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 IXa) in the presence of calcium ions and phospholipids. 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 chromogenic assay method consists of two consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme:

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the International Standard for Human Blood Coagulation Factor VIII concentrate as the standard, that the results obtained do not differ significantly.

Commercial assay kits are to be used in accordance with the manufacturers’ instructions; it is important to ascertain the suitability for the assay of the kit used.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, 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. Factor X is present in amounts giving a final concentration during the first step of the assay of 10-350 nmol/l, preferably 15-30 nmol/l. Factor IXa is prepared by activating purified factor IX to factor IXaβ using factor Xα, and by subsequent purification of factor IXaβ from the reaction mixture. Its final concentration during factor Xa generation is less than 30 per cent of the factor X concentration, usually 1-100 nmol/l, preferably 1-10 nmol/l. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous, complete activation of factor VIII in the assay. Phospholipids may be obtained from natural sources such as bovine brain or spinal cord or soya-bean extract, or synthetically prepared, and must consist to a substantial extent, usually 15 per cent to 35 per cent, of the species phosphatidylserine. The final phospholipid concentration during factor Xa generation is 1-50 μmol/l, preferably 10-35 μmol/l. The reagent contains calcium ions to give a final concentration of 5-15 mmol/l. The final factor Xa generation is performed in a solution containing at least 1 mg/ml of human or bovine albumin which is appropriately buffered, at a pH of 7.3-8.0. The components of the complete reagent are usually divided into at least two separate reagents each lacking the ability to generate factor Xa on its own. After reconstitution, these 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 second 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 three and five amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in water and used at a final concentration of 0.2-2 mmol/l. The substrate may further contain appropriate inhibitors to stop further factor Xa generation and to suppress thrombin activity, thereby improving selectivity for factor Xa.
ASSAY PROCEDURE

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of water R; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU/ml and 2.0 IU/ml.

The prediluent consists of plasma from a patient with severe haemophilia A, or of an artificially prepared reagent that gives results that do not differ significantly from those obtained employing haemophilic plasma and the same reference and test preparations. The prediluted materials must be stable beyond the time required for the assay, for at least 30 min at 20 °C and must be used within 15 min.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of human or bovine albumin and for example, tris(hydroxy-methyl)aminomethane or imidazole, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably with each one prepared in duplicate. Prepare the dilutions such that the final factor VIII concentration is below 0.03 IU/ml, and 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 without delay.

**Step 1.** Mix prewarmed dilutions of the factor VIII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents. Allow the activation of factor X to proceed for a suitable time, preferably terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

**Step 2.** Terminate the activation by addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as acetic acid (50 per cent V/V C₂H₄O₂) or a citrate solution (1 mol/l) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore in time. Appropriate hydrolysis times usually are between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, 5.3. Statistical analysis of results of biological assays and tests).

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**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 in 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 dilution of cephalin R to which has been added an appropriate activator such as kaolin so that a suitable blank recalcification time not exceeding 60 s is obtained. When kaolin is used, prepare just before use, a mixture of equal volumes of cephalin R and a 4 g/l suspension of light kaolin R in a 9 g/l solution of sodium chloride R. After exactly 2 min add 1 ml of a 3.7 g/l solution of calcium chloride R 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