

TFPI (Tissue Factor Pathway Inhibitor, type-I)

Determination of TFPI activity in plasma with S-2222 (microplate method)

Background

Human TFPI is a modular protein synthesized primarily by the vascular endothelium under normal physiologic conditions; small amounts are also expressed by monocytes and macrophages.

The regulatory activity of TFPI is directed towards the extrinsic initiation pathway of the coagulation cascade involving binding and direct inhibition of factor Xa. It also inhibits the Tissue Factor/ factor VIIa complex in a factor Xa dependent fashion. TFPI expression can be modulated in several cell types in response to various inflammatory stimuli. In vivo TFPI is distributed in three pools: 80-85% is associated with endothelial cell-surface, and it is released into plasma after injection of heparin; 10% circulates in plasma primarily in association with the lipoproteins and a small amount in free form, and 3% is found in platelets. TFPI levels are elevated in pregnancy and lower in the newborn than those in the adult, while recently a significant reduction was found in woman taking combined oral contraceptives, suggesting a potential underlying cause for an increased risk for thrombosis. The role of TFPI in several thrombotic, inflammatory and malignant conditions is already established.

Measurement Principle

TFPI activity assay is based on the ability of TFPI in the sample to inhibit TF/FVIIa catalytic activity, in presence of FXa. Plasma is incubated for a prolonged time, allowing the formation of inactive TF/FVIIa/TFPI/FXa complexes. Fibrin polymerization inhibitor, I-2882, is added to prevent formation of cross-linked fibrin. After first incubation, residual TF/FVIIa catalytic activity is determined by the addition of FX and a selective chromogenic substrate, S-2222.

Reagents

1. TBS/BSA/Polybrene® Buffer

(0.05 M Tris-HCL, 0.15 M NaCl, pH 7.5, supplemented with 2.0 mg/ml BSA and 2.0 µg/ml Polybrene®).

Dissolve 6.057 g Tris base, 8.766 g NaCl, 2.0 g BSA, and 2.0 mg Polybrene® in 900 ml H₂O, adjust the pH to 7.5 with 2 M HCl and top up to 1000 ml with H₂O

2. Acetic acid (50 % (V/V))

Add 125 ml 100 % Acetic acid to 125 ml H₂O

3. CaCl₂ (50 mM)

Dissolve 0.7351 g CaCl₂ · 2H₂O in 100.0 ml H₂O

4. I-2882 (10mg/ml) Art.No. 82 38 15 10

Dissolve the vial content (40 mg) with 4.0 ml H₂O

5. Factor Xa, bovine (7,1 nkat/ml) Art.No. 82 09 85

Dissolve the vial content (71 nkat) with 10.0 ml H₂O

6. Recombinant Human Tissue Factor

(RecombiPlasTin, Instrumentation Laboratory (Art.No. 49 73 27 50)

Dissolve the vial content with 5.0 ml diluent

7. Recombinant Factor VIIa (30 kIE/ml)

(NovoSeven, Novo Nordisk) Dissolve the vial content (240 kIE = 4.8 mg) with 8.5 ml diluent

8. Factor X, bovine (2 U/ml) Art.No. 82 22 39

Dissolve the vial content (2 U) in 1.0 ml H₂O

9. S-2222 (2.7 mM) Art.No. 82 03 16

Dissolve the vial content (25 mg) in 12.49 ml H₂O

10. HCl 2M

11. Normal plasma

Additional Material

Fridge or ice-bath

Thermostat at 37°C

Water bath at 37°C

Microplates, flat-bottom

Lids for microplates or Parafilm®

Spectrophotometer 405 nm

Storage Conditions

Store the Reagents 1 and 3-7 at 2-8°C or on ice until use it. Reagents 8 and 9 can be prepared during the first incubation step (30 min) and should have room temperature. All the reconstituted reagents can be used over a period of at least three weeks at 2-8°C. Avoid contamination by microorganism.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000xg for 20 minutes at 20-25°C. Separate plasma carefully from the blood cells. Plasma can be stored in aliquots at -70°C.

Standard Curve

1. Dilute Normal Plasma 1:10 with ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)
2. Dilute this 1:10 dilution (= 10 %) further to 7.5 %, 5.0 %, 3.75 %, 2.5 %, 1.25 %, 0.625 % and 0.313 % with ice cold TBS/BSA Buffer/Polybrene® (Reagent 1).

Note: an aliquot of 100 µl of each dilution (10 % - 0 %) is sufficient for the standard curve.

Sample Dilution

Normal Samples

10 µl Plasma + 390 µl ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)

Heparinized Samples

10 µl Plasma + 790µl ice cold TBS/BSA/Polybrene® Buffer (Reagent 1)

Preparation of the Combined Reagents

(sufficient for 1 plate)

1. Factor VIIa 10µl
2. Tissue Factor 125µl
3. Factor Xa 125µl
4. I-2882 125µl
5. CaCl₂ 2500µl
6. TBS/BSA Buffer 7115µl

Preparation of the Substrate Reagent

(sufficient for 1 plate)

1. Factor X 0.65 ml
2. TBS/BSA Buffer 1.95 ml
3. S-2222 2.60 ml

Microplate Assay Procedure

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|---|--------|
| | |
| Standard or Sample Dilution | 25 µl |
| Combined Reagent | 100 µl |
| Incubate the microplate covered with a lid for 30 min. at 37 °C | |
| Substrate Reagent | 50 µl |
| Incubate the microplate covered with a lid for 30 min. at 37 °C | |
| Acetic acidC | 50 µl |

Blank: For the Blank determined with the 10%-standard, 50 µl acetic acid are added to the wells before the Substrate Reagent.

Bibliography

1. A new sensitive Chromogenic substrate assay of tissue factor pathway Inhibitor type 1. Bjorn Bendez, Trine O. Andersen and per Morten Sandet. *Thrombosis research* 97 (2000) 463-472.
2. Total and free levels of tissue factor pathway inhibitor: a risk factor in pateients with factor V Leiden? P. van Dreden, M. Grosley and M. Cost. *Blood Coagulation and Fibrinolysis*, 1999; 10: 115-116.
3. Development of a method to separate lipoprotein-bound and lipoprotein-depleted tissue factor pathway inhibitor. Measurement of free tissue factor pathway inhibitor activity. F. Bridey, C. Lacombe, L. Sustendal, D. Moatti, F. Combe, O. Mammès and D. de Prost.
4. The 536C T. Transition in the human tissue factor pathway inhibitor (TFPI) gene is statistically associated with a higher risk for venous thrombosis. K. Kleesiek, M. Schmidt, C. Götting et al. *Thrombosis and Hemostasis* 1999; 82: 1-5.
5. Regulation of tissue factor initiated thrombin generation by the stoichiometric inhibitors tissue factor pathway inhibitor, antithrombin III, and heparin cafactor II. C. van't Veer and K.G. Mann. *The journal of Biological Chemistry* 1997; vol. 272 No 7, issue of february, pp. 4367-4377.
6. Tissue factor pathway inhibitor: from unknown coagulation inhibitor to major antithrombotic principle. A.K. Lindam. *cardiovascular research* 33 (1997) 286-291.
7. Tissue factor pathway inhibitor dose-dependently inhibits coagulation activation without influencing the fibrinolytic and cytokine response during human endotoxemia. E. de Jonge, P.E.P. Dekkers, A.A. Creasey, C.E. Hacr et al. *Blood*, 15 February 2000 - vol. 95, No. 4 (1124-1129).