07/2008:20725

2.7.25. ASSAY OF HUMAN PLASMIN INHIBITOR

Human plasmin inhibitor, also called human α_2-antiplasmin, is a plasma protein that inhibits the plasmin (a serine protease) pathway of fibrinolysis by rapidly forming a complex with free plasmin. Furthermore, upon blood coagulation, human plasmin inhibitor is cross-linked to fibrin strands by factor XIII, and interferes with binding of the proenzyme plasminogen to fibrin.

The potency of human plasmin inhibitor is estimated by comparing the ability of the preparation to be examined to inhibit the cleavage of a specific chromogenic substrate by plasmin with the same ability of a reference standard of human plasmin inhibitor. Plasmin cleavage of the chromogenic substrate yields a chromophore that can be quantified spectrophotometrically.

The individual reagents for the assay may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

REAGENTS

Dilution buffer pH 7.5. According to the manufacturer's instructions, a suitable buffer is used. Adjust the pH (*2.2.3*) if necessary.

Plasmin. A preparation of human plasmin that does not contain significant amounts of other proteases is preferably used. Reconstitute and store according to the manufacturer's instructions.

Plasmin chromogenic substrate. A suitable specific chromogenic substrate for plasmin is used: H-D-cyclohexylalanyl-norvalyl-lysyl-*p*-nitroaniline hydrochloride (H-D-CHA-Nva-Lys-*p*NA.HCl) or L-pyroglutamyl-L-phenylalanyl-L-lysine-*p*-nitroaniline hydrochloride (Glp-Phe-Lys-*p*NA.HCl). Reconstitute in *water R* to give a suitable concentration according to the manufacturer's instructions.

METHOD

Varying quantities of the preparation to be examined are mixed with a given quantity of plasmin and the remaining plasmin activity is determined using a suitable chromogenic substrate.

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.5 and prepare at least 2 independent series of 3 or 4 dilutions for both the preparation to be examined and the reference standard.

Mix 0.020 ml of each dilution with 0.020 ml of dilution buffer pH 7.5 and warm to 37 °C. Add 0.040 ml of a plasmin solution (test concentration in the range of 0.2 nkat/ml to 1.6 nkat/ml) previously heated to 37 °C and leave at 37 °C for 1 min. Add 0.020 ml of the chromogenic substrate solution, previously heated to 37 °C, to each mixture and measure the optical density at a wavelength of 405 nm. Substract the optical density of the blank (prepared with dilution buffer pH 7.5) from the optical density of the preparation to be examined. Check the validity of the assay and calculate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

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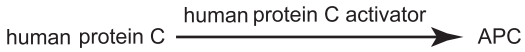
2.7.30. ASSAY OF HUMAN PROTEIN C

1. CHROMOGENIC ASSAY

Human protein C is a vitamin K-dependent plasma protein that, upon activation to activated protein C (APC), can inhibit blood coagulation through the proteolytic cleavage of factors Va and VIIIa. Human protein C activity is estimated using a two-step method: in the 1st step, human protein C in the preparation is activated by a specific activator from snake venom; in the 2nd step, APC cleaves a specific chromogenic substrate to form a chromophore that can be quantified spectrophotometrically.

Step 1



Step 2

The potency of human protein C is estimated by comparing the ability of the preparation to be examined to cleave a chromogenic substrate with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre plate end-point method.

REAGENTS

Dilution buffer pH 8.4. Dissolve 6.055 g of *tris(hydroxymethyl)aminomethane R* and 16.84 g of *caesium chloride R* in *water R* and adjust the pH (*2.2.3*) if necessary. Dilute to 1000.0 ml with *water R*.

Human protein C activator. Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions. Dilute to 0.25 U/ml with *water R* before use in the assay.

Activated protein C chromogenic substrate. Specific chromogenic substrate for APC, for example L-pyroglutamyl-L-prolyl-L-arginine-paranitroaniline hydrochloride (pyroGlu-Pro-Arg-pNA.HCl). Reconstitute with *water R* to give a concentration of 4.5 mmol/l. Further dilute to 1.1 mmol/l with dilution buffer pH 8.4 before use in the assay.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with *water R* to produce at least 3 separate dilutions for each preparation in the range 0.050-0.200 IU/ml, preferably in duplicate.

Step 1. Mix 0.025 ml of each dilution with 0.050 ml of the human protein C activator, both previously heated to 37 °C, and leave at 37 °C for exactly 10 min. For each dilution, prepare a blank in the same manner, using *water R* instead of the human protein C activator.

Step 2. Add 0.150 ml of diluted chromogenic substrate, previously heated to 37 °C, to each mixture and leave at 37 °C for exactly 10 min. The incubation time must be adjusted, if necessary, to ensure a linear development of chromophore with time. Terminate the reaction by adding 0.050 ml of a 50 per cent *V/V* solution of *glacial acetic acid R*.

Cleavage of the chromogenic substrate by APC causes release of the chromophore pNA, in proportion to the concentration of human protein C in the preparation. Measure the optical density at a wavelength of 405 nm. Subtract the optical density of the blank from the optical density of the test sample. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3.*).

2. CLOTTING ASSAY

Human protein C activity is estimated following cleavage to APC by a specific activator extracted from the venom of the viper *Agkistron contortrix contortrix*. The resulting APC inactivates factors Va and VIIIa, and thus prolongs the APTT (Activated Partial Thromboplastin Time) of a system in which all the coagulation factors are present, constant and in excess, except for human protein C, which is derived from the preparation being tested. Prolongation of the clotting time is proportional to the concentration of human protein C in the preparation.

The potency of human protein C is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

REAGENTS

Dilution buffer pH 7.4. Isotonic non-chelating buffer.

Human protein C-deficient plasma. Citrated human plasma with no measurable human protein C content. Reconstitute and store according to the manufacturer's instructions.

Human protein C activator. Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions.

Coagulation activator. A suitable APTT reagent containing phospholipids and a contact activator may be used. It may be combined with the human protein C activator.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.010-0.150 IU/ml, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein C-deficient plasma and 1 volume of the human protein C activator (combined with the APTT reagent where appropriate), all previously heated to 37 °C. Add 1 volume of *calcium chloride R* previously heated to 37 °C, and record the clotting time.

The clotting time is proportional to the concentration of human protein C in each dilution. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

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2.7.31. ASSAY OF HUMAN PROTEIN S

Human protein S is a vitamin K-dependent plasma protein that acts as a cofactor for the anticoagulant functions of activated protein C (APC). Human protein S activity may be determined by the clotting assay described below, which is sensitive to the ability of human protein S to accelerate the inactivation of factor Va by APC. In practice, the assay involves the addition of human protein S to a reagent mixture containing APC, factor Va and human protein S-deficient plasma. Prolongation of the clotting time is proportional to the concentration of human protein S in the preparation. Methods in which APC is added directly as a reagent are preferred to those in which APC is generated during the assay by the addition of a specific human protein C activator purified from snake venom. Activation of coagulation is initiated by the addition of an activating reagent such as thromboplastin or activated factor X, together with phospholipids and calcium chloride. During the assay, factor Va is generated from factor V in the human protein S-deficient plasma following the activation of coagulation. The assay procedure must ensure that human protein S is the only limiting factor.

The potency of human protein S is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein S calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein S. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

REAGENTS

Dilution buffer pH 7.4. Isotonic non-chelating buffer prepared as follows: dissolve 6.08 g of *tris(hydroxymethyl)-aminomethane R* and 8.77 g of *sodium chloride R* in *water R* and adjust the pH (*2.2.3*) if necessary; add 10 g of *bovine albumin R* or *human albumin R* and dilute to 1000.0 ml with *water R*.

Human protein S-deficient plasma. Citrated human plasma with no measurable human protein S content and, preferably, also free of C4b-binding protein.

Coagulation activator. This reagent is used to initiate coagulation in the human protein S-deficient plasma, and thereby also provides a source of activated factor V. The activator may consist of tissue factor, activated factor X, or an agent capable of directly activating factor X that may be purified from the venom of Russell's viper (*Vipera russelli*). The reagent also contains APC, phospholipids and *calcium chloride R*, or, alternatively, calcium chloride may be added separately after a timed activation period.