Fibrinogen Assay Test Protocol. The laboratory practitioner prepares a 1:10 dilution of each patient PPP and control with Owren buffer. Then 200 μL of each of the diluted PPPs is warmed to 37° C in each of two reaction tubes or cups for 3 minutes. After incubation, 100 μL of thrombin reagent is added, a timer is started, and the mixture is observed until a clot forms. The timer is stopped, values for duplicate runs are averaged, and the interval in seconds is compared with the graph. Results are reported in mg/dL of fibrinogen.

If the clotting time of the patient PPP dilution is short, indicating a fibrinogen level greater than 480 mg/dL, a 1:20 dilution is prepared and tested. The resulting fibrinogen concentration from the graph must be multiplied by 2 to compensate for the dilution. If the clotting time of the original 1:10 patient PPP dilution is prolonged, indicating less than 200 mg/dL of fibrinogen, a 1:5 dilution is prepared. The operator divides the resulting concentration reading from the graph by 2 to compensate for the greater concentration of the specimen.

Fibrinogen Assay Quality Control

All results for duplicate tests must agree within a coefficient of variation of less than 7%. The medical laboratory practitioner tests a normal control sample and an abnormal control sample with each batch of specimens for which fibrinogen levels are measured and records the results. The normal control results should be within the laboratory's reference interval. The abnormal control results should be less than 100 mg/dL. If either control result falls outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control is retested. The actions taken to correct out-of-limit tests are recorded. Control results are analyzed at regular intervals (weekly is typical) to determine the longitudinal validity of the procedure.

Specimen errors that affect the PTT likewise affect the fibrinogen assay and all factor assays (Table 42-7).

Fibrinogen Assay Results and Clinical Utility

One institution's reference interval for fibrinogen concentration is 220 to 498 mg/dL, although each local institution prepares its own interval. Hypofibrinogenemia, a fibrinogen level of less than 220 mg/dL, is associated with DIC and severe liver disease. Moderately severe liver disease, pregnancy, and a chronic inflammatory condition may cause an elevated fibrinogen level, greater than 498 mg/dL. Congenital afibrinogenemia leads to prolonged clotting times and is associated with a variable hemorrhagic disorder. Dysfibrinogenemia may give the same results as hypofibrinogenemia by this test method, because some abnormal fibrinogen species are hydrolyzed more slowly by thrombin than is normal fibrinogen. Some forms of dysfibrinogenemia may be associated with thrombosis.[81]

Fibrinogen values measured using immunologic assays and turbidimetric methods (Ellis-Stransky technique; PT-Fibrinogen HS Plus, Instrumentation Laboratory, Bedford, MA) are normal in dysfibrinogenemia. The fibrinogen concentration is estimated from reaction mixture turbidity and reported with each PT.

Fibrinogen Assay Limitations

Although antithrombotic effects are minimized by the dilution of PPP specimens, heparin levels greater than 0.6 units/mL and FDP levels greater than 100 μg/mL prolong the results and give falsely lowered fibrinogen results. The operator ensures that the thrombin reagent is pure and has not degenerated. Exposure to sunlight or oxidation results in rapid breakdown. The working dilution lasts only 1 hour at 1° C to 6° C and should remain cold until just before testing.

Single-Factor Assays Using the Partial Thromboplastin Time Test

Principle of Single-Factor Assays Based on Partial Thromboplastin Time

If the PTT is prolonged and the PT and TCT are normal, and there is no ready explanation for the prolonged PTT such as heparin therapy, LA, or a factor-specific inhibitor, the medical laboratory practitioner may suspect a congenital single-factor deficiency. Three factor deficiencies that give this reaction pattern and cause hemorrhage are factor VIII deficiency (hemophilia A), factor IX deficiency (hemophilia B), and factor XI deficiency, which causes a mild intermittent bleeding disorder called *Rosenthal syndrome* found primarily in Ashkenazi Jews.[82,83] These deficiencies are most often detected in childhood. The next step in diagnosis of a congenital single-factor deficiency is the performance of a one-stage single-factor assay based on the PTT system.

Although necessary for diagnosis, PTT-based single-factor assays are most often performed on specimens from patients with previously identified single-factor deficiencies. Their purpose is to monitor supportive therapy during bleeding episodes or invasive procedures. Because hemophilia A is the most common single-factor deficiency disorder, this discussion is confined to the factor VIII assay; however, the protocol may be generalized to the assays for factors IX and XI.

The medical laboratory practitioner uses the PTT system to estimate the concentration of functional factor VIII by incorporating commercially prepared factor VIII-depleted PPP in the test system (Cryo*check* Factor VIII Deficient Plasma; Precision BioLogic Inc, Dartmouth, Nova Scotia). Distributors collect plasma from normal donors and employ *immunodepletion*, relying on a monoclonal anti-factor VIII antibody bound to a separatory column, to prepare factor VIII-depleted plasma.[84]

In the PTT-based factor assay system, factor VIII-depleted PPP provides normal activity of all procoagulants except factor VIII. Tested alone, factor VIII-depleted PPP has a prolonged PTT, but when normal PPP is added, the PTT reverts to normal. In contrast, a prolonged result for a mixture of patient PPP and factor VIII-depleted PPP implies that the patient PPP is factor VIII deficient. The clotting time interval for the mixture of patient PPP and factor VIII-depleted PPP may be compared with a previously prepared reference curve to estimate the level of factor VIII activity in the patient PPP. The quantitative factor assay is typically performed on three or four dilutions of patient PPP—for instance, 1:10, 1:20, 1:40, and 1:80—and the results compared with mathematical manipulation. Multiple dilutions contribute to the accuracy of the results.

Factor VIII Assay Reference Curve

To prepare a reference curve for the factor VIII assay, the laboratory practitioner obtains a reference plasma such as CAP FVIIIc RM (College of American Pathologists, Northfield, IL) and prepares a series of dilutions with buffered saline.[85] Although laboratory protocols vary, most laboratory practitioners prepare a series of five dilutions, from 1:5 to 1:500. Each dilution is mixed with reagent factor VIII-depleted plasma and tested in duplicate using the PTT system. The duplicate results are averaged and plotted on log-log or log-linear graph paper (Figure 42-12). The 1:10 dilution is assigned the factor VIII assay activity value found on the package insert. When patient PPP is tested, the time interval obtained is entered on the vertical coordinate and converted to a percentage.[86]

Factor VIII Assay Procedure

The medical laboratory practitioner (or the automated coagulometer) prepares 1:10, 1:20, 1:40, and 1:80 dilutions of each patient PPP and control specimen and then mixes each dilution with equal volumes of factor VIII-depleted plasma and PTT reagent. In most cases, 100 µL of PTT reagent is mixed with 100 µL each of patient PPP dilution and factor VIII-depleted plasma mixture. All dilutions of each specimen or control are tested in duplicate. After incubation at 37° C for the manufacturer-specified time, typically 3 minutes, 100 µL of 0.025 M calcium chloride is added, and a timer is started. The interval is recorded in seconds, duplicates are averaged, the mean result is compared with the reference curve, and the percentage of factor VIII activity is reported. Factor activity results for the 1:20, 1:40, and 1:80 dilutions are multiplied by 2, 4, and 8, respectively, to compensate for the dilutions and should match the results of the 1:10 dilutions within 10%. If the results of the dilutions do not match within 10%, they are considered to be *nonparallel*. An LA may be present, and the assay cannot provide a reliable estimate of factor VIII activity.

Tests for factors IX and XI are performed using the same approach, except that the appropriate factor-depleted plasma is substituted for factor VIII-depleted plasma. Tests for the contact factors XII, prekallikrein, and high-molecular-weight kininogen are seldom requested because deficiencies are not associated with bleeding disorders. Acquired and congenital contact factor deficiencies are relatively common, however, and cause PTT prolongation. Factor XII, prekallikrein, and high-molecular-weight kininogen assays are available from hemostasis reference laboratories, and their use may be necessary to account for an unexplained prolonged PTT.

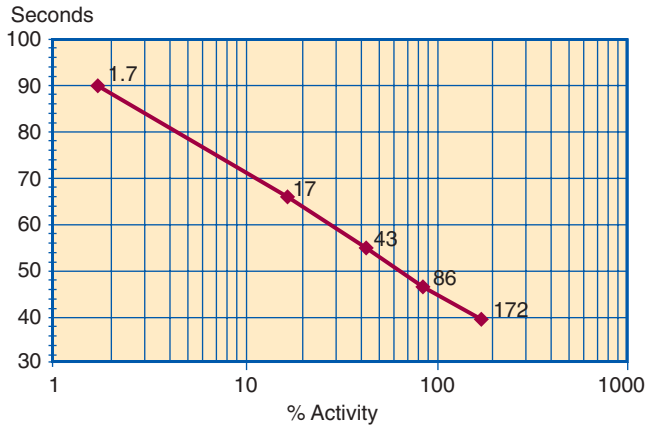

Figure 42-12 Factor VIII assay calibrator curve plotted on linear-log axes.

Expected Results and Clinical Utility of Single-Factor Assays

The reference interval for factor VIII activity is 50% to 186%. Spontaneous symptoms of hemophilia are evident at activity levels of 10% or less. The test is used most often to estimate the plasma level of factor VIII activity during therapy (Chapter 38). Chronically elevated factor VIII predicts an elevated risk of venous thrombotic disease (Chapter 39).

Single-Factor Assay Quality Control

All duplicate results must agree within 10%. The medical laboratory practitioner tests a normal and a deficient control specimen with each assay and records the results. The normal control results should fall within the reference interval. The deficient control results should be in the range of 10% factor VIII activity or below. If either control result falls outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control is retested. The practitioner records all actions taken to correct out-of-limit tests. Control results are analyzed at regular intervals (weekly is typical) to determine the longitudinal validity of the procedure.

Limitations of Single-Factor Assays

Interlaboratory coefficients of variation for the factor VIII assay reach 80%, which implies undesirable variation in the interpretation of therapeutic monitoring results from unrelated institutions. To reduce inherent variation, the medical laboratory practitioner uses assayed commercial plasma to prepare the reference curve and selects reference dilutions that correspond to only the linear portion of the curve. The laboratory must assay three or more dilutions of patient PPP to check for inhibitors. The practitioner also selects a matching reagent-instrument system with a demonstrated coefficient of variation of less than 5% and uses factor-depleted substrates with no trace of the depleted factor.[87] As with the PTT test, good specimen management is essential. Clotted, hemolyzed, icteric, or lipemic specimens are rejected because they give unreliable results. Reagents must be reconstituted with the correct diluents and volumes following manufacturer instructions. Reagents must be stored and shipped in accordance with manufacturer instructions and never used after the expiration date.

Bethesda Titer for Anti–Factor VIII Inhibitor

The Bethesda titer is used to confirm the presence of and quantify an anti-factor VIII inhibitor, which is typically an IgG4-class immunoglobulin.[88] In this method, 200 µL of patient PPP is incubated with 200 µL of reagent normal plasma for 2 hours at 37° C. A control specimen consisting of 200 µL of imidazole buffer at pH 7.4 mixed with 200 µL of reagent normal plasma is incubated simultaneously. During the incubation period,