

A Simple Method for the Assay of Factor VIII

By LUIS JOSÉ BERGNA

A SIMPLE and reliable method for the assay of factor VIII is needed for many reasons: Patients with “subhemophilia” and some hemophiliacs with the “mild” type of the disease can be diagnosed only by a quantitative estimation of factor VIII. Likewise, the diagnosis of angiohemophilia A (vascular hemophilia) in some patients can be made only by the evaluation of factor VIII in plasma. In any attempt at purification or concentration of the factor VIII, a method is required to test the biologic activity of different preparations obtained at various stages of their manufacture. There is need to measure the effect achieved by transfusing blood plasma or concentrated antihemophilic factor (AHF) so that hemophilic patients can be treated with more safety, particularly preceding a surgical procedure. The level of factor VIII in the relatives of a hemophilic subject may permit one to discover a female carrier and to learn more about the inheritance of this disorder. The clinical severity of the illness can be suspected by the degree of the deficiency. In normal people assay for factor VIII permits selection of donors with higher concentrations.

Development of a simple, accurate, and reproducible method for the assay of factor VIII has proved to be difficult. The first method proposed measured the effect on the *whole clotting time* produced by adding the unknown plasma to the blood of a severe hemophilic compared with the effect produced by different amounts of normal plasma^{1,21,23-25,37}; it proved to be too insensitive. Graham, Collins, Godwin and Brinkhous^{14,15} employed the *prothrombin utilization test* to assess the degree of correction. They observed that, within certain limits, the amount of prothrombin consumed in the whole hemophilic blood is linearly proportional to the amount of factor VIII added. Thus, by comparing the amount of the test plasma required to cause 50 per cent utilization of prothrombin in the hemophilic blood with the amount of control plasma required to produce the same change, the content of factor VIII can be expressed in per cent of the control. The prothrombin consumption test is also the basis for the more recent procedure proposed by Quick et al.^{16,31-33}; a straight-line proportion between the concentration of factor VIII and prothrombin consumption time is obtained in a system containing “erythrocytin” (a partial thromboplastin), fresh “native” hemophilic plasma and the diluted adsorbed test or normal plasma plus calcium. Nilsson et al.²² work with the *plasma recalcification time* technic; the effect produced by diluted normal plasma on the plasma of a patient with factor VIII deficiency is compared with the effect produced by the test plasma. Soulier and Larrieu³⁴⁻³⁶ have been using a similar method to assay AHF by adding heated platelets (which they have recently replaced by cephalin²⁰) to the system. Langdell, Wagner and Brinkhous¹⁷⁻¹⁹ use a procedure based on the *partial thromboplastin time*, in

From the Instituto de Investigaciones Hematológicas (Director Dr. A. Pavlovsky) de la Academia Nacional de Medicina, Buenos Aires, Argentina.

which the correcting effect of the diluted test or standard plasma on the prolonged cephalin time of a hemophilic plasma is recorded. Geiger et al.¹³ have added normal serum to the system for the purpose of improving the reproducibility of the method. A combination of these two procedures, stressing the importance of controlling the contact influence of glass upon the reagents, has been carefully worked out by Waaler, who claims to have developed a fairly reproducible and rapid method.³⁸ Bounameaux¹¹ has devised a method also based on the partial thromboplastin time, in which the hemophilic plasma is replaced by thrombopenic serum plus aged normal plasma. Pitney and Dacie²⁹ have reported a simple technic taken from the study of the *thrombin generation* in recalcified citrated plasma to evaluate the AHF. The "prothrombin conversion ratio," a more elaborate method described by Wolf,⁴⁰ also based on the principle of the thrombin generation test, has been found to be very satisfactory.

Biggs, Eveling and Richards⁷ and Biggs and Macfarlane⁸ have described a direct assay of factor VIII adapted from the *thromboplastin generation test* (Biggs and Douglas⁶). The principle of the method is based on the theory that the lack of factor VIII in the TGT causes a failure in the normal generation of thromboplastin, and the ability of factor VIII to correct thromboplastin formation can be assessed by a modification of the thromboplastin generation test. Several modifications have been introduced in Biggs, Eveling and Richard's method in order to simplify or improve it^{3-5,27,28,30,39}; apparently these methods are among the most accurate and reliable technics for the quantitative determination of factor VIII.

The majority of the methods described, although fairly sensitive, are rather complicated and time-consuming. With only a few exceptions, they all require hemophilic blood. This may make the test impracticable in some laboratories since it involves the collection of blood from a severe, noncomplicated hemophiliac at rather frequent intervals. Bounameaux's method does not require hemophilic blood, and further evaluation of this procedure will be very interesting. Likewise, in the methods of Biggs, Eveling and Richards, Wolf, and of Pool and Robinson, blood from a patient with hemophilia is not necessary; although these assays appear to be quite reliable, they are still complicated and time consuming since standardized materials are necessary and these are difficult to prepare, particularly in the first.

The method described below is also based on the TGT but does not require hemophilic blood nor special materials and is easily adapted to any laboratory interested in blood coagulation. In short, it consists in a mixture of calcium, cephalin and bovine serum (to provide all the clotting factors required for the generation of thromboplastin except factor VIII) added successively with diluted adsorbed unknown plasma and standard plasma. The correcting effect on the thromboplastin generation (measured in the usual way on a recalcified platelet-poor substrate) is compared; the amount of factor VIII can be expressed in per cent of the standard.

MATERIALS

Bovine serum.—Bovine blood is collected at the slaughter house from animals bled from a stab wound in the neck. The blood is allowed to flow directly into a measured amount

of anticoagulant (1 part of 1.85 per cent potassium oxalate to 10 parts of blood); these are mixed thoroughly, avoiding excessive foaming as much as possible. In the laboratory the blood is filtered through clean gauze and centrifuged at 2000 rpm for 30 minutes to remove the red cells; the plasma is aspirated. About 100 ml. of bovine plasma are recalcified with one-fourth of its volume of 0.1 M calcium chloride; it is allowed to clot at 37 C. and maintained at that temperature for 4 hours after coagulation, then placed in the ice box overnight. The next morning the serum is separated from the clot and stored at -20 C. in 1 ml. amounts until required. Absence of factor VIII activity was confirmed by inability of the serum to correct the abnormal thromboplastin generation, and the prothrombin consumption test of a hemophilic blood clotted with two-tenth volume of bovine serum. The different batches of bovine serum employed generally contain about 25 per cent of factors VII, IX and X, 200 per cent of factor V, and less than 1 per cent of prothrombin. The same supply of serum can be used for tests carried out over a period of several months. The day before a factor VIII assay is to be performed, 1 ml. of serum is diluted 1 in 5 with Veronal buffer at pH 7.35 and left at 4 C. overnight to permit complete activation which takes place after dilution.

Test plasma.—Blood is obtained from the patient by the two syringe technic with the use of clean, siliconized and precooled tubes and syringes. Nine volumes of blood are mixed immediately with one volume of 3.8 per cent trisodium citrate and spun in a refrigerated centrifuge at 3000 rpm for 30 minutes without any delay. The clear supernatant plasma is separated and adsorbed with one-tenth volume of aluminium-hydroxide gel; the mixture is shaken and incubated at 37 C. for 5 minutes. The tube is then spun at 3000 rpm for 10 minutes, and the supernatant is promptly aspirated into a cold, siliconized test tube. Three dilutions in citrated saline are then made, the choice of the dilution depending upon the probable content of factor VIII. For severe hemophiliacs, 1 in 10, 1 in 25 and 1 in 50 dilutions are the most suitable; for mild hemophiliacs, probable carriers and normal subjects, 1 in 25, 1 in 50 and 1 in 100 dilutions are utilized. The plasma dilutions and the other reagents are kept in a bath of melting ice until the moment of being used.

Standard plasma.—The standard plasma is collected and prepared as was outlined for the test plasma. Six dilutions, 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800 are commonly used for making the standard curve. The whole procedure should be done at approximately the same time as the test sample. Standard plasma may also be stored at -20 C. with the following precautions: after being separated from the blood in a refrigerated centrifuge, it is transferred without delay into small siliconized tubes, which are immersed in a CO₂-ice-alcohol mixture (with a temperature of approximately -70 C.) to freeze the specimens as quickly as possible. Similarly, the thawing of the frozen plasma should be performed rapidly, the tube being shaken with the frozen plasma in a waterbath at 37 C. until complete thawing has taken place. With these precautions it has been possible to preserve the AHF activity of the standard plasma for at least a month.

Cephalin.—Human brain extract prepared as described by Bell and Alton² is used as a platelet substitute. The suspension in saline is kept at -20 C. and diluted 1:50 in Veronal buffer before use. The suitable dilution must be determined by trial and error in the test.

Substrate.—Nine parts of human blood are mixed with one part of 3.8 per cent sodium citrate and centrifuged at 3000 rpm for 30 minutes to obtain a platelet-poor plasma. This is fractionated in convenient volumes and stored frozen until used.

METHOD AND STANDARD DILUTION CURVE

The reagents, kept in melting ice, are pipetted into a warm 10 x 75 mm. tube, maintained in a water-bath at 37 C., in the following order: (1) 0.1 ml. of cephalin; (2) 0.1 ml. of diluted bovine serum; (3) 0.1 ml. of diluted test or standard plasma; (4) 0.1 ml. of 0.025 M CaCl₂. At the moment of adding the calcium a stopwatch is started. Exactly 8 minutes after the incubation of the reagents, 0.1 ml. of the reacting mixture is transferred to a 10 x 75 mm. tube containing 0.1 ml. of substrate plasma, previously warmed in the water bath and to which 0.1 ml. of CaCl₂ was added 10 seconds before transferring the generating mixture. The clotting time is determined with a second stopwatch. It will be observed that 8 minutes elapse before the generating mixture is added to the substrate. By starting

successive generating tubes at intervals of one minute using the same stopwatch, it is possible to test the six dilutions of the standard plasma and the blank (in which the plasma dilution is replaced by the same amount of citrated saline) in 15 minutes. The duplication of the standard curve will take another 15 minutes. Then, in the next 15 minutes, the three dilutions of the plasma under study are tested in duplicate. Any subsequent determination will take another 15 minutes.

The average values of the two clotting times recorded with each dilution of the standard plasma are plotted on double logarithmic paper and the points are found to fall on a straight line, the "standard curve" (fig. 1). To figure out the factor VIII content of the patient's plasma, the average of the two thromboplastin times of each dilution are referred to the standard curve. On the standard curve one finds that concentration of factor VIII which corresponds to the clotting time obtained at a determined concentration of the unknown plasma. By a simple calculation the factor VIII activity expressed as per cent of the test plasma is found:

$$\frac{\text{concentration of standard plasma}}{\text{concentration of unknown plasma}} \times 100 = \text{Factor VIII activity in \%}$$

The mean of the factor VIII concentration in the test plasma is calculated by averaging the results obtained at the three dilutions assayed.

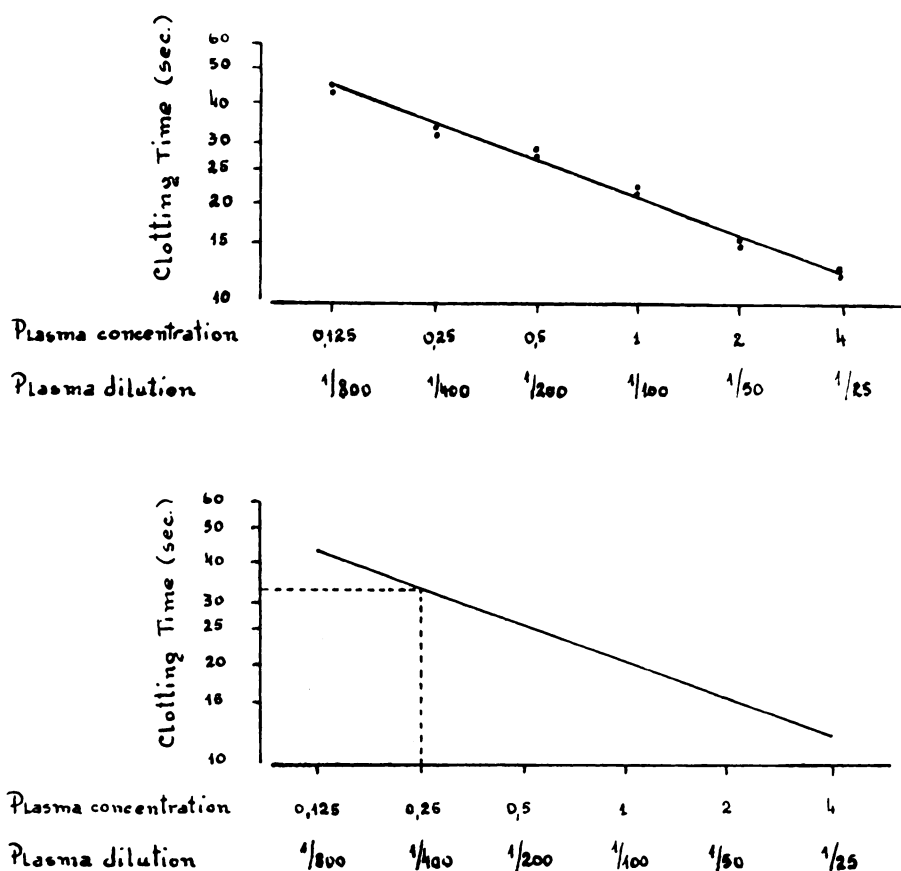


Fig. 1 (at top).—Standard dilution curve.

Fig. 2 (at bottom).—Assay of factor VIII. Calculation of factor VIII concentration in the unknown sample.

In the example of figure 2, the thromboplastin time of 32 seconds of the patient's plasma corresponds to 0.25 per cent on the curve of the standard plasma when tested at a concentration of 4.0 per cent (dilution 1:25). The AHF concentration of the unknown plasma calculated from this observation would be

$$\frac{0.25}{4.0} \times 100 = 6.25 \%$$

RESULTS AND DISCUSSION

The method described is relatively simple, particularly concerning preparation of the reagents required; plasma from a patient with factor VIII deficiency is not necessary and day-to-day reproducibility has been found to be sufficiently good. It has been observed that, in accordance with Pool and Robinson's findings, the plotting on double logarithmic paper of the dilutions of normal plasma produces a straight line when tested at a concentration of factor VIII between 4 and 0.125 per cent; at above or below these concentrations a flattening of the curve is observed. The most accurate part of the dilution standard curve has been found to be between concentrations 2 and 0.25 per cent (dilutions 1/50 and 1/400). Therefore, an appropriate dilution of the unknown plasma has to be made in order to have the generating time fall within those limits. The thromboplastin time given by the more concentrated dilution (1/25 corresponding to 4 per cent) has varied from 11 to 14 seconds, but mostly between 12 and 13 seconds. The citrated-saline control time for the system is usually above 60 seconds; the most convenient time of subsampling from the reacting mixture to obtain the minimum clotting time in the substrate was found to be 8 minutes (fig. 3). Although the slope of the curve is the same, within experimental limits, for a given plasma on different days, or for

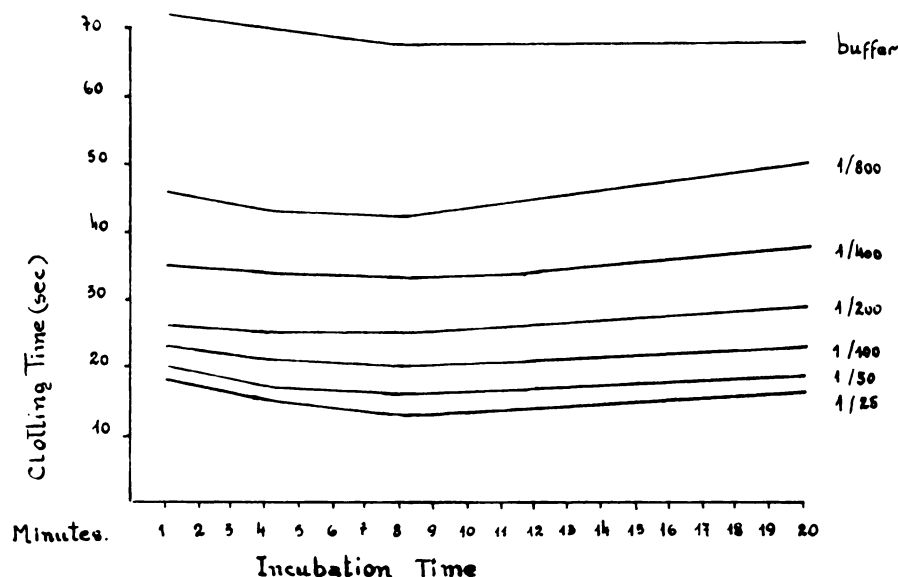


Fig. 3.—Thromboplastin time as a function of incubation time and plasma dilution in the factor VIII assay.

different plasmas on the same day, the absolute level of different plasmas varies from one to another, unless they have an equal concentration of factor VIII.

The validity of this method was demonstrated "in vitro" by diluting the standard plasma (or any normal plasma with a known concentration of factor VIII) in the plasma of a patient with severe hemophilia, to make a determined dilution; the expected concentration was found in the assay. Its reliability was also proved "in vivo" by transfusing a determined volume of plasma, with a known content of factor VIII, into a hemophiliac; the predicted rise of the level of factor VIII was observed 10 minutes after the transfusion, provided the patient had an "intermediate" or "mild" type of the disease; in severely affected patients, (with less than 1 per cent of factor VIII), the concentration of AHF observed after the transfusion was a little less than the expected (which is in accordance with the experience of other authors³⁰).

The selection of a standard for comparison is a very important part of the assay. Many donors, several per day, were tested simultaneously to assess their factor VIII content. Those who represented the mean value were considered to have 100 per cent of AHF. A fresh sample of any one of these selected donors was collected the day of the assay, and with this plasma the standard dilution curve was performed.

Certainly there is a limit to the frequency with which any one subject can be expected to give blood for use as standard plasma. To overcome this inconvenience we have found two possible alternatives. One is to store the standard plasma frozen at -20°C . in amounts sufficient for each test. In order to preserve the AHF activity, it is necessary to quick freeze at -70°C . by immersing the tube with the plasma in a CO_2 -ice alcohol mixture, and necessary also to thaw rapidly the frozen plasma in the water-bath until a complete thawing has been obtained. Careful collection of the blood with a clean rapid venepuncture is also required, with the use of precooled and siliconized glassware, as well as rapid mixing with the anticoagulant, immediate centrifugation and separation of the plasma from the red cells and platelets. The second alternative we have recently used is to employ a lyophilized fraction I-O prepared according to Blombäck and Blombäck^{9,10} and stored at -20°C . An appropriate dilution was prepared in order to find values which superimpose over those obtained with the standard plasma. With the same batch of lyophilized material it is possible to reproduce the results of the normal plasma by making the same starting dilution, since subsequent doubling dilutions give values corresponding with those obtained with the standard plasma.

The level of factor VIII in the plasma has been measured in about 50 normal males and females. The concentration range was found to be between 50 and 200 per cent, but most of the observations were between 80 and 120 per cent, values which agree with previous reports.^{7,12,18,22,27,28,30,38} The factor VIII content of the hemophiliacs studied ranged from 0 to 30 per cent; it was relatively common to find a decreased concentration of factor VIII in the mothers of hemophiliacs.²⁶

SUMMARY

A simple method for the assay of factor VIII activity, based on the thromboplastin generation test of Biggs and Douglas, has been described.

Bovine serum is used as a source of factor V and serum factors, and plasma from a hemophiliac is not required. The concentration of factor VIII of the test plasma is compared to that of a standard normal human plasma. The results obtained with the method have been fairly reproducible.

SUMMARIO IN INTERLINGUA

Es describe un simple methodo pro le essayage de activitate de factor VIII. Illo es basate super le test de generation de thromboplastina de Biggs e Douglas.

Sero bovin es usate como fonte de factor V e factores seral. Plasma ab un hemophilico non es requirite. Le concentration de factor VIII in le plasma sub investigation es comparate con normal plasma human como standard. Le resultatos obtenite per iste methodo se ha provate satis reproducibile.

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