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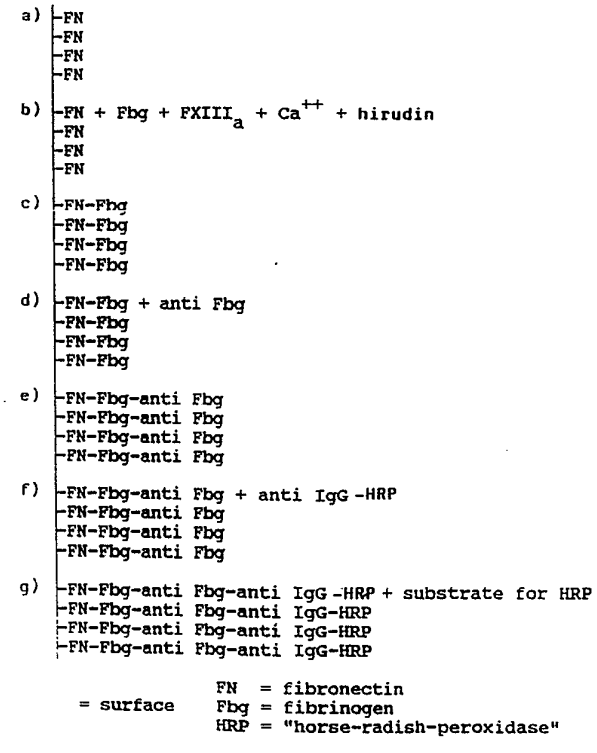
(54) Title: A METHOD OF DETERMINING QUANTITATIVELY FIBRINOGEN, FIBRONECTIN, α_2-ANTIPLASMIN OR A TRANSLUTAMINASE

(57) Abstract

Fibrinogen, fibronectin, α_2-antiplasmin and Factor XIII are determined quantitatively by a reaction process which involves reacting fibrinogen with fibronectin or α_2-antiplasmin with Factor XIII acting as a catalyst; predetermining the quantities of all reaction components used, with the exception of the sought quantity; and determining the sought quantity with the aid of an immunosorption technique in a known manner, particularly by adsorbing the fibrinogen or fibronectin on a surface and then reacting said fibrinogen or fibronectin with said other reaction component and then with an antibody specific against the substance to be determined, which antibody may be labelled, whereafter the amount of said antibody is measured in a known manner, optionally by reaction with a labelled secondary antibody.

A schematic illustration of the courses followed by the reactions that occur when determining fibrinogen concentration.



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A Method of Determining Quantitatively Fibrinogen, Fibronectin, α_2-Antiplasmin or a translutaminase

The present invention to a method of determining quantitatively fibrinogen, fibronectin, α_2-antiplasmin or a transglutaminase. A known and important transglutaminase is Factor XIII.

Fibrinogen, fibronectin, α_2-antiplasmin and Factor XIII are all important blood constituents and it is essential to be able to determine the concentration of these constituents in blood and in other tissues. It has been found that Factor XIII is a transglutaminase in blood, the primary role of which is to cross-link protein chains in the fibrin network subsequent to its formation. This results partly in cross-linking between the γ-chains in the fibrin and partly also between the α-chains themselves. Factor XIII is also able to catalyse specifically the incorporation of certain plasma proteins in the fibrin network. For instance, fibronectin and α_2-antiplasmin are linked to the fibrin by Factor XIII catalysis; amino groups in the fibrin fulfil a donator function in both cases.

The concentration of fibrinogen in the blood is also important, insomuch as a deficiency of fibrinogen will lead to bleeding conditions, whereas elevated concentrations predict cardiovascular diseases. Fibronectin is a protein which is apparently essential to cell proliferation and wound healing. The concentration of fibronectin is also lowered with infectious conditions, such as sepsis. α_2-antiplasmin is the most important inhibitor of fibrinolysis in blood. Increased concentrations of this inhibitor or increased incorporation in the fibrin coagulum has been observed in thrombotic conditions.

Consequently, it is essential to be able to determine the concentrations of these constituents or substances in blood and other tissues. Although methods are exist for determining the concentration of fibrinogen in blood, these methods are much too time-consuming and are non-responsive and/or non-specific. There are no functional methods by means of which the concentrations of fibronectin, α_2-antiplasmin and Factor XIII can be determined quantitatively.

B. Blombäck, R. Procyk, L. Adamson and B. Hessel (Thrombosis Research 37, pages 613-628, 1985) and R. Procyk, L. Adamson, M. Block and B. Blombäck (Thrombosis Research 40, pages 833-852, 1985) show that fibrinogen and fibronectin react in the presence of Factor XIIIa, particularly in the latter publication. This reaction takes place in bulk phase and there are no suggestions to the effect that this reaction can be used for the quantitative analysis of the reactants.

It is also shown in Biol. Chem. Hoppe Seyler 1987, June: 368 (6): 669-74, J. Biol. Chem. 1988 July: 25: 263 (21): 10464-9; Blood 1986 July: 68 (1): 95-101 and Biochim Biophys Acta 1988 November 17: 967 (2): 304-13 that thrombin-activated Factor XIII (Factor XIIIa) on polystyrene spheres in the presence of fibronectin is not able to bind fibrin to the spheres; but that fibronectin is able to cross-link to cellular locations on a matrix with Factor XIIIa acting as a catalyst. It is also shown that Factor XIII cross-links fibrin on itself and a limited number of substrates. Factor XIII also has a catalyzing effect in solutions containing fibrinogen and fibronectin, forming two types of cross-linked polymers, namely hybridoligomers and fibrinogenoligomers.

It has now surprisingly been found that these bulk phase reactions (reactions in solution) can be used in principle for assaying fibrinogen, fibronectin, α_2-antiplasmin and transglutaminase in practice. When the reactions are carried out as surface-bound reactions, it is surprisingly found that the reaction rate is increased considerably and that, at the same time, the concentrations of the reactants can be significantly decreased. The surface-bound reaction has also been found more specific.

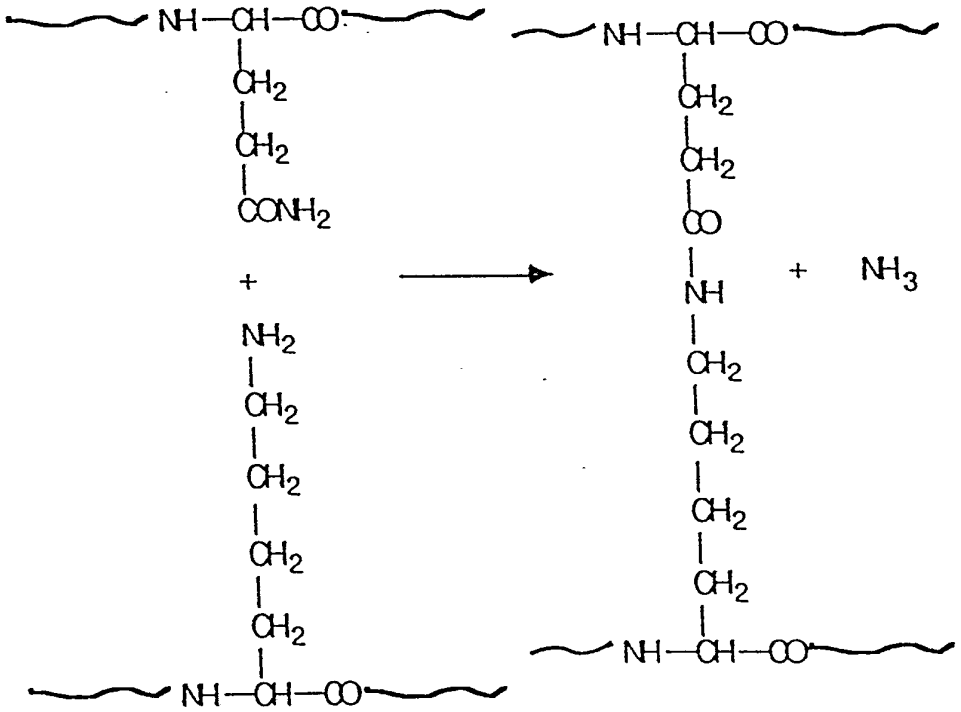
It has also been surprisingly found that fragments of the fibrinogen molecule will not disturb the surface-bound reaction. When quantitatively determining fibrinogen in a known manner, the fibrinolytic fragment of the fibrinogen molecule has had a disturbing effect on the assay analysis. This disturbance is not found when practicing the present method, despite the fact that potential cross-linking locations are found in such fragments as Ds. Heparin can also have a disturbing effect in several known assaying methods. This is not the case when practicing the present invention, however.

It has now been found that fibrinogen, fibronectin, α_2-antiplasmin or a transglutaminase, particularly Factor XIII can be assayed functionally in a simple, but very precise fashion by means of a reaction which involves:

- reacting fibrinogen with fibronectin or α_2-antiplasmin with a transglutaminase acting as a catalyst;
- using the reaction components in pre-determined quantities, with the exception of the quantity sought for; and
- determining the quantity sought for with the aid of

an immunosorption technique in a known manner, particularly by absorbing fibrinogen or fibronectin on a surface and then reacting the fibrinogen or fibronectin with the other reaction component andc then with a labelled antibody which is specific thereagainst the substance to be determined whereafter the amount of labelled antibody is measured in a known manner.

It has been found that several reactions take place in the organism which are dependent on enzymes which possess a transglutaminase activity. Such enzymes are present both in intracellular fluids and in tissue fluids, such as blood plasma and lymph. There are differences in specificity between these enzymes. A common feature of all these reactions, however, is the chemical reaction mechanism which involves the establishment of a covalent bond between a glutamin amino-acid rest (acceptor) in a polypeptide chain and a lysine amino acid rest (donator) in another polypeptide chain, in accordance with the following:



Thus, covalent bonds are obtained between the protein chains. Many different components possessing an amino function can serve as donators in this reaction, for instance dansyl cadaverine, putrescine, etc. Glutamine is required for the acceptor function. The specificity of the various enzymes is contingent partly on the amino acid sequence around the glutamic acid rest in a polypeptide chain, and partly on the structure of the amino function rest. The course followed by the reaction is accelerated when the reactant groups in the proteins are juxtaposed.

The invention will now be described in more detail with reference to the accompanying drawings, in which

Figure 1 is a schematic illustration of the course followed by the reaction when determining, or assaying, fibrinogen;
Figure 2 is a diagramme which illustrates the relationship between colour intensity and fibrinogen concentration;
Figure 3 is a diagramme which shows the relationship between colour intensity and fibronectin concentration, and also the effect of iodo acetamide on the reaction with Factor XIII;
Figure 4 is a diagramme which shows the relationship between colour intensity and activity for Factor XIIIa;
Figure 5 is a diagramme which shows the relationship between the colour intensity and the activity of Factor XIIIa.
Figure 6 illustrates the relationship between absorbency and fibrinogen concentration in plasma;
Figure 7 illustrates the relationship between absorbency and fibronectin concentration in plasma and serum; and
Figure 8 illustrates the activity of Factor XIII after

different reaction times.

Quantitative Determination of the Fibrinogen Concentration

When carrying out the analysis, fibronectin is adsorbed on a surface in a known manner (see Figure 1a). This surface may consist of a plastic material, such as polystyrene. Materials that are suitable for this purpose are available commercially, for example "Titertecplattor", latex spheres, etc. A sample solution containing fibrinogen is then added (Figure 1b)). A calcium chloride solution and Factor XIII, suitably in an activated form, for instance a thrombin activated form, is then added. A thrombin-activity inhibitor, for instance hirudin, is then added to inhibit the thrombin excess used in the activating process or possibly generated in the sample. Factor XIIIa (activated Factor XIII) now catalyses the incorporation of the fibrinogen into the surface-bound fibronectin (Figure 1c)). The fibronectin-bound fibrinogen is then caused to react with an antibody (for instance from goats) which is specific against fibrinogen, so-called antifibrinogen (Figure 1d)) and the antibody is combined with the fibrinogen on the surface (Figure 1e)). The resultant fibronectin-fibrinogen-antifibrinogen is now caused to react with a so-called secondary antibody (e.g. rabbit-anti-goat-IgG), labelled with peroxidase (Figure 1f), whereafter the enzyme is visibilized by adding a substrate for the peroxidase (Figure 1g) (HRP = "horse-radish-peroxidase"). The fibrinogen-bound peroxidase (HRP) splits or cleaves the substrate, therewith producing a yellowish colour. This part-stage of the process involving the use of an enzyme-labelled antibody and its visibilisation, is known as the enzyme immunosorption technique and is

well known.

Quantitative Determination of the Fibronectin Concentration

Fibrinogen that is essentially free from fibronectin and Factor XIII is adsorbed on a surface of the kind described with reference to the quantitative determination of fibrinogen. A fibronectin-containing solution is applied to the surface, together with calcium chloride, thrombin-activated Factor XIII and hirudin, this latter to neutralize excess thrombin. In this case, Factor XIIIa catalyses the incorporation of fibronectin into the surface-bound fibrinogen, analogously with that described with reference to the quantitative determination of fibrinogen. The fibrinogen-bound fibronectin is then caused to react with an antibody (e.g. from goats) which is specific against fibronectin, whereafter a reaction takes place with a secondary antibody labelled with peroxidase, analogously with that described with reference to the quantitative determination of fibrinogen. Should the sample solution containing fibronectin also contain fibrinogen, this fibrinogen shall be removed by coagulating said fibrinogen with a snake-venom enzyme, for instance batroxobine. This coagulation process should not be effected with thrombin, since Factor XIII may be present in the sample solution. In such case, the enzyme is activated and causes the fibronectin present in the sample solution to be incorporated in the precipitated coagulum. This drawback is not experienced when coagulation is effected with batroxobin.

Quantitative Determination of α_2-Antiplasmin

Fibrinogen is caused to be adsorbed on a surface in the

same manner as that described with reference to the quantitative determination of fibronectin. A solution containing α_2-antiplasmin is applied to the surface, followed by calcium chloride, thrombin-activated Factor XIII and hirudin, this latter to neutralize excess thrombin. Subsequent to a reaction time, the fibrinogen-bound α_2-antiplasmin is visibilised with a specific antibody against α_2-antiplasmin and a secondary antibody labelled with peroxidase, analogously with that described with reference to the quantitative determination of fibrinogen and fibronectin respectively. Should the sample solution contain fibrinogen and fibronectin, as is the case with blood plasma, it is necessary to remove the fibrinogen by coagulation with a snake-venom enzyme, and to remove fibronectin by adsorption on gelatine coupled to an appropriate polymer matrix, e.g. Sepharos®, in a known manner.

Determination of the Activity of Factor XIII

Fibrinogen that is essentially free from fibronectin and Factor XIII is adsorbed on a surface, analogous with the method described with reference to the quantitative determination of fibronectin and α_2-antiplasmin. An excess quantity of fibronectin is then added, together with calcium chloride and hirudin. A sample solution containing Factor XIII is then added. If active Factor XIII is present in the sample, the fibronectin will be incorporated on the surface of the fibrinogen and can be visibilised with a specific antibody against fibronectin and a secondary antibody labelled with peroxidase, e.g. HRP, as described with reference to the quantitative determination of fibronectin. The total activity of Factor XIII can be determined by first treating the sample solution with batroxobin, to remove the fibrinogen by coagulation.

Thrombin is then added, to convert Factor XIII to an active form.

The described embodiments concerned with the quantitative determination of fibrinogen, fibronectin, α_2-antiplasmin and Factor XIII have initially been the only embodiments and thus the preferred embodiments. However, it has recently been found possible to use a specific, labelled antibody, particularly an antibody labelled with "horse-radish-peroxidase", instead of using a specific antibody and a secondary antibody labelled with peroxidase. This latter embodiment is now the preferred embodiment for quantitatively determining the fibrinogen, fibronectin, α_2-antiplasmin concentration and the activity of Factor XIII.

Thus, when determining the fibrinogen concentration, there is now used an antibody which is specific against fibrinogen, a so-called antifibrinogen, which is labelled, preferably with "horse-radish-peroxidase" (HRP), a so-called primary antibody, instead of a specific antibody against fibrinogen which combines with the fibrinogen, whereafter the resultant product is reacted with a so-called secondary antibody.

Correspondingly, there is used when quantitatively determining the fibronectin concentration a labelled fibronectin antibody, while when determining the concentration of α_2-antiplasmin, there is used a labelled α_2-antiplasmin antibody. Thus, when determining the activity of Factor XIII, there is preferably used a primary labelled antifibronectin antibody. The primary antibody is preferably a HRP-labelled antibody.

The invention will now be described in more detail with reference to a number of Examples.

Example 1

Quantitative Determination of Fibrinogen

200 µl of a fibronectin solution (10 µg/ml) were introduced into the wells of a microtitre plate ("Titertec"). The plates were allowed to stand overnight at room temperature. The wells were then emptied and washed repeatedly with a solution of TNE-BSA-buffer (0.05 M-tris-0.10 M NaCl-1 mM EDTA, pH 7.4, containing 0.1% bovine serum albumin). A solution of bovine albumin (30 mg/ml) was then poured into the wells with the intention of blocking those locations on the well surfaces not saturated with fibronectin. Subsequent to washing with a solution of TNE-BSA-buffer, there were added 150 µl of a sample solution containing fibrinogen (between 0.15 and 20 µg/ml) and thereafter 20 µl $CaCl_2$ solution (0.2 M) and 5 µl hirudin (1000 ATU/ml) (ATU = antithrombin units). Finally, 25 µl thrombin activated Factor XIII (3.2 units/ml) were added, such that the final concentration was 0.4 E/ml. The plates were shaken slowly for 1.5 hours at a temperature of 37°C. The plates were then washed with a buffer solution and 200 µl of goat-antifibrinogen-IgG in suitable concentration were added. Subsequent to incubation at room temperature for 18 hours, the plates were washed with buffer solution and 200 µl rabbit-antigen-IgG labelled with peroxidase were added. Subsequent to incubation for 2 hours at room temperature, 100 µl of peroxidase substrate were added. The mixture was incubated for an additional 1.5 minutes, whereafter the reaction was interrupted by adding 100 µl of 1 M H_2SO_4 solution. The colour intensity was read-off at 490 nm. The adsorption relationship with the fibrinogen concentration in the solution is shown in Figure 2.

The experiment was repeated, but with the addition of iodo acetamide to the solution to a concentration of 0.5 mM. This addition was made prior to adding Factor XIII. Iodo acetamide inhibits Factor XIII. No discernible colour development occurred in this experiment. The experiment shows that the incorporation is dependent on the presence of Factor XIII.

Example 2

Determining Fibronectin Concentration

200 µl of fibrinogen (10 µg/ml), free from fibronectin and Factor XIII, were introduced into the wells of a microtitre plate. The plates were allowed to stand at room temperature overnight. The surface was washed and saturated with bovine serum albumin in the manner described with reference to Example 1. Subsequent to washing with buffer solution (as in Example 1), 150 µl of a sample solution containing fibronectin (0.02-2.5 µl/ml) were added, followed by 20 µl of a calcium chloride solution (0.2 M) and 5 µl of hirudin (1000 ATU/ml). Finally, 25 µl of thrombin-activated Factor XIII (3.2 units/ml) were added, such that the final concentration was 0.4 units/ml. The plates were shaken slowly for 2 hours at a temperature of 37°C. The plates were then washed with a buffer solution (as in Example 1) and 200 µl of goat-antifibronectin-IgG were added. Subsequent to incubation for 18 hours at room temperature, the wells were washed with buffer solution and 200 µl of rabbit-antigen-IgG labelled with peroxidase were added. Subsequent to incubation for 2 hours at 37°C, 100 µl of peroxidase substrate were added. The same method as that described with reference to Example 1 was then followed. The relationship of colour intensity to fibronectin concentration in the

sample will be seen from Figure 3.

An experiment was also carried out in this case in which iodo acetamide was added, analogously with the aforedescribed Example 1; no colour development was observed. Thus, this reaction is also dependent on the presence of Factor XIII (Figure 3).

Example 3

Determination of the Total Activity of Factor XIII When Using Secondary Antibodies Labelled with Peroxidase

Microtitre plates were treated with fibrinogen in the same manner as that described in Example 2. The plates were also blocked and washed in the same manner as that described in Example 2. 150 µl of fibronectin solution (2.5 µg/ml), 20 µl of calcium chloride (0.2 M) and 5 µl of hirudin (1000 ATU/ml) were then added. Finally, 25 µl of thrombin-activated Factor XIII (concentrations between 0.001 and 0.1 E/ml) were added and the plates incubated for 2 hours at 37°C, while slowly shaking the plates. The plates were then washed with buffer solution (in accordance with Example 1). Goat-antifibronectin IgG was then added. The process of determining the total activity was then continued in the manner described in Example 2. Figure 4 illustrates the relationship between colour intensity and activity of Factor XIII in the sample solution. Background colour was generated solely in the absence of Factor XIII.

Example 4

Determining the Total Activity of Factor XIII When Using Biotinylated Antifibronectin

Microtitre plates were treated with fibrinogen, in accordance with Example 2. The plates were also blocked and washed in the same manner as that described in Example 2. 150 µl of fibronectin solution (2.5 µg/ml), 20 µl of calcium chloride and 5 µl of hirudin (1000 ATU/ml) were then added. Finally, thrombin-activated Factor XIII (concentrations between 0.001.0.1 E/ml) was added and the plates incubated for 2.5 hours while slowly shaking the plates. The plates were then washed with a buffer solution (as in Example 1), whereafter 200 µl of biotinylated goat-antifibronectin-IgG (1 µg/ml) were added and the plates incubated room temperature for 18 hours. The plates were then washed with buffer solution (see Example 1), whereafter 200 µl avidine-HRP (0.5 µg/ml) were added, followed by a peroxidase substrate in accordance with the method described in Example 2. Figure 5 shows the relationship between the colour intensity and the activity of Factor XIIIa in the sample solution.

Example 5

Determining the Concentration of Fibrinogen in Blood

With the intention of determining the fibrinogen concentration in blood, the blood was introduced into a vessel containing an anticoagulant substance, for example 3.8% trisodium citrate or 0.1 M EDTA. The anticoagulant is normally present in a proportion of 1 part coagulant to 9 parts of blood. The blood was centrifuged (room temperature, 30 minutes, 1500 rpm) and blood plasma removed by suction. Prior to the analysis, the plasma was diluted with a TNE-BSA-buffer, (1:1000, 1:2000 and so on) (see Example 1).

Analysis: microtitre plates were treated with fibro-

nectin, as described in Example 1. The plates were also blocked and washed in the manner described in Example 1. 150 µl plasma diluted or thinned to different degrees, calcium chloride and hirudin were then added. 25 µl of thrombin-activated Factor XIII (final concentration 1 E/ml)(were then added and the plates incubated for one hour at 37°C, while slowly shaking the plates. The plates were washed with buffer solution, as described in Example 1. 200 µl of primary HRP-labelled fibrinogen-antibody were then added. Subsequent to incubation for one hour at 37°C, 200 µl of peroxidase substrate were added and the mixture was incubated for a further 2 minutes, whereafter the reaction was interrupted by adding 50 µl of 4 M H_2SO_4. The color intensity was read at 492 nm. The relationship between absorbence and fibrinogen concentration in plasma can be seen from Figure 6.

Example 6

A comparison was made between the determination of fibrinogen concentration according to the present invention and two known functional fibrinogen assaying methods.

Plasma taken from 23 different healthy persons was treated in the manner described in Example 5 and the fibrinogen content was determined in the manner described in Example 5 and also with the aid of said two known methods, the so-called syneresis method according to Bergström and the so-called "clot rate"-method according to Vermylen.

The results are set forth in the following table:
The fibrinogen concentration in plasma was determined with the aid of three different, functional

fibrinogen-assaying methods

Method	Mean value mg/ml	Spreading (SD) mg/ml
Cross-linking method*	2.89	0.444
Syneresis method (Bergström)	3.08	0.381
Clot-ratemethod (Vermylen)	2.76	0.537

* In accordance with the present invention.

Example 7

Analysis of the fibrinogen concentration in the same plasma on mutually different occasions.

Plasma from one single individual was treated in the manner described in Example 5 and analyzed (also in accordance with Example 5) on nine different occasions.

The results are set forth in the following Table:

Fibrinogen Concentration in the Same Plasma at Different Time Occasions

Date	Fibrinogen mg/ml
28 Nov. -90	2.938
29 Nov. -90	2.835
5 Dec. -90	2.785
17 Dec. -90	2.96
10 Jan. -91	2.874
14 Jan. -91	2.859
15 Jan. -91	2.764
8 Jan. -91	2.727
17 Jan. -91	2.924
Mean:	2.852
SD, %:	2.852

Example 8

Fibrinogen concentration in plasma and serum subsequent to adding fibrinogen, fibrinolysis products and heparin.

The plasma was treated in the manner described in Example 5. Serum was treated by treating a part of said plasma with the snake venom enzyme batroxobin (final concentration 0,5 E/ml for 60 minutes at 37°C) at room temperature, whereafter the fibrin coagulum was removed. Plasma standard is a mixed plasma from several individuals, plasma B.J. is plasma from a single individual. Fragment Ds and heparin were added to plasma and these plasmas were compared with intact plasma. Fragment Ds and fibrinogen were added to serum.

These plasmas and serums were analyzed in the manner described in Example 5.

The results are set forth in the following Table:

Sample	Fibrinogen mg/ml
Plasma standard	2.69
Serum	<0.03
Serum + 2 mg Fbg/ml	1.95
Serum + Fragment DS*	0.03
Plasma Stand. + Frg.DS**	2.66
Plasma B.J.	2.88
Plasma B.J. + Heparin***	2.85

* 100 µg/ml, **200 µg/ml

*** 5IE/ml

Example 9

Determining Fibronectin Concentration in Blood

Plasma was treated in the manner described in Example 5. The anticoagulant used comprised 0.1 M EDTA. Fibrinogen was extracted from this plasma by coagulation with the snake venom enzyme batroxobin (final concentration 0,5 E/ml, 37°C, for 60 minutes). The fibrin coagulum was removed and the supernatant (serum), and also the non-coagulated part of the plasma were used for analysis subsequent to diluting or thinning with TNE-BSA-buffer (see Example 5). The analysis of fibronectin was effected essentially in the same manner as that described in Example 2. Microtitre plates were treated with fibrinogen and the plates blocked and washed in the manner described in Example 2. 150 µl of diluted plasma 20 µl of calcium chloride and 5 µl of hirudin were then added. 25 µl of thrombin-activated Factor XIII (final concentrations between 0.001 and 0.1 E/ml) were then added and the plates incubated for one hour at 37°C, while shaking the plates slowly. The plates were washed with buffer solution (as in Example 1). 200 µl of HRP-labelled, primary fibronectin antibodies, were then added, whereafter the plates were incubated for one hour at 37°C; 200 µl of peroxidase substrate were then added. Incubation was continued for a further two minutes, whereafter the reaction was interrupted by adding 50 µl of 4 M H_2SO_4. The relationship between absorbence and fibronectin concentration i serum and plasma is set forth in Figure 7.

Example 10

The Activity of Factor XIII After Different Reaction Times

Microtitre plates were treated with fibrinogen, in the manner described in Example 2. Blocking and washing of the plates was also effected in the manner described in Example 2. 150 µl of fibronectin solution (10.0 µg/ml), 20 µl of calcium chloride (0.2 M) and 5 µl of hirudin (2 ATU/ml) were then added. Finally, 25 µl of thrombin- activated Factor XIII (concentrations between 0.001 and 0.1 E/ml) were added and the plates incubated over different time periods, namely 1, 1.5 and 4 hours at 37°C, while slowly shaking the plates.

The plates were then washed with buffer solution, as in Example 1. Monoclonal antifibronectin IgG was then added. The analysis was then continued in the same manner as that described in Example 2. Figure 8 shows the relationship between color intensity and the activity of Factor XIII in the sample solutions.

Example 11

The Total Activity of Factor XIII and the Spontaneous Activity in Normal Plasmas

Plasma from a number of individuals (12 persons) was treated in accordance with Example 5. The anticoagulant used consisted of 0.1 M EDTA. The plasmas were caused to coagulate by adding the snake venom enzyme batroxobin (0.5 E/ml in final concentration) over one hour at 37°. The coagulum was removed. These samples were analyzed with respect to the spontaneous activity of factor XIII in the manner described in Example 3. With

the intention of determining the total activity of Factor XIII, the batroxobin-coagulated plasmas were diluted or thinned in the ratio of 1:10 with a TNE-buffer (Example 1) and thereafter activated with thrombin (10 E/ml plasma) for from 10 to 60 minutes. The reaction was interrupted with hirudin, whereafter an analysis was carried out essentially in the manner described in Example 3.

The results obtained are set forth in the following Table:

Normal plasma	FXIII Total activity Units/ml	FXIII Spontaneous activity Units/ml
B.J. (Standard)	1.00	0.0040
B.B.	1.38	0.0050
K.S.	1.46	0.0030
K.A.	0.83	0.0060
M.G.	0.96	0.0040
I.A.	1.13	0.0000
W.G.	0.74	0.0020
E	1.14	0.0050
M	1.56	0.0095
K	0.83	0.0090
B.U.	1.39	0.0030
U.B.	1.13	0.0140
I.F.	0.75	0.0016
Mean:	1.1	0.0051
S.D.:	0.25	0.0038

Example 12

The total activity of Factor XIII and fibrinogen concentration prior to and subsequent to transfusion with plasma (from the father) on a girl having a Factor XIII

deficiency.

Plasma from a girl suffering from a Factor XIII deficiency and from her father were treated in the manner described in Example 5. The anticoagulant used consisted of 0.1 M EDTA. The girl received transfusion with plasma from the father and samples were subsequently taken. The plasmas were caused to coagulate and were activated in the same manner as that described in Example 11. The fibrinogen concentration was determined in the manner described in Example 5 and Factor XIII was determined in the manner described in Examples 3 and 11.

The results are set forth in the following Table:

Sample	FXIII U/ml	Fibrinogen mg/ml
Plasma standard	1.000	2.96
Pat. before transfusion	<0.003	4.21
Pat.30 min after transfusion	0.105	4.21
Pat.24 hours after transfusion	0.050	3.46
Pat.1 week after transfusion	0.032	3.74
Father's plasma	1.274	1.51

Claims

1. A method for quantitatively determining fibrinogen, fibronectin, α_2-antiplasmin or a transglutaminase, particularly Factor XIII c h a r a c t e r i z e d by carrying out a reaction process involving
   - reacting fibrinogen with fibronectin or α_2-antiplasmin with transglutaminase acting as a catalyst;
   - using all of the reaction components in predetermined quantities, with the exception of the sought quantity;
   - determining the sought quantity with the aid of a immunosorption techique in a known manner, particularly by adsorbing the fibrinogen or fibronectin on a surface and then reacting said fibrinogen or fibronectin with said other reaction component and then with an antibody specific against the substance to be determined, which antibody may be labelled, whereafter the amount of said antibody is measured in a known manner, optionally by reaction with a labelled secondary antibody.

2. A method according to Claim 1 for determining the fibrinogen concentration, c h a r a c t e r i z e d by
   - adsorbing fibronectin on a surface;
   - adding a sample solution containing fibrinogen to the surface on which the fibronectin is adsorbed;
   - adding calcium chloride and Factor XIIIa to said surface for the purpose of incorporating the fibrinogen;
   - reacting the fibronectin-bound fibrinogen with a specific antibody against fibrinogen;
   - reacting the resultant body with a so-called secondary antibody with peroxidase-labelled anti-IgG;

- visibilizing the amount of peroxidase bound to and quantitatively-proportional to the fibronectin-fibrinogen body, by adding a substrate for peroxidase, to produce a yellow colour;
- measuring the intensity of the yellow colour; and
- calculating the amount of fibrinogen present from said measured intensity.

3. A method according to Claim 1 for determining fibronectin, c h a r a c t e r i z e d by
- adsorbing fibrinogen on a surface;
- adding a sample solution containing fibronectin to the surface on which the fibrinogen is adsorbed;
- adding calcium chloride and Factor XIIIa to said surface for the purpose of incorporating the fibronectin;
- reacting the fibrinogen-bound fibronectin with an antibody which is specific against fibronectin;
- reacting the resultant body with a so-called secondary antibody with peroxidase labelled anti-IgG;
- visibilizing the amount of peroxidase bound to and quantity-wise proportional to the fibrinogen-fibronectin body, by adding a substrate for peroxidase, to form a colour;
- measuring the intensity of the colour; and
- calculating the amount of fibronectin present on the basis of the calculated colour intensity.

4. A method according to Claim 1 for determining α_2-antiplasmin, c h a r a c t e r i z e d by
- absorbing fibrinogen on a surface;
- adding a sample solution containing α_2-antiplasmin to the surface on which fibrinogen is absorbed;
- adding calcium chloride and Factor XIIIa to said surface for the purpose of incorporating the α_2-antiplasmin;

- reacting the fibrinogen-bound α_2-antiplasmin with a specific antibody against α_2-antiplasmin;
- reacting the resultant body with a so-called secondary antibody with peroxidase labelled anti-IgG;
- visibilizing the amount of peroxidase bound to and quantity-wise proportional to the fibrinogen-α_2-antiplasmin body, by adding a substrate for peroxidase, to form a colour;
- measuring the intensity of the colour; and
- calculating the amount of fibrinogen present from said measured colour intensity.

5. A method according to Claim 1 for determining the activity of Factor XIII, c h a r a c t e r i z e d by

- adsorbing fibrinogen on a surface;
- adding an excess quantity of fibronectin, and calcium chloride;
- adding to the surface a sample solution containing Factor XIII, wherein fibronectin is incorporated in the fibrinogen on said surface; and
- visibilizing fibronectin with a specific antibody against fibronectin and a secondary antibody labelled with peroxidase.

6. A method according to Claim 1 for determining the activity of Factor XIII, c h a r a c t e r i z e d by

- adsorbing fibronectin on a surface;
- adding an excess quantity of fibrinogen, and calcium chloride;
- adding to the surface a sample solution containing Factor XIII, wherein fibrinogen is incorporated in the fibronectin on said surface; and
- visibilizing fibrinogen with a specific antibody against fibrinogen and a secondary antibody labelled with peroxidase.

7. A method according to Claim 5 or 6 for determining the total activity of Factor XIII, c h a r a c - t e r i z e d by first removing fibrinogen from the sample solution by coagulation with a snake venom enzyme, such as batroxobin; and by converting Factor XIII to an activated form by adding an enzyme, such as thrombin.

8. A method according to any one of Claims 1-7, c h a r a c t e r i z e d by using a specific, labelled antibody, particularly an antibody labelled with "horse-radish-peroxidase", instead of using a specific antibody and a secondary antibody labelled with peroxidase.

Figure 1

A schematic illustration of the courses followed by the reactions that occur when determining fibrinogen concentration.

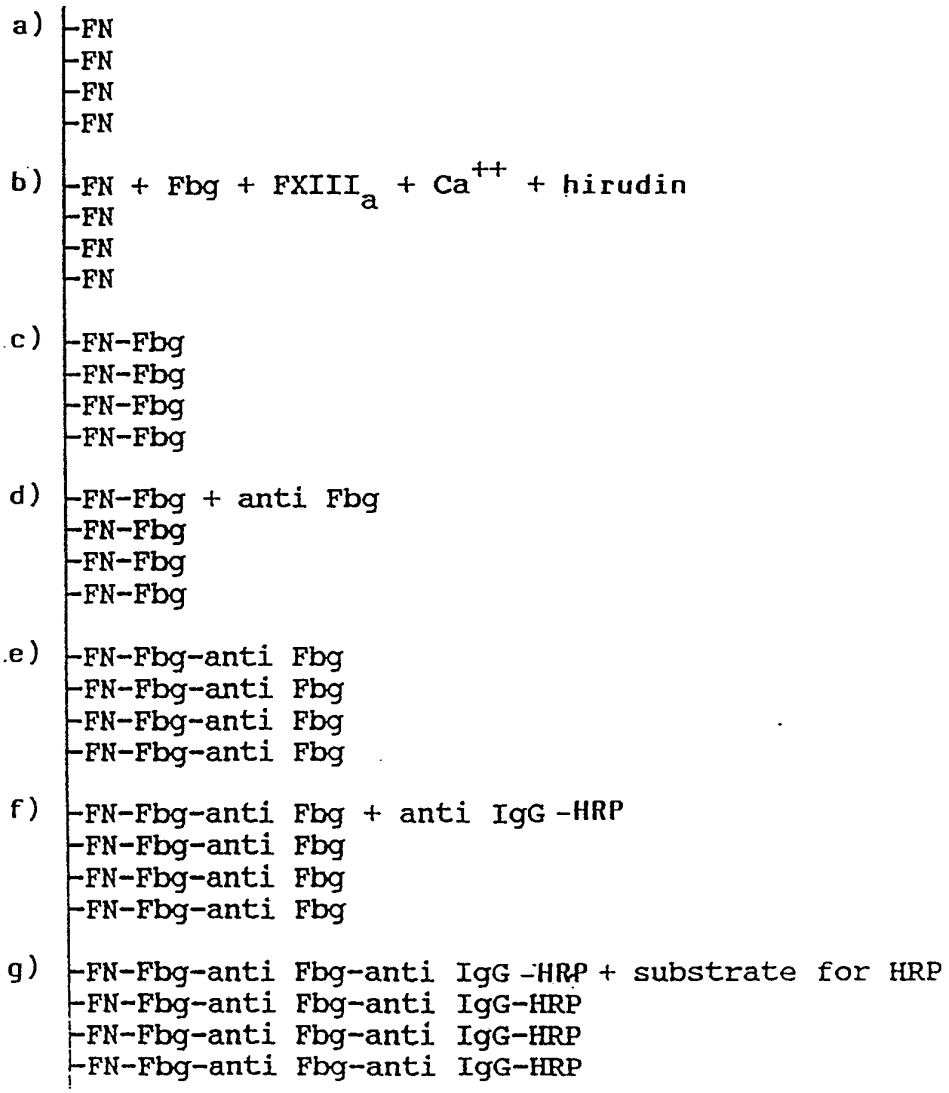


Figure 2

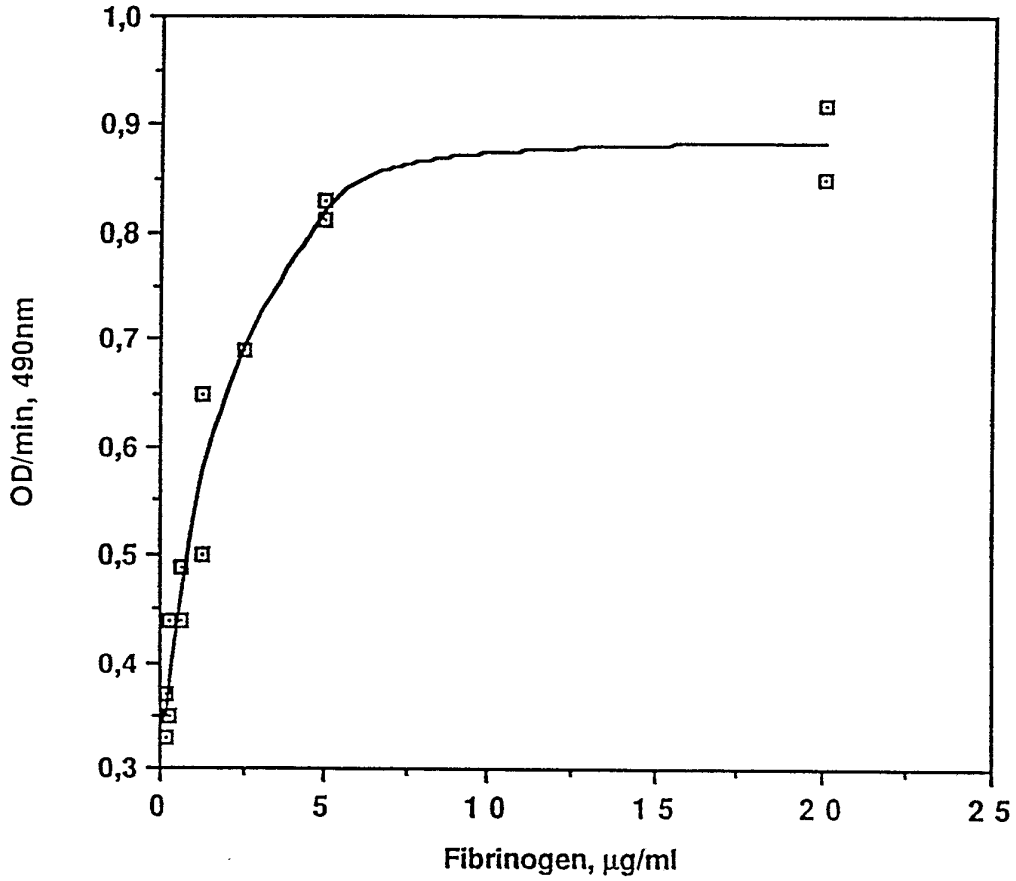


Figure 2. Relationship between colour intensity and fibrinogen concentration.

Figure 3

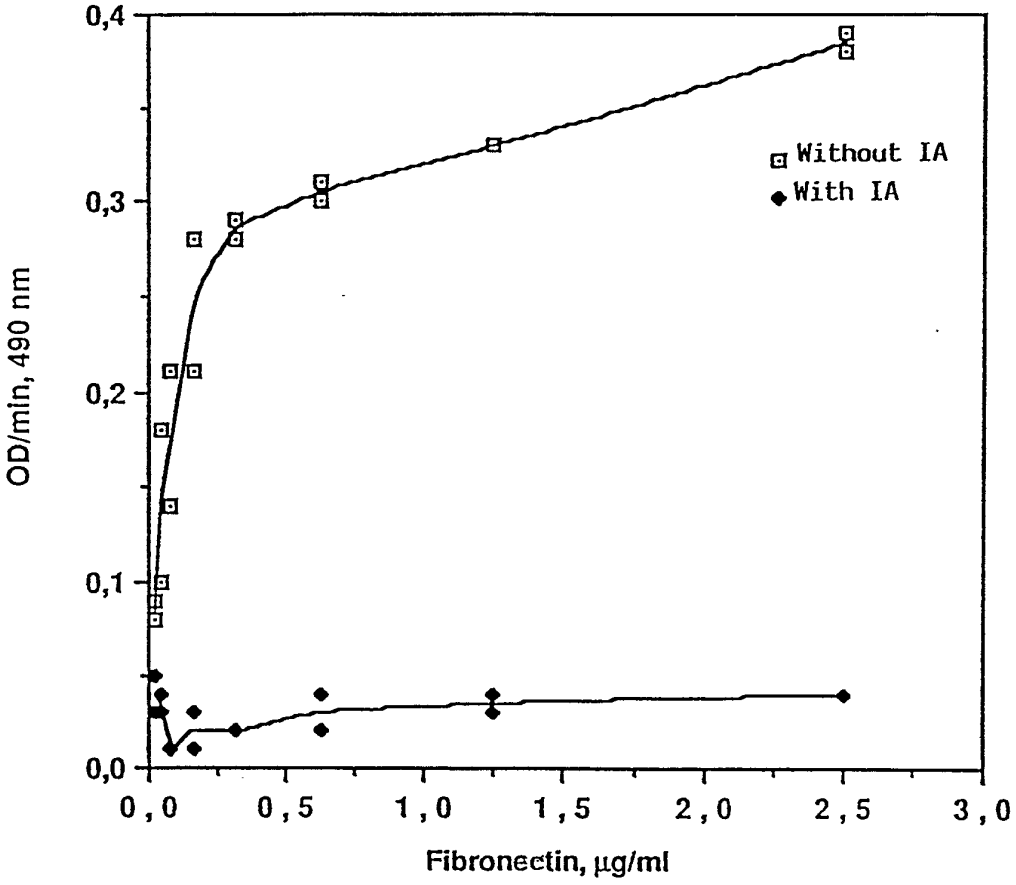


Figure 3. Relationship between colour intensity and fibronectin concentration and the effect of iodo acetamide (IA) on Factor XIII-reaction.

Figure 4

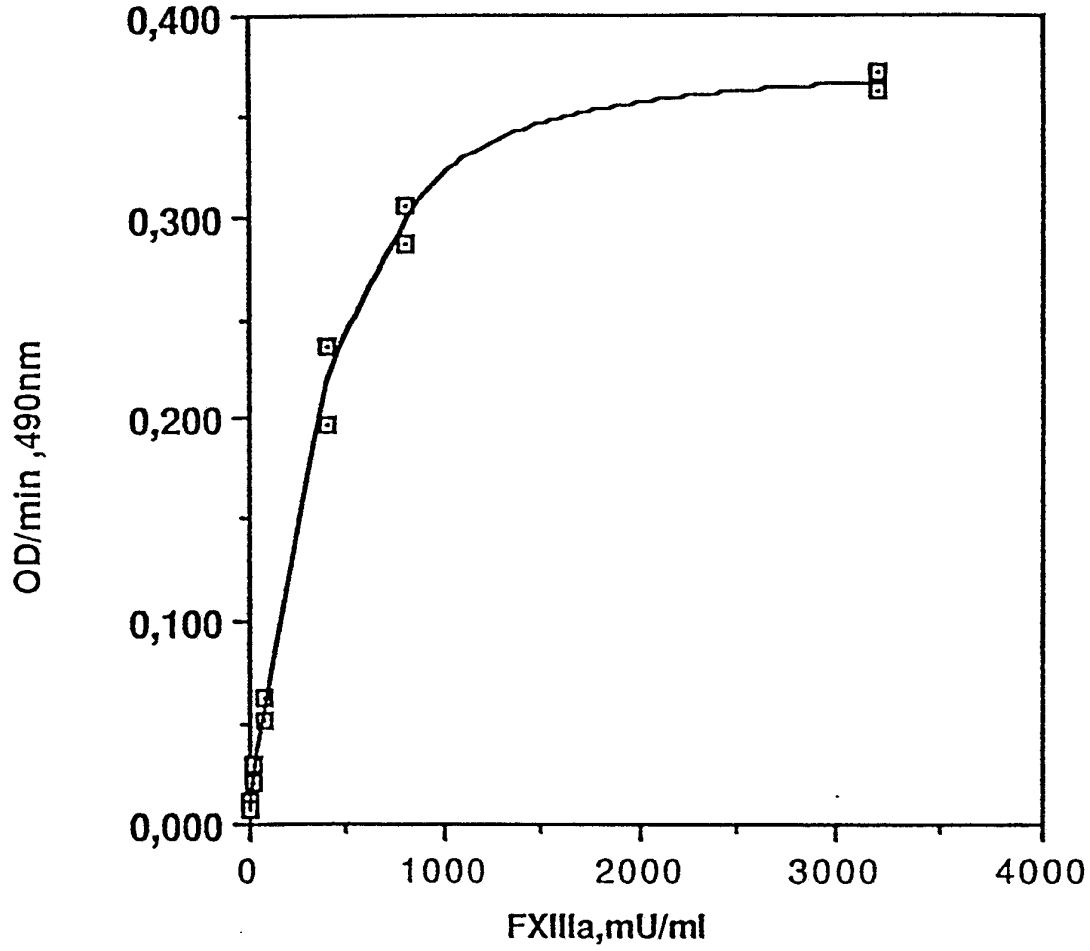


Figure 4. Relationship between colour intensity and Factor XIIIa-activity.

Figure 5

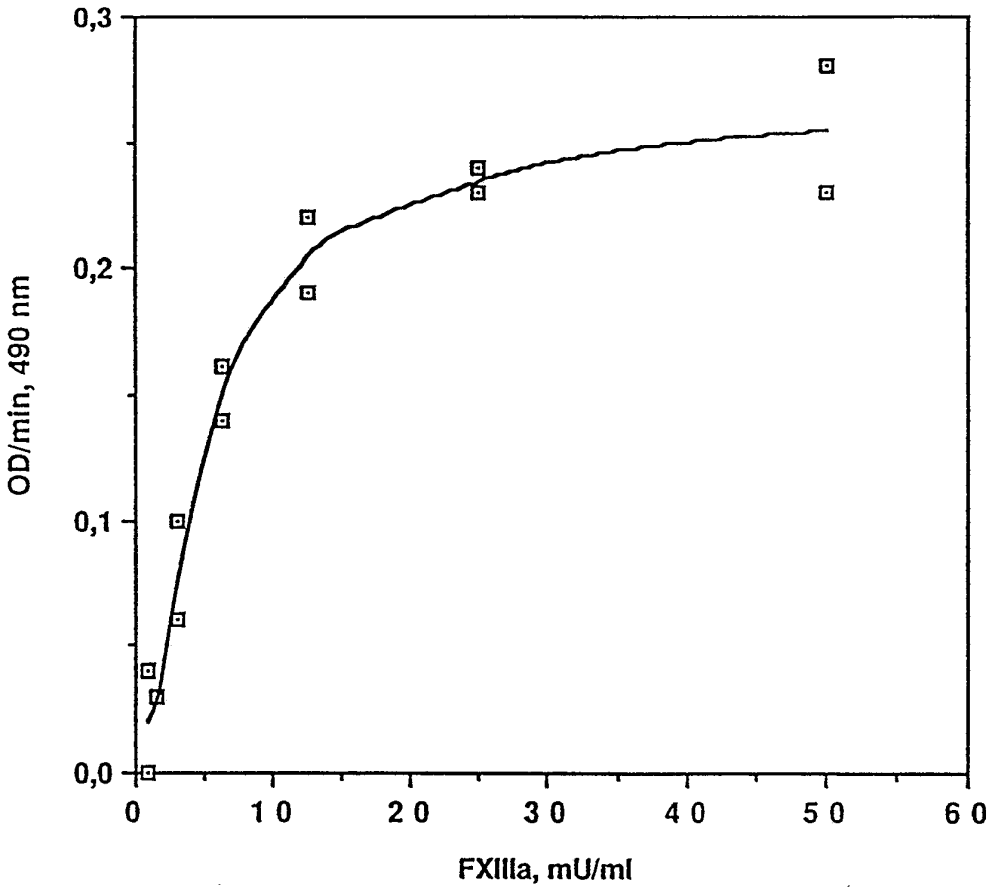


Figure 5. Relationship between colour intensity and Factor XIIIa-activity.

FIGURE 6

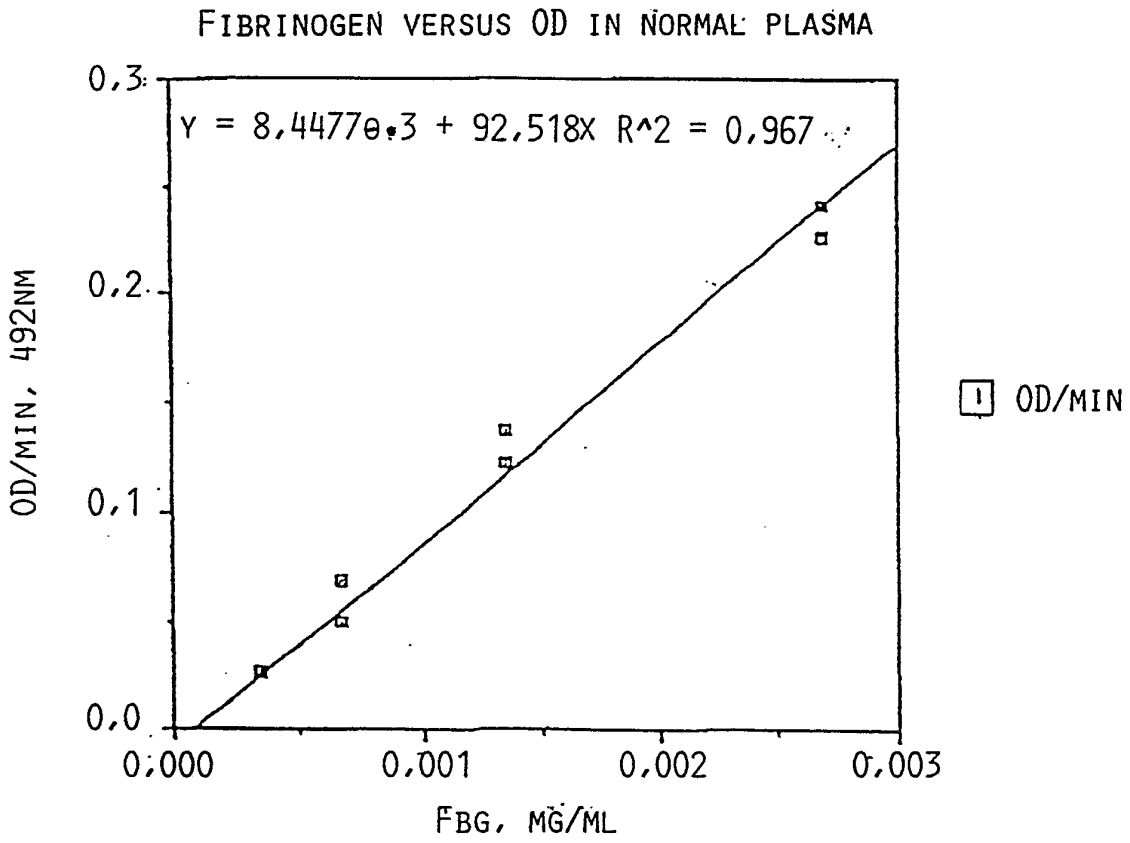

FIBRINOGEN VERSUS OD IN NORMAL PLASMA
Y = 8,4477e-3 + 92,518X R^2 = 0,967
OD/MIN, 492NM
0,3
0,2
0,1
0,0
0,000
0,001
0,002
0,003
FBG, MG/ML
OD/MIN

FIGURE 7

FIBRONECTIN CONCENTRATION IN PLASMA BEFORE AND AFTER COAGULATION WITH BATROXOBIN

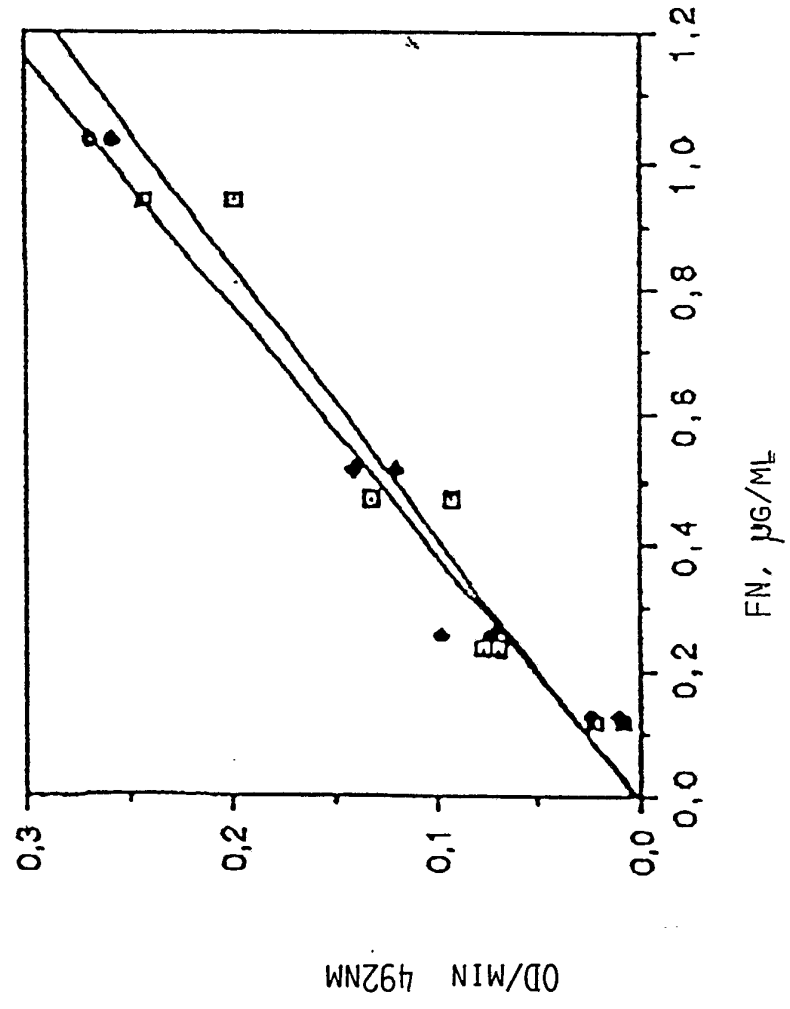
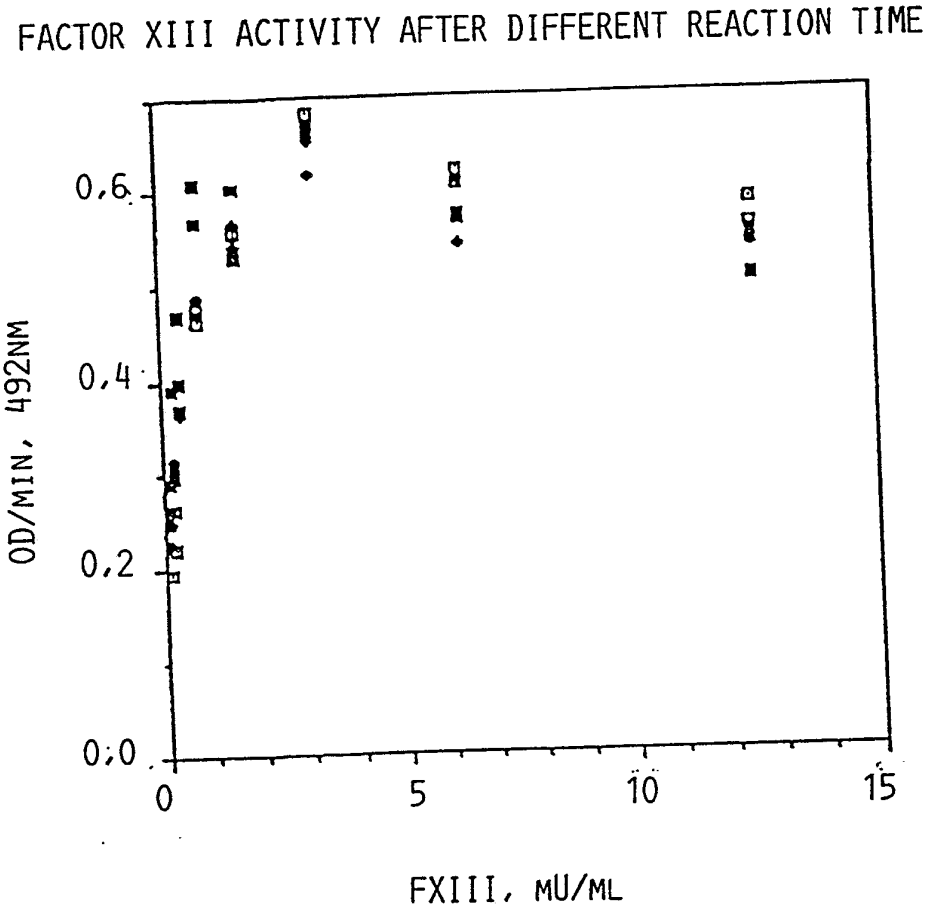


FIGURE 8


FACTOR XIII ACTIVITY AFTER DIFFERENT REACTION TIME
OD/MIN, 492NM
0,6
0,4
0,2
0;0
0
5
10
15
FXIII, MU/ML

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00318

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/573, 33/86

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	US, A, 4624927 (T. FUKUSHIMA ET AL.) 25 November 1986, see the whole document --	1,5-7
A	MEDLINE (NLM Database) Accession number 86243695, Blood 1986 Jul;68(1):95-101 (Hada M. et al.) "Covalent crosslinking of von Willebrand factor to fibrin". --	1-8
A	MEDLINE (NLM Database) Accession number 89051068, Biochim Biophys Acta 1988 Nov 17; 967(2):304-13 (Procyk R. et al.) "Factor XIII--induced crosslinking in solutions of fibrinogen and fibrinonectin". --	1-8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
9th July 1991	1991 -08- 02
International Searching Authority	Signature of Authorized Officer[14]
SWEDISH PATENT OFFICE	Carl-Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	MEDLINE (NLM Database) Accession number 88273153, J Biol Chem 1988 Jul 25; 263(21):10464-9 (Barry E.L. et al) "Factor XIII cross-linking of fibronectin at cellular matrix assembly sites". --	1,3
A	MEDLINE (NLM Database) Accession number 87299006, Biol Chem Hoppe Seyler 1987 Jun; 368(6):669-74 (Hormann H. et al) "N-terminal fibronectin 30-kDa fragment mediates the immobilization of soluble fibrin by factor XIIIa--coated polystyrene beads". --	1,3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00318

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-05-29
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4624927	86-11-25	CA-A- 1215923	86-12-30
		EP-A-B- 0122620	84-10-24
		JP-A- 59192961	84-11-01