Prothrombin

Method Sheet

Background

A number of studies during the last few years support the notion that venous thromboembolism (VTE) is a multifactorial disease most often triggered by circumstantial risk factors (trauma, surgery, pregnancy, oral contraceptives, immobilisation and age) in combination with one or more genetic or acquired coagulation disorders (see ref. 1 for a review). Elevated activity of prothrombin in the absence of a known underlying genetic disorder is also associated with an increased thrombotic risk (2). A mutation G → A in the untranslated 3'-region of the prothrombin gene at nucleotide position 20210 constitutes a risk factor for VTE with an odds ratio of 3-5 (2-10). About 90% of the carriers of this mutation have elevated levels (> 115%) of prothrombin activity (2, 7, 8). Levels above the upper limit of the normal range (75-130%) are common among hetero- and homozygotes (2, 7-9). So far, there is no explanation why a comparatively mild increase of prothrombin activity constitutes a risk factor for thrombosis and this is therefore an area of active clinical and biochemical research. Chromogenic methods for accurate determination of elevated activities of prothrombin and other coagulation factors, such as factor VIII (11, 12) are important tools for assessing the risk for VTE in patients and family members.

Measurement Principle

Prothrombin is activated to meizothrombin by the snake venom enzyme Ecarin from *Echis carinatus*. After a certain incubation time, the amount of meizothrombin formed is measured with the thrombin selective substrate S-2238, which also is cleaved by meizothrombin. The absorbance recorded at 405 nm is proportional to the prothrombin activity in the sample.

1. Prothrombin $\xrightarrow{\text{Ecarin}}$ Meizothrombin
2. S-2238 $\xrightarrow{\text{Meizothrombin}}$ pNA + peptide

Reagents

1. **Tris BSA Buffer** **Art. No. 82 35 18**
   20 ml stock solution
   Buffer for plasma sample dilution, containing 0.5 mol/l Tris•HCl pH 7.3, I = 2.0 with NaCl and 2% bovine serum albumin. An opened vial is stable for one month at 2-8°C. Before use, dilute the stock solution 1 + 9 with sterile water to obtain a buffer working solution. The buffer working solution should be prepared and used within the same day.

2. **Prothrombin Activator Diluent** **Art. No. 82 35 26**
   20 ml working solution
   Buffer for dilution of Ecarin, containing 0.05 mol/l Tris•HCl pH 7.6, I = 0.15 with NaCl, bovine serum albumin, polyethylene glycol and a fibrin polymerisation inhibitor.
   An opened vial is stable for one month at 2-8°C.

3. **Ecarin, 50 U (Sigma, E504)**
   Reconstitute with sterile water according to the Ecarin package insert. Freeze in suitable aliquots at -20°C or at -70°C. Stable for 3 months at both storage temperatures. Before use, dilute with Prothrombin Activator Diluent to obtain a concentration of 0.6 U/ml.
   Stable for 8 h at 20-25°C and for 1 week at 2-8°C.
   Note: *Echis carinatus* crude venom can also be used. A suitable final concentration of this reagent is approximately 5 µg/ml; however, this may vary between different sources. 10-20% loss of activity may occur upon freezing at -20°C.

4. **S-2238, 25 mg** **Art. No. 82 03 24**
   Reconstitute with 13 ml sterile water to obtain a 3 mmol/l solution. Stable for six months at 2-8°C.

Specimen Collection

Blood (9 volumes) is mixed with 0.1 mol/l sodium citrate (1 vol) and centrifuged at 2000 x g for 20 min at 20-25°C. Alternatively, freeze aliquots ≤ 1 ml at -20°C or below. Perform the analysis of frozen samples within two months when stored at -20°C or within one year when stored at -70°C or below. No significant loss of prothrombin activity occurs upon freezing or upon refreezing once, provided freezing is made in small aliquots (< 1 ml) and thawing is performed in a water bath or in an electric heater at 25-37°C.

Sample and Standard Dilutions

Standards
Calibrated normal plasma is diluted 1:23 -1:160 to provide standard concentrations of 25-175%. The following table provides a suggestion of standard dilutions.

Standard dilution	*Prothrombin activity*
1:23	*175%*
1:29	*138%*
1:40	*100%*
1:80	*50%*
1:160	*25%*

The standard dilution 1:40 corresponds to a nominal prothrombin activity of 100%. Calibration of the normal plasma should be made against a WHO International Standard or against the SSC Secondary Standard.

Samples
Plasma samples are diluted 1:40 in Tris BSA Buffer working solution for application on microplate and diluted 1:80 for application on ACL (see below).

Microplate Assay Procedure

Standard / sample dilution	50 μl
Incubate 2-4 min at 37°C	
Ecarin or *Echis carinatus* dilution, 37°C	50 μl
Incubate 3 min at 37°C	
S-2238, 3 mmol/l, 37°C	50 μl
Read kinetically or incubate 3 min at 37°C	
Acetic acid 20% or citric acid 2%	50 μl

Determine the absorbance difference A405-490 nm for the standard dilutions and the samples.
Draw a standard curve from the absorbances obtained for the standard dilutions.
Read the prothrombin activity for the samples from the standard curve.

Application on ACL

Use the plasminogen channel program. Prepare a standard dilution 1:40, which corresponds to a nominal prothrombin activity of 100% (see above regarding calibration). Standard dilutions corresponding to 25% and 50% are then automatically prepared by the instrument.
In order to allow determination of prothrombin activity up to 200%, sample plasma should be diluted 1:80 and the obtained result should be multiplied with two.

Expected Values

The normal range is 75-130% (mean 102% ± 2 SD) as determined from analysis in microplate and on the ACL 300 of 101 healthy individuals (49 men and 52 women; age range 20-68 years).
Analysis of plasma from 42 carriers of the G20210A mutation, who were not on oral anticoagulant treatment at the time of blood sampling, resulted in an activity range of 94-164% (mean 128% ± 2 SD).

Interference and Limitations

No influence in the assay is obtained from variation of antithrombin activity in the range 50-150% of normal.
Since meizothrombin is formed and measured, no influence in the assay is obtained from heparin levels ≤ 1 IU/ml plasma. since Ecarin also activates decarboxyprothrombin, which is produced during oral anticoagulant therapy with anti-vitamin K drugs, plasma from patients undergoing such treatment should not be analysed with this method.

Repeatability

The imprecision, expressed as CV, within and between series (7 series, 5 replicates in each series) is ≤ 4% at 50% and 100% prothrombin activity.

References

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