01/2015:20705

2.7.5. ASSAY OF HEPARIN

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 the reference preparation.

The assay of anti-factor Xa activity is carried out to determine the ratio of anti-factor Xa activity to anti-factor IIa activity.

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).

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 and 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_1, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 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 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, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_4, 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 a chromogenic substrate specific to factor IIa at a concentration suitable for the assay (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride dissolved in *water R* to give a 1.25 mM solution).

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 and 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, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 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, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_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 a chromogenic substrate specific to factor Xa at a concentration suitable for the assay (for example, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride dissolved in *water R* to give a 1 mM solution).

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*).