CHROMOGENIX RESEARCH METHODS - ANTITHROMBIN (anti-FIIa)

For research use only - Not for use in Diagnostic Procedures

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.

$\text{AT} + \text{Heparin (excess)} \rightarrow \text{AT-Heparin}$

$\text{[AT-Heparin]} + \text{Thrombin (excess)} \rightarrow \text{[AT-Heparin-Thrombin]} + \text{Thrombin (residual)}$

$\text{H-D-Phe-Pip-Arg-pNA} + \text{H}_2\text{O} \rightarrow \text{H-D-Phe-Pip-Arg-OH} + \text{pNA}$

(residual)

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, 54 ngat Art. No. DG86-1
   Reconstitute with 1.5 ml sterile water. The solution is stable for 4 weeks at 2-8°C.

3. Tris/Heparin Buffer, pH 8.4 (25°C)
   Tris 6.1 g (50 mmol/l)
   NaCl 10.2 g (175 mmol/l)
   Na₂EDTA - 2H₂O 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 3300 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.

4. Acid stopped method
   Diluted test plasma or standard 400 μl
   Incubate at 37°C 3.4 min
   Thrombin (20-25°C) 100 μl
   Mix and incubate at 37°C 30 sec
   Substrate (37°C) 300 μl
   Acetic acid 20% 300 μl

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:

<table>
<thead>
<tr>
<th>Antithrombin %</th>
<th>Normal plasma</th>
<th>Tris/Heparin buffer</th>
<th>μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>300</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>400</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Method

Dilute samples and standards as follows:

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

Initial rate method

<table>
<thead>
<tr>
<th>Diluted test plasma or standard 400 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubate at 37°C 3.4 min</td>
</tr>
<tr>
<td>Thrombin (20-25°C) 100 μl</td>
</tr>
<tr>
<td>Mix and incubate at 37°C 30 sec</td>
</tr>
<tr>
<td>Substrate (37°C) 300 μl</td>
</tr>
</tbody>
</table>

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.

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