CHROMOGENIC SUBSTRATE ASSAY FOR APROTININ IN SOLUTIONS AND HUMAN PLASMA

This kit is designed for the determination of aprotinin in human plasma. Plasma is treated with acetone to remove the effect of serine protease inhibitors, and then diluted in buffer containing inhibitors of α_2-macroglobulin and factor XIIa. Purified plasma kallikrein is added to acetone treated plasma or aqueous solutions containing aprotinin, and after an incubation period it complexes with the aprotinin in the test sample. Residual plasma kallikrein activity is then measured by its ability to cleave a chromogenic peptide substrate and liberate p-nitroaniline (pNA). The concentration of pNA is measured photometrically, and is inversely proportional to the aprotinin concentration[1].

REAGENTS

The reagents should be stored at 4°C until reconstituted.

1. *Human Plasma Kallikrein*
Dissolve in 10ml sterile distilled water. Store in aliquots at -20°C when not required.

2. *Kallikrein Substrate*
Dissolve in 10ml sterile distilled water. Stable for 8 hours at 4°C and 6 months at -20°C, if free from contamination.

3. *Buffer Concentrate*
Dilute contents of vial with 90ml sterile distilled water, store at 4°C.

4. *Aprotinin Standard*
Dissolve in 1ml distilled water (stock solution). Stable for 8 hours at 4°C and 6 months at -20°C.

5. *Corn Trypsin Inhibitor (CTI)*
Dissolve in 10ml distilled water.

6. *Corn Trypsin Inhibitor Containing Buffer (CTI Buffer)*
Add 10ml Corn Trypsin Inhibitor to 90ml dilute buffer, store at 4°C.

7. *Normal Plasma*
Dissolve in 3ml distilled water, leave for 5 minutes at room temperature and then gently mix until completely dissolved. Keep at room temperature and use within 4 hours or store at -20°C.

Reagents required but not provided: acetone, 50% acetic acid.

BLOOD COLLECTION AND PLASMA PREPARATION

Blood (9ml) is mixed with 0.106M Tri-sodium citrate (1ml) and centrifuged at 2000g for 15 minutes at room temperature. The plasma samples should be removed with plastic pipettes within two hours of blood collection and should be assayed immediately or stored frozen at -20°C.

PREPARATION OF STANDARD CURVES

Separate standard curves are prepared for aqueous solutions and plasma samples.

Standard Curve for Plasma Samples:

1. Primary Dilutions
From the stock Aprotinin solution prepare the following dilutions:

Aprotinin (KIU/ml)	Aprotinin Stock (µL)	CTI Buffer (µL)
A) 0	0	1000
B) 125	50	950
C) 250	100	900
D) 500	200	800
E) 2500	Use stock solution	

2. Secondary Dilutions:

Aprotinin (KIU/ml)	Aprotinin from 1.	Plasma (µL)
0	50 µL A	450
25	50 µL B	450
50	50 µL C	450
100	50 µL D	450
250	50 µL E	450

ACETONE TREATMENT

Mix 300µl of each test plasma or secondary standard dilution with 100µl acetone in a polypropylene or siliconised glass test tube. Leave for 15 minutes at 4°C, and centrifuge at 2000g for 5 minutes, then dilute 300µl acetone treated plasma with 1700µl CTI Buffer and keep on ice until assayed (within 1 hour).

Standard Curve for Aqueous Solutions of Aprotinin :

Prepare primary dilutions A, B, C, D, E as above. Prepare secondary dilutions as for plasma, but substituting buffer containing Corn Trypsin Inhibitor for plasma. Dilute the aqueous aprotinin test solutions appropriately, to bring the results into the range of the standard curve. No acetone treatment is required.

ASSAY METHOD

Have the substrate at 37°C. Into siliconised semi-micro cuvettes, siliconised glass or plastic tubes pipette:

Plasma dilution or buffer	200µl

Incubate at 37°C for 1 minute, add:

Plasma Kallikrein	200µl

Mix and incubate at 37°C for 5 minutes, add:

Kallikrein Substrate	200µl

Mix and record the change in optical density per minute at 405nm (rate assay), or incubate for exactly 10 minutes at 37°C, add:

Acetic acid (50%)	200µl

Mix and read optical density at 405nm (end point assay).

Blanks should be prepared when aprotinin is measured in plasma samples (to correct for differences in plasma colour and endogenous protease activities in plasma), by substituting 200µl of CTI Buffer for the Plasma Kallikrein.

CALCULATION

Subtract the blank values from the test values. Plot the results as log A_{405} against Aprotinin concentration and read the values for the test plasmas from the standard curve.

HAZARD WARNING

All materials of human origin were tested and found negative for the presence of HBsAG and anti-HIV antibody. However, as with all preparations of human origin, these products cannot be assumed to be free from infectious agents and suitable precautions should be taken in their use and disposal.

NOTE

The recommended standard and test dilutions may vary between different batches of this kit owing to differences in the specific activity of some batches of reagents.

REFERENCES

1. Gallimore MJ, Fuhrer G, Heller W, Hoffmeister HE. Augmentation of kallikrein and plasmin inhibition capacity by aprotinin using a new assay to monitor therapy. Kinins V: Advances in Experimental Medicine and Biology 1989; 247b: 55-60.

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