These batches are assayed using as reference standard a homologous production batch:

– by the currently approved *in vivo* assay for the vaccine;
– by the rat assay where this is not the currently approved *in vivo* assay;
– by the D-antigen assay.

Waiving of the *in vivo* assay is acceptable if the representative final bulk/lot complies with the *in vivo* and *in vitro* assays and the sub-potent batches fail to comply. If a sub-potent batch fails to comply with the D-antigen assay but complies with the *in vivo* assay, the latter may be repeated.

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2.7.21. ASSAY OF HUMAN VON WILLEBRAND FACTOR

The biological functions of human von Willebrand factor are numerous. At present, its ristocetin cofactor activity and its collagen binding activity can be utilised for assays. The potency of human von Willebrand factor is determined by comparing, in given conditions, its activity with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

The International Unit is the activity of a stated amount of the International Standard, which consists of a freeze-dried human von Willebrand factor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation (WHO).

RISTOCETIN COFACTOR ASSAY

The ristocetin cofactor activity of von Willebrand factor is determined by measuring agglutination of a suspension of platelets in the presence of ristocetin A. The assay can be carried out for quantitative determinations by using automated instruments, or for semi-quantitative determinations by visually assessing the endpoint of agglutination in a dilution series. Quantitative assays are preferred.

REAGENTS

Suspension of platelets. Use standardised and, for example, formaldehyde- or paraformaldehyde-fixed preparations of freshly isolated and washed human platelets. The suspension may also be freeze-dried. An appropriate amount of ristocetin A is added if necessary. Some platelet reagents may already contain ristocetin A.

Reference preparation. The reference preparation for von Willebrand factor is the WHO International Standard for von Willebrand factor concentrate.

METHOD

Semi-quantitative assay. Prepare suitable dilutions of the preparation to be examined and of the reference preparation, using as diluent a solution containing 9 g/l of *sodium chloride R* and 10-50 g/l of *human albumin R*. Add to each dilution an appropriate amount of the suspension of platelets and, if necessary, of ristocetin A. Mix on a glass slide by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

Quantitative Assay. Reconstitute the entire contents of 1 ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of the recommended diluent (for example *water R*); use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5-2.0 IU/ml. The prediluent consists of an isotonic non-chelating buffer containing, for example, 1-5 per cent of human or bovine albumin, and tris(hydroxymethyl)aminomethane or imidazole, appropriately buffered.

The test is performed in accordance with the manufacturer's instructions with at least 2 dilution series with as many dilutions as are needed to obtain a total of at least 3 different concentrations in the linear range of the assay.

Check the validity of the assay and calculate the potency of the test preparation using the usual statistical methods (for example, *5.3*).

COLLAGEN-BINDING ASSAY

Collagen-binding is determined by an enzyme-linked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

REAGENTS

Collagen. Use native equine or human fibrils of collagen type I or III. For ease of handling, collagen solutions may be used.

Collagen diluent. Dissolve 50 g of *glucose R* in *water R*, adjust to pH 2.7-2.9 with *1 M hydrochloric acid* and dilute to 1000 ml with *water R*.

Phosphate-buffered saline (PBS). Dissolve 8.0 g of *sodium chloride R*, 1.05 g of *disodium hydrogen phosphate dihydrate R*, 0.2 g of *sodium dihydrogen phosphate R* and 0.2 g of *potassium chloride R* in *water R*. Adjust to pH 7.2 using *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 1000 ml with *water R*.

Washing buffer. PBS containing 1 g/l of *polysorbate 20 R*.

Blocking reagent. PBS containing 1 g/l of *polysorbate 20 R* and 10 g/l of *bovine serum albumin R*.

Dilution buffer. PBS containing 1 g/l of *polysorbate 20 R* and 50 g/l of *bovine serum albumin R*.

Conjugate. Rabbit anti-human von Willebrand factor serum horseradish peroxidase conjugate. Use according to the manufacturer's instructions.

Substrate solution. Immediately before use, dissolve a tablet of *o*-phenylenediamine dihydrochloride and a tablet of urea hydrogen peroxide in 20 ml of *water R* or use a suitable volume of hydrogen peroxide. Protect from light.

Microtitre plates. Flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity.

METHOD

Test solutions. Reconstitute the preparation to be examined as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Reference solutions. Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen diluent to obtain a solution containing 30-75 µg/ml of collagen, mix gently to produce a uniform

suspension of collagen fibrils. Pipette 100 µl into each well of the microtitre plate. Cover the plate with plastic film and incubate at 37 °C overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times. Add 250 µl of blocking reagent to each well, cover the plate with plastic film and incubate at 37 °C for 1 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µl each of the test solutions or reference solutions to the wells. Add 100 µl of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Prepare a suitable dilution of the conjugate (for example, a dilution factor of 1 to 4000) with PBS containing 5 g/l of *bovine serum albumin R* and add 100 µl to each well. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µl of substrate solution to each of the wells and incubate at room temperature for 20 min in the dark. Add 100 µl of *1 M hydrochloric acid* to each of the wells.

Measure the absorbance at 492 nm. Use the absorbance values to estimate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

The assay is invalid if the absorbances measured for the negative controls are greater than 0.05.

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2.7.22. ASSAY OF HUMAN COAGULATION FACTOR XI

The principle of the assay is to measure the ability of a factor XI preparation to reduce the prolonged coagulation time of factor XI-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference preparation consisting of human normal plasma.

1 unit of factor XI is equal to the activity of 1 ml of human normal plasma. Human normal plasma is prepared by pooling plasma units from not fewer than 30 donors and stored at – 30 °C or lower.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor XI-deficient plasma (for example *plasma substrate R3*) to produce solutions containing 0.5-2.0 units/ml. Prepare at least 3 appropriate dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH 7.3 R*) containing 10 g/l of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or perform the assay with incubation tubes maintained in a water bath at 37 °C. Place in each tube 0.1 ml of factor XI-deficient plasma (for example *plasma substrate R3*) and 0.1 ml of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 ml of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37 °C. To each tube, add 0.1 ml of a 3.7 g/l solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency using the usual statistical methods (for example, *5.3*).

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2.7.23. NUMERATION OF CD34/CD45+ CELLS IN HAEMATOPOIETIC PRODUCTS

This chapter describes immunolabelling and analysis by flow cytometry (*2.7.24*) to determine the number of CD34/CD45+ cells contained in haematopoietic products. The determination is carried out by a single platform method using calibrated fluorospheres, after lysis of the sample red blood cells if necessary.

This method applies to all types of preparations and whole blood. However, its level of precision makes it particularly suitable for preparations containing very low percentages of CD34/CD45+ cells.

Graft quality assessment by CD34/CD45+ cell enumeration

A variety of studies have established that the 1-3 per cent of cells in the bone marrow that express the CD34 cell surface antigen are capable of reconstituting long-term, multilineage haematopoiesis after myeloablative therapy. CD34/CD45+ cells are also found in the peripheral circulation of normal individuals but are extremely rare (0.01-0.1 per cent). However, CD34/CD45+ cells may also be mobilised from marrow to the peripheral circulation in greater numbers by haematopoietic cytokines such as granulocyte colony-stimulating factor and/or chemotherapy.

The technique used for enumeration of CD34/CD45+ cells must meet the following requirements:

- high sensitivity, since haematopoietic stem cells are rare events;
- accuracy, to provide clinically relevant results;
- reproducibility, to provide clinically reliable results;
- speed, to provide real-time analysis.

Selection of parameters

The flow cytometry assay uses commercially available, directly conjugated fluorochrome-labelled monoclonal antibodies, routine staining and whole blood lysing procedures, and a gating strategy using light scatter and immunofluorescence analysis using a pan-CD45/CD34 monoclonal antibody combination.

It is possible to determine CD34/CD45+ cell viability by appropriate nucleic acid staining with a stain that does not cross the intact cell membrane (for example, with 7-aminoactinomycin D).