2.6.15. PREKALLIKREIN ACTIVATOR

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

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

REAGENTS

Prekallikrein activator in albumin BRP is calibrated in International Units by comparison with the International Standard.

Buffer A. Dissolve 6.055 g of tris(hydroxymethyl)aminomethane R, 1.17 g of sodium chloride R, 50 mg of hexadimethrine bromide R and 0.100 g of sodium azide R in water R. Adjust to pH 8.0 with 2 M hydrochloric acid R and dilute to 1000 mL with water R.

Buffer B. Dissolve 6.055 g of tris(hydroxymethyl)aminomethane R and 8.77 g of sodium chloride R in water R. Adjust to pH 8.0 with 2 M hydrochloric acid R and dilute to 1000 mL with water R.

PREPARATION OF PREKALLIKREIN SUBSTRATE

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or a 38 g/L solution of sodium citrate R) to which 1 mg/mL of hexadimethrine bromide R has been added. Centrifuge the mixture at 3600 g for 5 min. Separate the plasma and centrifuge again at 6000 g for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing agarose-DEAE for ion-exchange chromatography R that has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 mL/cm²/h. Collect the eluate in fractions and record the absorbance at 280 nm (2.2.25). Pool the fractions containing the 1st protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 7 g/L of sodium chloride R and filter through a membrane filter (nominal pore size 0.45 μm). Freeze the filtrate in portions and store at −25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

METHOD

The assay may be carried out using an automated enzyme analyser or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture.

Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate R or D-prolyl-1-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride R), dissolved in buffer B. Record the rate of change in absorbance per minute for 2-10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Depending on the method used, ΔA/min has to be corrected by subtracting the value obtained for the corresponding blank without the prekallikrein substrate. The results may be calculated using a standard curve, a parallel-line or a slope ratio assay or any other suitable statistical method. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined. During method validation, the spiking experiments must show that the sample matrix has no influence on the results. High blank values may impact assay validity and should be appropriately investigated.