A New Enzyme Immunoassay for Soluble Fibrin in Plasma, with a High Discriminating Power for Thrombotic Disorders

R. Bos¹, G. H. Laterveer-Vreeswijk¹, D. Lockwood², K. Szewczyk², W. Nieuwenhuizen¹

From ¹TNO Prevention and Health, Gaubius Laboratory, Leiden, The Netherlands and ²Organon Teknika Corporation, Durham NC, USA

Summary

Fibrin formation is a multistep process initiated by thrombin. At first thrombin converts fibrinogen to fibrin molecules which in vivo form soluble complexes with fibrinogen. Soluble fibrin is considered to be an early biochemical marker for intravascular fibrin formation and impending thrombotic events, such as deep venous thrombosis (DVT), pulmonary embolism (PE) and disseminated intravascular coagulopathy (DIC).

A new enzyme immunoassay (EIA) was developed on the basis of a monoclonal antibody directed against a fibrin specific neo-epitope located on the gamma-chain of fibrinogen; γ-(312-324). In addition, it was possible to prepare a lyophilized reference material of thrombin-generated soluble fibrin, that allowed for full antigen recovery after reconstitution with buffer. Assay conditions, e.g. solid phase-Ig concentration and buffer composition, sample and conjugate dilution, and incubation times were optimised.

The present assay was found to be specific (no interference of homologous antigens) and reproducible (intra-assay CV 4-9%, inter-assay CV 4-9%), and therefore highly suited for measuring soluble fibrin levels in a plasma milieu. The median normal value for soluble fibrin was determined in plasma samples obtained from apparently healthy volunteers (n = 81) and found to be 0.040 µg/ml, with a range (10-90 percentiles) of 0.026-0.059 µg/ml.

A retrospective study showed that soluble fibrin levels were highly significantly increased in patients with a confirmed diagnosis of DIC as determined by the Mann-Whitney U-Test.

The aim of the present study was to develop a new quantitative enzyme immunoassay (EIA) for SF in plasma that is specific, reproducible and accurate, and has a high predictive value for thrombotic events.

Materials and Methods

Monoclonal Antibodies

The EIA for soluble fibrin is based on a fibrin-specific monoclonal antibody (mAb) which was described earlier (22). The mAb was generated against a synthetic peptide with the sequence of a fibrin-specific site (neo-epitope) on the fibrinogen gamma chain: γ-(312-324). Earlier, we showed that this neo-epitope is hidden in fibrinogen, but becomes exposed in fibrin and thus becomes accessible to the mAb (22). The selected mAb, of the IgM/k-isotype, is purified from conditioned serum free culture medium by gel permeation chromatography and used to sensitize microtiter plates (see below). Binding of antigen to immobilized anti-γ-(312-324) is detected by the use of a second mAb, denoted G8, labelled with horseradish peroxidase (G8/HRP). The mAb G8 binds to an epitope located in the carboxyl-terminal domain of the fibrin(ogen) (A)α-chain (23).

Reference Material: Calibrator

Soluble fibrin reference material is prepared in two stages essentially as described earlier (20) with minor modifications. First a diluted stock of desAABB-fibrin is prepared. To this end, plasma is diluted to a predetermined...
fibrinogen concentration, i.e. 40 μg/ml, in a buffer that prevents fibrin aggregation, i.e. 0.005 M phosphate, 0.1 M glycine, 0.25 M sodium bromide, 0.07 M D-mannitol and 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 7.4. Then thrombin (Sigma, St. Louis MO, USA) is added to a final concentration of 3 NIH U/ml and allowed to react for 60 min at 37° C. Afterwards, thrombin activity is inhibited with PPACK (Bachem, Bubendorf, Switzerland), at a final concentration of 15 μM. The thrombin-mediated conversion of fibrinogen is monitored by an EIA for intact fibrinogen (Organon Teknika, Boxtel, The Netherlands) (23). This stock material is kept at 2-8° C and used within 2 h. The SF concentration assigned is equal to the starting fibrinogen concentration, i.e. 40 μg fibrinogen equivalent units (FEU)/ml.

The final reference material or SF calibrator that is to be used in the assay, is prepared by further diluting the freshly prepared stock material to the desired final concentration of 1.0 μg FEU/ml in freeze-dry medium: 0.005 M phosphate, 0.1 M glycine, 0.07 M D-mannitol, pH 7.4. The material is aliquoted in siliconized glass vials and lyophilized. The effect of several additives, e.g. sodium bromide, bovine serum albumin (BSA; Boseral, Kordia, Leiden, The Netherlands) or hydrolysed porcine skin collagen (Prionex; Pentapharm, Basel, Switzerland), on the recovery of antigen upon reconstitution was investigated.

For use in the assay, SF calibrator is first reconstituted with 5 mM phosphate, 0.15 M sodium chloride and 0.1% (v/v) Tween 20, pH 7.4 (PBST) to yield a concentration of 1.0 μg FEU/ml, and further diluted to the desired final concentrations with sample diluent (see below). A typical reference curve is prepared from serial two-fold dilutions and runs from 0.0125 to 0.20 μg FEU/ml (12.5 to 200 ng FEU/ml).

Specimen Collection and Preparation

Normal plasma samples were collected from apparently healthy volunteers after informed consent. Plasma samples from patients diagnosed as having DVT, DIC or PE were obtained from the University of North Carolina (Chapel Hill, NC), Clinisys Associates (Decatur, GA) and McMaster University (Hamilton, Canada). The presence of DIC was confirmed by general clinical laboratory evidence, i.e. prothrombin time, D-dimer antigen, antithrombin consumption or protamine sulphate-induced para-coagulation; episodes of DVT and PE were confirmed by ventilation and perfusion (V/Q) lung scanning, pulmonary angiography or trans-esophageal ultrasound.

Blood samples were collected by venepuncture into sodium citrate (0.11 M = 3.2% w/v) Vacutainers (Becton Dickinson, Meylan, France). After centrifugal separation of cells, the plasma was aliquoted in cryovials, frozen and stored at -70° C until further use. To prevent potential interference of cryo-precipitates, frozen samples were thawed thoroughly in a 37° C waterbath and mixed well prior to use.

**Fig. 1** Typical dose-response curve of SF reference material. Duplicate measurements are shown as determined in the present EIA. A curve was fitted assuming a 2nd-order polynomial relationship between SF antigen concentration (abscissa) and final response in the test (ordinate).
concentration, i.e. 100 μg FEU/ml, to either buffer or to plasma samples with 'low' and 'high' SF levels and their effect on the outcome of the EIA was determined.

Intra- and Inter-assay Variation

To determine the intra-assay (within-run) variation of the test, a 'low' and a 'high' SF sample were appropriately diluted and tested as 8 replicates on random locations in the microtiter plate. The inter-assay (run-to-run) variation was determined by measuring the same 'low' and 'high' SF plasma samples at the appropriate dilutions as 4 replicates in 4 consecutive and independent test runs. SF concentrations in each test were read from simultaneously run and independently prepared reference curves. Variability data were calculated on the basis of returned SF values as mean, standard deviation (SD) and coefficient of variation (CV).

Statistical Analysis

Soluble fibrin levels in healthy volunteers and in patients (DIC, DVT, PE) were analysed by non-parametric statistical analysis. For individual groups the median SF value and range (10-90 percentiles) were calculated, the significance of differences between healthy volunteers the respective patient groups was determined by the Mann-Whitney-U test.

Results

Reference Material; Calibrator

To prepare soluble fibrin reference material, plasma was first diluted in buffer to a final fibrinogen concentration of 40 μg/ml. Next, all fibrinogen was converted to (soluble) fibrin by incubating it with excess thrombin for 60 min at 37°C. Using an EIA for intact fibrinogen we were not able to detect residual fibrinogen (<0.01% of initial value) indicating that all fibrinogen was converted by the action of thrombin. Upon dilution to the appropriate concentrations, the material yielded a clear dose-response curve in the EIA for SF (Fig. 1). Experiments with lower amounts of thrombin, resulted in preparations with substantial amounts of residual fibrin, which negatively correlated with the relative response in the EIA for SF (not shown). The presence of both sodium bromide and EDTA in the buffer used to dilute the plasma, were shown to be essential for antigen recovery, since the absence of either yielded a preparation with little or no response in the EIA for SF (not shown), probably since a substantial part of the SF generated aggregated and/or was FXIIIa cross-linked, thus decreasing its solubility.

Next, attempts were made to prepare a lyophilized SF calibrator. For this, freshly prepared reference material was diluted to the desired final SF concentration, i.e. 1000 ng FEU/ml in freeze-dry medium, aliquoted and lyophilized. After reconstitution with an equal volume of PBST, the relative antigen recovery, as compared with freshly prepared, non-lyophilized SF calibrator (100% control), was assessed in the EIA for SF. Without any supplements to the freeze-dry medium, approximately 30% of SF antigen was recovered upon reconstitution. The presence of the relatively mild chaotropic agent sodium bromide, instrumental in the production of stock calibrator, had no effect on antigen recovery after reconstitution. The addition of 1.0% (w/v) BSA, a generally applied protein stabiliser, was shown to be detrimental to SF antigen recovery, since the relative response decreased to <10% after reconstitution. However, adding 1.0% (w/v) Prionex (partially hydrolysed porcine dermal collagen) was shown to be beneficial to SF at lyophilisation, since 100% antigen was recovered upon subsequent reconstitution.

Table 1 Analytical specificity; cross-reactivity

<table>
<thead>
<tr>
<th>sample</th>
<th>control</th>
<th>+ FbDP</th>
<th>+ FgDP</th>
<th>+ EDF</th>
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<td>buffer</td>
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<td>'low' SF plasma</td>
<td>0.123</td>
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<td>0.123</td>
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<td>'high' SF plasma</td>
<td>1.110</td>
<td>1.105</td>
<td>1.089</td>
<td>1.187</td>
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Effect of potentially cross-reacting homologous antigens, i.e. plasmin- and elastase-mediated fibrinogen (fibrin) degradation products, when added to a final concentration of 100 μg FEU/ml, on the outcome of various samples in the present EIA for soluble fibrin.

Analytical Performance

Recovery. One important aspect for the verification of the analytical specificity of any immunoassay, is full recovery of antigen added to the matrix of choice, independent of sample dilution (linearity). For the present test, citrated plasma was spiked at various levels with freshly prepared SF stock material. Recovered SF antigen levels were determined over a range of appropriate dilutions, reading SF concentrations from a simultaneously run reference curve. After linear regression of the final data, i.e. spiked against measured SF levels, the slope (a) of the resulting line (y = ax + b) indicates antigen recovery and should read about 1.0. The intercept with the ordinate (b) reflects the background SF value of the original plasma used to spike into and the coefficient of regression (r²) indicates linearity and should be close or equal to 1.0. It was shown that all SF antigen spiked into plasma was recovered, since theoretical and determined SF values were in full agreement (Fig. 2). These findings demonstrated that the assay is specific, since it allows for the determination of SF levels in a plasma background over a wide range of SF concentrations and the result is independent of sample dilution. In addition, such spiked plasma samples, after aliquoting and lyophilisation, also yielded full antigen recovery upon reconstitution in PBST (not shown), indicating that these samples may be suitable as a source of control plasmas.

Specificity. To study the potential interference of homologous antigens, FbDP, FgDP and EDF were added at 100 μg FEU/ml to buffer (PBST) or 'low' and 'high' SF plasma samples. The response of spiked and non-spiked (control) samples was determined in the EIA for SF (Table 1). In buffer, neither FgDP nor EDF yielded any notable response; only FbDP showed minor cross-reactivity (± 0.2% on a weight basis), possibly caused by residual non-degraded fibrin in the lysate used. In agreement with this, neither FgDP nor EDF had any apparent effect on SF readings when spiked to a 'low' SF sample, whereas FbDP resulted in a slightly higher reading as compared to the control. When added to a 'high' SF plasma sample, none of the preparations had any apparent effect on the assay outcome. These findings demonstrate that the presence of these related antigens in a

Table 2 Analytical specificity; reproducibility

<table>
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<th>sample</th>
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<th>inter-assay variation</th>
</tr>
</thead>
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<tr>
<td></td>
<td>mean SF</td>
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<td>low SF</td>
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<tr>
<td>high SF</td>
<td>1.205</td>
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</table>

Within-run and run-to-run variability of the present EIA for SF. Values for mean and SD are in μg FEU/ml.
plasma sample do not compromise the specificity of the test; they neither generate a response nor inhibit the final readings.

Reproducibility: In addition, assay-variability was determined. For the intra-assay (within-run) variation of the test, a ‘low’ and a ‘high’ SF sample were tested as 8 replicates. For the inter-assay (run-to-run) variation the same ‘low’ and ‘high’ SF plasma samples were tested as 4 replicates in 4 consecutive and independent test runs. SF concentrations in each test were read from simultaneously run and independently prepared reference curves. The intra-assay (within-run) variation of the test for a ‘low’ and a ‘high’ SF sample was 5.2% and 5.4%, respectively (Table 2). The inter-assay (run-to-run) variation of the test for a ‘low’ and a ‘high’ SF sample was 9.6% and 4.3%, respectively (Table 2).

Clinical Evaluation

Soluble fibrin levels were determined in plasma samples obtained from apparently healthy volunteers (‘normals’) and in various patient groups with a confirmed diagnosis of thrombotic disorders such as DIC, DVT and PE (Fig. 3). The median normal value for SF was found to be 0.040 μg FEU/ml (n = 81), with a relatively narrow distribution range (10-90 percentiles 0.026-0.059 μg/ml) (Table 3). Compared to the levels in the normal reference population, all three patient groups investigated had highly significantly increased SF levels as determined by the Mann-Whitney-U test (Table 3). Patients with a confirmed diagnosis of DIC (n = 21) yielded a median SF level of 1.042 μg FEU/ml (range 0.160-2.319 μg/ml), P < 0.0001 vs normals. Patients with a confirmed diagnosis of PE (n = 29) had a median SF level of 0.527 μg FEU/ml (range 0.084-1.234 μg/ml), P < 0.0001 vs normals. And patients with a confirmed diagnosis of DVT (n = 36) had a median SF level of 0.126 μg FEU/ml (range 0.059-0.878 μg/ml), P < 0.0001 vs normals.

Discussion

We have demonstrated here that the present test is an accurate and reproducible EIA for the specific assessment of soluble fibrin (SF) in plasma samples. The assay has a high discriminating power for thrombotic disorders, since confirmed patient groups yield highly significantly increased SF values as compared to healthy volunteers. The intra- and inter-assay coefficients of variation are low at both low and high antigen levels. Moreover, the analytical specificity of the test is high. As a result of the design of the test, homologous antigens such as fibrinogen degradation products do not notably interfere, since they lack the epitopes for either the solid-phase anti-γ-(213-324) and/or tagging G8/HRP antibodies.

The discussions around the quantitative determination of SF in plasma, are obfuscated by the heterogenous appearance of the analyte in vivo as a result of (local) variations in thrombin, FXIIa- and plasmin activity. It has been shown that in the plasma collected from DIC patients, SF occurs in complex with a variety of fibrinogen derivatives including intact fibrinogen, early and late fibrin degradation products, partly cross-linked by FXIIIa (28, 29). It is conceivable that the relative concentrations of the various fibrinogen derivatives within the complex are subject to major changes, possibly correlated with the disease state. That in turn may affect the outcome of SF immunnoassays as a result of the (combined) specificities of the mAbs employed.

Similar difficulties exist for alternative biochemical plasma markers often used for the diagnosis of thrombotic disorders, such as D-dimer, the prothrombin activation peptides F1.2 and thrombin-antithrombin (TAT) complexes (5, 6, 7, 9, 10, 18, 30). D-dimer reflects the plasmin-mediated proteolysis of FXIIa-cross linked fibrin; i.e. the combined effects of coagulation and fibrinolysis. Occasionally, a coagulopathy is correlated with an impaired fibrinolysis, rendering D-dimer a less suitable marker for thrombotic disorders of this nature. Similarly, the occurrence of F1.2 and TAT-complexes may only reflect (systemic) thrombin generation and not factual thrombin activity in vivo. In contrast, SF truly reflects thrombin generation and activity in vivo.

Extensive clinical studies will show which tests are, or which combination of tests is the most informative in diagnosing thrombotic disorders.

A major problem in the development of a reference material and kit calibrator for SF assays, is the ‘transient’ character of soluble fibrin which has a natural tendency to form insoluble aggregates. To keep the fibrin molecule in solution after its generation in vitro, typically chaotropic ions and/or sugars in high concentrations are employed and cross-linking by FXIIa is prevented by the addition of calcium chelating agents, e.g. EDTA. Alternatively, a different form of fibrin, i.e. desAA-fibrin, is generated by the use of snake venom-derived enzymes (14, 15, 17, 20). The latter strategy results in a functionally different and possibly also immunogenically different species of SF as compared to that generated by thrombin in vitro and in vivo.

In this study, it was demonstrated that it is possible to develop a thrombin-generated reference material or SF calibrator that is convenient to use; it may be lyophilized and allows for full antigen recovery upon reconstitution. For this, the presence of Prionex appeared to be instrumental. Moreover, the fibrinogen concentration of the plasma used as starting material can be measured accurately, e.g.

![Graphic representation of the distribution of soluble fibrin levels as determined in the plasma of healthy volunteers (norm) and patients with a confirmed diagnosis of disseminated intravascular coagulopathy (DIC), deep venous thrombosis (DVT) and pulmonary embolism (PE). Solid lines represent the median SF value of the respective group. Note the log-scale of the y-axis.](image-url)
gravimetrically. Since the conversion of the fibrinogen into fibrin is complete, an accurate value for SF can be assigned to the final reference material, and the concentration of SF in the calibrator may be expressed in fibrinogen equivalent units (FEU).

It was shown that all SF antigen was recovered when in vitro generated SF reference material was spiked to a normal plasma, independent of the initial value and the dilution factor in the test. No apparent matrix effects occurred, indicating the proper linearity of the test. In addition, it was possible to lyophilize the spiked plasma samples and still fully recover the antigen upon reconstitution. These samples may thus function as a reliable source for a Qualifying Control Plasma Panel with predetermined SF concentrations.

The present study of retrospective design, compared the SF levels determined in apparently healthy volunteers and in selected patient groups with a confirmed clinical diagnosis of DIC, DVT and PE, respectively. Our results show that the assay has at least the potential to predict thrombotic disorders, mainly because of the relatively low values and narrow distribution range of SF levels within the normal population. It is interesting to note that other SF assays frequently report relatively high SF values in normal individuals, often well in the $\mu$g/ml range (14-20). These findings seem to conflict with what is observed for alternative markers for thrombotic activity, i.e., F$_2$-TAT-complexes and D-dimer. These are either very low or not detectable in the plasma of healthy individuals. Part of the discrepancy may be explained by assay conditions and/or lack of analytical specificity, e.g., cross-reactivity with other fibrinogen(ogen) derivatives besides SF. Moreover, these findings may be a result of the value assigned to the calibrator employed. As mentioned earlier, a soluble fibrin reference material is difficult to prepare and it is possible that recovery and immunoreactivity of the in vitro prepared material is relatively poor. As a result, levels of in vitro generated antigen in the plasma sample will be overestimated. Besides an extensive assay comparison with patient samples, it would be interesting to cross-test the calibrators of the various tests that are currently available.

As mentioned, patients with a clinically confirmed thrombotic disorder had SF plasma values that were highly significantly increased as compared to apparently healthy volunteers. It would therefore be most interesting to investigate the clinical utility and cost-benefit ratio of the present assay as a diagnostic tool in prospective or management studies for diagnosing specified patients with suspected thrombotic disorders. In addition, it would be worthwhile investigating the potential use for predicting thrombotic events in patients at risk, e.g., post-operative or long-term hospitalised patients, testing on admission and/or at regular intervals after hospitalisation.

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References


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