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Fibrinopeptide A Radioimmunoassay: A Rapid and Sensitive Measurement of Fibrin Generation in vivo[1])

By *G. Stehle, J. Harenberg, H. Schmidt-Gayk* and *R. Zimmermann*

Medical University Hospital (Director: Prof. Dr. Dres. h.c. G. Schettler) Ruprecht-Karls-Universität, Heidelberg, FRG

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Summary: Fibrinopeptide A is cleaved from fibrinogen by thrombin. One mole of fibrinogen releases two moles of fibrinopeptide A during coagulation.

In the present paper we describe a rapid and sensitive method to detect human fibrinopeptide A immunoreactivity in plasma.

Double-antibody precipitation with a preincubation of first and second antiserum was used.

The limits of detection were extended to fibrinopeptide A levels between 0.3 μg/l and 160 μg/l and the time spent on the assay procedure was shortened from 14 hours to 2 hours.

Fibrinopeptide A levels in healthy volunteers were below 2.5 μg/l. This new technique correlates with our previously described method (r = 0.96; comparative determination of 100 patient plasmas).

Radioimmunassay für Fibrinopeptid A: Eine schnelle und empfindliche Bestimmung der Fibrinbildung in vivo

Zusammenfassung: Fibrinopeptid A wird vom Fibrinogenmolekül durch Thrombin abgespalten.

Ein Mol Fibrinogen gibt zwei Mol Fibrinopeptid A während des Gerinnungsprozesses ab.

In der vorliegenden Arbeit wird eine schnelle und sensible Untersuchungsmethode zur Messung von Fibrinopeptid A im Plasma beschrieben. Der Radioimmunoassay (RIA) wurde nach der Doppelantikörper-Methode mit gemeinsamer Vorinkubation des ersten und zweiten Antiserums durchgeführt. Die Nachweisgrenze des Assays konnte auf Fibrinopeptid A Konzentrationen von 0,3 μg/l bis 160 μg/l Fibrinopeptid A vergrößert werden; der Zeitaufwand der Untersuchung wurde von 14 h auf 2 h verkürzt.

Die Konzentration von Fibrinopeptid A lag bei gesunden Probanden unter 2,5 μg/l. Diese neue Untersuchungstechnik korreliert mit unserer früher beschriebenen Methode (r = 0,96; vergleichende Bestimmung von 100 Plasmaproben).

Introduction

Coagulation terminates when fibrinogen is converted to fibrin by the action of thrombin.

Thrombin cleaves arginine-glycine bonds of the A-α and B-β-chains near the aminoterminal ends of the fibrinogen molecule. During this process one molecule of fibrinogen loses two A peptides and two B peptides and the remaining fibrin monomers polymerize instantly into a fibrin clot under the influence of coagulation factor XIII (1).

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Fibrinopeptide A consists of 16 amino acids, the sequence of which is known.

Since it is possible to obtain a specific antiserum for fibrinopeptide A (2) the peptide's immunoreactivity can be measured in plasma samples by radioimmunoassay. Owing to its high rate of elimination from plasma (halflife from 3 to 5 min) Fibrinopeptide A can be considered a sensitive parameter for the indication of fibrin formation in vivo (3–19). However, all the radioimmunological procedures described so far are time-consuming (2, 13–17, 22). The use of shorter incubation times or radiolabelled second antibody lead to a loss of sensitivity related to increased coefficients of variation (16–18, 22). Specific fibrinopeptide A antisera[2]) and a RIA-KIT[3]) are available commercially.

We report a radioimmunological technique which permits the rapid determination of fibrinopeptide A without loss of sensitivity.

The method is based on the separation of the antibody-bound antigen by specific precipitation with a preincubated complex of first and second antibody and normal rabbit serum.

Materials and Method

Purified fibrinopeptide A (Lot No. 3057-1-2), deaminotyrosine-fibrinopeptide A (Lot No. 321-1-2), and fibrinopeptide A-antiserum from rabbit (Lot No. 135-F-64) were obtained from IMCO Corp., Stockholm, Sweden.

Albumin (Art. 11840) was purchased from Serva GmbH (Heidelberg), anti-rabbit-immunglobulin from goat from Behringwerke (Marburg/Lahn – Lot No. 150104 A) and anti-rabbit-immunglobulin from donkey from Wellcome GmbH (Burgwedel-Batch No. K 5579, K 7120, K 9237).

Normal rabbit serum was obtained by kaolin coagulation of rabbit plasma.

Metal plates with 96 cavities were used for the 0.45 ml capacity, plastic assay-tubes (Sarstedt, Nümbrecht, Art. No. 043 1742-8).

The radioimmunoassay was performed as previously described (17).

Samples were analysed in triplicate and the radioactivity was counted for 1 minute in a gamma counter.

Blood was collected by sterile venipuncture using steel needles (0.9 mm diameter, Braun Melsungen). After release of the short, slight tourniquet, applied during puncture, freely flowing blood was collected in a plastic tube containing 100 USP heparin per ml blood for anticoagulation.

The samples were centrifuged immediately at 1800 *g* at 4 °C for 10 min. The plasma was separated and then processed by ethanolic extraction as previously described (17).

The extracted plasma samples were stored at −24 °C until assayed.

[2]) Imco Corp., S-11330 Stockholm; Byk-Mallinckrodt, D-6057 Dietzenbach

[3]) Byk-Mallinckrodt.

Results

Normal rabbit serum

Without addition of normal rabbit serum to the assay reagents, the maximal binding was about 38% of the added amount of tracer (fig. 1). The standard deviation was calculated at 15%.

After addition of diluted normal rabbit serum the maximum binding increased to about 0.50 and the standard deviation decreased to 2.5%. Both second antibodies (from goat and from donkey) showed their most efficient binding at a final dilution of the normal rabbit serum of 1:900. This was adequate to a volume of 20 µl of a 1:50 dilution of normal rabbit serum per tube. Increasing the amount of normal rabbit serum led to a rapid decline in maximum binding (fig. 1).

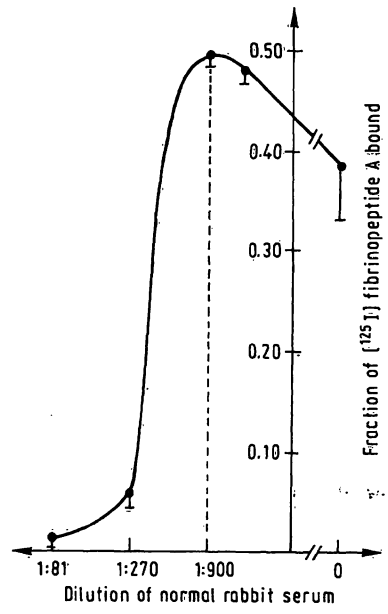

Fig. 1. Influence of different amounts of normal rabbit serum on the maximal binding rate of labelled fibrinopeptide A (ordinate, relative binding). On the abscissa the final dilutions of normal rabbit serum added are shown (0 = no normal rabbit serum). – – – = dilution of normal rabbit serum used in assay.

First and second antibody

Tests with the first antibody showed a maximum binding of about 0.50 at a dilution of 1:3333 (final dilution of 1:10000; fig. 2).

Ten microlitres of undiluted second antibody, produced from goat against rabbit-immunoglobulin, were required for binding (16).

The same binding rate was achieved for the second antibody from donkey by the addition of 4 µl in a total volume of 100 µl (1:24 dilution).

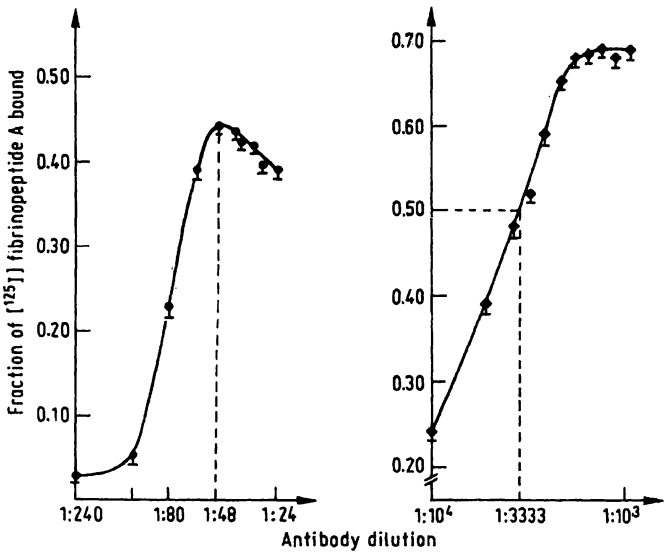

Fig. 2. The effect of different dilutions (abscissa) of the 1st (right figure) and the 2nd antibody (left figure) on the maximal binding of [^{125}I]fibrinopeptide A (Ordinate, relative binding) is shown. – – – = dilution of the antibodies used in assay.

Time of incubation

After an incubation period of 12 h both second antibodies showed about the same binding rate. When the equilibration times were shortened from 12 h to 2 h only the second antibody from donkey provided good results.

At the same time the second antibody from goat had only reached one third of its maximal binding (fig. 3).

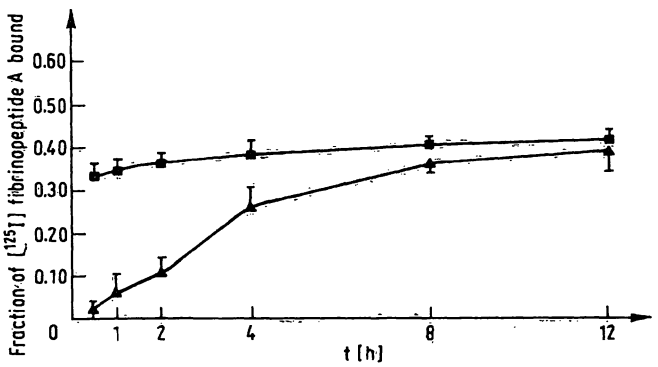

Fig. 3. Maximal binding of [^{125}I]fibrinopeptide A-1st antibody-complex by two different 2nd antibodies (donkey ■—■, goat ▲—▲) after different incubation times (abscissa, hours).

Preincubation of first and second antibody and normal rabbit serum

The first and the second antibody and normal rabbit serum were preincubated together before being added to the sample.

After combined incubation, the antiserum mixture immediately became turbid and visible aggregations of immunecomplexes were formed. Throughout the different incubation times the preincubation method showed a 5% higher binding in comparison to the separate addition of the antisera and the normal rabbit serum to labelled fibrinopeptide A.

When the reagents were preincubated for 0.4 or 12 hours no differences were observed on the fibrinopeptide A standard curve. Experiments with a preincubation time of about 150 h revealed an obvious loss in the binding rate (fig. 4).

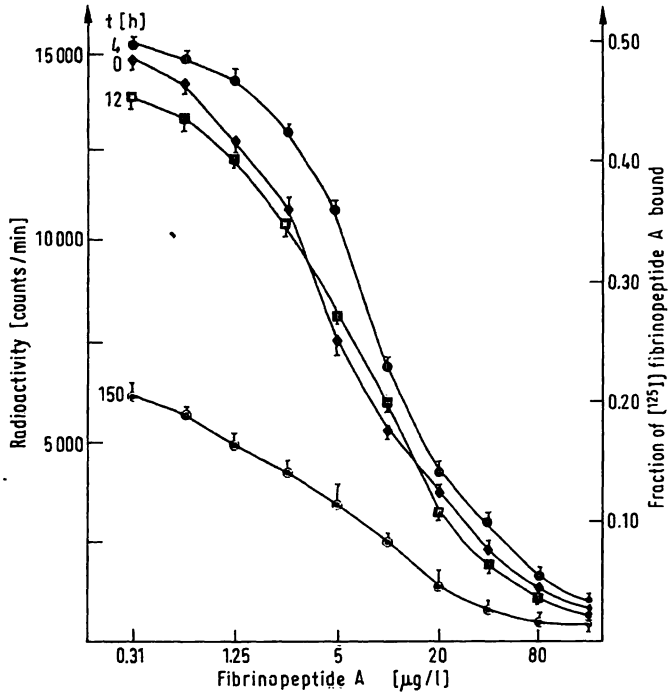

Fig. 4. The effect of different preincubation periods of the mixture (first antibody, second antibody and normal rabbit serum) on the standard curve is depicted.

Temperature and assay

The maximal binding rate of preincubated material was tested at 4 °C, 22 °C and 37 °C using an incubation period of 2 h. The binding rate decreased and the standard deviation increased substantially at 37 °C. Optimal results were achieved at 4 °C. However, there was no difference in the binding rate when the incubation was carried out at 22 °C in comparison to 4 °C (fig. 5).

These experiments further demonstrate that the antigen-antibody reaction had almost reached equilibrium after 10–20 min.

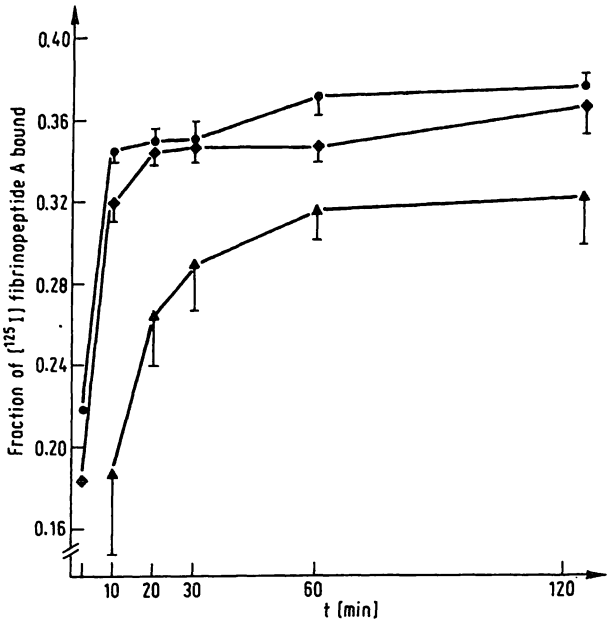

Fig. 5. The influence of the assay temperature (°C) on the maximal binding rate after different incubation periods (abscissa, minutes) is shown.
4 °C ○—○ 22 °C ◇—◇ 37 °C △—△

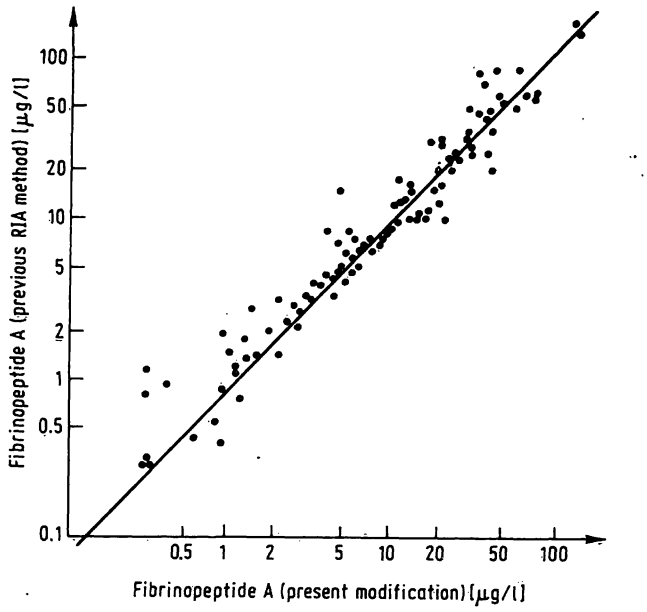

Fig. 6. The data for the determination of the correlation coefficient of the previous RIA method (2nd antibody from goat, 12 h (17)) and the present modification (2nd antibody from donkey, 2 h) are shown.
y = 1.06x − 0.08 r = 0.96

Validity of the method

The within assay coefficient of variation of the method was calculated at 3.0 (1 µg/l), 4.1 (5 µg/l), 4.4 (10 µg/l) and 6.0 (80 µg/l, n = 30).

The interassay coefficient of variation was 7% (n = 10, 7 determinations). The validity of the method was proved by comparison with our previously described procedure – 12 h incubation with the second antibody from goat and 2 h incubation with the second antibody from donkey. The correlation of 100 patient plasma samples was r = 0.96 in a range from 0.31 to 160 µg/l fibrinopeptide A (fig. 6).

Results showing the validity of the procedure are published elsewhere (11, 12).

Discussion

A sensitive and rapid method to test the hypercoagulability of plasma was not hitherto available in the clinical chemistry laboratory. Most of the previously described radioimmunological procedures for the measurement to fibrinopeptide A are time-consuming (2, 13–17, 22). Other methods lose sensitivity if incubation times are shortened (16, 17, 22). ^{125}I-labelling of the second antibody (18) or use of a solid-phase enzyme immunoassay system (19) manifest the same problem of decreased sensitivity. A decrease of sensitivity of the fibrinopeptide A RIA occurs mainly in the range of low concentrations (0.3–1.5 µg/l).

In contrast to the previously described methods, the fibrinopeptide A concentrations on the abscissa reflect the final amount in 1 ml plasma. Other methods always provide a correction of the fibrinopeptide A concentration shown on the abscissa by multiplication by a dilution factor, which ranges from 1.73 to 16.5.

Extraction of fibrinogen and fibrin(ogen) split products, which crossreact with the fibrinopeptide A antiserum, can be performed effectively by ethanolic extraction (2, 13, 16, 17) or by bentonite adsorption (15, 18, 19, own data not shown). Evaporation of the samples extracted with ethanol is than no longer necessary thus reducing the time spent on the assay to less than 2 hours (not shown).

Usually charcoal is used to precipitate antigen-bound and free tracer fibrinopeptide A. In 1979, we described a specific separation using a soluble second antibody (17) in the assay. Adding immunoglobulin as a carrier to the test system accelerates the binding of the first and second antibody (20). We introduced normal rabbit serum as a carrier resulting in an increase of 10–15% in binding. As a further modification and improvement, preformed precipitates composed of both first and second antibodies were used (21).

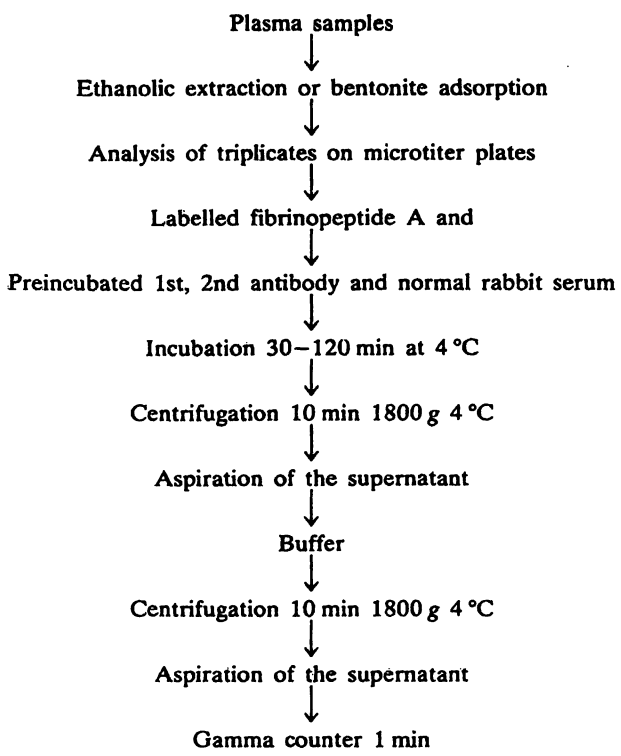

Fig. 7. Flow diagramm of the final fibrinopeptide A RIA procedure.

This technique was termed the "pre-precipitate method". In our approach both antibodies were preincubated together with the normal rabbit serum. Thus the preincubation period was shortened to a few minutes, when the immunocomplexes became visible. Previous results from the radioimmunological determination of thyroxine indicate that this modification can also be adapted for small molecular weight hormones.

The clinical application of the determination of fibrinopeptide A depends on an assay which can be completed in a single day. The technique described here seems to fulfill the requested requirements. In the present procedure the incubation times were shortened without losing sensitivity or any of the well known advantages of the second antibody separation. However, for the measurement of fibrinopeptide A an accurate collection of blood samples is required to avoid any fibrin generation in vitro. If this is guaranteed, the radioimmunological determination of fibrinopeptide A described here can be regarded as rapid, reliable and reproducible for the detection of intravascular fibrin formation in man.

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Dr. med. J. Harenberg
Medizinische Universitätsklinik
Bergheimerstraße 58
D-6900 Heidelberg 1