

# A Monoclonal Antibody-Based Quantitative Enzyme Immunoassay for the Determination of Plasma Fibrinogen Concentrations

E. Hoegge-de Nobel, M. Voskuilen, E. Briët\*, E. J. P. Brommer, and W. Nieuwenhuizen

From the Gaubius Institute TNO and the Academic Hospital\*, Leiden, The Netherlands

## Key words

Enzyme immunoassay – Fibrinogen – Monoclonal antibodies

## Summary

The most commonly used fibrinogen assays in the clinic are clotting rate assays, e.g. the Clauss method. Such functional assays may be disturbed by e.g. heparin, anticoagulant fibrinogen degradation products (FgDP) and in the case of a dysfibrinogenemia. Immunological methods would not suffer from these interferences. However, immunological assays for fibrinogen, which do not measure FgDPs, do not exist.

To set up such an enzyme immunoassay (EIA) we developed two monoclonal antibodies. The first monoclonal antibody (G8) has its epitope in the carboxyl-terminal 150 amino acid stretches of the fibrinogen A $\alpha$ -chains. G8 is used to coat the wells of microtitration plates, and is the capture antibody in this EIA. The second antibody (Y18) has been described by us previously (Blood 1985; 66: 503). It is directed against fibrinopeptide A, covalently bound to the  $\alpha$ -chains i.e. against the amino-terminal stretches of the A $\alpha$ -chains. Y18 is conjugated with horse-radish peroxidase, and used as tagging antibody.

The EIA does not react with, and is not interfered by FgDP such as purified fragments X and Y, up to a concentration of 800  $\mu$ g/ml. An FgDP mixture such as generated by Streptokinase treatment of plasma does not respond. Fibrin degradation products (whole blood lysate) up to 800  $\mu$ g/ml do not interfere nor do heparin, EDTA or oxalate. The time-to-result of the EIA is only 45 minutes. Some patient plasmas yielded dose-response curves which are not parallel with the calibration curve of the EIA. An explanation for this phenomenon could not be given. Our fibrinogen EIA may be especially suitable to monitor patients with conditions which favour proteolytic damage to fibrinogen such as thrombolytic therapies.

## Introduction

Fibrinogen determinations are frequently included in routine patient screening protocols in the clinic. In recent years the interest in plasma fibrinogen levels is growing, since these levels are related to the extent of coronary atherosclerosis (1, 2) in patients with manifest coronary heart disease, and since elevated fibrinogen levels are a risk factor in initially healthy subjects (3–6). The fibrinogen level has a prognostic significance comparable with that of serum cholesterol level and hypertension. Recently (7), substantial genetic control of the fibrinogen level has been shown.

Fibrinogen is a symmetrical glycoprotein, composed of three pairs of polypeptide chains: two A $\alpha$ -, two B $\beta$ - and two  $\gamma$ -chains, held together by disulphide bridges. Fibrinogen is highly susceptible to proteolysis. One of the very first events, when fibrinogen is attacked by proteases is the cleavage of considerable stretches from the carboxyl-terminal ends of the A $\alpha$ -chains. The heterogeneity of normal human plasma fibrinogen, described in the literature, may be the result of limited proteolysis in vivo. In plasma, three main fibrinogen fractions can be demonstrated with molecular weights 340 kD, 300 kD and 280 kD. These have been designated as the high molecular weight (HMW), the low molecular weight (LMW) and LMW' fractions, respectively.

HMW, LMW and LMW' are also known as fibrinogen bands I, II and III (11) or FI, FII and FIII (12). HMW, LMW and LMW' represent approximately 70%, 26% and 4% of the total fibrinogen. The HMW has been reported to have both A $\alpha$ -chains intact, LMW only one, and in LMW' both A $\alpha$ -chains have been proteolysed at the carboxyl-terminal ends (12, 13), i.e. 96% of all fibrinogen molecules have at least one intact A $\alpha$ -chain. The clottabilities of HMW, LMW and LMW' are 98%, 92% and 80%, respectively.

Many methods exist for the determination of fibrinogen levels. Amongst the clottable protein methods (14–19), the method described by Clauss (19) is the most commonly employed. Under the conditions of the assay, fibrinogen is the rate limiting factor in thrombin-induced clot formation. The concentrations of fibrinogen are then read from a calibration curve obtained with serial dilutions of plasma with a known concentration of fibrinogen. The clottable protein methods have several drawbacks. The Clauss method for instance may be disturbed by anything that affects the rate of fibrin formation such as the presence of anticoagulant plasmin-generated fibrinogen degradation products e.g. fragments X (20) and Y (21), heparin or an abnormal fibrinogen molecule. The fibrinogen assays which measure the amount of clotted protein after prolonged incubation with thrombin instead of the clotting rate, do not discriminate the (clottable) fibrinogen fragments X.

Several precipitation procedures have been described (e.g. 22–25). These are, however, somewhat less suitable for clinical use, and very few laboratories use these methods.

Existing quantitative immunological assays for fibrinogen (26–28) are not used by many investigators. Most are technically complicated, time consuming and also measure fibrinogen degradation products.

In this paper we describe a monoclonal antibody-based enzyme immunoassay (EIA) for fibrinogen. The EIA is based on a capture antibody which has its epitope in the carboxyl-terminal stretches of the A $\alpha$ -chain, and a tagging antibody, specific for fibrinopeptide A covalently linked with the fibrinogen A $\alpha$ -chain. As a result of specificities of the two used monoclonal antibodies, the EIA is specific for fibrinogen molecules having A $\alpha$ -chains which are intact both at their amino-terminal and carboxyl-terminal ends.

Correspondence to: Dr. W. Nieuwenhuizen, Gaubius Institute TNO, Post Box 612, 2300 AP Leiden, The Netherlands

## Materials and Methods

### Materials

Microtiter plates (Immulon, Dynatech) were purchased from Greiner (Alphen a/d Rijn, The Netherlands); 3,3',5,5'-tetramethylbenzidine (TMB) from Fluka (Buchs, Switzerland); N-succinimidyl-3-(2-pyridyl-dithio)propionate (SPDP) and protein A Sepharose from Pharmacia (Uppsala, Sweden); dimethylsulphoxide (DMSO) was bought from Baker Chemical Company (Philipsburg, USA); horse-radish peroxidase grade II (HRP) from Boehringer (Mannheim, FRG); Aprotinin solution (Trasyol) from Bayer (Leverkusen, FRG) and thrombin from Leo Pharmaceutical Products (Ballerup, Denmark).

Fibrinogen was purified as described before for rat fibrinogen (29).  $\text{A}\alpha$ -,  $\text{B}\beta$ - and  $\gamma$ -chains were separated and purified as described by Doolittle et al. (30). Fragments X and Y were prepared and purified as described by Nieuwenhuizen et al. (20, 21). A mixture of fibrin degradation products (FbDP) in a serum milieu was prepared by lysis of a whole non-anticoagulated blood clot with tissue-type plasminogen activator (t-PA) as described by Koopman et al. (31). A mixture of fibrinogen degradation products in plasma milieu was prepared by treatment of plasma with streptokinase (37).

Phosphate-buffered saline/Tween (PBST) was prepared by dissolving 1.4 g  $\text{Na}_2\text{HPO}_4$ , 0.215 g  $\text{KH}_2\text{PO}_4$ , 8.75 g NaCl and 0.5 ml Tween 20 in 1 liter of distilled water.

Plasma dilution buffer was prepared by dissolving 1.4 g  $\text{Na}_2\text{HPO}_4$ , 0.215 g  $\text{KH}_2\text{PO}_4$ , 8.75 g NaCl, 1 ml Tween 20 and 300 g urea in 1 liter of distilled water. This buffer is designated as PBSTU.

TMB/ $\text{H}_2\text{O}_2$  substrate solution was prepared as follows: 0.1 ml of a 42 mM solution of TMB in DMSO was added to 10 ml of a 0.1 M sodium acetate/citric acid buffer, pH 6.0, under constant agitation. Just before use 1.5  $\mu\text{l}$   $\text{H}_2\text{O}_2$  (30% v/v) was added.

### Methods

#### Production of Monoclonal Antibodies Y18 and G8

The production and characterization of Y18 has been described by us in detail before (32). Y18 reacts with fibrinogen and with fibrinopeptide A-containing fibrinogen fragments, but not with free fibrinopeptide A.

Monoclonal antibody G8 was produced by injecting Balb/c mice intraperitoneally with 25  $\mu\text{g}$  pure  $\text{A}\alpha$ -chain in complete Freund's adjuvant and then at 2-week intervals with 25  $\mu\text{g}$   $\text{A}\alpha$ -chain in Freund's incomplete adjuvant. Four days before fusion 10  $\mu\text{g}$   $\text{A}\alpha$ -chain in 0.5 ml 0.4 M urea was injected intravenously. On the day of fusion the immunized mice were killed in ether vapor, and their spleen cells harvested. Spleen cells ( $12 \times 10^7$ ) were fused with ( $2 \times 10^7$ ) non-producing SP2/OAG14 myeloma cells in the presence of 40% polyethylene glycol. Growth and selection media were essentially as described by Köhler and Milstein (33). For screening microtiter plates coated (by passive adsorption) with purified  $\text{A}\alpha$ -chain,  $\text{B}\beta$ -chain or  $\gamma$ -chain were used. Occasionally also plates coated with fragments X or Y were used. Culture supernatants were pipetted into the wells of these coated plates and incubated. Then the plates were washed and incubated with rabbit-anti-mouse Ig, conjugated with HRP. The amount of HRP bound to the plates was eventually quantitated using TMB/ $\text{H}_2\text{O}_2$  as a substrate as described under "standard EIA procedure". From our previous work (32) we know that our purified  $\text{B}\beta$ -chain preparation contains partially degraded  $\text{A}\alpha$ -chain, which comigrates with  $\text{B}\beta$ -chain upon SDS-polyacrylamide gel electrophoresis. The criteria for selection were: a positive reaction with intact  $\text{A}\alpha$ -chain, and no reaction with the  $\text{B}\beta$ -chain preparation (with partially degraded  $\text{A}\alpha$ -chain) and  $\gamma$ -chain. Occasionally we also checked for a negative reaction with purified fibrinogen fragments X and Y.

Cell lines producing antibodies with the required specificity were subcloned twice by limiting dilution (0.5 cells/well) as described by McKearn (34).

One cell line (G8) was selected which produces monoclonal antibodies that react strongly with intact  $\text{A}\alpha$ -chains and not with partially degraded  $\text{A}\alpha$ -chains, intact  $\text{B}\beta$ -chains or  $\gamma$ -chains, or with fibrinogen fragments X and Y. This cell line was injected into the peritoneal cavity of pristane-primed Balb/c mice for the production of ascites. G8 is an  $\text{IgG}_{1\text{K}}$  as assessed by immunoelectrophoresis according to Radl (35).

G8 was purified from ascites on protein A Sepharose CL-4B as described by Ey et al. (36). Elution was done with 0.1 M sodium citrate, adjusted to pH 4.0 with 0.1 M citric acid solution. Purified G8 was diluted with 0.04 M Tris buffer, pH 7.4 to a concentration of 10  $\mu\text{g}/\text{ml}$ .

#### HRP Conjugate of Y18

The purification and conjugation of Y18 (an  $\text{IgM}_{\text{K}}$ ) has been described by us in detail before (32, 37).

#### Calibration Material

Pooled fresh normal human plasma was used as a standard. The fibrinogen concentration was determined, using the gravimetric method, described by Astrup et al. (38).

#### Standard EIA Procedure

Aliquots of 135  $\mu\text{l}$  of a 10  $\mu\text{g}/\text{ml}$  solution of purified G8 in 0.04 M Tris/HCl, pH 7.4 were pipetted into the wells of microtitration plates, and incubated for 16 hours at 4° C. Plates were washed with PBST immediately before use. Eight-fold diluted plasma, and further 2-fold dilutions thereof in PBSTU (100  $\mu\text{l}$  aliquots) were pipetted into the wells of the G8-coated wells and incubated for 15 minutes at 37° C. After four washes with PBST (250  $\mu\text{l}/\text{well}$ ), the wells were filled with 100  $\mu\text{l}$  of Y18/HRP conjugate in PBST, and incubated for 15 minutes at 37° C. The wells were washed four times with PBST and 100  $\mu\text{l}$  aliquots of TMB/ $\text{H}_2\text{O}_2$  substrate solution were added. After 10 minutes at 37° C a blue colour has developed, which turns to yellow when the reaction is stopped by the addition of 100  $\mu\text{l}$  1 M  $\text{H}_2\text{SO}_4$ . The intensity of the colour is read at 450 nm with a Titertek multiscan (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland) and is dependent on the amount of fibrinogen in the plasma samples. The calibration curves are prepared using serial two-fold dilutions of pooled normal plasma with known fibrinogen concentrations. The fibrinogen concentration range for the calibration curves was chosen from 0 to 0.3 mg/ml.

## Results and Discussion

### Specificity of the Assay

The capture antibody G8 of our EIA for fibrinogen reacts with intact  $\text{A}\alpha$ -chains, and not with our preparations of purified  $\text{B}\beta$ -chain. We have demonstrated before (32), that our  $\text{B}\beta$ -chain preparations contain some  $\text{A}\alpha$ -chain, which co-migrates with  $\text{B}\beta$ -chain upon SDS-PAGE and still contains its fibrinopeptide A. We concluded that this form of  $\text{A}\alpha$  has been proteolysed at its carboxyl-terminal end (32). Since the intact  $\text{A}\alpha$ -chains and  $\text{B}\beta$ -chains are 610 and 461 amino acids long, respectively, the partially degraded  $\text{A}\alpha$ -chain in our  $\text{B}\beta$ -chain preparation, has lost approximately 150 amino acids at its carboxyl-terminal end. As a consequence, the epitope of G8 must be in the 150 amino acid residue carboxyl-terminal stretch of the intact  $\text{A}\alpha$ -chain. Y18, the tagging antibody of our EIA, is specific for fibrinogen and fibrinopeptide A-containing fibrinogen fragments i.e. for the amino-terminal part of the  $\text{A}\alpha$ -chain. It is expected that, on the basis of the specificities of G8 and Y18, the EIA will only react with fibrinogen with  $\text{A}\alpha$ -chain intact at both ends, and not with fibrinogen degradation products such as fragment X and Y. This is indeed the case.

Virtually no reactivity is seen for plasma treated *in vitro* with streptokinase, at an eight-fold dilution in our EIA. This streptokinase-treated plasma has a strong reactivity in our EIA, specific for fibrinogen degradation products, as we have shown before (37). The Clauss method showed a "fibrinogen" level of only 0.5% of the original value. The gravimetric determination showed a fibrinogen level of 42% of the original value. This indicates that virtually no intact fibrinogen is left, but that approximately half of the proteolysed molecules is still clottable.

A mixture of fibrin degradation products (see Materials) upto a concentration of 800 µg/ml, and fragments X and Y at a concentration of 800 µg/ml do not interfere in the fibrinogen EIA, when they are added to pooled normal plasma (results not shown).

When heparin, EDTA, oxalate or citrate are used as plasma anticoagulants, the same dilution curve as that depicted in Fig. 1 is obtained.

As shown in Fig. 2, the dose-response curves obtained with patient plasmas are parallel with the dose-response curve of normal pooled plasma.

#### Sensitivity of the Assay

The early versions of our fibrinogen EIA were too sensitive for routine use. In those versions we used PBST as plasma diluent. As a consequence of the high sensitivity in that milieu, plasma samples had to be diluted 4,000-fold, and this would make the assay less accurate in routine use. This was the reason to use PBSTU instead of PBST. The combined effects of 0.1% Tween 20 and 5 M urea suppress the immune reaction in the capture step of the EIA, and twenty-fold diluted plasma samples can be used.

For special purposes, when very low fibrinogen concentrations are anticipated e. g. in culture media of hepatocytes, PBSTU can be replaced by PBST in the standard EIA procedure described above.

#### Methodological Variation

The intra-assay variation of this EIA was determined from 12 dose-response curves of pooled normal plasma. For each curve the response was determined at 5 dilutions. Two microtitration plates were used. The standard deviation in the response varied with the used plasma dilution, and was between 0.022 and 0.052. This corresponds with a coefficient of variation in fibrinogen concentration (as read from the curve) between 3% and 7%. Blank values were low i.e. 0.051 with a standard deviation of 0.006.

The run-to-run variability was studied in a panel of one 30-fold diluted plasma and one 6-fold diluted plasma (each in triplicate). Each sample was measured on 4 different days. Between-run

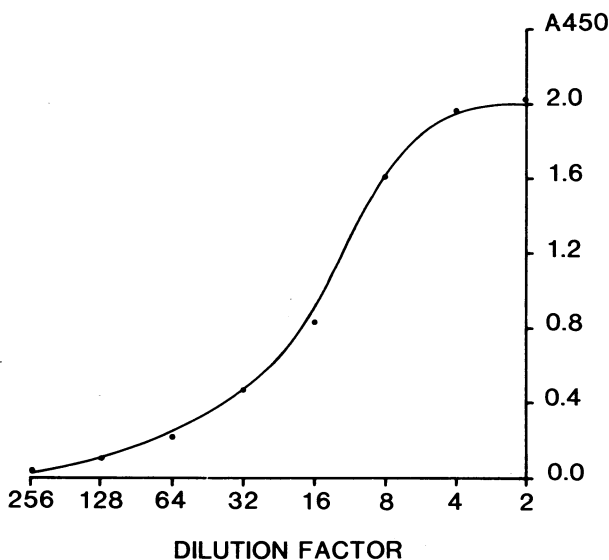


Fig. 1 Dose-response curves of fresh normal pooled plasma in the fibrinogen EIA. The fibrinogen concentration, as assessed gravimetrically (38) in the plasma was 2.4 mg/ml

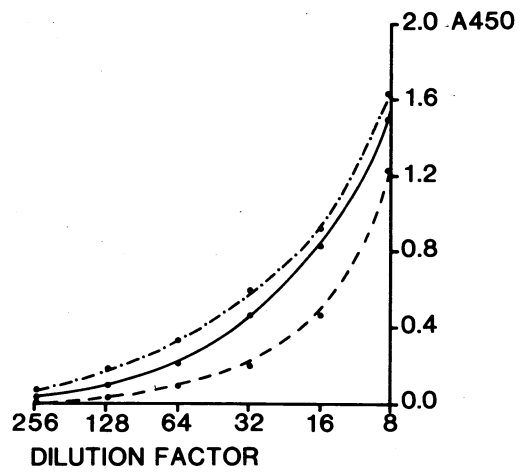


Fig. 2 Parallelism of dose-response curves of fresh normal pooled plasma (calibration material) and of two patient samples. Only the lower part of the curves have been depicted (compare Fig. 1)

coefficients of variation were 7.7% and 2.8% for the 30-fold and 6-fold diluted plasma, respectively.

#### Correlation with the Clauss Method (19)

Coded plasma samples from several patients referred to the Haematological Department of the Academic Hospital in Leiden (Dr. Briët) were given to us. The assessment of the fibrinogen levels of these samples had been performed at the Academic Hospital according to Clauss (19). The results were not known to us, when we performed our fibrinogen EIA. Fig. 3 shows the correlation between the results obtained with the two methods. The correlation is quite good, although the Clauss method measures clottable "fibrinogen" (see Introduction), whereas our fibrinogen EIA measures fibrinogen with intact A $\alpha$ -chain(s).

Some of the samples (not plotted in Fig. 3) yielded dose-response curves, which are not parallel with the calibration curve

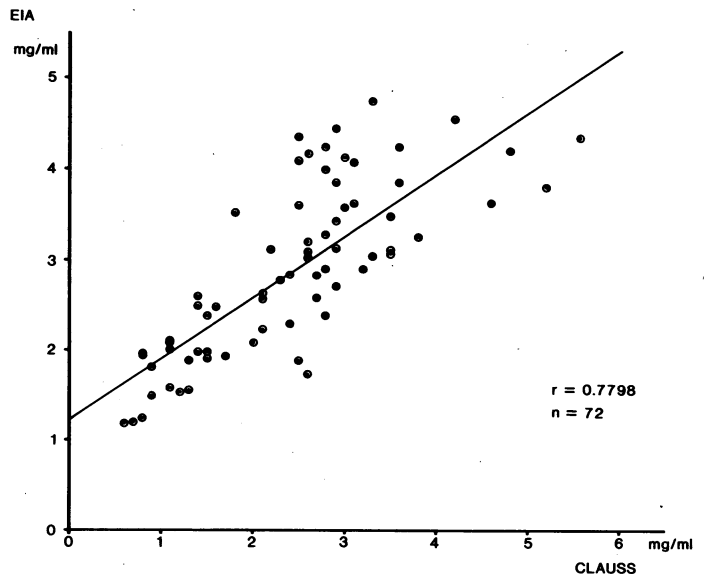


Fig. 3 Correlation between the fibrinogen levels of 72 plasma samples as assessed with the Clauss method and the fibrinogen EIA

of our EIA. As a consequence the fibrinogen level in these samples could not be determined with our EIA. Surprisingly, some of these samples showed also an abnormal dilution profile in the Clauss method. At present the nature of the factor(s) in these plasmas which have a similar effect on the functional assay and our immunological assay, is not known.

Although the statistical correlation between the EIA and the Clauss results is good, some individual samples (see Fig. 3) show an approximately 50% higher fibrinogen level as assessed with the EIA as compared with the Clauss method. These samples may contain factors which decrease the clotting rate. The nature of these factors is not known.

Since our fibrinogen EIA measures fibrinogen with intact A $\alpha$ -chain(s), it is particularly useful to monitor proteolytic damage to fibrinogen e.g. in patients undergoing thrombolytic therapy or in patients otherwise suspected of increased plasma proteolytic activity. The combination of a functional assay such as the Clauss method with an immunological assay such as our EIA may have more diagnostic value than each of the methods separately.

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