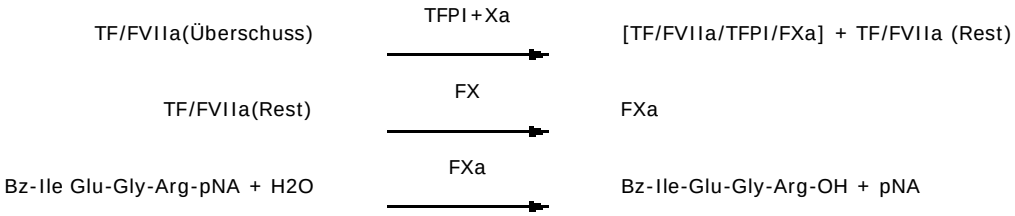
TFPI (Tissue Factor Pathway Inhibitor, type-I)

Determination of TFPI activity in plasma with chromogenic substrate BIOPHEN CS-11(22)

Measurement Principle

Human TFPI is a modular protein synthetized 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 new-born 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.

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, BIOPHEN CS-11(22).



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 H_2O, adjust the pH to 7.5 with 2 M HCl and top up to 1000 ml with H_2O

2. Acetic acid (50 % (V/V))
Add 125 ml 100 % Acetic acid to 125 ml H_2O

3. $CaCl_2$ (50 mM)
Dissolve 0.7351 g $CaCl_2$-$2H_2O$ in 100.0 ml H_2O

4. PefablocTH Thrombininhibitor (1,5 mg/ml) - Art. No. P381-01-5MG
Dissolve the vial content (40 mg) with 4.0 ml H_20

5. Factor Xa, bovine (7,1 nkat/ml) - Art.No. BE101O
Dissolve the vial content (71 nkat) with 10.0 ml H_20

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. BP102A
Dissolve the vial content (2 U) in 1.0 ml H 2 O

9. BIOPHENCS-11(22) (2.7 mM) - Art.No. 229015
Dissolve the vial content (25 mg) in 12.49 ml H 2 O

10. HCl 2M

11. Normal plasma - Art. No. 223602

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

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.

Preparation of the Combined Reagents

(sufficient for 1 plate)

1. Factor VIIa	10µl
2. Tissue Factor	125µl
3. Factor Xa	125µl
4. Pefabloc TH	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. CS-11(22)	2.60 ml

Method

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)

The test tube method or the Microplate method can be performed by the acid-stopped or the initial rate method.

Acid stopped method		
	A	B
Standard or Sample Dilution	50 µl	25 µl
Combined Reagent	200 µl	100 µl
Incubate at 37°C	30 min	30 min
Substrate Reagent	100 µl	50 µl
Incubate at 37°C	30 min	30 min
Acetic acid 20% or citric acid 2%	100 µl	50 µl

A: test tube method
B: microplate method

For the acid-stopped method: read the absorbance at 405 nm within 4 hours. If the plasma is icteric, hemolytic or lipemic, plasma blanks should be determined. Plasma blank is prepared by adding the reagents in reverse order starting with the acetic acid, without incubation. Subtract the absorbance of the blank from the absorbance of the corresponding sample.

For the initial rate method in test tubes: transfer sample immediately after addition of the substrate to a 1 cm semi-microcuvette (preheated at 37°C) for measurement of the absorbance change at 405 nm.

Calculation

Plot A or ΔA/min for the standards against their concentration of TFPI on linear graph paper. Read the TFPI value for the corresponding A or ΔA/min for the unknown test sample from the standard curve.

Bibliography

1. A new sensitive Chromogenic substrate assay of tissue factor patway 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 patway 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. Thrombosisi 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. Lindaml. 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).