using albumin barbital buffer solution, thereby obtaining the final adjusted volume $V_f = V_r \times A$ of sensitised human red blood cells and adjusting A to 1.0 ± 0.1 for a tenfold dilution.

Antibody binding of antigen-coated tanned human red blood cells. Prepare the following solutions in succession and in duplicate, using for each solution a separate half-micro cuvette (for example, disposable type) or test-tube.

(1) *Test solutions*. If necessary, adjust the immunoglobulin to be examined to pH 7.

Where method A is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 30 mg and 40 mg of immunoglobulin and adjust the volume to 900 µL with albumin barbital buffer.

Where method B is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 15 mg and 30 mg of immunoglobulin and adjust the volume to 1200 µL with albumin barbital buffer.

(2) *Reference solutions*. Prepare as for the test solutions using *human immunoglobulin (Fc function and molecular size) BRP*.

(3) *Complement control*. Albumin barbital buffer solution.

Where method A is performed, add to each cuvette/test-tube 100 µL of sensitised human red blood cells and mix well. Allow to stand for 15 min, add 1000 µL of albumin barbital buffer solution, collect the cells by centrifugation (1000 *g* for 10 min) of the cuvette/test-tube and remove 1900 µL of the supernatant. Replace the 1900 µL with albumin barbital buffer solution and repeat the whole of the washing procedure, finally leaving a volume of 200 µL. Test samples may be stored in sealed cuvettes/test-tubes at 4 °C for not longer than 24 h.

Where method B is performed, add to each test-tube 300 µL of sensitised human red blood cells and mix well (the final immunoglobulin concentration is in the range of 10-20 mg/mL). Allow to stand for 15 min, add 1500 µL of albumin barbital buffer solution and stir gently until homogeneous. Collect the cells by centrifugation (1000 *g* for 10 min) of the test-tube, remove the supernatant and add approximately 3 mL of albumin barbital buffer solution. Repeat this operation up to 4 times in total, leaving a final volume of 300 µL. Test samples may be stored in sealed test-tubes at 4 °C for not longer than 24 h.

Complement-initiated haemolysis.

To measure haemolysis where method A is performed, add 600 µL of albumin barbital buffer solution warmed to 37 °C to the test sample, resuspend the cells carefully by repeated pipetting (not fewer than 5 times) and place the cuvette in the thermostatted cuvette holder of a spectrophotometer. After 2 min, add 200 µL of diluted guinea-pig complement (125-200 CH_{50}/mL), mix thoroughly by pipetting twice and start immediately after the second pipetting the time-dependent recording of absorbance at 541 nm, using albumin barbital buffer solution as the compensation liquid. Stop the measurement if absorbance as a function of time has clearly passed the inflexion point.

To measure haemolysis where method B is performed, add 900 µL of albumin barbital buffer solution warmed to 37 °C to each test-tube and resuspend the cells carefully by repeated pipetting (not fewer than 5 times). The microtitre plate must be prewarmed to 37 °C before starting the test. Transfer 240 µL of each solution into 4 microtitre plate wells then incubate the microplate at 37 °C for 6 min, stirring gently every 10 s. To each microtitre plate well add 60 µL of diluted guinea-pig complement (150 CH_{50}/mL). Mix for 10 s and immediately start recording the absorbance at 541 nm at 37 °C, measuring every 20 s. Stop the measurement if the absorbance as a function of time has clearly passed the inflexion point.

Evaluation. For each cuvette/test-tube/well, determine the slope (S) of the haemolysis curve at the approximate inflexion point by segmenting the steepest section in suitable time intervals (for example, Δt = 1 min), and calculate S between adjacent intersection points, expressed as ΔA per minute. The largest value for S serves as S_{exp}. In addition, determine the absorbance at the start of measurement (A_s) by extrapolating the curve, which is almost linear and parallel to the time axis within the first few minutes. Correct S_{exp} using the expression:

$$S' = \frac{S_{exp}}{A_s}$$

Calculate the arithmetic mean of the values of S' for each preparation (test and reference solution). Calculate the index of Fc function (I_{Fc}) from the expression:

$$I_{Fc} = \frac{100 \times \left(\overline{S'} - \overline{S'_c}\right)}{\overline{S'_s} - \overline{S'_c}}$$

$\overline{S'}$ = arithmetic mean of the corrected slope for the preparation to be examined;

$\overline{S'_s}$ = arithmetic mean of the corrected slope for the reference preparation;

$\overline{S'_c}$ = arithmetic mean of the corrected slope for the complement control.

Calculate the index of Fc function for the preparation to be examined: the value is not less than that stated in the leaflet accompanying the reference preparation.

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2.7.10. ASSAY OF HUMAN COAGULATION FACTOR VII

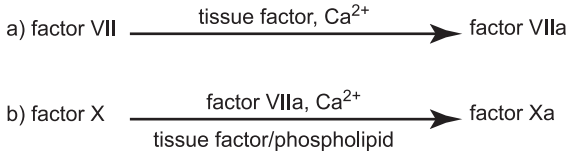
Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VII activity of a stated amount of the International Standard, which consists of freeze-dried plasma. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Human coagulation factor VII concentrate BRP is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of 2 consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ions, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarised in the following scheme.

Step 1



Step 2

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipid as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10-350 nmol/L, preferably 14-70 nmol/L. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipid component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of Ca^{2+} is 15-25 mmol/L. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3-8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water R* and used at a final concentration of 0.2-2 mmol/L. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

ASSAY PROCEDURE

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water R*; use within 1 h. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU and 2.0 IU of factor VII per millilitre.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of bovine or human albumin, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicate. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU/mL.

Prepare a control solution that includes all components except factor VII.

Prepare all dilutions in plastic tubes and use within 1 h.

Step 1. Mix dilutions of the factor VII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

Step 2. Terminate the activation by the addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by the addition of a suitable reagent, such as acetic acid (500 g/L $C_2H_4O_2$) or a citrate solution (1 mol/L) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

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

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2.7.11. ASSAY OF HUMAN COAGULATION FACTOR IX

The principle of the assay is to measure the ability of a factor IX preparation to reduce the prolonged coagulation time of factor IX-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, calibrated in International Units.

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

Human coagulation factor IX concentrate BRP is calibrated in International Units by comparison with the International Standard.

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 sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor IX-deficient plasma (for example *plasma substrate R2*) to produce solutions containing 0.5-2.0 IU/mL. Prepare at least 3 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 carry out the assay with incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 mL of factor IX-deficient plasma (for example *plasma substrate R2*) 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*).