heparin preparations used are given in Table 1.

*Inactivation of Factor Xa and Thrombin*

The heparin-catalyzed inactivations of factor Xa and thrombin by ATIII were studied under pseudo-first order conditions. Either of the enzymes was added to a mixture of ATIII and heparin in 50 mM Tris-HCl (pH 7.9) containing 175 mM NaCl, 4.0 mM $CaCl_2$ and 0.5 mg HSA/ml at 37° C. The initial concentrations were 30 nM factor Xa or thrombin and 400 nM ATIII. The heparin concentrations were variable. After 2 min (factor Xa) or 30 s (thrombin) residual enzyme concentrations were determined by transferring 20 µl samples to cuvettes containing 0.48 ml of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg HSA/ml and 0.22 mM of the appropriate chromogenic substrate (factor Xa: S2337; thrombin: S2238).

The rates of absorbance increase were measured on a thermostated (37° C) dual wavelength spectrophotometer at 405 nm (reference wavelength: 500 nm). From the residual factor Xa and thrombin concentrations the pseudo-first order rate constants were determined. The inactivation reactions were shown to follow pseudo-first order kinetics up to at least a 90% inhibition of the initial enzymatic activities. Under the conditions used, i.e. ATIII concentrations in excess over the heparin concentrations, and larger than the dissociation constant of the interaction between heparin and ATIII, essentially all heparin molecules containing the ATIII-binding region, are complexed with ATIII (12). Therefore, the pseudo-first order rate constants increase linearly with increasing heparin concentrations. The heparin-dependent increase of the rate constants, the anti-factor Xa and anti-thrombin specific catalytic activities, were estimated by linear regression analysis.

*Neutralization of heparin activity by PF4.* The heparin-catalyzed ATIII-dependent inactivation of factor Xa or thrombin was studied in the presence of variable PF4 concentrations. Pseudo-first order rate constants in the presence of PF4 ($k_{obs}$) were determined as outlined above. From these values we calculated the extents of neutralization (%) of the anti-protease activities as follows:

$$\text{NEUTRALIZATION} = (k - k_{obs})/(k - k_o) \times 100\%$$

where $k$ is the pseudo-first order rate constant obtained in the absence of PF4, and $k_o$ is the pseudo-first order rate constant obtained in the absence of heparin.

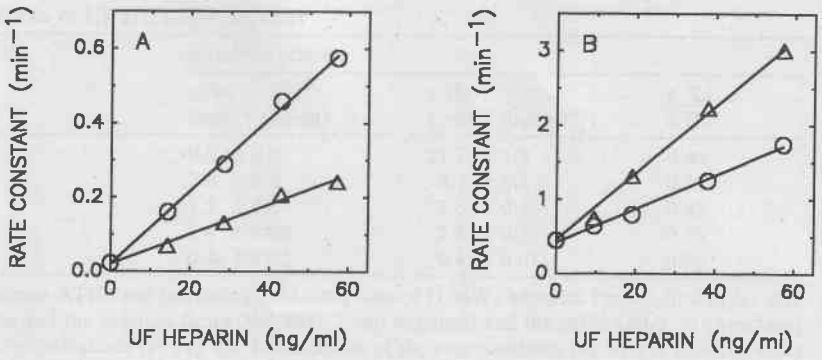


*Fig. 1* Rate constants of factor Xa and thrombin inactivation as function of the concentration of UF standard heparin. 30 nM factor Xa (panel A) or thrombin (panel B) was added to ATIII (400 nM) and heparin, either in the presence of 4.0 mM $CaCl_2$ (○) or in absence of $CaCl_2$ (△). Rate constants were determined as outlined in "Materials and Methods". Solid lines were obtained by linear regression analysis of the data

## Results

### *UF and LMW Heparin Catalyzed Inactivation of Factor Xa and Thrombin by ATIII*

The pseudo-first order rate constants of inhibition of factor Xa and thrombin as function of UF standard heparin concentrations are shown in Fig. 1. In addition, Fig. 1 also illustrates the effect of $Ca^{2+}$-ions on the heparin-dependent inhibition of thrombin and factor Xa. Linear regression analysis of the data obtained in the presence of 4 mM $CaCl_2$ gave an anti-factor Xa specific catalytic activity of 9.8 ± 0.4 (SE) $min^{-1}$ (μg UF heparin/ml)$^{-1}$ and an anti-thrombin specific catalytic activity of 21.2 ± 1.8 (SE) $min^{-1}$ (μg UF heparin/ml)$^{-1}$. The anti-factor Xa and anti-thrombin specific activities in the absence of $CaCl_2$ were 4.1 ± 0.2 (SE) and 44.6 ± 3.8 (SE) $min^{-1}$ (μg UF heparin/ml)$^{-1}$, respectively. When the same determinations were performed at a higher ATIII concentration (1 μM), the very same values were obtained, indicating that 0.40 μM ATIII was sufficiently high as to saturate all the ATIII-binding sites of heparin present (data not shown). Because of the stimulating effect of $Ca^{2+}$-ions on the UF heparin-catalyzed inhibition of factor Xa and its negative effect on the UF heparin-catalyzed inhibition of thrombin, all further experiments were performed in the presence of 4 mM $CaCl_2$.

The anti-factor Xa and anti-thrombin specific catalytic activities of a selection of LMW heparin preparations were determined in the same way, and are depicted in Table 2. The data show that, concomitant with the decrease of mean molecular weights, both the anti-thrombin *and* anti-factor Xa catalytic activities decrease. Hence, except for CY222, the heparins tested have rather invariable anti-factor Xa/anti-thrombin specific activity ratios. They were found to vary between 0.3 and 0.5. The ultra LMW heparin CY222 which, on a weight basis, consists for almost a quarter of saccharides with $M_r$ <2,500 (Table 1), has the lowest specific catalytic activities, and deviates from the other heparin preparations, in that it has a significantly higher anti-factor Xa over anti-thrombin ratio of 1.

Thus, although the different LMW heparin preparations include significant amounts of molecules consisting of 18 saccharide units and less ($M_r$ <5,400), the preparations show a striking similarity with respect to their anti-factor Xa over anti-thrombin activity ratios.

### *Molecular Weight Fractionation of CY216*

The fact that the anti-factor Xa over anti-thrombin activity ratios do not increase with decreasing molecular weights, as demonstrated in this study (Table 2), was unexpected. Besides a diminishing effect of $Ca^{2+}$-ions on the inhibition of factor Xa when heparins are used with decreasing molecular weights (11, 12), the anti-factor Xa specific activities will also be influenced by the actual distribution of saccharides with high affinity for ATIII. We therefore investigated the distribution of the anti-factor Xa and anti-thrombin activities, and of the ATIII-high affinity heparin species, of one of the LMW heparin preparations (CY216), over its range of molecular weights. To this end fractions obtained by gel filtration were studied. The mean molecular weights and molecular weight distributions of the obtained medium molecular weight (MMW), LMW, and ultra LMW fractions are given in Table 1. The anti-factor Xa and anti-thrombin catalytic activities as well as the ATIII-binding capacities of the three fractions, and of CY216 are given in Table 3.

It is seen that, on a weight basis, the ATIII-binding capacities of the fractions decrease with decreasing mean molecular weights.

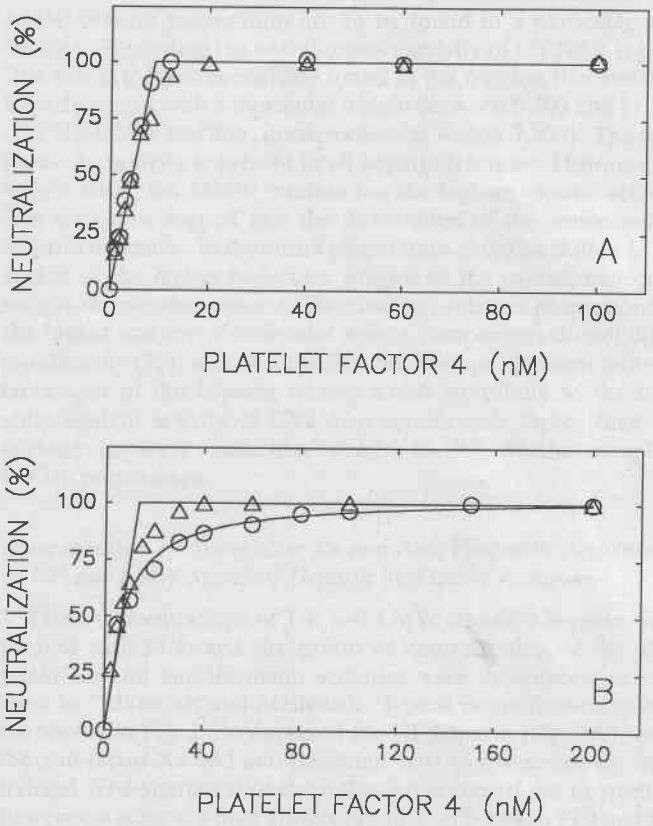


*Fig. 2* Neutralization of UF and LMW standard heparin by PF4. Pseudo-first order rate constants of inactivation of 30 nM factor Xa (○) or 30 nM thrombin (△) by 400 nM ATIII were determined in the presence of 100 ng/ml UF standard heparin (panel A) or 320 ng/ml LMW standard heparin (panel B), and variable concentrations of PF4. Neutralization of the anti-protease activities was assessed as described in "Materials and Methods". Solid lines are linear approximations of the data

*Table 2* Anti-factor Xa and anti-thrombin specific catalytic activities of UF and LMW heparins

| Heparin type | ATIII-binding capacity | Catalytic activity | | |
|---|---|---|---|---|
| | (nmol/mg) | a-Xa ($min^{-1}$ $(\mu g/ml)^{-1}$) | a-IIa ($min^{-1}$ $(\mu g/ml)^{-1}$) | a-Xa / a-IIa |
| UF standard heparin | 29 | 9.8 ± 0.4 | 21.2 ± 1.8 | 0.46 |
| LMW standard heparin | 43 | 2.8 ± 0.2 | 8.7 ± 0.4 | 0.32 |
| CY216 | 48 | 1.5 ± 0.1 | 3.6 ± 0.1 | 0.42 |
| Enoxaparin | 29 | 1.4 ± 0.05 | 2.8 ± 0.1 | 0.50 |
| CY222 | 41 | 0.44 ± 0.02 | 0.45 ± 0.01 | 0.98 |

Human factor Xa or thrombin (30 nM) was added to 400 nM human ATIII and increasing concentrations of (LMW) heparin. Pseudo-first order rate constants were determined from the initial enzyme concentrations and the residual factor Xa (after 2 min reaction) and thrombin (after 30 s reaction) concentrations. The rate constants were plotted vs. the heparin concentrations (cf. Fig. 1). The increase of the rate constants per weight concentration heparin were estimated by linear regression analysis, to reveal the anti-factor Xa (a-Xa) and anti-thrombin (a-IIa) specific catalytic activities. Errors represent the SE values of the estimates.

*Table 3* Anti-factor Xa and anti-thrombin specific catalytic activities of subfractions of CY216

| Heparin type | ATIII-binding capacity | Catalytic activity | | |
|---|---|---|---|---|
| | (nmol/mg) | a-Xa ($min^{-1}$ $(\mu g/ml)^{-1}$) | a-IIa ($min^{-1}$ $(\mu g/ml)^{-1}$) | a-Xa / a-IIa |
| CY216 | 48 | 1.5 ± 0.1 | 3.6 ± 0.1 | 0.42 |
| MMW fraction | 68 | 3.9 ± 0.2 | 20.3 ± 0.4 | 0.19 |
| LMW fraction | 49 | 1.0 ± 0.03 | 0.03 ± 0.01 | 33.3 |
| Ultra LMW fraction | 20 | 0.3 ± 0.05 | 0 | ∞ |

Experimental details as in Table 2.

We want to note here, that calculations on molar basis will not be realistic because of the dispersity of the ATIII-binding species in a certain fraction. However, a dramatic decrease of the ATIII-binding molarities with the decrease of the mean molecular weights of the fractions is evident. As a consequence of the decreased ATIII-binding capacities both the anti-factor Xa and anti-thrombin catalytic activities of the fractions will decrease with decreasing molecular weights. The more dramatic reduction of the anti-thrombin catalytic activity is the result of an abolished interaction between thrombin and heparin, due to the decreasing heparin chain lengths. As a result, the anti-factor Xa over anti-thrombin ratios of the fractions increase with decreasing mean molecular weights.

So far the data have clearly demonstrated a marked dispersity within a typical LMW heparin with respect to heparin species with different activities. The important question that remains to be answered, is whether or not the anticoagulant characteristics of a LMW heparin preparation are to be found in a particular subfraction. Regarding the anti-thrombin activity of CY216 it is clear that this activity is exclusively found in the fraction that contains heparin chains with a molecular weight between 6,100 and 11,200 (CY216 MMW fraction, mean molecular weight 7,900). The anti-factor Xa activity is present in all heparin fractions. However, on weight basis, the MMW fraction has the highest specific activity. The data thus suggest that the distribution of the anticoagulant heparin molecules in the entire preparation could be shifted to the region of the higher molecular weights of the overall molecular weight distribution pattern. Because the relative proportions of the higher and lower molecular weight components do not differ significantly (10), we conclude that the average apparent molecular weight of the heparin species which contribute to the total anticoagulant activity of CY216, is significantly higher than the average apparent molecular weight (5,100) of the complete CY216 preparation.

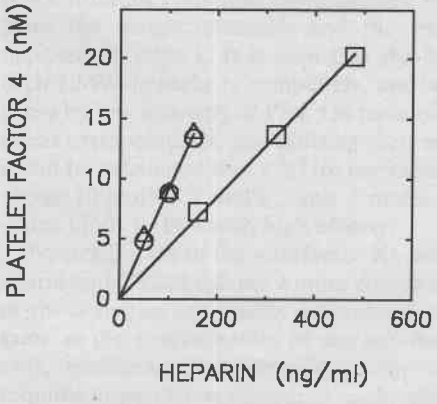


*Fig. 3* PF4-binding equivalents of UF and LMW standard heparin. Anti-factor Xa (○) and antithrombin (△) activities of variable concentrations of UF standard heparin, and antithrombin activities (□) of variable concentrations of LMW standard heparin were titrated with PF4, as in Fig. 2. Intersections of the linear extrapolations are shown as function of the heparin concentrations. Solid lines were obtained by linear regression analysis

*Neutralization of Anti-Factor Xa and Anti-Thrombin Activities of UF and LMW Standard Heparin by Platelet Factor 4*

Fixed concentrations of UF and LMW standard heparin were titrated with PF4, and the extent of neutralization of the anti-factor Xa and anti-thrombin activities were determined as outlined in "Materials and Methods". Typical neutralization curves are shown in Fig. 2. As expected for UF heparin (Fig. 2A), both the anti-factor Xa and anti-thrombin activities were readily neutralized. The sharpness of the inflection points of the neutralization curves suggest a high affinity interaction between PF4 and the heparin molecules with these anti-protease activities. Because of this sharp transition, the inflection points, obtained from neutralization experiments at varying heparin concentrations, were taken as measure for the PF4-binding normality of UF standard heparin. A replot of these values against the heparin concentrations is depicted in Fig. 3. From the slope, estimated by linear

regression analysis, it follows that the anti-factor Xa and anti-thrombin activities of 1 mg of UF standard heparin are abolished by 91 ± 2 nmol PF4. From stoichiometric titrations of ATIII (see "Materials and Methods"), it followed that 1 mg of UF standard heparin was equivalent to 29 nmol of ATIII-binding material. That a 3-times higher PF4-binding normality was found than could be expected on basis of the molar concentration of ATIII-binding heparin species can be readily explained. Although only about one-third of UF heparin binds to ATIII (24, 25), still UF standard heparin is largely (>95%) composed of heparin molecules containing 18 saccharide residues or more, therefore each heparin molecule can bind with high affinity to PF4, irrespective of whether it contains the ATIII-binding pentasaccharide region or not (15).

Typical curves of the neutralization of the anti-thrombin and anti-factor Xa activities of the LMW standard heparin are shown in Fig. 2B. It is seen, that both the anti-thrombin and anti-factor Xa activities can be completely abolished, although the neutralization of anti-factor Xa activity requires somewhat higher amounts of PF4.

The intersections of the linear extrapolations of the anti-thrombin neutralization curves, obtained at varying heparin concentrations, were replotted against the LMW standard heparin concentrations (Fig. 3). From the slope, estimated by linear regression analysis, it follows that the anti-thrombin activity of 1 mg of LMW standard heparin is abolished by an equivalent amount of 43 ± 1 nmol PF4. On basis of a mean molecular weight of 6,400 (Table 1), 1 mg of LMW standard heparin is estimated to correspond to about 160 nmol of material. Thus, in contrast with UF standard heparin, only a small fraction of LMW standard heparin (approximately 25 mol%) has a high affinity for PF4. The molar concentration of the ATIII-binding species, as determined by the intrinsic fluorescence enhancement (see "Materials and Methods"), was 43 nmol per mg LMW standard heparin. Evidently, part of the ATIII-binding saccharides have a lower affinity for PF4, which is (also) demonstrated by the more hyperbolic nature of the neutralization curves of the anti-factor Xa activities (Fig. 2B).

*Neutralization of Anti-Factor Xa and Anti-Thrombin Activities of CY216, Enoxaparin, and CY222*

Fixed amounts of CY216, enoxaparin, and CY222 were titrated with PF4. Neutralization of the anti-protease activities is shown in Fig. 4 as function of PF4 concentrations. The heparin concentrations employed corresponded to 100 nM, as calculated from the weight amounts and the mean molecular weights depicted in Table 1. It is seen that the anti-thrombin activity of each LMW heparin is completely, and with high affinity, abrogated by low amounts of PF4. On basis of the intersections of the linear extrapolations, and utilizing their mean molecular weights, it can be calculated that CY216, enoxaparin, and CY222 contain about 10 mol%, 7 mol%, and 5 mol% material, respectively, which binds to PF4 with high affinity.

Neutralization of the anti-factor Xa activities of CY216, enoxaparin and CY222 follows a more complex pattern. The first 50% of the activities are readily neutralized and seem to behave the same as the neutralization of the anti-thrombin activities. However, maximum neutralization of the anti-factor Xa activities requires more PF4 as compared to the complete neutralization of the anti-thrombin activities, due to a pronounced hyperbolic nature of the neutralization curves of anti-factor Xa activities. In contrast with the LMW standard heparin, the anti-factor Xa activities of CY216, enoxaparin and CY222 are not completely abolished. From these neutralization curves (Figs. 4B, D, and F), it is determined that about 5%, 10%, and 15% of the anti-factor Xa activities of CY216, enoxaparin, and CY222, respectively, cannot be neutralized by a large molar excess of PF4. This differential effect of increasing amounts of PF4 on the neutralization curves can be explained by increasing amounts of saccharides with anti-factor Xa and no anti-thrombin activities ($M_r$ <5,400), which have a lower affinity for PF4, as compared to that of the saccharides with both anti-factor Xa and anti-thrombin activities.

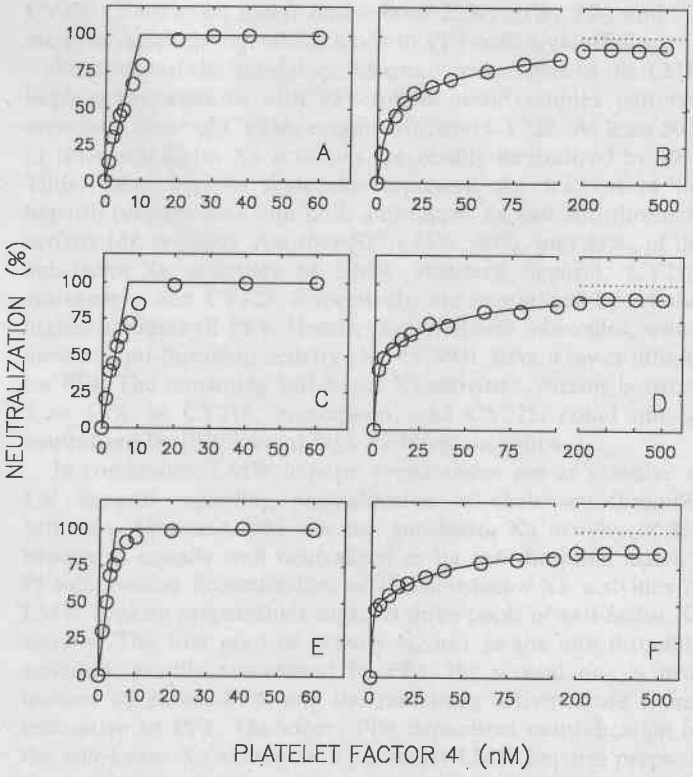


*Fig. 4* Neutralization of anti-thrombin and anti-factor Xa activities of LMW heparin preparations by PF4. The anti-thrombin (panels A, C, and E) and anti-factor Xa (panels B, D, and F) activities were neutralized by increasing amounts of PF4, and expressed relative to the activities as determined in the absence of PF4. The initial heparin concentrations were 510 ng CY216/ml (A and B), 380 ng enoxaparin/ml (C and D), and 380 ng CY222/ml (E and F)

We also investigated the extent to which the anti-factor Xa activities of the MMW, LMW, and ultra LMW fractions of CY216 could be neutralized by PF4. The relative amounts of the anti-factor Xa activities which could be neutralized were 100%, 40%, and 0%, respectively. This clearly demonstrates that as the heparin size goes from octadecasaccharide downwards, increasing amounts of anti-factor Xa activity resist neutralization by PF4.

## Discussion

It is generally accepted that ATIII-high affinity heparin oligosaccharides with molecular weights less than 5,400 (octadecasaccharide) have negligible anti-thrombin activities, while their potency to accelerate the inactivation of factor Xa is preserved (3, 4). The work of Lane et al. (3) has also indicated that the anti-thrombin and anti-factor Xa activities of these heparin molecules are less amenable to neutralization by PF4. We reasoned that regarding LMW heparin preparations mean molecular weights as such might have no predictive value for their anti-protease activities and their neutralization by PF4, because these preparations encompass heparin chains in a wide range of molecular weights with unknown distributions of the ATIII high affinity molecules over the range of molecular weights. There-

fore, we investigated in the present study whether or not LMW heparin preparations like the 1st International Standard for LMW heparin, CY216, enoxaparin, and CY222 with mean molecular weights of 6,400, 5,100, 3,800 and 3,800, respectively, have identical properties with respect to factor Xa and thrombin inactivation, and neutralization by PF4. We determined anti-factor Xa and anti-thrombin specific catalytic activities, i.e. rate constants normalized for the heparin concentrations, and evaluated the PF4-dependent neutralization of these activities. Because free calcium ions affect the rates of heparin-dependent inhibition of factor Xa and thrombin by ATIII (Fig. 1), 4.0 mM $CaCl_2$ was included in all our experiments.

We found that in a buffer system using purified proteases and ATIII of human origin the ratios of anti-factor Xa activity over anti-thrombin activity did not vary with average apparent molecular weights of the heparin preparations (Table 2). Even the ultra LMW heparin CY222, which is prepared by exhaustive nitrous acid depolymerization of pig mucosal heparin and has the highest number of molecules with $M_r$ <2,500, had only a 2-fold higher ratio than the other heparin preparations. The invariability of the specific activity ratios is caused by a parallel decrease of the anti-thrombin and anti-factor Xa activities with decreasing mean molecular weights.

Because catalysis of the inactivation of thrombin requires a heparin chain length of at least octadecasaccharide, it is obvious that a decrease of the number of molecules with $M_r$ <5,400 leads to a decrease of the anti-thrombin activity. At present two phenomena can be thought responsible for the decrease of the anti-factor Xa activity. First of all, we (12) and others (26) have shown that when the heparin molecular weights decrease from about 15,000 to 1,714 (pentasaccharide) the rate constants of inhibition of human factor Xa by human ATIII in the presence of $CaCl_2$ decrease. Secondly, and this in fact will contribute to the former effect, the $Ca^{2+}$-dependent enhancement of anti-factor Xa activity decreases as the heparin molecular weight decreases from 12,000 downwards (11). Consequently, it is well possible, that an increase of the number of heparin molecules with only anti-factor Xa activity ($M_r$ <5,400) is counterbalanced by a lesser enhancement of the inactivation reaction with factor Xa.

One of the LMW heparins (CY216) was fractionated by a gel permeation procedure. Analysis of the obtained MMW, LMW, and ultra LMW fractions showed that the anticoagulant heparin molecules present in CY216 have an average apparent molecular weight higher than the average apparent molecular weight of all (ATIII high and low affinity) heparin species. Support of our reasoning above is found in the anti-protease activities of the MMW and LMW fractions. The MMW fraction (6,100 <$M_r$ <11,200) has a relatively low anti-factor Xa activity as compared to its anti-thrombin activity (Table 3). The LMW fraction (3,300 <$M_r$ <5,500) which contributes for not more than 0.5% to the anti-thrombin activity of CY216 has considerable anti-factor Xa activity. Thus, admixture of the LMW fraction will restore the reduced anti-factor Xa specific activity of the MMW fraction, resulting in an unaltered specific anti-factor Xa/anti-thrombin ratio of CY216.

Thus, LMW heparin preparations are functionally equivalent to UF standard heparin on basis of their specific activity ratios. How does this compare with their sensitivity towards neutralization by PF4? First of all, we found that the anti-thrombin and anti-factor Xa activities of UF standard heparin were neutralized by stoichiometrical amounts of PF4. Neutralization of the anti-thrombin activities of the LMW heparin preparations followed the same pattern as obtained with UF standard heparin. From titrations with PF4 (Fig. 2B, and Figs. 4A, C, and E) it was estimated, that LMW standard heparin, CY216, enoxaparin, and CY222, contain on molar basis about 25%, 10%, 7%, and 5% material, respectively, which binds to PF4 with high affinity.

Titrations of the anti-factor Xa catalytic activities of the LMW heparin preparations with PF4 follow more complex patterns, especially those of CY216, enoxaparin, and CY222. At least 50% of their anti-factor Xa activities are readily neutralized by PF4. Thus, these heparin molecules represent the fraction of the heparin preparations with both anti-factor Xa and anti-thrombin activity ($M_r$ >5,400). Another 50%, 45%, 40%, and 35%, of the anti-factor Xa activities of LMW standard heparin, CY216, enoxaparin, and CY222, respectively, are neutralized by relative higher amounts of PF4. Hence, these heparin molecules, which have no anti-thrombin activity ($M_r$ <5,400), have a lower affinity for PF4. The remaining anti-factor Xa activities, varying between 5 to 15% of CY216, enoxaparin, and CY222, could not be neutralized by PF4 even at high PF4/heparin ratios.

In conclusion, LMW heparin preparations are as sensitive as UF heparin regarding neutralization of their anti-thrombin activities. However, whereas the anti-factor Xa activity of UF heparin is equally well neutralized as its anti-thrombin activity, PF4-dependent neutralization of the anti-factor Xa activities of LMW heparin preparations suggests three pools of anti-factor Xa activity. The first pool of activity is, just as the anti-thrombin activities, readily neutralized by PF4, the second one is neutralized by excess PF4, and the remaining activities are rather insensitive to PF4. Therefore, PF4 dependent neutralization of the anti-factor Xa activity of a particular LMW heparin preparation will strongly depend on the heparin molecular weight distribution pattern and on the local PF4 and heparin concentrations.

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