HEPARIN (anti-FIIa)

Determination of heparin in plasma with S-2238.

Measurement Principle

Heparin is analysed as a complex with antithrombin (AT) present in the sample. The concentration of this complex is dependent on the availability of AT. In order to obtain a more constant concentration of AT, purified AT is added to the test plasma. Thrombin in excess is neutralized in proportion to the amount of heparin, which determines the amount of heparin-AT complex. The remaining amount of thrombin hydrolyses the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S-2238) thus liberating the chromophoric group, pNA.
The colour is then read photometrically at 405 nm.

Heparin + AT ⟶ *[Heparin•AT]*

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

H-D-Phe-Pip-Arg-pNA —*Thrombin*→ *H-D-Phe-Pip-Arg-OH + pNA* (*residual*)

Reagents

1. S-2238, 25 mg Art. No. S820324
Reconstitute the substrate S-2238 (MW: 625.6) with 40 ml of distilled water.

2. Thrombin Art. Nos. EZ006A/B/O/K or DPGBT-1/10
Human thrombin or bovine thrombin can be used in 0.15 mol/l NaCl solution. The activity of the solution should be 14 nkat/l (about 6 NIH-U/ml).
If bovine thrombin 53 nkat from DiaPharma (Art. No. DPGBT-1) is used, dissolve the content of one vial with 3.8 ml saline.

3. Antithrombin, 10 IU Art. No. B820720
Reconstitute with 5 ml water to obtain a concentration of 2 IU/ml.

4. Tris Buffer, pH 8.4 (25°C)

Tris	6.1 g	(50 mmol/l)
NaCl	10.2 g	(175 mmol/l)
$Na_2EDTA\ H_2O$	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.

5. Normal plasma
Blood should be taken from normal donors. 10-30 ml of citrated blood (9 vol blood and 1 vol 0.1 mol/l sodium citrate) are taken from each donor. The first ml of blood is discarded and the tube is kept in an ice bath. Plasma is prepared by centrifugation at 2000 x g for 20 minutes at 4°C.
Equal amounts of plasma from the donors are mixed and dispensed in small volumes. The normal plasma is stable for 3 months at -20°C or below. Thaw at 37°C and then keep on ice.

6. 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

Blood (9 vol) is mixed with sodium citrate (1 vol) cooled to 0°C with ice and centrifuged at 2000 x g for 20 min at 4°C.
Dilute plasma 1:5 with Tris Buffer pH 8.4.

Standard curve

The same heparin as is used for the patient is diluted to 1 IU/ml with saline 0.9%. Then 100 µl dilution is further diluted with 1.9 ml buffer to obtain a concentration of 0.05 IU/ml.

Standard IU/ml	Buffer µl	AT µl	Normal plasma dil 1:5 µl	Heparin 0.05 IU/ml µl
0.00	800	100	100	0
0.25	700	100	100	100
0.50	600	100	100	200
0.75	500	100	100	300
1.00	400	100	100	400

Method

Initial rate method	Tube No. 1
Buffer	800 µl
AT	100 µl
Test plasma	100 µl
Mix	
	Tube No. 2
Standard or Tube No.1	200 µl
Incubate at 37°C	3-4 min
Thrombin	100 µl
Incubate at 37°C	30 sec
Substrate (37°C)	200 µl
Mix	

Transfer sample immediately to a 1 cm micro-cuvette (preheated at 37°C) for measurement of the absorbance change at 405 nm. Calculate ΔA/min.
Read the absorbance against a normal plasma blank in a photometer at 405 nm.

Acid stopped method	Tube No. 1
Buffer	800 µl
AT	100 µl
Test plasma	100 µl
Mix	
	Tube No. 2
Standard or Tube No.1	200 µl
Incubate at 37°C	3-4 min
Thrombin	100 µl
Incubate at 37°C	30 sec
Substrate (37°C)	200 µl
Incubate at 37°C	60 sec
Acetic acid 20 %	300 µl
Mix	

Blanks for acid stopped method	Normal plasma blank	Test Plasma blank
Standard 0 IU/ml	200 µl	-
Sample from Tube No.1	-	200 µl
Acetic acid 20%	300 µl	300 µl
Mix		
Distilled water	300 µl	300 µl
Mix		

Note: As a rule a normal plasma blank or even water is used as a blank. If bilirubin exceeds 100 µmol/l or the test plasma is opaque, read the test plasma sample against its own blank.

Calculation

Plot A or ΔA/min for the standards against their known heparin concentration.
Heparin concentration is determined by plotting the A or ΔA/min for the test sample on the standard curve and read the corresponding heparin value.

Bibliography

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