CHROMOGENIX RESEARCH METHODS - ANTITHROMBIN (anti-FIIa)

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Determination of antithrombin activity in plasma with S-2238.

Measurement Principle

The antithrombin activity in plasma is measured after addition of an excess of heparin, to form an AT•Heparin complex. An excess of thrombin is then added and allowed to react quantitatively in a 1:1 stoichiometric relationship with the AT Heparin complex present.
The residual thrombin splits off p-nitroaniline (pNA) from the substrate H-D-Phe-Pip-Arg-pNA (S-2238). The rate at which pNA is released is measured photometrically at 405 nm. This can be followed on a recorder (initial rate method) or read after stopping the hydrolysis with acid (acid stopped method). The correlation between the change in absorbance per minute (ΔA/min) or absorbance (A) and the AT activity is linear and inversely proportional in the 5-125% range of normal plasma.

AT + Heparin (excess) ⟶ *[AT•Heparin]*

[AT Heparin] + Thrombin (excess) ⟶ *[AT•Heparin•Thrombin] +Thrombin (residual)*

$$\text{H-D-Phe-Pip-Arg-pNA} + H_2O \xrightarrow{\text{Thrombin (residual)}} \text{H-D-Phe-Pip-Arg-OH} + \text{pNA}$$

Reagents

1. S-2238, 25 mg Art. No. S820324
Reconstitute the substrate S-2238 (MW: 625.6) in 53 ml of distilled water.
Note: Polybrene® can be added to the substrate solution at a final concentration of 0.33 mg/ml.

2. Thrombin, 53 nkat Art. No. DPGBT-1
Reconstitute with 1.5 ml sterile water. The solution is stable for 4 weeks at 2-8°C.

4. Tris/Heparin Buffer, pH 8.4 (25°C)

Tris	6.1 g	(50 mmol/l)
NaCl	10.2 g	(175 mmol/l)
$Na_2EDTA - 2H_2O$	2.8 g	(7.5 mmol/l)
Distilled water	800 ml	

Adjust the pH to 8.4 at 25°C by adding an appropriate amount (approx. 22 ml) of 1 mol/l HCl. Add 3000 IU of heparin. Fill up to 1000 ml with distilled water.
The buffer, if not contaminated, will remain stable for two months at 2-8°C.

5. Acetic acid 20%
Acetic acid is used in the acid-stopped method.

Equipment

1. Spectro- or filter-photometer, 405 nm
2. Semi-microcuvettes, 1 cm.
3. Thermostat, 37°C
4. Stop watch
5. Disposable plastic tubes
Additional equipment for the initial rate method:
6. Photometer with cuvette housing, thermostated at 37°C.

Specimen collection

Nine parts of freshly drawn venous blood are collected into one part trisodium citrate.
Centrifugation: 2000 x g for 10-20 min at 20-25°C.

Standard curve

Normal plasma has an antithrombin activity of 100%.
Two standards (e.g. 25% and 100%) made up fresh should be included in each test run. Check whether ΔA/min or A for the two standards correspond with the stored standard curve.
The tolerance limit is ± 0.1 Absorbance units.
Prepare the standards according to the table below:

Antithrombin %	Normal plasma µl	Tris/Heparin buffer µl
0	-	400
25	100	300
50	200	200
75	300	100
100	400	-

Method

Dilute samples and standards as follows:

Tris/Heparin Buffer	3000 µl
Test plasma or standard	50 µl

Initial rate method	
Diluted test plasma or standard	400 µl
Incubate at 37°C	3-6 min
Thrombin (20-25°C)	100 µl
Mix and incubate at 37°C	30 sec
Substrate (37°C)	300 µl

Transfer immediately to a 1 cm semi-microcuvette (preheated to 37°C) for measurement of the absorbance change in a photometer at 405 nm and at 37°C, calculate ΔA/min.

Acid stopped method	
Diluted test plasma or standard	400 µl
Incubate at 37°C	3-6 min
Thrombin (20-25°C)	100 µl
Mix and incubate at 37°C	30 sec
Substrate (37°C)	300 µl
Incubate at 37°C	30 sec
Acetic acid 20%	300 µl

Read the absorbance (A) of the sample against distilled water at 405 nm within 4 hours.

Limitations of the procedure

In some pathological states (DIC, sepsis) plasma alone may hydrolyse the substrate S-2238. This interfering reaction may be determined by assay of a test sample in the absence of added thrombin.
This activity rarely corresponds to more than 1% of that of the added thrombin.To improve the validity of the assay the value obtained in the absence of added thrombin can be subtracted from the sample value.
Bilirubin, haemoglobin and plasma from hyperlipaemic patients interfere in absorbance reading.
Patients plasma blanks are necessary in these instances for the acid stopped method only.
At concentrations below 25% AT it is recommended to double the plasma concentration (100 µl plasma + 3 ml buffer). The result is then divided by two.

Calculation

Plot A or ΔA/min for the standards against their known antithrombin activity. Percent of normal AT activity is determined by plotting the A or ΔA/min for the test sample on the standard curve and reading the corresponding AT value.

Bibliography

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