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Spectrophotometric Method for the Assay of Human Blood Coagulation Factor VIII

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Key Words. Factor VIII, spectrophotometric assay · Clotting assay, 1-stage · Chromogenic substrate

Abstract. A spectrophotometric method for the assay of human blood coagulation factor VIII in plasma is presented. The chromogenic assay for factor VIII:C in plasma is performed in 3 steps: (1) activation of factor VIII by thrombin; (2) activation of factor X in a mixture of factor X, factor IXa, phospholipids/ Ca^{2+} and plasma containing activated factor VIII, and (3) determination of the rate of factor Xa formation with the chromogenic substrate S2337. Within-assay variation was between 5 and 6.9% for factor VIII:C activities between 20 and 150%. Clotting and chromogenic factor VIII:C activities were compared in plasma of 50 normal healthy donors (coefficient of correlation $r = 0.83$).

Introduction

Factor VIII or antihemophilia globulin A is a plasma protein that is essential for normal hemostasis (see [1] for review). Its congenital absence is by far the most common cause of congenital bleeding tendency. The procoagulant protein (factor VIII:C) occurs in plasma tightly bound to the von Willebrand factor (factor VIII:vWF). Factor VIII:C and vWF together form the factor VIII complex. Factor VIII:C appears responsible for normal thrombin formation, whereas factor VIII:vWF is required for platelet-vessel wall interaction. (For reviews on factor VIII complex and vWF see [2, 3].)

If factor VIII:C is congenitally deficient, factor VIII:vWF is normal. If factor VIII:vWF is deficient, factor VIII:C is to a varying degree also.

A widely used method to determine factor VIII in plasma is the 1-stage clotting assay introduced in 1962 by Hardisty et al. [4]. This assay is based on the property of factor VIII to correct the prolonged clotting time of hemophiliac plasma to a degree determined by the amount of factor VIII present.

A new assay method for the determination of factor VIII using a chromogenic substrate for factor Xa appeared feasible after kinetic studies on the role of factor VIII in

the intrinsic factor-X-activating complex [5]. In this paper, we describe a spectrophotometric assay for factor VIII in human plasma and compare the properties and precision of the assay with the 1-stage clotting assay.

Materials and Methods

S2337, Bz-Ile-Glu(Piperidyl)-Gly-Arg-*p*-nitroanilide and S2238, H-D-Phe-Pip-Arg-*p*-nitroanilide were obtained from Kabi AB, Sweden. Egg-phosphatidylcholine (egg-PC) was from Koch-Light, UK, and brain-phosphatidylserine (brain-PS) was from Sigma, St. Louis, Mo., USA. Hirudin was from Pentapharm, Basel, Switzerland. Fatty acid free human albumin and soybean trypsin inhibitor were obtained from Sigma. Factor-deficient plasmas were purchased from George King Biomedical, Overland Park, Kans., USA. The batch numbers used were factor VIII, GK 837-314; high molecular weight kininogen, GK 1603-D30; factor XII, GK 1202-921; factor XI, GK 1110-N19; prekallikrein, GK 1704-124; passavoy GK 1501-625; factor V, GK 503-223, and von Willebrand, GK 1403-93. The factor-deficient plasmas from General Diagnostics were of the following lot numbers: factor VIII, 4G926; factor X, 4H699; factor VII, 4J759. Factor-VIII-deficient plasma (WH) and factor-IX-deficient plasma were obtained from local patients. Kaolin (light) was from Koch-Light. Inosithin was from Associated Concentrates, New York, USA.

Proteins

Bovine factor X (factor X₂ was used in this work), factor IXa and thrombin were purified and activated as published before [5]. Phospholipid vesicles consisting of 40% brain-PS and 60% egg-PC were made by sonication at 0 °C in a buffer of 50 mM tris-HCl, 175 mM NaCl, pH 7.9, as described before [6].

Human Donor Plasmas

Blood was obtained from healthy volunteers after informed consent by venipuncture using a 1.2 × 40 mm needle, discarding the first 2 ml. Blood (9 ml) was mixed with 1 ml of 0.1 M trisodium citrate in a plastic tube and centrifuged for 30 min at 2,000 g. The plasma was aspirated and made platelet-free by a centrifugation at 4 °C for 15 min at 14,000 g in the

Beckmann J-21 rotor. The plasma samples were frozen and stored at -70 °C in small portions. Reference pool plasma was made by mixing 1 ml of each of the donor plasmas (n = 50). It is assumed that factor VIII:C activity in the reference plasma is 1 U/ml. Different dilutions of the reference pool were used to obtain standard c-q reference curves.

Spectrophotometric Assay for Factor VIII (Standard Factor VIII Assay)

Forty microliters of human plasma (or a sample in which factor VIII must be determined) is added to a prewarmed mixture of 910 µl buffer containing 175 mM NaCl, 50 mM tris-HCl, pH 7.9, 0.5 mg/ml human albumin and 50 µl of 60 nM (6 NIH U/ml) of thrombin. After incubation for 1 min at 37 °C, 500 µl of the plasma, containing activated factor VIII, is added to a second tube containing 450 µl of a prewarmed (37 °C) mixture of phospholipids, CaCl₂ and factor IXa. Factor X activation is started by addition to the reaction mixture of 50 µl of 10 µM factor X, at the same time as the activated plasma. The final composition of the reaction mixture (1 ml) is: 20 µl of plasma, added as 500 µl thrombin-activated plasma; 0.5 µM factor X, 50 nM factor IXa, 20 µM phospholipid vesicles (40 mol% PS/60 mol% PC); 1.5 nM thrombin, 0.5 mg/ml human albumin in a buffer containing 175 mM NaCl, 50 mM tris-HCl (pH 7.9) and 6 mM CaCl₂.

After 1 and 2 min, samples from the reaction mixture (usually 0.1 ml) were added to a cuvette (37 °C) with 0.8 ml buffer of 175 mM NaCl, 50 mM tris-HCl, pH 7.9, 20 mM EDTA, 0.5 antithrombin units of hirudin and 0.5 mg/ml human albumin. After addition of 0.1 ml S2337 (2.35 mM; 25 A316 units/ml), the absorbance increase at 405 nm was recorded for 2–3 min using a Beckman Model 25 spectrophotometer. From the absorbance change, the amount of factor Xa present was calculated using a calibration curve made with a factor Xa preparation of known concentration. The rate of factor Xa formation was calculated from the amount of factor Xa present after 1 and 2 min and was corrected for the rate found in a blank experiment run without plasma.

Factor VIII Clotting Assay

A 1-stage clotting assay for factor VIII [4] was carried out by preincubating for 6 min at 37 °C 50 µl factor-VIII-deficient plasma, 50 µl kaolin-inosithin in Michaelis buffer, and 50 µl of the factor VIII sample.

After 6 min, 50 μ l 1/30 *M* CaCl_2 was added to start clot formation. The clotting time was determined by tilting the tubes. The amount of factor VIII present was determined from a reference curve. This curve is a double logarithmic plot of the clotting times (determined in duplicate) of 10 different dilutions of reference plasma. The composition of Michaelis buffer was 0.0285 *M* Na acetate, 0.0285 *M* Na barbiturate, 0.116 *M* NaCl (pH 7.4). The kaolin-inosithin suspension contained 5 mg/ml kaolin and 1 mg/ml inosithin.

Statistical Analysis of Data

The significance of differences between 2 values was tested by a paired Student *t* test. CV is the coefficient of variation, the standard deviation is expressed as a percentage of the mean.

Results

Determination of Optimal Assay

Conditions

In intrinsic factor X activation, factor X is cleaved to factor Xa by the enzyme factor IXa, and the rate of this reaction is strongly enhanced in the presence of the nonenzymatic accessory components, i.e. activated factor VIII and phospholipids. Factor VIIIa works by at least 2 different mechanisms: it increases the affinity of factor IXa for binding to phospholipids [7] by a factor of about 100 and it also increases the $V_{\max}$ turnover number of factor Xa formation by factor IXa about 1,000-fold [5]. The experimental conditions for the factor VIII assay in plasma as described in 'Materials and Methods' were based on the observations done in purified systems [5, 7]. The factor X concentration in the assay (0.5 μ M) was chosen well above the K_m for factor X (0.06 μ M). A phospholipid vesicle concentration of 20 μ M was selected in the assay because the rate of factor Xa formation strongly increases between 0 and 5 μ M, reaches a plateau between 10 and

50 μ M, and decreases at higher phospholipid concentration. The factor IXa concentration used in the assay is 50 nM and is based on the experiment shown in figure 1. The rate of factor Xa formation increases with the factor IXa concentration and levels off at higher factor IXa concentrations. In the absence of plasma factor VIII, a low rate of factor Xa formation proportional with factor IXa is found. When this rate is subtracted from the rate measured in the presence of plasma, a plateau level is obtained, indicating apparent saturation of the factor-X-activating system with factor IXa. A factor IXa concentration in the assay of 50 nM approaches saturation. When 20 μ l plasma is present in the assay, the background rate without plasma is about 10% of the rate measured in the presence of plasma.

Factor VIII must be activated before it can participate in factor X activation, and thrombin is a well-known activator of factor VIII. Activated factor VIII is not stable and rapidly inactivates [2, 6]. Therefore, the activation of factor VIII in plasma by thrombin was carried out in a 1-min period just before the start of each factor X activation reaction. Optimal conditions for activation were established by the experiments shown in figure 2. Plasma was incubated at varying thrombin concentrations and after 1 min, the activated plasma sample was transferred to a mixture of phospholipid, Ca^{2+} and factor IXa. Simultaneous with the activated plasma, factor X was added and the time course of factor Xa formation was determined during 3 min. As shown in figure 2, no factor X activation is found within 3 min when thrombin is omitted from the plasma activation mixture. The rate of factor Xa formation increases when increased amounts of thrombin are used (0–1.8 nM) to activate the

Fig. 1. Determination of the optimal factor IXa concentration. Forty microliter of reference pool plasma was added to 960 μ l of a prewarmed (37°C) solution of 3.0 nM thrombin in a buffer containing 50 mM tris-HCl, 175 mM NaCl, pH 7.9, and 0.5 mg/ml of human albumin. After exactly 1 min at 37°C , 500 μ l of the plasma thrombin mixture was transferred to a second prewarmed tube containing 450 μ l of a mixture of phospholipids, CaCl_2 and varying amounts of factor IXa. Together with the activated plasma, 50 μ l of factor X (10 μM) was added to start factor Xa formation. After 1 and 2 min, samples of 100 μ l were added to prewarmed (37°C) cuvettes containing 0.8 ml of a buffer containing 50 mM tris-

HCl, 175 mM NaCl, 20 mM EDTA, pH 7.9, and 0.5 mg/ml human albumin. To the cuvette was added 0.1 ml chromogenic substrate S2337 (2.35 mM). The absorbance change at 405 nm was measured and used to calculate the amount of factor Xa present as described in 'Materials and Methods'. The composition of the factor-X-activating mixture of 1 ml was: 20 μ l human plasma, 1.5 nM thrombin, 20 μM phospholipid vesicles consisting of 40% phosphatidylserine and 60% phosphatidylcholine, factor IXa as indicated in the figure, 0.5 μM factor X, 50 mM tris-HCl, 175 mM NaCl, 6 mM CaCl_2 , pH 7.9, and 0.5 mg/ml human albumin. ■ = Without plasma; ▲ = with reference pool plasma; ● = difference curve obtained by subtraction.

factor VIII present. No further increase (or decrease) in the time course of factor Xa formation was observed at thrombin concentrations between 1.8 and 6 nM. This result suggests that optimal activation of factor VIII in plasma is obtained under these conditions.

The amounts of thrombin did not influence the factor Xa generation by a factor-VIII-independent mechanism, as no activity was observed when factor VIII was absent (fig. 1) (see also below). Hence, in the standard assay procedure, plasma is activated for 1 min

Fig. 2. Determination of the optimal thrombin concentration for the activation of factor VIII in human plasma. Pool plasma (40 μ l) was added to 960 μ l of a buffer (37 $^{\circ}$ C) containing 50 mM tris-HCl, 175 mM NaCl, pH 7.9, 0.5 mg/ml human albumin and varying amounts of thrombin (0–6 nM) as indicated. After 1 min, 500 μ l of the thrombin-activated plasma was transferred to a reaction mixture for factor X activation, as described in the legend of figure 1. The rate of factor Xa formation was as determined as in figure 1 from the amounts of factor Xa present after 1 and 2 min. The final composition of the reaction mixture (1 ml) was: 20 μ l human plasma, 20 μ M phospholipid, 50 nM factor IXa, 0.5 μ M factor X, 50 mM tris-HCl, 175 mM NaCl, 6 mM CaCl_2 , pH 7.9, and 0.5 mg/ml human albumin.

at a thrombin concentration of 3 nM. The time course of factor Xa formation was linear for at least 2 min, and the rate of factor Xa formation was calculated from the amount of factor Xa present after 1 and 2 min.

Hirudin is present in the cuvettes to inhibit the activity of thrombin on the chromogenic substrate S2337. Thrombin is formed from prothrombin present in the plasma sample during the factor X activation reaction. In the standard factor VIII assay containing 20 μ l plasma, 27 nM thrombin is formed after 2 min. The thrombin concentration was determined using S2238 as the chromogenic substrate for thrombin in a cuvette that contained soybean trypsin inhibitor to inactivate factor Xa. Addition of hirudin (0.5 antithrombin units per cuvette) inhibited the thrombin activity on S2238 for 97.8%. A much larger

excess of hirudin (5–50 antithrombin units per cuvette) could not be used, since the hirudin preparation partially inhibited the activity of factor Xa on S2337. When in the standard factor VIII assay hirudin was omitted from the cuvettes, $3.2 \pm 2.4\%$ (mean $\pm$ SD; $n = 111$), higher rates of factor X activation were found, indicating that the contribution of thrombin to the total hydrolysis of S2337 is relatively small.

Properties of the Spectrophotometric Assay

Linearity of the Assay. Varying amounts of reference pool plasma were added to our standard test system. As shown in figure 3, a linear dependence between the rate of factor Xa formation and the plasma concentration is found between 5 and 30 μ l of plasma per milliliter reaction mixture. High amounts of plasma in the assay give rise to clot forma-

Fig. 3. Linearity of the assay. Varying amounts of reference pool plasma were incubated with 3.0 nM of thrombin in 1 ml. After 1 min at 37 °C, 500 μ l of the mixture was transferred to 500 μ l of a reaction mixture for factor X activation. The amount of factor Xa formed was determined after 1 and 2 min, as described in the legend of figure 1. The reaction mixture with the amounts of activated plasma as indicated contained 1.5 nM thrombin and further components for factor X activation as described in the legend of figure 2. An amount of 20 μ l of reference pool plasma in the factor X activation mixture was chosen as the condition for the standard factor VIII assay, the rate of factor Xa formation found was designated as the 1 U/ml value.

tion in the factor X activation mixture, which prevents accurate sampling. In the low range, a slight deviation from linearity is found. The same line is seen when the plasma samples are diluted in factor-VIII-deficient plasma. In the standard factor VIII assay, an amount of 20 μ l plasma per milliliter reaction mixture is present. If values of below 20% of normal are to be expected in the abnormal sample, the sample volume is increased so as to ensure the equivalent amount of at least 2 μ l of normal plasma factor VIII in the test.

To demonstrate that the spectrophotometric assay depends on factor VIII and not on other clotting factors, we determined the factor VIII activity with the 2 assays in various factor-deficient plasmas. The results shown in table I indicate that background activity is measured in 3 different factor-VIII-deficient plasmas and in plasma of a patient with severe von Willebrand's disease. All other plasmas show factor VIII activities in the 2 assays comparable to the activities of normal individuals (fig. 4). This

Table I. Factor VIII content of various factor-deficient plasmas determined with the chromogenic assay and a clotting assay

Deficient plasma	Factor VIII, U/ml	
	spectrophotometric assay	clotting assay
Factor XII	0.70	0.76
Factor XI	0.82	0.76
High molecular weight kininogen	0.46	0.60
Prekallikrein	1.05	0.76
Passovoy	0.42	0.68
Factor V	1.31	1.40
Factor IX	0.33	0.40
Factor VII	0.61	0.84
Factor X	0.79	0.80
von Willebrand	0.05	0.04
Factor VIII (WH)	0.05	0.01
Factor VIII (George King)	0.05	0.01
Factor VIII (Gen. Diagnostics)	0.05	0.02

Factor VIII was assayed as described in 'Materials and Methods'. Values in italics were obtained using undiluted plasma.

Fig. 4. Comparison of factor VIII activity determined by chromogenic and by clotting assay in 50 normal individuals. Blood was collected from healthy volunteers. Plasma preparation and the assays for factor VIII were carried out as described in 'Materials and Methods'. For 50 normal samples: $y = 0.0883 + 0.949x$; $r = 0.82$.

result and the observation that the plasma dilution curves made in buffer (fig. 3) and in factor-VIII-deficient plasma are overlapping indicate that the assay is not affected by other plasma components.

Within-Assay Precision and Between-Assay Precision. Precision of the 2 assays is shown in table II. Within-assay precision was estimated by testing several dilutions of the reference pool plasma 10 times each. Variation was expressed as percentage of the mean activity in plasma.

Between-assay precision of the chromogenic assay was determined by use of the reference pool plasma, stored in small portions at -70°C . On 10 consecutive days, 1 portion was assayed, from which the between-assay

variation was calculated. Considerably smaller assay variations are observed from the chromogenic factor VIII assay than for the factor VIII clotting assay.

Comparison of the Chromogenic Method with the Clotting Assay – Variation in Factor VIII Activity in Normal Individuals. We determined factor VIII activity with the chromogenic method and the clotting assay in plasma of 50 healthy volunteers (30 men and 20 women). The results are shown in figure 4. The coefficient of correlation between the 2 assay methods is 0.82. The mean, the range and the interindividual variation for the 2 assays are presented in table III. No significant differences in factor VIII activity were observed between males and females.

Table II. Within- and between-assay precision of the chromogenic and clotting assay

Assay	Within-assay variation, %						Between-assay variation, %
	0.10 U/ml	0.20 U/ml	0.50 U/ml	1.0 U/ml	1.5 U/ml	2.0 U/ml	
Factor VIII chromogenic assay	11.7	6.5	5.0	6.9	5.3	—	9.5
Factor VIII clotting assay	18.6	21.0	—	17.5	—	17.9	28.4

The 1 U/ml value of factor VIII in the chromogenic assay represents 20 μ l of human reference pool plasma in the final reaction mixture of 1 ml. The 100% value in the clotting assay is defined as the clotting time obtained when 0.1 ml of a 1:20 dilution of the reference pool plasma is clotted in a 1-stage clotting assay for factor VIII with a final reaction volume of 0.4 ml. Reference pool plasma was obtained from 50 subjectively healthy donors (30 men, 20 women). Within-assay variation: coefficient of variation for measurements on the same day. Between-assay variation: coefficient of variation for measurements on different days.

Table III. Variation in normal values

Assay	n	Mean factor VIII activity, U/ml	Inter-individual variation, %	Coefficient of variation assay, %	Range U/ml	Mean ΔA 405/min	Rate of Xa formation, nM/min
Factor VIII chromogenic assay	30 M	1.13	32.9		0.53–2.29	0.0203	2.85
	20 F	1.23	24.8		0.73–1.97	0.0221	3.10
	50	1.17	29.7	6.9	0.53–2.29	0.0210	2.95
Factor VIII clotting assay	30 M	1.07	26.2		0.68–1.87		
	20 F	1.23	24.8		0.78–1.86		
	50	1.14	26.3	17.5	0.68–1.87		

n = Number of donors; ΔA = absorbance change. The interindividual variation equals variation in normal values (see table II).

Discussion

In this paper, a spectrophotometric method for the determination of factor VIII in human plasma is presented. The method is based on the role of factor VIII as a non-enzymatic cofactor in intrinsic factor X activation. The assay consists of 3 steps: (1) incubation for 1 min of plasma (or a sample

containing factor VIII) with thrombin to convert factor VIII to factor VIIIa; (2) incubation for 2 min of factor VIIIa with factor IXa, factor X and phospholipid/ Ca^{2+} to allow factor Xa formation, and (3) determination of the amount of factor Xa in samples taken after 1 and 2 min. Factor Xa is measured with the chromogenic substrate S2337 in the presence of EDTA to stop further fac-

tor X activation. The rate of factor Xa formation is calculated and compared to the rate found with reference plasma. Factor VIII activity is expressed in units per milliliter, assuming that the factor VIII activity in reference pool plasma is 1 U/ml.

The components used in the chromogenic assay are bovine clotting factors IXa, X and thrombin that were prepared by standard procedures and commercial available phospholipids. Saturating (excess) concentrations of factors IXa, X and phospholipids were chosen to minimize variations due to a slight activity change that might occur in these preparations.

Factor VIII was activated in a separate step at an optimal concentration of thrombin. When thrombin was omitted, no measurable amount of factor Xa is formed within the assay period of 2 min. Factor Xa formation starts after 5–6 min, presumably as a result of factor VIII activation by traces of factor Xa and/or thrombin formed during the lag period. The finding that optimal factor VIII activation by thrombin could be obtained suggests that all factor VIII is converted to factor VIIIa. Consequently, the assay responds to the total concentration of factor VIII, irrespective of the activity state of factor VIII. By performing the factor VIII assay without thrombin, it might be possible to detect circulating factor VIIIa. The few plasma samples we have tested so far without thrombin showed no evidence for a presence of factor VIIIa.

The rate of factor Xa formation we find in plasma (3 nM Xa/min) is proportional to the plasma factor VIII concentration and can be used to calculate the plasma factor VIII concentration. The thrombin activation experiments and the factor IXa saturation curve indicate that all plasma factors VIII

participate in factor X activation. When it is assumed that factor VIII functions in a one-to-one stoichiometrical complex with factor IXa in the enzymatic unit, then the rate of factor Xa formation is proportional to the concentration of the limiting component in the complex, which is here the factor VIIIa concentration.

The maximal rate of factor Xa formation is 925 mol Xa/min/mol enzyme. This indicates that 3.0/925 nM factor VIII is present in the reaction mixture. In the assay, plasma is diluted 1:50, and hence, the plasma VIII concentration in plasma is $150/925 = 0.16 \text{ nM} \pm 32\%$. This value is in agreement with estimates by immunological methods [2].

The sensitivity of the chromogenic assay extends to plasma factor VIII levels of about 0.05 U/ml, but at factor VIII concentrations of 0.10 U/ml and lower, the coefficient of variation increases. Low levels of factor VIII can be detected by including larger sample volumes in the reaction mixture. When factor VIII is determined in plasma, not more than 40 μl plasma can be used if fibrin formation in the factor X activation mixture may prevent accurate sampling to the cuvette. Defibrination by Arvin is useful in that case. The absorbance change found after 2 min of factor Xa formation under standard conditions (20 μl plasma per milliliter reaction mixture and 100 μl sample per cuvette) is 0.0210 $\Delta A_{405}/\text{min}$. Up to 10-fold higher absorbance changes when a larger fraction of the reaction mixture is transferred to the cuvette.

The coefficient of variation of the chromogenic assay varies between 5–7%, whereas the precision of the clotting assay varies between 17 and 21%. The same large assay variations of the clotting method have

been observed in previous studies [4, 8, 9]. The diversity in reagents, methods and equipment that is used in clotting assays may account for the large interlaboratory variation observed in factor VIII determinations [10]. Introduction of the chromogenic assay with its superior reproducibility may allow standardization.

A spectrophotometric assay for factor VIII has been presented by Rosén et al. [11, 12] working at KabiVitrum Diagnostica. Details of their assay have not been disclosed, but as the assay presented here, their method is based on the factor X activation reaction. The precision and sensitivity of the Kabi assay is similar to ours.

Several groups have used immunoradiometric assays to quantify factor-VIII-coagulant antigen in plasma [13–17]. Human allogeneic antibodies are used in these assays obtained from hemophilia A patients with circulating anti-VIII:C antibodies that developed after infusions of factor VIII preparations or from individuals that developed these antibodies spontaneously. The radioimmune assays are very sensitive and have been used to detect factor VIII levels of 0.01–0.03 U/ml. The method depends on the availability of the antibodies which may prevent large-scale application; moreover, immunological methods are complicated by the fact that immunologically recognized factor VIII:C may not be functionally active in clotting reactions [13].

Factor VIII in normal individuals varied between 0.53 and 2.27 U/ml, what results in a variation in normals of 29.7%. Previously, we found a variation for factors VII, IX, X and prothrombin of 21.4, 11.0, 15.0, and 12.0%, respectively [18], suggesting that the factors VII and VIII present in plasma in the lowest concentration and the shortest half-

life tend to have the largest biological variation.

Factor VIII assays are carried out to detect hemophilia, to identify carriers of hemophilia, to check the potency of factor VIII preparations used to treat hemophilia and to control treatment.

For prenatal diagnosis of hemophilia [14], the assay can easily be scaled down such that the test can be carried out with small plasma volumes obtained by fetoscopy [17, 18].

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References

- 1 Hemker, H.C.: Why do hemophiliacs bleed. *Scand. J. Haemat.* (in press, 1984).
- 2 Hoyer, L.W.: The factor VIII complex: structure and function. *Blood* 58: 1–13 (1981).
- 3 Zimmerman, T.S.; Ruggeri, Z.M.: Von Willebrand's disease; in Spaet, Progress in hemostasis and thrombosis, vol 6, pp. 203–206 (Grune & Stratton, New York 1982).
- 4 Hardisty, R.M.; Macpherson, J.C.; Ingram, G.I.C.: A one-stage factor VIII assay and its use on venous and capillary plasma. *Thromb. Diath. haemorrh.* 7: 215–229 (1962).
- 5 van Dieijen, G.; Tans, G.; Rosing, J.; Hemker, H.C.: The role of phospholipid and factor VIIIa in the activation of bovine factor X. *J. biol. Chem.* 256: 3433–3442 (1981).
- 6 Rosing, J.; Tans, G.; Govers-Riemslog, J.W.P.; Zwaal, R.F.A.; Hemker, H.C.: The role of phospholipids and factor Va in the prothrombinase complex. *J. biol. Chem.* 255: 274–283 (1980).
- 7 van Dieijen, G.; van Rijn, J.L.M.L.; Govers-Riemslog, J.W.P.; Hemker, H.C.; Rosing, J.: Factor IXa-factor VIIIa interaction: enhanced factor IXa binding to phospholipids in the presence of

- factor VIIIa (Abstract). *Thromb. Haemostasis* 50: 256 (1983).
- 8 Palkuti, H.A.; Longberry, J.F.: A precision study of coagulation factor assay technics. *Am. J. clin. Path.* 59: 213 (1973).
- 9 Elödi, S.; Váradi, K.; Hollán, S.R.: Some sources of error in the one-stage assay of factor VIII. *Haemostasis* 7: 1-9 (1979).
- 10 Kocoshis, T.A.; Triplett, D.A.: CAP survey results for factor VIII assays (1977-1978). *Am. J. clin. Path.* 72: 346-361 (1979).
- 11 Rosén, S.; Oswaldsson, U.; Blombäck, M.; Larrieu, M.; Nilsson, I.M.; Vinazzer, H.: A collaborative comparison of a chromogenic FVIII:C method with one- and two-stage clotting methods (Abstract). *Thromb. Haemostasis* 50: 112 (1983).
- 12 Rosen, S.; Carlebjörk, G.; Oswaldsson, U.: Determination of factor VIII:C activity in microtiter plates using the chromogenic COATEST factor VIII kit. Poster presentation at the 20th Congr. Int. Soc. Hematol. (1984).
- 13 Lazarchick, J.; Hoyer, L.W.: Immunoradiometric measurement of the factor VIII procoagulant antigen. *J. clin. Invest.* 62: 1048-1052 (1978).
- 14 Peake, I.R.; Bloom, A.L.; Giddings, J.C.; Ludlam, A.L.: An immunoradiometric assay for procoagulant factor VIII antigen: results in haemophilia, van Willebrand's disease and fetal plasma and serum. *Br. J. Haematol.* 42: 269-281 (1979).
- 15 Reisner, H.M.; Barrow, E.S.; Graham, J.B.: Radioimmunoassay for coagulant factor-VIII-related antigen (VIII:CAg). *Thromb. Res.* 14: 235-239 (1979).
- 16 Muller, H.P.; van Tilburg, H.N.; Bertina, R.M.; Terwiel, J.Ph.; Veltkamp, J.J.: Immunoradiometric assay of procoagulant factor VIII antigen (VIII:CAg). *Clinica chim. Acta* 107: 11-19 (1980).
- 17 Hoyer, L.W.; Trabola, N.C.: Immunoradiometric assays for factor VIII-antigens: coagulant protein C (autohemophobic factor) and factor-VIII-related protein (von Hillebrand factor). *Meth. Enzym.* 84: 51-60 (1982).
- 18 van Dieijen-Visser, M.P.; van Wersch, J.; Brombacher, P.J.; Rosing, J.; Hemker, H.C.; van Dieijen, G.: Use of chromogenic peptide substrates in the determination of clotting factors II, VII, IX and X in normal plasma and in plasma of patients treated with oral anticoagulants. *Haemostasis* 12: 241-255 (1982).
- 19 Firshein, S.I.; Hoyer, L.W.; Lazarchick, J.; Forget, B.G.; Hobbins, J.C.; Clyne, L.P.; Pitlick, F.A.; Muir, W.A.; Merkat, I.R.; Mahoney, M.J.: Prenatal diagnosis of classic hemophilia. *New Engl. J. Med.* 300: 937-941 (1979).

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