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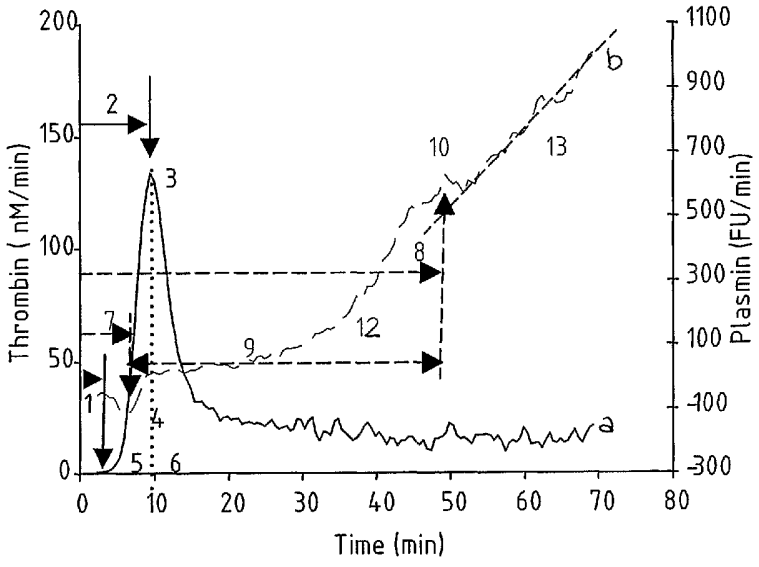


(57) Abstract: The invention relates to a hemostasis assay comprising the provision of a reaction mixture comprising a blood product to be tested, a trigger molecule for inducing thrombin generation, a thrombin-specific substrate that upon cleavage by thrombin produces a measurable thrombin-specific signal, a trigger molecule for inducing plasmin generation, a plasmin-specific substrate that upon cleavage by plasmin produces a measurable plasmin-specific signal, a phospholipid-containing surface and calcium ions, and determination of the amount of thrombin and the amount of plasmin generated in the reaction mixture in time starting at t=0 by measuring the thrombin-specific and plasmin-specific signals.

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# HEMOSTASIS ASSAY

The present invention relates to an assay for simultaneously determining two or more reaction parameters, in particular thrombin generation and plasmin generation. The invention further provides a kit for performing the assay.

Upon normal physiological conditions, hemostasis, the readiness of the blood to clot to prevent blood loss, is kept in hemostatic balance by feedback mechanisms. The hemostatic balance is dependent on both the pro and anticoagulant pathway as the fibrinolytic system and the vessel wall. In case the hemostatic balance is out of equilibrium, pathological clotting (vessel blockage) or bleeding (hemorrhage) can supplant normal hemostasis.

Abnormalities of the hemostatic system can be acquired or congenital. In such cases it is clinically essential to diagnose, monitor, and manage the patient in order to optimize therapeutic intervention.

Most coagulation testing involves end-point assays, which detect the clotting time of blood plasma or the real time clot lysis by means of turbidimetry. Although performed routinely, the currently available coagulation assays have inherent limitations that make them potentially unreliable as tools for monitoring increased coagulation. Moreover, there is not always a good correlation between the results of coagulation tests and the prevention of postoperative haemorrhage or recurrent thrombosis.

Most of the limitations relate to the fact that these are end-point tests that measure the time of clot formation *in vitro* and require the addition of exogenous reagents (such as tissue factor, kaolin and $Ca^{2+}$ ions to replenish those bound by an anticoagulant), and thus do not necessarily

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reflect the patient's thrombotic potential (clotting potential).

As compared to the tests decribed above EP-420 332 discloses an improved thrombin generation assay. In this assay not only information is gathered about the clotting of plasma but also about the total thrombin generation after clot formation. These assays were first performed with chromogenic substrates and later on with fluorogenic substrates. Furthermore, several thrombin generation assays with platelet poor and platelet rich plasma are disclosed.

In these assays again the limitation is the measurement of increased coagulability (thrombophilia). Thrombophilia is a term used to describe a group of conditions in which there is an increased tendency, often repeated and often over an extended period of time, for excessive clotting.

A need therefore exists for a new assay that does not have the above indicated drawbacks, that is more simple and can measure the fibrinolysis in dependency of thrombin generation. It is the object of the invention to provide such assay.

In the research that led to the invention a new assay, in particular a fluorimetric assay, was developed in which thrombin generation as well as plasmin generation can be determined in time, simultaneously in one single well. The hemostasis assay of the invention differs from existing assays in two ways. The assay of the invention provides simultaneous detection of the generation of both thrombin and plasmin in one single well. Furthermore, the assay uses thrombin generation dependent plasmin generation instead of the addition of thrombin or fibrin. This leads to the possibility to measure abnormalities in coagulation induced by aberrations in fibrinolysis.

The invention thus relates to a hemostasis assay comprising the provision of a reaction mixture comprising a blood product to be tested, a trigger molecule for inducing thrombin generation, a thrombin-specific substrate that upon cleavage by thrombin produces a measurable thrombin-specific signal, a trigger molecule for inducing plasmin generation, a plasmin-specific substrate that upon cleavage by plasmin produces a measurable plasmin-specific signal, calcium ions and a phospholipid-containing surface, and determining the amount of thrombin and the amount of plasmin generated in the reaction mixture in time starting at t=0 by measuring the thrombin-specific and plasmin-specific signals.

The assay of the invention can suitably be performed in one single container. For bulk testing of blood samples the container in which the assay is performed is suitably a well of a microtitre plate. Such microtitre plates can be automatically processed in equipment that is well-known in the art. In one embodiment, the reagents are suitably contained in the container before the blood sample is added and can for example be coated to the wall or be present in lyophilized form. This is in particular useful in kits. However, the other way round, i.e. first adding the blood product to the well and then the other reagents is also possible.

The assay of the invention can be used for determining the thrombin and plasmin generation in various blood products, such as plasma, whole blood, drain liquid and platelet-rich plasma.

The phospholipid-containing surface consists for example of phopholipid vesicles, cephalin, cells, in particular endothelial cells, blood platelets, bacteria, viruses, matrices of endothelial cells or microvessels or other surfaces known to the person skilled in the art.

The trigger molecule for inducing generation of thrombin is suitably tissue factor (TF). TF mediates hemostasis by complexing with factor VIIa to directly convert X to Xa (extrinsic pathway), or indirectly by generating Xa by converting IX to IXa, which, in turn, complexes with VIIIa to convert X to Xa. Factor Xa, once generated, complexes with its co-factor, Va, to convert prothrombin (II) to thrombin (IIa). TF is preferred because it is the same trigger that is found in the body for the extrinsic pathway. Triggers for the intrinsic route are for example surfaces like glass, kaolin, silica, or an acid.

Plasmin is formed by activation of the pro-enzyme, plasminogen, by plasminogen activators. Tissue plasminogen activators are found in many tissues. Tissue plasminogen activator is released by endothelial cells and activated platelets. The trigger molecule for inducing generation of plasmin in the assay of the invention is suitably tissue plasminogen activator (tPA) because it is also the trigger in the natural situation in the body. Examples of other triggers are urokinase and streptokinase.

The hemostasis assay of the invention uses preferably two fluorescent substrates, one for thrombin and one for plasmin, with different fluorescent excitation and emission spectra. After cleavage of the thrombin-specific fluorescent substrate by thrombin the fluorescent signal can be determined after excitation at the emission wavelength. The same holds true for the plasmin-specific fluorescent substrate.

Preferably two fluorescent substrates are chosen, that do not interfere with each other. In practice this means that the spectra of the different fluorescent substrates do not overlap. The assay of the invention can thus be performed to simultaneously measure both thrombin and plasmin

generation. Both products can be determined in real time in one single well by using a fluorimeter equipped with two sets of filters. Examples of suitable substrates are the following: thrombin specific substrate coupled to 7-amino-4-methylcoumarin (AMC) (Bz-ß-Ala-Gly-Arg-AMC-AcOH), and plasmin specific substrate coupled to rhodamine 110 (bis-(CBZ-L-phenylalanyl-L-arginine amide)).

The assay of the invention can be used to determine the effects of drugs, proteins, cells or other additives on both thrombin and plasmin generation and also the effect of a disturbed thrombin or plasmin generation on plasmin generation or thrombin generation, respectively. In order to measure the effect of such additives they can be added to the reaction mixture. These additives can also be coated to the wells in the container in which the assay is performed, such as the wells of 96-well plates. When endothelial cells are part of the reaction mixture they may be cultured in the wells.

Furthermore, different conditions can be chosen to start the reaction. For example, different concentrations of the reagents can be used.

The method of the invention has the following advantages compared to the currently available tests. Information about the extrinsic as well as intrinsic clotting pathways is obtained in one assay. To date this information can only be gathered by performing the APTT as well as the PT standard clotting assays. The invention further provides the possibility to determine also hypercoagulability, which is not possible with the standard thrombin generation assay performed in platelet poor plasma. With the assay of the invention straightforward analysis of thrombin generation without defibrination of plasma is possible. This pre-conditional step is initially required in the known thrombin

generation assay of Hemker. Furthermore, the assay of the invention provides direct and on-line analysis of both thrombin and plasmin generation, which allows direct interpretation of the results. Moreover, immediate results are obtained about fibrinolysis and the effect of fibrinolysis on coagulation. No pre-conditional steps have to be performed to analyse fibrinolysis. These steps are a prerequisite in the euglobulin clot lysis test.

In addition to the specific assay described above, the invention provides a more general assay in which two or more reactions are simultaneously analysed. The main requirement is that the spectra of the different substrates that are processed in the reaction to be tested do not overlap.

Such general method comprises provision of a reaction mixture comprising a surface, a sample to be tested, trigger molecules for inducing the reactions to be measured, substrates that produce a measurable signal that is the result of the reaction, and determination of the signals generated in the reaction mixture in time starting at t=0. The surface is in particular a phospholipid surface for thrombin generation and intrinsically formed fibrin for plasmin generation.

In the specific embodiment of the invention the reactions to be tested are thrombin generation and plasmin generation. Other pairs of reactions can also be tested, either with different pairs of coagulation factors and/or cells. An example is thrombin generation and release of platelet specific enzymes. The substrate depends on the enzyme to be tested. In general the system can accept even more than two analyses in one experiment as long as the spectra do not overlap.

The invention further relates to a kit for performing the assay of the invention comprising a container comprising one or more of the following reagents: a trigger molecule for inducing thrombin generation, a thrombin-specific substrate that upon cleavage by thrombin produces a measurable thrombin-specific signal, a plasmin-specific substrate that upon cleavage by plasmin produces a measurable plasmin-specific signal, a trigger molecule for inducing plasmin generation, a phospholipid surface and calcium ions. The other reagents of this list that are not present in the container are either part of the kit or can be added separately. It is important to have all reagents present upon performing the assay. The reaction is started when all reagents and the blood product to be tested are present in the reaction mixture.

The different embodiments of the various reagents have been described above.

In the Examples that follow the assay of the invention has been tested using different patient samples. Both intra- and inter-assay variations were established.

Reference is made to the following figures:

**Figure 1** shows a typical plot resulting from the assay of the invention. Line (a) represents the first derivative of thrombin generation expressed in nM/min (via a standard calculation curve). Line (b) represents the first derivative of plasmin generation expressed in FU/min. The following data can be generated out of the graph:

1: Thrombin generation lag time
2: Time to thrombin peak
3: Maximal thrombin generation
4: Thrombin potential
5: Procoagulant thrombin potential
6: Anticoagulant thrombin potential

7: Plasmin generation drop time (start clot formation)

8: Plasmin generation peak time (solubilized clot)

9: Clot lysis time

10: Plasmin generation level at plasmin generation peak time

12: Plasmin potential acceleration phase (clot lysis area)

13: Plasmin potential (measured over 10 minutes)

**Figure 2** shows the thrombin (A) and plasmin (B) generation in the presence of different hirudin concentrations using normal pooled citrated plasma.

**Figure 3** shows the effect of tPA titration on thrombin (A) and plasmin (B) generation using normal pooled citrated plasma.

**Figure 4** shows thrombin (A) and plasmin (B) generation after the addition of active protein C using normal pooled citrated plasma.

**Figure 5** shows thrombin (A) and plasmin (B) generation in the presence of different thrombomodulin (TM) concentrations using normal pooled citrated plasma.

**Figure 6** shows thrombin generation and plasmin generation in the presence of thrombomodulin (TM) and carboxypeptidase inhibitor (CPI), the inhibitor of TAFI using normal pooled citrated plasma.

**Figure 7** shows thrombin (A) and plasmin (B) generation in the presence of different plasmin concentrations using normal pooled citrated plasma.

**Figure 8** shows thrombin and plasmin generation in normal pooled citrated plasma of a patient with the alpha-2-anti-plasmin deficiency and a patient with a PAI-1 excess.

**Figure 9** shows thrombin and plasmin generation in plasma of a patient with Factor VII deficiency before and after Factor VIIa suppletion.

In **Figure 10** the lines a and d represent the thrombin (line a) and plasmin (lines d) generation measured using a normal pooled citrated plasma. The lines b and e represent plasma of a severe Hemophilia A patient before factor VIII suppletion whereas the lines c and f represents plasma of a HA patient after factor VIII suppletion.

**Figure 11** shows thrombin (line a) and plasmin (line c) generation in normal pooled plasma and plasma of a patient with a prothrombin deficiency (5%), lines b and d, respectively.

**Figure 12** shows Thrombin (lines a-e) and plasmin generation (lines f-j) in normal pooled plasma (lines a and f), and plasmas from a patient heterozygous for factor V Leiden (lines b and g), heterozygous for prothrombin mutation (lines c and h), protein S deficiency (lines d and i) and protein C deficiency (lines e and j).

## EXAMPLES

### MATERIALS AND METHODS

1. *Substrates*

The substrates used for testing were the thrombin specific substrate coupled to 7-amino-4-methylcoumarin (AMC) (Pentapharm, Bz-ß-Ala-Gly-Arg-AMC-AcOH, product number PF 082-21), and the plasmin specific substrate coupled to rhodamine 110 (Molecular Probes, rhodamine 110, bis-(CBZ-L-phenylalanyl-L-arginine amide), dihydrochloride. product number R-6502)

2. *Assay conditions*

The assay conditions were:

80 µl citrated plasma

2 µl cephalin (Roche product number 524298 (dissolved in 1 ml distilled water))

2 µl recombinant Tissue Factor (Innovin, Dade Behring) (final concentration 0.1 pM)

19 µl Tris buffered Saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4) containing

- thrombin-specific fluorogenic substrate (final concentration 833 µM)
- plasmin fluorogenic substrate (final concentration 33 µM)

17 µl TBS containing

- recombinant-tPA (Actilyse, Boehringer Ingelheim)(final concentration 150 IU/ml)
- $CaCl_2$ (final $Ca^{2+}$ concentration 16,7 mM)

3. *Method*

The fluorogenic signals were determined in a thermostated fluorimeter. For thrombin (line A), the excitation wavelength was 355 nm and the emission wavelength 460 nm. For plasmin (line B), the excitation wavelength was 485 nm and the emission wavelength 520 nm. The first derivatives were calculated and plotted. A typical plot is shown in **Figure 1**.

4. *Experiments*

In Example 1 the above described experiment was performed with variations in the concentration of the various reagents. In **Example 2** the plasma of various patient was tested.

**EXAMPLE 1**

Effect of different reagents

1. Hirudin

The thrombin and plasmin generation in the presence of different hirudin concentrations was tested. The results are shown in **Figure 2** and **Table 1.**

**Table 1**

| Hirudin | 0 U/ml | 2 U/ml | 8 U/ml | 17 U/ml | 33 U/ml | 83 U/ml |
|---|---|---|---|---|---|---|
| Lag Time Trombin Generation | 5,0 | 6,0 | 9,5 | 12,0 | 26,0 | N.D. |
| Time To Trombin Peak | 8,5 | 14,0 | 19,0 | 22,5 | 31,5 | N.D. |
| Trombin Peak Height | 273 | 220 | 187 | 130 | 89 | N.D. |
| Trombin Potential (ETP) | 2391 | 2233 | 1978 | 1425 | 933 | N.D. |
| ETP, procoagulant | 488 | 480 | 510 | 364 | 229 | N.D. |
| ETP, anticoagulant | 1903 | 1753 | 1469 | 1061 | 704 | N.D. |
| Plasmin Droptime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peaktime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Fibrin Lysis Time | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peak Height | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| (PPeakHeight-PDropHeight)/FLT | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, accel-phase | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, PPT+10min | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

In **Figure 2,** thrombin generation is presented in panel A whereas plasmin generation is shown in panel B. Different hirudin concentrations were used: 0 (line a), 2 (line b), 8 (line c), 17 (line d), 33 (line e) and 83 (line f) U/ml.

Hirudin is a direct inhibitor of thrombin. Increasing the hirudin concentration both inhibits and retards thrombin generation, probably due to the fact that both factor V, factor VIII and Factor XI are not activated by thrombin. In panel B it is shown that due to the inhibition of thrombin generation plasmin generation is only retarded as fibrin formation is retarded.

2. *Trypsin inhibitor*

The addition of Corn Trypsin Inhibitor (CTI) the inhibitor of the intrinsic pathway was also tested. CTI does not affect both thrombin generation and plasmin generation indicating that the reaction is completely tissue factor dependent (data not shown).

3. *tPA*

For a tPA titration in normal pooled plasma different tissue-plasminogen activator (t-PA) concentrations were used as follows: 600 (line a), 230 (line b), 150 (line c), 110 (line d), 75 (line e) and 0 (line f) IU/ml. The results are shown in **Figure 3** and **Table 2**. Panel A of **Figure 3** represents thrombin generation. Panel B represents plasmin generation.

**Table 2**

| t-PA titration | 600 IU/ml | 230 IU/ml | 150 IU/ml | 110 IU/ml | 75 IU/ml | 0 IU/ml |
|---|---|---|---|---|---|---|
| Lag Time Trombin Generation | 5.0 | 5.5 | 5.5 | 5.5 | 6.0 | 6.0 |
| Time To Trombin Peak | 10.0 | 12.0 | 14.0 | 13.0 | 11.5 | 13.5 |
| Trombin Peak Height | 257 | 196 | 151 | 160 | 195 | 111 |
| Trombin Potential (ETP) | 2095 | 2136 | 2024 | 1944 | 1945 | 1472 |
| ETP, procoagulant | 482 | 452 | 464 | 434 | 379 | 306 |
| ETP, anticoagulant | 1613 | 1684 | 1560 | 1510 | 1566 | 1166 |
| Plasmin Droptime | 5.0 | 5.5 | 7.0 | 5.5 | 6.0 | N.D. |
| Plasmin Peaktime | 13.0 | 18.0 | 24.0 | 38.5 | 45.0 | N.D. |
| Fibrin Lysis Time | 8.0 | 12.5 | 17.0 | 33.0 | 39.0 | N.D. |
| Plasmin Peak Height | 1418 | 901 | 716 | 703 | 74 | N.D. |
| (PPeakHeight-PDropHeight)/FLT | 169.7 | 71.0 | 43.0 | 21.4 | 1.4 | N.D. |
| Plasmin Potential, accel-phase | 4211 | 3808 | 4096 | 8052 | 1345 | N.D. |
| Plasmin Potential, PPT+10min | 12982 | 7721 | 6586 | 7469 | 391 | N.D. |

The higher the t-PA concentration the more plasmin and interestingly more thrombin is generated. Under normal conditions 150 IU/ml of t-PA (d) are used in the assay. Using this concentration allows the detection of thrombin-activatable fibrinolysis inhibitor (TAFI) activity. If the t-PA concentration is higher, the clot lysis is faster and less TAFI activity can be observed.

4. *Active protein C*

For testing thrombin (A) and plasmin (B) generation after the addition of active protein C, different APC concentrations were used as follows: 10.25 microgram/ml (line a), 5.1 (line b), 4.1 (line c), 2.6 (line d), 1.7 (line e), and 0 (line f) microgram/ml. The results are shown in **Figure 4** and **Table 3.**

**Table 3**

| APC | 10.25 microgr/ml | 5.1 microgr/ml | 4.1 microgr/ml | 2.6 microgr/ml | 1.7 microgr/ml | 0 microgr/ml |
|---|---|---|---|---|---|---|
| Lag Time Trombin Generation | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Time To Trombin Peak | 6.5 | 6.0 | 5.5 | 5.5 | 5.5 | 5.5 |
| Trombin Peak Height | 69 | 97 | 140 | 166 | 222 | 229 |
| Trombin Potential (ETP) | 982 | 1176 | 1487 | 1682 | 1974 | 2109 |
| ETP, procoagulant | 138 | 163 | 202 | 233 | 345 | 326 |
| ETP, anticoagulant | 844 | 1014 | 1285 | 1449 | 1630 | 1783 |
| Plasmin Droptime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peaktime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Fibrin Lysis Time | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peak Height | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| (PPeakHeight-PDropHeight)/FLT | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, accel-phase | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, PPT+10min | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

In **Figure 4,** the line (f) represents the situation without APC addition. Active protein C inactivates factor Va and factor VIIIa. As such APC is part of the anticoagulant pathway. As expected addition of APC solely affects the thrombin potential (panel A) and affects TAFI activity as TAFI needs relatively large concentration of thrombin to be activated. In the situation without APC (line f) fibrinolysis is retarded due to TAFI activity.

5. *Thrombomodulin*

Thrombin and plasmin generation was measured in the presence of different Thrombomodulin (TM) concentrations.

Thrombin generation is presented in panel A of **Figure 5** and **Table 4** whereas plasmin generation is schown in panel B. Different concentrations of TM were used as follows: 0 (line a), 0.085 nM (line b), 0.17 (line c), 0.34 (line d), 10.2 (line e), 51 (line f) and 102 nM (line g).

TM has two activities that are based on thrombin binding. First, TM activates Protein C to Active Protein C (APC). APC inactivates factor Va and Factor VIIIa and thus inhibits thrombin formation (A). The second activity is activation of the thrombin activatable fibrinolysis inhibitor (TAFI). TAFI inhibits fibrinolysis by removing carboxy terminal amino acids from fibrin and thereby removes the plasminogen binding site. The more TM the longer it takes to start plasmin generation which is expected to be an activity of TAFI (panel B). Activation of TAFI occurs at lower TM concentrations than activation of APC.

**Table 4**

| Thrombomodulin (TM) | 0 nM TM | 0.085 nM | 0.17 nM | 0.34 nM | 10.2 nM | 51 nM | 102 nM |
|---|---|---|---|---|---|---|---|
| Lag Time Trombin Generation | 5,0 | 4,5 | 6,0 | 5,5 | 4,5 | N.D. | N.D. |
| Time To Trombin Peak | 12,5 | 12,0 | 13,0 | 13,0 | 12,5 | N.D. | N.D. |
| Trombin Peak Height | 98 | 104 | 74 | 66 | 23 | N.D. | N.D. |
| Trombin Potential (ETP) | 1419 | 1392 | 1023 | 916 | 318 | N.D. | N.D. |
| ETP, procoagulant | 267 | 299 | 189 | 196 | 60 | N.D. | N.D. |
| ETP, anticoagulant | 1152 | 1093 | 834 | 720 | 258 | N.D. | N.D. |
| Plasmin Droptime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peaktime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Fibrin Lysis Time | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peak Height | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| (PPeakHeight-PDropHeight)/FLT | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, accel-phase | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, PPT+10min | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

6. *Carboxypeptidase inhibitor*

Thrombin generation and plasmin generation were measured in the presence of thrombomodulin (TM) and carboxy peptidase inhibitor (CPI), the inhibitor of TAFI. Line (a)

represent the normal situation without any addition. Line (b) represents the situation in the presence of 0.68 nM TM. Line (d) represent the situation with 33 microgram/ml CPI and line (c) the situation with TM and CPI. **Figure 6** and **Table 5** show the results.

Again, TM activates TAFI resulting in a retarded plasmin generation. In the presence of CPI, plasmin generation starts immediately. CPI also abolishes the effect of TM indicating that indeed TM activates TAFI. Thus, even in a normal situation there is TAFI activity.

**Table 5**

| figure 6 | CTAD NP | 0.68 nM TM | 0.68 nM TM + 33 microgr/ml CP | 33 microgr/ml CPI |
|---|---|---|---|---|
| Lag Time Trombin Generation | 5.0 | 5.5 | 5.0 | 4.5 |
| Time To Trombin Peak | 12.5 | 10.5 | 12.0 | 11.5 |
| Trombin Peak Height | 98 | 106 | 76 | 87 |
| Trombin Potential (ETP) | 1419 | 1260 | 1029 | 1369 |
| ETP, procoagulant | 267 | 228 | 205 | 227 |
| ETP, anticoagulant | 1152 | 1032 | 825 | 1143 |
| Plasmin Droptime | 9.0 | N.D. | 5.0 | 7.5 |
| Plasmin Peaktime | 37.5 | N.D. | 29.5 | 26.5 |
| Fibrin Lysis Time | 28.5 | N.D. | 24.5 | 19.0 |
| Plasmin Peak Height | 545 | N.D. | 457 | 459 |
| (PPeakHeight-PDropHeight)/FLT | 19.7 | N.D. | 19.6 | 26.5 |
| Plasmin Potential, accel-phase | 5434 | N.D. | 4210 | 3960 |
| Plasmin Potential, PPT+10min | 5842 | N.D. | 4750 | 4749 |

7. *Plasmin*

Thrombin and plasmin generation were measured in the presence of different plasmin concentrations. Thrombin generation is presented in panel A of **Figure 7**, whereas plasmin generation is shown in panel B (see also **Table 6**). The following final plasmin concentrations were used: 0 (line a), 1 (line b), 3 (line c), 6 (line d), 9 (line e), 13 (line

f), 16 (line g), 19 (line h), 22 (line i), and 25 (line j) microgram/ml.

To prove that plasmin affects thrombin generation plasmin was directly added to the reaction mixture. As expected, addition of plasmin directly had an effect on the plasmin generation curve. Moreover, plasmin affects also thrombin generation as it shortens the lag-time of thrombin generation and increases the maximal thrombin peak height.

**Table 6**

| series 1-10 | 25 microgr/ml plasmin | 22 microgr/ml plasmin | 19 microgr/ml plasmin | 16 microgr/ml plasmin | 13 microgr/ml plasmin | 9 microgr/ml plasmin | 6 microgr/ml plasmin | 3 microgr/ml plasmin | 1 microgr/ml plasmin | No plasmin |
|---|---|---|---|---|---|---|---|---|---|---|
| Lag Time Trombin Generation | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 4.0 | 5.0 | 5.0 | 5.0 |
| Time To Trombin Peak | 6.5 | 7.5 | 8.0 | 8.0 | 8.0 | 8.5 | 8.0 | 10.0 | 11.0 | 11.5 |
| Trombin Peak Height | 208 | 178 | 159 | 160 | 158 | 136 | 172 | 130 | 118 | 114 |
| Trombin Potential (ETP) | 1855 | 1739 | 1717 | 1927 | 2063 | 1960 | 2051 | 1839 | 1741 | 1729 |
| ETP, procoagulant | 403 | 439 | 418 | 428 | 398 | 307 | 340 | 324 | 290 | 296 |
| ETP, anticoagulant | 1452 | 1300 | 1299 | 1499 | 1666 | 1654 | 1711 | 1515 | 1451 | 1433 |
| Plasmin Droptime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peaktime | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Fibrin Lysis Time | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Peak Height | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| (PPeakHeight-PDropHeight)/FLT | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, accel-phase | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Plasmin Potential, PPT+10min | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

**EXAMPLE 2**

Testing of various clinical conditions

1. *Fibrinolysis patients*

**Figure 8** shows the thrombin (lines a-c) and plasmin (lines d-f) generation in normal pooled plasma (lines c, f) plasma of a patient with the alpha-2-anti-plasmin deficiency (lines b, e) and a patient with a PAI-1 excess (lines a, d) (see also **Table 7**).

**Table 7**

| >200, series 1-3 | PAI-1 excess | a2 anti def | NP plasma |
|---|---|---|---|
| Lag Time Trombin Generation | 6.0 | 3.5 | 4.5 |
| Time To Trombin Peak | 13.0 | 5.0 | 10.0 |
| Trombin Peak Height | 121 | 277 | 115 |
| Trombin Potential (ETP) | 1652 | 1786 | 1553 |
| ETP, procoagulant | 337 | 313 | 288 |
| ETP, anticoagulant | 1316 | 1473 | 1266 |
| Plasmin Droptime | N.D. | N.D. | N.D. |
| Plasmin Peaktime | N.D. | N.D. | N.D. |
| Fibrin Lysis Time | N.D. | N.D. | N.D. |
| Plasmin Peak Height | N.D. | N.D. | N.D. |
| (PPeakHeight-PDropHeight)/FLT | N.D. | N.D. | N.D. |
| Plasmin Potential, accel-phase | N.D. | N.D. | N.D. |
| Plasmin Potential, PPT+10min | N.D. | N.D. | N.D. |

In this example it was observed that the patient with alpha2-AP(lines b, e) had a very increased plasmin peak height and due to this a decreased lag-time in thrombin generation and even increased thrombin generation. The patient with a plasminogen-activator inhibitor type 2 (PAI-2)(lines a, d) excess shows an opposite effect. Plasmin generation is nearly absent and thrombin generation delayed. No effect on the total thrombin generation is observed.

2. *Factor VII deficiency*

Thrombin and plasmin generation was measured in plasma of a patient with Factor VII deficiency before (lines a, c) and after Factor VIIa suppletion (lines b, d). The line a in **Figure 9** represents the thrombin and the line c plasmin generation before factor VII(a) suppletion whereas the lines b and d are after 10 min of 40 microgram/kg factor VIIa suppletion, respectively. See also **Table 8**.

**Table 8**

| series 1-2 | before rVIIa infusion | after rVIIa infusion |
|---|---|---|
| Lag Time Trombin Generation | 5.5 | 3.5 |
| Time To Trombin Peak | 11.5 | 4.5 |
| Trombin Peak Height | 263 | 269 |
| Trombin Potential (ETP) | 2547 | 2519 |
| ETP, procoagulant | 506 | 238 |
| ETP, anticoagulant | 2041 | 2281 |
| Plasmin Droptime | 9.0 | 3.5 |
| Plasmin Peaktime | 32.0 | 29.0 |
| Fibrin Lysis Time | 23.0 | 25.5 |
| Plasmin Peak Height | 1081 | 1458 |
| (PPeakHeight-PDropHeight)/FLT | 49.3 | 58.8 |
| Plasmin Potential, accel-phase | 9039 | 12989 |
| Plasmin Potential, PPT+10min | 13257 | 18139 |

The patient has < 1% factor VII activity and shows a delayed thrombin generation curve and a delayed plasmin generation curves (lines a and c). Suppletion of factor VIIa decreases the lag-time of thrombin and plasmin generation (lines b and d). Interestingly, no effect is observed in the total thrombin generation (thrombin potential) indicating that the Bouma loop is not disturbed (which is the case in patients with severe hemophilia A (HA)).

Like the thrombin generation, after activation plasmin generation is delayed equally fast. This might be explained by the fact that plasmin generation is triggered after fibrin formation. TAFI the inhibitor of fibrinolysis is equally activated.

3. *Haemophilia A*

The plasma of a patient with severe hemophilia A was tested in the assay of the invention. **Figure 10** and **Table 9** show the results.

**Table 9**

| "28-12-2004, series 1-3 | NP plasma | before VIII infusion | 15 min after VIII infusion |
|---|---|---|---|
| Lag Time Trombin Generation | 4.5 | N.D. | 5.5 |
| Time To Trombin Peak | 8.5 | N.D. | 9.5 |
| Trombin Peak Height | 162 | N.D. | 111 |
| Trombin Potential (ETP) | 1756 | N.D. | 1483 |
| ETP, procoagulant | 266 | N.D. | 199 |
| ETP, anticoagulant | 1490 | N.D. | 1284 |
| Plasmin Droptime | 6.0 | N.D. | 7.5 |
| Plasmin Peaktime | 41.0 | N.D. | 28.0 |
| Fibrin Lysis Time | 35.0 | N.D. | 20.5 |
| Plasmin Peak Height | 543 | N.D. | 585 |
| (PPeakHeight-PDropHeight)/FLT | 19.1 | N.D. | 32.2 |
| Plasmin Potential, accel-phase | 5743 | N.D. | 4232 |
| Plasmin Potential, PPT+10min | 5870 | N.D. | 7076 |

In **Figure 10**, the lines represents the thrombin (a-c) and plasmin (d-f) generation measured using a normal pooled citrated plasma. The lines b and e represent plasma of a severe hemophilia A patient before factor VIII suppletion whereas the lines c and f represents plasma of a HA patient after factor VIII suppletion.

It can be seen that thrombin generation is clearly diminished in the Hemophilia A patient and partly restored by factor VIII suppletion. Next, plasmin generation is enhanced in the patient before suppletion and partly restored after factor VIII suppletion. The explanation might be that TAFI the inhibitor of fibrinolysis is not activated (due to the low thrombin level) and thus more plasmin will be generated.

4. *Prothrombin deficiency*

The assay of the invention was used to measure thrombin (line a and b in **Figure 11**) and plasmin (lines c and d) generation in normal pooled plasma (a and c) and plasma of

a patient with a prothrombin deficientie (5%)(b and d). See also **Table 10.**

**Table 10**

| new, series 1-2 | NP CTAD | prothrombin def. |
|---|---|---|
| Lag Time Trombin Generation | 6.5 | N.D. |
| Time To Trombin Peak | 16.0 | N.D. |
| Trombin Peak Height | 137 | N.D. |
| Trombin Potential (ETP) | 1750 | N.D. |
| ETP, procoagulant | 402 | N.D. |
| ETP, anticoagulant | 1348 | N.D. |
| Plasmin Droptime | 13.0 | N.D. |
| Plasmin Peaktime | 42.0 | N.D. |
| Fibrin Lysis Time | 29.0 | N.D. |
| Plasmin Peak Height | 567 | N.D. |
| (PPeakHeight-PDropHeight)/FLT | 18.9 | N.D. |
| Plasmin Potential, accel-phase | 5523 | N.D. |
| Plasmin Potential, PPT+10min | 5884 | N.D. |

The patient has a disturbed thrombin generation and as a consequence an increased fibrinolytic activity which might be explained partly by a lack of TAFI activation by thrombin. TAFI normally inhibits plasmin generation.

5. *Thrombophilia patients*

Thrombin (lines a to e) and plasmin (lines f to j) generation was measured in normal pooled plasma (lines a and f), and in plasmas from various thrombophilia patients, namely a patient heterozygous for factor V Leiden (lines b and g), heterozygous for a prothrombin mutation (lines c and h), a patient having a protein S deficiency (lines d and i) and a patient having protein C deficiency (lines e and j). The results are shown in **Figure 12** and **Table 11.**

**Table 11**

| NP serie 16/12, series 1-5 | NP Ctad | F5 het | F2 het | PS |
|---|---|---|---|---|
| Lag Time Trombin Generation | 4.5 | 4.0 | 4.0 | 3.5 |
| Time To Trombin Peak | 11.5 | 7.5 | 7.5 | 6.5 |
| Trombin Peak Height | 141 | 246 | 343 | 209 |
| Trombin Potential (ETP) | 1967 | 2354 | 2618 | 1924 |
| ETP, procoagulant | 339 | 399 | 592 | 351 |
| ETP, anticoagulant | 1628 | 1955 | 2026 | 1573 |
| Plasmin Droptime | 9.0 | 4.0 | 4.0 | 3.5 |
| Plasmin Peaktime | 29.5 | 34.5 | 38.5 | 33.5 |
| Fibrin Lysis Time | 20.5 | 30.5 | 34.5 | 30.0 |
| Plasmin Peak Height | 627 | 601 | 506 | 539 |
| (PPeakHeight-PDropHeight)/FLT | 31.3 | 20.0 | 12.6 | 16.7 |
| Plasmin Potential, accel-phase | 6151 | 5524 | 4612 | 4384 |
| Plasmin Potential, PPT+10min | 6923 | 6140 | 6333 | 5776 |

Interestingly, all thrombophilia patients have a decreased thrombin generation lag time, increased total thrombin generation (thrombin potential) and interestingly decreased plasmin generation, resulting in an increased clot lysis time. The explanation might be more TAFI activation and due to this a delayed plasmin generation. After a while, the activity of TAFI is gone and normal plasmin generation occurs.

**CLAIMS**

1. Hemostasis assay comprising the provision of a reaction mixture comprising a blood product to be tested, a trigger molecule for inducing thrombin generation, a thrombin-specific substrate that upon cleavage by thrombin produces a measurable thrombin-specific signal, a trigger molecule for inducing plasmin generation, a plasmin-specific substrate that upon cleavage by plasmin produces a measurable plasmin-specific signal, a phospholipid-containing surface and calcium ions, and determination of the amount of thrombin and the amount of plasmin generated in the reaction mixture in time starting at t=0 by measuring the thrombin-specific and plasmin-specific signals.

2. Assay as claimed in claim 1, wherein the blood product to be tested is selected from plasma, drain fluid, whole blood and platelet-rich plasma.

3. Assay as claimed in claim 1 or 2, wherein the trigger molecule for inducing generation of thrombin is an initiator of the extrinsic pathway, in particular tissue factor (TF) or of the intrinsic pathway, in particular, glass, kaoline, acid.

4. Assay as claimed in any one of the claims 1 to 3, wherein the trigger molecule for inducing generation of plasmin in the assay of the invention is selected from urokinase, streptokinase and tissue plasminogen activator and is preferably tissue plasminogen activator (tPA).

5. Assay as claimed in any one of the claims 1 to 4, wherein the substrates are compounds that upon contact with the molecule to be measured release a measurable group.

6. Assay as claimed in any one of the claims 1 to 4, wherein the substrates are fluorescent substrates.

7. Assay as claimed in claim 6, wherein the substrates have different excitation and emission spectra.

8. Assay as claimed in claim 7, wherein the thrombin specific substrate is coupled to 7-amino-4-methylcoumarin

9. Assay as claimed in claim 8, wherein the thrombin specific substrate is Bz-ß-Ala-Gly-Arg-AMC-AcOH.

10. Assay as claimed in claim 7, wherein the plasmin-specific substrate is coupled to rhodamine 110.

11. Assay as claimed in claim 10, wherein the plasmin-specific substrate is (bis-(CBZ-L-phenylalanyl-L-arginine amide)).

12. Assay as claimed in any one of the claims 1-11, wherein the phospholipid-containing surface is selected from cephalin, cells, in particular endothelial cells, blood platelets, bacteria, viruses, matrices of endothelial cells or microvessels.

13. Assay as claimed in any one of the claims 1 to 12 for use in the determination of the effects of drugs, proteins, cells or other additives on both thrombin and plasmin generation, comprising addition of the drug, protein, cell or other additive to the reaction mixture.

14. Kit for performing the assay as claimed in any one of the claims 1 to 12, comprising a container comprising one or more of the following reagents: a trigger molecule for inducing thrombin generation, a thrombin-specific substrate that upon cleavage by thrombin produces a measurable thrombin-specific signal, a plasmin-specific substrate that upon cleavage by plasmin produces a measurable plasmin-specific signal, a trigger molecule for inducing plasmin generation, a phospholipid-containing surface and calcium ions.

15. Kit as claimed in claim 14, wherein the trigger molecule for inducing generation of thrombin is an initiator

of the extrinsic pathway, in particular tissue factor (TF) or an initiator of the intrinsic pathway, in particular glass, kaolin, acid.

16. Kit as claimed in claim 14, wherein the trigger molecule for inducing generation of plasmin in the assay of the invention is selected from urokinase, streptokinase, and tissue plasminogen activator, and is preferably tissue plasminogen activator (tPA).

17. Kit as claimed in any one of the claims 14 to 16, wherein the substrates are compounds that upon contact with the molecule to be measured release a measurable group.

18. Kit as claimed in claim 14, 15 or 16, wherein the substrates are fluorescent substrates.

19. Kit as claimed in claim 18, wherein the substrates have different excitation and emission spectra.

20. Kit as claimed in claim 19, wherein the thrombin specific substrate is coupled to 7-amino-4-methylcoumarin

21. Kit as claimed in claim 19, wherein the thrombin specific substrate is Bz-ß-Ala-Gly-Arg-AMC-AcOH.

22. Kit as claimed in claim 19, wherein the plasmin-specific substrate is coupled to rhodamine 110.

23. Kit as claimed in claim 22, wherein the plasmin-specific substrate is (bis-(CBZ-L-phenylalanyl-L- arginine amide)).

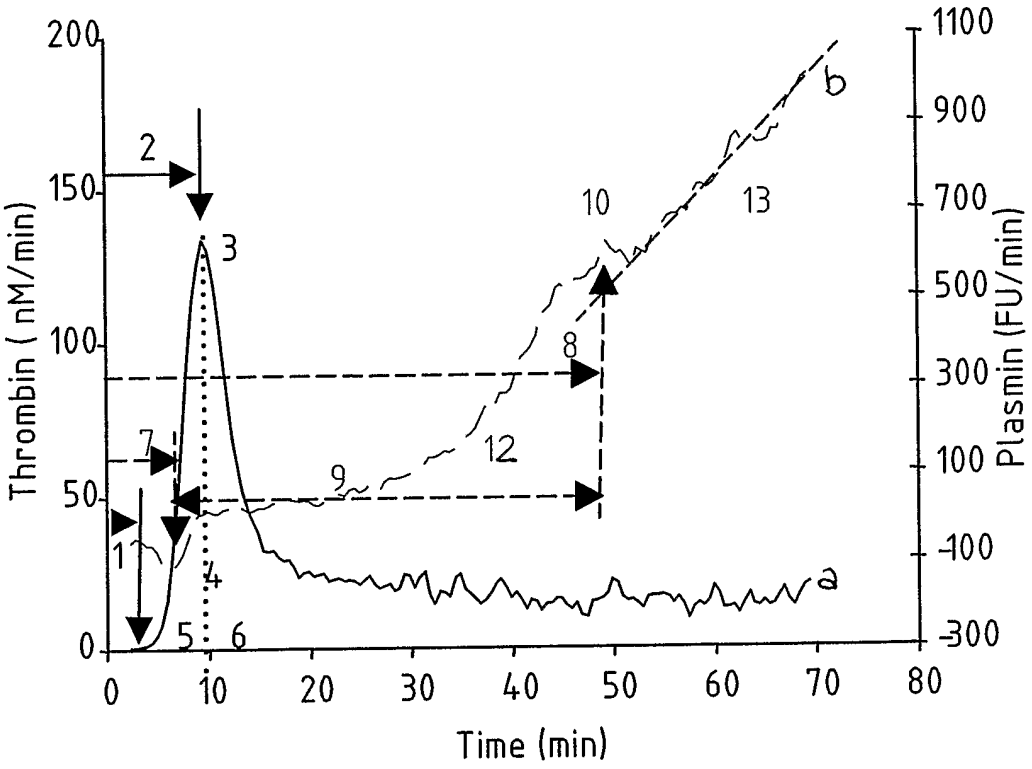
Thrombin ( nM/min)
Plasmin (FU/min)
Time (min)
200
150
100
50
0
1100
900
700
500
300
100
-100
-300
0
10
20
30
40
50
60
70
80
1
2
3
4
5
6
7
8
9
10
12
13
a
b

FIG. 1

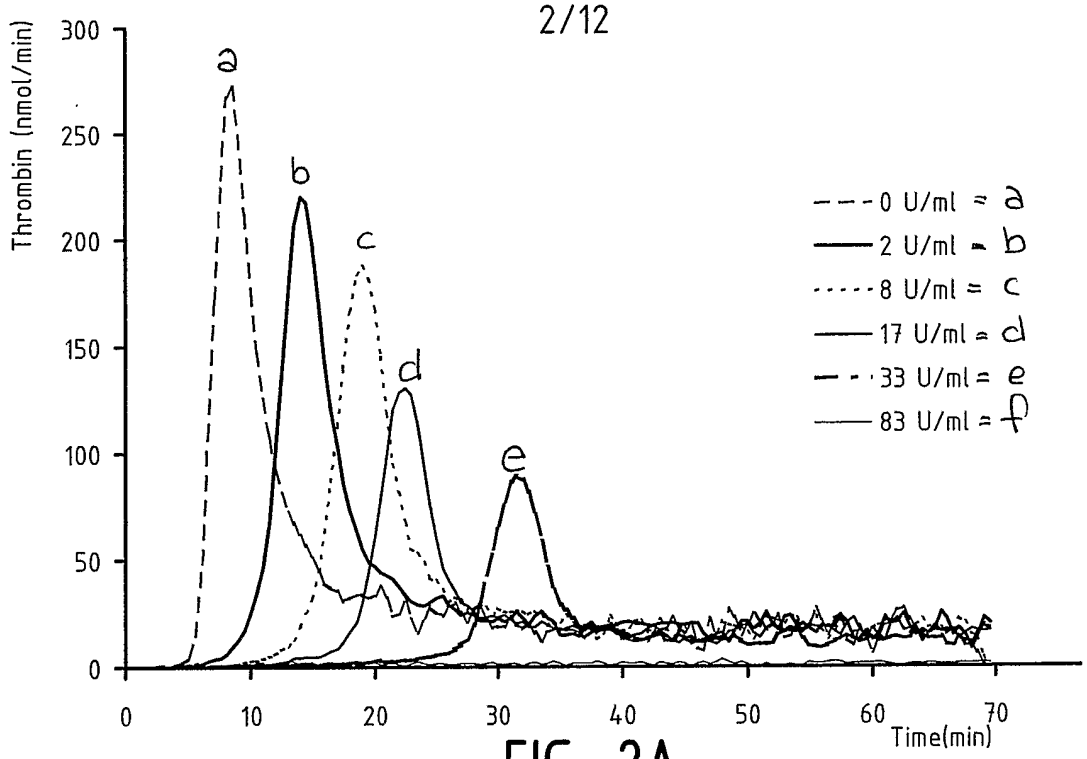
Thrombin (nmol/min)
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Time(min)
a
b
c
d
e
0 U/ml = a
2 U/ml = b
8 U/ml = c
17 U/ml = d
33 U/ml = e
83 U/ml = f

FIG. 2A

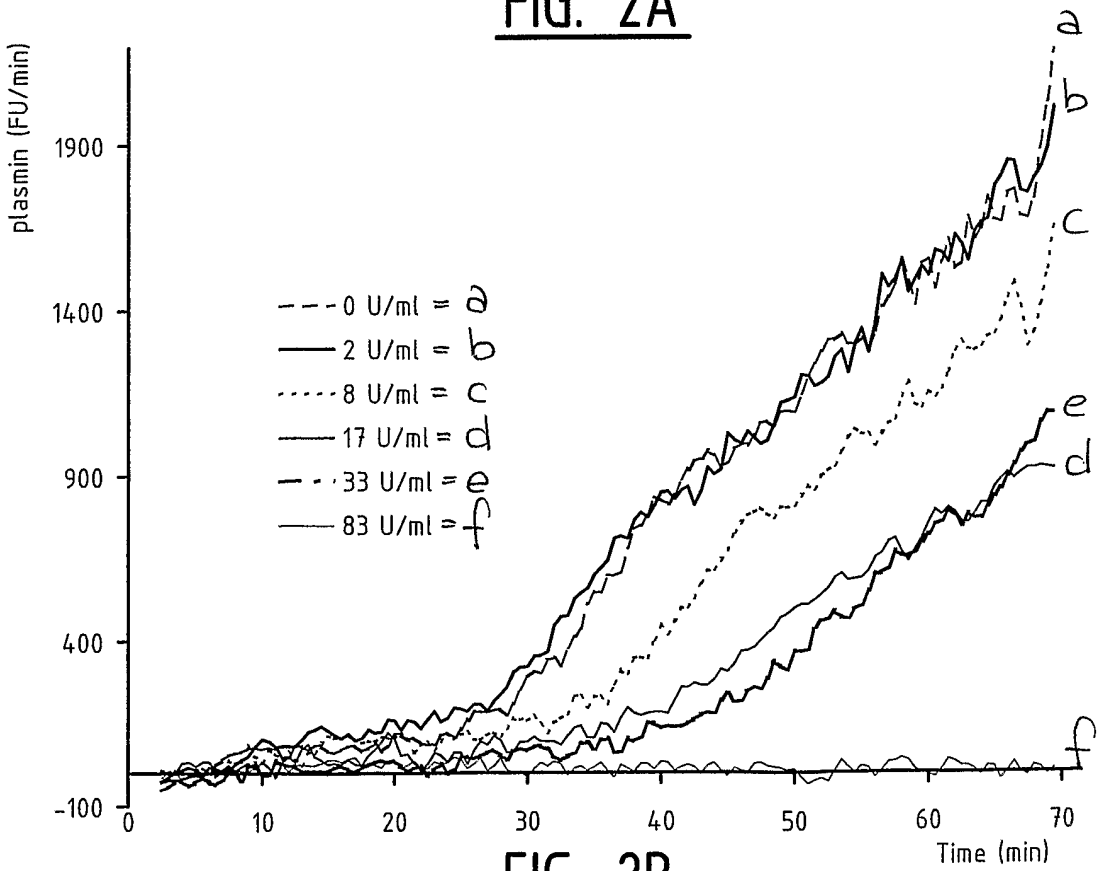
plasmin (FU/min)
1900
1400
900
400
-100
0
10
20
30
40
50
60
70
Time (min)
a
b
c
d
e
f
0 U/ml = a
2 U/ml = b
8 U/ml = c
17 U/ml = d
33 U/ml = e
83 U/ml = f

FIG. 2B

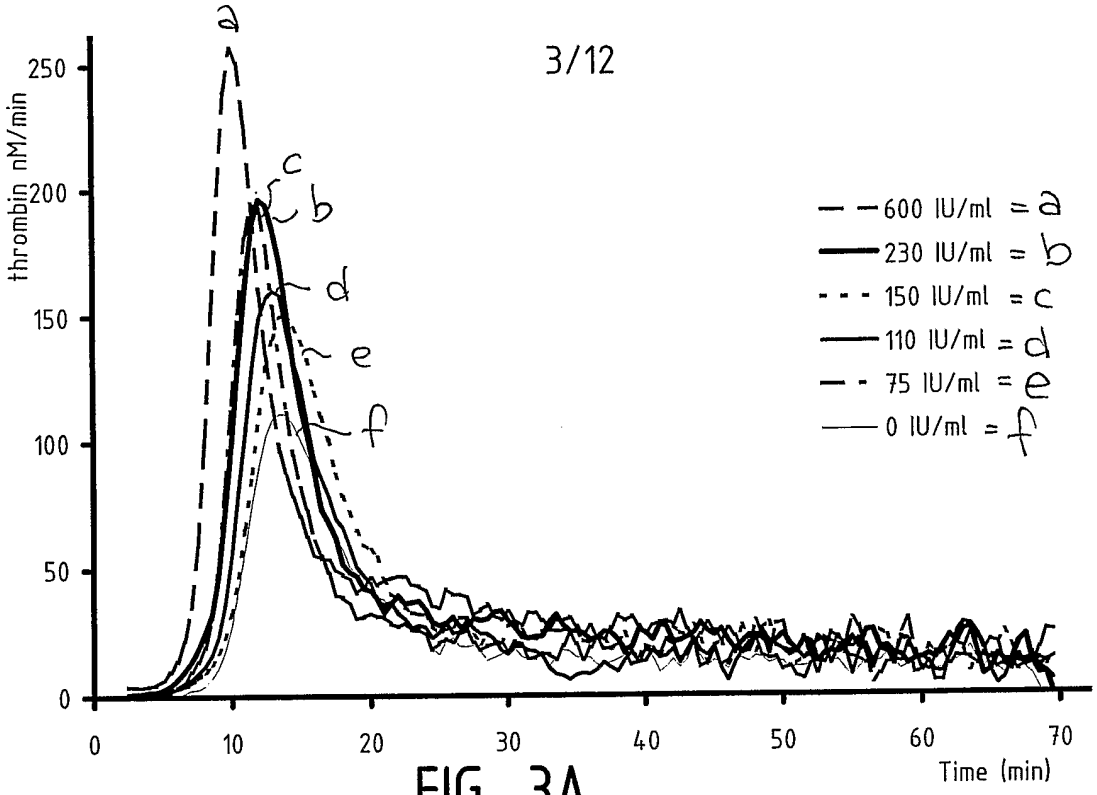
thrombin nM/min
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Time (min)
600 IU/ml = a
230 IU/ml = b
150 IU/ml = c
110 IU/ml = d
75 IU/ml = e
0 IU/ml = f

FIG. 3A

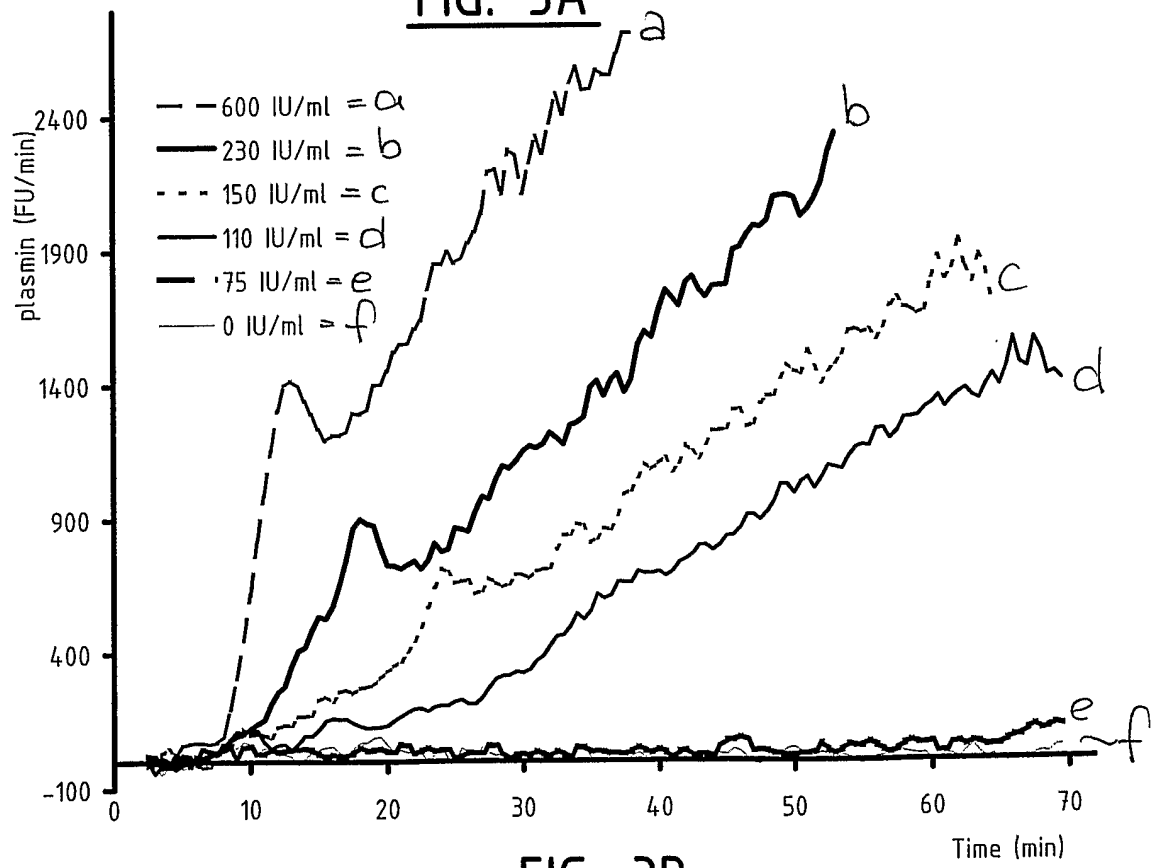
plasmin (FU/min)
2400
1900
1400
900
400
-100
0
10
20
30
40
50
60
70
Time (min)
600 IU/ml = a
230 IU/ml = b
150 IU/ml = c
110 IU/ml = d
75 IU/ml = e
0 IU/ml = f

FIG. 3B

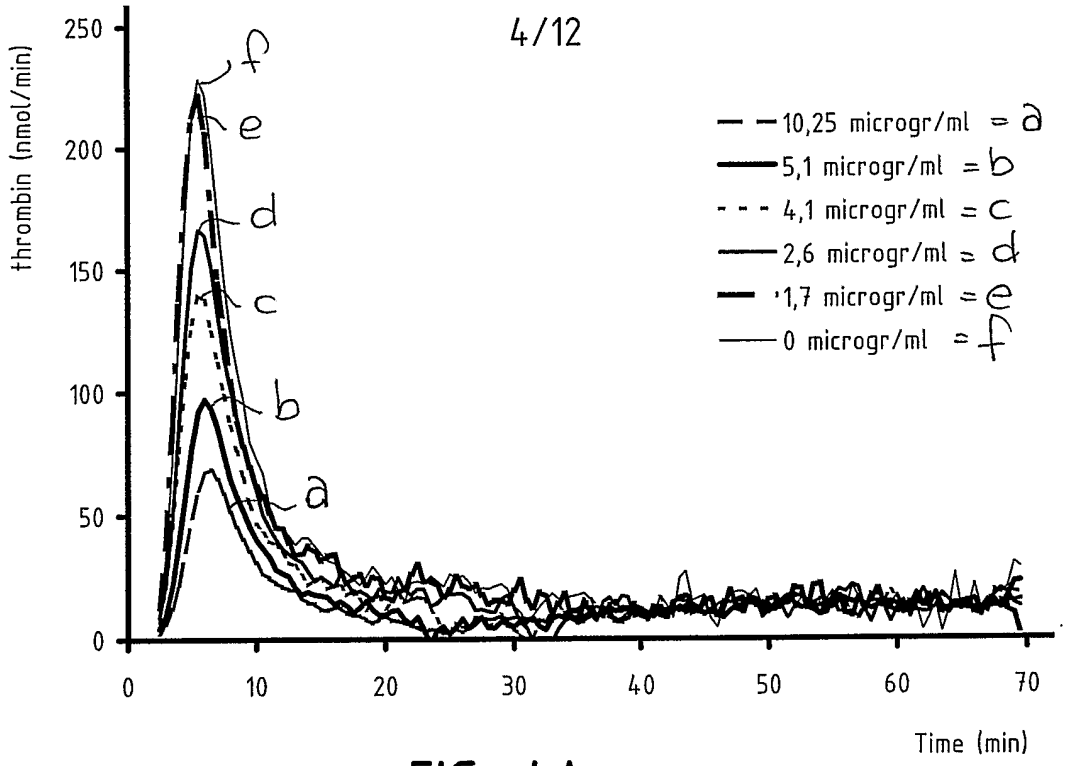
thrombin (nmol/min)
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Time (min)
10,25 microgr/ml = a
5,1 microgr/ml = b
4,1 microgr/ml = c
2,6 microgr/ml = d
1,7 microgr/ml = e
0 microgr/ml = f

FIG. 4A

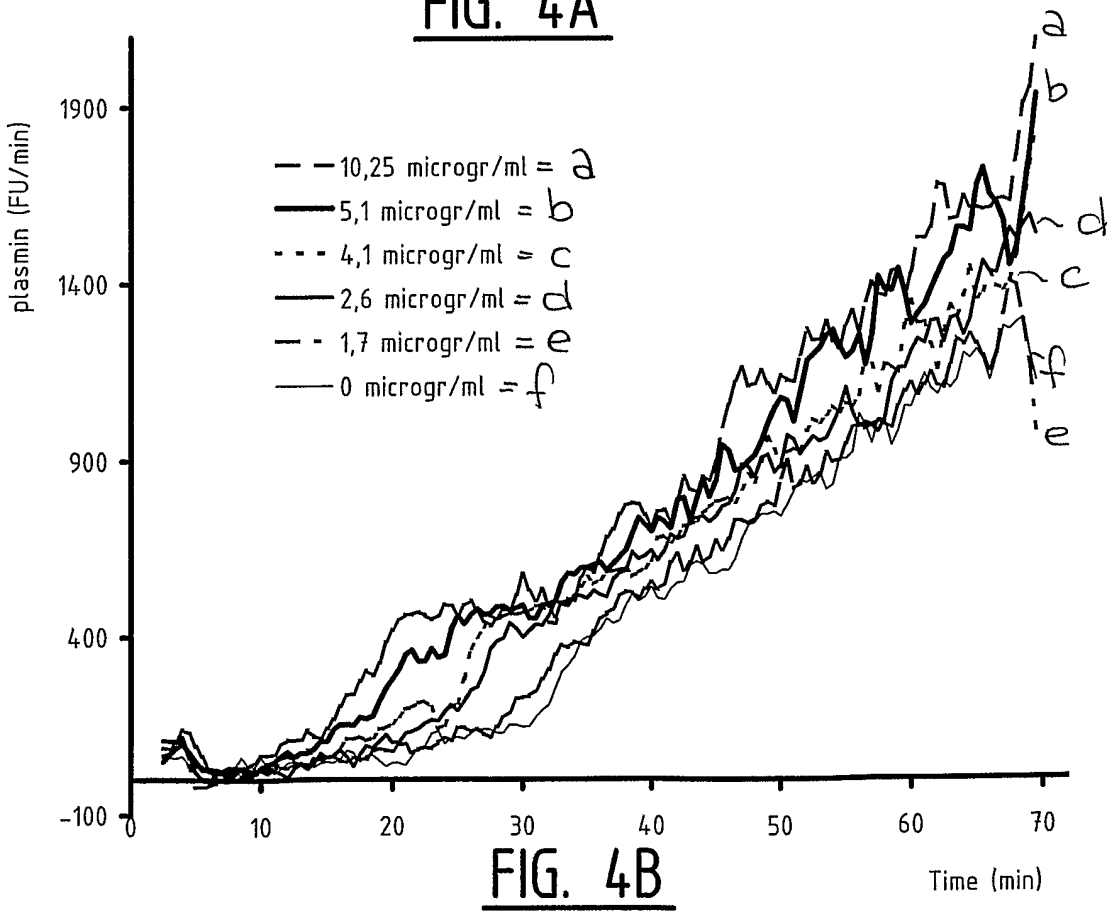
plasmin (FU/min)
1900
1400
900
400
-100
0
10
20
30
40
50
60
70
Time (min)
10,25 microgr/ml = a
5,1 microgr/ml = b
4,1 microgr/ml = c
2,6 microgr/ml = d
1,7 microgr/ml = e
0 microgr/ml = f

FIG. 4B

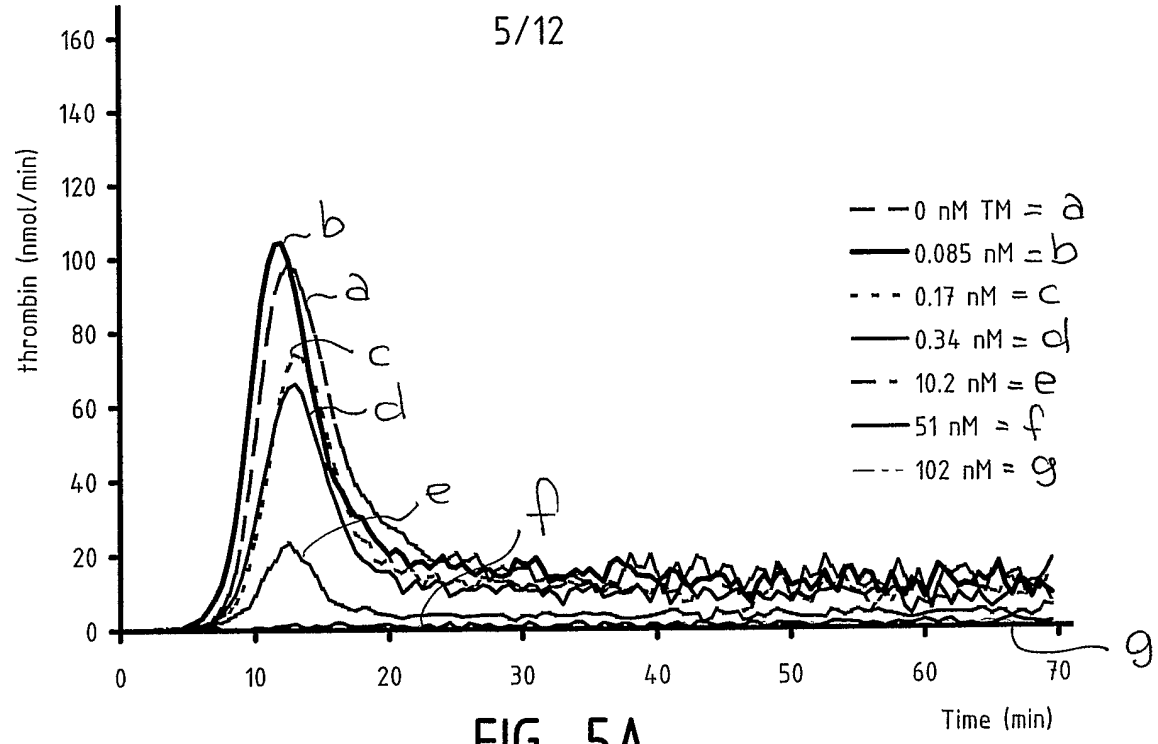
thrombin (nmol/min)
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Time (min)
0 nM TM = a
0.085 nM = b
0.17 nM = c
0.34 nM = d
10.2 nM = e
51 nM = f
102 nM = g
a
b
c
d
e
f
g

FIG. 5A

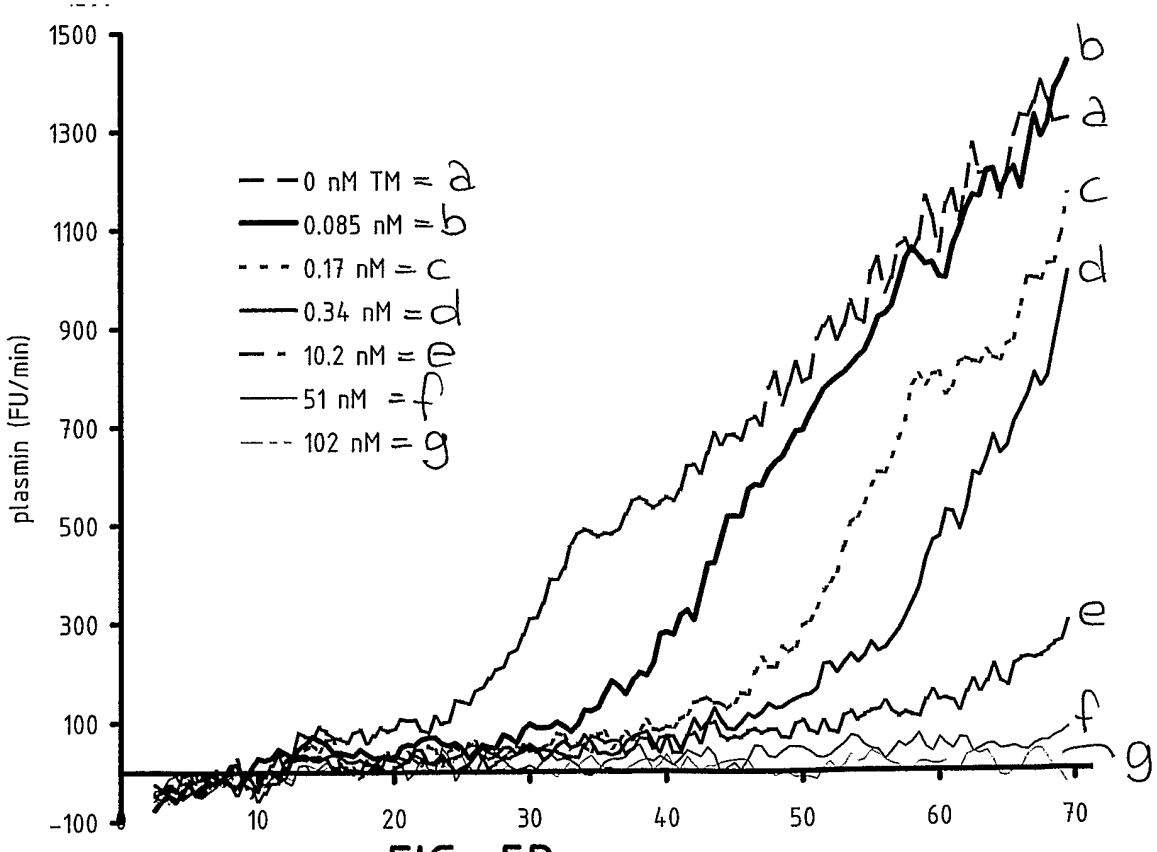
plasmin (FU/min)
1500
1300
1100
900
700
500
300
100
-100
10
20
30
40
50
60
70
Time (min)
0 nM TM = a
0.085 nM = b
0.17 nM = c
0.34 nM = d
10.2 nM = e
51 nM = f
102 nM = g
a
b
c
d
e
f
g

FIG. 5B

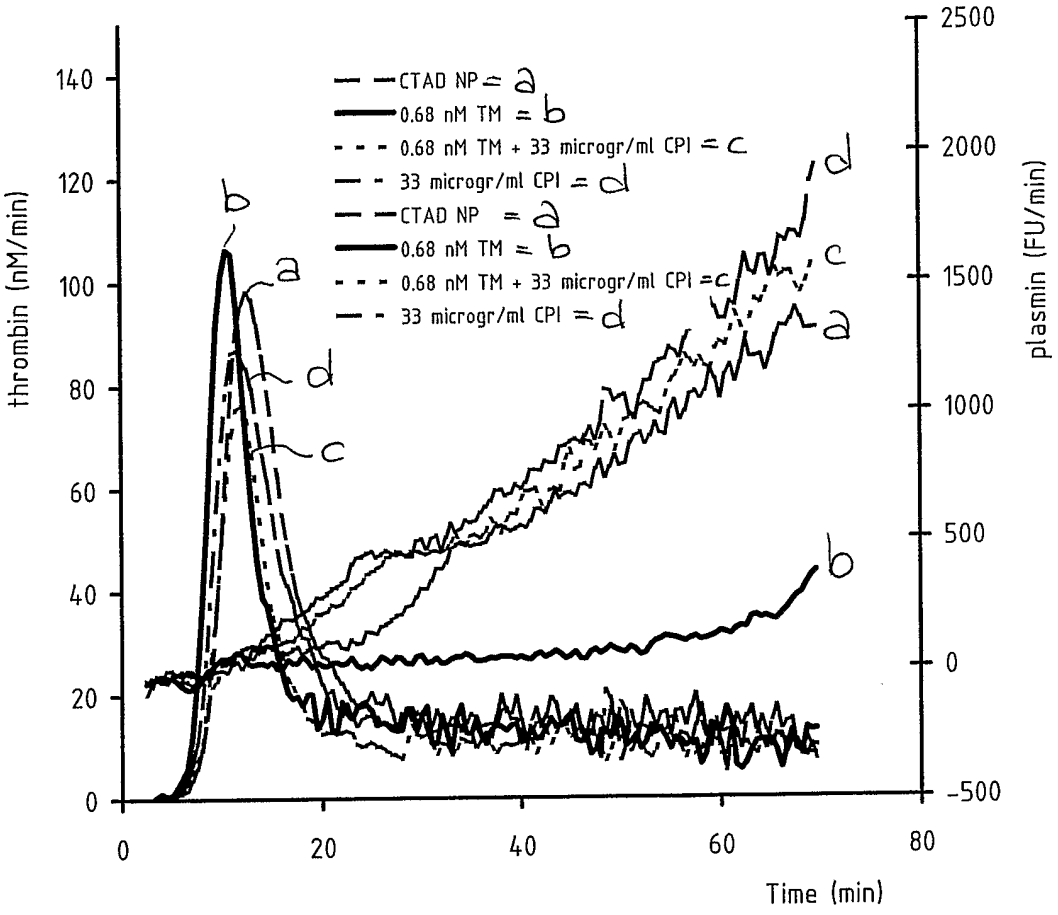
CTAD NP = a
0.68 nM TM = b
0.68 nM TM + 33 microgr/ml CPI = c
33 microgr/ml CPI = d
CTAD NP = a
0.68 nM TM = b
0.68 nM TM + 33 microgr/ml CPI = c
33 microgr/ml CPI = d
thrombin (nM/min)
plasmin (FU/min)
Time (min)
0
20
40
60
80
100
120
140
500
1000
1500
2000
2500
-500

FIG. 6

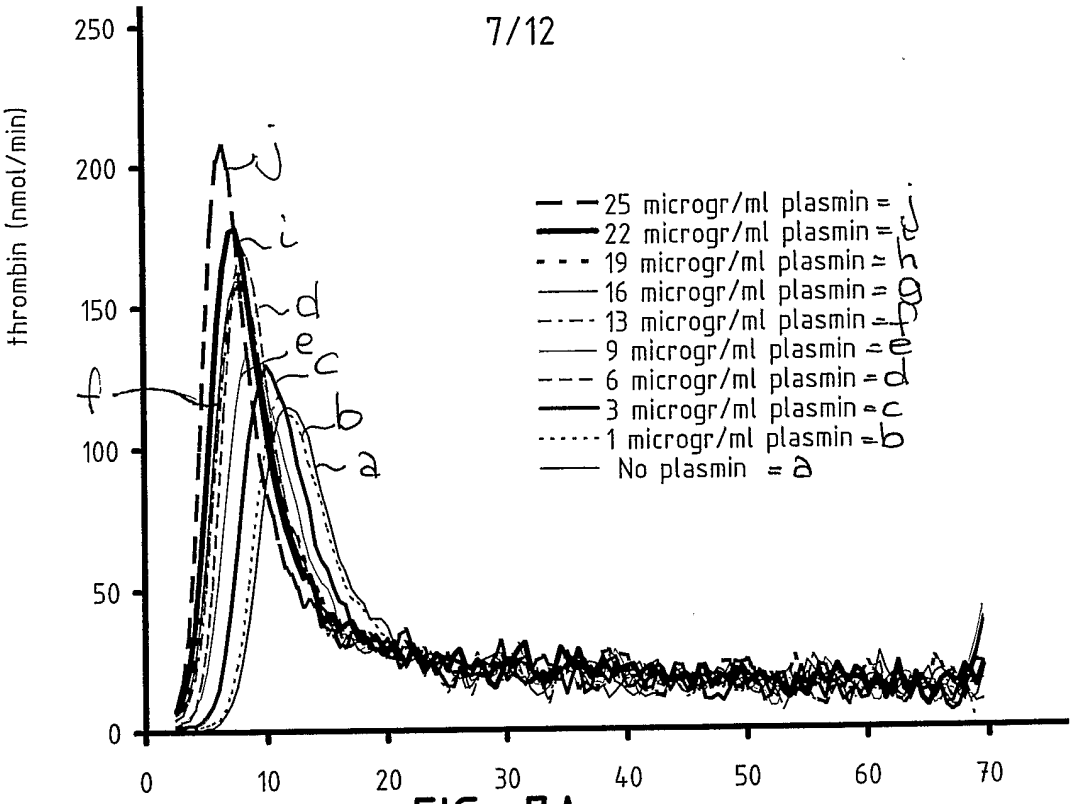
thrombin (nmol/min)
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Time (min)
25 microgr/ml plasmin = j
22 microgr/ml plasmin = i
19 microgr/ml plasmin = h
16 microgr/ml plasmin = g
13 microgr/ml plasmin = f
9 microgr/ml plasmin = e
6 microgr/ml plasmin = d
3 microgr/ml plasmin = c
1 microgr/ml plasmin = b
No plasmin = a

FIG. 7A

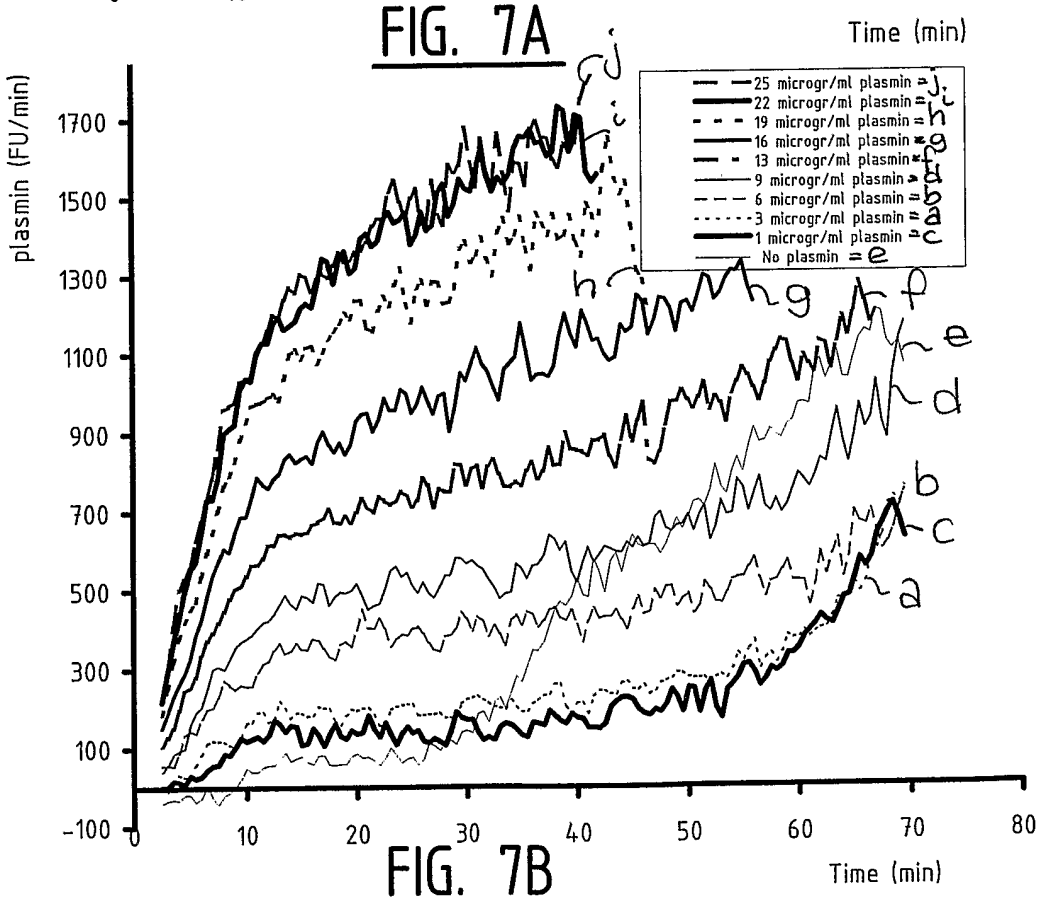
plasmin (FU/min)
1700
1500
1300
1100
900
700
500
300
100
-100
0
10
20
30
40
50
60
70
80
Time (min)
25 microgr/ml plasmin = j
22 microgr/ml plasmin = i
19 microgr/ml plasmin = h
16 microgr/ml plasmin = g
13 microgr/ml plasmin = f
9 microgr/ml plasmin = e
6 microgr/ml plasmin = d
3 microgr/ml plasmin = c
1 microgr/ml plasmin = b
No plasmin = a

FIG. 7B

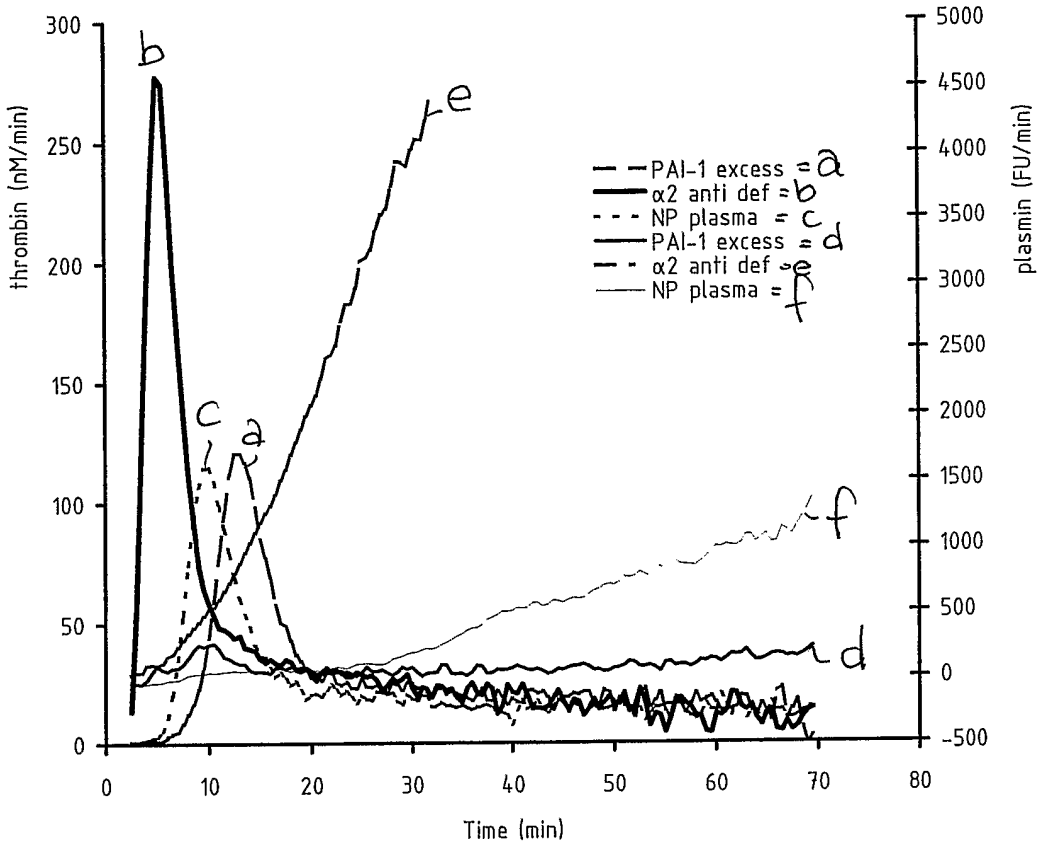
thrombin (nM/min)
plasmin (FU/min)
Time (min)
PAI-1 excess = a
α2 anti def = b
NP plasma = c
PAI-1 excess = d
α2 anti def = e
NP plasma = f
300
250
200
150
100
50
0
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0
10
20
30
40
50
60
70
80

FIG. 8

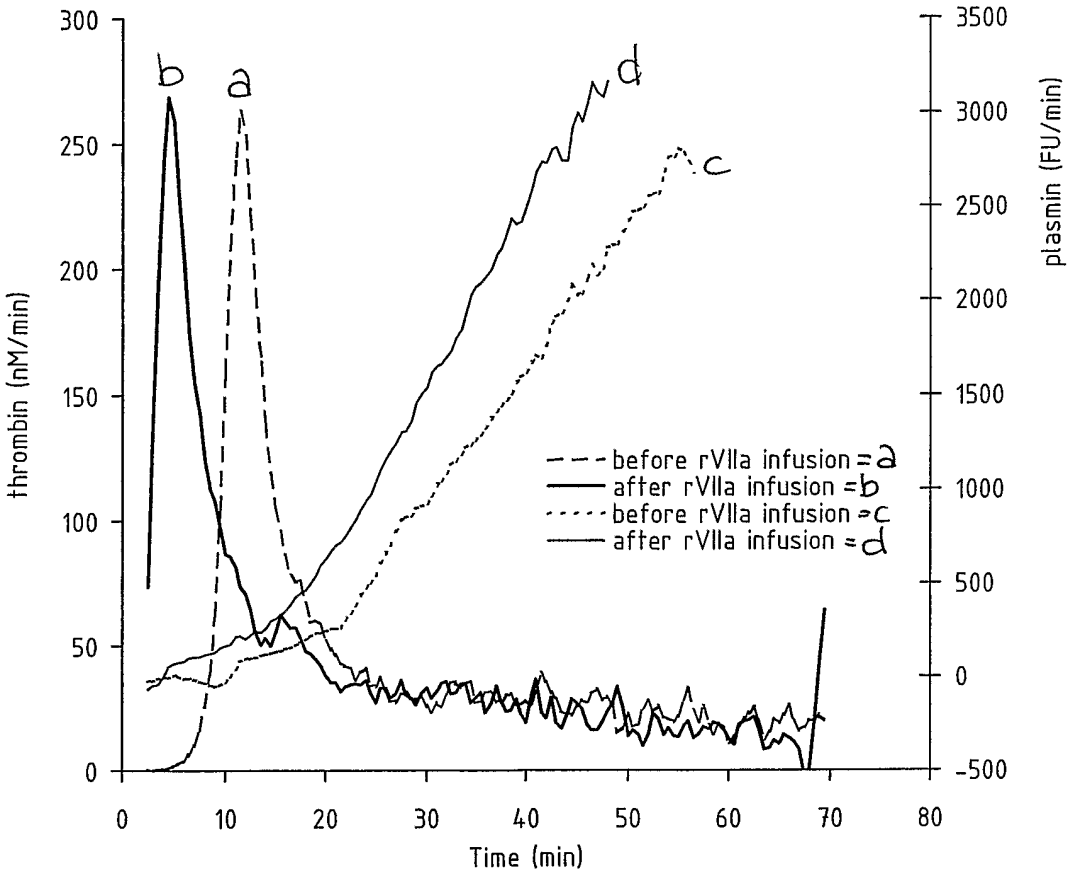
thrombin (nM/min)
plasmin (FU/min)
Time (min)
300
250
200
150
100
50
0
3500
3000
2500
2000
1500
1000
500
0
-500
0
10
20
30
40
50
60
70
80
a
b
c
d
before rVIIa infusion = a
after rVIIa infusion = b
before rVIIa infusion = c
after rVIIa infusion = d

FIG. 9

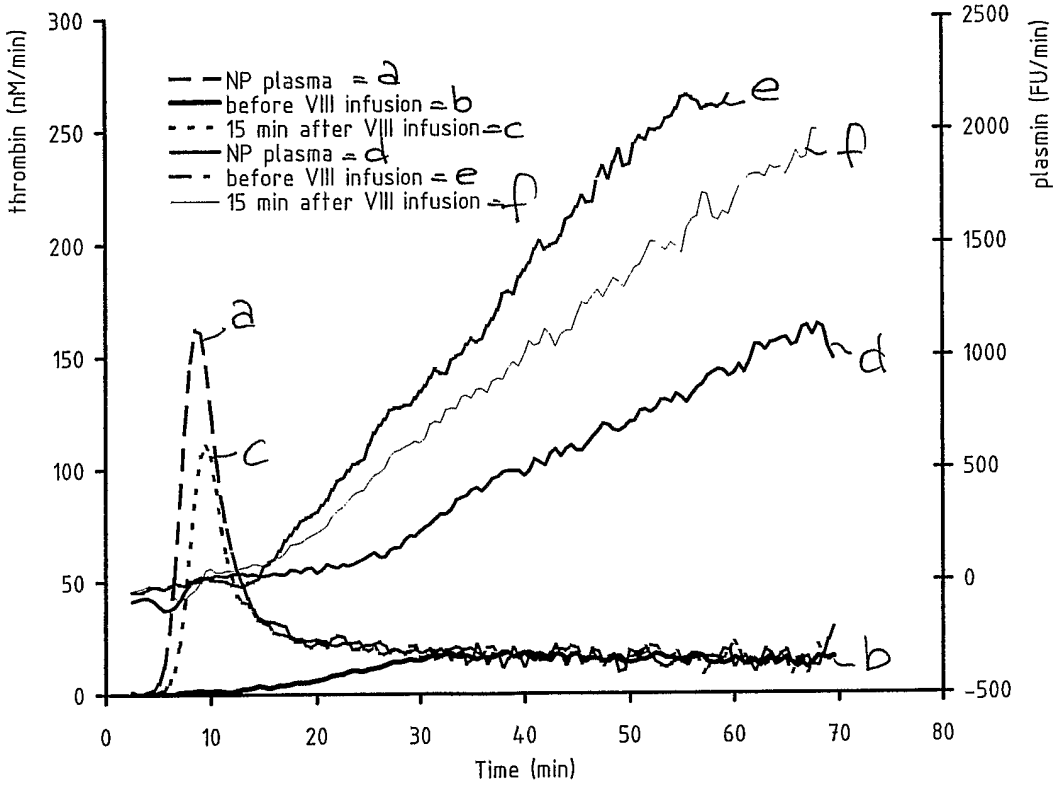
thrombin (nM/min)
plasmin (FU/min)
Time (min)
NP plasma = a
before VIII infusion = b
15 min after VIII infusion = c
NP plasma = d
before VIII infusion = e
15 min after VIII infusion = f
300
250
200
150
100
50
0
2500
2000
1500
1000
500
0
-500
0
10
20
30
40
50
60
70
80

FIG. 10

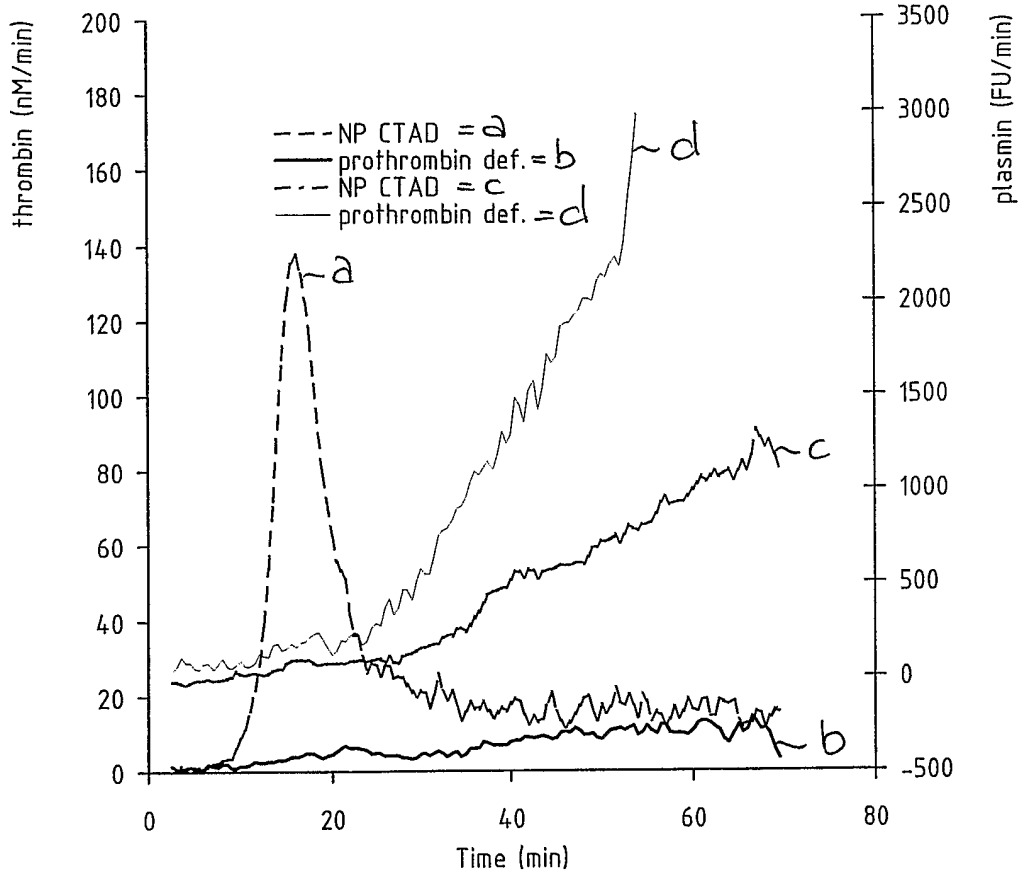
thrombin (nM/min)
plasmin (FU/min)
Time (min)
NP CTAD = a
prothrombin def. = b
NP CTAD = c
prothrombin def. = d
a
b
c
d
0
20
40
60
80
100
120
140
160
180
200
-500
0
500
1000
1500
2000
2500
3000
3500

FIG. 11

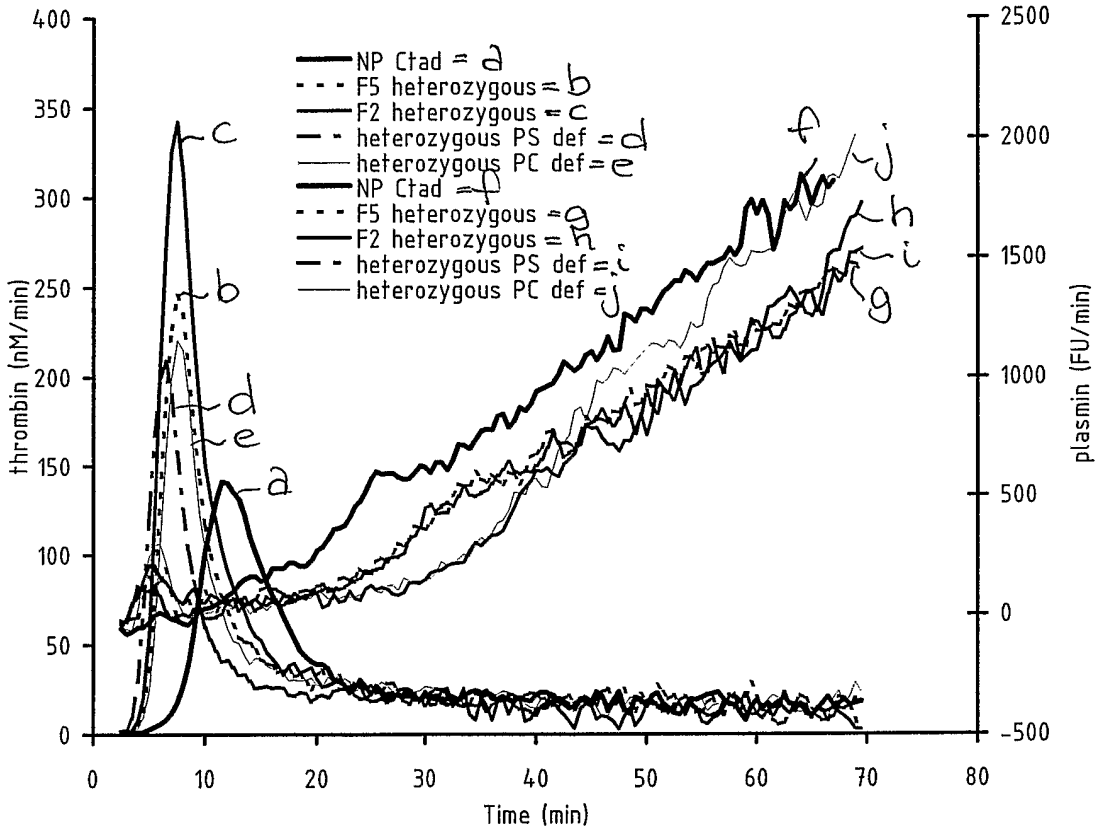
NP Ctad = a
F5 heterozygous = b
F2 heterozygous = c
heterozygous PS def = d
heterozygous PC def = e
NP Ctad = f
F5 heterozygous = g
F2 heterozygous = h
heterozygous PS def = i
heterozygous PC def = j
thrombin (nM/min)
plasmin (FU/min)
Time (min)
0
50
100
150
200
250
300
350
400
-500
500
1000
1500
2000
2500
10
20
30
40
50
60
70
80

FIG. 12

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/000184

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 03/093831 A (SYNAPSE B.V; GIESEN, PETER; HEMKER, HENDRICK, C; AL DIERI, RAED; BEGUI) 13 November 2003 (2003-11-13)<br>the whole document<br>in particular:<br>abstract<br>page 7, line 32 - line 34<br>page 8, line 1 - line 2<br>page 8, line 11<br>page 8, line 30 - line 35<br>page 9, line 1 - line 2<br>page 9, line 9 - line 10<br>page 9, line 12 - line 17<br>page 9, line 19 - line 34<br>page 11, line 35 - page 12, line 4<br>page 17, line 1 - line 5<br>-/-- | 1-23 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"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

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"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 when the document is taken alone

"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

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 31 March 2006 | 29/05/2006 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Gall, A-L |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/000184

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| T | PENTAPHARM: "Pefafluor uPA - highly sensitive fluorogenic peptide substrate for urokinase (uPA)" FLUOROGENIC SUBSTRATES, [Online] XP002375285 Retrieved from the Internet: URL:http://www.pentapharm.com/graphics/Pentapharm/download/diagnostics/Fluorogenic_Substrates/DSe_Fluorogenic_Substrates.pdf> [retrieved on 2006-03-31] code : 0821-21 page 3 ----- | 1-23 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/000184

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 03093831 A | 13-11-2003 | AU 2003232720 A1 | 17-11-2003 |
| | | CA 2484554 A1 | 13-11-2003 |
| | | CN 1650170 A | 03-08-2005 |
| | | JP 2005528097 T | 22-09-2005 |
| | | US 2006051828 A1 | 09-03-2006 |