Selection and distribution of the test animals. Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number suitable for the requirements of the assay.

Determination of potency of the vaccine to be examined. Using a 9 g/L solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine, prepare at least 3 dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 mL of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28-32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (*2.7.1*).

Calculations. Carry out the calculations by the usual statistical methods for an assay with a quantal response (*5.3*).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions. The test is not valid unless:

- for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement. The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

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2.7.18. ASSAY OF HUMAN COAGULATION FACTOR II

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor II activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human blood coagulation factor II. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

REAGENTS

Viper venom specific factor II activator (ecarin). A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor IIa chromogenic substrate. Specific chromogenic substrate for factor IIa such as: *H*-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, *H*-D-cyclohexylglycyl-α-aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 6.06 g/L of *tris(hydroxymethyl)aminomethane R*, 17.53 g/L of *sodium chloride R* and 1 g/L of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary, using *hydrochloric acid R*.

METHOD

Test solution. Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Reference solution. Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 25 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µL of dilution buffer, then 25 µL of ecarin and incubate for exactly 2 min. To each well add 25 µL of factor IIa chromogenic substrate.

Read the rate of change of absorbance (*2.2.25*) at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance (ΔA/min). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate ΔA/min as the slope of the line. From the ΔA/min values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (*5.3*).