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*, 2.79 g/L of *(ethylenedinitrilo)tetra-acetic acid 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 (Δ*A*/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 Δ*A*/min as the slope of the line. From the Δ*A*/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*).

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2.7.19. ASSAY OF HUMAN COAGULATION FACTOR X

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa 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 X activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human coagulation factor X. 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 X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

REAGENTS

Russell's viper venom specific factor X activator (RVV). A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor Xa chromogenic substrate. Specific chromogenic substrate for factor Xa such as: *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulfonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 3.7 g/L of *tris(hydroxymethyl)aminomethane R*, 18.0 g/L of *sodium chloride R*, 2.1 g/L of *imidazole R*, 0.02 g/L of *hexadimethrine bromide 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 0.18 IU of factor X 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 0.18 IU of factor X 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 12.5 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 µL of RVV and incubate for exactly 90 s. To each well add 150 µL of factor Xa chromogenic substrate, diluted 1 in 6 in dilution buffer.

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 (Δ*A*/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 Δ*A*/min as the slope of the line. From the Δ*A*/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*).

01/2008:20720

2.7.20. *IN VIVO* ASSAY OF POLIOMYELITIS VACCINE (INACTIVATED)

The capacity of the vaccine to induce the formation of neutralising antibodies is determined *in vivo* by one of the following methods.