solution or the reference solution and make up the volume in each well to 200 µl using dilution buffer (0.02-0.08 IU of heparin per millilitre in the final reaction mixture).

**End-point method.** Transfers 40 µl from each well to a second series of wells, add 20 µl of bovine factor Xa solution R and incubate at 37 °C for 30 s. Add 40 µl of a 1 mmol/l solution of factor Xa chromogenic substrate and incubate at 37 °C for 3 min. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20 per cent V/V solution of glacial acetic acid R and measure the absorbance at 405 nm (2.2.25). Appropriate reaction times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

**Kinetic method.** Transfers 40 µl from each well to a second series of wells, add 20 µl of bovine factor Xa solution R and incubate at 37 °C for 30 s. Add 40 µl of a 2 mmol/l solution of factor Xa chromogenic substrate, incubate at 37 °C and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (2.2.25), thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation by the usual statistical methods for a slope-ratio assay (for example, 5.3. Statistical analysis of results of biological assays and tests).

**2.7.13. ASSAY OF HUMAN ANTI-D IMMUNOGLOBULIN**

**METHOD A**

The potency of human anti-D immunoglobulin is determined by comparing the quantity necessary to produce agglutination of D-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity contained in a stated amount of the International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

**Human anti-D immunoglobulin BRP** is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Use pooled D-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not fewer than 4 group O Rf, donors. To a suitable volume of the cells, previously washed 3 times with a 5 g/l solution of sodium chloride R, allow to stand at 37 °C for 10 min, centrifuge, remove the supernatant liquid and wash 3 times with a 9 g/l solution of sodium chloride R. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 300 g/l solution of bovine albumin R and 45 volumes of a 9 g/l solution of sodium chloride R. Stand the resulting suspension in iced water, stirring continuously. Using a calibrated automated dilutor, prepare suitable dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 5 g/l of bovine albumin R and 9 g/l of sodium chloride R. Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils, at 15.0 °C. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 ml/min and a 3 g/l solution of methylcellulose 450 R at a rate of 0.05 ml/min. Introduce the dilutions of the preparation to be examined and the reference preparation at a rate of 0.1 ml/min for 2 min, followed by the diluent solution at a rate of 0.1 ml/min for 4 min before the next dilution is introduced.

Introduce air at a rate of 0.6 ml/min. Incubate at 37 °C for 18 min and then disperse the rouleaux by introducing at a rate of 1.6 ml/min a 9 g/l solution of sodium chloride R containing a suitable wetting agent (for example, polysorbate 20 R at a final concentration of 0.2 g/l) to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 ml/min and then at 0.6 ml/min. Lyse the unagglutinated red blood cells with a solution containing 5 g/l of octoxinol 10 R, 0.2 g/l of potassium ferricyanide R, 1 g/l of sodium hydrogen carbonate R and 0.05 g/l of potassium cyanide R at a rate of 2.5 ml/min. A ten-minute delay coil is introduced to allow for conversion of the haemoglobin. Continuously record the absorbance (2.2.25) of the haemolysate at a wavelength between 540 nm and 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between concentration and the resultant change in absorbance (△A). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation to be examined.

Calculate the potency of the preparation to be examined using the usual statistical methods (5.3).
solution of sodium edetate \( R \) and dilute to 10 ml with 0.067 M phosphate buffer solution \( pH \) 5.4 \( R \). Freeze in aliquots at \(-20 \) °C or below.

**Red blood cells.** Use pooled D-positive red blood cells obtained from not fewer than 3 group O \( R \), \( R \) donors. Wash the cells 4 times with PBS. Centrifuge the cells at 1800 \( g \) for 5 min, mix a suitable volume of prewarmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume has been found suitable) and incubate at 37 °C for 10 min. Wash the cells 4 times with PBS. Store at 4 °C in an appropriate stabiliser for up to 1 week.

**Biotinylated Brad-5.** Use according to instructions.

**Alkaline phosphatase-conjugated avidin/streptavidin reagent.** Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

**Substrate solution.** Use para-nitrophenyl phosphate according to instructions.

**Cell fixation buffer.** Dissolve 18.02 g of glucose \( R \), 4.09 g of sodium chloride \( R \), 1.24 g of boric acid \( R \), 10.29 g of sodium citrate \( R \) and 0.74 g of sodium edetate \( R \) in water \( R \). Adjust to \( pH \) 7.2-7.3 (2.2.3) using 1 M sodium hydroxide or 1 M hydrochloric acid, and dilute to 1000 ml with water \( R \). Use directly from storage at 4 °C.

**Glutaraldehyde.** Immediately before use, add 90 \( \mu l \) of a 250 g/l solution of glutaraldehyde \( R \) to 24 ml of cold PBS.

**Microtitre plates.** Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U or V-bottomed polystyrene or poly(vinyl chloride) plates.

**METHOD**

Prepare a 0.1 per cent ( \( V/V \) ) suspension of papain-treated red blood cells in cold cell fixation buffer. Pipette 50 \( \mu l \) into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 \( g \) for 3 min, preferably at 4 °C. Without removing the supernatant, gently add 100 \( \mu l \) of glutaraldehyde solution to each well and leave for 10 min.

Drain the wells by quickly inverting the plate and wash 3 times with 250-300 \( \mu l \) of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4 °C after draining off the PBS and adding 100 \( \mu l \) of cell fixation buffer per well and sealing with plastic film. Plates can be stored at 4 °C for up to 1 month.

**Test solutions.** For freeze-dried preparations, reconstitute as stated on the label. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU/ml in PBS containing 10 g/l of bovine albumin \( R \). If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

**Reference solutions.** Reconstitute the reference preparation according to instructions. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU/ml in PBS containing 10 g/l of bovine albumin \( R \).

Using U or V-bottomed microtitre plates, add 35 \( \mu l \) of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 \( \mu l \) of biotinylated Brad-5 at 250 ng/ml.

Empty the wells of the red cell-covered plate by inverting and draining on a paper towel. Add 250 \( \mu l \) of PBS containing 20 g/l of bovine albumin \( R \) and leave at room temperature for 30 min.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 \( \mu l \) from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 \( \mu l \) of PBS containing 10 g/l of bovine albumin \( R \) as negative control. Seal the plate with plastic film and incubate at room temperature for 1 h.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 \( \mu l \) of TBS.

Dilute the alkaline phosphatase-conjugated avidin/streptavidin reagent in TBS containing 10 g/l of bovine albumin \( R \) and add 50 \( \mu l \) to each well. Incubate for 30 min at room temperature.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 \( \mu l \) of TBS.

Add 100 \( \mu l \) of substrate solution to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 50 \( \mu l \) of 3 M sodium hydroxide to each of the wells.

Measure the absorbances at 405 nm. and subtract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation to be examined by the usual statistical methods (5.3).

**METHOD C**

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units. The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference preparation is stated by the World Health Organisation. Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

**MATERIALS**

Reagents not specified are of analytical grade.

PBS. Dissolve 8.0 g of sodium chloride \( R \), 0.76 g of disodium hydrogen phosphate \( R \), 0.2 g of potassium chloride \( R \) and 0.2 g of potassium dihydrogen phosphate \( R \) in water \( R \) and dilute to 1000 ml with the same solvent.

PBS-BSA solution. PBS containing 10.0 g/l of bovine albumin \( R \).

**Red blood cells.** Use D-positive red blood cells obtained from a group O \( R \), \( R \) donor within 2 weeks of collection. Store if necessary in an appropriate stabiliser at 4 °C. Wash the cells at least twice with PBS-BSA solution and prepare a suspension containing 1 \( \times 10^8 \) cells per microlitre but not more than 5 \( \times 10^6 \) cells per microlitre in PBS-BSA solution. Use D-negative red blood cells obtained from a group O \( R \) donor and prepared similarly.

**Secondary antibody.** Use a suitable fluorescent dye conjugated anti-IgG antibody-fragment specific for human IgG or parts of it. Store and use according to the manufacturer’s instructions.

**Microtitre plates.** Use flat-bottomed plates without surface treatment for enzyme immunoassays.

**METHOD**

**Test solutions.** For freeze-dried preparations, reconstitute as stated on the label. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting...
with a concentration in the range of 1.20–1.15 IU/ml using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting with a concentration in the range of 1.20–1.15 IU/ml using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µl of the D-positive red blood cells into each well of a microtitre plate. Add 50 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µl of PBS/BSA solution as negative control. Distribute 50 µl of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µl of the lowest dilution of the test preparation. To monitor spurious reactions distribute 50 µl of the D-positive red blood cells into 4 wells of the same microtitre plate and add 50 µl of PBS/BSA solution. Seal with plastic film and incubate at 37 °C for 40 min.

Centrifuge the plates at 50 g for 3 min, discard the supernatant and wash the cells with 200–250 µl of PBS/BSA solution. Repeat this at least once.

Centrifuge the plates at 50 g for 3 min, discard the supernatant and add 50 µl of the secondary antibody diluted with PBS/BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 min.

Centrifuge the plates at 50 g for 3 min, discard the supernatant and wash the cells with 200–250 µl of PBS/BSA solution. Repeat this at least once.

Centrifuge the plates at 50 g for 3 min, resuspend the cells into 200–250 µl of PBS. Transfer the cell suspension into a tube suitable for the flow cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate. Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose response curve to estimate the potency of the preparation to be examined by the usual statistical methods, 5.3.

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 three 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 to 32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assist the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (2.7.7).

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 ED50 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.

IN VITRO ASSAY

Carry out an immunochemical determination (2.7.7) of antigen content with acceptance criteria validated against the in vivo test. The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

Hepatitis A vaccine (inactivated, adsorbed) type A BRP, hepatitis A vaccine (inactivated, adsorbed) type B BRP and hepatitis A vaccine (inactivated, adsorbed) type C BRP are suitable for the in vitro assay of certain vaccines as described in the accompanying leaflet.

2.7.14. ASSAY OF HEPATITIS A VACCINE

The assay of hepatitis A vaccine is carried out either in vitro, by comparing in given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or in vivo, by an immunochemical determination of antigen content.

IN VIVO ASSAY

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

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.

2.7.15. ASSAY OF HEPATITIS B VACCINE (rDNA)

The assay of hepatitis B vaccine (rDNA) is carried out either in vitro, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or in vitro, by an immunochemical determination of the antigen content.