2.7.4. Assay of human coagulation factor VIII

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXα) in the presence of calcium ions and phospholipid. The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation. The International Unit is the factor VIII activity of a stated amount of the International Standard, which consists of a freeze-dried human coagulation factor VIII concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

**Human coagulation factor VIII BRP** is calibrated in International Units by comparison with the International Standard. The cosagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXα, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay. Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least 2 separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The 2nd step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between 3 and 5 amino acids, joined to a chromophore group. On cleavage of this group from the substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

**ASSAY PROCEDURE**

Reconstitute the entire contents of 1 ampoule of the reference preparation and of the preparation to be examined; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5-2.0 IU/ml.

The prediluent consists of haemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma. The prediluted materials must be stable beyond the time required for the assay.

Prepare further dilutions of the reference and test preparations using a non-chelating, appropriately buffered solution, for example, tris(hydroxymethyl)aminomethane or imidazole, containing 1 per cent of human or bovine albumin. Prepare at least 2 dilution series of at least 3 further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU/ml, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.
2.7.5. ASSAY OF HEPARIN

The anticoagulant activity of heparin is determined in vitro by comparing its ability in given conditions to delay the clotting of recalcified citrated sheep plasma with the same ability of a reference preparation of heparin calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of freeze-dried heparin sodium from pork intestinal mucosa. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Heparin sodium BRP is calibrated in International Units by comparison with the International Standard by means of the assay given below.

Carry out the assay using one of the following methods for determining the onset of clotting and using tubes and other equipment appropriate to the chosen method:

a) direct visual inspection, preferably using indirect illumination and viewing against a matt black background;
b) spectrophotometric recording of the change in optical density at a wavelength of approximately 600 nm;
c) visual detection of the change in fluidity on manual tilting of the tubes;
d) mechanical recording of the change in fluidity on stirring, care being taken to cause the minimum disturbance of the solution during the earliest phase of clotting.

ASSAY PROCEDURE

The volumes in the text are given as examples and may be adapted to the apparatus used provided that the ratios between the different volumes are respected.

Dilute heparin sodium BRP with a 9 g/l solution of sodium chloride R to contain a precisely known number of International Units per millilitre and prepare a similar solution of the preparation to be examined which is expected to have the same activity. Using a 9 g/l solution of sodium chloride R, prepare from each solution a series of dilutions with geometric progression such that the clotting time obtained with the lowest concentration is not less than 1.5 times the blank recalcification time, and that obtained with the highest concentration is such as to give a satisfactory log dose-response curve, as determined in a preliminary test.

Place 12 tubes in a bath of iced water, labelling them in duplicate: T₁, T₂ and T₃ for the dilutions of the preparation to be examined and S₁, S₂ and S₃ for the dilutions of the reference preparation. To each tube add 1.0 ml of thawed plasma substrate R₁ and 1.0 ml of the appropriate dilution of the preparation to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order S₁, S₂, S₃, T₁, T₂, T₃, transfer each tube to a water-bath at 37 °C, allow to equilibrate at 37 °C for about 15 min and add to each tube 1 ml of a suitable APTT (Activated Partial Thromboplastin Time) reagent containing phospholipid and a contact activator, at a dilution giving a suitable blank recalcification time not exceeding 60 s. After exactly 2 min add 1 ml of a 3.7 g/l solution of calcium chloride R previously heated to 37 °C and record as the clotting time the interval in seconds between this last addition and the onset of clotting determined by the chosen technique. Determine the blank recalcification time at the beginning and at the end of the procedure in a similar manner, using 1 ml of a 9 g/l solution of sodium chloride R in place of one of the heparin dilutions; the 2 blank values obtained should not differ significantly. Transform the clotting times to logarithms, using the mean value for the duplicate tubes. Repeat the procedure using fresh dilutions and carrying out the incubation in the order T₁, T₂, T₃, S₁, S₂, S₃. Calculate the results by the usual statistical methods (5.3).

Carry out not fewer than 3 independent assays. For each such assay prepare fresh solutions of the reference preparation and the preparation to be examined and use another, freshly thawed portion of plasma substrate.

Calculate the potency of the preparation to be examined, combining the results of these assays, by the usual statistical methods (5.3). When the variance due to differences between assays is significant at P = 0.01, a combined estimate of potency may be obtained by calculating the non-weighted mean of potency estimates.

2.7.6. ASSAY OF DIPHTHERIA VACCINE (ADSORBED)

The potency of diphtheria vaccine is determined by administration of the vaccine to guinea-pigs followed either by challenge with diphtheria toxin (method A or B) or by determination of the titre of antibodies against diphtheria toxin or toxoid in the serum of guinea-pigs (method C).

In both cases, the potency of the vaccine is calculated by comparison with a reference preparation, calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of diphtheria toxoid adsorbed on aluminium