METHOD OF LETHAL CHALLENGE

Selection and distribution of the test animals. Use in the test healthy guinea-pigs from the same stock, each weighing 250 g to 350 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 4 further groups of 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 not less than 100 LD₅₀ per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the lethal dose 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 approximately 100 LD₅₀ per millilitre. If necessary, dilute portions of the challenge toxin solution 1 in 32, 1 in 100 and 1 in 320 with the same diluent.

Determination of potency of the vaccine. Using a 9 g/l solution of sodium chloride R, prepare dilutions of the vaccine 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, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of diphtheria toxin prescribed for this test. 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, inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (100 LD₅₀).

Determination of the activity of the challenge toxin. If necessary, allocate the challenge toxin solution and the 3 dilutions made from it, 1 to each of the 4 groups of 5 guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that solution is allocated.

Reading and interpretation of results. Count the number of surviving guinea-pigs 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea-pigs, using the usual statistical methods.

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

- 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.

2.7.7. ASSAY OF PERTUSSIS VACCINE

The potency of pertussis vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of *Bordetella pertussis*, administered intracerebrally, with the quantity 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 dried pertussis vaccine. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Selection and distribution of the test animals. Use in the test, healthy mice less than 5 weeks old of a suitable strain from the same stock, the difference in mass between the heaviest and the lightest being not greater than 5 g. Distribute the mice in 6 groups of not fewer than 16 and 4 groups of 10. The mice must all be of the same sex or the males and females should be distributed equally between the groups.

Selection of the challenge strain and preparation of the challenge suspension. Select a suitable strain of *B. pertussis* capable of causing the death of mice within 14 days of intracerebral injection. If more than 20 per cent of the mice die within 48 h of the injection the strain is not suitable. Make one subculture from the strain and suspend the harvested *B. pertussis* in a solution containing 10 g/l of casein hydrolysate R and 6 g/l of sodium chloride R and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension. Prepare a series of dilutions in the same solution and allocate each dilution to a group of ten mice. Inject intracerebrally into each mouse a dose (0.02 ml or 0.03 ml) of the dilution allocated to its group. After 14 days, count the number of mice surviving in each group. From the results, calculate the expected opacity of a suspension containing 100 LD₅₀ in each challenge dose. For the test of the vaccine to be examined make a fresh subculture from the same strain of *B. pertussis* and prepare a suspension of the harvested organisms with an opacity corresponding to about 100 LD₅₀ in each challenge dose. Prepare 3 dilutions of the challenge suspension.

Determination of potency. Prepare 3 serial dilutions of the vaccine to be examined and 3 similar dilutions of the reference preparation such that in each the intermediate dilution may be expected to protect about 50 per cent of the mice from the lethal effects of the challenge dose of *B. pertussis*. Suggested doses are 1/8, 1/40 and 1/200 of the human dose of the vaccine to be examined and 0.5 IU, 0.1 IU and 0.02 IU of the reference preparation, each dose being contained in a volume not exceeding 0.5 ml. Allocate 6 dilutions one to each of the groups of not fewer than 16 mice and inject intraperitoneally into each mouse one dose of the dilution allocated to its group. After 14 to 17 days inject intracerebrally into each animal in the groups of not fewer than 16, one dose of the challenge suspension. Allocate the challenge suspension and the 3 dilutions made from it one to each of the groups of 10 mice and inject intracerebrally one dose of each suspension into each mouse in the group to which that suspension is allocated.
The potency of tetanus vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the paralysis method is not obligatory the LD$_{50}$ method may be used. For the LD$_{50}$ method, the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than paralysis.

The International Unit is the activity contained in a stated amount of the International Standard for tetanus toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Tetanus vaccine (adsorbed) BRP is calibrated in International Units with reference to the International Standard.

The method chosen for assay of tetanus 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 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 tetanus toxoid of the same origin, 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 vaccine. For combinations with a whole-cell pertussis component, a separate demonstration of equivalence must be made for the highest combination.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multidilution assays, it may be possible to decrease the number of animals needed to obtain a statistically significant result by applying 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 the dose-response curves and their linearity, parallelism and significant slope. The simplified model may lead to a considerable reduction in the number of animals required and its use must be considered 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:

- 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,
- antitoxin titres or scores of run controls (positive and negative serum samples),
- ratio of antitoxin titres or scores for the positive serum control and the serum samples corresponding to the reference vaccine.

METHOD A. CHALLENGE TEST IN GUINEA-PIGS

**SELECTION AND DISTRIBUTION OF THE TEST ANIMALS**

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 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 activity of the challenge toxin has to be determined, include 3 further groups of 5 guinea-pigs as unvaccinated controls.

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

**SELECTION OF THE CHALLENGE TOXIN**

Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

**PREPARATION OF THE CHALLENGE TOXIN SOLUTION**

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

**DILUTION OF THE TEST AND REFERENCE PREPARATIONS**

Using a 9 g/1 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, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.