Methods of Enzymatic Analysis

Third Edition

Editor-in-Chief: Hans Ulrich Bergmeyer
Editors: Jürgen Bergmeyer and Marianne Graßl

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Volume XII
Drugs and Pesticides

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Contents

1.18.2 Inhibition Assay

Marianne Jochum

General

The biomedical use of the low molecular-weight (M_r = 6500) proteinase inhibitor aprotinin (Trasylol®), from bovine tissue cells is based on its broad inhibitory specificity. Major target enzymes are trypsin, chymotrypsin, plasmin, tissue kallikrein and plasma kallikrein. Hence, administration of aprotinin to patients is recommended as part of the therapeutic regimen of various diseases, in particular in shock syndromes, hyperfibrinolysis or acute pancreatitis (for review cf. [1]). Serum or plasma levels of aprotinin obtained after intravenous injection decline rather rapidly due to distribution of the inhibitor in the extracellular fluid and subsequent accumulation, primarily in the kidney [2, 3]. Therefore, one major prerequisite for the optimization of the proteinase inhibitor therapy is the quantitative assessment of the inhibitor levels in patients' plasma or other body fluids.

Application of method: in biochemistry, clinical chemistry and pharmacology.

Substance properties relevant in analysis: aprotinin can be exposed to solutions with extreme pH values [1]. Hence, acid treatment of aprotinin-containing plasma samples allows a total recovery and thereby a specific measurement of aprotinin as the only tissue kallikrein inhibitor present in these specimens.

Methods of determination: so far, aprotinin has been measured in biological samples by the trypsin inhibition assay [1, 4] or by immunological methods [5, 6]. However, these assays are inappropriate for monitoring inhibitor levels in routine clinical diagnosis because of the limited specificity of the enzyme inhibition assay and the prolonged incubation (>40 h) necessary in the immunoassays. The enzymatic determination described here circumvents these difficulties and therefore can conveniently be used as a specific and rapid bedside control of high-dosage proteinase-inhibitor therapy [7].

An enzyme-linked immunosorbent assay was developed only recently ([8], cf. chapter 1.18.1) which provides a versatile means of measuring very precisely and relatively rapidly (4 h) large numbers of aprotinin-containing samples from plasma and other body fluids. This assay is suitable for special purposes, e.g. for monitoring lower dose inhibitor therapy in retrospective studies.

International reference method and standards: not yet available.

Assay

Method Design

Principle

(a) Aprotinin + tissue kallikrein (excess) $\xrightarrow{5\ \text{min}}$ aprotinin-tissue kallikrein + tissue kallikrein (remaining)

(b) D-Val-Leu-Arg-4-NA + H_2O $\xrightarrow{\text{tissue kallikrein (remaining)}}$ D-Val-Leu-ArgOH + 4-nitroaniline.

After incubation of acid-treated plasma with tissue kallikrein (EC 3.4.21.35) in excess, the remaining amount of the enzyme is determined by its amidolytic activity on the substrate D-Val-Leu-Arg-4-NA (S-2266). The initial rate at which 4-nitroaniline is released is measured photometrically at 405 nm.

The reaction rate decreases linearly with increasing concentration of aprotinin in the range of 20000 – 80000 KIU* per litre plasma. The concentration of aprotinin is calculated from a calibration curve prepared by diluting normal plasma to which aprotinin (2×10^5 KIU/l) has been added before acid treatment.

Optimized conditions for measurement: an enzyme activity of 1 KU** is used in the assay mixture; taking K_m into account, the substrate concentration of 0.15 mmol/l ensures minimal influence of substrate depletion. Pre-incubation of acid-treated aprotinin-containing samples with the enzyme for 5 min at pH 8.2 is sufficient to achieve complete complex formation. Since the kallikrein activity towards the chromogenic peptide substrate is increased by the addition of plasma, all dilutions of aprotinin-containing plasma samples have to be performed with aprotinin-free plasma instead of buffer: in this way, the same amount of plasma (e.g. 25 µl) is always added to the assay system.

Temperature conversion factors: the assay should be performed at 37 °C. Conversion factors for other temperatures have not yet been determined.

Equipment

Spectrophotometer or spectral-line photometer capable of exact measurement at 405 nm, provided with a thermostatted cuvette holder; water-bath, centrifuge (*Eppendorf* 5412); recorder or stopwatch; semi-microcuvettes.

* KIU biological kallikrein inhibitor unit (cf. [1]).

** KU biological kallikrein unit (cf. [1]).

Reagents and Solutions

Purity of reagents: all chemicals should be of the highest analytical grade commercially available.

Preparation of solutions (for about 280 determinations): all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. Buffer (Tris, 0.2 mol/l, pH 8.2):

   dissolve 24.2 g tris(hydroxymethyl)aminomethane in 800 ml water, adjust to pH 8.2 with HCl, 1 mol/l (approximately 100 ml), and dilute to 1000 ml with water (25 °C).

2. Substrate solution (D-Val-Leu-Arg-4-NA, 1.5 mmol/l):

   dissolve 25 mg S-2266 (from *AB Kàbi Diagnostica,* Stockholm, Sweden) in 28.8 ml water.

3. Porcine pancreatic kallikrein solution* (10^6 KU/l):

   dissolve the lyophilized enzyme with sterile physiological saline (adjusted to pH 7.5 with triethanolamine) to a stock solution of 10^6 KU/ml. Prepare further dilutions for the assay (5×10^4 KU/l ≙ 1 KU/20 µl) freshly each day.

4. Normal human plasma:

   take blood specimens from veins of at least ten healthy blood donors (9 vol blood and 1 vol sodium citrate solution, 0.1 mol/l). Prepare plasma by centrifugation at 2000 *g* for 20 min at 4 °C. Mix equal amounts of plasma from each donor and dispense in small volumes. Treat with perchloric acid (6) as described for samples under Collection and treatment of specimens.

5. Aprotinin standard solutions:

   use ampoules with 2×10^5 KIU/10 ml, pH 5, from *Bayer AG,* Leverkusen; add 10 µl (200 KIU) to 1 ml normal plasma (4) for preparation of the calibration curve. Dilute this stock solution (10^5 KIU/l) with aprotinin-free normal plasma (4) according to the following scheme:

* Research product from *Bayer AG,* Wuppertal-Elberfeld.

Stock solution (10^5 KIU/l) µl	Normal plasma µl	KIU per assay	KIU ($\times 10^3$) per l untreated plasma
10	190	0.125	10
10	90	0.25	20
20	80	0.50	40
30	70	0.75	60
40	60	1.00	80
50	50	1.25	100

6. Perchloric acid solution (PCA, 30 g/l):

   dilute 8.57 ml 70% (w/v) PCA with 191.43 ml water.

7. Potassium carbonate solution (K_2CO_3, 5 mol/l):

   dissolve 6.91 g K_2CO_3 in 8 ml water and dilute to 10 ml with water.

Stability of solutions: store all solutions at 2 °C to 8 °C. Solutions (1) and (7) are stable for two months if not contaminated with micro-organisms. Solution (2) is stable for six months if prepared with sterile water and kept in the dark. Solution (3) is stable for at least two weeks. If not contaminated, solutions (5) and (6) are stable indefinitely. Normal plasma (4) is stable for at least 3 – 4 months at – 20 °C or more than 6 months at – 70 °C. Commercially available lyophilized normal plasma may also be used.

Procedure

Collection and treatment of specimens: take blood specimens from the vein without stasis (9 vol blood + 1 vol sodium citrate solution, 0.1 mol/l). Prepare plasma by centrifugation at 2000 *g* for 20 min at 4 °C.

Acid treatment of plasma samples: incubate plasma (e.g. 0.5 ml) for 10 min at 25 °C with an equal volume of PCA solution (6). Centrifuge for 10 min at 10000 *g*, neutralize ca. 0.980 ml supernatant with ca. 20 µl K_2CO_3 solution (7), keep at 4 °C to 8 °C for further 30 min and thereafter centrifuge again for 10 min. Freeze the resulting supernatant at – 20 °C or below.

Stability of aprotinin in the sample: aprotinin in plasma is stable at room temperature at least for 1 day or at 4 °C for 2 days. Aprotinin solutions of pH < 4 should be used within a few hours. Neutralized aprotinin-containing plasma samples can be stored for 1 month at – 20 °C or for 6 months at – 70 °C. Repeated freezing and thawing (n = 10) has no influence on the inhibitory activity of aprotinin in plasma.

Details for measurements in other samples: the same procedure may be used for measurements in serum and other specimens.

Assay conditions: wavelength Hg 405 nm; light path 10 mm; final volume 1 ml; temperature 37 °C (thermostatted cuvette holder). Measure against air. Before starting the assay, adjust temperature of solutions to 37 °C.

Establish a calibration curve using aprotinin solution (5) instead of sample.

Measurement

Pipette successively into the cuvette:		blank	sample	concentration in assay mixture
Tris buffer	(1)	0.855 ml	0.855 ml	Tris 171 mmol/l
normal plasma	(4)	0.025 ml	–	
sample or standard	(5)	–	0.025 ml	aprotinin up to 1250 KIU/l
kallikrein solution	(3)	0.020 ml	0.020 ml	kallikrein 1000 KU/l
mix thoroughly with a plastic spatula, wait for 5 min,				
substrate solution	(2)	0.100 ml	0.100 ml	D-Val-Leu-Arg-4-NA 0.15 mmol/l
mix and read absorbance each min or monitor the reaction on a recorder over a period of 5 min.				

If the amount of aprotinin in the sample exceeds 1.0 KIU per assay, plasma samples must be diluted with aprotinin-free, acid-treated normal plasma (4).

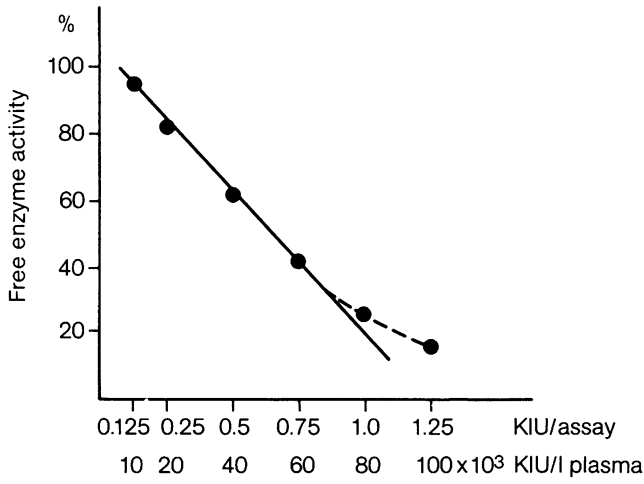

Fig. 1. Typical calibration curve for the assay of aprotinin in plasma.

Calibration curve: calculate $(\Delta A/\Delta t)_{sample}$ in percent of $(\Delta A/\Delta t)_{blank}$. Plot the percent of free enzyme activities of the standards against their activity concentrations of aprotinin (Fig. 1).

Calculation: the inhibitory activity of aprotinin in the sample (patients' plasma) is taken from the calibration curve.

Validation of Method

Precision, accuracy, detection limit and sensitivity: the within-run imprecision for aprotinin, 4×10^4 KIU/l was 1.8 – 10.8% in 8 series; the between-series imprecision ($n = 8$) was 4.4%. The recovery for 2×10^4 to 8×10^4 KIU/l (working range) was 90 – 125%. Data on accuracy are not available since standard reference material is not established yet.

The detection limit is 0.125 KIU per assay, i.e. 10^4 KIU/l of untreated plasma. Sensitivity is found to be $\Delta A/\Delta t = 0.002$/min at 405 nm (*Eppendorf* photometer). the mean 1.23-fold increase caused by pooled plasma. With an individual increase in

Sources of error: since kallikrein activity is increased 1.23-fold ($n = 90$; RSD = 7.2%) by addition of acid-treated normal plasma, all dilutions of aprotinin-containing plasma samples must be made with aprotinin-free, acid-treated plasma. Plasma samples without acid treatment cannot be used because of a rather high and irregular stimulation of the kallikrein activity in the assay system. The same holds true for serum. Dilution of aprotinin-containing plasma samples with buffer or isotonic saline solution instead of plasma yields too low inhibitor concentrations.

Occassionally, aprotinin-free plasma samples from a single individual may stimulate kallikrein activity to a lesser (up to 1.1-fold) or greater (up to 1.5-fold) extent than kallikrein activity lower than 1.15-fold or higher than 1.3-fold, the calibration curve and dilutions of aprotinin-containing plasma samples should be performed with the individual patient's plasma (pre-aprotinin infusion sample) instead of pooled plasma.

Addition of NaN_3 to aprotinin-containing plasma as a preservative before acid treatment partly destroys the inhibitor activity during the subsequent procedure.

Specificity: under the given conditions the substrate S-2266 is split specifically by the tissue kallikrein used in the assay.

Therapeutic ranges: continuous i.v. infusion of 250000 KIU/h in polytraumatized patients resulted in a mean plasma concentration of 4.5×10^4 KIU/l [7].

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