

Reference: PA/PH/Exp. 6/T (12) 39 ANP

NOTE ON THE GENERAL CHAPTER

It is proposed to replace the clotting assay in sheep plasma by more specific chromogenic assays for anti-factor IIa activity and anti-factor Xa activity. To prepare for this revision, heparin sodium BRP has been recalibrated in a collaborative study, to be used with the new methods. In addition, the impact of this recalibration has been assessed by heparin manufacturers.

XXXX:20705

2.7.5. ASSAY OF HEPARIN

The anticoagulant activity of heparin is determined *in vitro* by comparing its ability in given conditions to delay the clotting of recalcified citrated sheep plasma with the same ability of a reference preparation of heparin calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of freeze-dried heparin sodium from pork intestinal mucosa. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Heparin sodium BRP is calibrated in International Units by comparison with the International Standard by means of the assay given below.

Carry out the assay using one of the following methods for determining the onset of clotting and using tubes and other equipment appropriate to the chosen method:

- a) direct visual inspection, preferably using indirect illumination and viewing against a matt black background;
- b) spectrophotometric recording of the change in optical density at a wavelength of approximately 600 nm;
- c) visual detection of the change in fluidity on manual tilting of the tubes;
- d) mechanical recording of the change in fluidity on stirring, care being taken to cause the minimum disturbance of the solution during the earliest phase of clotting.

ASSAY PROCEDURE

The volumes in the text are given as examples and may be adapted to the apparatus used provided that the ratios between the different volumes are respected.

Dilute *heparin sodium BRP* with a 9 g/L solution of *sodium chloride R* to contain a precisely known number of International Units per millilitre and prepare a similar solution of the preparation to be examined which is expected to have the same activity. Using a 9 g/L solution of *sodium chloride R*, prepare from each solution a series of dilutions in geometric progression such that the clotting time obtained with the lowest concentration is not less than 1.5 times the blank recalcification time, and that obtained with the highest concentration is such as to give a satisfactory log dose-response curve, as determined in a preliminary test.

Place 12 tubes in a bath of iced water, labelling them in duplicate: T_4, T_2 and T_3 for the dilutions of the preparation to be examined and S_4, S_2 and S_3 for the dilutions of the reference preparation. To each tube add 1.0 mL of thawed *plasma substrate R1* and 1.0 mL of the appropriate dilution of the preparation to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order $S_4, S_2, S_3, T_4, T_2, T_3$, transfer each tube to a water-bath at 37 °C, allow to equilibrate at 37 °C for about 15 min and add to each tube 1 mL of a suitable APTT (Activated Partial Thromboplastin Time) reagent⁽¹⁾ containing phospholipid and a contact activator, at a dilution giving a suitable blank recalcification time not exceeding 60 s. After exactly 2 min add 1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C and record as the clotting time the interval in seconds between this last addition and the onset of clotting determined by the chosen technique. Determine the blank recalcification time at the beginning and at the end of the procedure in a similar manner, using 1 mL of a 9 g/L solution of *sodium chloride R* in place of one of the heparin dilutions; the 2 blank values obtained should

(1) CK-Prest kits are suitable.

not differ significantly. Transform the clotting times to logarithms, using the mean value for the duplicate tubes. Repeat the procedure using fresh dilutions and carrying out the incubation in the order $T_4, T_2, T_3, S_4, S_2, S_3$. Calculate the results by the usual statistical methods (5.3).

Carry out not fewer than 3 independent assays. For each such assay prepare fresh solutions of the reference preparation and the preparation to be examined and use another, freshly thawed portion of plasma-substrate.

Calculate the potency of the preparation to be examined, combining the results of these assays, by the usual statistical methods (5.3). When the variance due to differences between assays is significant at $P = 0.01$, a combined estimate of potency may be obtained by calculating the non-weighted mean of potency estimates.

The anticoagulant activity of heparin is determined *in vitro* by its ability to accelerate the inhibition of thrombin, factor IIa (anti-IIa assay), by antithrombin. The International Unit is the activity contained in a stated amount of the International Standard for Unfractionated Heparin. *Heparin sodium BRP*, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as reference preparation.

For anti-IIa and anti-Xa assays, carry out the assay by determining the absorbance (end-point method) or the change of absorbance per minute (kinetic method) using tubes, microtitre plates and other automated equipment as appropriate to the chosen method.

ANTI-FACTOR IIa ACTIVITY

Reference and test solutions. Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of *heparin sodium BRP* in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*; the concentration range must be within 0.005 IU to 0.03 IU per millilitre and the dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

Procedure. Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_{11}, T_{12}, T_{13}, T_{14}$ for each of the 4 series of dilutions of the substance to be examined and $S_{11}, S_{12}, S_{13}, S_{14}$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 100 μL of *antithrombin III solution R5* and 50 μL of either *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* or the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_{11}, S_{12}, S_{13}, S_{14}, T_{11}, T_{12}, T_{13}, T_{14}, S_{21}, S_{22}, S_{23}, S_{24}, T_{21}, T_{22}, T_{23}, T_{24}, S_{31}, S_{32}, S_{33}, S_{34}, T_{31}, T_{32}, T_{33}, T_{34}, S_{41}, S_{42}, S_{43}, S_{44}, T_{41}, T_{42}, T_{43}, T_{44}$, allow to equilibrate at 37 °C (water-bath or heating block) for at least 1 min and add to each tube 25 μL of *human thrombin solution R2*. Incubate for exactly 1 min and add 50 μL of *chromogenic substrate R6*.

For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (2.2.25) at 405 nm using a suitable reading device.

For the end-point method, stop the reaction after exactly 4 min by adding 50 μL of a 20 per cent (V/V) solution of *glacial acetic acid R*. Assess whether exactly 4 min of incubation with the chromogenic substrate yields the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (2.2.25) at 405 nm using a suitable reading device. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (5.3).

ANTI-FACTOR Xa ACTIVITY

Reference and test solutions. Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of *heparin sodium BRP* in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*; the concentration range must be within 0.03 IU to 0.375 IU per millilitre and the dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

Procedure. Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_{1,1}, T_{2,1}, T_{3,1}, T_{4,1}$ for each of the 4 series of dilutions of the substance to be examined and $S_{1,1}, S_{2,1}, S_{3,1}, S_{4,1}$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 μL of *antithrombin III solution R6* and 50 μL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_{1,1}, S_{2,1}, S_{3,1}, S_{4,1}, T_{1,1}, T_{2,1}, T_{3,1}, T_{4,1}, S_{1,2}, S_{2,2}, S_{3,2}, S_{4,2}, T_{1,2}, T_{2,2}, T_{3,2}, T_{4,2}, S_{1,3}, S_{2,3}, S_{3,3}, S_{4,3}, T_{1,3}, T_{2,3}, T_{3,3}, T_{4,3}, S_{1,4}, S_{2,4}, S_{3,4}, S_{4,4}$, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 μL of *bovine factor Xa solution R2*. Incubate for exactly 2 min and add 100 μL of *chromogenic substrate R7*. For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (2.2.25) at 405 nm using a suitable reading device. For the end-point method, stop the reaction after exactly 4 min by adding 50 μL of a 20 per cent (V/V) solution of *glacial acetic acid R*. Assess whether exactly 4 min⁻¹ of incubation with the chromogenic substrate yields² the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (2.2.25) at 405 nm using a suitable reading device. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (5.3).

Reagents

Tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1. XXXXXXXX.

Dissolve 10.20 g of *sodium chloride R*, 6.10 g of *tris(hydroxymethyl)aminomethane R*, 2.80 g of *sodium edetate R* and 1.00 g of macrogol 6000 or 2.00 g of *bovine albumin R* or of *human albumin R* in 800 mL of *water R*. Adjust to pH 8.4 with *hydrochloric acid R* and dilute to 1.0 L with *water R*.

Antithrombin III solution R5. XXXXXXXX.

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.125 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

Thrombin solution, human R2. XXXXXXXX.

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

Chromogenic substrate R6. XXXXXXXX.

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 1.25 mM solution.

Antithrombin III solution R6. XXXXXXXX.

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 1.0 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

Factor Xa solution, bovine R2. XXXXXXXX.

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* to obtain a solution that gives an absorbance between 0.65 and 1.25 at 405 nm when assayed according to general chapter 2.7.5 using the end-point method.

Chromogenic substrate R7. XXXXXXXX.

Dissolve *N*- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 1 mM solution.