HIRUDIN

Determination of hirudin levels in plasma with the chromogenic substrate S-2366.

Background

Hirudin is a protein originating from the medical leech[1,2]. It is the most potent thrombin inhibitor with a dissociation constant below 10^{-12} mol/l. Due to the production of hirudin through recombinant technology, this protein is now readily available in abundant amounts and hence it has become an interesting candidate as a new antithrombotic agent[3-6]. The therapeutic range of hirudin is roughly 0.5 – 2.5 µg/ml. Hirudin may be determined by ELISA methods, by clotting methods (APTT, thrombin time or Ecarin clotting time (ECT)) or by chromogenic methods[7,8]. The latter is insensitive to oral anticoagulants[9] and is suitable for automation. The ECT method is the most competitive of the clotting methods being essentially insensitive towards heparin. However it shows some sensitivity to changes in plasma concentrations of prothrombin and fibrinogen[9, 10]. The chromogenic methods described here may be used for the monitoring of hirudin levels in plasma samples. The same principle may be used for the potency assessment of hirudin preparations. In this case the potency of hirudin is expressed in thrombin inhibitory units (TIU)[11] and the international standard for thrombin must be used.

Measurement principle

Thrombin is added in excess to the sample.
Thrombin activity is neutralised in proportion to the amount of hirudin contained in the sample, and the remaining amount hydrolyses the chromogenic substrate S-2366.
The pNA released upon hydrolysis of the substrate is then monitored photometrically at 405 nm.

1. *Thrombin (excess) + Hirudin* ⟶ *[Thrombin•Hirudin] + Thrombin (residual)*

2. *S-2366* —*Thrombin (residual)*→ *Peptide + pNA*

Reagents

1. S-2366, 25 mg Art. No. S821090
Reconstitute with 20 ml sterile water.

2. Thrombin Art. Nos. EZ006A/B/O/K or DPGBT-1/10
Reconstitute with sterile water to obtain a concentration of 1.4 NIH-U/ml or 35 nkat/ml. Solution stable 4 wks at 2-8°C

3. Polybrene® (Sigma, H-9268)
Dissolve the substance with water to obtain 1 mg/ml.

4. Tris EDTA Buffer Art. No. AR015B
10 ml stock solution
Buffer containing 0.5 mol/l Tris pH 8.4, 1.5 mol/l NaCl, 70 mmol/l Disodium-EDTA. An opened vial is stable for 2 months at 2-8°C. Before use, dilute 10 ml of the stock solution with 88.7 ml sterile water. Add 1.26 ml polybrene solution.

5. Hirudin Art. Nos. SC020K or SC020L
Prepare a stock solution of 500 µg/ml hirudin.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 minutes at 20-25°C. Separate plasma carefully from the blood cells.

Standard and Sample Dilutions

Standards
Low range (0 – 2 µg/ml)

	Predilution		Final dilution	
Hirudin µg/ml	Hirudin Stock µl	Water µl	Diluted Hirudin µl	Plasma µl
2.0	200	300	10	1000
1.5	150	350	10	1000
1.0	100	400	10	1000
0.5	50	450	10	1000
0	-	-	-	1000

High range (0 – 10 µg/ml)

Hirudin µg/ml	Hirudin Stock µl	Plasma µl
10.0	20	1000
7.5	15	1000
5.0	10	1000
2.5	5	1000
0	-	1000

Samples and Standards

Dilution	Low dose range	High dose range
Sample/Standard	50 µl	25 µl
Buffer	800 µl	2000 µl

Microplate Assay Procedure

	Sample	Blank
Sample/Standard	50 µl	50 µl
Incubate at 37°C	3-4 min	-
Thrombin (20-25°C)	50 µl	
Incubate at 37°C	2 min	-
S-2366 (37°C)	50 µl	
Incubate at 37°C	2 min	-
Acetic acid, 20%	50 µl	50 µl
Water	-	100 µl

Read the absorbance at 405 nm, using a reference wavelength of 490 nm. The colour is stable for at least 4 hours.
Subtract the absorbance for the blank from the absorbance of the corresponding standards and test plasma sample.
Plot the corrected absorbances for the standards against hirudin concentrations in a lin-lin graph and draw the standard curve from linear regression. The concentration of hirudin in the test sample is calculated from the standard curve.

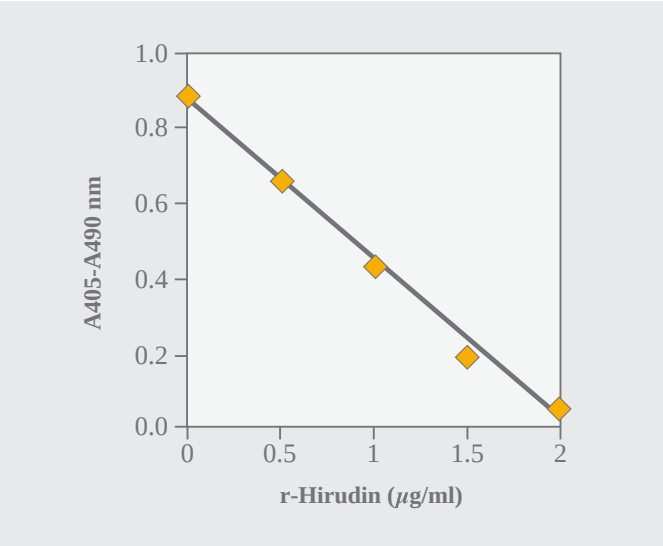

Fig. 1. Low range standard curve with the microplate method. Recombinant hirudin was used.

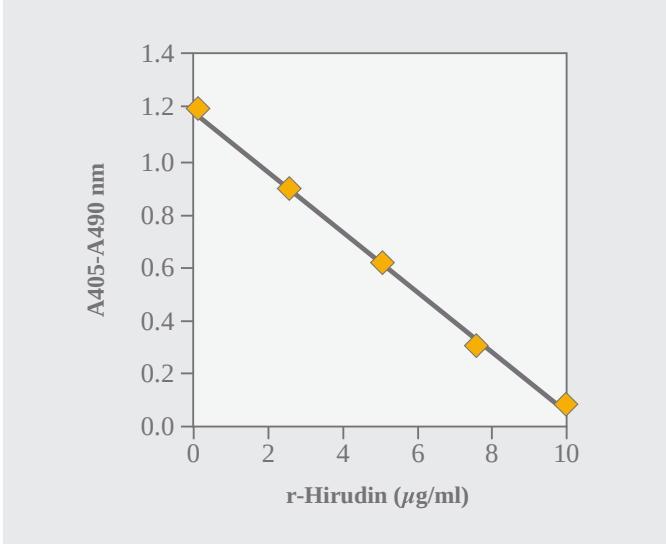

Fig. 2. High range standard curve with the microplate method. Recombinant hirudin was used.

Bibliography

1. Markwardt F. Untersuchungen über hirudin. Naturwissenschaften 42; 537-538 (1955).
2. Markwardt F. Hirudin as an inhibitor of thrombin. Meth Enzymol 19; 924-931 (1970).
3. Markwardt F, Hauptmann J, Nowak G, Klessen C, Walsmann P. Pharmacological studies on the antithrombotic action of hirudin in experimental animals. Thromb Haemost 47; 226-229 (1982).
4. Walenga J, Pifarré R, Fareed J. Recombinant hirudin as an antithrombotic agent. Drugs of the Future 15; 267-280 (1990).
5. Johnson PH, Sze P, Winant R, Payne PW, Lazar JB. Biochemistry and genetic engineering of hirudin. Semin Thromb Haemost 15; 302-315 (1989).
6. Märki WE, Grossenbacher H, Grutter MG, Liersch MH, Meyhack B, Heim J. Recombinant hirudin: genetic engineering and structure analysis. Semin Thromb Haemost 17; 88-93 (1991).
7. Lindhoff-Last E, Mosch G, Rabe FW, Schenk J, Breddin HK. Chromogenic substrate assay for automatic determination of high dose and low dose hirudin levels in plasma. GTH (Gesellschaft für Thrombose- und Hämostaseforschung), 1993.
8. Lindhoff-Last E, Bauersachs R, Mosch G, Piechottka GP, Rabe FW, Ehrly AM. A chromogenic method for the determination of hirudin in plasma. GTH (Gesellschaft für Thrombose- und Hämostaseforschung), 1999.
9. Lindhoff-Last E, Piechottka GP, Rabe F, Bauersach R. Hirudin determination in plasma can be strongly influenced by the prothrombin level. Trom. Res. 100, 55-60 (2000)
10. Nowak G. Monitoring of the action of antithrombin agents by Ecarin clotting time. In: New Anticoagulants for the Cardiovascular Patient. Ed Pifarré. Hanley and Belfus Inc. Philadelphia PA, USA (1997).
11. Longstaff C, Wong M, Gaffney PJ. An international collaborative study to investigate standardisation of hirudin potency. Thromb Haemost 69; 430-435 (1993).