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Development of a Rapid and Sensitive Chromogenic Heparin Assay for Clinical Use

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Key Words
Chromogenic heparin assay
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Dermatan sulphate
Pentosan polysulphate
Pentasaccharide

Abstract

A new sensitive chromogenic heparin assay is developed, which is well suited for clinical use. For the assay two reaction mixtures are required which can be lyophilized and reconstituted on the day of use. These reagents are stable during at least 6 h. Only two time-dependent pipetting steps are necessary. Any compound that inactivates thrombin, or can potentiate thrombin inactivation by an inhibitor, can be measured with this assay, including standard heparin, low molecular weight heparins, hirudin, α-NAPAP, pentosan polysulphate and dermatan sulphate.

It is shown that heparin can be measured accurately in whole blood and in plasma. By addition of dextran sulphate to one of the reagents a platelet factor 4-insensitive assay is developed, so heparin can be measured even in blood that is partially activated and thus contains platelet factor 4 which neutralizes heparin.

Introduction

The mode of action of unfractionated heparin or classical heparin in plasma is described by several authors [1–6]. Unfractionated heparin is an antithrombotic drug, which is able to accelerate the inactivation of activated clotting factors by antithrombin III [1–7]. Heparin primarily acts on thrombin and secondary on thrombin-dependent activation reactions [1, 5], viz platelet activation, factor VIII and factor V activation. In case of extrinsic blood coagulation only factor V activation is important (besides platelet activation), whereas in case of intrinsic blood coagulation both factor VIII and factor V activations are important. For this reason it can be understood that intrinsic blood coagulation is more sensitive for heparin than extrinsic blood coagulation. Thus the activated partial thromboplastin time (APTT) is more sensitive for heparin than the prothrombin time.

The action of low molecular weight (LMW) heparin in blood coagulation is comparable with that of standard heparin [2, 5, 8]. However, LMW heparins have less affinity for thrombin, but retain their affinity for factor Xa [2, 5, 8].

Presently, heparin is measured in different ways. In the anti-factor Xa test [9–13] a known amount of factor Xa is added to plasma and the disappearance of factor Xa in time is measured. The more heparin is present in the plasma the more rapid factor

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Xa is inactivated by antithrombin III. A similar test is the anti-thrombin test, in which disappearance of exogenous added thrombin in plasma is measured [13, 14]. Both the anti-factor Xa and the anti-thrombin test, however, do not reflect the mode of action of heparin in plasma, because the feedback reactions in plasma, viz factor VIII and factor V activation by initial trace amounts of thrombin, are not measured with these tests. Also it was shown that exogenous added thrombin is faster inactivated by antithrombin III than endogenous formed thrombin [1]. As unfractionated heparin, LMW heparins are measured with the anti-thrombin and anti-factor Xa tests. In those cases one observes that the anti-factor Xa test is more sensitive for LMW heparins than anti-thrombin tests [15–17], and that the sensitivity for one LMW heparin is different from another one [17].

A relatively new test is the so-called heptest [18–22]. In this test factor Xa is added to plasma and the clotting time is recorded. This test resembles the APTT, which up to now is the best assay for heparins. In this test, heparin-containing plasma is activated with a contact activator in the presence of phospholipids and the clotting time is registered. The APTT correlates well with the APTT-like British pharmacopoeial assay [23], in which sheep plasma is used instead of human plasma. Recently, a chemical assay for heparin has been described in which heparin correlates with a decrease in color yield [24].

The above-described assays have different disadvantages. The chemical assay is laborious. The APTT is difficult to standardize, because the clotting time is dependent on both the contact activator (ellagic acid, kaolin, sulphatides) and the used plasma. Because the heptest is a clotting assay it shows rather high variations from one individual to another [20]. Also the correlation between the tests is not too well [18, 19]. Keeping in mind all these disadvantages of the present heparin tests, there is a great need for a chromogenic heparin test which measures the biological activity of heparin, which is reproducible, easy to perform and thus can be automated.

To develop such an assay a mixture was prepared with the clotting factors of the common pathway of blood coagulation and antithrombin III. Then the cascade was started well controlled by factor IXa and the effect of heparin on thrombin formation was determined. By choosing the right clotting factor concentrations a sensitive heparin assay was developed which fulfils the criteria mentioned above. The pipetting steps were minimized by preparing reaction mixtures of clotting factors, which do not react before mixing. The sensitivity of the assay for standard heparin, LMW heparins, pentasaccharide, hirudin, α-NAPAP, pentosan polysulphate and dermatan sulphate was studied. It was also shown that heparin can be measured accurately in activated whole blood. When platelets are activated platelet factor 4 is released [25, 26], which binds heparin and neutralizes its action. Thus, to measure heparin accurately, one either should avoid platelet activation during blood sampling, or one should have a heparin assay which is insensitive to platelet factor 4. Lyon et al. [27] showed that heparin can be detected in the presence of platelet factor 4 in the presence of a polysulphated dextran (8,000). In this article it is shown that by addition of dextran sulphate (500,000) to the reaction mixture a platelet factor 4-insensitive assay is obtained.

Besides platelet factor 4 the tissue factor pathway inhibitor (TFPI) might affect the assay. This TFPI is present in plasma [28], but a heparin-releasable form of this inhibitor has recently been discovered [29]. Besides, TFPI directly inhibits factor Xa [30–32] and thus might induce some inhibition of the assay in case plasma samples are tested, which is not

due to heparin. In the Discussion this point will be argued further.

Methods and Materials

Proteins

All proteins were isolated from bovine plasma. Factor V was isolated according to Lindhout et al. [33]. Factor VIII was prepared as described by Wagenvoord et al. [34]. Factors X and II were isolated according to Fujikawa et al. [35] and Owen et al. [36], respectively. Thrombin was prepared from prothrombin by activation with prothrombinase as described elsewhere [37]. Factor IXa was prepared by activating factor IX (see [38] for isolation procedure) with contact product [39, 40], according to Fujikawa et al. [41]. Antithrombin III was isolated as described by Thaler and Schmer [42]. Heparin cofactor II was prepared according to Tollefsen et al. [43].

Phospholipids

Phosphatidyl choline and phosphatidyl serine were extracted from egg yolks and bovine brains, respectively, according to Bligh and Dyer [44] and purified as described by Comfurius and Zwaal [45]. Vesicles were prepared by mixing 25 mol-% phosphatidyl serine and 75 mol-% phosphatidyl choline and by sonication of the mixture as described elsewhere [46].

Materials

FXa substrate (CH_3OCO-D-CHG-glycyl-arginine-*p*-nitroanilide acetate) and α-NAPAP [N α-(2-naphtylsulfonylglycyl)-*D,L*-amidinophenyl-alanine-piperidide hydroidide] were obtained from Pentapharm, Switzerland. S2337 [N-benzoyl-*L*-isoleucyl-*L*-glutamyl-(γ-piperidyl)-glycyl-*L*-arginine-*p*-nitroanilide hydrochloride] and S2238 (*D*-phenylalanyl-*L*-pipecolyl-*L*-arginine-*p*-nitroanilide dihydrochloride) were from Kabi, Sweden. Normal pooled plasma from 15 healthy donors was kindly donated by T. Janssen-Claessen.

Protein Concentrations

Factor VIII was measured according to Wagenvoord et al. [46]. Factor X was measured by mixing 100 µl of a mixture containing 300 n*M* thrombin, 15 m*M* $CaCl_2$, 60 µ*M* phospholipid vesicles and 300 n*M* factor IXa with 100 µl diluted factor X (0.3–1 n*M*) and 100 µl factor VIII (20 n*M*). After 10 min at 37 °C the factor Xa formation was measured with FXa substrate containing α-NAPAP [46]. Factor V was measured as described by Lindhout et al. [33] and prothrombin by activation with *Echis carinatus* venom [47] and by measuring the formed activation product with S2238. Antithrombin III was measured by its capacity to inactivate thrombin. To 800 µl 1.25 n*M* thrombin were added 100 µl antithrombin III or buffer. After 30 min inactivation at 37 °C 100 µl S2238 were added. From the difference between the blanc and the antithrombin III sample the concentration of antithrombin III was calculated. Factor IXa was measured by mixing 100 µl 300 n*M* thrombin, 15 m*M* $CaCl_2$, 60 µ*M* phospholipid vesicles with 100 µl factor IXa (1–2 n*M*). After 2 min preincubation at 37 °C of a mixture containing 5 n*M* factor VIII and 1 µ*M* factor X were added to start the reaction. After 2 min 200-µl samples were pipetted into 700 µl 10 m*M* EDTA to measure formed factor Xa. By comparison with a known factor IXa standard the concentration of the unknown samples was determined.

All dilutions were made in 175 m*M* NaCl, 50 m*M* Tris-HCl, 0.5 mg/ml ovalbumin (pH 7.9), unless otherwise stated.

Results

Development of the Chromogenic Heparin Assay

In the Introduction we discussed the conditions necessary for a biological heparin assay. For that reason we prepared two reagents. Reagent A contained the clotting factors of the common pathway of blood coagulation in similar concentrations as in plasma, phospholipids and antithrombin III. Reagent B contained factor IXa to activate the cascade and $CaCl_2$. Then a calibration curve was made as described in figure 1.

Figure 1 shows that the amidolytic activity of the samples decreases at increasing amounts of heparin in the sample. To stabilize factor VIII 0.1 m*M* $CaCl_2$ was added to reagent A [34].

The reaction time in figure 1 was 5 min. By using longer reaction times (up to 30 min), the amidolytic activities became much lower and there was no good correlation with the heparin concentration. For that reason the incubation time was kept on 5 min.

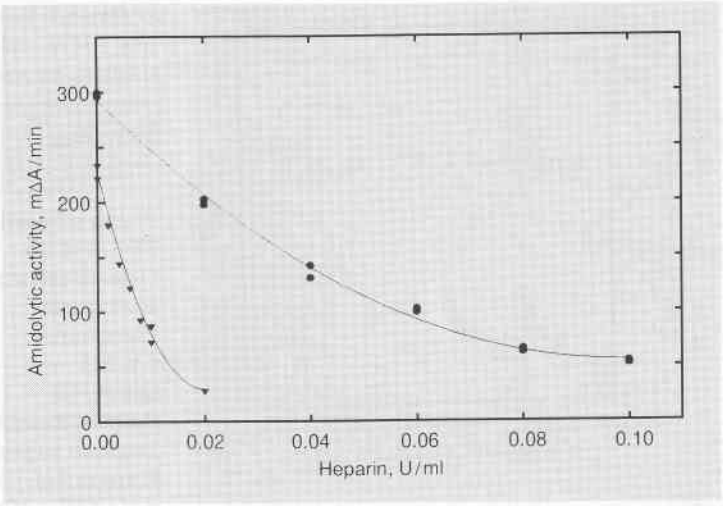

Fig. 1. Heparin calibration curves at two different factor VIII concentrations. Two reagents were prepared. Reagent A contained 200 n*M* factor X, 5 n*M* (●) or 1.2 n*M* (▼) factor VIII, 500 n*M* prothrombin, 10 n*M* factor V, 500 n*M* antithrombin III, 60 μ*M* phospholipid vesicles, 0.1 m*M* $CaCl_2$ and 2 mg/ml ovalbumin. Reagent B contained 10 n*M* factor IXa and 15 m*M* $CaCl_2$. 50 μl reagent A was mixed with 50 μl heparin (Organon reference). The heparin concentrations of the samples are those given in the x-axis of the figure, thus the final concentrations in the reaction mixture are three times lower. This mixture and reagent B were prewarmed during 5 min at 37 °C. Then 50 μl reagent B was added to the mixture (t = 0 min) and at t = 5 min a 100-μl sample was added to a cuvette containing 850 μl 10 m*M* EDTA. Formed thrombin was measured by addition of 50 μl S2238 (3.88 m*M*).

The concentrations of the clotting factors in reagent A were chosen on basis of their concentration in plasma. However, we have varied their concentration to study the effect on the heparin assay. Changing the factor X concentration has no effect on the assay, but when the concentration is lower than 40 n*M* factor X, the formed thrombin becomes less. Changing the prothrombin concentration has no effect on the sensitivity of the assay; however, the formed thrombin is more or less proportional with the present amount of prothrombin, assuming that antithrombin III is present in the same concentration as prothrombin. The effect of changing the concentration of factors VIII and V on the assay is larger. We found that at suboptimal concentrations of these factors the assay is the most sensitive. This effect is most pronounced for factor VIII. Figure 1 shows that by using 1.2 n*M* factor VIII instead of 5 n*M* the assay is about 6 times more sensitive.

Figure 2 shows the effect of factor VIII on thrombin formation. Above 1.6 n*M* factor VIII a plateau is reached and at lower factor VIII concentration less thrombin is formed.

We also have determined the optimal factor IXa concentration in reagent B. It was shown that a concentration as low as 25 p*M* factor IXa was sufficient to activate the cas-

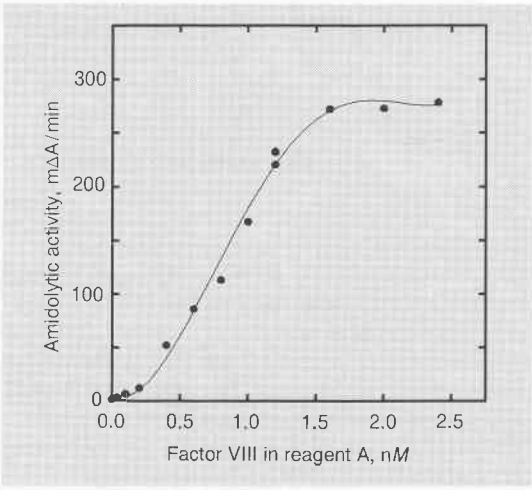

Fig. 2. Effect of factor VIII on thrombin formation in the chromogenic heparin assay. Reagent A and B described in figure 1 were used; however, reagent A contained variable amounts of factor VIII as indicated in the figure. Then, the procedure described in figure 1 was followed.

Table 1. Sensitivity of the heparin assay for heparins, LMW heparins, pentasaccharide, pentosan polysulphate, hirudin and α-NAPAP

Heparin	Concentration causing 50% inhibition
Fourth international standard	0.048
FF-32038	0.035
Organon reference heparin	0.059
Enoxaparin	0.31
Fraxiparin	0.48
CY-222	0.88
Org 10172	15
Pentasaccharide	> 100 (plateau at 75%)
Pentosan polysulphate	1.04
Hirudin, n*M*	200
α-NAPAP, μ*M*	4
Dermatan sulphate	0.15
Effect of plasma on the amount standard heparin causing half maximal inhibition	
No plasma	0.048
Plasma 1 in 100	0.054
Plasma 1 in 50	0.057
Plasma 1 in 20	0.069

Amount of compound causing 50% inhibition of the assay. The effect of plasma on the sensitivity of the assay is also shown. Values are in μg/ml unless otherwise stated.

cade: thrombin formation was not less than in fig. 1 and 2. However, this amount is marginal, and decreasing either the factor IXa concentration or a component in reagent A causes a lower amount of formed thrombin. For that reason we decided to use an excess of factor IXa: 1 n*M*.

To study the effect of plasma on the assay we have made calibration curves in the absence and presence of diluted normal pooled plasma. Samples were prepared with 0–0.02 U/ml standard heparin to which were added plasma with final dilutions of 1 in 100, 1 in 50 and 1 in 20, respectively. Then calibration curves were made. In case of a plasma dilution of 1 in 20 in some cases a clot was formed after the 5-min reaction time; however, pipetting was usually still possible. The effect of plasma on the calibration curve was very small. The most pronounced effect was an increase of the blanc value (no heparin in sample) of 5–10%; however, the sensitivity of the assay for heparin was affected only slightly (see table 1). By making reference curves in the presence of plasma it is well possible to correct for these minor changes in sensitivity. Table 1 shows the effect of plasma on the sensitivity of the assay for standard heparin.

Lyophilization of Reagents for the Heparin Assay

Table 2 shows that the proteins in the reagents for the heparin assay (reagents A and B) do not loose their activity during lyophilization. The reagents prepared in this way were used in further tests. They were reconstituted on the day of use.

Table 2. Effect of lyophilization on the components of reagents A and B

Protein concentration, n*M*	Before lyophilization	After lyophilization
Factor VIII	2.5	2.45
Factor X	40	40.3
Factor V	5	4.83
Prothrombin	500	486
Antithrombin III	500	506
Factor IXa	1	0.935

Reagent A contained 40 n*M* factor X, 2.5 n*M* factor VIII, 500 n*M* prothrombin, 5 n*M* factor V, 500 n*M* antithrombin III, 60 μ*M* phospholipid vesicles, 0.1 m*M* $CaCl_2$ and 20 mg/ml ovalbumin. Reagent B contained 1 n*M* factor IXa, 15 m*M* $CaCl_2$ and 0.5 mg/ml ovalbumin. The reagents were divided into 2-ml amounts and lyophilized. After reconstitution the clotting factor concentrations were determined.

Stability of the Chromogenic Heparin Assay

Figure 3a shows that the assay is stable during at least 6 h at room temperature (about 22 °C). One can notice that the activity of the assay increases somewhat during the first 3 h of incubation and then decreases very slowly. Stability experiments were done also at 0 and 37 °C with comparable results as shown in figure 3a. We have made reference curves at each time point, which showed that the sensitivity for heparin was not changed. Figure 3b shows two of these curves, one prepared immediately after reconstitution of the reagents and one after 6 h incubation at room temperature.

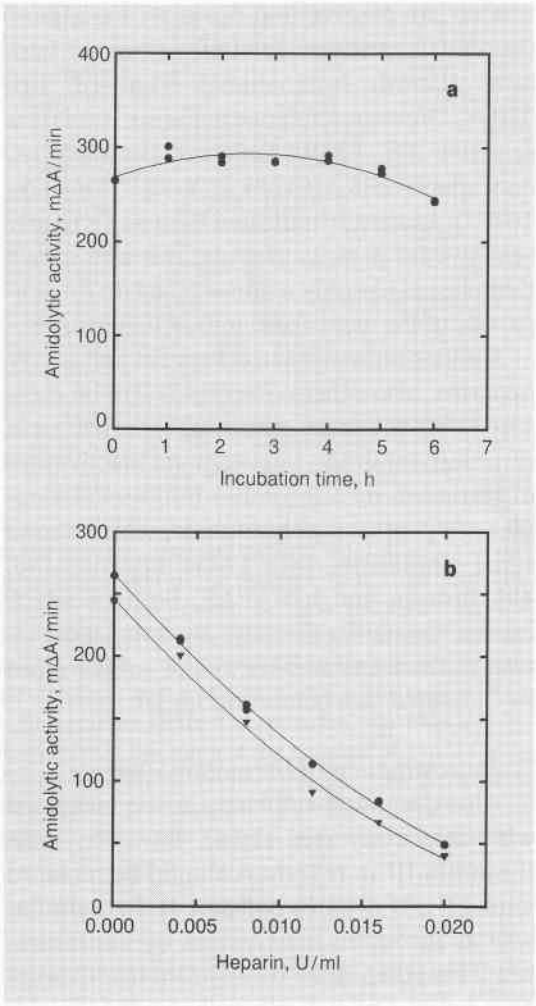

Fig. 3. Stability of lyophilized heparin assay reagents. Lyophilized reagents A and B were reconstituted and kept at 22 °C (ambient temperature). **a** The standard protocol was done at 0, 1, 2, 3, 4, 5 and 6 h incubation time with samples containing no heparin. **b** At t = 0 (●) and t = 6 (▼) h calibration curves were made with the fourth international standard heparin.

Sensitivity of the Assay for Several Heparins and Other Compounds

We have determined the sensitivity of the chromogenic heparin assay for different standard heparins, LMW heparins, pentasaccharide and other compounds. In all cases similar calibration curves were obtained with one exception: pentasaccharide, it was impossible to inhibit the assay for more than 25% even at concentrations up to 100 μg/ml. Although similar calibration curves were obtained, the amount of heparin needed for 50% inhibition varied quite a lot, which is shown in table 2.

One can observe that the assay has almost the same sensitivity for unfractionated heparins (Fourth International Standard, FF-32038, Organon reference heparin), but is less sensitive for LMW heparins [Fraxiparine, enoxaparin (PK 10169), CY-222, Organon 10172]. As already indicated the assay is hardly sensitive for pentasaccharide. It is also shown that plasma has only a minor effect on the sensitivity of the assay for standard heparin.

Besides unfractionated heparin and LMW heparins, also other compounds can be measured with the assay, provided that they are a thrombin inhibitor, or potentiate inactivation of thrombin by a thrombin inhibitor. Examples are pentosan polysulphate, which shows some similarities with LMW heparins [48, 49], hirudin and α-NAPAP, both of which inhibit thrombin directly. By small adaptation of the assay also dermatan sulphate can be measured (see below and fig. 4).

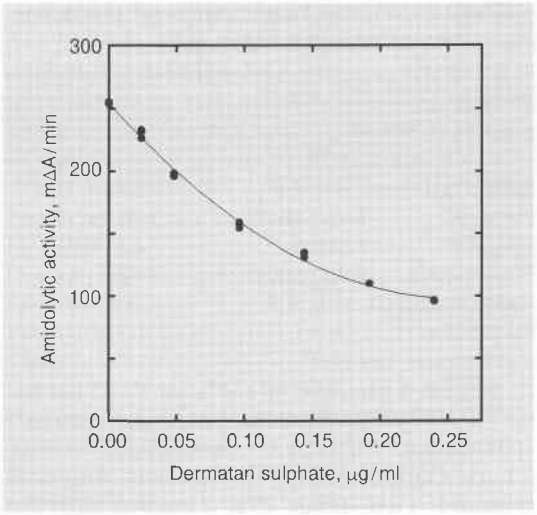

Fig. 4. Calibration curve of dermatan sulphate. Reagents A and B were prepared as described in table 2. However, antithrombin III in reagent A was replaced with 1.0 μ*M* heparin cofactor II. The further procedure was not changed.

Determination of Dermatan Sulphate

Also dermatan sulphate can be measured with this assay (see fig. 4); however, antithrombin III in reagent A should be replaced with 1.0 μ*M* heparin cofactor II. In a similar way as thrombin inactivation by antithrombin III is potentiated by heparin, inactivation of thrombin by heparin cofactor II is potentiated by dermatan sulphate [50]. In this specific case dermatan sulphate inhibits 50% at a concentration of 0.15 μg/ml (see also table 2).

Platelet Factor 4-Insensitive Heparin Assay

Lyon et al. [27] showed that dextran sulphate is able to prevent binding of heparin to platelet factor 4. In figure 5 is shown that by addition of dextran sulphate to the assay, heparin can be measured reliably even in the presence of platelet factor 4. The addition of dextran sulphate does not affect the sensitivity of the assay for heparin (fig. 5a). Figure 5b shows that in the presence of platelet factor 4, heparin (0.1 U/ml) cannot be detected, but by addition of dextran sulphate it can. By addition of 0.8 μg/ml dextran sulphate to reagent A, 0.092 U/ml heparin could be detected, which is close to the theoretical amount of 0.1 U/ml. By addition of dextran sulphate to reagent B the same result is found.

Some Tests with Heparin-Containing Plasma and Blood

To test the clinical applicability of the chromogenic heparin assay we have measured heparin in plasma and in whole blood. In the first place we have prepared plasma samples (normal pooled plasma) to which was added heparin (fourth international standard) in concentrations of 0–2 U/ml. Then the plasma samples were diluted 100 times and a reference curve was made as described in figure 3b. To calculate the heparin in an unknown sample we have made a reference

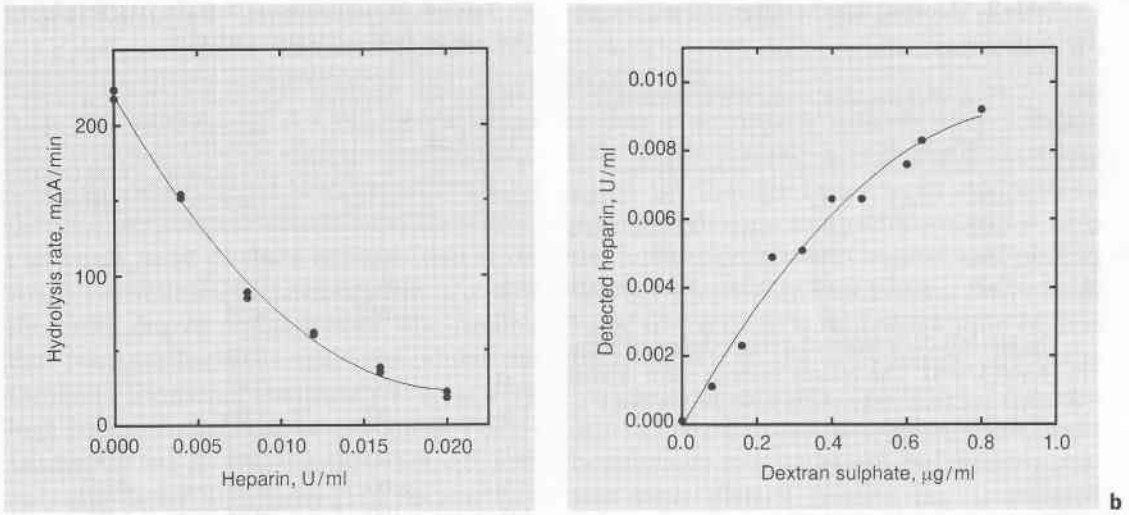

Fig. 5. Effect of dextran sulphate on the heparin assay and heparin determination in the presence of platelet factor 4. **a** Heparin determination in the presence of dextran sulphate. Reagent A (see table 2) was prepared with 0.8 µg/ml dextran sulphate 500,000. Then for heparin a reference curve was prepared in the same way as in figure 1. **b** The effect of dextran sulphate on the determination of heparin in the presence of platelet factor 4. Samples with 0.01 U/ml heparin (fourth international standard) and a platelet factor 4 preparation (4 µl per sample of 100 µl) were prepared. The platelet factor 4 preparation was obtained by activation of platelets (3×10^8 cells/ml) with 5 n*M* thrombin in the presence of 5 m*M* $CaCl_2$ during 10 min and subsequent centrifugation (3 min at 10,000 *g*) of the cells. Reagent A contained variable amounts of dextran sulphate (0–0.8 µg/ml). The further procedure is described above. The amount of heparin plotted in the figure was determined from the reference curve.

curve simulation. Straight lines were obtained by plotting the logarithm of the formed thrombin against the heparin concentration. With the constants of the simulation the heparin concentration in the samples was calculated. Unknown plasma samples from patients who received a single dosage of heparin (4,000 Units) were tested. Samples before and 1–5 h after administration of the heparin were tested (table 3).

One can notice that the half-life of heparin is about 1 h and that the amount of heparin found in the patients' plasma varies a lot, which was to be expected because a fixed dosage of heparin was given and not an amount per body weight.

Another point of practical importance is the measurement of heparin in whole blood. For that reason we have taken blood from a vena puncture, which was obtained directly after putting the needle in the vena. The blood was collected in a tube containing 3.8% sodium citrate (9 parts blood and 1 part citrate). In this way we obtained blood that very likely was activated and thus should contain platelet factor 4. To obtain unactivated blood the first blood after vena puncture was discarded. To the blood we added heparin in concentrations given in table 4. Then the heparin was measured with reagents A and B described in table 2. When dextran sulphate was present, it was added to reagent B in a concentration of 0.8 µg/ml.

Table 3. Heparin determination (U/ml) in patients' plasma

Patient	Time after the administration of heparin, h					
	before	1	2	3	4	5
1	0.04	0.85	0.52	0.22	0.04	−0.09
2	0.05	1.49	0.95	0.65	0.38	0.10
3	0.06	0.61	0.39	0.15	0.07	
4	0.09	1.13	0.67	0.24	0.13	0.05

The patients daily received a single dosage of heparin (4,000 Units). The plasma samples were diluted 100 times and then measured as described in figure 1.

Table 4 shows that heparin determination in whole blood is well possible. When the blood was drawn carefully the measured amounts of heparin were close to the theoretical amounts. When the blood was not drawn carefully and probably activated to some extent, the heparin detected in the samples was always lower than the added amounts, when no dextran sulphate was added. Having present dextran sulphate in the assay system, especially at low heparin concentrations, the detected amounts of heparin were close to the added amounts. At high heparin concentration the effect of dextran sulphate was less; however, in each case more heparin was detected, thus also in these cases the dextran sulphate was able to cancel the effect of platelet factor 4.

Discussion

In the first part of this article the development of a chromogenic heparin assay was described. In figure 1 is shown that by addition of factor IXa plus $CaCl_2$ to a mixture of clotting factors of the common pathway of blood coagulation, phospholipid vesicles and antithrombin III a heparin-sensitive system is obtained.

Table 4. Measurement of heparin in whole blood that was probably activated partially

Added heparin U/ml	Dilution times	Calculated heparin in the sample, U/ml	
		no dextran sulphate	with dextran sulphate
0	100	−0.12	
0.5	100	0.17	
1.0	100	0.59	
0[1]	100	−0.05	
0.5[1]	100	0.46	
1.0[1]	100	1.00	
0	100	0.01	0.00
0.5	100	0.46	0.65
1.0	100	0.86	1.03
0	20	−0.01	0.00
0.1	20	0.05	0.12
0.2	20	0.16	0.21

[1] Blood that was carefully drawn to prevent activation. Effect of dextran sulphate on the determination.

The sensitivity of the heparin assay was improved by changing the composition of the reaction mixtures (reagents A and B). It was shown that the sensitivity of the system especially depends on the factor VIII concentration. By changing the factor VIII concentration from 5 to 1.2 n*M* the sensitivity increased 5-fold (fig. 1). Lower concentrations of factor VIII caused a lower rate of thrombin formation (see fig. 2), but made the system even more sensitive for heparin.

It is important that the reagents used in the assay can be lyophilized without loosing their activities. The sensitivity of the assay for different heparins was studied with reconstituted lyophilized reagents. In table 2 is shown that the sensitivity of the assay is almost the same for standard heparins (unfractionated heparins), that it is less for LMW heparins and that it is hardly sensitive for pentasaccharide. To obtain 50% inhibition one needs

about 0.05 µg/ml standard heparin, about 0.3 µg/ml enoxaparin, about 0.5 µg/ml Fraxiparine, about 0.9 µg/ml CY-222 and about 15 µg/ml Organon 10172. It was not possible to obtain 50% inhibition with pentasaccharide. One can notice that LMW heparin can be detected very well with this assay, however, one needs more of these heparins (based on weight) than unfractionated heparin to obtain the same degree of inhibition. The finding that pentasaccharide is unable to inhibit the thrombin formation completely even at very high concentrations can be explained. Pentasaccharide has no anti-thrombin activity and only anti-factor Xa activity [51]. Because factor Va stimulates the activity of the prothrombinase enormously [52], a small trace of factor Xa already is sufficient for a high rate of thrombin formation. Possibly, the pentasaccharide is unable to inhibit factor Xa formation completely and the residual amount of formed factor Xa binds to the formed factor Va to form the highly active complete prothrombinase complex, which activates rapidly prothrombin. A similar finding is reported by Walenga et al. [53], who showed that intrinsic started thrombin generation could not be inhibited for more than 50% with pentasaccharide.

In the Introduction the possibility was mentioned that TFPI might inhibit the assay, because it directly inhibits factor Xa. However, in view of the finding that pentasaccharide is unable to inhibit the assay for more than 25% and the concluded factor Xa inhibitor insensitivity of the assay, it is unlikely that the assay will be affected by the presence of TFPI, also if we take in mind the high plasma dilutions that will be used in practice.

Stability tests have shown that the reagents A and B are stable for several hours even at 37 °C. This is important for clinical use. It is not necessary to make a reference curve at every measurement. However, it is advisable to do a blanc at each measurement, because there is some drift in the assay of maximally 10%. This drift does not affect the sensitivity of the assay.

The experiments with dextran sulphate have shown that the assay can be made insensitive for platelet factor 4. By addition of 0.8 µg/ml dextran sulphate to reagent A or B the interaction between heparin and platelet factor 4 is broken and the heparin can be measured in the usual way. The addition of dextran sulphate to the assay hardly affects the sensitivity of the assay for heparin. Thus heparin can be measured in samples, which might be activated to some extent and possibly contain some platelet factor 4 that is released from the platelets.

The presence of plasma in the sample has only a small effect on the calibration curve and thus heparin in plasma samples can be well measured, with almost the same sensitivity as in the absence of plasma (table 1). In practice, plasma should be diluted at least 30 times to avoid clot formation. From this fact and the sensitivity for heparin shown in figure 1, one has some indication on the minimal amount of heparin that can be detected in plasma, i.e. $\approx 0.0005 \times 30 = 0.015$ U/ml.

It is also possible to measure heparin in whole blood. In the case, however, one needs a sensitive spectrophotometer, because the absorption of the red cells is high. It was also shown that heparin could be measured in blood that contained platelet factor 4, which was released from activated platelets. In that case, however, dextran sulphate should be added to reagent A or B.

It is shown that the assay is also sensitive for other compounds besides heparin (table 2). Pentosan polysulphate, hirudin and α-NAPAP can be measured without changing the reagents, whereas for the determination of dermatan sulphate a small adaptation is necessary, the replacement of antithrombin III in reagent A by heparin cofactor II (see fig. 4).

During the period that the article was reviewed by the referees it was shown that the assay was not sensitive for aprotinin (Trasylol) up to 500 kallikrein inactivator units per ml in the sample.

In this article is described the development of a precise chromogenic heparin assay, which is suited for clinical use, because of its simplicity. The assay can be modified for specific demands. For instance by changing the prothrombin concentration in reagent A the final amount of formed thrombin can be modulated. The size of the signal can also be changed by using another chromogenic substrate. A simplification of the procedure can be obtained by stopping the reaction not by subsampling, but by addition of stop buffer plus chromogenic substrate to the reaction mixture and immediate measurement of formed thrombin. In the last case it is necessary to use less prothrombin, because otherwise the hydrolysis rate of chromogenic substrate will be too high.

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