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QUANTITATIVE CLOTTING ASSAY FOR ACTIVATED FACTOR VII

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(57) Claim

1. A method for measuring the concentration of coagulation Factor VIIa in a patient's plasma sample, comprising the steps of:

(a) mixing a quantity of a plasma sample with a first reagent, said first reagent comprising a soluble truncated tissue factor characterized by its inability to bind to phospholipids and its inability to support conversion of Factor VII to Factor VIIa, to form a first mixture;

(b) either simultaneously with said first reagent or thereafter, adding a second reagent comprising an effective amount of calcium ions to initiate clotting of said first mixture;

(c) measuring a plasma clotting time of said first mixture from a time of addition of said second reagent to a time of clot formation; and

(d) comparing said plasma clotting time to a standards clotting time determined for Factor VIIa of a known concentration.

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(57) Abstract

An assay for activated factor VII (factor VIIa) has been developed using truncated tissue factor (TF), a soluble mutant form of TF that retains the cofactor function of TF toward factor VIIa. Unlike full-length TF, however, tTF appears not to support the conversion of factor VII to VIIa. As a result, the tTF assay for factor VIIa is free from interference from factor VII in the plasma and is therefore specific for factor VIIa. The assay is much simpler than existing assays, because it is a single-stage clotting assay performed almost identically to a PT assay. It is also considerably more sensitive than current assays for factor VIIa in plasma. Since the tTF assay is calibrated against a factor VIIa standard, it yields an absolute concentration of factor VIIa in ng/ml.

QUANTITATIVE CLOTTING ASSAY FOR ACTIVATED FACTOR VII

Background of the Invention

The United States government has rights in this invention by virtue of a grant from the National Institutes of Health.

Blood coagulation results from the production of thrombin, a proteolytic enzyme inducing platelet aggregation and cleaving fibrinogen to fibrin, which stabilizes the platelet plug. A number of proenzymes and procofactors circulating in the blood interact in this process through several stages during which they are sequentially or simultaneously converted to the activated form, ultimately resulting in the activation of prothrombin to thrombin by activated factor X (fXa) in the presence of factor Va, ionic calcium, and platelets.

Factor X can be activated by either of two pathways, termed the extrinsic and intrinsic pathways. The intrinsic pathway, or surface-mediated activation pathway, consists of a series of reactions where a protein precursor is cleaved to form an active protease, beginning with activation of factor XII to factor XIIa, which converts factor XI to factor XIa, which, in the presence of calcium, converts factor IX to factor IXa. Factor IX can also be activated via the extrinsic pathway by tissue factor (TF) in combination with activated factor VII (factor VIIa; fVIIa). The activated factor IX, in the presence of calcium, phospholipid (platelets), and factor VIIIa, activates factor X to factor Xa.

Physiologically, the major pathway involved in coagulation is believed to be the extrinsic pathway, an essential step of which is activation of factor VII to factor VIIa. Clotting assays (and other activity assays) designed to measure factor VII and VIIa generally must employ TF, the cofactor required for factor VIIa coagulant activity. Most commonly, TF is provided as a relatively crude preparation known as

thromboplastin. Tissue factor is an integral membrane glycoprotein having a protein and a phospholipid component. It has been isolated from a variety of tissues and species and reported to have a molecular mass of between 42,000 and 53,000. The original crude tissue abstracts referred to the fraction containing the tissue factor as thromboplastin. DNA encoding tissue factor and methods for expression of the protein have now been reported, for example, in European Patent Application 0 278 776 by Genentech, Inc. and by J. H. Morrissey, et al. Cell 50, 129-135 (1987).

The complex of factor VIIa and TF is the most potent known trigger of the clotting cascade. Factor VII is present in plasma at a concentration of 0.5 μg/ml plasma. In contrast, measured as described herein, factor VIIa is present in plasma at trace levels of roughly 1 ng/ml. Accordingly, factor VII is normally in considerable excess over factor VIIa. As there are presently no immunoassays capable of discriminating between factor VII and VIIa, current assays attempt to exploit the difference in enzymatic activity between the two, relative to total factor VII protein.

Factor VIIa circulates with a relatively long half-life of about two hours in plasma. This is an unusual property among activated coagulation enzymes, which typically are inactivated very rapidly by various protease inhibitors in plasma. Some studies have suggested that elevated levels of plasma factor VIIa are associated with increased risk of thromboembolic disease. This may be due to a direct effect of factor VIIa in promoting thrombosis, or because elevated plasma factor VIIa is a by-product of a "prethrombotic state". In either case, there is clinical interest in measuring plasma factor VIIa levels.

Two basic types of assays are routinely employed to assess coagulation function: clotting assays and chromogenic substrate assays. Clotting assays compare clotting times relative to normal controls (time in seconds to clot formation versus percent normal control, also referred to as one unit). Chromogenic substrate assays are typically used only with the purified enzymes and the amount of active factor calculated based on the amount of product generated in a given time as determined by absorbance of light transmitted through the sample at a particular wavelength. Immunological assays involving antigen-antibody reactions are also sometimes used to measure the total amount of protein present.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays are routinely employed clinically in the assessment of hemostatic function. PT is performed by adding a preparation of phospholipid, tissue factor and calcium to the patient's citrated plasma and determining the time required for a clot to form. This test measures the aggregate activity of factors II, VII, X, V and fibrinogen. The PT is often used to follow oral anticoagulant therapies, i.e., coumarin and anti-vitamin K drugs. The aPTT test is performed by adding a preparation of celite (or other contact activator), phospholipid, and calcium to the patient's citrated plasma and determining the time required for a clot to form. This test measures the aggregate activity of factors XII, XI, IX, VIII, X, II, V and fibrinogen. The aPTT is commonly used as a coagulation screening test for factor dysfunction in hospitalized patients.

In contrast to these general assays, quantitative assays of specific individual coagulation factors are usually performed using a plasma deficient in the factor to be assayed and comparing clotting times relative to controls. Typically, the amount of

factor VII/factor VIIa present in a patient's plasma would be determined using factor VII deficient plasma, adding a dilution of patient plasma, TF, and calcium, usually in the form of calcium chloride, and determining the time required for the plasma mixture to clot. The result is a measure of what is called "factor VII coagulant activity", or FVIIc. In reality, FVIIc is a function of the concentrations of both factor VII and VIIa. It is difficult to assess the amount of factor VII relative to the amount of factor VIIa, however, since TF greatly accelerates the activation of factor VII, and the newly converted factor VIIa contributes to the measured activity. A further complication of the significance of FVIIc is the recent finding of an unusual, lipoprotein-associated form of factor VII that has elevated FVIIc activity. The nature of this lipoprotein-associated form of factor VII is poorly understood at the present time.

These factors limit the sensitivity of existing assays and complicate their interpretation. In conventional clotting assays, human factor VIIa has approximately 25-fold more activity than factor VII on an equimolar basis. This means that prior assays have had difficulty detecting concentrations of factor VIIa protein that are much below 1/25 of the concentration of factor VII. The normal plasma concentration of factor VII is approximately 0.5 μg/ml, and the lower limit of detection of factor VIIa in normal plasma using the conventional factor VII clotting assay is between 1 and 10 ng/ml.

In order to try to control for possible variation of plasma factor VII levels, another assay is usually performed in parallel that is designed to measure total factor VII plus VIIa. This can be an immunoassay or a chromogenic substrate assay in which factor VII is converted to factor VIIa. The final

result is then expressed as a ratio of factor VII clotting activity (FVIIc) to total factor VII, and an increase in the ratio above normal is taken as evidence of an increased level of factor VIIa in the test plasma. These assays do not really measure factor VIIa directly and specifically, nonetheless, nor do these assays give a measure of absolute level of factor VIIa. It is thought that elevated FVIIc can be due to the presence of elevated circulating factor VIIa levels. Alternatively, elevated FVIIc can be due to an increased concentration of factor VII in plasma. In any case, the results from conventional assays of FVIIc depend heavily on the source of thromboplastin, as reported by Poggio, et al. Thromb. Haemost. 65,160-164 (1991), making it difficult to compare the results of such assays from one laboratory with another.

In summary, prior art methods for measuring factor VIIa levels in plasma are limited by the interference of factor VII which is also present in plasma. There are therefore three main drawbacks to the available assays: (1) it is extremely difficult to measure absolute levels of factor VIIa in plasma, as compared with levels of factor VII; (2) low sensitivity of available assays means that only factor VIIa levels substantially increased over normal can be measured; and (3) it is difficult to directly compare the results obtained in different studies.

It is therefore an object of the present invention to provide a sensitive assay which measures the absolute levels of factor VIIa, not factor VII, in plasma.

It is further object of the present invention to provide an assay for factor VIIa which is reproducible and can be performed with existing methodologies and most reagents, manually or automatically, and commercially viable.

Summary of the Invention

An *in vitro* assay for detecting and quantitating factor VIIa (the activated form of coagulation factor VII) in human plasma has been developed. The single-stage clotting assay is performed in a manner nearly identical to the Prothrombin Time (PT) clotting assay using a small sample of blood drawn into a citrate anticoagulant, from which plasma is prepared by centrifugation. A modified clotting assay is then performed on a sample of the plasma using a preparation of truncated tissue factor and phospholipid vesicles in place of thromboplastin. The truncated derivative of tissue factor retains cofactor activity toward factor VIIa, but, unlike normal tissue factor, does not stimulate the conversion of factor VII to factor VIIa. As a result, unactivated factor VII, which is also present in plasma, does not interfere with the assay. The clotting time obtained in this assay is directly related to the level of plasma factor VIIa and is quantitated by comparison to a standard curve generated using factor-VII deficient plasma supplemented with known quantities of factor VIIa. The useful range of the assay is 10 pg factor VIIa/ml to 10 μg factor VIIa/ml in plasma. The lower limit of detection of factor VIIa in the truncated tissue factor-based assay is calculated to be less than 10 pg factor VIIa/ml.

The assay is useful in accurately measuring plasma levels of factor VIIa in individuals. Recent studies have indicated a possible correlation between elevated factor VIIa levels and the risk of such disorders as ischemic heart disease and fetal growth retardation. The assay can also be used to monitor factor VIIa therapy in hemophiliacs and other individuals undergoing factor VIIa therapy.

Brief Description of the Drawings

Figure 1 is the amino acid and nucleotide sequence encoding a soluble form of truncated tissue factor. Nucleotide sequence is numbered on the left. Amino acid sequence is given above the nucleotide sequence using the standard one-letter code. The signal peptide is underlined.

Figure 2 is an exemplary factor VIIa standard curve generated using an automated coagulometer (ACL™ 300 Automated coagulation Laboratory) run in the PT mode, using a high-sensitivity tTF reagent. Human factor VII-deficient plasma was supplemented with varying concentrations of recombinant human factor VIIa spanning the anticipated normal range of FVIIa concentrations. The tTF reagent contained 1 μM tTF. Data (clotting times vs. added factor VIIa concentration) were plotted on log-log axes and fit to a fourth order polynomial by regression analysis.

Figure 3 is an exemplary plot of clotting times obtained in the tTF assay for factor VIIa with a low-sensitivity tTF reagent and normal human plasma supplemented with factor VIIa at levels that mimic the therapeutic range. The tTF reagent contained 50 nM tTF. Data (clotting times vs. added factor VIIa concentration) were plotted on log-log axes and fit to a fourth order polynomial by regression analysis.

Figure 4 is an exemplary factor VIIa standard curve generated by the manual tilt tube method and employing a high-sensitivity tTF reagent. Human factor VII-deficient plasma was supplemented with varying concentrations of recombinant human factor VIIa (spanning 10 pg/ml to 10 μg/ml f.VIIa). The tTF reagent contained 100 nM tTF. Data (clotting times vs. added factor VIIa concentration) were plotted on log-log axes and fit to a fourth order polynomial by regression analysis.

Detailed Description of the Invention

Clotting of citrate-anticoagulated, platelet-poor plasma is initiated by a combination of truncated tissue factor, phospholipid vesicles and calcium ions. The clotting time is an indicator of the level of factor VIIa present in the plasma.

There are two preferred embodiments of the assay for activated factor VII: a manual version, using 100 nM tTF and a relatively low concentration of phospholipids, and an assay for use in automated coagulometers and fibrometers, which most clinical labs in the US are using. The manual assay can be manipulated to have a million-fold range in sensitivity, ranging from 10 pg/ml to 10 μg/ml. This is not practical using the automated coagulometers due to small but significant reagent carry-over. Accordingly, two "stock" solutions of the tTF reagent, containing different amounts of tTF and/or phospholipid, are maintained: one, for measuring the normal range of fVIIa levels, approximately 0.1 to 10 ng/ml, and a second for measuring the therapeutic range of fVIIa levels, up to about 4 μg/ml.

Reagents for use in Activated factor VII assays.

The reagents common to all embodiments of the assay are the plasma sample, a source of phospholipid, a source of clotting factors other than factor VIIa, truncated tissue factor (tTF), and calcium.

1. Phospholipid Vesicles.

In the preferred embodiment, the formulation consists of 100 nM truncated tissue factor reagent (2.55 μg/ml) plus 80 μg mixed brain phospholipid vesicles/ml suspended in TBS/BSA (50 mM Tris.HCl pH 7.4, 100 mM NaCl, 0.02% sodium azide, 0.1% bovine serum albumin). Phospholipid vesicles (600 μM) can be prepared by the deoxycholate method of Carson and Konigsberg, Thromb. Haemostasis 44, 12-15 (1981). Phospholipid vesicles are not absolutely required, but

their addition increases the sensitivity of the assay. It is believed that the presence of the vesicles decreases variability in the assay, since coagulation is less dependent on the residual content of lipoprotein particles, platelets and cell membrane fragments in the plasma samples.

Cephalin, prepared according to Bell and Alton, Nature, London, 174:880-881 (1954), the teachings of which are incorporated herein, and available commercially from a number of suppliers, including, for example, Rabbit Brain Cephalin from Sigma Chemical Company, St. Louis, MO, can also be used as the source of phospholipid vesicles. The vesicle preparations are reconstituted according to the manufacturer's instructions and employed at the final concentration recommended for use in the partial thromboplastin time (PTT) assay, described by Langdell, et al., J. Lab. Clin. Med. 41:637 (1953).

Other preparations of phospholipid vesicles suitable for use as a "platelet substitute" in PTT assays or thrombin generation time (TGT) assays are acceptable for use in the truncated TF assay for factor VIIa, including vesicles prepared from crude phospholipid extracts of tissues as well as vesicles prepared from purified or synthetic phospholipids. Such vesicle preparations must not contain tissue factor or additives that activate the contact pathway of coagulation. Examples of unsuitable preparations of vesicles are commercial thromboplastins designed for use in PT assays and "activated partial thromboplastins", cephalin preparations admixed with activating agents such as elegiac acid, kaolin, or venom extracts.

2. The tTF Reagent.

As used herein, "truncated tissue factor" is a soluble tissue factor having the extracellular domain, which is not bound to a phospholipid membrane surface,

and therefore does not support conversion of fVII to fVIIa. In the preferred embodiment, truncated tissue factor is a recombinant protein produced *in vitro* in cell culture using a human cell line (293 cells), ATCC CRL 1573. These cells are stably transfected with a mutant form of the human tissue factor cDNA carried in a commercially available expression vector, and secrete a form of tissue factor consisting only of amino acids 1-219 (numbered according to Morrissey, et al., Cell 50:129-135 (1987), the teachings of which are incorporated herein).

The recombinant, truncated tissue factor is purified from the culture medium using an immobilized monoclonal antibody to human tissue factor, such as TF9-5B7, described in Morrissey, et al. Thromb. Res. 52:247-261 (1988). Hybridomas for production of monoclonal antibodies can be propagated by ascites growth and the monoclonal antibodies (MAbs) purified from ascites fluid using the BioRad MAPS II system for mAb purification, as described by Morrissey, et al., Thromb. Res. 52:247-261 (1988).

The TF9-5B7 is coupled to Affigel™ beads. Detergent is not used during the purification of TF_{219}. After removal of cellular debris by centrifugation, the culture medium is made 25 mM in Tris.HCl (pH 7.4), 10 mM in sodium EDTA (pH 7.4), and 0.1% in sodium azide by the addition of concentrated stock solutions. In order to remove proteins that bind to agarose beads non-specifically, the culture medium is gently agitated for 4 hr at 4°C with AffiGel™-10 beads that had previously been blocked chemically with glycine ethyl ester (GEE-AffiGel). The GEE-AffiGel beads are removed by filtration through a sintered glass funnel, and the supernatant is agitated overnight at 4°C with the MAb TF9-5B7 coupled to AffiGel beads (typically 2 ml of beads). The TF9-5B7-AffiGel beads are collected on a sintered glass funnel, and the beads are washed

on the funnel with 100 ml of TBS-EDTA (TBS = 100 mM NaCl, 50 mM Tris.HCl pH 7.4, 0.02% sodium azide; TBS-EDTA = TBS with 10 mM EDTA included). The beads are then transferred to a chromatography column and washed with 40 ml TBS followed by 40 ml of a solution consisting of 1 M NaCl, 10 mM Tris.HCl pH 7.4, 0.02% sodium azide. Truncated TF is eluted from the beads using 100 mM glycine.HCl pH 2.5, with 1 ml fractions being collected into tubes containing 57 µl 1 M Tris base (to immediately neutralize the acidic glycine buffer). Fractions containing protein are detected using the BCA protein assay (Pierce), pooled, dialyzed against TBS, and then stored at -70°C. Protein concentrations are determined using standard methods such as the BCA assay (Pierce Chemical Co.) based on a bovine serum albumin standard of known concentration.

The TF_{1-219} deletion mutant used in the preferred embodiment was constructed and stably expressed in eukaryotic cells as follows. A deletion mutant of human TF cDNA was created that contained the coding sequence for the predicted leader peptide and extracellular domains, but lacked the predicted transmembrane and cytoplasmic domains. Starting with clone pCTF543 (described by Mackman et al., Biochemistry, 28, 1755-1762 (1989)), a 775 bp fragment of TF cDNA was released by digestion with *Eco*RI. This fragment extended from the 5′ noncoding sequence to the *Eco*RI site in the coding sequence (nucleotides 1 through 775 as numbered according to Morrissey et al., Cell, 50, 129-135 (1987)). To each end of the 775 bp cDNA fragment was ligated a double-stranded synthetic oligonucleotide made by annealing together the following two single-stranded oligonucleotides (sequences listed 5′ to 3′): AATTTAGAGAATAAGAATTCGGG and CCCGAATTCTTATTCTCTA. On the 3′ end of the insert, the adaptor took advantage of redundancy in the codon for phenylalanine by changing the codon for Phe^{217} from

TTC to TTT, thereby destroying the original *Eco*RI site but preserving the encoded amino acid sequence through Glu^{219}. The codon for Ile^{220} was replaced with a TAA stop codon, and a new *Eco*RI site was generated at the 3′ end for cloning purposes. Amino acid 219 is the last amino acid upstream of the predicted transmembrane domain, so this construct encodes the whole extracellular domain of TF (referred to here as rTF_{1-219}). The double-stranded oligonucleotide was also ligated to the 5′ end of the clone, but as it has no initiator ATG codon, it is predicted to have no effect on expression of the recombinant protein.

The resulting 805 bp TF_{1-219} cDNA was digested with *Eco*RI and subcloned into the *Eco*RI site of the mammalian expression vector pCDNAI (available from Invitrogen, San Diego, CA) in the proper orientation for expression. The resulting construct was introduced into 293 cells by liposome-mediated transfection using Lipofectin Reagent (available from Gibco/BRL, Gaithersburg, MD, and used according to the manufacturer's directions). Stable clones were selected by co-transfection of the construct with the plasmid pMAM-neo (available from Clontech, San Francisco, CA) in a 10:1 molar ratio. Stably transfected cell lines expressing truncated TF were selected initially by treating the cells with the neomycin analog G418 (Geneticin from Gibco/BRL, Gaithersburg, MD) at 400 μg/ml. Subsequently, culture supernatants of surviving drug-resistant colonies were screened for high levels of expression of truncated TF using a two-site, sandwich, enzyme-linked immunoassay based on two MAbs described by Morrissey, et al. Thromb. Res. 52:247-261 (1988). One stable, clonally-derived cell line was chosen that secreted truncated TF at levels of up to 1 mg per liter per day in roller bottle culture.

For production of recombinant truncated TF, cells are typically grown to confluence in roller bottles in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% iron-supplemented calf serum (HyClone Laboratories, Logan, Utah). Upon reaching confluence, the cultures are shifted to DMEM containing 2% serum, and culture medium is collected every four days thereafter.

The nucleotide and amino acid sequence of truncated tissue factor (tTF) is shown in Figure 1. The truncated tissue factor protein lacks the predicted transmembrane and cytoplasmic domains of tissue factor. This version of the protein retains cofactor activity, as reported by Ruf, et al., Thromb. Haemost. 62, 347 (abstract) (1989) and Ruf, et al., J. Biol. Chem. 266: 2158-2166 (1991). However, it has now been discovered that this truncated form of tissue factor fails to support conversion of factor VII to VIIa, allowing it to be used in a specific clotting assay for factor VIIa free from interference by factor VII.

The essential difference between truncated tissue factor and wild-type tissue factor is that truncated tissue factor is no longer tethered to the phospholipid membrane surface. It is therefore expected that other methods for preparing truncated tissue factor can be used to generate a soluble form of tissue factor that retains some cofactor activity while no longer stimulating conversion of factor VII to factor VIIa. Methods include enzymatic digestion of wild-type tissue factor to separate the predicted extracellular domain from the transmembrane region. Recombinant human TF is available from Calbiochem. Precise positioning of the stop codon following amino acid 219 is believed to not be essential to make functional truncated TF, and other placements of a stop codon near amino acid 219 are predicted to yield

an equivalent product with respect to its utility in the factor VIIa assay described herein.

A factor VIIa standard curve must be generated for each new batch of tTF reagent, and the curve is applicable only for the assay configuration tested (e.g., for the specific manner of performing the manual tilt-tube method, or for the specific automated or semi-automated coagulation measuring device used to generate the standard curve). Changing from one measuring device to another, or changing batches of tTF reagent, will necessitate generating a new standard curve.

3. Purified Proteins and Deficient Plasmas

Factor VII can be prepared as described by Fair, Blood 62, 784-791 (1983). FVII can be converted to fVIIa by incubation of the purified protein with factor Xa immobilized on Affi-Gel™ 15 beads (Bio-Rad). Conversion can be monitored by SDS-polyacrylamide gel electrophoresis of reduced samples. Free factor Xa in the fVIIa preparation can be detected with the chromogenic substrate methoxycarbonyl-D-cydohexylglycyl-glycyl-arginine-*p*-nitroanilide acetate (Spectrozyme™ FXa, American Diagnostica, Greenwich, CT) at 0.2 mM final concentration in the presence of 50 mM EDTA. Recombinant fVIIa can also be purchased from Novo Biolabs (Danbury, CT).

In the preferred embodiment, test plasma consists of platelet-poor, citrated plasma, although other anticoagulants can be used such as ethylenediamine tetracetic acid (EDTA) or sodium or potassium oxalate, and platelets do not have to be removed. This is prepared using standard hematologic techniques by drawing a small sample of blood by venipuncture into a citrate anticoagulant (ACD or CPD), from which plasma is prepared by centrifugation.

In the preferred embodiment, the reference plasmas from which the calibration standard curve is generated consist of known quantities of purified, recombinant human factor VIIa added to human factor VII-deficient plasma. The human factor VII-deficient plasma can be from congenitally deficient donors (available from George King Biomedical, Overland Park, Kansas) or can be immunodepleted plasma (available from American Diagnostica (Greenwich, CT). Concentrations of added factor VIIa range from 1 pg/ml to 10 µg/ml. A log-log plot of clotting time vs. factor VIIa concentration is prepared and a curve (4th order polynomial) is fit using regression analysis. Clotting times for unknown plasma samples are converted to factor VIIa concentrations using graphical analysis or a computer program.

Values obtained using the preferred embodiment of this assay for plasma levels of factor VIIa range from 0.34 to 4.4 ng/ml.

The plasma must be handled and stored in containers made of materials known not to promote activation of the contact system of coagulation. This includes the materials the plasma comes in contact with during the assay. The manual assay typically employs tubes formed of plastics such as polystyrene, polyethylene and polypropylene. An example of an unacceptable material is untreated glass, well-known for its ability to activate the contact coagulation system. Glass treated by siliconization, or by other methods known to eliminate contact activation of plasma, are also acceptable.

4. Ionic calcium

Typically, ionic calcium is provided as a 25 mM solution of $CaCl_2$. Calcium chloride is available from a number of commercial sources.

Assays for activated factor VII using the tTF

The assay can be performed using the reagents described above and obtained individually, or as a kit to be used in clinical coagulation laboratories, alone or in combination with other assays such as antigen-antibody assays. The essential components of the kit are the truncated tissue factor reagent and a set of factor VIIa standards consisting of factor VII-deficient (or factor VII-depleted) human plasma supplemented with known concentrations of factor VIIa. In most cases, the kit would be used with existing equipment such as coagulometers. Since the assay is conducted in a manner similar to that for the PT assay, minimal training of personnel is required.

The sensitivity of the tTF reagent to factor VIIa levels, expressed as the decrease in clotting time of factor VIIa-containing plasma when assayed in the presence versus the absence of tTF reagent, is a function of the concentrations of both tTF and phospholipid vesicles (cephalin). Therefore, the sensitivity of the reagent can be increased, i.e., the clotting time at a given factor VIIa concentration shortened, by increasing the concentration of tTF or cephalin in the reagent. Sensitivity of the reagent is adjusted by varying the concentrations of tTF or cephalin to achieve the desired range of clotting times relative to the range of factor VIIa levels to be measured. Preferably, the cephalin concentration is kept constant at the strength normally employed for PTT or TGT assays, and the sensitivity of the tTF reagent is adjusted by varying only the concentration of tTF.

For example, high sensitivity tTF reagents can be prepared using rabbit brain cephalin and between 100 nM and 1 μM tTF. As illustrated in Figure 2, the high sensitivity reagent is most useful for manual, semi-automated, or automated detection of factor VIIa

levels in the anticipated normal range (approximately 0.1 to 10 ng factor VIIa/ml). A low sensitivity tTF reagent can be prepared using rabbit brain cephalin and between 10 and 50 nM tTF. As illustrated in Figure 3, the low sensitivity reagent is most useful for manual, semi-automated, or automated detection of factor VIIa levels in the therapeutic range, up to approximately 5 μg factor VIIa/ml.

1. Manual clotting assays

In the preferred embodiment, the manual assay is performed as follows:

1. Pre-warm 12 X 75 mm polystyrene test tubes to 37°C in a water bath.

2. Add 0.1 ml truncated tissue factor reagent per tube and allow to warm to 37°C for at least 3 min.

3. Add 0.1 ml test plasma, mix and warm for exactly 30 sec.

4. Add 0.1 ml pre-warmed (37°C) 25 mM $CaCl_2$ solution, mix immediately, and determine clotting time from point of calcium addition using the manual tilt-tube method. The endpoint is when a visible clot is formed in the tube.

Figure 4 is a graph of a factor VIIa standard curve generated by the manual tilt tube method employing tTF.

2. Automated Measuring Devices for Coagulation Testing.

Automated and semi-automated devices for coagulation testing are available from a variety of suppliers. An example of a fully automated, photometric-based detection instrument suitable for the tTF assay for factor VIIa is the ACL™ 300 Automated Coagulation Laboratory available from Instrument Laboratory Division of Fisher Scientific Co. (Lexington, MA). In principle, any device capable of determining prothrombin times can be used for the

tTF assay for factor VIIA. This includes devices that detect clots photometrically (coagulometers) or using electrodes (fibrometers). The devices should be run in PT mode, and the data should be recorded in seconds. A variety of automated and semi-automated coagulation testing devices suitable for use with the tTF assay for factor VIIa are available from Scientific Products Division of Baxter Diagnostics, Inc. (McGaw Park, IL).

3. Application of tTF in Chromogenic Substrate Assays and Other Assay Configurations.

The tTF assay for plasma factor VIIa levels can be adapted for any assay configuration that employs TF or thromboplastin by substituting the tTF reagent for thromboplastin. The resulting assay will then be capable of measuring factor VIIa levels independent of the level of inactivated factor VII. An example of such an adaptation is substitution of tTF reagent for thromboplastin in the chromogenic substrate assay (also known as coupled amidolytic assay) for factor VII (FVIIam; see, for example, Seligsohn et al., Blood 52:978, 1978). Another example is substitution of tTF reagent for thromboplastin in alternative PT assays such as those performed on whole, citrated blood, for example, the Coumatrak™ Protime Test System marketed by Du Pont Company, Wilmington, DE.

4. tTF Assay for Factor VIIa Levels in Plasmas with Abnormal Prothrombin Times.

The tTF clotting assay for plasma factor VIIa depends on adequate levels of factors II, V, X and fibrinogen. Deficiencies in any of these factors, or the presence of inhibitors of these factors in plasma, could result in inaccurate measurement of factor VIIa levels. Accordingly, the tTF assay for factor VIIa levels should be run in parallel with a standard PT assay. Plasmas that exhibit PT values outside the acceptable normal range must be diluted ten-fold or one hundred-fold with factor VII deficient plasma and

re-assayed. The resulting value for plasma factor VIIa is then multiplied by the dilution factor to calculate the level of factor VIIa present in the original, undiluted plasma.

5. FVIIag Levels.

FVIIag levels can be measured with a commercial ELISA method, as described by Amiral, et al., Clin. Chem., 30:1512, (1984) and available from Diagnostica Stago (Asnieres, France).

Applications of the Assay

The assay is designed for the measurement of plasma levels of factor VIIa in individuals, with primary application as a clinical diagnostic agent, particularly as a screening test in order to identify those individuals with elevated levels of factor VIIa. Such individuals are thought to have increased risk of thrombotic disorders. Furthermore, there may be other associations between elevated factor VIIa levels and disease that could be established with the use of this assay.

Another application for this assay is to monitor plasma factor VIIa levels in hemophiliacs and other individuals undergoing factor VIIa therapy. Recombinant factor VIIa therapy is currently in clinical trials.

1. Monitor Plasma Factor VIIa Levels in Patients Treated with Recombinant Factor VIIa.

Clinical trials of recombinant factor VIIa therapy for bleeding disorders are currently underway, but without a reliable and specific assay for measuring the resulting factor VIIa levels. The tTF assay provides a means for setting dose and monitoring efficacy in these patients.

2. Monitor Plasma Factor VIIa Levels in Patients Taking Oral Anticoagulants, Particularly Low-Dose Coumadin Therapy.

Patients on low-dose coumadin therapy show little change in clotting times in standard clotting

assays, making it difficult to determine efficacy of the dose. For patients who respond to oral anticoagulant therapy with depressed factor VIIa levels, the tTF assay provides a means of monitoring such patients for efficacy, adjusting dose, etc.

3. Identify Individuals in the General Population with Increased Risk of Ischemic Heart Disease, Stroke, or Other Thrombotic Disease.

Recent studies have indicated a possible correlation between elevated factor VIIa levels and risk of such disorders as ischemic heart disease, as reported by de Sousa, et al., Thromb. Res. 51: 165-173 (1988); Hoffman, et al., J. Lab. Clin. Med. 111: 475-481 (1988); Gordon, et al., J. Lab. Clin. Med. 109: 409-413 (1987); and Mitropoulos, Sem. Thromb. Hemost. 14: 246-252 (1988).

4. Screen Women During Pregnancy to Identify Those with Increased Risk of Complications of Pregnancy.

Factor VII coagulant activity normally rises during pregnancy. The assay may therefore be of use in screening the blood of pregnant women, since there are suggestions of a correlation between excessively elevated plasma factor VIIa levels and fetal growth retardation, as described by Scarabin, et al., Thromb. Res. 45: 845-850 (1987).

5. Screen Women Taking Oral Contraceptives to identify Risk Group for Thrombotic Complications.

Since FVIIc normally rises in pregnancy, it may also rise during use of oral contraceptives. It is possible that elevation of plasma factor VIIa could be an underlying cause of the thrombotic episodes that occur in some women using oral contraceptives. Alternatively, elevated factor VIIa levels in these women may be a marker of a "prethrombotic state". In either case, it is of clinical value to identify reliably a subpopulation of women who experience an

excessive rise in factor VIIa levels when taking oral contraceptives.

6. Identify Individuals with Undetected Malignancy.

Fibrin deposition and other indicators of activation of the coagulation system are frequently associated with malignant tumors. Elevated plasma factor VIIa levels may constitute a sensitive means of indicating the presence of otherwise undetected tumors.

7. Identify and/or Monitor Patients with Inflammatory Diseases, Sepsis, and Incipient DIC.

Activation of coagulation often accompanies inflammation, sepsis, and forms the basis of disseminated intravascular coagulation (DIC). Diagnosis of elevated plasma factor VIIa could serve as an early marker for these conditions.

8. Assess degree of thrombotic risk in patients who have diseases that are known or suspected to be associated with increased tendency toward thrombosis.

Plasma factor VIIa levels serve as an extremely sensitive indicator for activation of the clotting cascade in individuals; therefore, measuring factor VIIa can be used to help assess the actual thrombotic risk in patients who have diseases known to be associated with increased tendency toward thrombosis. Measuring factor VIIa levels may also be of value for monitoring effectiveness of anticoagulant therapy (or prophylaxis) in such individuals. Diseases with a known or suspected risk of thrombotic episodes include preeclampsia, hypertension, certain malignancies, hyperlipidemia, hypercholesterolemia, protein C deficiency, and unexplained recurrent venous and arterial thrombosis. In addition, monitoring factor VIIa levels may be of use during thrombolytic therapy of patients with acute myocardial infarction or

stroke, and during and following vascular surgery, including angioplasty.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Morrissey, James H.

(ii) TITLE OF INVENTION: Quantitative Clotting Assay for Activated Factor VII

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kilpatrick & Cody
(B) STREET: 100 Peachtree Street
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: USA
(F) ZIP: 30303

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/683682
(B) FILING DATE: 10-APR-1991
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: OMRF122

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (404) 572-6508
(B) TELEFAX: (404) 572-6555

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 795 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCGCGCCCCG AGACCGCCGT CGCTCGGACG CTCCTGCTCG GCTGGGTCTT CGCCCAGGTG 120

GCCGGCGCTT CAGGCACTAC AAATACTGTG GCAGCATATA ATTTAACTTG GAAATCAACT 180

AATTTCAAGA CAATTTTGGA GTGGGAACCC AAACCCGTCA ATCAAGTCTA CACTGTTCAA 240

ATAAGCACTA AGTCAGGAGA TTGGAAAAGC AAATGCTTTT ACACAACAGA CACAGAGTGT 300

GACCTCACCG ACGAGATTGT GAAGGATGTG AAGCAGACGT ACTTGGCACG GGTCTTCTCC 360

TACCCGGCAG GGAATGTGGA GAGCACCGGT TCTGCTGGGG AGCCTCTGTA TGAGAACTCC 420

CCAGAGTTCA CACCTTACCT GGAGACAAAC CTCGGACAGC CAACAATTCA GAGTTTTGAA 480

CAGGTGGGAA CAAAAGTGAA TGTGACCGTA GAAGATGAAC GGACTTTAGT CAGAAGGAAC 540

AACACTTTCC TAAGCCTCCG GGATGTTTTT GGCAAGGACT TAATTTATAC ACTTTATTAT 600

TGGAAATCTT CAAGTTCAGG AAAGAAAACA GCCAAAACAA ACACTAATGA GTTTTTGATT 660

GATGTGGATA AAGGAGAAAA CTACTGTTTC AGTGTTCAAG CAGTGATTCC CTCCCGAACA 720

GTTAACCGGA AGAGTACAGA CAGCCCGGTA GAGTGTATGG GCCAGGAGAA AGGGGAATTT 780

AGAGAATAAG AATTC 795

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 251 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..32
(D) OTHER INFORMATION: /label= leader

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
        35                  40                  45

Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
    50                  55                  60

Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
65                  70                  75                  80

Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
                85                  90                  95

Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
            100                 105                 110

Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
        115                 120                 125

Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
    130                 135                 140

Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
145                 150                 155                 160
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Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
                  165                 170                 175

Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
             180                 185                 190

Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
         195                 200                 205

Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
     210                 215                 220

Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
225                 230                 235                 240

Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu
                  245                 250
```

CLAIMS

1. A method for measuring the concentration of coagulation Factor VIIa in a patient's plasma sample, comprising the steps of:

(a) mixing a quantity of a plasma sample with a first reagent, said first reagent comprising a soluble truncated tissue factor characterized by its inability to bind to phospholipids and its inability to support conversion of Factor VII to Factor VIIa, to form a first mixture;

(b) either simultaneously with said first reagent or thereafter, adding a second reagent comprising an effective amount of calcium ions to initiate clotting of said first mixture;

(c) measuring a plasma clotting time of said first mixture from a time of addition of said second reagent to a time of clot formation; and

(d) comparing said plasma clotting time to a standards clotting time determined for Factor VIIa of a known concentration.

2. The method of Claim 1, wherein said first reagent or said second reagent further comprises a phospholipid agent.

3. The method of Claim 1 or 2, wherein said plasma sample is diluted between about 1:10 and 1:100 with Factor VII-deficient plasma prior to step (a).

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4. The method of Claim 1, 2, or 3, wherein the concentration of said soluble truncated tissue factor in said first mixture is from about 10 nanomolar to about 1 micromolar.

5. The method of Claims 1, 2, 3 or 4, wherein said standards clotting time is determined for a known concentration of Factor VIIa diluted in Factor VII-deficient plasma by adding a like quantity of said first reagent and said second reagent as that added to said plasma sample to said known concentration of Factor VIIa diluted in Factor VII-deficient plasma and measuring said standards clotting time from a time of addition of said second reagent to a time of clotting, and correlating said known concentration of Factor VIIa with said standards clotting time to form a standard curve.

6. The method of Claims 1, 2, 3, 4, or 5 wherein said effective amount of calcium ions is a concentration of at least 3mM.

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7. A kit ~~for use in measuring~~ to measure Factor VIIa in a plasma sample according to the method of claim 1 comprising:

(a) a truncated tissue factor reagent comprising truncated tissue factor solubilized in an aqueous medium, said truncated tissue factor characterized by inability to bind to a phospholipid membrane surface and inability to support conversion of Factor VII to Factor VIIa, provided in a first container;

(b) a Factor VII-deficient plasma provided in a second container; and

(c) a Factor VIIa standard provided in a third container.

8. The kit of Claim 7, further comprising:

(d) a fourth container containing a calcium ion source.

9. The kit of Claim 7 or 8, further comprising a phospholipid agent either in a fifth container or added to one or more of the other containers.

10. The kit of Claims 7-9, further comprising:

a plurality of vials, each of said vials containing a known concentration of Factor VIIa in a diluent comprising Factor VII-deficient plasma, said plurality of vials providing a range of known Factor VIIa concentrations from about 1 picogram per milliliter to about 10 microgram per milliliter.

AUSTRALIA PATENT OFFICE MB

```
                                                  M  E   T  P  A  W
  1   CGTTCCGCTC GATCTCGCCG CCAACTGGTA GACATGGAGA CCCCTGCCTG

       P  R  V   P  R  P   E  T  A  V   A  R  T   L  L  L
 51   GCCCCGGGTC CCGCGCCCCG AGACCGCCGT CGCTCGGACG CTCCTGCTCG

      G  W  V  F   A  Q  V   A  G  A   S  G  T  T   N  T  V
101   GCTGGGTCTT CGCCCAGGTG GCCGGCGCTT CAGGCACTAC AAATACTGTG

       A  A  Y   N  L  T  W   K  S  T   N  F  K   T  I  L  E
151   GCAGCATATA ATTTAACTTG GAAATCAACT AATTTCAAGA CAATTTTGGA

        W  E  P   K  P  V   N  Q  V  Y   T  V  Q   I  S  T
201   GTGGGAACCC AAACCCGTCA ATCAAGTCTA CACTGTTCAA ATAAGCACTA

      K  S  G  D   W  K  S   K  C  F   Y  T  T  D   T  E  C
251   AGTCAGGAGA TTGGAAAAGC AAATGCTTTT ACACAACAGA CACAGAGTGT

       D  L  T   D  E  I  V   K  D  V   K  Q  T   Y  L  A  R
301   GACCTCACCG ACGAGATTGT GAAGGATGTG AAGCAGACGT ACTTGGCACG

        V  F  S   Y  P  A   G  N  V  E   S  T  G   S  A  G
351   GGTCTTCTCC TACCCGGCAG GGAATGTGGA GAGCACCGGT TCTGCTGGGG

      E  P  L  Y   E  N  S   P  E  F   T  P  Y  L   E  T  N
401   AGCCTCTGTA TGAGAACTCC CCAGAGTTCA CACCTTACCT GGAGACAAAC

       L  G  Q   P  T  I  Q   S  F  E   Q  V  G   T  K  V  N
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        V  T  V   E  D  E   R  T  L  V   R  R  N   N  T  F
501   TGTGACCGTA GAAGATGAAC GGACTTTAGT CAGAAGGAAC AACACTTTCC

      L  S  L  R   D  V  F   G  K  D   L  I  Y  T   L  Y  Y
551   TAAGCCTCCG GGATGTTTTT GGCAAGGACT TAATTTATAC ACTTTATTAT

       W  K  S   S  S  S  G   K  K  T   A  K  T   N  T  N  E
601   TGGAAATCTT CAAGTTCAGG AAAGAAAACA GCCAAAACAA ACACTAATGA

        F  L  I   D  V  D   K  G  E  N   Y  C  F   S  V  Q
651   GTTTTTGATT GATGTGGATA AAGGAGAAAA CTACTGTTTC AGTGTTCAAG

      A  V  I  P   S  R  T   V  N  R   K  S  T  D   S  P  V
701   CAGTGATTCC CTCCCGAACA GTTAACCGGA AGAGTACAGA CAGCCCGGTA

       E  C  M   G  Q  E  K   G  E  F   R  E
751   GAGTGTATGG GCCAGGAGAA AGGGGAATTT AGAGAATAAG AATTC
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FIGURE 1

WO 92/18870 PCT/US92/02898

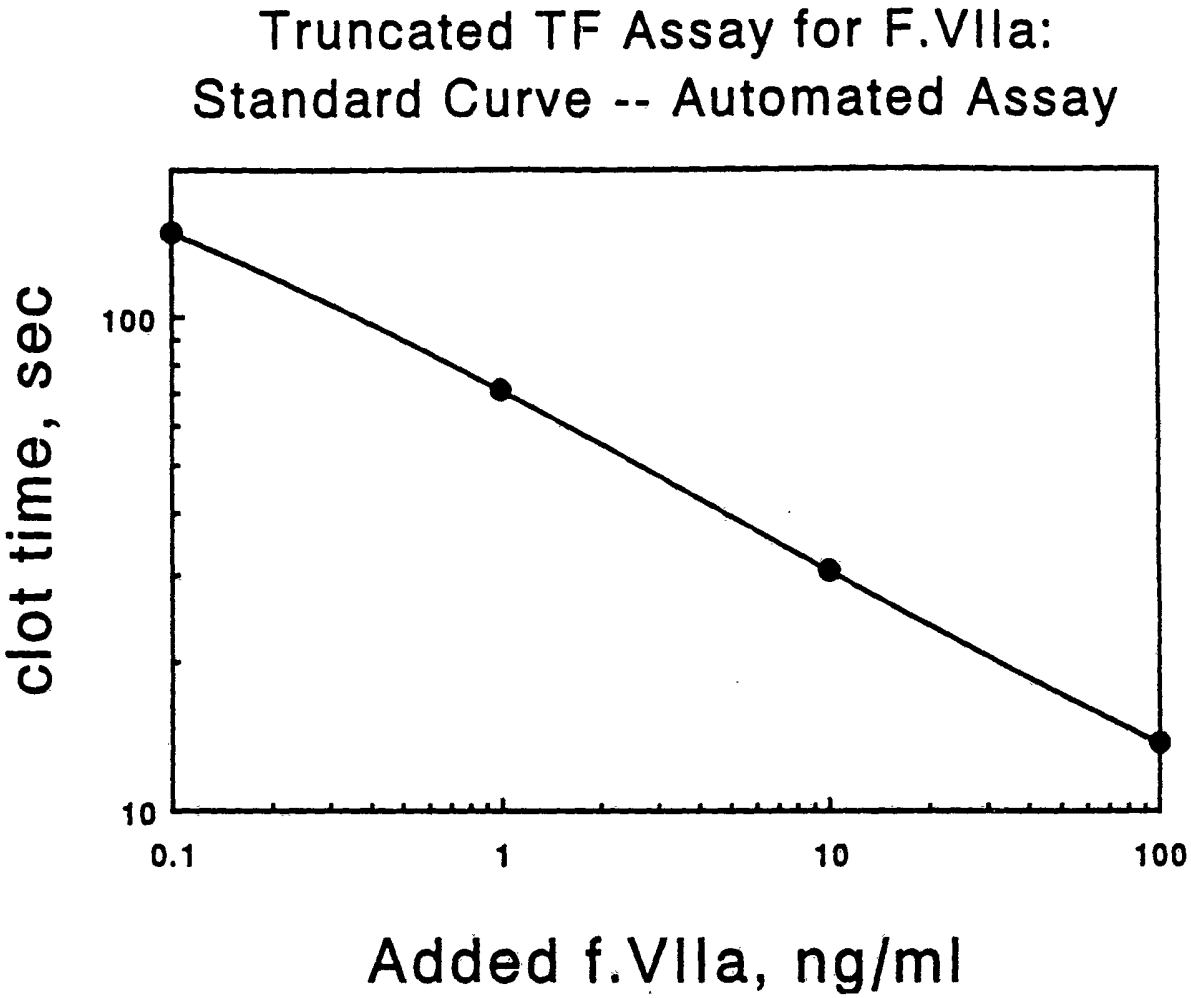

FIGURE 2

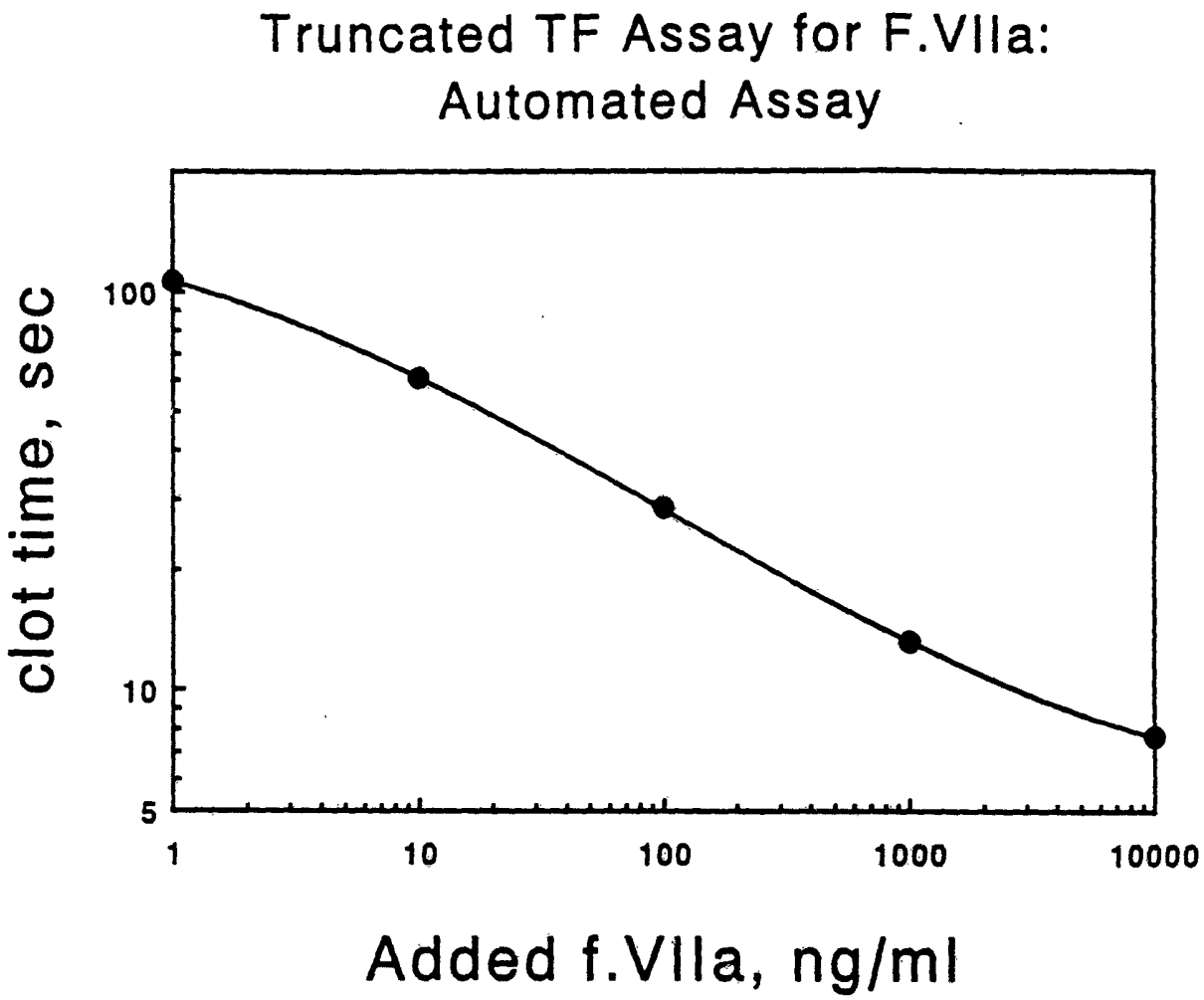
Truncated TF Assay for F.VIIa:
Automated Assay
clot time, sec
100
10
5
1
10
100
1000
10000
Added f.VIIa, ng/ml

FIGURE 3

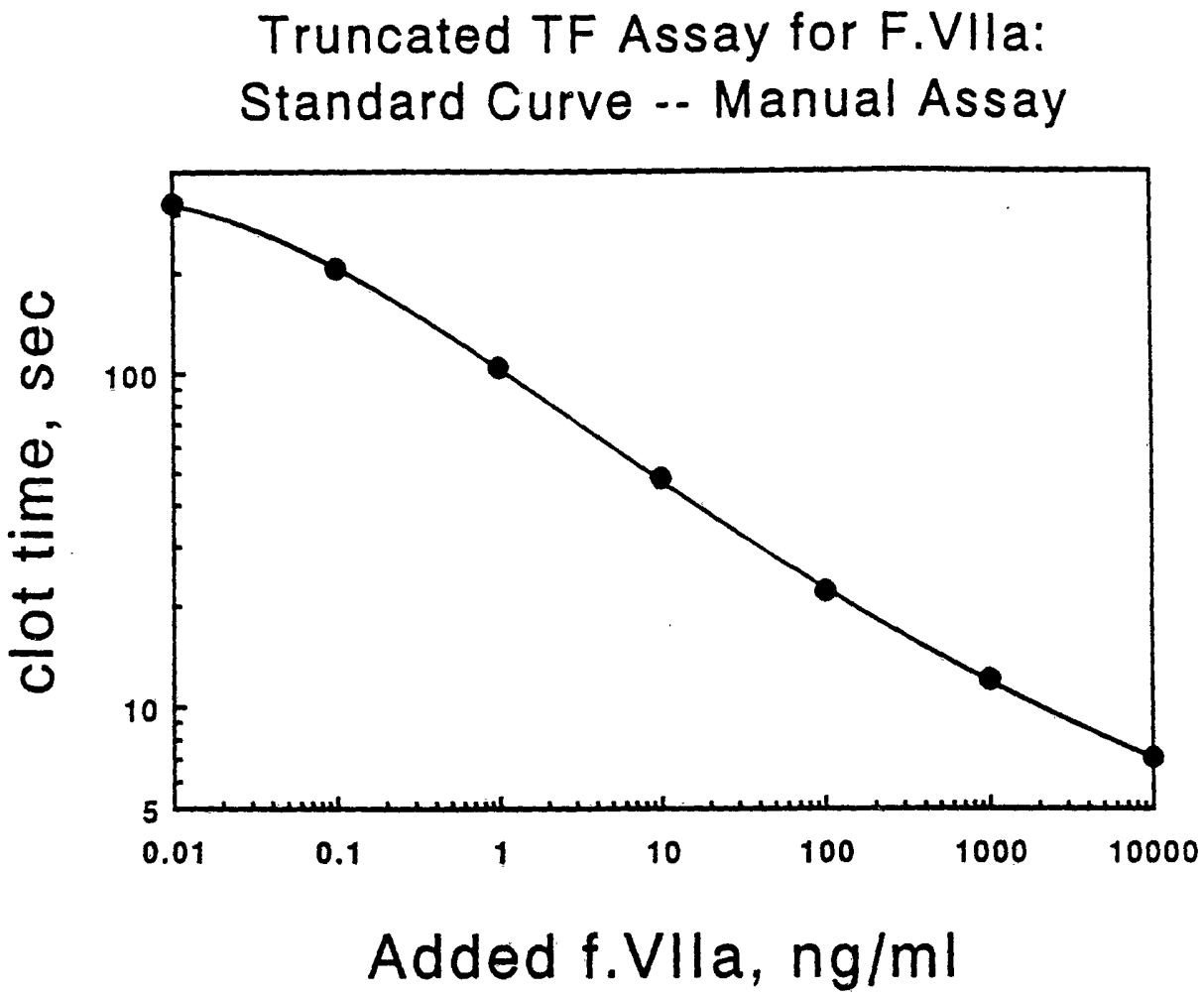
Truncated TF Assay for F.VIIa:
Standard Curve -- Manual Assay
clot time, sec
100
10
5
0.01
0.1
1
10
100
1000
10000
Added f.VIIa, ng/ml

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02898

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

Int.Cl. 5 G01N33/86; G01N33/68; G01N33/92

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; C07K

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

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]
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 4, 5 February 1991, BALTIMORE US pages 2158 - 2166; W. RUFF; A. REHEMTULLA, AND T.S. EDGINGTON: 'Phospholipid-indpendent and -dependent Interactions Required for Tissue Factor Receptor and Cofactor Function' cited in the application	1-21
Y	see the whole document	1-21

P,Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 267, no. 7, 5 March 1992, BALTIMORE US pages 4834 - 4843; J.H. LAWSON; S. BUTENAS, AND K.G. MANN: 'The Evaluation of Complex-dependent Alterations in Human Factor VIIa' see the whole document	1-21

	-/--	

° 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
01 SEPTEMBER 1992	21. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	DÖPFER K.P.

Form PCT/ISA/210 (second sheet) (January 1985)

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	BLOOD vol. 71, no. 1, January 1988, ORLANDO, FL,US pages 1 - 8; YALE NEMERSON: 'Tissue Factor and Hemostasis' see the whole document	1-21
A	BIOCHEMISTRY. vol. 25, no. 14, 15 July 1986, EASTON, PA US pages 4007 - 4020; R. BACH, R. GENTRY, AND Y. NEMERSON: 'Factor VII Binding to Tissue Factor in Reconstituted Phospholipid Vesicles: Induction of Cooperativity by Phosphatidylserine' see abstract see page 4012, left column, line 1 - page 4019, right column, line 29; figure 10	1-21
A	BIOCHEMISTRY. vol. 28, no. 4, 21 February 1989, EASTON, PA US pages 1755 - 1762; N. MACKMAN, J.H. MORRISSEY, B. FOWLER, AND T.S. EDGINGTON: 'Complete Sequence of the Human Tissue Factor Gene, a Highly Regulated Cellular Receptor That Initiates the Coagulation Protease Cascade' cited in the application see the whole document	1-21
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 257, no. 10, 25 May 1982, BALTIMORE US pages 5623 - 5631; M. ZURT, R.D. RADCLIFFE, J. OBERDICK, AND Y. NEMERSON: 'The Dual Role of Factor VII in Blood Coagulation'	