PRINCIPLE
The one-stage assay for FVIII is described in this section. The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of a plasma known to be totally deficient in FVIII but containing all other factors required for normal clotting. For factors IX, XI, and XII, the assay is essentially the same and is performed by substituting the relevant deficient plasma for FVIII-deficient plasma, and after selection of the appropriate reference plasma.

REAGENTS

- Platelet-poor citrated test and standard plasma
  The standard plasma used should be either a locally prepared plasma pool kept at -70°C or lower, or a commercial standard plasma. In either case, this reference plasma must be calibrated against an international standard for FVIII. It is not acceptable to assume that a pooled normal plasma has 100 U/dl FVIII:C.

- FVIII-deficient plasma
  This is available commercially or may be collected from a donor whose FVIII level is less than 1 U/dl, who has no anti-FVIII antibodies, who has received no treatment for two weeks, and who has normal liver function tests. Abnormal liver function could lead to reduction in other clotting factors, which affect the specificity of the assay. This plasma can be stored in aliquots at -37°C. It is preferable to use FVIII-deficient plasma produced by immunodepletion of FVIII from normal plasma using a monoclonal antibody. This type of material is available commercially and has the advantage of viral safety compared with plasmas from hemophiliacs who have been treated with plasma products. However, not all immunodepleted plasmas are found to be <1 U/dl, and care should be taken to check this. Some experts hold the view that the presence of normal concentrations of VWF in FVIII-deficient plasma may be an advantage, and there is evidence to support this in relation to FVIII assays performed as part of FVIII inhibitor determinations.

- APTT reagent

- Owren’s buffered saline (OBS or glyoxaline buffer; see Section 9)

- 25mM CaCl₂
METHOD

1. Make 1/10 dilutions of standard and test plasma in buffered saline in plastic tubes. (If the test plasma is expected to have a very low level of factor VIII, start at a 1/5 dilution).

2. Using 0.2 ml volumes, make doubling dilutions in OBS of standard and test plasma from 1/10 to 1/40 in plastic tubes. (Mix each dilution well before transferring to next tube.) Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on wet ice prior to testing.

3. Pipette 0.1 ml of each standard dilution into a 75 × 10 mm glass tube.

4. Add 0.1 ml of FVIII-deficient plasma and transfer to 37°C water bath.

5. Add 0.1 ml of APTT reagent and incubate for 5 minutes.

6. At 5 minutes add 0.1 ml CaCl₂ and record the clotting time.

   A “blank” should also be set up as follows:
   • 0.1 ml OBS
   • 0.1 ml FVIII-deficient plasmas
   • 0.1 ml APTT reagent
   • Incubate 5 minutes
   • 0.1ml CaCl₂

   The clotting time of the blank should be longer than the time of 1% FVIII activity of standard from the calibration graph. If the time is shorter, this indicates that the substrate plasma is not totally deficient in FVIII and thus is not a suitable substrate plasma.

RESULTS

Plotting of results is described in Section 22, requiring double logarithmic or logarithmic/linear scale graph paper.

The 1/10 dilution is arbitrarily assigned a value of 100%, the 1/20 dilution a value of 50%, and the 1/40 dilution a value of 25%.

Straight lines, parallel to each other, should be obtained.

Read off concentration of test sample as shown in Figure 22.1 (Section 22). In this example, the FVIII concentration in the test sample is 7% of that in the standard. If the standard has a concentration of 85 IU/dl, the test sample has a concentration of 85 IU/dl × 7% = 6 IU/dl.

If the lines are not parallel, the assay should be repeated.
Non-parallel lines may occur due to technical error. If technical error has been eliminated, it may be due to the presence of an inhibitor, which may act specifically against FVIII or may be of the “lupus type”, showing a converging pattern. Diverging lines are typical of an activated sample.

NOTES

• If the test plasma FVIII (or FIX, FXI, or FXII) concentration is close to zero (i.e., the clotting times of all dilutions are similar to the blank), non-parallel lines may occur.

• The normal range should be established locally but often has a lower limit of 50–65 IU/dl in the case of FIX or FXI.

• In relation to FXI, the International Unit has only recently been established. At the time of writing, there are few data on the normal levels of FXI in IU. Publications that predate the establishment of the IU have indicated that the lower limit of normality for FXI is in the range 63–80 U/dl (see Bolton-Maggs et al. 2004 for a review).

**Figure 23.1. Graph of FVIII assay**

**REFERENCE**