It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa generation in order to determine the time taken to reach 50 per cent of the maximal factor Xa generation.

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. 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 peptide 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.

Step 1. Mix prewarmed dilutions of the factor VIII reference preparation and of 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. Allow the activation of factor X to proceed for a suitable time, terminating the reaction (step 2) when the factor Xa concentration has reached approximately 50 per cent of the maximal (plateau) level. Appropriate activation times are usually between 2 min and 5 min.

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 a 50 per cent *V/V* solution of acetic acid, or a 1 M pH 3 citrate buffer solution. Adjust the hydrolysis time to achieve a linear development of chromophore over time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

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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: T_1, T_2 and T_3 for the dilutions of the preparation to be examined and S_1, S_2 and S_3 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 S_1, S_2, S_3, T_1, T_2, T_3, 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 T_1, T_2, T_3, S_1, S_2, S_3. 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.

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corrected 6.0

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 hydroxide. The equivalence in International Units of the International Standard is stated by the World Health Organisation (WHO).

Diphtheria vaccine (adsorbed) BRP is suitable for use as a reference preparation.

The method chosen for the assay of diphtheria vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from diphtheria toxoid of the same origin, and with comparable levels (expressed in Lf/mL) of the same diphtheria toxoid, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccines. Any combinations containing a whole-cell pertussis component or containing haemophilus type b conjugate vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier in the same vial must always be assessed separately.

For combinations containing diphtheria and tetanus components, the serological assay (method C) can be performed with the same group of animals used for the serological assay of the tetanus vaccine (adsorbed) (*2.7.8*) when the common immunisation conditions for the diphtheria and the tetanus components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Once the analyst has sufficient experience with this method for a given vaccine, it is possible to apply a simplified model such as a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required, but does not give information on linearity, parallelism and the dose-response curve. The simplified model allows for a considerable reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- the mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation;
- the antitoxin titres or scores of run controls (positive and negative serum samples);
- the ratio of antitoxin titres or scores for the positive serum control to the serum samples corresponding to the reference vaccine.

METHOD A: INTRADERMAL CHALLENGE TEST IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy, white guinea-pigs from the same stock and of a size suitable for the prescribed number of challenge sites, the difference in body mass between the heaviest and the lightest animal being not greater than 100 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 5 guinea-pigs as unvaccinated controls.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of diphtheria toxin containing 67 to 133 lr/100 in 1 Lf and 25 000 to 50 000 minimal reacting doses for guinea-pig skin in 1 Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 mL. Prepare from this a further series of 5 four-fold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf in 0.2 mL.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, will result in an intradermal score of approximately 3 when the animals are challenged.