bromide R has been added. Centrifuge the mixture at 3600 *g* for 5 min. Separate the plasma and centrifuge again at 6000 *g* for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing *agarose-DEAE for ion exchange chromatography R* which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 ml/cm^2/h. Collect the eluate in fractions and record the absorbance at 280 nm (*2.2.25*). Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 7 g/l of *sodium chloride R* and filter using a membrane filter (porosity 0.45 µm). Freeze the filtrate in portions and store at – 25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

METHOD

The assay may be carried out using an automated enzyme analyser or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate R* or *D-prolyl-L-phenylalanyl-L-arginine-4-nitroanilide-dihydrochloride R*), dissolved in buffer B. Record the rate of change in absorbance per minute for 2-10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Depending on the method used, ΔA/min has to be corrected by subtracting the value obtained for the corresponding blank without the prekallikrein substrate. The results may be calculated using a standard curve, a parallel-line or a slope ratio assay or any other suitable statistical method. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined.

01/2008:20616

2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

In those tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (*5.2.2*).

VIRUS SEED LOT

Take samples of the virus seed lot at the time of harvesting and, if they are not tested immediately, keep them at a temperature below – 40 °C.

Adult mice. Inoculate each of at least ten adult mice, each weighing 15 g to 20 g, intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the virus seed lot. Observe the mice for at least 21 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice which are observed for 21 days. The virus seed lot complies with the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Suckling mice. Inoculate each of at least twenty mice, less than 24 h old, intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the virus seed lot. Observe the mice daily for at least 14 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice which are observed daily for 14 days. The virus seed lot passes the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Guinea-pigs. Inoculate intraperitoneally into each of at least five guinea pigs, each weighing 350 g to 450 g, 5.0 ml of the virus seed lot. Observe the animals for at least 42 days for signs of disease. Carry out an autopsy of all guinea-pigs that die after the first 24 h of the test, or that show signs of illness and examine macroscopically; examine the tissues both microscopically and culturally for evidence of infection. Euthanise animals that survive the observation period and examine in a similar manner. The virus seed lot passes the test if no guinea-pig shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the guinea-pigs survive the observation period.

VIRUS SEED LOT AND VIRUS HARVESTS

Take samples at the time of harvesting and, if not tested immediately, keep them at a temperature below – 40 °C.

Bacterial and fungal sterility. A 10 ml sample complies with the test for sterility (*2.6.1*).

Mycoplasmas. A 10 ml sample complies with the test for mycoplasmas (*2.6.7*).

Mycobacteria (*2.6.2*). A 5 ml sample is tested for the presence of *Mycobacterium spp.* by culture methods known to be sensitive for the detection of these organisms.

Test in cell culture for other extraneous agents. Neutralised samples equivalent, unless otherwise prescribed, to 500 human doses of vaccine or 50 ml, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures. If the virus is grown in human diploid cells, the neutralised virus harvest is also tested on a separate

culture of the diploid cells. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. The virus seed lot or harvest passes the tests if none of the cell cultures shows evidence of the presence of any extraneous agents not attributable to accidental contamination. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Avian viruses (only required for virus propagated in avian tissues). Neutralise a sample equivalent to 100 human doses or 10 ml, whichever is the greater. Using 0.5 ml per egg, inoculate a group of fertilised SPF eggs, 9 to 11 days old, by the allantoic route and a second group, 5 to 7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes, examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

PRODUCTION CELL CULTURE: CONTROL CELLS

Examine the control cells microscopically for freedom from any virus causing cytopathic degeneration throughout the time of incubation of the inoculated production cell cultures or for not less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the tests described below.

Test for haemadsorbing viruses. Examine not fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20 °C to 25 °C for 30 min. No evidence of haemadsorbing agents is found.

Tests in cell cultures for other extraneous agents. Pool the supernatant fluids from the control cells and examine for the presence of extraneous agents by inoculation of simian kidney and human cell cultures. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, but from a separate batch, are also inoculated. In each cell system, at least 5 ml is tested. Incubate the inoculated cultures at a temperature of 36 ± 1 °C and observe for a period of 14 days. No evidence of extraneous agents is found.

If the production cell culture is maintained at a temperature different from 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells as used for growth of the virus.

Avian leucosis viruses (required only if the virus is propagated in avian tissues). Carry out a test for avian leucosis viruses using 5 ml of the supernatant fluid from the control cells.

CONTROL EGGS

Haemagglutinating agents. Examine 0.25 ml of the allantoic fluid from each egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 ml sample of the pooled amniotic fluids from the control eggs in 0.5 ml volumes into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

Avian leucosis viruses. Use a 10 ml sample of the pooled amniotic fluids from the control eggs. Carry out amplification by five passages in leucosis-free chick-embryo cell cultures; carry out a test for avian leucosis using cells from the fifth passage. The control eggs comply with the test if no evidence of the presence of avian leucosis viruses is found.

Other extraneous agents. Inoculate 5 ml samples of the pooled amniotic fluids from the control eggs into human and simian cell cultures. Observe the cell cultures for 14 days. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

01/2008:20617

2.6.17. TEST FOR ANTICOMPLEMENTARY ACTIVITY OF IMMUNOGLOBULIN

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH_{50}) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The haemolytic unit of complement activity (CH_{50}) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitised red blood cells.

Magnesium and calcium stock solution. Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 ml with the same solvent.

Barbital buffer stock solution. Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 ml of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid*. Add 12.5 ml of magnesium and calcium stock solution and dilute to 5000 ml with *water R*. Filter through a membrane filter (pore size 0.22 µm). Store at 4 °C in glass containers.

Gelatin solution. Dissolve 12.5 g of *gelatin R* in about 800 ml of *water R* and heat to boiling in a water-bath. Cool to 20 °C and dilute to 10 litres with *water R*. Filter through a membrane filter (pore size: 0.22 µm). Store at 4 °C. Use clear solutions only.

Citrate solution. Dissolve 8.0 g of *sodium citrate R*, 4.2 g of *sodium chloride R* and 20.5 g of *glucose R* in 750 ml of *water R*. Adjust to pH 6.1 using a 100 g/l solution of *citric acid R* and dilute to 1000 ml with *water R*.

Gelatin barbital buffer solution. Add 4 volumes of gelatin solution to 1 volume of barbital buffer stock solution and mix. Adjust to pH 7.3, if necessary, using *1 M sodium hydroxide* or *1 M hydrochloric acid*. Maintain at 4 °C. Prepare fresh solutions daily.

Stabilised sheep blood. Collect one volume of sheep blood into one volume of citrate solution and mix. Store at 4 °C for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

Haemolysin. Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)