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 C₂H₃O₂) 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).

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 recalculated 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...
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.

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 by combining the results of these assays by the usual statistical methods. 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 (adsorbed) is determined by comparing the dose of the vaccine required to protect guinea-pigs from the effects of either an erythrogenic dose of diphtheria toxin administered intradermally or a lethal dose of diphtheria toxin administered subcutaneously with the dose of a reference preparation, calibrated in International Units, needed to give the same protection.

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.

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

The design of the assay described below follows a parallel-line model with 3 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 using 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 leads to a considerable reduction in the number of experimental 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.

**METHOD OF INTRADERMAL CHALLENGE**

**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. 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. Use guinea-pigs of the same sex or with males and females equally distributed between the groups.

**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.

**Determination of potency of the vaccine.** 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. Allocate the dilutions 1 to each of the groups of guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, shave both flanks of each guinea-pig and inject 0.2 ml of each of the 6 toxin dilutions intradermally into 6 separate sites on each of the vaccinated guinea-pigs in such a way as to minimise interference between adjacent sites.

**Determination of the activity of the challenge toxin.** If necessary, inject the unvaccinated control animals with dilutions containing 80, 40, 20, 10 and 5 millionths of an Lf of the challenge toxin.

**Reading and interpretation of results.** Examine all injection sites 48 h after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intra-dermal challenge score. Tabulate together the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as (score)\(^2\) or arcsin((score/6)\(^2\)), to obtain an estimate of the relative potency for each of the test preparations by parallel-line quantitative analysis.

**Requirements for a valid assay.** The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the mean score obtained at the lowest dose level is less than 3 and the mean score at the highest dose level is more than 3,
- if applicable, the toxin dilution that contains 40 millionths of an Lf gives a positive erythema in at least 80 per cent of the control guinea-pigs and the dilution containing 20 millionths of an Lf gives a positive erythema in less than 80 per cent of the guinea-pigs (if these criteria are not met a different toxin has to be selected),
- the confidence limits (\( P = 0.95 \)) are not less than 50 per cent and not more than 200 per cent of the estimated potency,
- the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.