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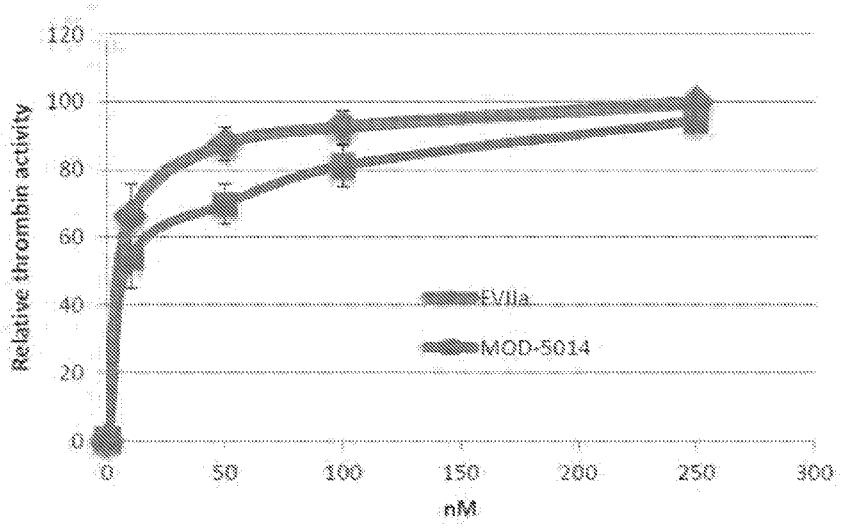

FIGURE 119

(57) Abstract: Polypeptides comprising at least one carboxy- terminal peptide (CTP) of chorionic gonadotropin attached to the carboxy terminus but not to the amino terminus of a coagulation factor and polynucleotides encoding the same are disclosed. Pharmaceutical compositions and pharmaceutical formulations comprising the polypeptides and polynucleotides of the disclosure and methods of using and producing same are also disclosed.

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LONG-ACTING COAGULATION FACTORS AND METHODS OF PRODUCING SAME

FIELD OF DISCLOSURE

[001] Polypeptides comprising at least one carboxy-terminal peptide (CTP) of chorionic gonadotropin attached to the carboxy terminus of a coagulation factor and polynucleotides encoding the same are disclosed. Pharmaceutical compositions and pharmaceutical formulations comprising the polypeptides and polynucleotides of the disclosure and methods of using and producing same are also disclosed.

BACKGROUND

[002] The development of coagulation factor replacement therapy has transformed the lives of many individuals with hemophilia. Hemophilia is a group of hereditary genetic disorders that impair the body's ability to control blood clotting or coagulation. Patients with hemophilia do not produce adequate amounts of Factor VIII or Factor IX proteins, which are necessary for effective blood clotting. In severe hemophiliacs even a minor injury can result in blood loss that continues for days or weeks, and complete healing may not occur, leading to the potential for debilitating permanent damage to joints and other organs, and premature death.

[003] Hemophilia is an inherited, X-chromosome-linked hemorrhagic disorder caused by defects in, or the absence of, critical factors in the coagulation cascade. In hemophilia patients, thrombin generation and fibrin clot formation are severely compromised, leading to spontaneous bleeding episodes most commonly in joints and internal organs, and excessive bleeding during and following surgery or trauma. Frequent bleeding can also cause joint swelling, joint damage, severe deformity, frequent infections, and reduced mobility in hemophilia patients (Mayo Clinic). Hemophilia A is caused by defects in or lack of Factor VIII expression, while hemophilia B is caused by defects in or lack of Factor IX expression.

[004] Hemophilia B results in a deficiency of the procoagulant activity of FIX. Hemophilia B patients have spontaneous soft tissue hemorrhages and recurrent hemarthroses that often lead to a crippling arthopathy. Current treatment for these patients includes an intravenous administration of recombinant FIX. However issues of cost and relatively rapid clearance of FIX from the circulation make developing a long-acting FIX a challenging task.Commercial availability of FVIII and FIX has led to improved control of life-threatening bleedings episodes. Many patients receive prophylactic therapy, which reduces the risk of bleeding and

its associated complications. However, a significant proportion of patients (10-30%) develop inhibitory antibodies to exogenously administered FVIII and FIX. Administration of FVIIa, which is a bypassing product, can induce homeostasis and provide an effective treatment for patients with inhibitory Abs.

[005] Recombinant FVIIa (NovoSeven®) is commercially available and was approved in 1996 for treatment of bleeding episodes in hemophilia patients with inhibitors. However, rFVIIa is rapidly cleared with a terminal half-life of 2.5 hours. As a result, patients generally require multiple, frequent infusions (2-3 doses given in 2-3 hour intervals) to achieve adequate homeostasis following a mild to moderate bleed. Consequently, there is much interest in developing a long-acting form of FVIIa that would prolong the duration of haemostatic activity following a single dose and allow much less frequent dosing. A long-acting FVIIa would also increase the feasibility of long-term prophylactic therapy.

[006] Various technologies are being developed for prolonging the half-life of FVIIa. However, there remains a need to achieve a prolonged half-life of this protein while preserving its biological activity and ensuring that the modifications do not induce significant immunogenicity. The present invention addresses this need by attaching gonadotropin carboxy terminal peptides (CTPs) to FVIIa, thereby modifying it to prolong its half-life and biological activity.

SUMMARY OF THE DISCLOSURE

[007] In one aspect, the present invention provides a CTP-modified polypeptide consisting of a Factor VII coagulation factor and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein said polypeptide does not include a signal peptide. In another aspect, the present invention provides the CTP-modified polypeptide, wherein the coagulation factor is activated FVII (FVIIa). In another aspect, the sequence of the CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

[008] In another aspect, the CTP-modified polypeptide comprising an activated FVIIa-CTP_3 comprises a light chain and a heavy chain linked by a disulfide bond. In another aspect, separation of the light chain and the heavy chain on an SDS-PAGE gel occurs under denaturing conditions, wherein the light chain migrates at about 25 kDa molecular weight and the heavy chain migrates at about 60 kDa molecular weight.

[009] In one aspect, this invention provides a pharmaceutical formulation comprising a buffer, an amino acid, wherein in another embodiment is glycine, a tonicity agent, and a CTP-modified polypeptide consisting of a Factor VII coagulation factor and three chorionic

gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein the polypeptide does not include a signal peptide. In another aspect, the buffer comprises 20 mM citrate. In another aspect, the tonicity agent is 150 mM sodium chloride. In another aspect, the formulation is a liquid formulation. In another aspect, the formulation is at a pH of about 6.4.

[010] In another aspect, this invention provides a formulation comprising 20 mM citrate, 13.3 mM Glycine, 150 mM sodium chloride, at a pH of about 6.4.

[011] In another aspect, this invention provides a pharmaceutical formulation wherein the coagulation factor is activated FVII (FVIIa). In another aspect, the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46. In another aspect, the pharmaceutical formulation comprising the polypeptide is administered daily, every other day, every three days, once weekly, twice weekly, or once every other week, or any combination thereof. In another aspect, administration of the pharmaceutical formulation is intravenous or subcutaneous administration.

[012] In another aspect, this invention provides a pharmaceutical composition comprising the CTP-modified polypeptide as described herein, and a pharmaceutically acceptable carrier.

[013] In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutical formulation as described herein..

[014] In one aspect, this invention provides a method of extending the biological half-life of a Factor VII (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby extending the biological half-life of said coagulation factor, wherein an activated form of the coagulation factor does not include a signal peptide. In another aspect, the coagulation factor is activated FVII (FVIIa). In another aspect, the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

[015] In one aspect, this invention provides a method of improving the area under the curve (AUC) of a Factor VII (FVII) coagulatiIn on factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby improving the AUC of said coagulation factor, wherein an activated form of the coagulation factor does not include a signal peptide. In another aspect, the coagulation factor is activated FVII (FVIIa). In another aspect, the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

[016] In one aspect, this invention provides a method of reducing the dosing frequency of a Factor VIIa (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII

coagulation factor, thereby reducing the dosing frequency of said coagulation factor, wherein said activated form of the FVII coagulation factor does not include a signal peptide. In another aspect, the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

[017] In one aspect, this invention provides a method of preventing or treating a blood clotting or coagulation disorder in a subject, the method comprising the step of administering an activated CTP-modified coagulation factor polypeptide to the subject, wherein said coagulation factor is FVIIa-CTP_3, thereby preventing or treating a blood clotting or coagulation disorder in said subject. In another aspect the disorder is hemophilia. In another aspect, the hemophilia comprises hemophilia A or hemophilia B with inhibitors. In another aspect, administering is via a subcutaneous route. In another aspect, administering is via the intravenous route.

[018] In another aspect, this invention provides a method of reducing bleeding episodes.

[019] Other features and advantages of the present invention will become apparent from the following detailed description, examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the disclosure are given by way of illustration only, since various changes and modification within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[020] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[021] **Figure 1A.** Shows a bar graph showing harvests limited, diluted, transfected, and selected cells with FIX-CTP and FIX-CTP-CTP variants in the presence of 5µg/ml of Vitamin K3. The level of FIX was quantified using Human FIX ELISA kit (Affinity Biologicals; Cat. No. FIX-AG RUO), and the calculated protein concentration (µg/ml) is the average of two independent runs.

[022] **Figures 1B.** Shows SDS-PAGE gel micrographs of FIX Ab recognition and depicts recognition of anti-FIX antibody in Western-blot; Lane 1 in Figures **1B** was loaded with a sample containing recombinant FIX; Lane 2 in Figures 1B was loaded with a sample containing FIX-CTP harvests. Lane 3 in Figures **1B** was loaded with a sample containing FIX-$(CTP)_2$ harvest.

[023] **Figure 1C.** Shows SDS-PAGE gel micrographs of FIX Ab recognition. **Figure 1C**

depicts recognition of anti-γ carboxylation antibody in Western-blot. Lane 1 in Figure 1C was loaded with a sample containing recombinant FIX. Lane 2 in **Figure 1C** was loaded with a sample containing FIX-CTP harvests. Lane 3 in **Figure 1C** was loaded with a sample containing FIX-$(CTP)_2$ harvest.

[024] **Figure 2.** Shows a graph showing FIX-CTP and FIX-$(CTP)_2$ harvests comparative chromogenic activity (measured by a the EC_{50}. concentration) compared to rhFIX (American Diagnostics).

[025] **Figure 3.** Shows a graph showing PK profile of rhFIX, harvest of FIX-CTP-CTP, and harvest of FIX-CTP.

[026] **Figure 4.** Shows a bar graph showing harvests of FIX-CTP and FIX-CTP-CTP harvests and FIX-CTP-CTP purified protein FIX antigen level as determined using Human FIX ELISA kit (Affinity Biologicals; cat. No. FIX-AG RUO). The calculated protein concentration (μg/ml) is the average of two independent runs.

[027] **Figure 5A.** Shows SDS-PAGE gel micrographs of FIX Ab recognition and depicts a coomassie blue staining. Lane 1 was loaded with a sample containing FIX-$(CTP)_2$. Lane 2 was loaded with a sample containing unbound FIX-$(CTP)_2$. Lane 3 was loaded with a sample containing a concentrated elution of FIX-$(CTP)_2$.

[028] **Figure 5B.** Shows SDS-PAGE gel micrographs of FIX Ab recognition and depicts recognition of anti-FIX antibody in Western-blot. Lane 1 was loaded with a sample containing FIX-$(CTP)_2$. Lane 2 was loaded with a sample containing unbound FIX-$(CTP)_2$. Lane 3 was loaded with a sample containing a concentrated elution of FIX-$(CTP)_2$.

[029] **Figure 5C.** Shows SDS-PAGE gel micrographs of FIX Ab recognition and depicts recognition of anti-γ carboxylation antibody in Western-blot. Lane 1 was loaded with a sample containing FIX-$(CTP)_2$. Lane 2 was loaded with a sample containing unbound FIX-$(CTP)_2$. Lane 3 was loaded with a sample containing a concentrated elution of FIX-$(CTP)_2$.

[030] **Figure 6.** Shows a graph showing FIX-$(CTP)_2$ chromogenic activity (sample concentration/O.D.) compared to human normal pool plasma and rhFIX (American Diagnostics).

[031] **Figure 7.** Shows a graph showing the PK profile of purified FIX-CTP-CTP, rhFIX, harvest of FIX-CTP-CTP, and harvest of FIX-CTP.

[032] **Figure 8A.** Shows an anti-CTP and anti-gamma carboxylation antibodies Western blots of FIX fused to three, four or five CTPs. FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 harvests were loaded on 12% Tris-Glycine gel using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immuno-blot using anti-CTP polyclonal Ab (Adar Biotech Production).

[033] **Figure 8B.** Shows an anti-CTP and anti-gamma carboxylation antibodies Western blots of FIX fused to three, four or five CTPs. FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 harvests were loaded on 12% Tris-Glycine gel using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immuno-blot using anti-Gla Ab (American Diagnostica).

[034] **Figure 9.** Shows a Coomassie blue detection of FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5. After a purification process utilizing Jacalin column (immunoaffinity purification of glycosylated proteins), FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE was stained by Coomassie blue dye for sample detection.

[035] **Figure 10.** Shows FIX Chromogenic activity. A comparative assessment of the *in vitro* potency of fully purified (HA column) FIX-CTP_3 FIX-CTP_4 and FIX-CTP_5 versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). All samples were serially diluted and the potency was assessed by comparing a dose response curve to a reference preparation consisting of normal human plasma.

[036] **Figure 11.** Shows the comparative pharmacokinetic (PK) profile of FIX-CTP_3 FIX-CTP_4 and FIX-CTP_5. FIX concentration in plasma samples were quantified using human FIX Elisa kits (Affinity Biologicals). Pharmacokinetic profile was calculated and is the mean of 3 animals at each time point. Terminal half-lives were calculated using PK Solutions 2.0 software.

[037] **Figure 12A**. Shows the FIX-CTP_3 SDS-PAGE analysis - Coomassie SDS-PAGE. FIX-CTP_3 γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE Coomassie analysis was performed by staining the gel with Coomassie blue reagent (800 ng of protein).

[038] **Figure 12B**. Shows the FIX-CTP_3 SDS-PAGE analysis - Coomassie SDS-PAGE. FIX-CTP_3 γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). A Western immunoblot was performed using 100 ng of protein with anti-human FIX polyclonal Ab.

[039] **Figure 12C**. Shows the FIX-CTP_3 SDS-PAGE analysis - Coomassie SDS-PAGE. FIX-CTP_3 γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). A Western immunoblot was performed using 100 ng of protein with anti-human gamma carboxylation monoclonal antibody (American Diagnostics Cat #499, 3570).

[040] **Figure 12D**. Shows the FIX-CTP_3 SDS-PAGE analysis - Coomassie SDS-PAGE. FIX-CTP_3 γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). A Western immunoblot was performed using 100 ng of protein with anti-FIX pro-peptide polyclonal Ab (Figure 12D).

[041] **Figure 12E**. Shows the FIX-CTP_3 SDS-PAGE analysis - Coomassie SDS-PAGE. FIX-CTP_3 γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). A Western immunoblot was performed using 100 ng of protein with anti-CTP polyclonal Ab.

[042] **Figure 13.** Shows the FIX-CTP_3 chromogenic activity. A comparative assessment of the *in vitro* potency of FIX-CTP_3 harvest and FIX-CTP_3 γ-carboxylated enriched protein, versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). FIX-CTP_3 harvest and protein were serially diluted, and the potency was assessed by comparing a dose-response curve to a reference preparation consisting of normal human plasma.

[043] **Figure 14.** Shows the comparative clotting time. An *in vitro* aPTT (activated Partial Thrombin Time Assay) was performed comparing the clotting activity of FIX-CTP_3 to BeneFIX. The proteins were serially diluted and spiked into human FIX-depleted plasma, and the clotting time was evaluated.

[044] **Figure 15.** Shows FIX-CTP_3 comparative PK profile. FIX concentration was quantitated using human FIX ELISA kits (Affinity Biologicals; Cat. # FIX-AG RUO). The pharmacokinetic profile was calculated for each protein and is the mean of 3 animals at each time point.

[045] **Figure 16A.** In parallel to PK sampling, FIX-deficient animals administered with FIX-CTP_3, citrated plasma samples, were evaluated for their clotting activity by aPTT assay, which was translated to % activity. The % activity at each collection point was calculated as the current clotting time/clotting time of normal pool mice plasma*100.

[046] **Figure 16B.** In parallel to PK sampling, FIX-deficient animals administered with either BeneFIX®, citrated plasma samples, were evaluated for their clotting activity by aPTT assay, which was translated to % activity. The % activity at each collection point was calculated as the current clotting time/clotting time of normal pool mice plasma*100.

[047] **Figure 17A.** Shows a first challenge bleeding parameters. FIX-deficient mice were administered a single intravenous injection of 100 IU/Kg of BeneFIX® or rFIX-CTP_3. The tail vein was slightly clipped 48 hours post-dosing and tail vein bleeding time (TVBT) was evaluated. A second bleeding challenge was performed 15 minutes after reaching

homeostasis, and the same parameters were measured.

[048] **Figure 17B.** Shows a first challenge bleeding parameters. FIX-deficient mice were administered a single intravenous injection of 100 IU/Kg of BeneFIX® or rFIX-CTP_3. The tail vein was slightly clipped 48 hours post-dosing and tail vein bleeding time (TVBT) was evaluated. A second bleeding challenge was performed 15 minutes after reaching homeostasis, and the same parameters were measured.

[049] **Figure 17C.** Shows a first challenge bleeding parameters. FIX-deficient mice were administered a single intravenous injection of 100 IU/Kg of BeneFIX® or rFIX-CTP_3. The tail vein was slightly clipped 48 hours post-dosing and bleeding intensity (hemoglobin OD) was evaluated. A second bleeding challenge was performed 15 minutes after reaching homeostasis, and the same parameters were measured.

[050] **Figure 17D.** Shows a first challenge bleeding parameters. FIX-deficient mice were administered a single intravenous injection of 100 IU/Kg of BeneFIX® or rFIX-CTP_3. The tail vein was slightly clipped 48 hours post-dosing and bleeding intensity (hemoglobin OD) was evaluated. A second bleeding challenge was performed 15 minutes after reaching homeostasis, and the same parameters were measured.

[051] **Figure 18A.** Shows a second challenge bleeding parameters. Once the first bleeding described in the legend to Figure 19 was spontaneously or manually stopped, a second bleeding challenge was performed 15 minutes following the first one, and the time was re-measured.

[052] **Figure 18B.** Shows a second challenge bleeding parameters. Once the first bleeding described in the legend to Figure 19 was spontaneously or manually stopped, a second bleeding challenge was performed 15 minutes following the first one, and the time was re-measured.

[053] **Figure 18C.** Shows a second challenge bleeding parameters. Once the first bleeding described in the legend to Figure 19 was spontaneously or manually stopped, a second bleeding challenge was performed 15 minutes following the first one, and the bleeding intensity was re-measured.

[054] **Figure 18D.** Shows a second challenge bleeding parameters. Once the first bleeding described in the legend to Figure 19 was spontaneously or manually stopped, a second bleeding challenge was performed 15 minutes following the first one, and the bleeding intensity was re-measured.

[055] **Figure 19A.** Shows a diagram illustrating the rFVII-CTP construct.

[056] **Figure 19B.** Shows a diagram illustrating the rFVII-CTP-CTP construct.

[057] **Figure 19C.** Shows a diagram illustrating the rFIX-CTP construct.

[058] **Figure 19D.** Shows a diagram illustrating the rFIX-CTP-CTP construct.

[059] **Figure 20A.** Shows a bar graph showing harvests limited diluted clone transfected and selected cells with FVII-CTP variants in the presence of 5µg/ml of Vitamin K3. The level of FVII was quantified using FVII ELISA (AssayPro).

[060] **Figure 20B.** Shows a bar graph showing harvests of limited diluted transfected and selected cells with FVII-CTP variants in the presence of 5µg of Vitamin K3.activity. FVII activity was quantified using FVII chromogenic activity assay (AssayPro).

[061] **Figure 20C.** Shows a bar graph showing harvests of limited diluted transfected and selected cells with FVII-CTP variants in the presence of 5µg of Vitamin K3. The specific activity of FVII was calculated for each version by dividing the activity value by the harvest FVII concentration.

[062] **Figure 20D.** Shows a graph showing PK profile of FVII, FVII-CTP-CTP, and FVII-CTP harvests.

[063] **Figure 21A**. Shows western blots of FVII fused to three, four and five CTPs, detected using anti-FVII, anti-CTP, and anti-gamma carboxylation antibodies. FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 harvests were loaded on 12% Tris -Glycine gel (*expedeon*) using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immunoblot using anti-FVII.

[064] **Figure 21B**. Shows western blots of FVII fused to three, four and five CTPs, detected using anti-FVII, anti-CTP, and anti-gamma carboxylation antibodies. FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 harvests were loaded on 12% Tris -Glycine gel (*expedeon*) using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immunoblot using anti-CTP polyclonal Ab (Adar Biotech Production).

[065] **Figure 21C**. Shows western blots of FVII fused to three, four and five CTPs, detected using anti-FVII, anti-CTP, and anti-gamma carboxylation antibodies. FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 harvests were loaded on 12% Tris -Glycine gel (*expedeon*) using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immunoblot using anti-Gla Ab (American Diagnostica).

[066] **Figure 22**. Shows the FVII Activity – Chromogenic activity. A comparative assessment of the *in vitro* potency of HA purified (highly gamma carboxylated fraction) FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 versus normal human pool plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221304). All samples were serially diluted and the potency was assessed by comparing a dose response curve to a reference preparation consisting of normal human plasma.

[067] **Figure 23.** Shows a first comparative pharmacokinetic (PK) profile-FVII 3, 4 and 5

CTPs. FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 (Group A, B and C, respectively) were administered in a single intravenous injection to Sprague Dawley rats (six rats per treatment) in a dose of 250 µg/kg body weight. Blood samples were drawn retro-orbitally from 3 rats alternately at 0.083, 0.5 2, 5, 8, 24, 48, 72 and 96 hours post dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis. FVII-CTP_5 demonstrated a superior profile as compared to the two other versions.

[068] **Figure 24.** Shows a second comparative PK profile-FVII 3, 4 and 5 CTPs. FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 following FVII selection and the HA purification process (Group A, B and C, respectively) were administered in a single intravenous injection to Sprague Dawley rats (three rats per substance) in a dose of 29.45 µg/kg body weight. Blood samples were drawn retro-orbital at 0.083, 0.5 2, 8, 24, 48, and 72 hours post-dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis.

[069] **Figure 25A**. Shows a schematic diagram of FVII-CTP_3 purification process. Batch 31 was produced for the PK/PD study.

[070] **Figure 25B**. Shows a schematic diagram of FVII-CTP_3 purification process. Batch 38 was produced for the survival study.

[071] **Figure 26A**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 10 µg (Batch 31) or 5 µg (Batch 38) were loaded in each lane of Coomassie stained SDS-PAGE. 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain. All three antibodies detect FVII.

[072] **Figure 26B**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 10 µg (Batch 31) or 5 µg (Batch 38) were loaded in each lane of Coomassie stained SDS-PAGE 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain.

[073] **Figure 26C**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 10 µg (Batch 31) or 5 µg (Batch 38) were loaded in each lane of Coomassie stained SDS-PAGE 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain.

[074] **Figure 26D**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 10 µg (Batch 31) or 5 µg (Batch 38) were loaded in each lane of Coomassie stained SDS-PAGE 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain.

[075] **Figure 26E**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 10 µg (Batch 31) or 5 µg (Batch 38) were loaded in each lane of Coomassie stained SDS-PAGE 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain.

[076] **Figure 26F**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa. 1 µg protein was loaded in each lane of Western blot. 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain. All three antibodies detect FVII. FVIIa light chain is

detected with both α-FVII.

[077] **Figure 26G**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa**.** 1 µg protein was loaded in each lane of Western blot. 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain. All three antibodies detect FVII. FVIIa heavy chain was detected by α-CTP.

[078] **Figure 26H**. Shows an SDS -PAGE and Western blot of Final FVII and FVIIa**.** 1 µg protein was loaded in each lane of Western blot. 1. FVII-CTP_3 polypeptide; 2. Heavy chain, including 3x CTP; 3. Light Chain. All three antibodies detect FVII. FVIIa heavy chain was detected by α-Gla.

[079] **Figure 27**. Shows that FVII-CTP_3 chromogenic activity is enhanced as a result of purification on ceramic hydroxyapatite (HA) column. A comparative assessment of the *in vitro* potency of FVII-CTP_3 harvest, in-process fractions, and purified FVII-CTP_3 versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221304). FVII-CTP_3 harvest and protein were serially diluted and the potency was assessed by comparing a dose-response curve to a reference preparation of normal human plasma.

[080] **Figure 28.** Shows the PK profile of FVIIa-CTP_3 vs. NovoSeven® in FVIII-deficient mice**.** FVIIa-CTP_3 was produced following **FVII** selection, HA purification process and activation. FVIIa-CTP_3 or NovoSeven® was administered in a single intravenous injection to FVIII-/- hemophilic mice. Blood samples were drawn retro-orbitally at 0.083, 0.5 2, 8, 24, 48, and 72 hours post-dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis, and a PK profile was established based on FVIIa clotting activity using a STACLOT commercial kit.

[081] **Figure 29A**. Shows that FVIIa-CTP_3 was produced following FVII selection, HA purification process and activation. FVIIa-CTP_3 or NovoSeven® was administered in a single intravenous injection to FVIII-/- hemophilic mice. Blood samples were drawn retro-orbitally at 0.083, 0.5 2, 8, 24, 48, and 72 hours post-dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis. Thrombin generation parameters were evaluated during the PK experiment, and parameters including maximal amount to peak was evaluated.

[082] **Figure 29B**. Shows that FVIIa-CTP_3 was produced following FVII selection, HA purification process and activation. FVIIa-CTP_3 or NovoSeven® was administered in a single intravenous injection to FVIII-/- hemophilic mice. Blood samples were drawn retro-orbitally at 0.083, 0.5 2, 8, 24, 48, and 72 hours post-dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis. Thrombin generation

parameters were evaluated during the PK experiment, and parameters including amount of thrombin to time point was evaluated.

[083] **Figure 29C**. Shows that FVIIa-CTP_3 was produced following FVII selection, HA purification process and activation. FVIIa-CTP_3 or NovoSeven® was administered in a single intravenous injection to FVIII-/- hemophilic mice. Blood samples were drawn retro-orbitally at 0.083, 0.5 2, 8, 24, 48, and 72 hours post-dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20°C until analysis. Thrombin generation parameters were evaluated during the PK experiment, and parameters including rate of thrombin generation was evaluated.

[084] **Figure 30A.** Shows hemophilic mice survival curves post tail vain transection (TVT). TVT was performed 15 min post administration. Mice Survival was observed for 24 hours after TVT and recorded every single hour for the first 12 hours, and after 24 hours. Control group data (vehicle) is the sum of the 3 experiments with 5 mice/experiment.

[085] **Figure 30B.** Shows hemophilic mice survival curves post tail vain transection (TVT). TVT was performed 24 hours post administration. Mice Survival was observed for 24 hours after TVT and recorded every single hour for the first 12 hours, and after 24 hours. Control group data (vehicle) is the sum of the 3 experiments with 5 mice/experiment.

[086] **Figure 30C.** Shows hemophilic mice survival curves post tail vain transection (TVT). TVT was performed 48 hours post administration. Mice Survival was observed for 24 hours after TVT and recorded every single hour for the first 12 hours, and after 24 hours. Control group data (vehicle) is the sum of the 3 experiments with 5 mice/experiment.

[087] **Figure 30D.** Summarizes mouse survival as recorded 24 hours post TVT.

[088] **Figure 31A.** Shows FVII – 3- CTP and FVII- 5 CTP immune-blots, blotted for GLA.

[089] **Figure 31B.** Shows FVII – 3- CTP and FVII- 5 CTP immune-blots, blotted for FVII.

[090] **Figure 31C.** Shows FVII – 3- CTP and FVII- 5 CTP immune-blots, blotted for CTP.

[091] **Figure 32.** Shows a comparative PK profile-FVII 3 & 5 CTP- from select and HA column purification (FVIIS vs. FVII HA).

[092] **Figure 33.** Shows a comparative PK profile-FVII 3 & 5 CTP-The second study (IV vs. SC).

[093] **Figure 34.** Shows hemophilic mice survival curves post tail vain transection (TVT) following SC administration. TVT was performed 12 hours post administration. Mice Survival was observed for 24 hours after TVT and recorded every single hour for the first 12 hours, and after 24 hours.

[094] **Figure 35A.** Shows the PK profile of MOD-5014 vs. NovoSeven® following IV administration.

[095] **Figure 35B.** Shows the PK profile of MOD-5014 vs. NovoSeven® following SC administration.

[096] **Figure 36.** Shows the PK profile of MOD-5014 (Clone 61 #75, #81) vs. NovoSeven® following single SC administration.

[097] **Figure 37.** Shows that warfarin increases PT and aPTT values. SD-rats received 10 mg/Kg warfarin per-os, and blood samples were taken at the designated time point. Plasma was prepared and PT and aPTT values were determined.

[098] **Figure 38.** Acute effect of IV injection of MOD-5014 and NovoSeven® on Warfarin treated rats.

[099] **Figure 39.** Shows the response of Warfarin treated rats to a wide range of MOD-5014 and NovoSeven® doses, 24 hours post injection.

[0100] **Figure 40.** Shows that MOD-5014 restored PT values to normal up to 48 hours post dosing, while the effect of NovoSeven® no longer exists after 24 hours.

[0101] **Figure 41.** Shows IV injection of MOD-5014 reduce bleeding time in warfarin treated rats as compared to NovoSeven® 24 and 48 hours post injection.

[0102] **Figure 42.** Shows that MOD-5014 is able to restore PT values to normal up to 48 hours post dosing, while the effect of NovoSeven® no longer exists after 24 hours

[0103] **Figure. 43.** Shows superiority over NovoSeven® by keeping the blood loss at low level for 48 hours after administration.

[0104] **Figure 44.** Shows that Tissue Factor Pathway Inhibitor (TFPI) inhibits MOD-5014 and NovoSeven® in a similar dose-dependent manner.

[0105] **Figure 45.** Shows that anti-thrombin III inhibited MOD-5014 and NovoSeven® in a similar manner.

[0106] **Figure 46.** Shows the results of Factor X activation by MOD-5014 and NovoSeven®, which were almost identical.

[0107] **Figure 47.** Shows the Factor X activation in the presence of TFPI (20 µg/ml to 0.002ng/ml) by MOD-5014 and NovoSeven®, present at 0.6 ng/ml.

[0108] **Figure 48.** Shows the Factor X activation in the presence of TFPI (20 µg/ml to 0.002ng/ml) by MOD-5014 and NovoSeven®, present at 4.0 ng/ml.

[0109] **Figure 49.** Shows Factor X activation in the presence of TFPI and Heparin, wherein MOD-5014 and NovoSeven® exhibited similar activation.

[0110] **Figure 50.** Shows Factor X activation in the presence of anti-thrombin III, wherein MOD-5014 and NovoSeven® exhibited similar activation.

[0111] **Figure 51.** Shows the Factor X activation by MOD-5014 and NovoSeven® in the presence of Heparin, wherein similar moderate inhibition was observed.

[0112] **Figure 52.** Shows similar Factor X activation by MOD-5014 and NovoSeven® in the presence of anti-thrombin and heparin.

[0113] **Figure 53.** Shows a MOD-5014 thrombin generation profile as compared to commercially available NovoSeven® at high phospholipid (PL) concentration.

[0114] **Figure 54.** Shows a MOD-5014 peak thrombin generation profile as compared to commercially available NovoSeven® at low phospholipid (PL) concentration.

[0115] **Figures 55A and B.** Show the thromboelastography results for MOD-5014 and NovoSeven®, wherein both decreased clotting time and increased the rate of clot formation.

[0116] **Figure 56.** Shows the results of NovoSeven® thrombin generation (TG) following re-calcification. (Run #1)

[0117] **Figure 57.** Shows the results of MOD-5014 thrombin generation (TG) following re-calcification. (Run #1)

[0118] **Figure 58 A-E.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) TG results at similar concentrations. **(A)** provides results at 1.25 µg/ml. **(B)** provides results at 5 µg/ml. **(C)** provides results at 15 µg/ml. **(D)** provides results at 2.5 µg/ml. **(E)** provides results at 10 µg/ml. (Run #1)

[0119] **Figure 59 A-D.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) TG results at different concentrations. **(A)** shows results of NS at 1.25 µg/ml and PRO at 2.5 µg/ml. **(B)** shows results of NS at 5 µg/ml and PRO at 10 µg/ml **(C)** shows results of NS at 2.5 µg/ml and PRO at 5 µg/ml. **(D)** shows results of NS at 10 µg/ml and PRO at 15 µg/ml. (Run #1)

[0120] **Figure 60.** Shows the results of NovoSeven® (NS) thrombin generation (TG) following re-calcification for NS at different concentrations. (Run #2)

[0121] **Figure 61.** Shows the results of MOD-5014 (PRO) thrombin generation (TG) following re-calcification for PRO at different concentrations. (Run #2)

[0122] **Figure 62 A-E.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) TG results at similar concentrations. **(A)** provides results at 1.25 µg/ml. **(B)** provides results at 5 µg/ml. **(C)** provides results at 15 µg/ml. **(D)** provides results at 2.5 µg/ml. **(E)** provides results at 10 µg/ml. (Run #2)

[0123] **Figure 63 A-D.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) TG results at different concentrations. **(A)** shows results of NS at 1.25 µg/ml and PRO at 2.5 µg/ml. **(B)** shows results of NS at 5 µg/ml and PRO at 10 µg/ml. **(C)** shows results of NS at 2.5 µg/ml and PRO at 5 µg/ml. **(D)** shows results of NS at 10 µg/ml and PRO at 15 µg/ml. (Run #2)

[0124] **Figure 64.** Shows data for TG by NovoSeven® following re-calcification at low TF

concentration. (Run #1)

[0125] **Figure 65.** Shows data for TG by MOD-5014 following re-calcification at low TF concentration. (Run #1)

[0126] **Figure 66 A-E.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) results at similar concentrations, following re-calcification at low TF. **(A)** provides results at 1.25 µg/ml. **(B)** provides results at 5 µg/ml. **(C)** provides results at 15 µg/ml. **(D)** provides results at 2.5 µg/ml. **(E)** provides results at 10 µg/ml. (Run #1)

[0127] **Figure 67.** Shows data for TG by NovoSeven® following re-calcification at low TF concentration. (Run #2)

[0128] **Figure 68.** Shows data for TG by MOD-5014 following re-calcification at low TF concentration. (Run #2)

[0129] **Figure 69 A-E.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) results at similar concentrations, following re-calcification at low TF. **(A)** provides results at 1.25 µg/ml. **(B)** provides results at 5 µg/ml. **(C)** provides results at 15 µg/ml. **(D)** provides results at 2.5 µg/ml. **(E)** provides results at 10 µg/ml. (Run #2)

[0130] **Figure 70 A-C.** Provide an overlay analysis of NovoSeven® (NS) results versus MOD-5014 (PRO) results at different concentrations, following re-calcification at low TF. **(A)** shows results of NS at 1.25 µg/ml and PRO at 5 µg/ml. **(B)** shows results of NS at 5 µg/ml and PRO at 15 µg/ml. **(C)** shows results of NS at 2.5 µg/ml and PRO at 10 µg/ml. (Run #2)

[0131] **Figure 71.** Shows complete thrombin generation by FVIII, as a comparison for the results with NovoSeven® and MOD-5014 in the presence and absence of low TF.

[0132] **Figure 72.** Provides an overlay analysis of thrombin generation by FVIII in the presence and absence of low TF.

[0133] **Figure 73.** Provides an overlay analysis of NovoSeven® (NS; 1.25 µg/kg) TG results versus MOD-5014 (PRO; 1.25 µg/kg) TG results at escalating concentrations of TF. (Run #1)

[0134] **Figure 74 A-E.** Provide an overlay analysis of NovoSeven® (NS; 1.25 µg/kg) TG results versus MOD-5014 (PRO; 1.25 µg/kg) TG results at escalating concentrations of TF. **(A)** provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM TF. **(D)** provides results at 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #1)

[0135] **Figure 75.** Provides an overlay analysis of NovoSeven® (NS; 2.5 µg/kg) TG results versus MOD-5014 (PRO; 2.5 µg/kg) TG results at escalating concentrations of TF. (Run #1)

[0136] **Figure 76 A-E.** Provide an overlay analysis of NovoSeven® (NS; 2.5 µg/kg) TG results versus MOD-5014 (PRO; 2.5 µg/kg) results at escalating concentrations of TF. **(A)** provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM

TF. **(D)** provides results 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #1)

[0137] **Figure 77.** Provides an overlay analysis of NovoSeven® (NS; 5 µg/kg) TG results versus MOD-5014 (PRO; 5 µg/kg) results at escalating concentrations of TF. (Run #1)

[0138] **Figure 78 A-E.** Provide an overlay analysis of NovoSeven® (NS; 5 µg/kg) TG results versus MOD-5014 (PRO; 5 µg/kg) TG results at escalating concentrations of TF. **(A)** provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM TF. **(D)** provides results 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #1)

[0139] **Figure 79 A-C.** Show a dose dependent TG response in the presence of NovoSeven® (NS) at escalating concentrations of TF. **(A)** provides results at 1.25 µg/kg NS. **(B)** provides results at 5 µg/kg NS. **(C)** provides results at 2.5 µg/kg NS. (Run #1)

[0140] **Figure 80 A-C.** Show a dose dependent TG response in the presence of MOD-5014 (PRO) at escalating concentrations of TF. **(A)** provides results at 1.25 µg/kg PRO. **(B)** provides results at 5 µg/kg PRO. **(C)** provides results at 2.5 µg/kg PRO. (Run #1)

[0141] **Figure 81.** Provides an overlay analysis of NovoSeven® (NS; 10 µg/kg) TG results versus MOD-5014 (PRO; 10 µg/kg) TG results at escalating concentrations of TF. (Run #2)

[0142] **Figure 82.** Provides an overlay analysis of NovoSeven® (NS; 2.5 µg/kg) TG results versus MOD-5014 (PRO; 2.5 µg/kg) TG results at escalating concentrations of TF. (Run #2)

[0143] **Figure 83.** Provides an overlay analysis of NovoSeven® (NS; 5 µg/kg) TG results versus MOD-5014 (PRO; 5 µg/kg) results at escalating concentrations of TF. (Run #2)

[0144] **Figure 84 A-C.** Show a dose dependent TG response in the presence of NovoSeven® (NS) at escalating concentrations of TF. **(A)** provides results at 2.5µg/ml NS. **(B)** provides results at 10 µg/ml NS. **(C)** provides results at 5 µg/ml NS. (Run #2)

[0145] **Figure 85 A-C.** Show a dose dependent TG response in the presence of MOD-5014 (PRO) at escalating concentrations of TF. **(A)** provides results at 2.5µg/ml PRO. **(B)** provides results at 10 µg/ml PRO. **(C)** provides results at 5 µg/ml PRO. (Run #2)

[0146] **Figure 86 A-E.** Provide an overlay analysis of NovoSeven® (NS; 10 µg/kg) TG results versus MOD-5014 (PRO; 10 µg/kg) TG results at escalating concentrations of TF. **(A)** provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM TF. **(D)** provides results 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #2)

[0147] **Figure 87.** Provides an overlay analysis of NovoSeven® (NS; 2.5 µg/kg) TG results versus MOD-5014 (PRO; 2.5µg/kg) TG results at escalating concentrations of TF **(A)** provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM TF. **(D)** provides results 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #2)

[0148] **Figure 88.** Provides an overlay analysis of NovoSeven® (NS; 5µg/kg) TG results versus MOD-5014 (PRO; 5 µg/kg) TG results at escalating concentrations of TF. **(A)**

provides results at 0 pM TF. **(B)** provides results at 1 pM TF. **(C)** provides results at 5 pM TF. **(D)** provides results 0.5 pM TF. **(E)** provides results at 2.5 pM TF. (Run #2)

[0149] **Figure 89.** Shows graphs of WBCT Following MOD-5014 Spiking Canine Blood.

[0150] **Figure 90.** Shows a schematic Drawing of Two-compartment Pharmacokinetic Model.

[0151] **Figure 91.** Provides a graph of mean Plasma MOD-5014 Concentrations vs Time Following IV Infusion in Dogs.

[0152] **Figure 92.** Provides a graph of mean Plasma MOD-5014 Activity vs Time Following IV Infusion in Dogs.

[0153] **Figures 93A-B.** Show comparison data of MOD-5014 Plasma Concentration and Activity Following IV Infusion of 50μg/kg in Dogs. **Figure 93(A)** shows the results for dog P14 and **Figure 93(B)** shows the results for dog N06.

[0154] **Figures 94A-D.** Show comparison data of MOD-5014 Plasma Concentration and Activity Following IV Infusion of 200μg/kg in Dogs. **Figure 94(A)** shows the results for dog Blondie; **Figure 94(B)** for dog Josie; **Figure 94(C)** for dog N06; and **Figure 94(D)** for dog P14.

[0155] **Figures 95A-B.** Show comparison data of MOD-5014 Plasma Concentration and Activity Following IV Infusion of 400μg/kg in Dogs. **Figure 95(A)** shows the results for dog Blondie and **Figure 95(B)** shows the results for dog Josie.

[0156] **Figures 96A-B.** Show comparison data of MOD-5014 Plasma Concentration and Activity Following IV Infusion of 600μg/kg in Dogs. **Figure 96(A)** shows the results for dog N05 and **Figure 96(B)** shows the results for dog Joanie.

[0157] **Figures 97A-J.** Show plots of Plasma MOD-5014 Concentrations versus Time. Points represent observed plasma concentrations and the line represents the terminal slope used to calculate $T_{1/2}$. **Figure 97(A)** shows the results for dog N06 after more than 30 hours; **Figure 97(B)** for dog P14 after more than 30 hours; **Figure 97(C)** for dog Blondie after about 50 hours; **Figure 97(D)** for dog Josie after about 50 hours; **Figure 97(E)** for dog N06 after almost 100 hours; **Figure 97(F)** for dog P14 after almost 100 hours; **Figure 97(G)** for dog Blondie after almost 100 hours; **Figure 97(H)** for dog Josie after almost 100 hours; **Figure 97(I)** for dog Joanie after almost 100 hours and **Figure 97(J)** for dog N05 after almost 100 hours.

[0158] **Figures 98A-J.** Show plots of plasma MOD-5014 Activity versus Time. Points represent observed plasma concentrations and the line represents the terminal slope used to calculate $T_{1/2}$. **Figure 98(A)** shows the results for dog N06 after more than 30 hours; **Figure 98(B)** for dog P14 after more than 30 hours; **Figure 98(C)** for dog Blondie after almost 50

hours; **Figure 98(D)** for dog Josie after almost 50 hours; **Figure 98(E)** for dog N06 after almost 50 hours; **Figure 98(F)** for dog P14 after almost 50 hours; **Figure 98(G)** for dog Blondie after almost 50 hours; **Figure 98(H)** for dog Josie after almost 50 hours; **Figure 98(I)** for dog Joanie after almost 50 hours and **Figure 98(J)** for dog N05 after almost 50 hours.

[0159] **Figures 99A-J.** Show the results of modeling – Plasma Concentrations. Points represent observed plasma concentrations and the solid line represents concentrations predicted by model. **Figure 99(A)** shows the observed and predicted results for dog N06; **Figure 99(B)** for dog P14; **Figure 99(C)** for dog Blondie; **Figure 99(D)** for dog Josie; **Figure 99(E)** for dog N06 after almost 100 hours; **Figure 99(F)** for dog P14 after almost 100 hours; **Figure 99(G)** for dog Blondie after almost 100 hours; **Figure 99(H)** for dog Josie after almost 100 hours; **Figure 99(I)** for dog Joanie after almost 100 hours and **Figure 99(J)** for dog N05 after almost 100 hours.

[0160] **Figures 100A-J.**Show the results of modeling – Activity. Points represent observed plasma activity and the solid line represents activity predicted by model. **Figure 100(A)** shows the observed and predicted results for dog N06 after at least 32 hours; **Figure 100(B)** for dog P14 after at least 32 hours; **Figure 100(C)** for dog Blondie after at least 48 hours; **Figure 100(D)** for dog Josie after at least 48 hours; **Figure 100(E)** for dog N06 after at least 48 hours; **Figure 100(F)** for dog P14 after at least 48 hours; **Figure 100(G)** for dog Blondie after at least 48 hours; **Figure 100(H)** for dog Josie after at least 48 hours; **Figure 100(I)** for dog Joanie after at least 48 hours and **Figure 100(J)** for dog N05 after at least 48 hours.

[0161] **Figure 101A-D.** Show the dose-dependent change in kaolin-initiated TEG kinetics following administration of 50, 200 or 400µg/kg MOD-5014 in dog NO6. **(A)** shows the R-time (Reaction Time); **(B)** shows the K-time (time from the end of R until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0162] **Figure 102A-D.** Show the dose-dependent change in kaolin-initiated TEG kinetics following administration of 50 and 200 µg/kg MOD-5014 in dog P-14. **(A)** shows the R-time (Reaction Time; **(B)** shows the K-time (time from the end of R until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0163] **Figure 103A-D.** Show the dose-dependent change in kaolin-initiated TEG kinetics following administration of 200 and 400µg/kg MOD-5014 in dog Blondie. **(A)** shows the R-time (Reaction Time); **(B)** shows the K-time (time from the end of R until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as

the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0164] **Figure 104A-D.** Show the dose-dependent change in kaolin-initiated TEG kinetics following administration of 200 and 400µg/kg MOD-5014 in dog Josie. **(A)** shows the R-time (Reaction Time); **(B)** shows the K-time (time from the end of R until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0165] **Figure 105A-D.** Show the dose-dependent change in kaolin-initiated TEG kinetics following administration of 50, 200 or 400µg/kg MOD-5014 in dog Joanie. **(A)** shows the R-time (Reaction Time); **(B)** shows the K-time (time from the end of R until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0166] **Figure 106A-D.** Show the kinetics of TEG over time following MOD-5014 administration in dog NO5. **(A)** shows the R-time (Reaction Time); **(B)** shows the K-time (time from the end of RT until the clot reaches 20mm, the speed of clot formation); **(C)** shows the Angle (the tangent of the curve made as the K is reached); and **(D)** shows the MA (Maximum Amplitude).

[0167] **Figure 107 A-D.** Show the change in TEG performance following administration of 270µg/kg of rhFVIIa **(A)** shows R-Time (Reaction Time). **(B)** shows Angle (the tangent of the curve made as the K is reached). **(C)** shows K-Time (time from the end of RT until the clot reaches 20 mm, the speed of clot formation). **(D)** shows the MA (Maximum Amplitude).

[0168] **Figure 108A-D.** Show MOD-5014 extended TEG effect, representative data from an individual animal (Blondie). **(A)** shows R-Time (Reaction Time). **(B)** shows K-Time (time from the end of RT until the clot reaches 20 mm, the speed of clot formation). **(C)** shows the Angle (the tangent of the curve made as the K is reached). **(D)** shows the MA (Maximum Amplitude). Arrows in **(B), (C)** and **(D)** are pointing to MOD-5014 TEG values 4 hours post dosing.

[0169] **Figure 109.** Shows map of pCI-dhfr-MOD-5014 plasmid.

[0170] **Figure 110.** Shows combined representative PK-PD Profile of rat toxicology studies, wherein rate of change of plasma concentration (ng/ml) over time is designated by the open squares and rate of change of activity (mU/ml) over time is designated by the open circles.

[0171] **Figure 111.** Shows combined representative PK-PD Profile of monkey toxicology studies, wherein rate of change of plasma concentration (ng/ml) over time is designated by the open circles and rate of change of activity (mU/ml) over time is designated by the open squares.

[0172] **Figure 112**. Mean Plasma MOD-5014 Concentration vs Time Following IV Bolus Administration in Male Cynomolgus Monkeys.

[0173] **Figure 113.** Mean MOD-5014 Clotting Activity vs Time Following IV Bolus Administration in Male Cynomolgus Monkeys.

[0174] **Figure 114.** Comparison of MOD-5014 Plasma Concentration and Clotting Activity Following IV Bolus Injection of 1 mg/kg in Male Cynomolgus Monkeys.

[0175] **Figure 115.** Comparison of MOD-5014 Plasma Concentration and Clotting Activity Following IV Bolus Injection of 7.5 mg/kg in Male Cynomolgus Monkeys.

[0176] **Figure 116.** Comparison of MOD-5014 Plasma Concentration and Clotting Activity Following IV Bolus Injection of 15 mg/kg in Male Cynomolgus Monkeys.

[0177] **Figures 117A-117R.** Individual Plasma MOD-5014 Concentrations and Clotting Activity versus Time.

[0178] **Figure 118**. Mean Fluorescence Intensity of MOD-5014 or FVIIa-treated Platelets.

[0179] **Figure 119**. Thrombin Generation in MOD-5014 or FVIIa-treated Platelets.

[0180] **Figure 120.** Shows a comparison of substrate (Pefachrome FVIIa) cleavage activity between FVIIa (NovoSeven) and CTP-modified factor VIIa (MOD-5014).

[0181] **Figure 121.** Shows a comparison of substrate (Pefachrome FVIIa) activity between FVIIa (NovoSeven) and CTP-modified factor VIIa (MOD-5014) when bound to tissue factor.

[0182] **Figures 122.** Shows a comparison of the generation of activated Factor X by FVIIa (NovoSeven) or CTP-modified FVIIa (MOD-5014), in view of Factor VIIa concentration.

[0183] **Figure 123.** Shows a comparison of the generation of activated Factor X by FVIIa (NovoSeven) or CTP-modified FVIIa (MOD-5014), in view of Factor X concentration.

[0184] **Figures 124A and 124B.** Shows a comparison of the rate of generation of activated Factor X by FVIIa (NovoSeven) or CTP-modified FVIIa (MOD-5014), in the absence of tissue factor and in view of lipid concentration (124A). Show a comparison of the generation of activated Factor X by FVIIa (NovoSeven) or CTP-modified FVIIa (MOD-5014), in the absence of tissue factor and in view of lipid concentration (124B).

[0185] **Figure 125.** Show the a comparison of the generation of activated Factor X between FVIIa (NovoSeven) and MOD-5014, in the absence of tissue factor and in view of Factor X concentration.

[0186] **Figure 126.** Show a comparison of the inhibition of substrate (Pefachrome FVIIa) cleavage by FVIIa (NovoSeven) and CTP-modified FVIIa (MOD-5014) in view of polybrene.

[0187] **Figures 127A-127C.** Show a comparison of the inhibition of substrate (Pefachrome FXa) cleavage by FVIIa (NovoSeven) and CTP-modified FVIIa (MOD-5014) in view of TFPI concentration (Figure 127A) and duration of TFPI exposure for FVIIa (Figure 127B) and MOD-5014 (127C).

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0188] In one embodiment, the present invention provides long-acting coagulation factors and methods of producing and using same. In another embodiment, long-acting coagulation factors comprise a carboxy terminal peptide (CTP, also referred to as CTP unit). In another embodiment, long-acting polypeptides which comprise a coagulation factor further comprise a carboxy terminal peptide (CTP) of human Chorionic Gonadotropin (hCG). In another embodiment, CTP acts as a protectant against the degradation of a coagulation factor. In another embodiment, CTP extends the C_{max} of a coagulation factor. In another embodiment, CTP extends the T_{max} of a coagulation factor. In another embodiment, CTP extends the circulatory half-life of a coagulation factor. In some embodiments, CTP enhances the potency of a coagulation factor.

[0189] In another embodiment, provided herein is a method of extending the biological half-life of a coagulation factor, comprising the step of attaching one to ten CTPs to the carboxy terminus of the coagulation factor, thereby extending the biological half-life of the coagulation factor. In another embodiment, provided herein is a method of extending the biological half-life of a coagulation factor, comprising the step of attaching one to five CTPs to the carboxy terminus of the coagulation factor, thereby extending the biological half-life of the coagulation factor. In another embodiment, the present invention provides a method for extending the circulatory half-life of a coagulation factor. In another embodiment, the present invention provides a method for increasing the half-life of a coagulation factor. In another embodiment, the present invention provides a method for extending the half-life of a coagulation factor.

[0190] In one embodiment, the disclosure relates to a pharmaceutical composition comprising a CTP-modified coagulation factor.

[0191] In one embodiment, the disclosure relates to a pharmaceutical formulation comprising a buffer, a tonicity agent, and a CTP-modified polypeptide consisting of a coagulation factor and three chorionic gonadotropin CTPs attached to the carboxy terminus of said coagulation factor.

[0192] In one embodiment, the disclosure relates to a formulation for a once a week administration to a subject having hemophilia A or B. In another embodiment, the subject has a coagulation factor deficiency. In another embodiment, the subject has acquired hemophilia. In another embodiment, the disclosure relates to a process for making a pharmaceutical formulation for a once a week administration to a subject having a

coagulation factor deficiency, or having a hemophilia, the process comprising the steps of:

a) modifying a coagulation factor by attaching three chorionic gonadotropin CTPs to the carboxy terminus of said coagulation factor;
b) mixing the modified coagulation factor in step a. with said buffer, and said tonicity agent at a pH of about 6.4; and,
c) pre-filling a syringe with said formulation.

[0193] In one embodiment, the disclosure relates to a process for filling a syringe with a formulation provided herein comprising the steps of:

a) formulating a once a week dosage form of said CTP-modified coagulation factor having a pre-determined amount of CTP-modified coagulation factor; and
b) filling the syringe with said formulation.

[0194] In one embodiment, a "pharmaceutical composition" or a "pharmaceutical formulation" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition or a "pharmaceutical formulation" is to facilitate administration of a compound to an organism. In certain embodiments, a "pharmaceutical composition" or a "pharmaceutical formulation" provides the pharmaceutical dosage form of a drug. "Pharmaceutical compositions" or "pharmaceutical formulations" in certain embodiments include slow release technologies, transdermal patches, or any known dosage form in the art.

[0195] Coagulation Factor VII (FVII) is a 444 amino acid glycoprotein (50KDa) secreted by hepatocytes into the bloodstream as an inactive pro-enzyme. Upon tissue injury and exposure to circulating blood, FVII forms a complex with Tissue Factor (TF) which is a true receptor protein to FVII and is expressed by various cells localized in the deeper layers of the vessel wall. The formation of this FVII-TF complex leads to activation of FVII. Activated FVII (FVIIa) initiates the extrinsic coagulation pathway by activating Factor IX and Factor X.

[0196] FVII belong to a group of Vitamin K-dependent glycoproteins associated with the coagulation system. Besides FVII, this group consists of Factor IX, Factor X, Protein C and prothrombin. These proteins have similar domain organizations and are synthesized as precursors with an N-terminal propeptide followed by a mature amino acid sequence. The propeptide contains a docking site for gammacarboxylase which converts glutamic acids (Glu) into gamma carboxy glutamic acids (Gla). This domain is followed by two epidermal growth factor-like (EGF) domains, a connecting region (CR) and a C-terminal serine protease domain. Prior to secretion, FVII propeptide is cleaved forming a 406 amino acid single chain zymogen FVII glycoprotein. After secretion, the protein can be activated into a disulfide-

linked two chain heterodimer, FVIIa, by cleavage in the CR. The plasma concentration of FVII is 10 nM and approximately 1% circulates in the active form in healthy individuals.In other embodiments, a CTP-modified FVII comprises occupancy of at least one O-linked glycosylation site. In other embodiments, a CTP-modified FVIIa comprises occupancy of at least one O-linked glycosylation site.

[0197] Factor IX (FIX) is a 415 Amino acid (55KDa) glycoprotein; it belongs to a group of vitamin K dependent glycoproteins associated with the coagulation system. FIX has a similar domain organization as factor FVII, Factor X, Protein C and prothrombin that are synthesized as precursors with an N-terminal propeptide followed by a mature amino acid sequence.

[0198] FIX is secreted as a single chain molecule that undergoes complex post-transcriptional modifications, many of which are critical to its biochemical and pharmacokinetic properties. Among all the post-transcriptional modifications, 12 glutamic acid residues near the amino terminus of FIX that are gamma carboxylated by the vitamin K-dependent gamma carboxylase are the most crucial ones. Carboxylation is required for the interaction of FIX with the phospholipid surfaces and for optimal FIX activity. The amino terminus propeptide serves as a recognition site for the gamma carboxylase and thus, following gamma carboxylation, it is cleaved off by the Golgi apparatus serine protease known as Paired basic Amino acid Cleave Enzyme (PACE/Furin). Four additional post-transcriptional modifications might occur at the Golgi apparatus: sulfation of tyrosine 155, phosphorylation of serine 158, O- glycosylation on Ser 63 and on 61 and finally, N-glycosylation on Asn 157 and 16, but were shown not to be necessary for proper activity of FIX.

[0199] FIX circulates in the plasma (average concentration of 5 µg/ml) as a single chain inactive zymogen. Upon proteolytic cleavage at two peptide bonds: Arg 145 and Arg 180 by either one or two physiological activators, FVIIa-TF complex or FIXa, the activation peptide is removed, converting FIX to a fully active enzyme consisting of a light and heavy chain held together by a single disulfide bond. The N-terminal light chain contains the non-catalytic gamma carboxyglutamic acid (Gla) and two epidermal growth factor-like domains, while the C-terminal heavy chain contains the trypsin-like catalytic domain of the molecule. FIXa alone is characterized by poor catalytic activity. However when complexed with FVIII, its proteolytic activity increase by 4-5 orders of magnitude towards its natural substrate FX.

[0200] In another embodiment, provided herein is a method of extending the biological half-life or a method of improving the area under the curve (AUC) of a coagulation factor, comprising the step of attaching one to ten CTPs to the carboxy terminus of the coagulation factor, thereby extending the biological half-life or improving the AUC of the coagulation factor. In another embodiment, provided herein is a method of extending the biological half-

life or a method of improving the area under the curve (AUC) of a coagulation factor, comprising the step of attaching one to five CTPs to the carboxy terminus of the coagulation factor, thereby extending the biological half-life or improving the AUC of the coagulation factor. In another embodiment, provided herein is a method of extending the biological half-life or a method of improving the area under the curve (AUC) of FIX, comprising the step of attaching one to five CTPs to the carboxy terminus of the FIX, thereby extending the biological half-life or improving the AUC of the FIX. In another embodiment, provided herein is a method of extending the biological half-life or a method of improving the area under the curve (AUC) of FVII or FVIIa, comprising the step of attaching one to five CTPs to the carboxy terminus of FVII or FVIIa, thereby extending the biological half-life or improving the AUC of FVII or FVIIa.

[0201] In another embodiment, the present invention provides a method of extending the biological half-life of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby extending the biological half-life of said FIX polypeptide. In another embodiment, the present invention further provides a method of extending the biological half-life of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching from one and up to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby extending the biological half-life of said FVIIa polypeptide. In one embodiment, three chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said FVIIa polypeptide. In another embodiment, four chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said FVIIa polypeptide. In another embodiment, five chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said FVIIa polypeptide.

[0202] In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby improving the AUC of said FIX polypeptide. In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching up to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby improving the AUC of said FVIIa polypeptide. In one embodiment, three chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said FVIIa polypeptide. In another embodiment, four chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said

FVIIa polypeptide. In another embodiment, five chorionic gonadotropin carboxy terminal peptides (CTPs) are attached to the carboxy terminus of said FVIIa polypeptide.

[0203] In another embodiment, a coagulation factor of the disclosure is a protein. In another embodiment, a coagulation factor of the disclosure is a peptide. In another embodiment, a coagulation factor of the disclosure is a polypeptide. In another embodiment, the coagulation factor is an enzyme. In another embodiment, the coagulation factor is a serine protease. In another embodiment, the coagulation factor is a glycoprotein. In another embodiment, the coagulation factor is a transglutaminase. In another embodiment, the coagulation factor is an inactive zymogen. In another embodiment, the coagulation factor is any coagulation factor known to one of skill in the art.

[0204] In another embodiment, the coagulation factor is Factor VIII (FVIII). In another embodiment, the coagulation factor is Factor V (FV). In another embodiment, the coagulation factor is Factor XIII (FXIII). In another embodiment, the coagulation factor is Factor X (FX). In another embodiment, the coagulation factor is fibrin.

[0205] In another embodiment, the coagulation factor is Factor VIIa (FVIIa). In another embodiment, the coagulation factor is Factor VII (FVII). In another embodiment, the coagulation factor is Factor IX (FIX). In another embodiment, the coagulation factor is Factor X (FX). In another embodiment, the coagulation factor is Factor XIa (FXIa). In another embodiment, the coagulation factor is Factor XII (FXII). In another embodiment, the coagulation factor is Factor Xa (FXa). In another embodiment, the coagulation factor is Factor Va (FVa). In another embodiment, the coagulation factor is prothrombin. In another embodiment, the coagulation factor is thrombin. In another embodiment, the coagulation factor is Factor XI (FXI). In another embodiment, the coagulation factor is Von Willebrand factor (vWF). In another embodiment, the coagulation factor is Factor VIIIa (FVIIIa). In another embodiment, the coagulation factor is B-deleted Domain FVIII (FVIIIBDD). In another embodiment, the coagulation factor is B domain-deleted FVIII (FVIIIBDD). In another embodiment, the coagulation factor is Beta domain-deleted FVIII (FVIIIBDD). In another embodiment, the coagulation factor is Factor IXa (FIXa). In another embodiment, the coagulation factor is prekallikrein. In another embodiment, the coagulation factor is kallikrein. In another embodiment, the coagulation factor is Factor XIIa (FXIIa). In another embodiment, the coagulation factor is Fibrinogen. In another embodiment, the coagulation factor is thrombomodulin. In another embodiment, the coagulation factor is Factor II (FII).

[0206] In another embodiment, the coagulation factor is a glycoprotein. In another embodiment, the coagulation factor is a vitamin K-dependent glycoprotein. In another embodiment, the coagulation factor is a vitamin K-independent glycoprotein.

[0207] In another embodiment, the coagulation factor is a recombinant protein. In another embodiment, the coagulation factor is a recombinant glycoprotein. In another embodiment, the coagulation factor is a recombinant glycoprotein FV. In another embodiment, the coagulation factor is a recombinant FVI. In another embodiment, the coagulation factor is a recombinant FVII. In another embodiment, the coagulation factor is a recombinant FVIII. In another embodiment, the coagulation factor is a recombinant FIX. In another embodiment, the coagulation factor is a recombinant FX. In another embodiment, the coagulation factor is a recombinant FXI. In another embodiment, the coagulation factor is a recombinant FXII. In another embodiment, the coagulation factor is a recombinant FvW. In another embodiment, the coagulation factor is a recombinant FII. In another embodiment, the coagulation factor is a recombinant FIXa. In another embodiment, the coagulation factor is a recombinant FXIa. In another embodiment, the coagulation factor is a recombinant fibrin. In another embodiment, the coagulation factor is a recombinant FVIIa. In another embodiment, the coagulation factor is a recombinant FXa. In another embodiment, the coagulation factor is a recombinant FVa. In another embodiment, the coagulation factor is a recombinant prothrombin. In another embodiment, the coagulation factor is a recombinant thrombin. In another embodiment, the coagulation factor is a recombinant FVIIIa. In another embodiment, the coagulation factor is a recombinant prekallikrein. In another embodiment, the coagulation factor is a recombinant kallikrein. In another embodiment, the coagulation factor is a recombinant FXIIa. In another embodiment, the coagulation factor is any known recombinant coagulation factor. In another embodiment, the coagulation factor comprising a signal peptide is any known recombinant coagulation factor. In another embodiment, a recombinant coagulation factor does not comprise a signal peptide. In another embodiment, an activated coagulation factor does not comprise a signal peptide.

[0208] In another embodiment, a coagulation factor comprises 1-10 CTP repeats attached to the C-terminus and no CTPs attached to the N-terminus. In another embodiment, a coagulation factor comprises at least one CTP attached to the C-terminus and no CTPs attached to the N-terminus. In another embodiment, a coagulation factor comprising 1-10 CTP repeats attached to the C-terminus and no CTPs attached to the N-terminus is an engineered coagulation factor. In another embodiment, a coagulation factor comprising at least one CTP attached to the C-terminus and no CTPs attached to the N-terminus is an engineered coagulation factor. In another embodiment, a coagulation factor comprising 1-10 CTP repeats attached to the C-terminus and no CTPs attached to the N-terminus is a conjugated coagulation factor. In another embodiment, a coagulation factor comprising at least one CTP attached to the C-terminus and no CTPs attached to the N-terminus is a

conjugated coagulation factor.

[0209] In one embodiment, the present invention provides a CTP-modified Factor IX (FIX) polypeptide consisting of a FIX polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said CTP-modified FIX polypeptide.

[0210] In another embodiment, the present invention further provides a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa. In another embodiment, the present invention further provides a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0211] In another embodiment, the coagulation factor is a coagulation factor comprising a domain organization similar or identical to the domain organization of FIX, FVII, Factor X, Protein C, or prothrombin. In another embodiment, the coagulation factor is synthesized as a precursor with an N-terminal propeptide. In another embodiment, the coagulation factor as used herein is in an inactive pro-enzyme form. In another embodiment, the coagulation factor is produced in hepatocytes. In another embodiment, the coagulation factor comprises a docking site for gammacarboxylase which converts glutamic acids (Glu) into gamma carboxy glutamic acids (Gla). In another embodiment, the coagulation factor as used herein is a commercially available coagulation factor.

[0212] In one embodiment, the nucleic acid sequence encoding Factor VII comprises the following nucleic acid sequence:

ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga
ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt
gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg
atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc
ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga
ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata
tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg
ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg
cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga
gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg
caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt
ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac
ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag
caaggactcctgcaaggggacagtggaggcccacatgccacccactaccggggcacgtggtacctgacgggcatcgtcagctgg

ggccagggctgcgcaaccgtgggccactttggggtgtacaccagggtctcccagtacatcgagtggctgcaaaagctcatgcgctca
gagccacgcccaggagtcctcctgcgagccccatttccctgaggatgcggccgc (SEQ ID NO: 11).

[0213] In another embodiment, the amino acid sequence of Factor VII comprises the following amino acid sequence:
MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECK
EEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFE
GRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEY
PCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVS
AAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRL
HQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRL
MTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGI
VSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFP (SEQ ID NO: 9).

[0214] In another embodiment, the amino acid sequence of Factor VII comprises the following amino acid sequence:
MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECK
EEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFE
GRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEY
PCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVS
AAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRL
HQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRL
MTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGI
VSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFP*GCGR (SEQ ID
NO: 10).

[0215] In another embodiment, the nucleic acid sequence encoding Factor VII-CTP (attached to the carboxy terminus) comprises the following nucleic acid sequence:
ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga
ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt
gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg
atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc
ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga
ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata
tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg
ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg
cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga
gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg

caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt
ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac
ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag
caaggactcctgcaagggggacagtggaggcccacatgccacccactaccggggcacgtggtacc
tgaccggcatcgtgagctggggccagggctgcgccaccgtgggccacttcggcgtgtacaccagggtgtcccagtacatcgagtgg
ctgcagaaactgatgagaagcgagcccagacccggcgtgctgctgagagccccttccccagcagcagctccaaggcccctcccc
ctagcctgcccagccctagcagactgcctgggcccagcgacacccccatcctgccccagtgaggatccgcggccgc (SEQ ID NO: 12).

[0216] In another embodiment, the amino acid sequence of Factor VII-CTP (attached to the carboxy terminus) comprises the following amino acid sequence: MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECK EEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFE GRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEY PCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVS AAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRL HQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRL MTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGI VSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFPSSSSKAPPPSLPS PSRLPGPSDTPILPQ* (SEQ ID NO: 13).

[0217] In another embodiment, the nucleic acid sequence encoding Factor VII-CTP-CTP (attached to the carboxy terminus) comprises the following nucleic acid sequence: ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag caaggactcctgcaagggggacagtggaggcccacatgccacccactaccggggcacgtggtacctgaccggcatcgtgagctgg

ggccagggctgcgccaccgtgggccacttcggcgtgtacaccagggtgtcccagtacatcgagtggctgcagaaactgatgagaag
cgagcccagacccggcgtgctgctgagagccccccttccccagcagcagctccaaggcccctcccccctagcctgcccagccctagca
gactgcctgggccctccgacacaccaatcctgccacagagcagctcctctaaggcccctcctccatccctgccatcccccctcccggct
gccaggcccctctgacacccctatcctgcctcagtgatgaaggtctggatccgcggccgc (SEQ ID NO: 14).

[0218] In another embodiment, the amino acid sequence of Factor VII-CTP-CTP (attached to the carboxy terminus) comprises the following amino acid sequence: MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECK EEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFE GRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEY PCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVS AAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRL HQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRL MTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGI VSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFPSSSSKAPPPSLPS PSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQ** (SEQ ID NO: 15).

[0219] In another embodiment, the nucleic acid sequence encoding Factor VII-CTP-CTP-CTP (attached to the carboxy terminus) comprises the following nucleic acid sequence: ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag caaggactcctgcaaggggacagtggaggcccacatgccacccactaccggggcacgtggtacctgaccggcatcgtgagctgg ggccagggctgcgccaccgtgggccacttcggcgtgtacaccagggtgtcccagtacatcgagtggctgcagaaactgatgagaag cgagcccagacccggcgtgctgctgagagccccccttccccagcagcagctccaaggcccctcccccctagcctgcccagccctagca gactgcctgggcccagtgacacccctatcctgcctcagtccagctccagcaaggccccacccccctagcctgccttctccttctcggctg cctggccccagcgatactccaattctgccccagtcctccagcagtaaggctccccctccatctctgccatcccccagcagactgccag

gcccttctgatacacccatcctcccacagtgatgaggatccgcggccgcttaattaa (SEQ ID NO: 24).

[0220] In another embodiment, the amino acid sequence of Factor VII-CTP-CTP-CTP (attached to the carboxy terminus) comprises the following amino acid sequence: MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECK EEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFE GRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEY PCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVS AAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRL HQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRL MTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGI VSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFPSSSSKAPPPSLPS PSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGP SDTPILPQ (SEQ ID NO: 25). In another embodiment, amino acids 1-38 of SEQ ID NO: 25 comprise a signal sequence.

[0221] In another embodiment, the amino acid sequence of Factor VII-CTP-CTP-CTP (attached to the carboxy terminus) lacking a signal peptide comprises the following amino acid sequence: ANAFLEELRP GSLERECKEE QCSFEEAREI FKDAERTKLF WISYSDGDQC ASSPCQNGGS CKDQLQSYIC FCLPAFEGRN CETHKDDQLI CVNENGGCEQ YCSDHTGTKR SCRCHEGYSL LADGVSCTPT VEYPCGKIPI LEKRNASKPQ GRIVGGKVCP KGECPWQVLL LVNGAQLCGG TLINTIWVVS AAHCFDKIKN WRNLIAVLGE HDLSEHDGDE QSRRVAQVII PSTYVPGTTN HDIALLRLHQ PVVLTDHVVP LCLPERTFSE RTLAFVRFSL VSGWGQLLDR GATALELMVL NVPRLMTQDC LQQSRKVGDS PNITEYMFCA GYSDGSKDSC KGDSGGPHAT HYRGTWYLTG IVSWGQGCAT VGHFGVYTRV SQYIEWLQKL MRSEPRPGVL LRAPFPSSSS KAPPPSLPSP SRLPGPSDTP ILPQSSSSKA PPPSLPSPSR LPGPSDTPIL PQSSSSKAPP PSLPSPSRLP GPSDTPILPQ (SEQ ID NO: 46).

[0222] In another embodiment, the amino acid sequence of activated Factor VII-CTP-CTP-CTP (attached to the carboxy terminus) (FVIIa-CTP_3) lacks a signal peptide and comprises the amino acid sequence as put forth in SEQ ID NO: 46. In another embodiment, FVIIa-CTP_3 lacks a signal peptide and comprises a homolog of SEQ ID NO: 46. In another embodiment, FVIIa-CTP_3 lacks a signal peptide and comprises a variant of SEQ ID NO: 46. In another embodiment, the amino acid sequence of FVIIa-CTP_3 is cleaved between arginine (R) at residue 152 and isoleucine (I) at residue 153. In another embodiment, the amino acid sequence of FVIIa-CTP_3 is structurally present as a disulfide-linked two chain heterodimer

comprising a disulfide S-S bridge between cysteine residues present on each of the chains. In another embodiment, the amino acid sequence of FVIIa-CTP_3 is structurally present as a heterodimer comprising a light-chain and a heavy-chain linked by a disulfide –S-S- bond between a cysteine residue present in the light-chain and a cysteine residue present in the heavy chain. In another embodiment, the light chain comprises an N-terminal fragment of the FVIIa-CTP3 amino acid sequence and the heavy chain comprises a C-terminal fragment of the FVIIa-CTP3 amino acid sequence. In another embodiment, the cysteine residues may be any cysteine residue in either chain. In another embodiment, the amino acid sequence of FVIIa-CTP_3 is structurally present as a disulfide-linked two chain heterodimer comprising an S-S bridge between cysteine residue 135 and cysteine residue 262 of SEQ ID NO: 46, wherein said two chains comprise a light chain comprising amino acids 1-152 and a heavy chain comprising amino acids 153-490.

[0223] In another embodiment, a light chain migrates at about 25 kDA in an SDS-PAGE under denaturing conditions. In another embodiment, a heavy chain migrates at about 50 kDA in an SDS-PAGE under denaturing conditions. In another embodiment, a heavy chain migrates at about 60 kDA in an SDS-PAGE under denaturing conditions.

[0224] In another embodiment, the nucleic acid sequence encoding Factor VII-$(CTP)_4$ (attached to the carboxy terminus) comprises the following nucleic acid sequence:

ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga
ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt
gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg
atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc
ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga
ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata
tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg
ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg
cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga
gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg
caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt
ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac
ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag
caaggactcctgcaaggggggacagtggaggcccacatgccacccactaccggggcacgtggtacctgaccggcatcgtgagctgg
ggccagggctgcgccaccgtgggccacttcggcgtgtacaccagggtgtcccagtacatcgagtggctgcagaaactgatgagaag
cgagcccagacccggcgtgctgctgagagcccccttccccagcagcagctccaaggcccctccccctagcctgcccagccctagca
gactgcctgggcccagtgacacccctatcctgcctcagtccagctccagcaaggccccacccccctagcctgccttctccttctcggctg

cctggccccagcgatactccaattctgccccagtcctccagcagtaaggctccccctccatctctgccatcccccagcagactgccag gcccttctgatacacccatcctcccacagtgatgaggatccgc (SEQ ID NO: 26).

[0225] In another embodiment, the amino acid sequence of Factor VII-$(CTP)_4$ (attached to the carboxy terminus) comprises the following amino acid sequence: LEDMVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERE CKEEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLP AFEGRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPT VEYPCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIW VVSAAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIAL LRLHQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNV PRLMTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWY LTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFPSSSSKAPPP SLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRL PGPSDTPILPQ (SEQ ID NO: 27).

[0226] In another embodiment, the nucleic acid sequence encoding Factor VII-$(CTP)_5$ (attached to the carboxy terminus) comprises the following nucleic acid sequence: ctcgaggacatggtctcccaggccctcaggctcctctgccttctgcttgggcttcagggctgcctggctgcagtcttcgtaacccagga ggaagcccacggcgtcctgcaccggcgccggcgcgccaacgcgttcctggaggagctgcggccgggctccctggagagggagt gcaaggaggagcagtgctccttcgaggaggcccgggagatcttcaaggacgcggagaggacgaagctgttctggatttcttacagtg atggggaccagtgtgcctcaagtccatgccagaatgggggctcctgcaaggaccagctccagtcctatatctgcttctgcctccctgcc ttcgagggccggaactgtgagacgcacaaggatgaccagctgatctgtgtgaacgagaacggcggctgtgagcagtactgcagtga ccacacgggcaccaagcgctcctgtcggtgccacgaggggtactctctgctggcagacggggtgtcctgcacacccacagttgaata tccatgtggaaaaatacctattctagaaaaaagaaatgccagcaaaccccaaggccgaattgtgggggggcaaggtgtgccccaaagg ggagtgtccatggcaggtcctgttgttggtgaatggagctcagttgtgtgggggggaccctgatcaacaccatctgggtggtctccgcgg cccactgtttcgacaaaatcaagaactggaggaacctgatcgcggtgctgggcgagcacgacctcagcgagcacgacggggatga gcagagccggcgggtggcgcaggtcatcatccccagcacgtacgtcccgggcaccaccaaccacgacatcgcgctgctccgcctg caccagcccgtggtcctcactgaccatgtggtgcccctctgcctgcccgaacggacgttctctgagaggacgctggccttcgtgcgctt ctcattggtcagcggctggggccagctgctggaccgtggcgccacggccctggagctcatggtcctcaacgtgccccggctgatgac ccaggactgcctgcagcagtcacggaaggtgggagactccccaaatatcacggagtacatgttctgtgccggctactcggatggcag caaggactcctgcaaggggacagtggaggcccacatgccacccactaccggggcacgtggtacctgaccggcatcgtgagctgg ggccagggctgcgccaccgtgggccacttcggcgtgtacaccagggtgtcccagtacatcgagtggctgcagaaactgatgagaag cgagcccagacccggcgtgctgctgagagccccccttccccagcagcagctccaaggcccctccccctagcctgcccagccctagca gactgcctgggccctctgacacccctatcctgcctcagtccagctcctctaaggctccaccaccttccctgcctagcccttcaagactgc caggccctagcgatacaccaattctgccccagtcctccagcagcaaggctcccccacctagcctgccttctccatcaaggctgcctgg cccatccgataccccaattttgcctcagagcagctctagcaaggcacctccccccagtctgccctctccaagcagactccctggcccctt

cagacactccaatcctcccacagtcctctagctctaaagctccacctcccagcctgcccagccctagtagactccccggaccttctgata ccccccatcttgccccagtgatgaggatccgc (SEQ ID NO: 28).

[0227] In another embodiment, the amino acid sequence of Factor VII-$(CTP)_5$ (attached to the carboxy terminus) comprises the following amino acid sequence: LEDMVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERE CKEEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLP AFEGRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPT VEYPCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIW VVSAAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIAL LRLHQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNV PRLMTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWY LTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFPSSSSKAPPP SLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRL PGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPI LPQGS (SEQ ID NO: 29).

[0228] In another embodiment, the nucleic acid sequence encoding Factor IX comprises the following nucleic acid sequence: gcgatcgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccattgcctttaggatatctactcagtgctgaat gtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtttgttcaaggg aaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaactgaattttgg aagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctatgaatgttggtg tccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaaaaatagtgctg ataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccatttccatgtggaa gagtttctgtttcacaaacttctaagctcacccgtgctgagactgtttttcctgatgtggactatgtaaattctactgaagctgaaaccattttg gataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaattcccttggcag gttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgttgaaactggt gttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaattattcctcac cacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagctacgttacac ctatttgcattgctgacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtcttccacaaagg gagatcagctttagttctccagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatctataacaacat gttctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggaccccatgttactgaagtggaagggaccagtt tcttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgtcaactggat taaggaaaaaacaaagctcacttgaacgcggccgc (SEQ ID NO: 16).

[0229] In another embodiment, the amino acid sequence of Factor IX comprises the following amino acid sequence:

MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQ
GNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINS
YECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLAENQKSC
EPAVPFPCGRVSVSQTSKLTRAETVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVV
GGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNI
EETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNI
FLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNNMFCAGFH
EGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEK
TKLT* (SEQ ID NO: 17).

[0230] In another embodiment, the nucleic acid sequence encoding Factor IX-CTP (attached to the carboxy terminus) comprises the following nucleic acid sequence: gcgatcgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccatctgccttttaggatatctactcagtgctgaa tgtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtttgttcaagg gaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaactgaattttg gaagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctatgaatgttggt gtccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaaaaatagtgct gataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccatttccatgtgga agagtttctgtttcacaaacttctaagctcacccgtgctgagactgtttttcctgatgtggactatgtaaattctactgaagctgaaaccatttt ggataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaattcccttggca ggttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgttgaaactgg tgttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaattattcctcac cacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagctacgttacac ctatttgcattgctgacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtcttccacaaagg gagatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatctataacaacat gttctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggaccccatgttactgaagtggaagggaccagtt tcttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgtcaactggat taaggaaaaaacaaagctcactagctccagcagcaaggcccctcccccgagcctgccctccccaagcaggctgcctgggccctccg acacaccaatcctgccacagtgatgaaggtctggatccgcggccgc (SEQ ID NO: 18).

[0231] In another embodiment, the amino acid sequence of Factor IX-CTP (attached to the carboxy terminus) comprises the following amino acid sequence: MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQ
GNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINS
YECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLAENQKSC
EPAVPFPCGRVSVSQTSKLTRAETVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVV
GGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNI

EETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNI
FLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNNMFCAGFH
EGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEK
TKLTSSSSKAPPPSLPSPSRLPGPSDTPILPQ(SEQ ID NO: 19).

[0232] In another embodiment, the nucleic acid sequence encoding Factor IX-CTP-CTP (attached to the carboxy terminus) comprises the following nucleic acid sequence: gcgatcgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccatctgccttttaggatatctactcagtgctgaa tgtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtttgttcaagg gaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaactgaattttg gaagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctatgaatgttggt gtccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaaaaatagtgct gataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccatttccatgtgga agagtttctgtttcacaaacttctaagctcacccgtgctgagactgtttttcctgatgtggactatgtaaattctactgaagctgaaaccatttt ggataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaattcccttggca ggttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgttgaaactgg tgttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaattattcctcac cacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagctacgttacac ctatttgcattgctacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtcttccacaaaggg agatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatctataacaacatgt tctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggaccccatgttactgaagtggaagggaccagtttc ttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgtcaactggatta aggaaaaaacaaagctcactagctccagcagcaaggcccctccccgagcctgccctccccaagcaggctgcctgggccctccga cacaccaatcctgccacagagcagctcctctaaggcccctcctccatccctgccatccccctcccggctgcctggcccctctgacacc cctatcctgcctcagtgatgaaggtctggatccgcggccgc (SEQ ID NO: 20).

[0233] In another embodiment, the amino acid sequence of Factor IX-CTP-CTP (attached to the carboxy terminus) comprises the following amino acid sequence: MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQ
GNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINS
YECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLAENQKSC
EPAVPFPCGRVSVSQTSKLTRAETVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVV
GGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNI
EETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNI
FLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNNMFCAGFH
EGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEK
TKLTSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQ

(SEQ ID NO: 21).

[0234] In another embodiment, the nucleic acid sequence encoding Factor IX-$(CTP)_3$ (attached to the carboxy terminus) comprises the following nucleic acid sequence: tctagagtcgaccccgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccatctgccttttaggatatctactc agtgctgaatgtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtt tgttcaagggaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaa ctgaattttggaagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctat gaatgttggtgtccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaa aaatagtgctgataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccatt tccatgtggaagagtttctgtttcacaaacttctaagctcacccgtgctgaggcagtttttcctgatgtggactatgtaaattctactgaagct gaaaccattttggataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaat tcccttggcaggttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgt tgaaactggtgttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaat tattcctcaccacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagc tacgttacacctatttgcattgctgacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtctt ccacaaagggagatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatcta taacaacatgttctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggacccatgttactgaagtggaag ggaccagtttcttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgt caactggattaaggaaaaaacaaagctcactagctccagcagcaaggcccctccccgagcctgccctccccaagcaggctgcctg ggcccagtgacaccctatcctgcctcagtccagctccagcaaggccccaccccctagcctgccttctccttctcggctgcctggcccc agcgatactccaattctgccccagtcctccagcagtaaggctccccctccatctctgccatcccccagcagactgccaggcccttctgat acacccatcctcccacagtgatgaggatccgcggccgc (SEQ ID NO: 30).

[0235] In another embodiment, the amino acid sequence of Factor IX-$(CTP)_3$ (attached to the carboxy terminus) comprises the following amino acid sequence: MQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKLEEFVQ GNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSCKDDINS YECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLAENQKSC EPAVPFPCGRVSVSQTSKLTRAEAVFPDVDYVNSTEAETILDNITQSTQSFNDFTRVV GGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVVAGEHNI EETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIADKEYTNI FLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNNMFCAGFH EGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEK TKLTSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSS SKAPPPSLPSPSRLPGPSDTPILPQ (SEQ ID NO: 31).

[0236] In another embodiment, the nucleic acid sequence encoding Factor IX-$(CTP)_4$

(attached to the carboxy terminus) comprises the following nucleic acid sequence: tctagagtcgaccccgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccatctgccttttaggatatctactc agtgctgaatgtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagtt tgttcaagggaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaa ctgaattttggaagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctat gaatgttggtgtccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaa aaatagtgctgataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccatt tccatgtggaagagtttctgtttcacaaacttctaagctcacccgtgctgaggcagtttttcctgatgtggactatgtaaattctactgaagct gaaaccattttggataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaat tcccttggcaggttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgt tgaaactggtgttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaat tattcctcaccacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagc tacgttacacctatttgcattgctgacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtctt ccacaaagggagatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatcta taacaacatgttctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggaccccatgttactgaagtggaag ggaccagtttcttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgt caactggattaaggaaaaaacaaagctcactagctccagcagcaaggcccctccccgagcctgccctccccaagcaggctgcctg ggccctctgacacccctatcctgcctcagtccagctcctctaaggccccaccaccttccctgcctagcccttcaagactgccaggccct agcgatacaccaattctgccccagtcctccagcagcaaggctcccccacctagcctgccttctccatcaaggctgcctggcccatccg ataccccaattttgcctcagagcagctctagcaaggcacctcccccagtctgccctctccaagcagactccctggcccttcagacact cccattctgccacagtgatgaggatccgcggccgc (SEQ ID NO: 32).

[0237] In another embodiment, the amino acid sequence of Factor IX-$(CTP)_4$ (attached to the carboxy terminus) comprises the following amino acid sequence: SRVDPAMQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGK LEEFVQGNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSC KDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLA ENQKSCEPAVPFPCGRVSVSQTSKLTRAEAVFPDVDYVNSTEAETILDNITQSTQSFN DFTRVVGGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVV AGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIA DKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNN MFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRY VNWIKEKTKLTSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDT PILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQGS AA (SEQ ID NO: 33).

[0238] In another embodiment, the nucleic acid sequence encoding Factor IX-$(CTP)_5$

(attached to the carboxy terminus) comprises the following nucleic acid sequence: ctagagtcgaccccgccatgcagcgcgtgaacatgatcatggcagaatcaccaggcctcatcaccatctgccttttaggatatctactca gtgctgaatgtacagtttttcttgatcatgaaaacgccaacaaaattctgaatcggccaaagaggtataattcaggtaaattggaagagttt gttcaagggaaccttgagagagaatgtatggaagaaaagtgtagttttgaagaagcacgagaagtttttgaaaacactgaaagaacaac tgaattttggaagcagtatgttgatggagatcagtgtgagtccaatccatgtttaaatggcggcagttgcaaggatgacattaattcctatg aatgttggtgtccctttggatttgaaggaaagaactgtgaattagatgtaacatgtaacattaagaatggcagatgcgagcagttttgtaaa aatagtgctgataacaaggtggtttgctcctgtactgagggatatcgacttgcagaaaaccagaagtcctgtgaaccagcagtgccattt ccatgtggaagagtttctgtttcacaaacttctaagctcacccgtgctgaggcagtttttcctgatgtggactatgtaaattctactgaagct gaaaccattttggataacatcactcaaagcacccaatcatttaatgacttcactcgagttgttggtggagaagatgccaaaccaggtcaat tcccttggcaggttgttttgaatggtaaagttgatgcattctgtggaggctctatcgttaatgaaaaatggattgtaactgctgcccactgtgt tgaaactggtgttaaaattacagttgtcgcaggtgaacataatattgaggagacagaacatacagagcaaaagcgaaatgtgattcgaat tattcctcaccacaactacaatgcagctattaataagtacaaccatgacattgcccttctggaactggacgaacccttagtgctaaacagc tacgttacacctatttgcattgctgacaaggaatacacgaacatcttcctcaaatttggatctggctatgtaagtggctggggaagagtctt ccacaaagggagatcagctttagttcttcagtaccttagagttccacttgttgaccgagccacatgtcttcgatctacaaagttcaccatcta taacaacatgttctgtgctggcttccatgaaggaggtagagattcatgtcaaggagatagtgggggaccccatgttactgaagtggaag ggaccagtttcttaactggaattattagctggggtgaagagtgtgcaatgaaaggcaaatatggaatatataccaaggtatcccggtatgt caactggattaaggaaaaaacaaagctcactagctccagcagcaaggcccctccccgagcctgccctccccaagcaggctgcctg ggccctctgacacccctatcctgcctcagtccagctcctctaaggctccaccaccttccctgcctagcccttcaagactgccaggcccta gcgatacaccaattctgccccagtcctccagcagcaaggctcccccacctagcctgccttctccatcaaggctgcctggcccatccgat accccaattttgcctcagagcagctctagcaaggcacctcccccagtctgccctctccaagcagactccctggcccttcagacactcc aatcctcccacagtcctctagctctaaagctccacctcccagcctgcccagccctagtagactccccggaccttctgataccccatctt gccccagtgatgaggatccgcggccgc (SEQ ID NO: 34).

[0239] In another embodiment, the amino acid sequence of Factor IX-$(CTP)_5$ (attached to the carboxy terminus) comprises the following amino acid sequence: RVDPAMQRVNMIMAESPGLITICLLGYLLSAECTVFLDHENANKILNRPKRYNSGKL EEFVQGNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDGDQCESNPCLNGGSC KDDINSYECWCPFGFEGKNCELDVTCNIKNGRCEQFCKNSADNKVVCSCTEGYRLA ENQKSCEPAVPFPCGRVSVSQTSKLTRAEAVFPDVDYVNSTEAETILDNITQSTQSFN DFTRVVGGEDAKPGQFPWQVVLNGKVDAFCGGSIVNEKWIVTAAHCVETGVKITVV AGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKYNHDIALLELDEPLVLNSYVTPICIA DKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQYLRVPLVDRATCLRSTKFTIYNN MFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTGIISWGEECAMKGKYGIYTKVSRY VNWIKEKTKLTSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDT PILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSSSKAPPPSLPSPSRLPGPSDTPILPQSSS SKAPPPSLPSPSRLPGPSDTPILPQGSAA (SEQ ID NO: 35).

[0240] In another embodiment, furin is added to a cell expressing the coagulation factor-CTP of the disclosure. In another embodiment, furin increases the production efficiency of a coagulation factor-CTP of the disclosure in a cell. In another embodiment, furin is co-transfected with the vector comprising the coding sequence of the coagulation factor-CTP of the disclosure. In another embodiment, furin is encoded by a separate vector. In another embodiment, furin and a coagulation factor-CTP are encoded by one vector. In another embodiment, the coding sequence of furin is inserted into pCI-DHFR. In another embodiment, the coding sequence of furin is engineered in pCI-dhfr/smaI+NotI, Furin/AsisI F.I.+NotI.

[0241] In another embodiment, the nucleic acid sequence encoding furin comprises the following nucleic acid sequence:

tctagagtcgaccccgccatggagctgaggccctggttgctatgggtggtagcagcaacaggaaccttggtcctgctagcagctgatg
ctcagggccagaaggtcttcaccaacacgtgggctgtgcgcatccctggaggcccagcggtggccaacagtgtggcacggaagcat
gggttcctcaacctgggccagatcttcggggactattaccacttctggcatcgaggagtgacgaagcggtccctgtcgcctcaccgcc
cgcggcacagccggctgcagagggagcctcaagtacagtggctggaacagcaggtggcaaagcgacggactaaacgggacgtgt
accaggagcccacagaccccaagtttcctcagcagtggtacctgtctggtgtcactcagcgggacctgaatgtgaaggcggcctggg
cgcagggctacacagggcacggcattgtggtctccattctggacgatggcatcgagaagaaccacccggacttggcaggcaattatg
atcctggggccagttttgatgtcaatgaccaggaccctgacccccagcctcggtacacacagatgaatgacaacaggcacggcacac
ggtgtgcgggggaagtggctgcggtggccaacaacggtgtctgtggtgtaggtgtggcctacaacgcccgcattggaggggtgcgc
atgctggatggcgaggtgacagatgcagtggaggcacgctcgctgggcctgaaccccaaccacatccacatctacagtgccagctg
gggccccgaggatgacggcaagacagtggatgggccagcccgcctcgccgaggaggccttcttccgtggggttagccagggccg
agggggggctgggctccatctttgtctgggcctcggggaacgggggccgggaacatgacagctgcaactgcgacggctacaccaac
agtatctacacgctgtccatcagcagcgccacgcagtttggcaacgtgccgtggtacagcgaggcctgctcgtccacactggccacg
acctacagcagtggcaaccagaatgagaagcagatcgtgacgactgacttgcggcagaagtgcacggagtctcacacgggcacctc
agcctctgccccccttagcagccggcatcattgctctcaccctggaggccaataagaacctcacatggcgggacatgcaacacctggtg
gtacagacctcgaagccagcccacctcaatgccaacgactgggccaccaatggtgtgggccggaaagtgagccactcatatggcta
cgggcttttggacgcaggcgccatggtggccctggcccagaattggaccacagtggcccccagcggaagtgcatcatcgacatcct
caccgagcccaaagacatcgggaaacggctcgaggtgcggaagaccgtgaccgcgtgcctgggcgagcccaaccacatcactcg
gctggagcacgctcaggcgcggctcaccctgtcctataatcgccgtggcgacctggccatccacctggtcagccccatgggcacccg
ctccaccctgctggcagccaggccacatgactactccgcagatgggtttaatgactgggccttcatgacaactcattcctgggatgagg
atccctctggcgagtgggtcctagagattgaaaacaccagcgaagccaacaactatgggacgctgaccaagttcaccctcgtactctat
ggcaccgcccctgaggggctgcccgtacctccagaaagcagtggctgcaagaccctcacgtccagtcaggcctgtgtggtgtgcga
ggaaggcttctccctgcaccagaagagctgtgtccagcactgccctccaggcttcgccccccaagtcctcgatacgcactatagcacc
gagaatgacgtggagaccatccgggccagcgtctgcgcccccctgccacgcctcatgtgccacatgccaggggccggccctgacag
actgcctcagctgccccagccacgcctccttggaccctgtggagcagacttgctcccggcaaagccagagcagccgagagtccccg

ccacagcagcagccacctcggctgcccccggaggtggaggcggggcaacggctgcgggcagggctgctgccctcacacctgcct gaggtggtggccggcctcagctgcgccttcatcgtgctggtcttcgtcactgtcttcctggtcctgcagctgcgctctggctttagttttcg gggggtgaaggtgtacaccatggaccgtggcctcatctcctacaaggggctgccccctgaagcctggcaggaggagtgcccgtctg actcagaagaggacgagggccggggcgagaggaccgcctttatcaaagaccagagcgccctctgaacgcggccgc (SEQ ID NO: 22).

[0242] In another embodiment, the amino acid sequence of furin comprises the following amino acid sequence: MELRPWLLWVVAATGTLVLLAADAQGQKVFTNTWAVRIPGGPAVANSVARKHGFL NLGQIFGDYYHFWHRGVTKRSLSPHRPRHSRLQREPQVQWLEQQVAKRRTKRDVY QEPTDPKFPQQWYLSGVTQRDLNVKAAWAQGYTGHGIVVSILDDGIEKNHPDLAGN YDPGASFDVNDQDPDPQPRYTQMNDNRHGTRCAGEVAAVANNGVCGVGVAYNAR IGGVRMLDGEVTDAVEARSLGLNPNHIHIYSASWGPEDDGKTVDGPARLAEEAFFRG VSQGRGGLGSIFVWASGNGGREHDSCNCDGYTNSIYTLSISSATQFGNVPWYSEACS STLATTYSSGNQNEKQIVTTDLRQKCTESHTGTSASAPLAAGIIALTLEANKNLTWRD MQHLVVQTSKPAHLNANDWATNGVGRKVSHSYGYGLLDAGAMVALAQNWTTVA PQRKCIIDILTEPKDIGKRLEVRKTVTACLGEPNHITRLEHAQARLTLSYNRRGDLAIH LVSPMGTRSTLLAARPHDYSADGFNDWAFMTTHSWDEDPSGEWVLEIENTSEANNY GTLTKFTLVLYGTAPEGLPVPPESSGCKTLTSSQACVVCEEGFSLHQKSCVQHCPPGF APQVLDTHYSTENDVETIRASVCAPCHASCATCQGPALTDCLSCPSHASLDPVEQTCS RQSQSSRESPPQQQPPRLPPEVEAGQRLRAGLLPSHLPEVVAGLSCAFIVLVFVTVFLV LQLRSGFSFRGVKVYTMDRGLISYKGLPPEAWQEECPSDSEEDEGRGERTAFIKDQS AL (SEQ ID NO: 23).

[0243] In one embodiment, the term coagulation factor further includes a homologue of a known coagulation factor. In one embodiment, the homologue has a coagulating activity. In some embodiments, homology according to the present invention also encompasses deletion, insertion, or substitution variants, including an amino acid substitution, thereof and biologically active polypeptide fragments thereof. In one embodiment, the variant comprises conservative substitutions, or deletions, insertions, or substitutions that do not significantly alter the three dimensional structure of the coagulation factor. In another embodiment, the deletion, insertion, or substitution does not alter the function of interest of the coagulation factor, which in one embodiment, is binding to a particular binding partner.

[0244] In another embodiment, the disclosure includes a homologue of a coagulation factor. In another embodiment, the disclosure includes a homologue of a coagulation factor having a coagulation activity. In another embodiment, the disclosure includes a homologue of a coagulation factor having functional binding. In another embodiment, the disclosure includes

homologues of a coagulation factor as described herein having a coagulation activity. In another embodiment, the disclosure includes homologues of a coagulation factor as described herein having functional binding. In another embodiment, homologues e.g., polypeptides which are at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 87%, at least 89%, at least 91%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homologous to a coagulation factor as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters.

[0245] In another embodiment, the disclosure includes homologues of furin. In another embodiment, the disclosure includes homologues of furin maintaining a function of interest, which in one embodiment is cleaving of a precursor protein. In another embodiment, homologues e.g., polypeptides which are at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 87%, at least 89%, at least 91%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homologous to a furin as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters.

[0246] In another embodiment, provided herein is a polypeptide comprising a coagulation factor and one to ten gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and one to three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and one to five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor having at least one CTP on its carboxy terminus.

[0247] In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and one to five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor.

[0248] In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and one to five CTPs attached to the carboxy terminus of the coagulation factor.

[0249] It is to be understood that the compositions, formulations and methods of the present invention comprising the elements or steps as described herein may, in another embodiment, consist of those elements or steps, or in another embodiment, consist essentially of those elements or steps. In some embodiments, the term “comprise” refers to the inclusion of the

indicated active agent, such as the CTP-modified coagulation factor, as well as inclusion of other active agents, and pharmaceutically acceptable carriers, excipients, emollients, stabilizers, etc., as are known in the pharmaceutical industry. In some embodiments, the term "consisting essentially of" refers to a composition, whose only active ingredient is the indicated active ingredient, however, other compounds may be included which are for stabilizing, preserving, etc. the formulation, but are not involved directly in the therapeutic effect of the indicated active ingredient. In some embodiments, the term "consisting essentially of" may refer to components which facilitate the release of the active ingredient. In some embodiments, the term "consisting" refers to a composition, which contains the active ingredient and a pharmaceutically acceptable carrier or excipient.

[0250] In one embodiment, the present invention provides a polypeptide comprising a coagulation factor and two gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to three CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and two to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0251] In one embodiment, the present invention provides a polypeptide comprising a coagulation factor and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a

coagulation factor and three to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and three to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0252] In one embodiment, the present invention provides a polypeptide comprising a coagulation factor and four gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and four to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0253] In one embodiment, the present invention provides a polypeptide comprising a coagulation factor and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and five to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and five to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and five to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and five to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide comprising a coagulation factor and five to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0254] In one embodiment, the present invention provides a polypeptide consisting of a coagulation factor and two gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to three CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and two to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0255] In one embodiment, the present invention provides a polypeptide consisting of a coagulation factor and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and three to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0256] In one embodiment, the present invention provides a polypeptide consisting of a

coagulation factor and four gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and four to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0257] In one embodiment, the present invention provides a polypeptide consisting of a coagulation factor and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and five to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and five to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and five to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and five to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting of a coagulation factor and five to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0258] In one embodiment, the present invention provides a polypeptide consisting essentially of a coagulation factor and two gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to three CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to six CTPs

attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and two to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0259] In one embodiment, the present invention provides a polypeptide consisting essentially of a coagulation factor and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to four CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and three to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0260] In one embodiment, the present invention provides a polypeptide consisting essentially of a coagulation factor and four gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and four to five CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and four to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and four to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided

herein is a polypeptide consisting essentially of a coagulation factor and four to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and four to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and four to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0261] In one embodiment, the present invention provides a polypeptide consisting essentially of a coagulation factor and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and five to six CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and five to seven CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and five to eight CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and five to nine CTPs attached to the carboxy terminus of the coagulation factor. In another embodiment, provided herein is a polypeptide consisting essentially of a coagulation factor and five to ten CTPs attached to the carboxy terminus of the coagulation factor.

[0262] In another embodiment, provided herein is a polypeptide comprising, consisting essentially of, or consisting of a coagulation factor having no CTPs on its amino terminus. In another embodiment, provided herein is a polypeptide comprising, consisting essentially of, or consisting of a coagulation factor lacking a CTP on its amino terminus. In another embodiment, provided herein is a polypeptide comprising, consisting essentially of, or consisting of a coagulation factor having at least one CTP on its carboxy terminus and no CTPs on its amino terminus. In another embodiment, provided herein is a polypeptide comprising, consisting essentially of, or consisting of a coagulation factor having the number of CTPs on its carboxy terminus as described herein and no CTPs on its amino terminus.

[0263] In another embodiment, the present invention provides a polynucleotide encoding a polypeptide as described hereinabove.

[0264] In another embodiment, the present invention further provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor IX (FIX) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide.

[0265] In another embodiment, the present invention further provides a polynucleotide

encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide. In another embodiment, the present invention further provides a composition comprising an expression vector comprising a polynucleotide encoding a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide. In one embodiment, the CTP-modified FVIIa includes a signal peptide. In another embodiment, the CTP-modified FVIIa does not include a signal peptide.

[0266] In one embodiment, the present invention provides a recombinant coagulation factor as described hereinabove. In one embodiment, the present invention provides an engineered coagulation factor as described hereinabove. In one embodiment, the engineered coagulation factor as described hereinabove is referred to as a CTP-modified coagulation factor.

[0267] In one embodiment, the CTPs that are attached to the carboxy terminus of the coagulation factor are attached in tandem to the carboxy terminus.

[0268] In one embodiment, an engineered coagulation factor as described herein has equivalent or improved biological activity compared to the non-CTP-modified coagulation factor. In another embodiment, an engineered coagulation factor as described herein has equivalent or improved pharmacological measurements compared to the non-CTP-modified coagulation factor. In another embodiment, an engineered coagulation factor as described herein has equivalent or improved pharmacokinetics compared to the non-CTP-modified coagulation factor. In another embodiment, an engineered coagulation factor as described herein has equivalent or improved pharmacodynamics compared to the non-CTP-modified coagulation factor.

[0269] In one embodiment, the present invention provides a method of preventing or treating a clotting or coagulation disorder. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified coagulation factor of the present invention. In another embodiment, the present invention provides a method of preventing and treating hemophilia in a subject comprising administering a CTP-modified coagulation factor of the present invention. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VII of the present invention.

[0270] In one embodiment, hemophilia is hemophilia A. In another embodiment, hemophilia is hemophilia B. In another embodiment, methods of this invention for prevention or treating a clotting or coagulation disorder prevent or treat hemophilia in patients having hemophilia A or B with inhibitors to FVIII or FIX, respectively. In another embodiment,

methods of this invention are for preventing or treating patients with acquired hemophilia (hemophilia without inhibitors). In another embodiment, methods of this invention for prevention or treating a clotting or coagulation disorder prevent or treat hemophilia A or B without inhibitors. In another embodiment, hemophilia is severe hemophilia. In another embodiment, hemophilia is moderate hemophilia. In another embodiment, hemophilia is moderate to severe hemophilia with or without inhibitors. It will be appreciated by a skilled artisan that the term "moderate to severe hemophilia" refers to a subject having less than or equal to 3% FVIII or FIX.

[0271] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX of the present invention. In one embodiment, hemophilia is hemophilia B. In one embodiment, hemophilia B is known as factor IX deficiency or Christmas disease. In one embodiment, the hemophilia is severe hemophilia, which in one embodiment, describes hemophilia in which the coagulation factor levels are 0-1%. In another embodiment, the hemophilia is moderate hemophilia, which in one embodiment, describes hemophilia in which the coagulation factor levels are 1-5%. In another embodiment, the hemophilia is mild hemophilia, which in one embodiment, describes hemophilia in which the coagulation factor levels are 5-50%.

[0272] In another embodiment, the present invention provides a method of preventing or treating a clotting or coagulation disorder in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby preventing or treating a clotting or coagulation disorder in said subject. In another embodiment, the present invention provides a method of preventing or treating a clotting or coagulation disorder in a subject comprising administering a CTP-modified Factor VII (FVII) polypeptide comprising a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide to said subject, thereby preventing or treating a clotting or coagulation disorder in said subject.

[0273] In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor VIIa (FVIIa) polypeptide comprising a FVIIa

polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide to said subject, thereby preventing or treating hemophilia in said subject.

[0274] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering one or more CTP-modified coagulation factors as described herein to said subject. Thus, in one embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide and a CTP-modified Factor VIIa (FVIIa) polypeptide comprising a FVIIa polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide to said subject, thereby treating hemophilia in said subject. In one embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in the same composition at the same time. In another embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in separate compositions at the same time. In another embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in separate compositions at separate times.

[0275] In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and four chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and three

to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) and a CTP-modified Factor VII polypeptide comprising a FIX and a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX and said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) and a CTP-modified Factor VII polypeptide comprising a FIX and a FVII polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX and said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject.

[0276] In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and four chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) or a CTP-modified Factor VII polypeptide comprising a FIX or a FVII polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX or said FVII polypeptide to said subject, thereby preventing or treating hemophilia

in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) and a CTP-modified Factor VII polypeptide comprising a FIX and a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX and said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject. In another embodiment, the present invention provides a method of preventing or treating hemophilia in a subject comprising subcutaneously or intravenously administering a CTP-modified Factor IX (FIX) and a CTP-modified Factor VII polypeptide comprising a FIX and a FVII polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX and said FVII polypeptide to said subject, thereby preventing or treating hemophilia in said subject.

[0277] In some embodiments, provided herein is a method of preventing or treating a hemophilia in a subject, the method comprising the step of administering to the subject a CTP-modified coagulation factor, comprising three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor polypeptide, wherein the sequence of said CTP-modified coagulation factor is selected from the group consisting of SEQ ID NO: 25, 27, or 29, thereby preventing hemophilia in said subject. In another embodiment, said CTP-modified coagulation factor is selected from the group consisting of SEQ ID NOP 25, 27, 29 or 46. In another embodiment, said CTP-modified coagulation factor consists of SEQ ID NO: 46.

[0278] In one embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and three to five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide. In another embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide. In another embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide.

[0279] In one embodiment, the term “three to five” when referring to gonadotropin carboxy terminal peptides (CTPs), refers to attaching three, four, or five CTPs to the carboxy terminal of a coagulation factor polypeptide provided herein

[0280] In one embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and three to five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide. In another embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide. In another embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VII (FVII) polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide.

[0281] In one embodiment, the present invention provides a method of extending the biological half-life of a Factor VII (FVII) polypeptide, comprising the step of attaching three to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby extending the biological half-life of said FVII polypeptide. In another embodiment, the present invention provides a method of extending the biological half-life of a Factor VII (FVII) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby extending the biological half-life of said FVII polypeptide. In another embodiment, the present invention provides a method of extending the biological half-life of a Factor VII (FVII) polypeptide, comprising the step of attaching five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby extending the biological half-life of said FVII polypeptide.

[0282] In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor VII (FVII) polypeptide, comprising the step of attaching three to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby improving the AUC of said FVII polypeptide. In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor VII (FVII) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby improving the AUC of said FVII polypeptide. In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor VII (FVII) polypeptide, comprising the step of attaching five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said

FVII polypeptide, thereby improving the AUC of said FVII polypeptide.

[0283] In one embodiment, the present invention provides a method of reducing the dosing frequency of a Factor VII (FVII) polypeptide, comprising the step of attaching three to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the dosing frequency of said FVII polypeptide. In another embodiment, the present invention provides a method of reducing the dosing frequency of a Factor VII (FVII) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the dosing frequency of said FVII polypeptide. In another embodiment, the present invention provides a method of reducing the dosing frequency of a Factor VII (FVII) polypeptide, comprising the step of attaching five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the dosing frequency of said FVII polypeptide.

[0284] In one embodiment, the present invention provides a method of reducing the clearance rate of a Factor VII (FVII) polypeptide, comprising the step of attaching three to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the clearance rate of said FVII polypeptide. In another embodiment, the present invention provides a method of reducing the clearance rate of a Factor VII (FVII) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the clearance rate of said FVII polypeptide. In another embodiment, the present invention provides a method of reducing the clearance rate of a Factor VII (FVII) polypeptide, comprising the step of attaching five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby reducing the clearance rate of said FVII polypeptide.

[0285] In one embodiment, the present invention provides a method of producing a CTP-modified Factor VII (FVII) polypeptide, comprising the step of attaching three to five chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby producing a CTP-modified FVII polypeptide. In another embodiment, the present invention provides a method of producing a CTP-modified Factor VII (FVII) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide, thereby producing a CTP-modified FVII polypeptide. In another embodiment, the present invention provides a method of producing a CTP-modified Factor VII (FVII) polypeptide, comprising the step of attaching five chorionic gonadotropin carboxy terminal peptides (CTPs) to the

carboxy terminus of said FVII polypeptide, thereby producing a CTP-modified FVII polypeptide.

[0286] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VII (FVII) polypeptide comprising a FVII polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VII (FVII) polypeptide comprising a FVII polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VII (FVII) polypeptide comprising a FVII polypeptide and five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVII polypeptide to said subject, thereby treating hemophilia in said subject.

[0287] In another embodiment, the methods provided herein further comprise the step of attaching four chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVII polypeptide.

[0288] In other embodiments, the engineered coagulation factor is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 3 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 4 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 5 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In another embodiment, coagulation Factor IX comprising 1 CTP repeat in its carboxy terminus is for the treatment of hemophilia B patients. In other embodiments, the engineered coagulation factor can reduce the number of infusions required for a patient, reduce the required doses for a patient, or a combination thereof.

[0289] In one embodiment, coagulation Factor IX comprising 3 CTPs in tandem in its carboxy terminus exhibits an improved PK profile while maintaining its coagulation activity vs. FIX-CTP-CTP harvest, FIX-CTP harvest or rhFIX. In one embodiment, the elimination half-life of rFIX-CTP3 is 2.5- to 4-fold longer than rFIX in rats and in FIX-deficient mice. In

one embodiment, the administration of rFIX-CTP3 significantly prolonged the procoagulatory effect in FIX-deficient mice for at least 76 hr after dosing. In one embodiment, the administration of rFIX-CTP3 produced a higher activity peak than rFIX in FIX-deficient mice. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus exhibits an improved PK profile while maintaining its coagulation activity vs. FIX-CTP harvest or rhFIX. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus exhibits 3-fold increase in half-life and 4.5-fold higher AUC compared to rhFIX.

[0290] In another embodiment, SC administration results in higher bioavailability of CTP-modified FVII as compared to recombinant FVII. In another embodiment, half-life is longer and bioavailability (AUC SC/AUC IV) is higher following FVIIa-CTP3 and CTP5 SC administration when compared to SC administration of NovoSeven®. In another embodiment, subcutaneously injected MOD-5014 and MOD-5019 shows improved mice survival in comparison to recombinant FVII (NovoSeven®) (see Example 8 below).

[0291] In one embodiment, MOD-5014 is FVIIa-CTP_3 (having three CTP peptides attached at the C-terminus end). In one embodiment, MOD-5014 provides a long-acting coagulation factor. In one embodiment, MOD-5014 provides a more sustained and prolonged blood clotting response compared with recombinant human FVIIa. (*See* for example, Example 14). It will be appreciated by a skilled artisan that the terms MOD-5014 or FVIIa-CTP_3 may be used interchangeably having all the same qualities and meanings, and refer in one embodiment, to a disulfide-linked two chain heterodimer structure comprising the amino acid SEQ ID NO: 46. Further, a skilled artisan would appreciate that in describing CTP-modified coagulation factors, for example FVII-CTP_3, the term FVII-CTP_3 may in certain instances refer to the inactive form of FVII-CTP_3.The skilled artisan would certainly recognize which form is being referred to based on associated details such as activity. Similarly, while the term MOD-5014 is interchangeable with the term FVIIa-CTP_3, i.e., represents the active form of the CTP-modified coagulation factor, in certain instances the term MOD-5014 may be used to denote an active form of FVII or a nucleotide sequence encoding a FVII-CTP_3, which will then be expressed and secreted from a cell, and purified and activated in vitro, resulting in the active form of FVIIa being present in a MOD-5014 molecule.

[0292] In one embodiment, deactivation of MOD-5014 by tissue factor pathway inhibitor (TFPI) is dose-dependent. In one embodiment, deactivation of MOD-5014 by TFPI shows a similar dose-dependent deactivation pattern to that of recombinant FVIIa (NovoSeven®) by TFPI. In one embodiment, MOD-5014 is inhibited by anti-thrombin III. In one embodiment, inhibition of MOD-5014 by anti-thrombin III is augmented in the presence of heparin. In one

embodiment, inhibition of MOD-5014 by anti-thrombin III shows similar inhibition pattern to that of recombinant FVIIa (NovoSeven®), in the presence or absence of heparin. (see Example 11 below)

[0293] In one embodiment, MOD-5014 generates thrombin in a dose-dependent manner. In one embodiment, MOD-5014 decreases lag phase of thrombin generation. In one embodiment, MOD-5014 decreases blood clotting time. In one embodiment, MOD-5014 increases efficiency of blood clot formation. In one embodiment, administration of MOD-5014 decreases blood clotting time in a subject. In one embodiment, administration of MOD-5014 increases efficiency of blood clot formation in a subject. In one embodiment, the generation of thrombin by MOD-5014 is similar to that produced by recombinant FVIIa (NovoSeven®). In one embodiment, decrease of lag-phase of thrombin generation by MOD-5014 is similar to that produced by recombinant FVIIa (NovoSeven®). In one embodiment, decrease blood clotting time by MOD-5014 is similar to that produced by recombinant FVIIa (NovoSeven®). In one embodiment, increased efficiency of blood clot formation by MOD-5014 is similar to that produced by recombinant FVIIa (NovoSeven®) (see Example 12 below).

[0294] As provided herein, CTP attachments to blood clotting factors, for example factors FVII, FVIIA and FX, increase the half-life of the blood clotting factor. Examples 11, 12 and 13 show that CTP attachments, for example three CTPs attached to FVIIA, do not appear to affect blood clotting activities. In one embodiment, CTP attachments to FVII do not interfere with blood clot formation. In one embodiment, CTP attachments to FVII do not interfere with increased efficiency of blood clot formation. In one embodiment, CTP attachments to FVII do not interfere with decreased in blood clotting time. In one embodiment, binding of phospholipid to FVII is maintained following attachment of CTPs to the blood clotting factor. In one embodiment, CTP attachments to FVIIA do not interfere with blood clot formation. In one embodiment, CTP attachments to FVIIA do not interfere with increased efficiency of blood clot formation. In one embodiment, CTP attachments to FVIIA do not interfere with decreased blood clot formation. In one embodiment, binding of phospholipid to FVIIA is maintained following attachment of CTPs to the blood clotting factor.

[0295] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a

CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VIIa (FVIIa) polypeptide comprising a FVIIa polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide to said subject, thereby treating hemophilia in said subject.

[0296] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering one or more CTP-modified coagulation factors as described herein to said subject. Thus, in one embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide and a CTP-modified Factor VIIa (FVIIa) polypeptide comprising a FVIIa polypeptide and three to five chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide to said subject, thereby treating hemophilia in said subject. In one embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in the same composition at the same time. In another embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in separate compositions at the same time. In another embodiment, the CTP-modified FIX and the CTP-modified FVIIa are administered in separate compositions at separate times.

[0297] In other embodiments, the engineered coagulation factor is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 3 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 4 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In one embodiment, coagulation Factor IX comprising 5 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus is for the treatment of hemophilia B patients. In another embodiment, coagulation Factor IX comprising 1 CTP repeat in its carboxy terminus is for the treatment of

hemophilia B patients. In other embodiments, the engineered coagulation factor can reduce the number of infusions required for a patient, reduce the required doses for a patient, or a combination thereof.

[0298] Example 14 shows the results of administering MOD-5014 to a large mammal (dogs). MOD-5014 administration provided an effective and safe long-acting FVIIa for blood coagulation. Treatment using MOD-5014 may be prophylactic or on-demand. In one embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering MOD-5014 to said subject, thereby treating hemophilia in said subject. In one embodiment, the present invention provides a method of preventing excess bleeding in a subject comprising administering MOD-5014 to said subject, thereby preventing excess bleeding in said subject. In one embodiment, the present invention provides a method of prophylactically treating hemophilia in a subject comprising administering MOD-5014 to said subject, thereby prophylactically treating hemophilia in said subject.

[0299] In one embodiment, treating hemophilia in a subject with MOD-5014 comprises a reduced frequency of administration of MOD-5014, as compared with recombinant FVIIa (NovoSeven®). In one embodiment, prophylactically treating hemophilia in a subject with MOD-5014 comprises a reduced frequency of administration of MOD-5014, as compared with recombinant FVIIa (NovoSeven®). In one embodiment, preventing excess bleeding in a subject with MOD-5014 comprises a reduced frequency of administration of MOD-5014, as compared with recombinant FVIIa (NovoSeven®).

[0300] In one embodiment, coagulation Factor IX comprising 3 CTPs in tandem in its carboxy terminus exhibits an improved PK profile while maintaining its coagulation activity vs. FIX-CTP-CTP harvest, FIX-CTP harvest or rhFIX. In one embodiment, the elimination half-life of rFIX-CTP3 is 2.5- to 4-fold longer than rFIX in rats and in FIX-deficient mice. In one embodiment, the administration of rFIX-CTP3 significantly prolonged the procoagulatory effect in FIX-deficient mice for at least 76 hr after dosing. In one embodiment, the administration of rFIX-CTP3 produced a higher activity peak than rFIX in FIX-deficient mice. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus exhibits an improved PK profile while maintaining its coagulation activity vs. FIX-CTP harvest or rhFIX. In another embodiment, coagulation Factor IX comprising 2 CTPs in tandem in its carboxy terminus exhibits 3-fold increase in half-life and 4.5-fold higher AUC compared to rhFIX.

[0301] In one embodiment, coagulation Factor VII comprising 3 CTPs in tandem in its carboxy terminus exhibits an improved PK profile while maintaining its coagulation activity vs. NovoSeven® (see Table 59 and Figure 36).

[0302] In another embodiment, the terms "CTP peptide," "carboxy terminal peptide" and "CTP sequence" are used interchangeably herein. In another embodiment, the carboxy terminal peptide is a full-length CTP. Each possibility represents a separate embodiment of the disclosure.

[0303] In another embodiment, a signal peptide is attached to the amino terminus of the CTP, as described in US 7,553,940, which is incorporated by reference herein in its entirety. In another embodiment, no signal peptide is attached to the amino terminus of the CTP.

[0304] In other embodiments, the term engineered coagulation factor refers to the amino acid sequence of a matured coagulation factor. In other embodiments, the term engineered coagulation factor refers to the amino acid sequence of the coagulation factor including its signal sequence or signal peptide.

[0305] In another embodiment, "signal sequence" and "signal peptide" are used interchangeably herein having all the same qualities and meanings. In another embodiment, "sequence" when in reference to a polynucleotide molecule can refer to a coding portion. In another embodiment, an engineered coagulation factor comprising at least one CTP as described herein has enhanced in vivo biological activity compared the same coagulation factor without at least one CTP. In one embodiment, the enhanced biological activity stems from the longer half-life of the engineered coagulation factor while maintaining at least some biological activity. In another embodiment, the enhanced biological activity stems from enhanced biological activity resulting from the CTP modification. In another embodiment, the enhanced biological activity stems from both a longer half-life and from enhanced functionality of the CTP-modified coagulation factor.

[0306] In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor provides enhanced protection against degradation of a coagulation factor. In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor provides enhanced protection against clearance. In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor provides prolonged clearance time. In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor enhances its Cmax. In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor enhances its Tmax. In some embodiments, at least one CTP sequence at the carboxy terminal end of the coagulation factor prolongs its T½.

[0307] In another embodiment, a conjugated coagulation factor of this invention is used in the same manner as an unmodified conjugated coagulation factor. In another embodiment, a conjugated coagulation factor of this invention has an increased circulating half-life and

plasma residence time, decreased clearance, and increased clinical activity in vivo. In another embodiment, due to the improved properties of the conjugated coagulation factor as described herein, this conjugate is administered less frequently than the unmodified form of the same coagulation factor.

[0308] In another embodiment, decreased frequency of administration will result in improved treatment strategy, which in one embodiment, will lead to improved patient compliance leading to improved treatment outcomes, as well as improved patient quality of life. In another embodiment, compared to conventional conjugates of coagulation factors, it has been found that conjugates having the molecular weight and linker structure of the conjugates of this invention have an improved potency, improved stability, elevated AUC levels, and enhanced circulating half-life.

[0309] In another embodiment, the present invention further provides a pharmaceutical composition or a pharmaceutical formulation comprising a CTP-modified Factor IX (FIX) polypeptide consisting of a FIX polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said CTP-modified FIX polypeptide.

[0310] In another embodiment, the present invention further provides a pharmaceutical composition or a pharmaceutical formulation comprising a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0311] In another embodiment, the present invention further provides a pharmaceutical composition or a pharmaceutical formulation comprising a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and four gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0312] In another embodiment, the present invention further provides a pharmaceutical composition or a pharmaceutical formulation comprising a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0313] In another embodiment, provided herein is a composition comprising a conjugated coagulation factor as described herein. In another embodiment, provided herein is a pharmaceutical composition comprising the conjugated coagulation factor as described herein. In another embodiment, provided herein is a pharmaceutical composition comprising a therapeutically effective amount of the conjugated coagulation factor as described herein. In one embodiment, a therapeutically effective amount of a conjugated coagulation factor is determined according to factors such as the specific condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition.

[0314] In one embodiment, the disclosure provides a pharmaceutical formulation for use in compositions, formulations, and methods of the disclosure. In another embodiment, the disclosure provides a pharmaceutical formulation comprising a polypeptide consisting of a coagulation factor and three chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor. In another embodiment, the pharmaceutical formulation further comprises a buffer and a tonicity agent. In another embodiment, the buffer is 20mM citrate and13.3 mM Glycine, and the tonicity agent is 150 mM NaCl. In another embodiment, the formulation is at about a pH of 6.4. In another embodiment, the buffer is 20mM citrate and 13.3 mM Glycine, and the tonicity agent is 150 mM NaCl, and the pH is 6.4. In another embodiment, the formulation is a liquid formulation. In another embodiment, the formulation is a lyophilized formulation. In another embodiment, the liquid formulation may be formed using a lyophilized CTP-modified coagulation factor. In another embodiment, the CTP-modified coagulation factor is FVII-CTP-CTP-CTP. In another embodiment, the CTP-modified coagulation factor is FVIIa-CTP-CTP-CTP.

[0315] In another embodiment, provided herein is a once weekly dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is a once daily dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is an every other day dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is an every third day dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is a twice weekly dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is twice weekly dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is a weekly dosage form comprising the pharmaceutical formulation provided herein. In another embodiment, provided herein is a bi-weekly (every two weeks) dosage form comprising the pharmaceutical formulation provided herein.

[0316] In another embodiment, the present invention provides a formulation comprising a polypeptide consisting of a coagulation factor and three-five chorionic gonadotropin CTPs attached to the carboxy terminus of said coagulation factor, and wherein said polypeptide optionally consists of a signal peptide, wherein said formulation has increased stability. In one embodiment, the formulation is stable for at least one year. In another embodiment, the formulation is stable for at least two years.

[0317] In one embodiment, a coagulation factor modified by CTPs is formulated in a liquid formulation. In another embodiment, a Factor VII modified by CTPs is formulated in a liquid formulation. In another embodiment, a Factor VIIa modified by CTPs is formulated in a

liquid formulation. In another embodiment, a Factor IX modified by CTPs is formulated in a liquid formulation. In another embodiment, coagulation factor modified by CTPs is formulated in an intranasal dosage form. In another embodiment, coagulation factor modified by CTPs is formulated in an injectable dosage form.

[0318] In another embodiment, the methods of the disclosure include increasing the compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a coagulation factor modified by CTPs, thereby increasing compliance in the use of coagulation factor therapy.

[0319] In another embodiment, a coagulation factor modified by CTPs is administered to a subject once a day. In another embodiment, a polypeptide comprising a coagulation factor modified by CTPs is administered to a subject once every two days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every three days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every four days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every five days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every six days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every week. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every 7-14 days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every 10-20 days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every 5-15 days. In another embodiment, a coagulation factor modified by CTPs is administered to a subject once every 15-30 days.

[0320] In one embodiment, the preparation of the present invention is formulated in liquid formulations for injection via a syringe or Pen device.

[0321] In one embodiment, the formulations provided herein also comprise preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0322] In one embodiment, a coagulation factor as described herein is a human coagulation factor.

[0323] In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with a coagulation or clotting disorder. In another embodiment, the coagulation or clotting disorder is Hemophilia. In another embodiment, a conjugated coagulation factor as described herein is useful in the prophylactic therapy of Hemophilia thus reducing the risk of bleeding and associated complications. In another embodiment, reducing the risk of bleeding and associated complications reduces the risk of spontaneous bleeding. In another embodiment, reducing the risk of bleeding and associated complications reduces the risk of excessive bleeding. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with Hemophilia while reducing the risk of developing inhibitory antibodies to exogenously administered coagulation factors. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with Hemophilia thus inducing homeostasis.

[0324] In one embodiment, a CTP-modified coagulation factor of the present invention has therapeutic uses. In another embodiment, a CTP-modified coagulation factor of the present invention has prophylactic uses.

[0325] In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects experiencing excessive bleeding or bruising or having a prolonged Prothrombin Time (PT) or Partial Thromboplastin Time (PTT). In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects having an acquired condition that is causing bleeding, such as vitamin K deficiency or liver disease. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects having deficiencies in coagulation factors that are acquired (due to other diseases) or inherited, mild or severe, permanent or temporary. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with hemophilia A. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with hemophilia B. In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects having acquired deficiencies due to chronic diseases, such as liver disease or cancer; to an acute condition such as disseminated intravascular coagulation (DIC), which uses up clotting factors at a rapid rate; or to a deficiency in vitamin K or treatment with a vitamin K antagonist like warfarin (the production of factors II, VII, IX, and X require vitamin K). In another embodiment, a conjugated coagulation factor as described herein is useful in the treatment of subjects afflicted with a disease in which causes clotting imbalances such as but not limited to: a liver disease, uremia, a cancer, a bone marrow disorder, an exposure to snake

venom, a vitamin K deficiency, an anticoagulation therapy, an accidental ingestion of the anticoagulant warfarin, multiple blood transfusions (stored units of blood lose some of their clotting factors), or a combination thereof. In another embodiment, the present invention provides a method of treating deep vein thrombosis in a subject comprising administering a CTP-modified coagulation factor of the present invention. In another embodiment, the present invention provides a method of preventing uncontrolled bleeding in a subject with hemophilia comprising administering a CTP-modified coagulation factor of the present invention. In another embodiment, the present invention provides a method of preventing bleeding episodes in a subject with hemophilia comprising administering a CTP-modified coagulation factor of the present invention. In another embodiment, the present invention provides a method of controlling bleeding episodes in a subject with hemophilia B (congenital factor IX deficiency).

[0326] In one embodiment, a composition of this invention comprises a formulation as described herein. In another embodiment, a method of this invention comprises administering a formulation as described herein. In another embodiment, a method of this invention comprises administering a composition comprising a formulation as described herein.

[0327] In another embodiment, the compositions, formulations and methods of the present invention are for the treatment of bleeding episodes in hemophilia A or B patients with inhibitors to FVIII or FIX and in patients with acquired hemophilia; prevention of bleeding in surgical interventions or invasive procedures in hemophilia A or B patients with inhibitors to FVIII or FIX and in patients with acquired hemophilia; treatment of bleeding episodes in patients with congenital FVII deficiency and prevention of bleeding in surgical interventions or invasive procedures in patients with congenital FVII deficiency. Acquired hemophilia is a spontaneous autoimmune disorder in which patients with previously normal hemostasis develop autoantibodies against clotting factors, most frequently FVIII. The development of autoantibodies against FVIII leads to FVIII deficiency, which results in insufficient generation of thrombin by factor IXa and the factor VIIIa complex through the intrinsic pathway of the coagulation cascade. The following conditions may be associated with acquired hemophilia A: idiopathic, pregnancy, autoimmune disorders, inflammatory bowel disease, ulcerative colitis, dermatologic disorders (eg, psoriasis, pemphigus), respiratory diseases (eg, asthma, chronic obstructive pulmonary disease), allergic drug reactions, diabetes, acute hepatitis B infection, acute hepatitis C infection, malignancies-solid tumors (prostate, lung, colon, pancreas, stomach, bile duct, head and neck, cervix, breast, melanoma, kidney), hematologic malignancies. It will be appreciated by the skilled artisan that autoimmune disorders may include rheumatoid arthritis, systemic lupus erythematosus,

multiple sclerosis, temporal arteritis, sjögren syndrome, autoimmune hemolytic anemia, goodpasture syndrome, myasthenia gravis, graves' disease, autoimmune hypothyroidism. It will be appreciated by the skilled artisan that allergic reactions may occur from a subject being administered penicillin and its derivatives, sulfamides, phenytoin, chloramphenicol, methyldopa, depot thioxanthene, interferon alfa, fludarabine, bacille calmette-guérin (BCG) vaccination, desvenlafaxine. It will be appreciated by the skilled artisan that hematologic malignancies may include chronic lymphocytic leukemia, non-Hodgkin lymphoma, multiple myeloma, waldenstrom macroglobulinemia, myelodysplastic syndrome, myelofibrosis, and erythroleukemia. Hence, and in one embodiment, provided herein is a method for treating acquired hemophilia in a subject, comprising administering to the subject any of the compositions provided herein.

[0328] In another embodiment, the compositions, formulations, and methods of the present invention are for the treatment or prevention of muscle bleeds. In another embodiment, the compositions, formulations, and methods of the present invention are for the treatment or prevention of joint bleeds. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of epistaxis and gum bleeding, mucous membrane bleeding, bleeding into the central nervous system. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of gastrointestinal or cerebral bleeding. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of low frequency mild bleeds. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of low frequency moderate bleeds. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of high frequency mild bleeds. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of high frequency moderate bleeds.

[0329] In one embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of asymptomatic hemophilia. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of mild to moderate hemophilia. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of severe hemophilia.

[0330] In one embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of hemorrhage, which in one

embodiment, is uncontrollable hemorrhage, and, in another embodiment, intracerebral hemorrhage. In another embodiment, the compositions, formulations, and methods of the present invention provide therapeutic or prophylactic treatment of neonatal coagulopathies; severe hepatic disease; high-risk surgical procedures; traumatic blood loss; bone marrow transplantation; thrombocytopenias and platelet function disorders; urgent reversal of oral anticoagulation; congenital deficiencies of factors V, VII, X, and XI; or von Willebrand disease, in one embodiment, von Willebrand disease with inhibitors to von Willebrand factor.

[0331] In one embodiment, a CTP-modified coagulation factor of the present invention is for the treatment of hemophilia or a related disease as described herein in a subject. In one embodiment, the subject is human. In another embodiment, the subject is a human child. In another embodiment, the subject is a domesticated animal. In another embodiment, the subject is a mammal. In another embodiment, the subject is a farm animal. In another embodiment, the subject is a monkey. In another embodiment, the subject is a horse. In another embodiment, the subject is a cow. In another embodiment, the subject is a mouse. In another embodiment, the subject is a rat. In another embodiment, the subject is canine. In another embodiment, the subject is feline. In another embodiment, the subject is bovine, ovine, porcine, equine, murine, or cervine. In one embodiment, the subject is male. In another embodiment, the subject is female. In one embodiment, the subject is a child, in another embodiment, an adolescent, in another embodiment, an adult or, in another embodiment, an elderly subject. In another embodiment, the subject is a pediatric subject, in another embodiment, a geriatric subject.

[0332] In another embodiment, a [(CTP)n>1-coagulation factor] as described herein comprises a full length coagulation factor or an active fragment thereof connected via a peptide bond on its carboxy terminus to at least one CTP unit with no CTPs on its amino terminus. In another embodiment, a [(CTP)n>1-coagulation factor] as described herein comprises a coagulation factor or an active fragment thereof connected via a peptide bond to at least one CTP unit which is connected to an additional CTP unit via a peptide bond with no CTPs on its amino terminus. In another embodiment, one nucleic acid molecule encodes an engineered coagulation factor comprising at least one CTP attached to its C-terminus and no CTPs on its amino terminus.

[0333] In another embodiment, the CTP is attached to the coagulation factor via a linker. In another embodiment, the linker which connects the CTP sequence to the coagulation factor is a covalent bond. In another embodiment, the linker which connects the CTP sequence to the coagulation factor is a peptide bond. In another embodiment, the linker which connects the CTP sequence to the coagulation factor is a substituted peptide bond. In another embodiment,

the CTP sequence comprises: DPRFQDSSSSKAPPPSLPSPSRLPGPSDTPIL (SEQ ID NO: 1). In another embodiment, the CTP sequence comprises: SSSSKAPPPSLPSPSRLPGPSDTPILPQ (SEQ ID NO: 2). In another embodiment, the CTP sequence comprises an amino acid sequence selected from the sequences set forth in SEQ ID NO: 1 and SEQ ID NO: 2.

[0334] In another embodiment, the carboxy terminal peptide (CTP) peptide of the present invention comprises the amino acid sequence from amino acid 112 to position 145 of human chorionic gonadotropin, as set forth in SEQ ID NO: 1. In another embodiment, the CTP sequence of the present invention comprises the amino acid sequence from amino acid 118 to position 145 of human chorionic gonadotropin, as set forth in SEQ ID NO: 2. In another embodiment, the CTP sequence also commences from any position between positions 112-118 and terminates at position 145 of human chorionic gonadotropin. In some embodiments, the CTP sequence peptide is 28, 29, 30, 31, 32, 33 or 34 amino acids long and commences at position 112, 113, 114, 115, 116, 117 or 118 of the CTP amino acid sequence.

[0335] In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 1-5 conservative amino acid substitutions as described in U.S. Pat. No. 5,712,122, which is incorporated herein by reference. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 1 conservative amino acid substitution. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 2 conservative amino acid substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 3 conservative amino acid substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 4 conservative amino acid substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 5 conservative amino acid substitutions.

[0336] In another embodiment, the CTP peptide amino acid sequence of the present invention is at least 70% homologous to the native CTP amino acid sequence or a peptide thereof. In another embodiment, the CTP peptide amino acid sequence of the present invention is at least 80% homologous to the native CTP amino acid sequence or a peptide thereof. In another embodiment, the CTP peptide amino acid sequence of the present invention is at least 90% homologous to the native CTP amino acid sequence or a peptide thereof. In another embodiment, the CTP peptide amino acid sequence of the present invention is at least 95% homologous to the native CTP amino acid sequence or a peptide thereof. In another embodiment, the CTP peptide amino acid sequence of the present invention is at least 98%

homologous to the native CTP amino acid sequence or a peptide thereof.

[0337] In another embodiment, the polynucleotide encoding the CTP peptide of the present invention is at least 70% homologous to the native human CTP DNA sequence or a peptide thereof. In another embodiment, the polynucleotide encoding the CTP peptide of the present invention is at least 80% homologous to the native human CTP DNA sequence or a peptide thereof. In another embodiment, the polynucleotide encoding the CTP peptide of the present invention is at least 90% homologous to the native CTP DNA sequence or a peptide thereof. In another embodiment, the polynucleotide encoding the CTP peptide of the present invention is at least 95% homologous to the native CTP DNA sequence or a peptide thereof. In another embodiment, the polynucleotide encoding the CTP peptide of the present invention is at least 98% homologous to the native CTP DNA sequence or a peptide thereof.

[0338] In one embodiment, at least one of the chorionic gonadotropin CTP amino acid sequences is truncated. In another embodiment, both of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, 2 of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, 3 of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, 4 of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, 5 of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, 2 or more of the chorionic gonadotropin CTP amino acid sequences are truncated. In another embodiment, all of the chorionic gonadotropin CTP amino acid sequences are truncated. In one embodiment, the truncated CTP comprises the first 10 amino acids of SEQ ID NO: 3. In another embodiment, SEQ ID NO: 3 comprises the following amino acid (AA) sequence: SSSSKAPPPSLP.

[0339] In one embodiment, the truncated CTP comprises the first 10 amino acids of SEQ ID NO: 2. In another embodiment, SEQ ID NO: 2 comprises the following amino acid (AA) sequence: SSSSKAPPPSLPSPSRLPGPSDTPILPQ.

[0340] In one embodiment, the truncated CTP comprises the first 11 amino acids of SEQ ID NO: 2. In one embodiment, the truncated CTP comprises the first 12 amino acids of SEQ ID NO: 2. In one embodiment, the truncated CTP comprises the first 8 amino acids of SEQ ID NO: 2 or SEQ ID NO: 3. In one embodiment, the truncated CTP comprises the first 13 amino acids of SEQ ID NO: 2. In one embodiment, the truncated CTP comprises the first 14 amino acids of SEQ ID NO: 2. In one embodiment, the truncated CTP comprises the first 6 amino acids of SEQ ID NO: 2 or SEQ ID NO: 3. In one embodiment, the truncated CTP comprises the first 5 amino acids of SEQ ID NO: 2 or SEQ ID NO: 3.

[0341] In one embodiment, at least one of the chorionic gonadotropin CTP amino acid

sequences is glycosylated. In another embodiment, both of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, 2 of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, 3 of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, 4 of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, 5 of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, 2 or more of the chorionic gonadotropin CTP amino acid sequences are glycosylated. In another embodiment, all of the chorionic gonadotropin CTP amino acid sequences are glycosylated.

[0342] In one embodiment, the CTP sequence of the present invention comprises at least one glycosylation site. In one embodiment, the CTP sequence of the present invention comprises 2 glycosylation sites. In one embodiment, the CTP sequence of the present invention comprises 3 glycosylation sites. In one embodiment, the CTP sequence of the present invention comprises 4 glycosylation sites. In one embodiment, one or more of the chorionic gonadotropin CTP amino acid sequences is fully glycosylated. In another embodiment, one or more of the chorionic gonadotropin CTP amino acid sequences is partially glycosylated. In one embodiment, partially glycosylated indicates that one of the CTP glycosylation sites is glycosylated. In another embodiment, two of the CTP glycosylation sites are glycosylated. In another embodiment, three of the CTP glycosylation sites are glycosylated.

[0343] In some embodiments, the CTP sequence modification is advantageous in permitting the usage of lower dosages. In some embodiments, the CTP sequences modification is advantageous in permitting fewer dosages. In some embodiments, the CTP sequences modification is advantageous in permitting a safe, long-acting effect.

[0344] In some embodiments, "polypeptide", "engineered coagulation factor", or "protein" as used herein encompasses native polypeptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides) and peptidomimetics (typically, synthetically synthesized polypeptides), as well as peptoids and semipeptoids which are polypeptide analogs, which have, in some embodiments, modifications rendering the polypeptides comprising a coagulation factor even more stable while in a body or more capable of penetrating into cells.

[0345] In some embodiments, modifications include, but are limited to C terminus modification, polypeptide bond modification, including, but not limited to, CH2-NH, CH2-S, CH2-S=O, O=C-NH, CH2-O, CH2-CH2, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A.

Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

[0346] In some embodiments, polypeptide bonds (-CO-NH-) within the polypeptide are substituted. In some embodiments, the polypeptide bonds are substituted by N-methylated bonds (-N(CH3)-CO-). In some embodiments, the polypeptide bonds are substituted by ester bonds (-C(R)H-C-O-O-C(R)-N-). In some embodiments, the polypeptide bonds are substituted by ketomethylen bonds (-CO-CH2-). In some embodiments, the polypeptide bonds are substituted by α-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH2-NH-). In some embodiments, the polypeptide bonds are substituted by hydroxyethylene bonds (-CH(OH)-CH2-). In some embodiments, the polypeptide bonds are substituted by thioamide bonds (-CS-NH-). In some embodiments, the polypeptide bonds are substituted by olefinic double bonds (-CH=CH-). In some embodiments, the polypeptide bonds are substituted by retro amide bonds (-NH-CO-). In some embodiments, the polypeptide bonds are substituted by polypeptide derivatives (-N(R)-CH2-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom. In some embodiments, these modifications occur at any of the bonds along the polypeptide chain and in one embodiment at several (2-3 bonds) at the same time.

[0347] In some embodiments, natural aromatic amino acids of the polypeptide such as Trp, Tyr and Phe, are substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr. In some embodiments, the polypeptides of the present invention include one or more modified amino acid or one or more non-amino acid monomers (e.g. fatty acid, complex carbohydrates etc).

[0348] In one embodiment, "amino acid" or "amino acid sequence" is understood to include the 20 naturally occurring amino acid; those amino acid often modified post-translationally in vivo, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acid including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. In one embodiment, "amino acid" includes both D- and L-amino acids.

[0349] In some embodiments, the polypeptides of the present invention are utilized in therapeutics which requires the polypeptides comprising a coagulation factor to be in a soluble form. In some embodiments, the polypeptides of the present invention include one or more non-natural or natural polar amino acid, including but not limited to serine and threonine which are capable of increasing polypeptide solubility due to their hydroxyl-containing side chain.

[0350] In some embodiments, the engineered coagulation factor of the present invention is utilized in a linear form, although it will be appreciated by one skilled in the art that in cases where cyclicization does not severely interfere with engineered coagulation factors characteristics, cyclic forms of the engineered coagulation factors can also be utilized.

[0351] In some embodiments, the engineered coagulation factors of the present invention are biochemically synthesized such as by using standard solid phase techniques. In some embodiments, these biochemical methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, or classical solution synthesis.

[0352] In some embodiments, recombinant protein techniques are used to generate the engineered coagulation factors of the present invention. In some embodiments, recombinant protein techniques are used for the generation of relatively long polypeptides (e.g., longer than 18-25 amino acids). In some embodiments, recombinant protein techniques are used for the generation of large amounts of the engineered coagulation factors of the present invention. In some embodiments, recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463, which are incorporated herein by reference in their entirety.

[0353] In another embodiment, the disclosure provides a polynucleotide molecule comprising the coding portion of a gene encoding a polypeptide comprising a coagulation factor and gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove. In another embodiment, the disclosure provides a polynucleotide molecule consisting of the coding portion of a gene encoding a polypeptide comprising a coagulation factor and gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove. In another embodiment, the disclosure provides a polynucleotide molecule consisting essentially of the coding portion of a gene encoding a polypeptide comprising a coagulation factor and gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove.

[0354] In another embodiment, the disclosure provides a polynucleotide encoding a polypeptide comprising a coagulation factor and three gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove. In another embodiment, the disclosure provides a polynucleotide encoding a polypeptide

consisting of a coagulation factor and three gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove. In another embodiment, the disclosure provides a polynucleotide encoding a polypeptide consisting essentially of a coagulation factor and three gonadotropin carboxy terminal peptides attached to the carboxy terminus of the coagulation factor, as described hereinabove. In one embodiment, the polynucleotide is a polynucleotide sequence. In one embodiment, the polynucleotide is a polynucleotide molecule.

[0355] In another embodiment, the disclosure provides an expression vector comprising a polynucleotide molecule as described herein. In another embodiment, the present invention provides an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor IX (FIX) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide. In another embodiment, the present invention provides an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three to five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0356] In another embodiment, the disclosure provides a cell comprising the expression vector as described herein. In another embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor IX (FIX) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide. In another embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0357] In another embodiment, the disclosure provides a composition comprising the expression vector as described herein. In another embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor IX (FIX) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide. In another embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0358] In another embodiment, the disclosure provides a composition comprising the cell as

described herein. In another embodiment, the cell is a eukaryotic cell. In another embodiment, the cell is a prokaryotic cell.

[0359] In another embodiment, the present invention provides a method of producing a CTP-modified coagulation factor, comprising the step of attaching one to ten chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said coagulation factor, thereby producing a CTP-modified coagulation factor. In another embodiment, the present invention provides a method of producing a CTP-modified coagulation factor, comprising the step of attaching one to ten polynucleotide sequences encoding a chorionic gonadotropin carboxy terminal peptide (CTP) to the carboxy terminus of a polynucleotide sequence encoding said coagulation factor, thereby producing a CTP-modified coagulation factor. In another embodiment, the present invention provides a method of producing a CTP-modified Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby producing a CTP-modified FIX polypeptide. In another embodiment, the present invention provides a method of producing a CTP-modified Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby producing a CTP-modified FVIIa polypeptide.

[0360] In another embodiment, the engineered coagulation factors of the present invention are synthesized using a polynucleotide molecule encoding a polypeptide of the present invention. In some embodiments, the polynucleotide molecule encoding the engineered coagulation factors of the present invention is ligated into an expression vector, comprising a transcriptional control of a cis-regulatory sequence (e.g., promoter sequence). In some embodiments, the cis-regulatory sequence is suitable for directing constitutive expression of an engineered coagulation factor of the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing tissue-specific expression of the engineered coagulation factors of the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing inducible expression of the engineered coagulation factors of the present invention.

[0361] In some embodiment, tissue-specific promoters suitable for use with the present invention include sequences which are functional in one or more specific cell populations. Examples include, but are not limited to, promoters such as albumin that is liver-specific [Pinkert et al., (1987) Genes Dev. 1:268-277], lymphoid-specific promoters [Calame et al., (1988) Adv. Immunol. 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) EMBO J. 8:729-733] and immunoglobulins; [Banerji et al. (1983) Cell 33729-740],

neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) Proc. Natl. Acad. Sci. USA 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) Science 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Inducible promoters suitable for use with the present invention include, for example, the tetracycline-inducible promoter (Srour, M.A., et al., 2003. Thromb. Haemost. 90: 398-405).

[0362] In one embodiment, the phrase "a polynucleotide molecule" refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

[0363] In one embodiment, a "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA-dependent DNA polymerase. In one embodiment, the sequence can be subsequently amplified in vivo or in vitro using a DNA polymerase.

[0364] In one embodiment, a "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

[0365] In one embodiment, a "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. In one embodiment, a composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. In one embodiment, the intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. In one embodiment, intronic sequences include cis-acting expression regulatory elements.

[0366] In one embodiment, following expression and secretion, the signal peptides are cleaved from the precursor engineered coagulation factors resulting in the mature engineered coagulation factors lacking a signal peptide.

[0367] In some embodiments, polynucleotides of the present invention are prepared using PCR techniques, or any other method or procedure known to one skilled in the art. In some embodiments, the procedure involves the ligation of two different DNA sequences (See, for example, "Current Protocols in Molecular Biology", eds. Ausubel et al., John Wiley & Sons, 1992).

[0368] In one embodiment, polynucleotides of the present invention which encode the engineered coagulation factors are inserted into expression vectors (i.e., a nucleic acid construct) to enable expression of the recombinant polypeptide. In one embodiment, the

expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes. In one embodiment, the expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in eukaryotes. In one embodiment, the expression vector of the present invention includes a shuttle vector which renders this vector suitable for replication and integration in both prokaryotes and eukaryotes. In some embodiments, cloning vectors comprise transcription and translation initiation sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals).

[0369] In one embodiment, a variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the coagulation factors of the present invention. In some embodiments, these include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the polypeptide coding sequence; yeast transformed with recombinant yeast expression vectors containing the polypeptide coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the polypeptide coding sequence.

[0370] In some embodiments, non-bacterial expression systems are used (e.g. mammalian expression systems such as CHO cells) to express the coagulation factors of the present invention. In one embodiment, the expression vector used to express polynucleotides of the present invention in mammalian cells is pCI-DHFR vector comprising a CMV promoter and a neomycin resistance gene. Construction of the pCI-dhfr vector is described, according to one embodiment, in Example 1.

[0371] In some embodiments, in bacterial systems of the present invention, a number of expression vectors can be advantageously selected depending upon the use intended for the polypeptide expressed. In one embodiment, large quantities of polypeptide are desired. In one embodiment, vectors that direct the expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified are desired. In one embodiment, certain fusion proteins are engineered with a specific cleavage site to aid in recovery of the polypeptide. In one embodiment, vectors adaptable to such manipulation include, but are not limited to, the pET series of E. coli expression vectors [Studier et al., Methods in Enzymol. 185:60-89 (1990)].

[0372] In one embodiment, yeast expression systems are used. In one embodiment, a number

of vectors containing constitutive or inducible promoters can be used in yeast as disclosed in U.S. Pat. Application. No: 5,932,447, which is incorporated by reference herein in its entirety. In another embodiment, vectors which promote integration of foreign DNA sequences into the yeast chromosome are used.

[0373] In one embodiment, the expression vector of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

[0374] In some embodiments, mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

[0375] In some embodiments, expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses are used in the present invention. SV40 vectors include pSVT7 and pMT2. In some embodiments, vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0376] In some embodiments, recombinant viral vectors are useful for in vivo expression of the coagulation factors of the present invention since they offer advantages such as lateral infection and targeting specificity. In one embodiment, lateral infection is inherent in the life cycle of, for example, a retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. In one embodiment, the result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. In one embodiment, viral vectors are produced that are unable to spread laterally. In one embodiment, this characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

[0377] In one embodiment, various methods can be used to introduce the expression vector of the present invention into cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York

(1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992, incorporated herein by reference, for positive-negative selection methods.

[0378] In some embodiments, introduction of nucleic acid by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

[0379] In one embodiment, it will be appreciated that the engineered coagulation factors of the present invention can also be expressed from a nucleic acid construct administered to the individual employing any suitable mode of administration, described hereinabove (i.e., in vivo gene therapy). In one embodiment, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (i.e., ex vivo gene therapy).

[0380] In one embodiment, plant expression vectors are used. In one embodiment, the expression of a polypeptide coding sequence is driven by a number of promoters. In some embodiments, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV [Brisson et al., Nature 310:511-514 (1984)], or the coat protein promoter to TMV [Takamatsu et al., EMBO J. 6:307-311 (1987)] are used. In another embodiment, plant promoters are used such as, for example, the small subunit of RUBISCO [Coruzzi et al., EMBO J. 3:1671-1680 (1984); and Brogli et al., Science 224:838-843 (1984)] or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B [Gurley et al., Mol. Cell. Biol. 6:559-565 (1986)]. In one embodiment, constructs are introduced into plant cells using Ti plasmid, Ri plasmid, plant viral vectors, direct DNA transformation, microinjection, electroporation and other techniques well known to the skilled artisan. See, for example, Weissbach & Weissbach [Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463 (1988)]. Other expression systems such as insects and mammalian host cell systems, which are well known in the art, can also be used by the present invention.

[0381] It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the polypeptide), the expression construct of the present invention can also include sequences engineered to

optimize stability, production, purification, yield or activity of the expressed polypeptide.

[0382] In some embodiments, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant engineered coagulation factors. In some embodiments, effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. In one embodiment, an effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. In some embodiments, a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. In some embodiments, cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. In some embodiments, culturing is carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. In some embodiments, the determination of culturing conditions are within the expertise of one of ordinary skill in the art.

[0383] In some embodiments, depending on the vector and host system used for production, resultant engineered coagulation factors of the present invention either remain within the recombinant cell, are secreted into the fermentation medium, are secreted into a space between two cellular membranes, such as the periplasmic space in E. coli; or are retained on the outer surface of a cell or viral membrane.

[0384] In one embodiment, following a predetermined time in culture, recovery of the recombinant engineered coagulation factor is effected.

[0385] In one embodiment, the phrase "recovering the recombinant engineered coagulation factor" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

[0386] In one embodiment, engineered coagulation factors of the present invention are purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

[0387] In one embodiment, to facilitate recovery, the expressed coding sequence can be engineered to encode the engineered coagulation factor of the present invention and fused cleavable moiety. In one embodiment, a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. In one embodiment, a cleavage site is engineered

between the engineered coagulation factor and the cleavable moiety and the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth et al., Immunol. Lett. 19:65-70 (1988); and Gardella et al., J. Biol. Chem. 265:15854-15859 (1990)].

[0388] In one embodiment, the engineered coagulation factor of the present invention is retrieved in "substantially pure" form.

[0389] In one embodiment, the phrase "substantially pure" refers to a purity that allows for the effective use of the protein in the applications described herein.

[0390] In one embodiment, the engineered coagulation factor of the present invention can also be synthesized using in vitro expression systems. In one embodiment, in vitro synthesis methods are well known in the art and the components of the system are commercially available.

[0391] In some embodiments, the recombinant engineered coagulation factors are synthesized and purified; their therapeutic efficacy can be assayed either in vivo or in vitro. In one embodiment, the binding activities of the recombinant engineered coagulation factors of the present invention can be ascertained using various assays as known to one of skill in the art.

[0392] In another embodiment, the engineered coagulation factor of the present invention can be provided to the individual per se. In one embodiment, the engineered coagulation factor of the present invention can be provided to the individual as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

[0393] In another embodiment, a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[0394] In another embodiment, "active ingredient" refers to the polypeptide sequence of interest, which is accountable for the biological effect.

[0395] In another embodiment, any of the compositions of the present invention will comprise at least one CTP sequence bound only to the carboxy terminus of an engineered coagulation factor of interest, in any form. In one embodiment, the present invention provides combined preparations. In one embodiment, "a combined preparation" defines especially a "kit of parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners i.e., simultaneously, concurrently, separately or sequentially. In some embodiments, the parts of the kit of parts can then, e.g., be administered simultaneously or

chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners, in some embodiments, can be administered in the combined preparation. In one embodiment, the combined preparation can be varied, e.g., in order to cope with the needs of a patient subpopulation to be treated or the needs of the single patient which different needs can be due to a particular disease, severity of a disease, age, sex, or body weight as can be readily made by a person skilled in the art.

[0396] In another embodiment, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which are interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. In one embodiment, one of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979)).

[0397] In another embodiment, "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. In one embodiment, excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

[0398] Techniques for formulation and administration of drugs are found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

[0399] Various embodiments of dosage ranges are contemplated by this invention. The dosage of the engineered coagulation factor of the present invention, in one embodiment, is in the range of 0.005-100 mg/day. In another embodiment, the dosage is in the range of 0.005-5 mg/day. In another embodiment, the dosage is in the range of 0.01-50 mg/day. In another embodiment, the dosage is in the range of 0.1-20 mg/day. In another embodiment, the dosage is in the range of 0.1-10 mg/day. In another embodiment, the dosage is in the range of 0.01-5 mg/day. In another embodiment, the dosage is in the range of 0.001-0.01 mg/day. In another embodiment, the dosage is in the range of 0.001-0.1 mg/day. In another embodiment, the dosage is in the range of 0.1-5 mg/day. In another embodiment, the dosage is in the range of 0.5-50 mg/day. In another embodiment, the dosage is in the range of 0.2-15mg/day. In another embodiment, the dosage is in the range of 0.8-65 mg/day. In another embodiment, the dosage is in the range of 1-50 mg/day. In another embodiment, the dosage is in the range of 5-10 mg/day. In another embodiment, the dosage is in the range of 8-15 mg/day. In another

embodiment, the dosage is in a range of 10-20mg/day. In another embodiment, the dosage is in the range of 20-40 mg/day. In another embodiment, the dosage is in a range of 60-120 mg/day. In another embodiment, the dosage is in the range of 12-40 mg/day. In another embodiment, the dosage is in the range of 40-60 mg/day. In another embodiment, the dosage is in a range of 50-100mg/day. In another embodiment, the dosage is in a range of 1-60 mg/day. In another embodiment, the dosage is in the range of 15-25 mg/day. In another embodiment, the dosage is in the range of 5-10 mg/day. In another embodiment, the dosage is in the range of 55-65 mg/day.

[0400] In another embodiment, the dosage is in a range of 50-500 mg/day. In another embodiment, the dosage is in a range of 50-150 mg/day. In another embodiment, the dosage is in a range of 100-200 mg/day. In another embodiment, the dosage is in a range of 150-250 mg/day. In another embodiment, the dosage is in a range of 200-300 mg/day. In another embodiment, the dosage is in a range of 250-400 mg/day. In another embodiment, the dosage is in a range of 300-500 mg/day. In another embodiment, the dosage is in a range of 350-500 mg/day.

[0401] In one embodiment, the dosage is 20 mg/day. In one embodiment, the dosage is 30 mg/day. In one embodiment, the dosage is 40 mg/day. In one embodiment, the dosage is 50 mg/day. In one embodiment, the dosage is 0.01 mg/day. In another embodiment, the dosage is 0.1 mg/day. In another embodiment, the dosage is 1 mg/day. In another embodiment, the dosage is 0.530 mg/day. In another embodiment, the dosage is 0.05 mg/day. In another embodiment, the dosage is 50 mg/day. In another embodiment, the dosage is 10 mg/day. In another embodiment, the dosage is 20-70 mg/day. In another embodiment, the dosage is 5 mg/day.

[0402] In one embodiment, the dosage of the CTP-modified coagulation factor is 1-5 mg/day. In one embodiment, the dosage of the CTP-modified coagulation factor is 1-3 mg/day. In another embodiment, the dosage of the CTP-modified coagulation factor is 2 mg/day.

[0403] In another embodiment, the dosage is 1-90 mg/day. In another embodiment, the dosage is 1-90 mg/2 days. In another embodiment, the dosage is 1-90 mg/3 days. In another embodiment, the dosage is 1-90 mg/4 days. In another embodiment, the dosage is 1-90 mg/5 days. In another embodiment, the dosage is 1-90 mg/6 days. In another embodiment, the dosage is 1-90 mg/week. In another embodiment, the dosage is 1-90 mg/9 days. In another embodiment, the dosage is 1-90 mg/11 days. In another embodiment, the dosage is 1-90 mg/14 days.

[0404] In another embodiment, the coagulation factor dosage is 10-50 mg/day. In another embodiment, the dosage is 10-50 mg/2 days. In another embodiment, the dosage is 10-50

mg/3 days. In another embodiment, the dosage is 10-50 mg/4 days. In another embodiment, the dosage is 10-50 micrograms mg/5 days. In another embodiment, the dosage is 10-50 mg/6 days. In another embodiment, the dosage is 10-50 mg/week. In another embodiment, the dosage is 10-50 mg/9 days. In another embodiment, the dosage is 10-50 mg/11 days. In another embodiment, the dosage is 10-50 mg/14 days.

[0405] In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is formulated in an intranasal dosage form. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is formulated in an injectable dosage form. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 0.0001 mg to 0.6 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 0.001 mg to 0.005 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 0.005 mg to 0.01 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 0.01 mg to 0.3 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose in a dose ranging from 0.2 mg to 0.6 mg. In another embodiment, the coagulation factor is free of CTPs on its amino terminus.

[0406] In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 1-100 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 10-80 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 20-60 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 10-50 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 40-80 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 10-30 micrograms. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 30-60 micrograms.

[0407] In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 0.2 mg to 2 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is

administered to a subject in a dose ranging from 2 mg to 6 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 4 mg to 10 mg. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject in a dose ranging from 5 mg and 15 mg.

[0408] In one embodiment, coagulation factor modified by CTPs is administered to a subject in a dose ranging from 10μg/kg-1000μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose ranging from 25μg/kg-600μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose ranging from 50μg/kg-400μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 25μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 50μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 100μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 200μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 300μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 400μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 500μg/kg. In another embodiment, coagulation factor modified by CTPs is administered to a subject in a dose of about 600μg/kg.

[0409] In one embodiment, the dosage of the CTP-modified FIX comprises 50% of the amount of FIX administered in the recommended dosage of recombinant FIX (e.g., Benefix®, Wyeth or Mononine®, CSL Behring) to patients over the same period of time. In one embodiment, the dosage of the CTP-modified FVIIa comprises 50% of the amount of FVIIa administered in the recommended dosage of recombinant FVIIa (e.g., NovoSeven®) to patients over the same period of time. In one embodiment, the dosage of the CTP-modified FVII comprises 50% of the amount of FVII administered in the recommended dosage of recombinant FVII to patients over the same period of time. For example, if NovoSeven® is given at a dose of 90 mcg/kg every two hours to a patient pre- or post-operatively (i.e., 7.65 mg every two hours or 45.9 mg in six doses over a 12 hour period, for an 85 kg patient), a CTP-modified coagulation factor of the present invention may be given at a dose that is 50% of the patient's 12-hour dose of recombinant FVIIa (i.e., at a dose of 23 mg given once over a 12-hour period).

[0410] In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 45% of the amount of the coagulation factor than that administered using the non-

CTP-modified coagulation factor. In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 10% of the amount of the coagulation factor than that administered using the non-CTP-modified coagulation factor. In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 25% of the amount of the coagulation factor than that administered using the non-CTP-modified coagulation factor. In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 35% of the amount of the coagulation factor than that administered using the non-CTP-modified coagulation factor. In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 75% of the amount of the coagulation factor than that administered using the non-CTP-modified coagulation factor. In another embodiment, the dosage of CTP-modified coagulation factor is such that it contains 100% of the amount of the coagulation factor than that administered using the non-CTP-modified coagulation factor. However, even if the dosage contains the same amount of coagulation factor (e.g. FIX) as non-CTP-modified coagulation factor, it is still advantageous to subjects in that it will be administered less frequently because of its increased half-life compared to recombinant coagulation factors.

[0411] In another embodiment, a therapeutically effective amount of a conjugated coagulation factor is between 50-500 IU per kg body weight administered once a day to once a week for FIX or 10μg/Kg-500μg/Kg for FVIIa. In another embodiment, a therapeutically effective amount of a conjugated coagulation factor is 150-250 IU per kg body weight, administered once a day. In another embodiment, a pharmaceutical composition comprising a conjugated coagulation factor is formulated at a strength effective for administration by various means to a human patient.

[0412] In one embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 20-30 IU/dL in a subject. In another embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 25-50 IU/dL in a subject. In another embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 50-100 IU/dL in a subject. In another embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 100-200 IU/dL in a subject. In another embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 10-50 IU/dL in a subject. In another embodiment, FIX is administered in an amount effective to bring circulating Factor IX activity to 20-100 IU/dL in a subject.

[0413] In one embodiment, the CTP-modified coagulation factor is administered to a subject on a weekly basis. In another embodiment, the CTP-modified coagulation factor is administered to a subject twice a week. In another embodiment, the CTP-modified

coagulation factor is administered to a subject on a fortnightly (once every two weeks) basis. In another embodiment, the CTP-modified coagulation factor is administered to a subject twice a month. In another embodiment, the CTP-modified coagulation factor is administered to a subject once a month. In another embodiment, the CTP-modified coagulation factor is administered to a subject on a daily basis. In another embodiment, the CTP-modified coagulation factor is administered to a subject every two days.

[0414] In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every three days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every four days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every five days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every six days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every 7-14 days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every 10-20 days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every 5-15 days. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is administered to a subject once every 15-30 days.

[0415] In another embodiment, the methods of the disclosure include increasing the compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and at least one chorionic gonadotropin carboxy terminal peptide (CTP) attached to the carboxy terminus of the coagulation factor, thereby increasing compliance in the use of coagulation factor therapy.

[0416] In another embodiment, the methods of the disclosure include increasing the compliance of patients afflicted with chronic illnesses that are in need of a coagulation factor therapy. In another embodiment, the methods of the disclosure enable reduction in the dosing frequency of a coagulation factor by modifying the coagulation factor with CTPs as described hereinabove.

[0417] In another embodiment, the present invention provides a method of reducing the dosing frequency of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby reducing the dosing frequency of said FIX polypeptide. In another embodiment, the present invention provides a method of reducing the dosing frequency of a

Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby reducing the dosing frequency of said FVIIa polypeptide.

[0418] In another embodiment, the term compliance comprises adherence. In another embodiment, the methods of the disclosure include increasing the compliance of patients in need of a coagulation factor therapy by reducing the frequency of administration of the coagulation factor. In another embodiment, reduction in the frequency of administration of the coagulation factor is achieved due to the CTP modifications which render the CTP-modified coagulation factor more stable. In another embodiment, reduction in the frequency of administration of the coagulation factor is achieved as a result of increasing T½ of the coagulation factor. In another embodiment, reduction in the frequency of administration of the coagulation factor is achieved as a result of increasing the clearance time or reducing the clearance rate of the coagulation factor.

[0419] In another embodiment, the present invention provides a method of reducing the clearance rate of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby reducing the clearance rate of said FIX polypeptide. In another embodiment, the present invention provides a method of reducing the clearance rate of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby reducing the clearance rate of said FVIIa polypeptide.

[0420] In another embodiment, reduction in the frequency of administration of the coagulation factor is achieved as a result of increasing the AUC measure of the coagulation factor.

[0421] In another embodiment, provided herein is a method of reducing the dosing frequency of a coagulation factor, comprising the step of attaching one to ten CTPs to the carboxy terminus of the coagulation factor, thereby reducing a dosing frequency of the coagulation factor. In another embodiment, provided herein is a method of reducing the dosing frequency of a coagulation factor, comprising the step of attaching one to five CTPs to the carboxy terminus of the coagulation factor, thereby reducing a dosing frequency of the coagulation factor. In another embodiment, provided herein is a method of reducing the dosing frequency of a coagulation factor, comprising the step of attaching three CTPs to the carboxy terminus of the coagulation factor, thereby reducing a dosing frequency of the coagulation factor. In another embodiment, provided herein is a method of reducing the dosing frequency of a coagulation factor, comprising the step of attaching three to five CTPs to the carboxy

terminus of the coagulation factor, thereby reducing a dosing frequency of the coagulation factor.

[0422] In another embodiment, provided herein is a method of increasing compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and one to ten chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby increasing compliance in the use of coagulation factor therapy. In another embodiment, provided herein is a method of increasing compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and one to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby increasing compliance in the use of coagulation factor therapy. In another embodiment, provided herein is a method of increasing compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby increasing compliance in the use of coagulation factor therapy. In another embodiment, provided herein is a method of increasing compliance in the use of coagulation factor therapy, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby increasing compliance in the use of coagulation factor therapy.

[0423] In another embodiment, provided herein is a method of preventing or treating a blood clotting or coagulation disorder in a subject, comprising providing to said subject a polypeptide comprising a coagulation factor and one to ten chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby treating a blood clotting or coagulation disorder in said subject. In another embodiment, provided herein is a method of preventing or treating a blood clotting or coagulation disorder in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and one to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing or treating a blood clotting or coagulation disorder in said subject. In another embodiment, provided herein is a method of preventing or treating a blood clotting or coagulation disorder in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing or treating a blood clotting or coagulation disorder in said subject. In another embodiment, provided herein is a method of preventing or treating a

blood clotting or coagulation disorder in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing or treating a blood clotting or coagulation disorder in said subject.

[0424] In another embodiment, provided herein is a method of preventing hemophilia in a subject, comprising providing to said subject a polypeptide comprising a coagulation factor and one to ten chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing hemophilia in said subject. In another embodiment, provided herein is a method of preventing hemophilia in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and one to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing hemophilia in said subject. In another embodiment, provided herein is a method of preventing hemophilia in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing hemophilia in said subject. In another embodiment, provided herein is a method of preventing hemophilia in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby preventing hemophilia in said subject.

[0425] In another embodiment, the present invention shows that the compositions provided herein are surprisingly more effectively absorbed into the bloodstream after SC administration (see Examples 7-9 herein). To be able to administer FVIIa subcutaneously serves as an advantage as it can be used for prophylactic applications. Subcutaneous injections are also much easier for patients to self-inject, and are advantage when the patients are very young and their veins are small and difficult to find.

[0426] In another embodiment, provided herein is a method of treating hemophilia in a subject, comprising providing to said subject a polypeptide comprising a coagulation factor and one to ten chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby treating hemophilia in said subject. In another embodiment, provided herein is a method of treating hemophilia in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and one to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby treating hemophilia in said subject. In another embodiment, provided herein is a method of treating hemophilia in a subject, comprising providing to a

subject in need thereof, a polypeptide comprising a coagulation factor and three chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby treating hemophilia in said subject. In another embodiment, provided herein is a method of treating hemophilia in a subject, comprising providing to a subject in need thereof, a polypeptide comprising a coagulation factor and three to five chorionic gonadotropin carboxy terminal peptides attached to the carboxy terminus of a coagulation factor, thereby treating hemophilia in said subject.

[0427] Oral administration, in one embodiment, comprises a unit dosage form comprising tablets, capsules, lozenges, chewable tablets, suspensions, emulsions and the like. Such unit dosage forms comprise a safe and effective amount of the desired coagulation factor of the disclosure, each of which is in one embodiment, from about 0.7 or 3.5 mg to about 280 mg/70 kg, or in another embodiment, about 0.5 or 10 mg to about 210 mg/70 kg. The pharmaceutically-acceptable carriers suitable for the preparation of unit dosage forms for peroral administration are well-known in the art. In some embodiments, tablets typically comprise conventional pharmaceutically-compatible adjuvants as inert diluents, such as calcium carbonate, sodium carbonate, mannitol, lactose and cellulose; binders such as starch, gelatin and sucrose; disintegrants such as starch, alginic acid and croscarmelose; lubricants such as magnesium stearate, stearic acid and talc. In one embodiment, glidants such as silicon dioxide can be used to improve flow characteristics of the powder-mixture. In one embodiment, coloring agents, such as the FD&C dyes, can be added for appearance. Sweeteners and flavoring agents, such as aspartame, saccharin, menthol, peppermint, and fruit flavors, are useful adjuvants for chewable tablets. Capsules typically comprise one or more solid diluents disclosed above. In some embodiments, the selection of carrier components depends on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of this invention, and can be readily made by a person skilled in the art.

[0428] In one embodiment, the oral dosage form comprises predefined release profile. In one embodiment, the oral dosage form of the present invention comprises an extended release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises a slow release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises an immediate release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form is formulated according to the desired release profile of the pharmaceutical active ingredient as known to one skilled in the art.

[0429] Peroral compositions, in some embodiments, comprise liquid solutions, emulsions,

suspensions, and the like. In some embodiments, pharmaceutically-acceptable carriers suitable for preparation of such compositions are well known in the art. In some embodiments, liquid oral compositions comprise from about 0.001% to about 0.933% of the desired compound or compounds, or in another embodiment, from about 0.01% to about 10%.

[0430] In some embodiments, compositions for use in the methods of this invention comprise solutions or emulsions, which in some embodiments are aqueous solutions or emulsions comprising a safe and effective amount of the compounds of the present invention and optionally, other compounds, intended for topical intranasal administration. In some embodiments, h compositions comprise from about 0.001% to about 10.0% w/v of a subject compound, more preferably from about 00.1% to about 2.0, which is used for systemic delivery of the compounds by the intranasal route.

[0431] In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is injected into the muscle (intramuscular injection). In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is injected below the skin (subcutaneous injection). In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is injected into the muscle. In another embodiment, a polypeptide comprising a coagulation factor and at least one CTP unit is injected into the skin. In another embodiment, a coagulation factor as described herein is administered via systemic administration. In another embodiment, a coagulation factor as described herein is administered by intravenous injection. In another embodiment, administration can be parenteral, pulmonary, oral, topical, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, transnasal, intraocular, ophthalmic, epidural, buccal, rectal, transmucosal, intestinal or parenteral delivery, including intramedullary injections as well as intrathecal or direct intraventricular administration.

[0432] In another embodiment, the preparation is administered in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

[0433] In one embodiment, the route of administration may be enteral. In another embodiment, the route may be conjunctival, transdermal, intradermal, intra-arterial, vaginal, rectal, intratumoral, parcanceral, transmucosal, intramuscular, intravascular, intraventricular, intracranial, intra-nasal, sublingual, or a combination thereof.

[0434] In another embodiment, the pharmaceutical compositions and pharmaceutical formulations are administered by intravenous, intra-arterial, or intramuscular injection of a liquid preparation. In some embodiments, liquid formulations include solutions, suspensions,

dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions and pharmaceutical formulations are administered intravenously, and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions and pharmaceutical formulations are administered intra-arterially, and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions and pharmaceutical formulations are administered intramuscularly, and are thus formulated in a form suitable for intramuscular administration.

[0435] Further, in another embodiment, the pharmaceutical compositions and pharmaceutical formulations are administered topically to body surfaces, and are thus formulated in a form suitable for topical administration. Suitable topical formulations include gels, ointments, creams, lotions, drops and the like. For topical administration, the compounds of the present invention are combined with an additional appropriate therapeutic agent or agents, prepared and applied as solutions, suspensions, or emulsions in a physiologically acceptable diluent with or without a pharmaceutical carrier.

[0436] In one embodiment, pharmaceutical compositions and pharmaceutical formulations of the present invention are manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0437] In one embodiment, pharmaceutical compositions and pharmaceutical formulations for use in accordance with the present invention are formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. In one embodiment, formulation is dependent upon the route of administration chosen.

[0438] In one embodiment, injectables of the disclosure are formulated in aqueous solutions. In one embodiment, injectables of the disclosure are formulated in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. In some embodiments, for transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0439] In one embodiment, the preparations described herein are formulated for parenteral administration, e.g., by bolus injection or continuous infusion. In some embodiments, formulations for injection are presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. In some embodiments, compositions are suspensions, solutions or emulsions in oily or aqueous vehicles, and contain formulatory

agents such as suspending, stabilizing and/or dispersing agents.

[0440] The compositions also comprise, in some embodiments, preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise, in some embodiments, local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0441] In some embodiments, pharmaceutical compositions and pharmaceutical formulations for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients, in some embodiments, are prepared as appropriate oil or water based injection suspensions. Suitable lipophilic solvents or vehicles include, in some embodiments, fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions contain, in some embodiments, substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. In another embodiment, the suspension also contains suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

[0442] In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez- Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; J. E. Diederichs and al., Pharm./nd. 56 (1994) 267- 275).

[0443] In another embodiment, the pharmaceutical composition delivered in a controlled release system is formulated for intravenous infusion, implantable osmotic pump, transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump is used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984). Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990).

[0444] In some embodiments, the active ingredient is in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use. Compositions are formulated, in some embodiments, for atomization and inhalation administration. In another embodiment, compositions are contained in a container with attached atomizing means.

[0445] In one embodiment, the preparation of the present invention is formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

[0446] In some embodiments, pharmaceutical compositions and pharmaceutical formulations suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. In some embodiments, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

[0447] In one embodiment, determination of a therapeutically effective amount is well within the capability of those skilled in the art.

[0448] Some examples of substances which can serve as pharmaceutically-acceptable carriers or components thereof are sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and methyl cellulose; powdered tragacanth; malt; gelatin; talc; solid lubricants, such as stearic acid and magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, mannitol, and polyethylene glycol; alginic acid; emulsifiers, such as the Tween™ brand emulsifiers; wetting agents, such sodium lauryl sulfate; coloring agents; flavoring agents; tableting agents, stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline; and phosphate buffer solutions. The choice of a pharmaceutically-acceptable carrier to be used in conjunction with the compound is basically determined by the way the compound is to be administered. If the subject compound is to be injected, in one embodiment, the pharmaceutically-acceptable carrier is sterile, physiological saline, with a blood-compatible suspending agent, the pH of which has been adjusted to about 7.4.

[0449] In addition, the compositions further comprise binders (e.g. acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g. cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (e.g., Tris-HCI., acetate, phosphate) of various pH and ionic strength, additives such as

albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g. sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g. hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents(e.g. carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweeteners (e.g. aspartame, citric acid), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), lubricants (e.g. stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g. colloidal silicon dioxide), plasticizers (e.g. diethyl phthalate, triethyl citrate), emulsifiers (e.g. carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g. ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[0450] Typical components of carriers for syrups, elixirs, emulsions and suspensions include ethanol, glycerol, propylene glycol, polyethylene glycol, liquid sucrose, sorbitol and water. For a suspension, typical suspending agents include methyl cellulose, sodium carboxymethyl cellulose, cellulose (e.g. Avicel™, RC-591), tragacanth and sodium alginate; typical wetting agents include lecithin and polyethylene oxide sorbitan (e.g. polysorbate 80). Typical preservatives include methyl paraben and sodium benzoate. In another embodiment, peroral liquid compositions also contain one or more components such as sweeteners, flavoring agents and colorants disclosed above.

[0451] The compositions also include incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance.

[0452] Also comprehended by the disclosure are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

[0453] In some embodiments, compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline. In another embodiment, the modified compounds exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds. In one embodiment, modifications also increase the

compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. In another embodiment, the desired in vivo biological activity is achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

[0454] In some embodiments, preparation of effective amount or dose can be estimated initially from in vitro assays. In one embodiment, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

[0455] In one embodiment, toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. In one embodiment, the data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. In one embodiment, the dosages vary depending upon the dosage form employed and the route of administration utilized. In one embodiment, the exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

[0456] In one embodiment, depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

[0457] In one embodiment, the amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

[0458] In one embodiment, compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier are also prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

[0459] In another embodiment, a coagulation factor as described herein is lyophilized (i.e., freeze-dried) preparation in combination with complex organic excipients and stabilizers such as nonionic surface active agents (i.e., surfactants), various sugars, organic polyols and/or human serum albumin. In another embodiment, a pharmaceutical composition comprises a lyophilized coagulation factor as described in sterile water for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized coagulation factor as described in sterile PBS for injection. In another embodiment, a pharmaceutical composition comprises a lyophilized coagulation factor as described in sterile 0.9% NaCl for injection.

[0460] In another embodiment, the pharmaceutical composition comprises a coagulation factor as described herein and complex carriers such as human serum albumin, polyols, sugars, and anionic surface active stabilizing agents. In another embodiment, the pharmaceutical composition comprises a coagulation factor as described herein and lactobionic acid and an acetate/glycine buffer. In another embodiment, the pharmaceutical composition comprises a coagulation factor as described herein and amino acids, such as arginine or glutamate that increase the solubility of interferon compositions in water. In another embodiment, the pharmaceutical composition comprises a lyophilized coagulation factor as described herein and glycine or human serum albumin (HSA), a buffer (e g. acetate) and an isotonic agent (e.g NaCl). In another embodiment, the pharmaceutical composition comprises a lyophilized coagulation factor as described herein and phosphate buffer, glycine and HSA.

[0461] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein is stabilized when placed in buffered solutions having a pH between about 4 and 7.2. In another embodiment, the pharmaceutical composition comprising a coagulation factor is in a buffered solution having a pH between about 4 and 8.5. In another embodiment, the pharmaceutical composition comprising a coagulation factor is in a buffered solution having a pH between about 6 and 7. In another embodiment, the pharmaceutical composition comprising a coagulation factor is in a buffered solution having a pH of about 6.5. In another embodiment, the pharmaceutical composition comprising a coagulation factor is in a buffered solution having a pH of about 6.4. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein is stabilized with an amino acid as a stabilizing agent and in some cases a salt (if the amino acid does not contain a charged side chain).

[0462] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein is a liquid composition comprising a stabilizing agent at between about 0.3% and 5% by weight which is an amino acid.

[0463] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein provides dosing accuracy and product safety. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein provides a biologically active, stable liquid formulation for use in injectable applications. In another embodiment, the pharmaceutical composition comprises a non-lyophilized coagulation factor as described herein.

[0464] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein provides a liquid formulation permitting storage for a long period

of time in a liquid state facilitating storage and shipping prior to administration.

[0465] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises solid lipids as matrix material. In another embodiment, the injectable pharmaceutical composition comprising a coagulation factor as described herein comprises solid lipids as matrix material. In another embodiment, the production of lipid microparticles by spray congealing was described by Speiser (Speiser and al., Pharm. Res. 8 (1991) 47-54) followed by lipid nanopellets for peroral administration (Speiser EP 0167825 (1990)). In another embodiment, lipids, which are used, are well tolerated by the body (e. g. glycerides composed of fatty acids which are present in the emulsions for parenteral nutrition).

[0466] In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises polymeric microparticles. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises nanoparticles. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises liposomes. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises lipid emulsion. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises microspheres. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises lipid nanoparticles. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises lipid nanoparticles comprising amphiphilic lipids. In another embodiment, the pharmaceutical composition comprising a coagulation factor as described herein comprises lipid nanoparticles comprising a drug, a lipid matrix and a surfactant. In another embodiment, the lipid matrix has a monoglyceride content which is at least 50% w/w.

[0467] In one embodiment, compositions of the present invention are presented in a pack or dispenser device, such as an FDA approved kit, which contain one or more unit dosage forms containing the active ingredient. In one embodiment, the pack, for example, comprise metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, in one embodiment, is labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

[0468] In one embodiment, it will be appreciated that the coagulation factors of the present invention can be provided to the individual with additional active agents to achieve an improved therapeutic effect as compared to treatment with each agent by itself. In another embodiment, measures (e.g., dosing and selection of the complementary agent) are taken to avoid adverse side effects which are associated with combination therapies.

[0469] In another embodiment, the present invention provides a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0470] In another embodiment, the present invention provides a pharmaceutical composition comprising a CTP-modified Factor VIIa (FVIIa) polypeptide consisting of a FVIIa polypeptide and five gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa.

[0471] In another embodiment, the present invention provides a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0472] In another embodiment, the present invention provides an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0473] In another embodiment, the present invention provides a cell comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0474] In another embodiment, the present invention provides a composition comprising an expression vector comprising a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor VIIa (FVIIa) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide.

[0475] In another embodiment, the present invention provides a method of extending the biological half-life of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby extending the biological half-life of said FVIIa polypeptide.

[0476] In another embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy

terminus of said FVIIa polypeptide, thereby improving the AUC of said FVIIa polypeptide.

[0477] In another embodiment, the present invention provides a method of reducing the dosing frequency of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby reducing the dosing frequency of said FVIIa polypeptide.

[0478] In another embodiment, the present invention provides a method of reducing the clearance rate of a Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby reducing the clearance rate of said FVIIa polypeptide.

[0479] In another embodiment, the present invention provides a method of producing a CTP-modified Factor VIIa (FVIIa) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FVIIa polypeptide, thereby producing a CTP-modified FVIIa polypeptide.

[0480] In another embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor VIIa (FVIIa) polypeptide comprising a FVIIa polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FVIIa polypeptide to said subject, thereby treating hemophilia in said subject.

[0481]

[0482] In one embodiment, the present invention provides a CTP-modified Factor IX (FIX) polypeptide consisting of a FIX polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said CTP-modified FIX polypeptide. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein the sequence of said CTP-modified FIX polypeptide is the sequence set forth in SEQ ID NO: 31. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein at least one CTP is truncated. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a CTP-modified FIX polypeptide, wherein said linker is a peptide bond.

[0483] In one embodiment, the present invention provides a pharmaceutical composition comprising the CTP-modified FIX polypeptide.

[0484] In one embodiment, the present invention provides a polynucleotide encoding a CTP-modified polypeptide consisting of a Factor IX (FIX) polypeptide and three gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide. In another embodiment, the present invention provides a polynucleotide, wherein the sequence of said polynucleotide is as set forth in SEQ ID NO: 30. In another embodiment, the present invention provides a polynucleotide, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a polynucleotide, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a polynucleotide, wherein at least one CTP is truncated. In another embodiment, the present invention provides a polynucleotide, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a polynucleotide, wherein said linker is a peptide bond. An expression vector comprising the polynucleotide.

[0485] In one embodiment, the present invention provides a cell comprising the expression vector.

[0486] In one embodiment, the present invention provides a composition comprising the expression vector.

[0487] In one embodiment, the present invention provides a method of extending the biological half-life of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby extending the biological half-life of said FIX polypeptide. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0488] In one embodiment, the present invention provides a method of improving the area under the curve (AUC) of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby improving the AUC of said FIX polypeptide. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded

by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0489] In one embodiment, the present invention provides a method of reducing the dosing frequency of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby reducing the dosing frequency of said FIX polypeptide. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0490] In one embodiment, the present invention provides a method of reducing the clearance rate of a Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX polypeptide, thereby reducing the clearance rate of said FIX polypeptide. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FVII polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0491] In one embodiment, the present invention provides a method of producing a CTP-modified Factor IX (FIX) polypeptide, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTPs) to the carboxy terminus of said FIX

polypeptide, thereby producing a CTP-modified FIX polypeptide. In another embodiment, the present invention provides a method, wherein the sequence of said CTP-modified FIX polypeptide is the sequence set forth in SEQ ID NO: 31. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0492] In one embodiment, the present invention provides a method of treating hemophilia in a subject comprising administering a CTP-modified Factor IX (FIX) polypeptide comprising a FIX polypeptide and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said FIX polypeptide to said subject, thereby treating hemophilia in said subject. In another embodiment, the present invention provides a method, wherein the sequence of said CTP-modified FIX polypeptide is the sequence set forth in SEQ ID NO: 31. In another embodiment, the present invention provides a method, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2. In another embodiment, the present invention provides a method, wherein at least one CTP is glycosylated. In another embodiment, the present invention provides a method, wherein at least one CTP is truncated. In another embodiment, the present invention provides a method, wherein at least one CTP is attached to said FIX polypeptide via a linker. In another embodiment, the present invention provides a method, wherein said linker is a peptide bond.

[0493] As is generally known in the art, the modified peptides and proteins of the disclosure may be coupled to labels, drugs, targeting agents, carriers, solid supports, and the like, depending on the desired application. The labeled forms of the modified biologicals may be used to track their metabolic fate; suitable labels for this purpose include, especially, radioisotope labels such as iodine 131, technetium 99, indium 111, and the like. The labels may also be used to mediate detection of the modified proteins or peptides in assay systems; in this instance, radioisotopes may also be used as well as enzyme labels, fluorescent labels, chromogenic labels, and the like. The use of such labels is particularly helpful if the peptide or protein is itself a targeting agent such as an antibody or a receptor ligand.

[0494] Similar linking techniques, along with others, may be employed to couple the modified peptides and proteins of the disclosure to solid supports. When coupled, these

modified peptides and proteins can then be used as affinity reagents for the separation of desired components with which specific reaction is exhibited.

[0495] Finally, the modified peptides and proteins of the disclosure may be used to generate antibodies specifically immunoreactive with these new compounds. These antibodies are useful in a variety of diagnostic and therapeutic applications, depending on the nature of the biological activity of the unmodified peptide or protein. It is to be understood that the disclosure provides antibodies that are immunoreactive with CTP-modified FIX, FVII, or FVIIa as described herein. In one embodiment, such antibodies may be used to distinguish or identify CTP-modified coagulation factors that were administered from endogenous coagulation factors. In another embodiment, the antibodies may be used to localize administered CTP-modified coagulation factors.

[0496] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

[0497] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available

immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference. Other general references are provided throughout this document.

EXAMPLE 1

Generation and Utilization of Coagulation Factor IX

[0498] **Cloning and expression of recombinant FIX molecule**:

[0499] Factor IX clones were constructed in our eukaryotic expression vector pCI-neo (Promega, catalog no. E1841). ORF Clone of Homo sapiens coagulation factor IX was ordered from "OriGene" (RC219065). Primers were ordered from **Sigma-Genosys**.

[0500] **Construction of 301-1-pCI-neo-p200-11 (Factor IX-ctp x2):**

[0501] Primer 101: 5' GTTTAGTGAACCGTCAGAAT 3' (SEQ ID NO: 36)

[0502] Primer $103^{R:}$ 5' TTGAGGAAGATGTTCGTGTA 3' (contains the SspI site of factor IX) (SEQ ID NO: 37)

[0503] A PCR reaction was conducted with primer 101 and primer 103^{R} and plasmid DNA, cDNA clone of Factor IX (OriGene" RC219065) as a template; as a result of the PCR amplification , a ~ 1085 bp (pcr 10) product was formed and purified from the gel (the fragment containing the amino terminus of Factor IX sequence).

[0504] Primer 98: 5' ATTACAGTTGTCGCAGGTGA 3' (SEQ ID NO: 38)

[0505] Primer 99^{R} : 5' GCTGGAGCTAGTGAGCTTTGTTTTTTCCTT 3' (SEQ ID NO: 39)

[0506] Primer 100: 5' GCTCACTAGCTCCAGCAGCAAGGCC 3' (SEQ ID NO: 40)

[0507] Primer 27^{R}: 5' TTTTCACTGCATTCTAGTTGTGG 3' (SEQ ID NO: 41)

[0508] Three PCR reactions were performed. The first reaction was conducted with primer 98 and primer 99^{R} and plasmid DNA, cDNA clone of Factor IX (OriGene" ,RC219065) as a template; as a result of the PCR amplification, a ~ 540 bp product was formed.

[0509] The second reaction was conducted with primer 100 and primer 27^R and plasmid DNA of 402-2-p72-3 (hGH-CTP-CTP) as a template; as a result of the PCR amplification, a ~ 258 bp product was formed.

[0510] The last reaction (pcr 3) was conducted with primers 98 and 27^R and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a ~ 790 bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01). SspI –EcoRI fragment was isolated (TA 3-3).

[0511] Another PCR reaction was conducted (pcr 12) with primer 101 and primer 27^R and a mixture of the products of pcr 10 and SspI-EcoRI fragment from pcr 3 as a template; as a result of the PCR amplification, a ~ 1700 bp product was formed (Factor IX-ctp-ctp) and ligated into TA cloning vector (Invitrogen, catalog K2000-01) (lig 180).

[0512] A mistake was found in the Factor IX sequence so fragments were replaced in order to form an insert of Factor IX-ctp-ctp with the correct DNA sequence.

[0513] TA- pcr 3-3 was digested with SspI and XbaI and the large fragment was isolated (vector). TA 180-4 was digested with SspI and XbaI and the small fragment (insert) was isolated and ligated to the isolated large fragment of TA-pcr-3-3digested with SspI and XbaI. The new plasmid TA-183-2 was digated with Sal I and NotI, and the Factor IX-CTP-CTP insert was isolated (~1575 bp). This fragment was inserted into eukaryotic expression vector pCI-neo (digested with Sal I and Not I) to yield the 301-2-p200-11 clone.

[0514] **pCI-dhfr –Factor 9- ctpx2 (p223-4) construction:** Vector pCI-dhfr (p6-1) was digested with SmaI and NotI. Factor IX-CTP-CTP (p200-11) was digested with ASisI F.I. and NotI. The two fragments were ligated.

[0515] **pCI-dhfr Factor 9-ctp x3 (p225-7) construction:** Vector pCI-dhfr OXM-CTP×3 (p216-4) was digested with XbaI and ApaI. Factor IX-CTP-CTP (223-4) was digested with XbaI and ApaI. The two fragments were ligated.

[0516] **pCI-dhfr Factor 9-ctp x3 T148A (p243-2) construction:** Plasmid p225-7 contained Threonine at position 148, since the more common version of FIX contains Alanine at this position, Thr was replaced to Ala using site directed mutagenesis method.

[0517] Primer 75: ctcccagttcaattacagct (SEQ ID NO: 42)

[0518] Primer 122r: ggaaaaactgcctcagcacgggtgagc (SEQ ID NO: 43)

[0519] Primer 123: gtgctgaggcagtttttcctgatgtggactat (SEQ ID NO: 44)

[0520] Primer 124r: caacacagtgggcagcag (SEQ ID NO: 45)

[0521] Three PCR reactions were performed. The first reaction was conducted with primer 75 and primer 122r and plasmid DNA p225-7 as a template; as a result of the PCR amplification, a ~ 692 bp product was formed and purified from the gel. A second PCR reaction was

conducted with primer 123 and primer 124r and plasmid DNA p225-7 as a template; as a result of the PCR amplification, a ~237 bp product was formed and purified from the gel. The third - overlap PCR reaction was conducted with primers 75 and 124r, and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a ~ 910 bp product was formed. This overlap PCR product was digested with XbaI and NsiI and re ligated into p225-7 plasmid (digested with XbaI and NsiI) to yield Factor IX-ctpx3 T148A designated p243-2.

[0522] **FIX-4CTP (p259-4) construction:** 3.5CTP fragment was isolated from oxym-4CTP (p254-3) by restriction enzymes Apa1 and Xba1. FIX+0.5CTP fragment was isolated from FIX-3CTP (p243-2) with restriction enzymes Apa1 and Xba1. The two fragments were ligated.

[0523] **FIX-5CTP (p260-18) construction:** 4.5CTP fragment was isolated from oxym-5CTP (255-1) by restriction enzymes Apa1 and Xba1. FIX+0.5CTP fragment was isolated from FIX-3CTP (p243-2) using enzymes Apa1 and Xba1. The two fragments were ligated.

[0524] Dg44 cells were plated in 100mm tissue culture dishes and grown to 50-60% confluence. A total of 2 µg (microgram) of FIX cDNA was used for the transfection of one 100mm plate using the FuGene reagent (Roche) in protein-free medium (Invitrogene CD Dg44). The media was removed 48 hours after transfection and replaced with a protein-free medium (Invitrogene CD Dg44) without nucleosides and in the presence of 800 µg/ml of G418 (Neomycin). After 14 days, the transfected cell population was transferred into T25 tissue culture flasks, and selection continued for an additional 10-14 days until the cells began to grow as stable clones. High expressing clones were selected. Approximately $2x10^7$ cells were used to inoculate 300 ml of growth medium in a 1700 cm^2 roller bottle (Corning, Corning NY) supplemented with 5 ng/ml of Vitamin K3 (menadione sodium bisulfate; Sigma). The production medium (harvest) was collected after a rapid decrease in cell viability to about 70%. The production medium was first clarified and then concentrated approximately 20-fold and dialyzed with PBS using flow filtration cassette (10KDa MWCO; Millipore Corp.).

[0525] **Determination of FIX antigen level:** FIX-CTP harvest antigen levels were determined using AssayMax Human FIX ELISA kit (AssayPro-EF1009-1). The calculated protein concentration is the average of three different dilutions in two independent runs (**Figure 1A**, Table 1).

Table 1: Calculated protein concentration

	FIX-CTP	FIX-CTP-CTP
FIX Ag level (µg/ml)	41.9	19.2

SD	8.76	3.67
%CV	20.92	19.15

[0526] **FIX SDS-PAGE - immune blot:** FIX-CTP harvests or purified rhFIX (American Diagnostics), 100 ng of protein, were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immunoblot using anti-human FIX polyclonal antibody and anti-human gamma carboxylation monoclonal antibody (American Diagnostics). As previously reported, rhFIX migrated at 55KDa, while FIX fused to two CTPs migrated at 75KDa. Both variants of FIX-CTP proteins were shown to be gamma carboxylated, an essential post-translation modification for FIX activity and function (Figure 1B).

[0527] **Determination of FIX chromogenic activity:** A comparative assessment of the *in vitro* potency of FIX-CTP harvests versus rhFIX protein (American Diagnostics) was performed using the commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). In the presence of thrombin, phospholipids, calcium, excess amounts of FXIa activates sampled FIX into FIXa. FIXa forms an enzymatic complex with thrombin, activated FVIII:C (supplied in an excess amounts), phospholipids, and calcium and activates Factor X, present in the assay system, into FXa. The activity directly correlates with the amount of FIX, which is the limiting factor. The generated FXa is then measured by its specific activity on FXa chromogenic substrate (pNA). The amount of pNA generated is directly proportional to FIXa activity. rhFIX and FIX-CTP harvests were serially diluted, and the potency was assessed by comparing a dose-response curve of the FIX harvests to a reference preparation consisting of rhFIX or human plasma. The average EC50 of FIX was 21 ng/ml, while the FIX-$(CTP)_2$ harvest calculated EC50 was 382 ng/ml, and the FIX-CTP harvest calculated EC50 was 1644 ng/ml. An approximately 15-fold decrease in the enzymatic activity of the FIX-$(CTP)_2$ harvest was observed (Figure 2).

[0528] **FIX Clotting activity (aPTT):** The activated partial thromboplastin time (aPTT) is a measure of the integrity of the intrinsic and common pathways of the coagulation cascade. The aPTT is the time, in seconds, for plasma to clot following the addition of an intrinsic pathway activator, phospholipid and calcium. The aPTT reagent is called a partial thromboplastin because tissue factor is not included with the phospholipid as it is with the protime (PT) reagent. The activator initiates the system and then the remaining steps of the intrinsic pathway take place in the presence of phospholipid. Reference aPTT range varies from laboratory to laboratory, but is usually in the range of 27-34 seconds.

[0529] The principal of the assay was to quantitate the ability of FIX-CTP harvests to restore

the clotting activity of FIX-depleted human plasma by the addition of rhFIX. 300 µl of FIX-deficient human plasma was mixed with 100µl of rhFIX or FIX-CTP harvests and serially diluted. Following a 60 second incubation at 37°C, thromboplastin, $CaCl_2$, and phospholipids were added to the mixture, and clotting time in seconds was determined (performed by American Medical Laboratories). The potency was assessed by comparing a dose-response curve of the FIX harvests to a reference preparation consisting of rhFIX or human plasma. One unit of FIX activity corresponds to the FIX concentration that equals the activity of one ml normal human plasma. The presented aPTT results indicate that FIX-$(CTP)_2$ exhibit a 5.7-fold reduction in its specific coagulation activity compared to rhFIX (Table 2). Moreover, the aPTT results together with the chromogenic activity *in vitro* assay suggest that FIX-$(CTP)_2$ harvest has an improved enzymatic activity vs. FIX-CTP harvest (Table 2). An improved activity of FIX-CTP proteins can be obtained following optimization of the expression system (i.e. co-transfection with Furin and optimization of Vitamin K3 medium concentration), which was strengthened following super-transfection with Furin (data not shown).

Table 2: FIX clotting activity

rhFIX(AD) (µg/ml)	PTT(Sec)	FIX-CTP (µg/ml)	PTT (Sec)	FIX-CTP-CTP (µg/ml)	PTT (Sec)
5	31.3	9	45.2	4	47.5
1.25	35.7	2.25	53.3	1	55.9
0.3125	43	0.5625	64.1	0.25	67
0.078125	52.1	0.140625	76.3	0.0625	77.4

[0530] **Pharmacokinetic study:** rhFIX (American Diagnostic) and FIX-CTP harvests were administered in a single intravenous injection to Sprague-Dawley rats (six rats per substance) at a dose of 75 µg/kg body weight (Table 3).

Table 3: PK study plan of operation

Treated Groups	Test Article	No. of animals/ group	Dose Route	Gender	Dose Level (µg/kg)	Dose Level (µg per animal)	Injected Vol. (µl)	Con. (µg/ml)	*Time-Points (hours post-dose)
1	rFIX	*6*	IV	M	75	15	500	30	0 (Pre-dose) 0.083, 0.5, 1.5, 4, 8, 24, 48, 72.

2	rFIX-CTP	*6*	IV	M	75	15	500	30	0 (Pre-dose) 0.083, 0.5, 1.5, 4, 8, 24, 48, 72.
3	rFIX-CTP-CTP	*6*	IV	M	75	15	1000	15	0 (Pre-dose) 0.083, 0.5, 1.5, 4, 8, 24, 48, 72.

[0531] Blood samples were drawn retro-orbitally from 3 rats alternately at 0.083, 0.5 1.5, 4, 8, 24, 48, and 72 hours post-dosing. Plasma was prepared immediately after sampling and stored at -20ºC until analysis. FIX concentration was quantitated by FIX ELISA-specific assay (AssayPro). A pharmacokinetic profile was calculated for each protein and represents the mean of 3 animals at each time point (Figure 3). The terminal half-lives were calculated using PK solutions 2.0 software. Table 4 summarizes the observed FIX concentrations at the different sampling time points.

Table 4: Observed FIX concentrations

Time (Hr)	FIX-AD (ng/ml)	FIX-CTP (ng/ml)	FIX-CTP-CTP (ng/ml)
0.083	1506.7	1477.5	1914.8
0.5	1949.8	1150.1	1830.1
1.5	2189.4	1009.0	1264.3
4	733.90	709.33	1000.00
8	319.80	167.20	1234.67
24	BLQ	54.625	230
48	BLQ	BLQ	120.9

[0532] The PK profile and summary of the terminal half-lives are summarized in Table 5. FIX-CTP harvests exhibit an improved $T_{½\beta}$ values compared to rhFIX (2- and 5-fold increases, respectively). Since in FIX dosing collection, animal serum concentrations of FIX at 24hr were below limit of quantitation (BLQ), additional PK parameters were not calculated.

Table 5: Summary of PK parameters

Product	Terminal half-life- (hr)	Ratio (FIX-(CTP)$_X$/rhFIX)
rhFIX (American Diagnostics)	2.62	–
FIX-CTP	5.55	2.11
FIX-CTP (FIX-CTP-CTP)	12.9	4.92

[0533] In this study, a novel approach was described for prolonging FIX half-life while retaining the therapeutic potency. Adding a CTP peptide to an active protein has a harmful potential in interfering with the protein's activity. Therefore, the generation of an active recombinant FIX-CTP by adding a CTP sequence at the C-terminus of the FIX is unexpected.

Characterization of an immunoaffinity purified FIX-CTP-CTP

FIX-CTP-CTP purification

[0534] In order to evaluate a protein at high grade content with increased activity whose PK profile mimics and can be extrapolated to a clinical setting, FIX-CTP-CTP is a FIX modified with 2 CTP units in tandem in its carboxy-terminal. FIX-CTP-CTP was purified using matrix-bound monoclonal antibody against γ carboxyglutamyl (Gla) residues present in the N-terminal region of FIX (American Diagnostics Cat. # 3570MX). The monoclonal antibody was bound to Sepharose CL-4B. The FIX-CTP-CTP harvest at a concentration of 88 µg/ml was dialyzed against 20mM Tris, 150Mm NaCl and 10mM EDTA at PH =7.4. The loading rate was 0.5 ml/min, elution was performed using 20Mm Tris-HCl, 350 mM NaCl and 50 mM CaCl, and the unbound fraction was recycled five times. Finally, the elution fraction was dialyzed with PBS, pulled and concentrated.

[0535] **Determination of FIX antigen level:** FIX-CTP harvests, FIX-$(CTP)_2$ harvests, and FIX-$(CTP)_2$ purified protein levels were determined using the Human FIX ELISA kit (Affinity Biologicals; Cat. #FIX-AG RUO). The calculated protein concentration (µg/ml) is the average of two independent runs (**Figure 4**, Table 6).

Table 6: Calculated protein concentration

	FIX-CTP	FIX-CTP-CTP	FIX-CTP-CTP (purified)
FIX Ag level (µg/ml)	125.78	88.53	172.9

SD	17.28	21.31	2.63
%CV	13.74	24.08	1.52

[0536] Additionally, FIX-CTP-CTP was quantitated by Bradford assay. The calculated concentration was 202 µg/ml, which is similar to the concentration obtained by human FIX ELISA.

[0537] **SDS-PAGE blots:** FIX-CTP-CTP harvest, unbound fraction and purified protein, were loaded on a 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE Coomassie analysis was performed by staining the gel with Coommasie blue reagent (800ng of protein). A Western immunoblot was performed with 100 ng of protein, anti-human FIX polyclonal antibody (Ab), and anti-human gamma carboxylation monoclonal Ab (American Diagnostics Cat #499 and #3570). The immunoaffinity purification procedure significantly enriched the FIX-CTP-CTP portion while reduced impurity (**Figure 5**).

[0538] **N-terminal sequencing**: FIX-CTP-CTP purified protein was separated by 12% Tris-Glycine SDS-PAGE and subsequently electro-blotted to PVDF membrane. The band of interest was cut out and put on a purified Biobrene treated glass fiber filter. The N-terminal sequence analysis was carried out by Edmann degradation using a pulsed liquid protein sequencer equipped with a 140 C HPLC micro-gradient system. N-terminal sequencing revealed that FIX-CTP-CTP is a mixture of incomplete and complete pro-peptide cleaved proteins. Inadequate pro-peptide cleavage was shown to reduce FIX coagulation activity. By co-transfection with Furin, the pro-peptide cleavage process can be an improved.

[0539] **Determination of FIX chromogenic activity:** A comparative assessment of the *in vitro* potency of FIX-CTP-CTP purified protein versus rhFIX (American Diagnostics) and a pool of human normal plasma was performed using the commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). In the presence of thrombin, phospholipids and calcium, excess amounts of FXIa activates FIX into FIXa. FIXa forms an enzymatic complex with thrombin (supplied in excess amounts), phospholipids and calcium activates Factor X, present in the assay system, into FXa. The activity directly correlates with the amount of FIX, which is the limiting factor. The generated FXa was measured by its specific activity on FXa chromogenic substrate (pNA). The amount of pNA generated was directly proportional to FIXa activity. rhFIX, human plasma and FIX-CTP-CTP were serially diluted, and potency was assessed by comparing a dose-response curve (Figure 6). The average EC_{50} of rhFIX was 68.74 ng/ml while FIX-CTP-CTP calculated EC_{50} was 505 ng/ml. An approximately 7-fold decrease in the enzymatic activity of FIX-CTP-CTP was observed

vs. recombinant FIX and a 16.5-fold decrease versus normal human pulled plasma. This reduced activity could be explained by inadequate cleavage of N-terminal pro-peptide, which was identified by N-terminal analysis.

[0540] **FIX Clotting activity (aPTT):** The activated partial thromboplastin time (aPTT) is a measure of the integrity of the intrinsic and common pathways of the coagulation cascade. The aPTT is the time (measured in seconds) it takes plasma to clot following the addition of an intrinsic pathway activator, phospholipid and calcium.

[0541] The assay quantitated the ability of the FIX-CTP-CTP protein to restore the clotting activity of FIX depleted human plasma by the addition of rhFIX. 300 µl of FIX-deficient human plasma was mixed with 100 µl of rhFIX, FIX-CTP-CTP (FIX-CTP-CTP (the CTP are in tandem at the C-terminal)), or normal pool human plasma which was further diluted. Following a 60 second incubation at 37°C, Tissue Factor (TF), $CaCl_2$, and phospholipids were added to the mixture. Clotting time in seconds was determined. Potency was assessed by comparing a dose-response curve of FIX-CTP-CTP to a reference preparation of rhFIX or human plasma. One unit of FIX was defined as the amount of FIX which equals to the activity of 1 ml human normal plasma.

[0542] The aPTT results indicate that FIX-CTP-CTP coagulation activity is only 1.4 less than normal pool human plasma and similar to the rhFIX. The aPTT results together with the chromogenic activity *in vitro* assay suggest that FIX-CTP-CTP purification did not damage its activity.

[0543] **Pharmacokinetic activity of FIX-CTP-CTP:** Purified FIX-CTP-CTP, rhFIX (American Diagnostic) and harvests containing FIX-CTP-CTP and FIX-CTP were administered in a single intravenous injection to Sprague-Dawley rats (eight rats per substance) in a dose of 100µg/kg body weight (Table 7).

Table 7: PK study outline

Treated Groups	Test Article	No. of animals/ group/ time point	Dose Level (µg/kg)	Dose Level (µg per animal)	Injected Vol. (µl)	Con. (µg/ml)	Time-Points (hours post-dose)
A	rFIX	8	100	20	500	40	0 (Pre-dose) 0.083, 0.5, 1, 2, 4, 7, 10, 24, 48, 72.
B	rFIX-CTP (harvest)	8	100	20	500	40	0 (Pre-dose) 0.083, 0.5, 1, 2, 4, 7, 10, 24, 48, 72.

Treated Groups	Test Article	No. of animals/ group/ time point	Dose Level (µg/kg)	Dose Level (µg per animal)	Injected Vol. (µl)	Con. (µg/ml)	Time-Points (hours post-dose)
C	rFIX-CTP-CTP(harvest)	6	100	20	500	40	0 (Pre-dose) 0.083, 0.5, 1, 2, 4, 7, 10, 24, 48, 72.
D	rFIX-CTP-CTP (purified)	4	100	20	500	40	0.083, 0.5 1, 2, 4, 7, 10, 24, 4, 8, 72.

[0544] Blood samples were drawn retro-orbitally from 4 rats alternately at 0.083, 0.5, 2, 4, 7 10, 24, 48, and 72 hours post-dosing. Citrated plasma (0.32%) was prepared immediately after sampling and stored at -20ºC until analysis. FIX concentration was quantitated using a human FIX ELISA kit (Affinity Biologicals). The pharmacokinetic profile was calculated for each protein as the mean of 4 animals at each time point (Figure 7). The terminal half-life was calculated using PK Solutions 2.0 Software. Table 8 summarizes the observed FIX concentrations at different sampling time points.

Table 8: Observed FIX concentrations

Time (hr)	**FIX-CTP harvest ng/ml**	**FIX-$(CTP)_2$ harvest ng/ml**	**rhFIX ng/ml**	**Purified FIX-CTP-CTP ng/ml**
0.085	1038.97	1123.62	325.05	886.48
0.5	939.12	956.80	274.58	670.92
1	791.97	843.85	222.90	674.17
2	304.98	673.31	186.00	503.91
4	315.37	525.50	109.69	357.36
7	171.45	384.36	67.62	257.02
10	50.34	250.73	40.20	158.66
24	10.07	78.50	BLQ	52.13
48	BLQ	23.40	BLQ	18.07

[0545] A summary of the PK parameters are presented in Table 9.

Table 9: Summary of PK parameters

	T½ (hr)	**AUC ng-hr/ml**	**MRT (hr)**	**Vd ml/Kg**	**CL Ml/hr/Kg**

FIX-CTP harvest	4.17	3622	4.5	155.1	27.6
FIX-$(CTP)_2$ harvest	10.44	9105.7	12	165.4	10.9
rhFIX	3.72	1416.8	5.1	373.8	70.183
Purified FIX-CTP-CTP	11.14	6314.2	12.3	254.5	15.83

[0546] The FIX-CTP-CTP harvest demonstrated an improved PK profile compared to FIX-CTP harvest. Furthermore, purified FIX-CTP-CTP exhibited a 3-fold increase in $T½_{\beta}$ value and a 4.5-fold increase in AUC compared to rhFIX.

[0547] The reduced amount of secreted FIX fused to tandem CTP molecules versus fusion of a single CTP appears to be due to the addition of an extra CTP and not to reduced detection by ELISA, because the Bradford-purified FIX-CTP-CTP calculated concentration was similar to the ELISA-calculated concentration.

[0548] FIX-CTP-CTP clotting activity was similar to pooled human plasma; however, its *in vitro* chromogenic activity was significantly lower when compared to rhFIX or pooled human plasma. The chromogenic activity assay was reported as a very sensitive assay compared to the coagulation assay. The reason for reduced activity of FIX-CTP-CTP may vary. Addition of CTP may decrease the affinity of FIX to FXIa or reduce post-transcriptional modifications (e.g. 12-10 GLA residues and pro-peptide cleavage). N-terminal analysis revealed that the proteolytic cleavage of the FIX-CTP-CTP pro-peptide was not fully completed prior to secretion. Since this post-transcriptional modification is crucial for the normal enzymatic activity of the protein, co-transfection with Furine-PACE plasmid is favorable and may improve FIX-CTP-CTP activity.

[0549] Finally, FIX-CTP-CTP comparative PK study in rats demonstrated that fusion of two tandem CTPs to the C-terminal of FIX generated a FIX with an extended half-life.

[0550] **FIX depleted mouse model**: In order to assess the *in vivo* activity, FIX knockout mice are obtained, and a breeding colony is established. 10µg of either commercial recombinant hFIX (BeneFIX®) or rFIX-$(CTP)_2$ (FIX-CTP-CTP) are injected into the tail vein of an anaesthetized FIX knockout mouse (22-28g). The amount of injected protein equals to the required concentration of FIX in normal plasma (5µg/ml). Blood samples are taken from the clipped tail into heparinized capillary tubes at specific time points. Plasma samples are assessed for FIX levels by ELISA and efficacy is measured by aPTT coagulation assay.

[0551] **Increasing FIX Propeptide cleavage efficacy:** CTP peptide cDNA was fused to the 3' end of human FIX cDNA. The corresponding rFIX and Furin expressing constructs were

co-transfected into Dg44 cells; a human rFIX cDNA was also co-transfected with the Furin plasmid as a control. Secretion of high level of FIX leads to secretion of a mixture of pro-factor and a mature factor FIX, due to limited amount of the Furin protease in the cell. Co-transfection of a Furin expressing vector with a pro-factor expressing vector increases the recovery and result in the secretion of fully processed FIX in to the medium.

[0552] Following FIX-$(CTP)_2$ and Furin co-transfection, stable clones are generated and harvest is collected for pro-peptide cleavage evaluation. 100 ng of protein, are loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE analysis is performed by Western immunoblot using anti-human FIX polyclonal Ab (American Diagnostics) and anti-pro-peptide polyclonal antibody. As previously reported, rhFIX migrated at 55KDa, while FIX fused to two CTPs migrated at 75 kDa. Both variants of FIX proteins are shown to undergo a proper, full pro-peptide cleavage.

[0553] To determine whether proper pro-peptide cleavage improves FIX-$(CTP)_2$ enzymatic activity, a comparative assessment of chromogenic and coagulation activity of FIX-$(CTP)_2$ harvest cotransfecated with Furin is performed. A significant improvement in FIX-$(CTP)_2$ specific activity is observed, which is similar to rhFIX.

[0554] In conclusion, the results described herein suggest that FIX-CTP-CTP can be used efficiently for treating Hemophilia B patients. FIX fused to CTP constructs benefit from improved *in vivo* pharmacologic performance that overcomes the drawback in certain *in vitro* measures. This proposed treatment is advantageous over previous treatments as the rate of infusions and the amount of required doses are reduced.

[0555] It is important to notice that when an albumin-fused molecule strategy was used to improve the FIX half-life, the recombinant FIX became inactive. The present novel approach lead to the design and purification of a novel recombinant FIX-fused protein that presents an improved long-lasting activity. Since mere size modifications did not improve the pharmacokinetics of injected FIX, the finding that CTP fused to FIX facilitates pharmacokinetic parameters was unexpected. The presence of highly glycosylated peptide-sialic acid residues stabilized the protein and protected it from interactions with vascular receptors without abrogating key determinants of FIX function.

[0556] FIX-CTP has a similar therapeutic efficacy to rFIX in hemophilia B patients and required less frequent dosing. A single injection of FIX-CTP is sufficient to control bleeding episodes and reduce the number of injections that are needed during surgical intervention in hemophilia B patients.

[0557] The CTP technology was utilized for the development of a long-acting FIX. Specifically, extending the half-life of recombinant rFIX molecule was performed by fusion

of at least one human CTP to FIX. The recombinant FIX-CTP was expressed in mammalian cells and characterized *in vitro* and *in vivo*. It was demonstrated that the *in vitro* activity of rFIX-CTP was comparable to rFIX. Pharmacokinetics and efficacy studies in rats demonstrated improved properties of the rFIX-CTP. The results of this study demonstrate that it is feasible to develop a half-life extended rFIX molecule having similar haemostatic properties to the wild type enzyme.

EXAMPLE 2

Comparative Assessment of Purified FIX-CTP$_3$ vs. FIX-CTP$_4$ and FIX-CTP$_5$

[0558] **2.1 Study objective**

[0559] A comparative assessment of the pharmacokinetic parameters of FIX-CTP$_4$ and FIX-CTP$_5$ versus FIX-CTP$_3$ following a partial purification process.

[0560] **2.2 Production of FIX-CTP$_4$ and FIX-CTP$_5$ harvests**

[0561] FIX cDNA (OriGene RC219065) fused at the C-terminal to four or five tandem CTP sequences was expressed in Dg44 cells using Excellgene expression system in the presence of 10 ng/L of vitamin K3 (Sigma, Mennadion). The harvests were collected (300ml), filtered and frozen.

[0562] **2.3 Production of FIX-CTP$_3$ harvest**

[0563] FIX-CTP$_3$ was expressed in-house in CHO cells using pCI-DHFR vector, clone 196, BR-9 in the presence of 25 ng/L of vitamin K3 (Sigma). The harvests were collected and filtered.

[0564] All FIX-CTP samples (3, 4 and 5 CTP) were purified only by Jacalin column because of a lack of material.

[0565] **2.4 Determination of FIX antigen level**

[0566] FIX antigen level was determined using Human FIX ELISA kit (Affinity Biologicals; Cat. # FIX-AG RUO). The calculated protein concentration is the average of four independent runs. FIX-CTP$_3$ concentration was slightly higher as compared to the two additional versions (Table 10).

Table 10: FIX antigen level

	3 CTP Final Jacalin40	**4 CTP Final Jacalin40**	**5 CTP Final Jacalin40**
Av. (ng/ml)	1016.69	4644.11	**1686.82**
SD	225.41	925.63	**160.07**

%CV	22.17	19.93	**9.49**

[0567] **2.5 FIX-CTP Coomassie stain and immune-blot**

[0568] FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 harvests were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immuno-blot using anti-CTP polyclonal Ab (Adar Biotech Production) or anti-Gla Ab (American Diagnostica).

[0569] As previously reported, FIX fused to three CTPs migrated at 80 kDa while FIX fused to four or five CTPs migrated at 85 KDa or 90 KDa, respectively. As expected, FIX-CTP_4 and FIX-CTP_5 harvests from Excellgene showed very low levels of gamma carboxylation compared to FIX-CTP_3 harvest, which was produced at Prolor (Figure 8).

[0570] After a purification process utilizing Jacalin column (immunoaffinity purification of glycosylated proteins), FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE was stained by Coomassie blue Dye for samples detection. All variants showed much cleaner band profiles (Figure 9), suggesting an improved purity.

[0571] **2.6 Determination of FIX chromogenic activity**

[0572] A comparative assessment of the *in vitro* potency of fully purified (HA column) FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). All samples were serially diluted, and the potency was assessed by comparing a dose-response curve to a reference preparation of normal human plasma. The reduced chromogenic activity of FIX-CTP_4 and FIX-CTP_5 (Figure 10) as compared to plasma can be a consequence of improper post-transcriptional modifications of FIX proteins, e.g. inappropriate gamma carboxylation and pro-peptide cleavage or, alternatively, due to the addition of CTP cassettes. The fluctuation in the FIX-CTP_4 and FIX-CTP_5 activity (Table 11) might be caused by inappropriate quantitation capabilities of the FIX ELISA due to CTP masking of the antigen site.

Table 11: Sample/plasma EC50 ratio

Sample	Sample/plasma EC50 ratio
Plasma	**1**
3 CTP Final HA	**2**
4 CTP Final HA	**5.35**

5 CTP Final HA	**2.73**

[0573] **2.7 Pharmacokinetic study**

[0574] Jacalin-purified FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 (Group A, B and C, respectively) were administered in a single intravenous injection to Sprague-Dawley rats (six rats per treatment group) at a dose of 250 µg/kg body weight. Blood samples were drawn retro-orbitally from 3 rats alternately at 0.083, 0.5 2, 5, 8, 24, 48, 72 and 96 hours post-dosing (Table 12). Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20℃ until analysis.

Table 12: PK study plan of operation

Treatment Group	Treatment	No. of animals/ group	Dose Route	Dose Level (µg per animal)	Injected Vol. (µl)	Conc. (µg/ml)	Time-Points (hr post-dose)
A	FIX-CTP*3 Jacalin 40	6	IV	50	200	250	0.083, 0.5, 2, 5, 8, 24, 48, 72, 96
B	FIX-CTP*4 Jacalin 40	6	IV	50	200	250	0.083, 0.5, 2, 5, 8, 24, 48, 72, 96
C	FIX-CTP*5 Jacalin 40	6	IV	50	200	250	0.083, 0.5, 2, 5, 8, 24, 48, 72, 96

[0575] FIX concentration in plasma samples were quantified using human FIX ELISA kits (Affinity Biologicals). The pharmacokinetic profile was calculated and is the mean of 3 animals at each time point. Terminal half-lives were calculated using PK Solutions 2.0 Software. Table 13 below summarizes the calculated FIX concentrations at the different sampling time points.

Table 13: Calculated FIX concentrations

Time (hr)	**Av. 3 CTP ng/ml**	**SD 3 CTP**	**Av. 4 CTP ng/ml**	**SD 4 CTP**	**Av. 5 CTP ng/ml**	**SD 5 CTP**
0.083	1087.82	72.39	904.54	21.06	1097.23	82.24
0.5	774.18	86.31	736.82	66.93	998.79	70.43
2	562.23	3.70	627.09	32.47	747.85	14.02

5	357.44	8.63	431.23	29.41	576.49	27.36
8	239.20	7.82	327.46	30.26	394.96	36.48
24	77.08	4.26	107.38	5.18	142.42	16.13
48	27.73	2.02	39.83	1.85	53.66	3.33
72	12.55	1.48	21.53	1.55	23.54	3.32
96	6.66	1.23	10.63	0.13	18.54	3.39

[0576] The PK profile and a summary of the PK parameters are presented in Table 14 below and in Figure 11. A full PK analysis profile at all time points suggested that addition of 4 or 5 CTP cassettes to FIX did not increase its half-life as compared to FIX-CTP_3. The AUC following FIX-CTP_5 administration increased by 1.4- to 1.6-fold versus FIX-CTP_3, which was not statistically significant.

Table 14: PK profile and a summary of the PK parameters

24- 96hr	**3 CTP**	**4 CTP**	**5 CTP**
Half-life (hr)	20.43	22.02	23.96
AUC (ng-hr/ml)	8218.38	10504.49	13329.41
Vd (ml/kg)	700.76	586.02	494.89
CL (ml/hr/kg)	23.77	18.45	14.32

[0577] Since 96 hr post-dosing samples were shown to have very low FIX concentrations, which were at the lower limit of quantification of the assay, the terminal half-life was recalculated providing a more precise and scientifically appropriate calculation (Table 15). According to this calculation, even smaller differences were obtained between the half-life of FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5.

Table 15: Recalculated terminal half-life

8-72 hr	**3 CTP**	**4 CTP**	**5 CTP**
Half-life (hr)	15.38	16.63	16.04

[0578] **2.8 Conclusions:**

[0579] In this study, the pharmacokinetic parameters and potential clotting activity of FIX-CTP_3, FIX-CTP_4, and FIX-CTP_5 were assessed. Fusion of 4 and 5 CTPs to FIX did not provide a superior or improved half-life extension, as compared to FIX-CTP_3, and reduced chromogenic activity was observed. Table 16 below summarizes the percent improvement of half-life for the different FIX-CTP fused variants (1 to 5 CTPs). Fusion of CTP to FIX improved its pharmacokinetic behavior, but, unpredictably, this improvement was limited. Surprisingly, following fusion of 3, 4 or 5 CTPs in tandem to FIX, a similar half-life value was calculated.

Table 16: Summary of the percent improvement of half-life

FIX Version	T½(8-72hr) % increase
rhFIX vs. 1CTP	112
1CTP vs. 2CTP	141
2CTP vs. 3CTP	37
3CTP vs. 4CTP	6
4CTP vs. 5CTP	0

[0580] These data suggest that fusion of 3 CTPs to FIX produces a maximal improvement in protein half-life, confirming that FIX-CTP_3 is the optimal variant in terms of half-life, structure and potential clotting activity for further clinical development.

EXAMPLE 3

FIX-CTP_3 TREATMENT OF FIX-/- HEMOPHILIC MOUSE MODEL

[0581] As described above, a study testing FIX-CTP, FIX-CTP_2 and FIX-CTP_3 harvest PK profile and coagulation activity vs. rhFIX was conducted. FIX-CTP_3 exhibited an improved PK profile while maintaining its coagulation activity vs. FIX-CTP_1 and FIX-CTP_2 harvests or rhFIX. To further evaluate this result, FIX-CTP_3 γ-Carboxyglutamate protein was purified. FIX-CTP_3 exhibits a 3-fold increase in half-life and 4.5-fold higher AUC compared to rhFIX in normal rats following a single IV administration. FIX-CTP_3 demonstrated a reduced *in vitro* chromogenic and clotting activity, most likely due to insufficient cleavage of N-terminal

pro-peptide and in appropriate post-transcriptional modifications (PTMs), such as appropriate gamma carboxylation.

[0582] In the current study, the pharmacokinetic and pharmacodynamic properties of human recombinant FIX fused to three tandem CTPs were tested in FIX-deficient mice.

[0583] **Study purpose:**

[0584] To determine the pharmacokinetic and pharmacodynamic parameters of rFIX-$(CTP)_3$ vs. commercial rhFIX (BeneFIX®) in FIX-deficient mice following a single IV administration of FIX-$(CTP)_3$ at a similar specific activity and dose (similar specific activity to PD and similar FIX constant for PK).

[0585] **Production of FIX-CTP_3 harvest:**

[0586] FIX cDNA (OriGene RC219065-Thr 148) fused at the C-terminal to three tandem CTP sequences was expressed in Dg44 cells using Excellgene expressing system in the presence of 25 ng/ml of Vitamin K3 (Sigma, Mennadion). Five separate batches containing 5 liters of cell suspension was cultured (total of twenty-five liters) and harvested following viability decline to 60-70%. The harvest was filtered and frozen at -70°C.

[0587] **Determination of harvest FIX antigen level:**

[0588] Harvest FIX antigen level was determined using a human FIX ELISA kit (Affinity Biologicals; Cat. # FIX-AG RUO). The antigen level was calculated per each batch. The FIX concentration was maintained through the different batches (Table 17).

Table 17: FIX antigen level

	FIX antigen level		
Batch	**#1**	**Bat #2**	**Bat #3**
Av (µg/ml)	28.81	32.74	42.9
STD	2.5	2.69	4.0
%CV	8.84	8.38.2	9.4

[0589] **FIX-CTP_3 purification process:**

[0590] Following a short purification study, a purification process using the following 3 columns was performed: DEAE Sepharose, Heparin Sepharose and HA Bio Rad Ceramic Hydroxyapatite type 1 (40 µm), FIX-CTP_3. γ-carboxylated enriched protein was purified. In brief: Five liters of clarified harvest was thawed at 4°C over a 4 day period. For each purification batch, the clarified harvest (2 liters) was concentrated 4-fold and dialyzed against 20 mM Tris-HCl pH 8.2 using a disposable hollow fiber cartridge with a nominal molecular

weight cutoff size of 10 KDa. This process (UFDF1) was performed twice, and one liter of UFDF1 was loaded on DEAE Sepharose column, and Factor IX was eluted with 20 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2 pH 8.2. The product was diluted 1:1 with 20 mM Tris-HCl, 10 mM CaCl2 pH 7.5, and the pH was adjusted to 7.5 before loading on Heparin Sepharose column. The elution was performed with 20 mM Tris-HCl, 300 mM NaCl, and 10 mM CaCl2 pH 7.5. The eluted product was concentrated and dialyzed against 10 mM phosphate pH 6.8 using a Pellicon XL cassette 10 KDa cutoff membrane (UFDF2). The product was loaded on an HA column, and the activated fraction of Factor IX was eluted with 150 mM phosphate pH 6.8. The purification product was concentrated to a target concentration of 2 mg/ml and dialyzed against TBS pH 7.45, divided in aliquots and stored at -70°C.

[0591] The purification process was repeated five times, on a weekly basis in order to purify the total volume (25 liters). The purification processes were named HA# 6-10. Each purification product was separately evaluated (App # 1-5). At the end of the purification process, the different batches were pooled and further concentrated to a target concentration of 4 mg/ml.

[0592] **FIX-CTP$_3$ analytical properties:**

[0593] Determination of FIX antigen level

[0594] FIX-CTP$_3$ γ-carboxylated enriched protein antigen level was determined using a human FIX ELISA kit (Affinity Biologicals; Cat. # FIX-AG RUO). The calculated protein concentration is the average of two independent runs (Table 18).

Table 18: FIX-CTP$_3$ antigen level

FIX-CTP$_3$ HA purified pool ELISA #1				FIX-CTP$_3$ HA purified pool- ELISA #2				Final Av
Dil.	1	2	Av.	Dil.	1	2	Av.	
130000	3412240	3781830	3597035	130000	3692260	3568240	3630250	3613643
260000	3915600	4158440	4037020	260000	3706820	3595540	3651180	3844100
520000	4158544	4334096	4246320	520000	3831464	3530748	3681106	3963713
1040000	4096352	4004104	4050228	1040000	3863392	3684304	3773848	3912038
Av. (ng/ml)	3895684	4069618	3982651	Av. (ng/ml)	3773484	3594708	3684096	3833373
STD	338367.5	234486.7	274313.5	STD	86576.66	65369.65	63369.86	154459.6
%CV	8.685703	5.761884	6.887712	%CV	2.294343	1.818497	1.720092	4.029338
Av. (mg/ml)	3.895684	4.069618	3.982651	Av. (mg/ml)	3.773484	3.594708	3.684096	3.833373

FIX-CTP$_3$ HA purified pool ELISA #1				FIX-CTP$_3$ HA purified pool- ELISA #2				Final Av
Dil.	1	2	Av.	Dil.	1	2	Av.	
130000	3412240	3781830	3597035	130000	3692260	3568240	3630250	3613643
260000	3915600	4158440	4037020	260000	3706820	3595540	3651180	3844100
520000	4158544	4334096	4246320	520000	3831464	3530748	3681106	3963713
1040000	4096352	4004104	4050228	1040000	3863392	3684304	3773848	3912038
Av. (ng/ml)	3895684	4069618	3982651	Av. (ng/ml)	3773484	3594708	3684096	3833373
STD	338367.5	234486.7	274313.5	STD	86576.66	65369.65	63369.86	154459.6
%CV	8.685703	5.761884	6.887712	%CV	2.294343	1.818497	1.720092	4.029338
Av. (mg/ml)	3.895684	4.069618	3.982651	Av. (mg/ml)	3.773484	3.594708	3.684096	3.833373

[0595] SDS-PAGE blots:

[0596] FIX-CTP$_3$ γ-carboxylated enriched protein, rhFIX and rFIXa (activated FIX) were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE Coomassie analysis was performed by staining the gel with Coomassie blue reagent (800 ng of protein) (Figure 12). A Western immunoblot was performed using 100 ng of protein with anti-human FIX polyclonal Ab (Figure 12B), anti-human gamma carboxylation monoclonal antibody (American Diagnostics Cat #499, 3570) (Figure 12C), anti-FIX pro-peptide polyclonal Ab (Figure 12D), and anti-CTP polyclonal Ab (Figure 12E). As previously reported, FIX-CTP$_3$ migrated at 75KDa.

[0597] The purification procedure significantly enriched FIX-CTP$_3$ portion while reducing impurities. The purification process yield was very low ranging around 2-3% (data not shown) due to the requirement to collect only the γ-carboxylated FIX-CTP$_3$ fractions, as demonstrated in the anti-Gla immunoblot (Figure 12B). Based on the Coomassie and FIX immunoblot, the FIX-CTP$_3$ portion is only around 60-70%, and additional lower molecular weight bands, presumably with lower glycosylation forms, were also detected.

[0598] **FIX-CTP$_3$ clotting activity:**

[0599] FIX-CTP$_3$ chromogenic activity:

[0600] A comparative assessment of the *in vitro* potency of FIX-CTP$_3$ harvest and FIX-CTP$_3$ γ-carboxylated enriched protein, versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221802). FIX-CTP$_3$ harvest and protein were serially diluted, and the potency was assessed by comparing a dose-response curve to a reference preparation consisting of normal human plasma. As previously demonstrated, FIX-CTP$_3$ harvest was 50 times less active then human pool plasma (Table 19, Figure 13). Following FIX-CTP$_3$ purification, the chromogenic activity was significantly improved and was only 4.72 times less active then human pool plasma (Table 19, Figure 13). Harvest reduced chromogenic activity can be a consequence of improper post-transcriptional modifications of FIX protein variants, e.g. inappropriate gamma

carboxylation and pro-peptide cleavage. Following purification and enrichment of the FIX-CTP_3 γ-carboxylated fraction, the activity was improved, demonstrating the important contribution of γ-carboxylation to FIX activity.

Table 19: FIX-CTP_3 chromogenic activity

Sample	EC_{50} (ng/ml)	Sample /plasma EC_{50} ratio
FIX-CTP_3 Harvest	741.3	54.4
Pur. FIX-CTP_3	64.6	4.72
Plasma	13.63	1

[0601] One stage clotting assay (aPTT):

[0602] The activated partial thromboplastin time (aPTT) is a measure of the integrity of the intrinsic and common pathways of the coagulation cascade. The aPTT is the time, in seconds, for plasma to clot following the addition of an intrinsic pathway activator, phospholipid and calcium. The principal of the assay was to quantitate the ability of FIX-CTP_3 to restore the clotting activity of FIX-depleted human plasma by the addition of rhFIX. 200 µl of FIX-deficient human plasma was mixed with 25 µg/ml of FIX-CTP_3 and further diluted in TBS. Following a 60 second incubation at 37°C, 50 µl of PTT activator (Actin FS) and 50 µl of calcium 25 mM were added to the mixture, and the clotting time in seconds was determined using a Sysmex® CA 1500 Coagulator (performed by Sheba hospital, National Coagulation Center using validated aPTT assay). The potency was assessed by comparison of FIX-CTP_3 to the dose-response curve of a reference preparation of normal human pool plasma. The results are expressed in percent of activity interpolated from the standard curve covering FIX levels of <1-110%. FIX-CTP_3 exhibited a 15-20-fold reduction in its coagulation activity versus normal human pool plasma since the activity at 5 µg/ml, which is the normal value of FIX in the body, was shown to be 6.5% (Table 20).

Table 20: FIX-CTP_3 clotting activity

FIX-CTP_3	**FIX Concentration by provider (mg/ml)**	**Concentration in tested sample (µg/ml)**	**FIX % of activity** (normalized to human normal pool plasma)
	3.83	25	34.7

		5	6.5

[0603] FIX-CTP$_3$ also exhibited increased clotting time compared to BeneFIX® (Table 21 and Figure 14).

Table 21: Comparative clotting time (aPTT)

Clotting time		
	FIX-CTP$_3$	BeneFIX®
38ug/ml	77.6	
19ug/ml	83.4	
7.6ug/ml	93.2	50.6
3.8ug/ml	104.8	57.6
1.9ug/ml	112.2	63.7
0.95ug/ml	122.6	71.5
0.475ug/ml		83.7
0.238ug/ml		94.3

[0604] An additional clotting assay was performed independently in FIX-deficient mice by Dr. Paul Monahan at University of North Carolina prior to the initiation of the PK-PD study. The aPTT results suggested that FIX-CTP$_3$ coagulation activity is 40 times less than normal pooled human plasma as demonstrated by the longer period (as measured in seconds) and higher concentration that are required for proper clotting activity (Table 22).

Table 22: Comparative clotting activity

FIX activity (Units)		
	FIX-CTP$_3$	BeneFIX®
38ug/ml	13.9	
19ug/ml	8.8	
7.6ug/ml	4	116.8
3.8ug/ml	1.6	67.4
1.9ug/ml	0.9	41.7
0.95ug/ml	0.4	22.4
0.475ug/ml		8.5
0.238ug/ml		3.7

[0605] The specific activity (u/ml), which was based on FIX antigen level as calculated by ELISA for FIX-CTP$_3$ and BeneFIX®, was 4.46 and 198.9 respectively.

[0606] The inconsistency in the calculated FIX-CTP$_3$ activity as demonstrated in the

chromogenic vs. aPTT assays can be explained by the superior sensitivity of the aPTT assay and *in vivo* relevance. In the chromogenic activity assay, an excess amount of reagents and enzymes are present which can activate less potent FIX versions. The difference in the FIX-CTP specific activity values can be explained by the use of different reagents and automated machines. The activity value as calculated at University of North Carolina was used for the PK-PD study design.

FIXa Protein detection:

[0607] In order to confirm that following the purification process, FIX activation (FIXa) did not occur, a FIXa detection assay was performed using FIXa Biophen Chromogenic Assay (Cat. # Ref. 221812). The assay measures the amount of FIXa present in a specific sample using the chromogenic activity cascade, as previously described. FIX-CTP_3 and rhFIX were diluted and FIXa levels were evaluated. FIX-CTP_3 wasn't activated through purification or storage (Table 23).

Table 23: FIXa detection

Sample	FIX-CTP_3	rhFIX
Initial Con.(mg/ml)	1000	5.7
rFIXa (mg/ml)	BLQ	0.00487
%FIXa in sample	BLQ	0.085

[0608] **FIX-CTP_3 PK-PD study:** FIX-CTP_3 and rhFIX (BeneFIX®) were administered in a single intravenous injection to C57BI FIX-deficient mice in a dose of 625 μg/kg body weight containing 100 IU FIX/kg body weight. Blood samples were drawn retro-orbitally from 3 mice alternately at 0.25, 4, 24, 48, 72, and 96 hours post-dosing. Citrated plasma (0.32%) was prepared immediately after sampling and stored at -20ºC until analysis. hFIX antigen level was evaluated, and a detailed PK analysis was performed. In order to evaluate the ability of FIX-CTP_3 to elongate the clotting activity of FIX-deficient animals compared to BeneFIX®, FIX activity in citrated plasma samples, collected from the FIX-/- treated mice, was calculated using an automated FIX activity assay (Table 24).

Table 24: Study outline

	Product	Administration	Dose	# mice	Collection Points (hr post-dosing)	Required amount
**Cohort 1	FIX-CTP_3	Single dose: IV	100IU/Kg 2.5IU/mouse (553µg/mouse)	12 mice	0.25, 1, 4,8, 16, 24, 48	**6636µg**
Cohort 2	FIX-CTP_3	Single dose: IV	**472µg/Kg 12.57µg/mouse	18 mice	*0.25,1*, 4*,8*,16*, 24*, 48*, 72*,96*	**200µg 12.57µg/mouse**
**Cohort 3	BeneFIX®	Single dose: IV	100IU/Kg 2.5IU/mouse	18 mice,	0.25, 1,4,8,16, 24, 48, *72,*96	**226.3 µg 12.57µg/mouse**

** PK collection points only*

*** Tail vein bleeding at T=48 post-dosing; cohorts 1 & 3*

[0609] FIX-CTP_3 Pharmacokinetic profile in $FIX^{-/-}$ mice

[0610] FIX concentration was quantitated using human FIX ELISA kits (Affinity Biologicals; Cat. # FIX-AG RUO). The pharmacokinetic profile was calculated for each protein and is the mean of three animals at each time point. Table 25 below and Figure 15 summarize the calculated FIX concentrations at the different sampling time points for Cohorts 1 & 3. The PK profile and a summary of the PK parameters are presented below (Tables 26 & 27). A PK analysis was also performed for Cohort #2 in order to verify exposure (data not shown).

Table 25: FIX concentrations

Time point(hr)	FIX-CTP_3 ng/ml	BeneFIX® ng/ml
0.25	3645.397	2823.023
1	2411.09	2416.248
4	1703.205	1506.228
8	1139.736	864.764
16	415.32	347.465
24	238.37	158.7973
36	141.0105	94.40067
48	95.461	42.28833
72	76.90953	11.87567
96	24.955	BLQ

[0611] A two-compartmental module was used (WinLin software) to determine AUC0-inf,

$T_{terminal}$ and clearance (CL). The PK parameters are described below in Table 26.

Table 26: PK properties

FIX Version	T½α (1/hr)	T½ β (1/hr)	AUC ng/ml*hr	CL ml/Kg/hr	MRT (hr)	Vss (ml/Kg)
BeneFIX®	3.4	12.7	22428	29	11.5	320.8
FIX-CTP$_3$	4	28.7	31770	19	22	425.2

[0612] The addition of the three CTP "cassettes" to rhFIX elongated FIX half-life *in vivo* by at least 2.5-fold. AUC following *in vivo* FIX-CTP$_3$ administration increased 2-fold versus rhFIX. FIX-CTP$_3$-injected mice demonstrated an improved PK profile compared to BeneFIX®-injected mice.

[0613] FIX-CTP$_3$ Pharmacodynamic profile in FIX-deficient mice:

[0614] In parallel to PK sampling, FIX-deficient animals administered with either BeneFIX® or FIX-CTP$_3$, citrated plasma samples, were evaluated for their clotting activity by aPTT assay, which was translated to % activity. The % activity at each collection point was calculated as the current clotting time/clotting time of normal pool mice plasma*100. Table 27 summarizes the activity values following administration of either BeneFIX® or FIX-CTP$_3$.

[0615] Following FIX-CTP$_3$ administration, significant clotting activity was detected one hour after administration reaching 96% activity at four hours post-dosing, while BeneFIX® highest activity value was 40% (Table 27, Figure 16). FIX-CTP$_3$ clotting activity was maintained for a longer period of time, demonstrating elongated activity. Clotting activity for the BeneFIX®-treated mice was undetectable at time points later than 36 hours, while FIX-CTP$_3$-treated mice continued to retain measurable activity at 72 hours post-dosing (Table 27, Figure 16). Analysis of the % clotting pharmacokinetic profile suggest that FIX-CTP$_3$ clotting activity is maintained for a significantly longer period and its half-life is almost 2-fold higher than Benefix® (Table 28).

Table 27: FIX % of activity

Hr post-dosing	BeneFIX® % of activity	FIX-CTP$_3$ % of activity
0.25	39.9	1.0
1	33.4	15.5
4	24.9	93.6
8	18.8	65.2
16	10.3	39.9
24	1.7	11.9
36	1.4	11.0
48	<1	4.6
72	<1	1.4

Table 28: Clotting Activity

FIX Version	T½α (1/hr)	T½ β (1/hr)
BeneFIX®	5.7	-----
FIX-CTP$_3$	7.3	16

[0616] 9.3 FIX-deficient mice bleeding challenge

[0617] FIX-deficient mice were administered a single intravenous injection of 100 IU/kg of BeneFIX® or rFIX-CTP$_3$. The tail vein was slightly clipped 48 hours post-dosing, and tail vein bleeding time (TVBT) and bleeding intensity (hemoglobin OD) were evaluated. A second bleeding challenge was performed 15 minutes after reaching homeostasis, and the same parameters were measured. Following the first bleeding challenge, FIX-CTP$_3$-administered animals' bleeding was significantly less intense then BeneFIX® bleeding as demonstrated by the Hemoglobin OD values (Figure 17).

[0618] Since it was previously reported that during the first bleeding challenge in hemophilic mice, the bleeding time does not necessarily correlate with treatment efficacy, it is recommended to evaluate the homeostasis following additional bleeding. Once the first bleeding was spontaneously or manually stopped, a second bleeding challenge was performed 15 minutes following the first one, and the time and bleeding intensity were re-measured. During the second bleeding episode FIX-CTP$_3$-administered animals had reduced bleeding time and intensity, demonstrating that FIX-CTP$_3$ was potent at a later time points (Figure 18).

[0619] Finally, the animals were further observed for the 12 hours following the second

bleeding challenge, and all recurring bleeding events were documented. FIX-CTP_3-administered animals were able to maintain blood homeostasis for the next 12 hours with no re-occurring bleeding events. In contrast, 50% of BeneFIX®-treated mice had spontaneous bleeding episodes from the tail (Table 29).

Table 29: Outcome 12 hours after tail transection

Mouse group	Delayed rebleeding	Death or Distress Requiring Euthanasia
FIX-CTP_3 (100IU/kg)	**0/5 (0%)**	**0/5**
BeneFIX® (100IU/kg)	**3/6 (50%)**	**0/6**
FIX-/- (untreated)	**5/6 (100%)**	**1/6**

[0620] Recombinant FIX-CTP_3, a fusion protein comprised of a single molecule of FIX fused to three CTP "cassettes" in tandem was developed to address the short half-life of currently available FIX products used to treat patients with hemophilia B. The results herein demonstrated that the elimination half-life of rFIX-CTP_3 was consistently 2.5- to 4-fold longer than rFIX in rats (as previously reported) and in FIX-deficient mice.

[0621] Without being bound by theory, the fusion protein reduces clearance of FIX and protects FIX from protease activity, degradation by masking and reduces the affinity of FIX for hepatic receptors. Taken together these characteristics of the CTP domain extend the half-life of FIX.

[0622] In addition to pharmacokinetic analysis of rFIX-CTP_3, we examined the pharmacodynamic properties of FIX-CTP_3 in FIX-deficient mice. rFIX-CTP_3 and rFIX, were administered at comparable doses (in units) to compensate for the clotting deficiency levels in FIX-deficient mice. However, the effect of rFIX-CTP_3 in FIX-deficient mice was significantly prolonged to at least 76 hr after dosing, reaching a higher activity peak. FIX-CTP_3 clotting activity began after a 1-hour delay compared to BeneFIX®. FIX activation may be required since the addition of three tandem CTPs might theoretically mask the activation site and delay cascade onset. Following FIX-CTP_3 administration, a 100% peak activity was observed, while BeneFIX® activity was only 40%. The superior initial activity is a very important parameter and demonstrates that addition of 3 CTPs has the potential to improve

recovery.

[0623] Prophylactic FIX replacement therapy for patients with hemophilia B aims to maintain plasma levels of 1–2% normal clotting activity. The tail vein bleeding assay is a sensitive *in vivo* test that measures the ability to maintain bleeding homeostasis at low activity values mimicking human bleeding homeostasis model. In response to tail vein bleeding challenge 48 hours post-dosing, rFIX-CTP_3-administered animals maintained blood homeostasis with shorter and less severe bleeding episodes, demonstrating sustained clotting activity.

[0624] FIX is a complex protein that contains a number of functional domains which undergo extensive post-translational modifications. One of the essential post-translational modifications for FIX activity is gamma-carboxylation of the first 12 glutamic acids in the Gla domain by vitamin K-dependent γ-glutamyl carboxylase. This modification facilitates the binding of FIX to phospholipid membranes and, thus, is critical to its function. FIX that is not gamma-carboxylated is not functional, and hence gamma-carboxylation is a rate-limiting step.

[0625] This PK-PD study was conducted using transiently transfected cells. An extensive analytical evaluation of post-translational modifications is performed on the stable FIX-CTP_3 protein produced and secreted from stable optimized clone.

[0626] Based on the presented data, FIX-CTP_3 coagulation factor has the potential to reduce the frequency of injections in patients receiving routine prophylactic doses of FIX replacement therapy. It is anticipated that rFIX-CTP_3 can confer prolonged protection from bleeding following each dose of factor, decrease the overall units of factor needed to treat bleeding episodes, and/or maintain adequate hemostasis during surgical procedures with fewer injections.

EXAMPLE 4

Generation and utilization of Coagulation Factor FVII

[0627] A long-acting version of activated Factor VII (FVIIa) coagulation factor will be useful for the treatment of patients with hemophilia A and B. FVIIa-CTP_3 recombinant protein has the clinical potential to improve the treatment of hemophilia patients by reducing the frequency of infusions and even by reducing the drug load, enabling a prophylactic treatment approach which can significantly improves a patient's quality of life, avoid spontaneous bleeding episodes and accumulated damage to the joint and other organs.

[0628] The generation of a recombinant FVIIa-CTP molecule with an extended half-life based on fusion of FVII to a human CTP is described herein. The recombinant FVIIa-CTP was expressed in mammalian cells and characterized *in vitro* and *in vivo*. It was demonstrated that rFVIIa-CTP activity was comparable to rFVIIa. Pharmacokinetic and efficacy studies in

rats demonstrated improved properties of rFVIIa-CTP. The results of this study demonstrated that it is feasible to develop a half-life extended rFVIIa molecule with very similar haemostatic properties to the wild-type enzyme.

[0629] **Cloning and expression of recombinant FVII molecule**: Several Factor VII clones were constructed in our eukaryotic expression vector (pCI-dhfrr) (Figure 19). Human MGC verified FL cDNA clone (IRCM) containing the sequence of *homo sapiens* coagulation Factor VII was ordered from "Open Biosystems" (OB-MHS4426). The following primers were synthesized by Sigma-Genosys in the following sequence: Primer 67: 5'CTCGAGGACATGGTCTCCCAGGCCC3' (contains the 5' end of Factor VII DNA and the restriction site of XhoI) (SEQ ID NO: 5); Primer 68^R: 5' TCTAGAATAGGTATTTTTCCACATG3' (contains the restriction site of XbaI) (SEQ ID NO: 6); Primer 69**:** 5' TCTAGAAAAAAGAAATGCCAGC3' (contains the restriction site of XbaI) (SEQ ID NO: 7); and Primer 70^R: 5'GCGGCCGCATCCTCAGGGAAATGGGGCTCGCA3' (contains the 3' end of Factor VII DNA and the restriction site of NotI) (SEQ ID NO: 8).

[0630] Cloning was performed in two sets of PCR reactions. The first reaction was conducted with primer 67 and primer 68^R using a cDNA plasmid with the Factor VII sequence (OB-MHS4426) as a template; as a result of the PCR amplification, a ~534 bp product was formed, isolated and ligated into a TA cloning vector (Invitrogen, Catalog No: K2000-01). A XhoI –XbaI fragment containing the amino terminus of the Factor VII sequence was isolated. The second reaction was conducted with primer 69 and primer 70^R and again, a cDNA plasmid with the Factor VII sequence (OB-MHS4426) was used as a template. As a result of the PCR amplification, a ~813 bp product was formed and ligated into TA cloning vector (Invitrogen, Catalog No: K2000-01). A XbaI-NotI fragment containing the carboxy terminus of Factor VII sequence was isolated. The two fragments were inserted into our eukaryotic expression vector pCI-dhfr (triple ligation) to yield the 501-0-p136-1 clone.

[0631] Plasmid 501-p136-1 (Factor VII in pCI-dhfr vector) was digested with restriction enzymes XhoI and KpnI. A fragment of ~1186 bp was isolated. A partial Factor VII clone (1180 bp-1322 bp) followed by a CTP sequence, termination sequence and NotI sequence that was synthesized by GeneArt (0721543) was digested with restriction enzymes KpnI and NotI. A fragment of ~253 bp was isolated. The two fragments were inserted into our eukaryotic expression vector pCI-dhfr (triple ligation) to yield the 501-1-p137-2 clone. pCI-dhfr-701-2-p24-2 was digested with restriction enzymes XhoI and ApaI, and the large fragment (vector) was isolated.

[0632] pCI-dhfr-501-2-p137-2 (Factor VII-ctp x1) was digested with restriction enzymes

XhoI and ApaI, and a ~1200 bp insert was isolated. The vector and insert were ligated to yield 501-2-p139-2. Dg44 cells were plated in 100 mm tissue culture dishes and grown to confluence of 50-60%. A total of 2 μg of DNA was used for transfection of one 100 mm plate using the FuGene reagent (Roche) in protein-free medium (Invitrogen CD Dg44). The medium was removed 48 hours post-transfection and replaced with a protein-free medium (Invitrogen CD Dg44) without nucleosides. After 14 days, the transfected cell population was transferred into T25 tissue culture flasks, and the selection was continued for 10-14 days until the cells began to grow well as a stable clone. High-expressing clones were selected and approximately $2x10^7$ cells were used to inoculate 300 ml of growth medium in a $1700cm^2$ roller bottle (Corning, Corning NY) supplemented with 5 ng/ml of Vitamin K3 (menadione sodium bisulfate; Sigma). The production medium (harvest) was collected after a rapid decrease in the cell viability to around 70%. The production medium was first clarified and then concentrated approximately 20-fold and dialyzed to PBS using flow filtration cassette (10KDaMWCO; Millipore Corp, Billerica, MA).

[0633] **Determination of FVII antigen level**

[0634] The cDNA coding the CTP peptide was fused to the 3' end of the cDNA coding human FVII. The corresponding rFVII construct was transfected into Dg44 cells. As a control, a human rFVII cDNA was utilized. The production medium (harvest) was collected, concentrated and the secreted recombinant FVII was further evaluated. rFVII, rFVII-CTP and rFVII-CTP-CTP antigen levels were determined by AssayMax Human FVII ELISA kit (AssayPro) (Figure 20A). There was no significant difference in the secretion level of rFVII-CTP and rFVII-$(CTP)_2$ compared to native rFVII.

[0635] **SDS-PAGE blots**

[0636] SDS-PAGE analysis was done by loading 50 ng of either harvest, purified or activated rFVII protein. Samples were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE analysis was done by performing a Western immunoblot using an anti-human FVII monoclonal antibody (Ab) (R&D systems) or anti-CTP polyclonal antibody generated in Rabbit.

[0637] The level of rFVII antigen correlated with the detected protein level in a SDS-PAGE immunoblotted with anti-FVII Ab. rFVII-CTP migrated as a single band, while the corresponding molecular weight of the FVII control was approximately 52 KDa (data not shown). Both proteins reacted with antibodies specific for FVII on immunoblots. The rFVII-CTP also reacted with antibodies specific for CTP. rFVII was secreted in its zymogene form with no trace of activated protein.

[0638] **FVII Chromogenic activity**:

[0639] rFVII, rFVII-CTP and rFVII-$(CTP)_2$ harvest activities were determined using a commercially available chromogenic test kit (AssaySense Human FVII Chromogenic Activity Assay Kit (AssayPro). For functional characterization of the rFVII-CTP and its ability to be further activated (FVIIa), concentrated rFVII-CTP (harvests) were placed in a commercially available chromogenic test kit that measure the ability of TF/FVIIa to activate Factor X to Factor Xa that in the presence of FXa specific substrate releases a quantitated signal (AssayPro). The addition of the CTP peptide at the C-terminal of the rFVII protein did not impair the FVII serine protease activity (**Figures 20B and 20C**).

[0640] **FVII clotting activity**:

[0641] Prothrombin time (PT) measures the extrinsic pathway of coagulation. The PT is the time (measured in seconds) it takes plasma to clot following the addition of an extrinsic pathway activator, phospholipid and calcium. It is used to determine the clotting tendency of blood, specifically in the measure of warfarin dosage, liver damage, and vitamin K status. The reference range for prothrombin time is usually around 12–15 seconds. Specifically, the assay quantitated the ability of FVII-CTP and FVII-$(CTP)_2$ harvest to restore the clotting activity of FVII-depleted human plasma by the addition of rhFVII. 300 µl of FVII-deficient human plasma was mixed with 100 µl of FVII, FVII-CTP and FVII-$(CTP)_2$ harvests at specific concentrations, or normal pool human plasma and were further diluted. Following a 60 second incubation at 37°C, Tissue Factor (TF), $CaCl_2$, and phospholipids were added to the mixture. The clotting time in seconds was determined. Potency was assessed by comparing a dose-response curve of FVII-CTP and FVII-$(CTP)_2$ harvests to a reference preparation consisting of rhFVII or human pool plasma. One unit of active FVII was defined as the amount of FVII which equals to the activity of one ml human normal plasma. The PT Clotting activity of rFVII and rFVII-CTP was measured on a coagulometer (Instrumentation Laboratory).

[0642] As previously shown, the addition of a CTP peptide at the C-terminal of the rFVII protein did not damage its serine protease activity and lead to the initiation and activation of a native Factor X and Factor IX in human plasma. Following the insertion of an additional CTP at the C terminal, there was a three-fold reduction in the serine protease activity (data not shown).

[0643] **Pharmacokinetics study:**

[0644] rFVII, rFVII-CTP, and rFVII-$(CTP)_2$ harvests were administered intravenously to Sprague-Dawley rats (six rats per substance) with a dose of 100µg/kg body weight. For all of the *in vivo* experiments, the amount of the respective protein was determined on the basis of FVII ELISA kit. For each FVII test substance, the injected amount was calculated by taking

into account the differences in the molecular weight of rFVII versus rFVII-CTP, which leads to a different molar concentration.

[0645] Blood samples were drawn retro-orbitally using an altering sampling scheme to minimize interference of the sampling procedure levels to be quantified: from 3 rats at 30 and 90 min and at 2, 6, and 48 hrs, and from the remaining three rats at 15 and 60 min and at 1.5, 4, and 24 hrs alternately. Plasma was prepared immediately after sampling and stored at -20ºC until analysis. FVII concentration was quantified by FVII ELISA specific assay. Half-life and area under the curve (AUC) were calculated using a linear trapezoidal rule. Comparison of these clearance parameters revealed that the *in vivo* half-life and rFVII-$(CTP)_2$ AUC are significantly higher than those of rFVII (Table 30).

Table 30: PK study parameters

Group	Route	Dose	T½	AUC_{0-t}	CL/F	MRT
		µg/kg	min	ng/min/mL	mL/min/kg	min
FVII	IV	60	4.07	3314.7	6.195	6.2
FVII-CTP	IV	60	ß=51.06	31353.9	0.287	73.7
FVII-CTP-CTP	IV	60	ß=13.66	7626.8	1.18	15.4

[0646] **Characterization of recombinant FVIIa-CTP**:

[0647] During activation, FVII is cleaved at R152 resulting in heavy and light chain domains that are held together by a single disulfide bridge. rFVIIa-$(CTP)_2$ is purified and activated by an ion exchange column purification process. In order to fully evaluate rFVIIa-$(CTP)_2$, the protein is loaded on SDS-PAGE under reducing conditions to commercial FVIIa (NovoSeven®). The heavy and the light chain domains are separated and migrate as separated bands of molecular weights 55 and 25 KDa. Both proteins react with antibodies specific for FVII, but the heavy chain of the rFVIIa-CTP specifically reacts with anti-CTP-specific antibodies, indicating that this band represents the FVII heavy chain fused to CTP. The light chain reacts specifically with anti-gamma carboxylase Ab. The FVIIa protein concentration is determined by FVIIa-specific ELISA kit.

[0648] **FVIIa N-terminal sequencing**:

[0649] rFVII-CTP-CTP in activated or zymogene purified proteins is separated by SDS-PAGE (on 12% Tris-Glycine) and subsequently electroblotted to a PVDF membrane. The bands of interest are cut out and put on a purified Biobrene-treated glass fiber filter. The N-terminal sequence analysis is carried out by Edmann degradation using a pulsed liquid protein

sequencer equipped with a 140C HPLC microgradient system. The identity of the recombinant protein and proper pro-peptide cleavage is further verified by N-terminal sequencing.

[0650] **FVIIa clotting activity:**

[0651] In order to evaluate FVII-$(CTP)_2$ coagulation activity, activated partial thromboplastin time assay (aPTT) is performed. FVII-deficient plasma sample is substituted with rFVIIa (NovoSeven®) or rFVIIa-$(CTP)_2$. 300 µl of FVII deficient human plasma is mixed with 100 µl of FVIIa or rFVIIa-$(CTP)_2$ at specific concentrations, or normal pooled human plasma which is further diluted. Following 60 seconds incubation at 37°C. Tissue Factor (TF), $CaCl_2$, and phospholipids are added to the mixture. Clotting time in seconds is determined. Potency is assessed by comparing a dose-response curve of rFVIIa-$(CTP)_2$ to a reference preparation consisting of rhFVIIa or human pool normal plasma. One unit of FVIIa is defined as the amount of FVIIa which equals to the activity of 1 ml human normal plasma. The aPTT clotting activity of rFVII and rFVIIa-$(CTP)_2$ is measured on a coagulometer (Instrumentation Laboratory). The aPTT clotting activity of rFVIIa and rFVIIa-$(CTP)_2$ is similar.

[0652] **Pharmacokinetics studies in rats:**

[0653] In order to characterize the influence of the CTP addition to the rFVIIa on its longevity potential, a comparative pharmacokinetic study in rats is performed. NovoSeven® (rFVIIa) and rFVIIa-$(CTP)_2$ in TBS are injected IV to 6 SD rats. The levels of FVIIa over time are detected using a FVIIa ELISA kit. The half-life and AUC are calculated for each protein. Comparison of these clearance parameters reveals that the *in vivo* measures of half-life, recovery, and AUC of the rFVIIa-$(CTP)_2$ are superior to those of NovoSeven®.

[0654] **FVIIa-CTP *in vivo* efficacy model (FVIII-deficient mouse model of hemophilia):**

[0655] In order to assess the *in vivo* activity model, FVIII knockout mice are obtained, and a breeding colony is established. 10 µg of either commercial recombinant hFVIIa (NovoSeven®) or rFVIIa-$(CTP)_2$ are injected into the tail vein of an anaesthetized FVIII knockout mouse (22-28g). The amount of injected protein equals to the required concentration of FVIII in normal plasma (5µg/ml). Blood samples are taken from the clipped tail into heparinized capillary tubes at specific time points. Plasma samples are assessed for FVIIa levels by ELISA, and efficacy is measured by a PTT coagulation assay.

[0656] In this study, a fusion construct of FVII with CTP is generated. This recombinant protein is the basis for a treatment that provides a prolonged half-life and retention of therapeutic potency.

[0657] These results suggest that rFVIIa-$(CTP)_2$ has a similar therapeutic efficacy to rFVIIa

in hemophilia patients. Moreover, this technology requires less frequent dosing. It appears that a single injection of rFVIIa-$(CTP)_2$ is sufficient to control bleeding episodes and reduce the number of injections that are needed during surgical intervention. This recombinant protein may be used as a long term prophylactic treatment.

EXAMPLE 5

Comparative assessment of Purified FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5

[0658] **5.1 Study objective**

[0659] Comparative assessment of pharmacokinetic parameters and clotting activity of FVII-CTP_4 and FVII-CTP_5 versus FVII-CTP_3.

[0660] **5.2 Production of FVII-CTP_4 and FVII-CTP_5 harvests**

[0661] FVII cDNA fused at the C-terminal to four or five tandem CTP sequences was expressed in Dg44 cells using the Excellgene expressing system in the presence of 20 µg/L of vitamin K3 (Sigma, Mennadion). The harvest was collected (300 ml), filtered and frozen.

[0662] **5.3 Production of FVII-CTP_3 harvest**

[0663] FVII-CTP_3 was expressed in-house in mammalian expressing system, CHO cells, using pCI-DHFR vector (**Figure 109**). While the form of the nucleotide cassette encoding FVII-CTP_3 includes nucleotide sequences encoding the signal peptide, for example comprising a nucleic acid sequence as is put forth in SEQ ID NO: 24, the cassette is labeled as MOD-5014 since the expression, purification and activation of the polypeptide encoded therein results in the active FVIIa-CTP_3 form of the coagulation factor. Stable transfected pool #71 was grown in shake flasks, in the presence of 25 ng/L of vitamin K3 (Sigma). The harvests were collected and filtered.

[0664] All FVII-CTP harvests (3, 4 and 5 CTPs) were concentrated and dialyzed against TBS (50 mM Tris, 150mM NaCl, pH 7.4) using Pellicon XL MWCO 10kDa.

[0665] **5.4 Determination of FVII antigen level**

[0666] FVII antigen level was determined using Human FVII ELISA kit (Zymotest HyPhen) (Table 31). The calculated protein concentration is the average of two independent runs.

Table 31: FVII antigen level

	FVII-CTP_3	FVII-CTP_4	FVII-CTP_5
Av. (ng/ml)	224357.3	87884.1	589423
SD	44789.5	3248.7	5309
%CV	20.0	3.7	9

[0667] **5.5 FVII-CTP immune-blot**

[0668] FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 harvests were loaded on 12% Tris-Glycine gel (*expedeon*) using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by Western immune-blot using anti-CTP polyclonal Ab (Adar Biotech Production) or anti-Gla Ab (American Diagnostica).

[0669] FVII fused to three, four and five CTP migrated at 80, 90 and 100kDa, respectively. As expected, FVII-CTP_4 and FVII-CTP_5 harvests from Excellgene contain low gamma carboxylation content as compared to FVII-CTP_3 harvest which was produced at Prolor since the production process wasn't optimized (Figure 21).

[0670] **5.6 Comparative assessment of FVII *in vitro* potency**

[0671] A comparative assessment of the *in vitro* potency of HA purified (highly gamma carboxylated fraction) FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 versus normal human pool plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221304). All samples were serially diluted, and the potency was assessed by comparing a dose-response curve to a reference preparation consisting of normal human plasma. FVII-CTP_3 and FVII-CTP_5 demonstrated chromogenic activity lower than pooled normal plasma (**Figure 22**). FVII-CTP_4 demonstrated higher activity as reflected by EC50 ratios, compared to FVII-CTP_3 and FVII-CTP_5 (Table 32).

Table 32: FVII *In Vitro* Clotting Activity

Sample	EC50 (ng/ml)	Sample /plasma EC50 ratio
Plasma	0.05	
FVII 3CTP	0.12	2.72
FVII 4CTP	0.03	0.71
FVII 5CTP	0.06	1.35

[0672] **5.7 FVII *In Vitro* Clotting Activity:**

[0673] Factor VII (FVII) activity assay, which was performed in Sheba Medical Center, the Israel National Coagulation Center, is a prothrombin (PT)-based assay using immuno-adsorbed plasma deficient in Factor VII (Siemens). The PT reagent is innovin, and the assay is performed in the Sysmex® CA 1500 instrument. FVII normal range is within 55-145%.

Table 33: FVII *In Vitro* Chromogenic Activity

Sample	FVII % of activity	Concentration in tested sample (µg/ml)	Concentration (µg/ml)
FVII 3CTP	36	0.5	224.2
	18	0.25	
	6	0.125	
FVII 4 CTP	334	0.5	87.9
	176	0.25	
	93	6.25	
FVII 5 CTP	38	0.5	58.9
	19	0.25	
	10	0.125	

[0674] Since the normal level of circulating FVII in the body is around 0.5 µg/ml, FVII-CTP_3 and FVII-CTP_5 harvests exhibit 3-fold reductions in their coagulation activity versus normal human pool plasma; this result correlates with the obtained chromogenic activity (Table 33).

[0675] The FVII-CTP_4 harvest exhibits a 3-fold increase in its potential coagulation activity versus normal human pool plasma as observed in the chromogenic activity assay (Table 33). The activity percentage of FVII-CTP_4 is much higher compared to activity percentage of FVII-CTP_3 and FVII-CTP_5. Methodological limitations of the ELISA method may limit the accuracy of Ag level calculations of FVII-CTP_4.

[0676] **5.8 Pharmacokinetic study**

[0677] Two pharmacokinetic studies were performed in order to determine the FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 pharmacokinetics (PK) parameters. During the first study, FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 (Group A, B and C, respectively) were administered in a single intravenous injection to Sprague Dawley rats (six rats per treatment) in a dose of 250 µg/kg body weight. Blood samples were drawn retro-orbitally from 3 rats alternately at 0.083, 0.5 2, 5, 8, 24, 48, 72 and 96 hours post-dosing (Table 34). Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20ºC until analysis.

Table 34: Pharmacokinetic Study Design – Concentrated Harvest

Treatment Group	Test Article	No. of animals/ group/ time point	Dose Route	Dose Level (µg per animal)	Injected Vol. (µl)	Conc. (µg/ml)	Time-Points (hours post-dose)
A	FVII-CTP*3	6	IV	50	200	250	0 (Pre-dose) 0.083, 0.5, 2, 5, 8, 24,48,72,96

B	FVII-CTP*4	6	IV	50	200	250	0 (Pre-dose) 0.083, 0.5, 2, 5, 8, 24,48,72,96
C	FVII-CTP*5	6	IV	50	200	250	0 (Pre-dose) 0.083, 0.5, 2, 5, 8, 24,48,72,96

[0678] FVII concentration in plasma samples were quantified using human FVII Elisa kits (Zymutest FVII-Biophen). The pharmacokinetic profile was calculated and is the mean of 3 animals at each time point. Terminal half-life values were calculated using PK Solutions 2.0 Software. Table 35 below summarizes the calculated FVII concentrations at the different sampling time points. The PK profile (**Figures 23-24**) and a summary of the PK parameters (Table 36) are also presented below. FVII-CTP_5 demonstrated a superior profile as compared to FVII-CTP_3 and FVII-CTP_4 (Table 36).

Table 35: First Pharmacokinetic Study - FVII Concentrations

Time (hr)	AVE-FVII-3-CTP (ng/ml)	SD	AVE-FVII-4-CTP (ng/ml)	SD	AVE-FVII-5-CTP (ng/ml)	SD
0.083	**4214**	583	**3600**	427	**4888**	504
0.5	**3386**	892	**5213**	1682	**5384**	2549
2	**1138**	219	**3603**	1338	**3082**	289
5	**1390**	374	**2726**	1127	**2480**	561
8	**333**	167	**1349**	44	**2316**	633
24	**133**	12	**476**	98	**788**	34
48	**38**	3	**165**	24	**384**	61
72	**12**	2	**91**	62	**167**	31
96	**26**	1	**42**	8	**93**	49

Table 36: Pharmacokinetic Analysis

	FVII 3-CTP	FVII-4-CTP	FVII-5CTP
half-life (0.083-8 hr) (hr)	2.5	4.9	6.6
half-life (8-72hr) (hr)	13.3	16.6	17.7
AUC (ng-hr/ml)(8-72hr)	18374.6	51224.4	72954.2
Vd (ml/kg)(8-72hr)	203.7	91.9	67.7

CL(ml/hr/kg) (8-72hr)	10.6	3.8	2.7

[0679] The addition of four or five CTPs significantly elongated FVII half-life as compared to 3 CTPs by 2- and 3-fold, respectively (Table 36). This superiority was more significant in the initial part of the study (0.083-8 hr), suggesting a potential improved protein recovery and reduced extra vascular clearance. AUC following FVII-CTP_4 and FVII-CTP_5 administration increased by 3- and 4-fold, respectively, versus FVII-CTP_3. Clearance was also reduced while adding 4 and 5 CTPs to FVII (Table 36).

[0680] As observed in the study, the addition of four and five CTPs significantly elongated FVII half-life as compared to 3 CTPs, both in the initial and terminal half-life. The half-life values in the first and second study are different due to a different analysis approach which was effected by the dose and study duration, nevertheless the overall trend was maintained. The AUC following FVII-CTP_4 and FVII-CTP_5 administration increased by 2.5- and 7-fold, respectively, versus FVII-CTP_3.

[0681] **5.9 Conclusions:**

[0682] In this study, the PK parameters and potential clotting activity of FVII-CTP_3, FVII-CTP_4, and FVII-CTP_5 were assessed. Fusion of 4 and 5 CTPs to FVII provided a superior and improved half-life, exposure and reduced clearance as compared to FVII-CTP_3 while maintaining a similar chromogenic and *in vitro* clotting activity. These results were observed at different concentrations of protein and were consistent for both harvest and purified protein. While evaluating the overall effect of fusion of CTP at the C terminus to FVII, fusion of 1-5 CTPs considerably increased the half-life and AUC of FVII in a CTP proportional manner, suggesting that as the CTP portion of the molecule increases, FVII longevity and stability is significantly improved while maintaining its initial *in vitro* clotting activity, as summarized in Table 37 hereinbelow.

Table 37:

Comparative assessment	$T_{1/2}$ percent increase	AUC percent increase
FVII vs. FVII-CTP_2	268	200
FVII-CTP_2 vs. FVII-CTP_3	67	57.8
FVII-CTP_3 vs. FVII-CTP_4	24	178
FVII-CTP_4 vs. FVII-CTP_5	6	42

[0683] As previously reported, FVII half-life correlates with the half-life of the activated form of FVII (FVIIa) both in humans and animals. Therefore, it is anticipated that a similar improvement in half-life will be obtained for the activated versions following CTP fusion.

EXAMPLE 6

FVII-CTP$_3$ feasibility studies in FVIII-deficient hemophilic mice

[0684] Studies described hereinabove testing FVII-CTP, FVII-CTP$_2$ and FVII-CTP$_3$ harvest PK profile and coagulation activity vs. a commercial FVII were conducted. FVII-CTP$_3$ exhibited an improved PK profile while maintaining its coagulation activity vs. FVII-CTP and FVII-CTP$_2$ harvests or rhFVII. In order to further characterize FVII-CTP$_3$ *in vitro* and *in vivo* properties, a mini stable pool expressing and secreting the protein was generated, and purification and activation processes were developed.

[0685] In the current study, the pharmacokinetic and pharmacodynamic properties of FVIIa-CTP$_3$ were tested in FVIII-deficient mice. The PK profile of the protein was evaluated. A FVIIa specific activity-based PK profile was established and compared to commercial product NovoSeven®. In addition, the long-lasting *in vivo* hemostatic capabilities of FVIIa-CTP$_3$ to induce coagulation in FVIII-deficient mice after a tail vain transection (survival study) were tested.

[0686] **Study Objectives:**

[0687] To evaluate the pharmacokinetic and pharmacodynamic parameters of FVIIa-CTP$_3$ vs. commercial rhFVIIa (NovoSeven®) in FVIII-deficient mice following a single IV administration at a similar activity dose.

[0688] To determine the *in vivo* ability of FVIIa-CTP$_3$ to maintain homoeostasis in FVIII-deficient mice by a single IV administration of FVIIa-CTP$_3$ and NovoSeven® at a similar activity dose followed by a challenge of tail vein transection (survival study).

[0689] **Production of FVII-CTP$_3$ harvest:**

[0690] FVII-CTP$_3$ was expressed in-house in Dg44 cells using a pCI-DHFR vector. Stable transfected pool #71 was grown in shake flasks, in the presence of 25 ng/L of Vitamin K3 (Sigma). Cell suspension was cultured and harvested following viability decline to 60-80%. The harvest was filtered and frozen at -70°C.

[0691] **Determination of harvest FVII antigen level:**

[0692] FVII antigen level was determined using human FVII ELISA kit (Zymotest HyPhen) (Table 38). The antigen level was calculated per each pooled harvest batch.

Table 38: FVII-CTP$_3$ antigen level

FVII antigen level

	PK-PD study		Survival study
	harvest 31A	harvest 31B	harvest 38
Av (µg/ml)	16.0	15.9	16.6
STD	1.5	0.0	0.8
%CV	9.1	0.1	4.9

[0693] **FVII-CTP$_3$ purification process (Figure 25)**

[0694] **Process outline**

[0695] Following a short purification study, the following purification process using 2 columns was performed. VII-Select affinity column (GE) and Ceramic Hydroxyapatite type 1 (HA), 40 µm (Bio Rad), FVII-CTP$_3$ γ-carboxylated enriched protein was purified. Auto-activation was induced by incubation of purified FVII-CTP$_3$ in the presence of $CaCl_2$ overnight at 2-8°C. The purification process is in its final developmental stage and is being optimized, thus although most of the purification steps are similar, some part of the purification steps are not identical in the two batches.

[0696] **Ultra-filtration/diafiltration (UFDF) using 10kDa hollow fiber or Pellicon cassette**

[0697] Clarified harvest was thawed at 4°C over the weekend (2-3 days).

[0698] In Batch 31, clarified harvest (12 liters) was concentrated 4-fold (in two successive runs) using a hollow fiber cartridge (GE Healthcare Catalog # UFP-10-C-4X2MA) with a 10 KDa molecular weight cut-off. Concentrated harvest was dia-filtrated against 1-2 volumes of TBS (50mM Tris 150mM NaCl pH 7.4).

[0699] In Batch 38, clarified harvest (8.5 liters) was concentrated 4-fold using a Pellicon 2 (Millipore) cassette with a 10 KDa molecular weight cut-off. Concentrated harvest was directly loaded on VII-Select column.

[0700] Both ultra-filtrations were performed on ice with ice cold buffers. UFDF samples were filtered 0.22 µm before loading.

[0701] **Capture on FVII-Select column**

[0702] The UFDF or concentrated harvest was loaded on VII-Select column (XK16/20, CV 18ml), pre-equilibrated with TBS pH 7.4. The column was washed with 50 mM Tris-HCl, 0.5M NaCl pH 7.5, and FVII-CTP$_3$ was eluted with 50 mM Tris-HCl, 1M NaCl 50% (v/v), Propylene Glycol pH 7.5. The process was performed in two successive cycles utilizing the same column.

[0703] **Gamma carboxylation-based separation on a ceramic hydroxyapatite column**

[0704] The eluted product was diluted 1:10 with 10 mM sodium phosphate pH 6.8 and loaded on ceramic hydroxyapatite columns (XK16/20, CV 24ml). The column was washed with 59 mM sodium phosphate pH 6.8 and the γ-carboxylated rich fraction of Factor VII was eluted with 500mM sodium phosphate pH 6.8. This process was performed in two successive cycles on the same column. At each batch, the eluates of the two cycles were pooled and concentrated to 1.7-2 mg/ml and dia-filtered with 20 mM Tris-HCl, 100 mM NaCl pH 8.2 to reduce volume and prepare the material for the activation step.

[0705] **FVII activation**

[0706] Purified FVII-CTP_3 was diluted to 1 mg/ml and incubated in 20 mM Tris-HCl, 100 mM NaCl and 1mM $CaCl_2$ pH 8.2 at 2-8°C for 24 hours. Activation was terminated by buffer exchange (UFDF) to preliminary formulation buffer (20 mM Citric buffer, 240 mM NaCl, 13.3 mM Glycine, pH 6.9).

[0707] **FVII-CTP_3 and FVIIa-CTP_3 analytical properties:**

[0708] **SDS-PAGE and Western blots**

[0709] Purified FVII-CTP_3, and FVIIa-CTP_3 were loaded on 12% Tris-Glycine gel using Precision Plus Dual Color Protein Marker (Bio-Rad). The SDS-PAGE Coomassie analysis was performed by staining the gel with Coomassie brilliant blue reagent (5 or 10 µg of protein/lane). Western blot analysis was performed (1 µg of protein/ lane) using anti-human FVII polyclonal Ab (R&D systems; AF2338), anti-human gamma carboxylation monoclonal antibody (American Diagnostics Catalog #499, 3570), and anti-CTP polyclonal Ab. Under reduced conditions, FVII-CTP_3 migrated at 75KDa, and FVIIa-CTP_3 migrated as two main bands: a heavy chain at 50 kDa, and a light chain at 25 kDa, represented in **Figure 26** as Bands 2 and 3, respectively.

[0710] The purification procedure significantly enriched the FVII-CTP_3 portion while reducing impurities. The purification process yield was 25-30% FVII (according to ELISA). Most of the protein lost during purification had low FVII chromogenic activity or no activity. Based on Coomassie-stained SDS-PAGE, the reduced FVIIa-CTP_3 contains more than the predicted bands. A band migrating to around ~75 kDa represents non-activated FVII (**Figure 26**, Band 1). This band consists of two bands with minor MW differences, which might reflect different γ-carboxylation content. Additional bands with MW lower than 20 kDa were observed. This was previously reported to be degradation products of the heavy chain.

[0711] **FVII-CTP_3 chromogenic activity:**

[0712] A comparative assessment of the *in vitro* potency of FVII-CTP_3 harvest, in-process fractions, and purified FVII-CTP_3 versus human pool normal plasma was performed using a commercially available chromogenic activity test kit, BIOPHEN (Hyphen BioMed 221304).

FVII-CTP_3 harvest and protein were serially diluted and the potency was assessed by comparing a dose-response curve to a reference preparation of normal human plasma. Following FVII-CTP_3 purification, the chromogenic activity was significantly improved, and non-active fractions were separated mainly by HA column (Figure 27). A strong correlation between FVII chromogenic activity and detection of FVII with monoclonal anti-Gla antibodies in Western blot was observed. The potency of FVII chromogenic activity as reflected by EC50 value in harvest is affected from both carboxylated and non-carboxylated FVII fractions. Following purification and enrichment of FVII-CTP_3 γ-carboxylated fraction, the activity was improved, demonstrating the important contribution of γ-carboxylation to FVII activity (Figure 27). This parameter is crucial for proper FVII *in vivo* activity and will be further addressed in a clone development program.

[0713] **Protein determination by A280**

[0714] The theoretical extinction coefficient of FVIIa-CTP_3 and NovoSeven® was calculated using the ProtParam algorithm (http://web.expasy.org/protparam). The calculation is based on amino acid sequence. The calculated extinction coefficients for FVII-CTP_3 and NovoSeven® is 1.186 and 1.406, respectively. These values represent the absorbance of 1 g/L at 280 nm.

[0715] The extinction coefficient difference between the two proteins derives solely from the increase in molecular weight of FVIIa-CTP_3 compared to NovoSeven®, since CTP lacks aromatic and cysteine residues, thus does not contribute to the absorbance.

[0716] Protein determination by A280 is used for final FVII, and for purified in-process samples, starting from the elution of VII-Select column.

[0717] **Determination of FVIIa antigen level**

[0718] FVIIa antigen level was determined using Human FVIIa ELISA kit (IMUBIND, American Diagnostica). The antigen level was calculated per each batch. However, this tool was not useful for the determination of the dose for injection, since it did not represent the amount of active product.

[0719] **Clotting assay of FVIIa- Staclot® VIIa-rTF**

[0720] FVIIa is derived from an intra-chain cleavage of the single-chain FVII. Native tissue factor (TF) is a cofactor of FVIIa. Upon binding to TF, FVII mediates the activation of Factor X to Xa, while itself is transformed to FVIIa. The soluble tissue factor is the extracellular part of native tissue factor. It can no longer activate FVII by auto-activation, but the FVIIa bound to tissue factor can activate FX to FXa.

[0721] The recombinant soluble tissue factor (rsTF) used in this assay utilizes the FVIIa specificity to construct a FVIIa clotting test. rsTF, in the presence of FVIIa, calcium and phospholipids leads to coagulation of plasma, without activating FVII to FVIIa.

[0722] The observed clotting time in this system has an inverse relationship with the FVIIa content in the tested sample, with no interference of FVII presence in the sample.

[0723] The assay was performed by Omri Laboratories (Nes-Ziona, Israel). FVIIa activity was evaluated for both NovoSeven® following reconstitution and FVIIa-CTP_3 prior to each study. NovoSeven® activity did not correlate with the anticipated activity as reported on the vial, but the discrepancy might be due to a different approach for activity evaluation. Table 39 summarizes the FVIIa clotting activity per volume without considering the protein concentration.

Table 39: FVIIa clotting activity of batch products

	PK study		Survival Study	
	FVIIa-3*CTP (FVIIa 31)	NovoSeven®	FVIIa-3*CTP (FVIIa 38)	NovoSeven®
Activity (U/ml)	1.3E+06	2.5E+05	1.3E+06	7.4E+05

[0724] **Specific activity of FVIIa-CTP_3**

[0725] FVIIa specific activity (which is calculated as the activity/ml divided by protein concentration) was calculated based on A280 and is presented in Table 40. When comparing the specific activity of the two molecules, which differ in MW, compensation must be made in order to normalize the activity (i.e. because of the molecular weight difference, the number of active sites in 1 mg of NovoSeven® is 1.185-fold higher than in FVIIa-CTP_3). Calculation of the conversion factor is presented in the following equation:

$$\text{Normalized_SA} = \frac{SA(FVIa - CTP_3)}{MW.(FVII \quad CTP_3)} \times MW(\text{Native_FVII}) =$$

$$= \frac{SA(FVIIa \quad CTP_3)}{53419.5Da} \times 45079.1Da = SA(FVIIa - CTP_3) * 1.185$$

Table 40: FVIIa-CTP_3 specific activity compared to NovoSeven®

Sample	Average A280	STDV (n=9)	%CV	Extinction coefficient	Prot conc. (mg/ml)	U/ml	Specific Activity		Fold decrease from NovoSeven®
							U/mg protein	U/mg FVIIa	
NovoSeven®	1.274	0.031	2.398	1.406	0.906	8.36E+05	9.23E+05	9.23E+05	1.0
FVIIa-CTP_3	4.396	0.092	2.094	1.186	3.706	7.23E+05	1.95E+05	2.31E+05	4.0

[0726] **FVIIa-CTP$_3$ PK-PD study:**

[0727] **Study outline**

[0728] FVIIa-CTP$_3$ and rhFVIIa (NovoSeven®, NS) were administered in a single intravenous injection to C57B FVIII-deficient mice at a dose of 6.4E6 U/kg body weight (160,000 U/animal). Blood samples were drawn retro-orbitally from 4 mice alternately at 0.166, 0.5, 2, 4, 8, 12, 24, 34, 48, 58, and 72 hours post-dosing (Table 41). Citrated plasma (0.32%) was prepared immediately after sampling and stored at -20°C until analysis. FVIIa clotting activity level was evaluated, and a detailed PK analysis was performed. The study was performed by Omri Laboratories (Nes-Ziona, Israel).

Table 41: Study outline

Treated Groups	Test Article	No. of animals/ group/ timepoint	Dose Route	Amount of Units/ animal	Injected Vol. (µl)	Time-Points (hours post-dose)
A	rhFVIIa	4	IV	1.6e5	200	0 (Pre-dose) 0.166, 0.5, 2, 4, 8, 12, 24, 34, 48, 58, 72
B	FVIIa-CTP$_3$	4	IV	1.6e5	200	0 (Pre-dose) 0.166, 0.5, 2, 4, 8, 12, 24, 34, 48, 58, 72

[0729] **FVIIa-CTP$_3$ PK profile in FVIII-deficient mice**

[0730] FVIIa activity in blood samples was quantitated using a Staclot® VIIa-rTF kit (Stago, Parsippany, NJ). The pharmacokinetic profile was calculated for each protein and represents the mean of 4 animals at each time point. Figure 28 presents the PK profile of FVIIa throughout the experiment. FVIIa recovery is presented in Table 43. A summary of the PK parameters is presented in Table 44.

[0731] Table 42 summarizes the clotting activity values following administration of either NovoSeven® or FVIIa-CTP$_3$. FVIIa-CTP$_3$ and NovoSeven® reached maximal activity half an hour post-dosing. NovoSeven®'s highest activity value reached only 43% of FVIIa-CTP$_3$'s maximal activity value. FVIIa-CTP$_3$ clotting activity was maintained for a longer period of time, demonstrating elongated activity. Clotting activity for the NovoSeven®-treated mice was undetectable at time points later than 12 hours, while FVII-CTP$_3$ treated mice continued to retain measurable activity at 48 hours post dosing (Table 42 and Figure 28).

[0732] The addition of three tandem CTP copies to FVIIa elevated recovery by 100% (Table 43), as measured by the highest activity post-dosing and compared to the anticipated activity based on *in vitro* analysis, and increased the half-life and mean resident time (MRT) 5-fold. The exposure time (AUC) was increased 3-fold (Table 44).

Table 42: FVIIa clotting activity following single IV injection

Time after administration (hours)	**Average FVIIa Clotting Activity (U/ml)**	
	FVIIa-CTP$_3$	**NovoSeven®**
0.16	6.8E+07	3.2E+07
0.5	9.7E+07	4.3E+07
2	2.1E+07	3.9E+06
4	7.7E+06	7.3E+05
8	2.7E+06	4.2E+04
12	3.7E+05	6.2E+03
24	2.4E+04	BLQ
34	4.6E+03	BLQ
48	1.5E+03	BLQ

Table 43: FVIIa-CTP$_3$ recovery

Treated. Groups	**Test Article**	**Amount of Units/ animal**	**Practical administered dose (U/ml)**	***Anticipated Cmax (U/ml blood)**	**Cmax (U/ml)**	**%Recovery**
A	rFVIIa	1.60E+05	1.20E+06	1.40E+05	4.25E+04	**30**
B	FVIIa-CTP$_3$	1.60E+05	1.29E+06	1.50E+05	9.74E+04	**64.6**

*anticipated Cmax is derived from administered dose divided in blood volume

Table 44: PK parameters of FVIIa-CTP$_3$ vs. NovoSeven®

PK Parameters	**NovoSeven®**	**FVIIa-CTP$_3$**
Half-life-$_\alpha$ (0.5-12hr)	**0.94**	1.57
Half-life-$_\beta$ (12-48hr)	NA	**4.62**
AUC (mU*hr/ml)	5.80E+07	1.80E+08
Vd/Kg (ml/Kg)	1408	2375
CL/Kg (ml/hr/Kg)	1034	356
MRT (hr)	1.3	6.7

[0733] **Thrombin generation assay (TGA)**

[0734] The generation of thrombin is a fundamental part of the clotting cascade and as such

an estimate of how well a particular individual can generate thrombin may correlate with either a risk of bleeding or thrombosis. Commonly measured variables when analyzing thrombin generation include: the lag time, the time to peak thrombin generation, the peak, the endogenous thrombin potential [ETP] (i.e., the area under the curve and the tail), the time course of the thrombogram ("TG"). After a lag time, a burst of thrombin is observed. However, clotting occurs at the end of the lag time, when more than 95% of all thrombin has not yet formed. The thrombin generation assay was performed at Omri Laboratories, using Thrombinoscope reagents supplemented with human hemophilic plasma. TGA reflects of the clotting ability in mice plasma, derived from injection of NovoSeven® and FVIIa-CTP_3. Figure 29 presents TGA parameter values for mice plasma following administration of either FVIIa-CTP_3 or NovoSeven®. Following FVIIa-CTP_3 administration, all three parameters (rate of thrombin generation, maximal amount of generated thrombin and KIIa) demonstrate an advantage of FVII-CTP_3 over NovoSeven® treatment. This further strengthens the notion of potential long-acting superiority of FVII-CTP_3 as compared to NovoSeven®.

[0735] **FVIIa-CTP_3 Tail Vain Transection (TVT) study:**

[0736] **Study outline**

[0737] The data obtained from the PK/PD test for FVIIa-CTP_3 provided insight into the functionality of FVIIa-CTP_3, and demonstrated that FVIIa-CTP_3 had a pharmacokinetic advantage when compared with NovoSeven®. However, the ability of the protein to induce a clot *in vivo*, after a traumatic event has not yet been demonstrated. In order to evaluate the ability of FVIIa-CTP_3 to stop bleeding, the same FVIII-deficient mice model was employed for a bleeding challenge.

[0738] FVIII-deficient mice were administered a single intravenous injection of FVIIa-CTP_3 or NovoSeven®. The mice were dosed with drug in amounts that provided equivalent FVIIa activity (1.6E05 units, 200 µl), calculated according to the potency of each drug evaluated in the FVIIa clot activity assay (Table 45). The administered doses were 9 mg/kg of NovoSeven®, and 40 mg/kg of FVII-CTP_3 due to the reduced activity of FVIIa-CTP_3. A control group was injected with 200 µl vehicle.

[0739] The tail vein was transected 2.7 cm from the tail tip 15 min (injection 1), 24 hours (injection 2) or 48 hours (injection 3) post-administration, and mice survival was recorded for 24 hours.

Table 45: Evaluation of injected samples

	NovoSeven®	***FVIIa-CTP_3***

Injection No.	protein conc. (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)	protein conc. (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)	Specific Activity (normalized)
1	0.91	8.0E+05	8.8E+05	3.63	6.6E+05	1.8E+05	2.2E+05
2	0.92	8.3E+05	9.0E+05	3.81	7.8E+05	2.0E+05	2.4E+05
3	0.89	8.8E+05	9.9E+05	3.68	7.3E+05	2.0E+05	2.3E+05

[0740] Protein concentration was determined by A280.

[0741] **Results**

[0742] Data from the vehicle-injected control groups for the three injections (5 animals x 3 injections), were summarized and are presented in Figure 30. 30% survival was observed 24 hours after tail vein transection.

[0743] NovoSeven® and FVIIa-CTP_3-treated mice demonstrated proper hemostatic activity after tail vein transection performed 15 min after FVIIa administration. A 100% survival rate was observed in FVIIa-CTP_3 and NovoSeven® treated animals (Figure 30).

[0744] The reduced clearance rate of FVII-CTP_3 which was demonstrated in the PK/PD study is most clearly appreciated after a tail vein transection performed 24 hours post-administration. A decline in the survival rate of NovoSeven® is observed. Similar to the control group, 50% death is observed within 10 hours. Meanwhile, 90% of FVIIa-CTP_3 treated mice survived (Figure 30). This result emphasizes the long-lasting efficacy of the FVIIa-CTP_3 treatment.

[0745] 48 hours after administration, a decline in survival rate is demonstrated in groups treated with either FVIIa-CTP_3 or NovoSeven® (Figure 30C). A slight improvement in FVIIa-CTP mice was observed, but the difference did not reach statistical significance.

[0746] **Discussion:**

[0747] CTP fusion to recombinant proteins extends the circulatory half-life of proteins while maintaining comparable activity. While the mechanism behind the reduced clearance of protein above a threshold size of 70 KDa is well understood with respect to renal clearance, additional protection is achieved following CTP fusion. CTP fusion is believed to sweep around the protein shield and protect it from proteolytic cleavage, to increase its radial molecular weight due to the highly negative charge and to reduce its affinity to hepatic clearance receptors.

[0748] The present study was aimed to provide specific insight on the impact of CTP fusion

to FVII on protein half-life and clearance and also address the paradigm of its specific activity following this modification. FVIII-deficient mice were administered with a single IV injection of FVIIa-CTP_3 or recombinant commercial FVIIa (NovoSeven®) at similar dose (unit based) and a PK activity-based analysis was performed. FVIIa-CTP_3 demonstrated a superior longevity as reflected by 5- and 3.5-fold increase in its half-life and AUC, respectively. The specific activity (U/mg) of FVIIa-CTP as calculated by the Staclot® activity kit divided by the protein concentration measured by A280 was shown to be 4-5 times lower than the specific activity of NovoSeven®.

[0749] To build on the understanding of how CTP affects the haemostatic effects of FVIIa *in vivo*, the ability of FVIIa-CTP_3 to reduce bleeding was investigated. In the tail vein transection bleeding model in hemophilic mice model, rFVIIa administration can improve the survival rate of challenged animals and avoid their bleeding to death. In the study described herein, animals were administered with FVIIa-CTP_3 or NovoSeven®. Both molecules were able to maintain homeostasis when the transection was performed 0.25 hours post-dosing. A significantly prolonged duration of activity was demonstrated for the FVIIa-CTP_3-treated group when the tail transection was performed 24 hr post dosing. The vehicle-treated group's survival rate was higher than anticipated and higher than that obtained in previous studies (50% vs. 20% in previous studies, data not shown). The percent survival of treated animals at is further evaluated at earlier time points, including at 36 hr post dosing.

[0750] In conclusion, it was demonstrated that FVIIa-CTP_3 has an increased duration of activity in hemophilic mice which translates into a longer duration of haemostatic effect when compared to NovoSeven®. The data gathered suggest that fusion of CTP to FVII is a technology with the potential to significantly improve prophylactic treatment in patients with hemophilia.

EXAMPLE 7: COMPARATIVE ASSESSMENT OF PURIFIED FVII-CTP_3 vs. FVII-CTP_5 PROFILE FOLLOWING SINGLE IV or SC INJECTION TO SD RATS

Study objective

[0751] Two studies were carried out:

[0752] The first study objective was to determine the pharmacokinetic parameters of rFVII-CTP3 versus rFVII- CTP5 following FVII select- and HA-column purification in male Spargue Dawley rats, after a single intravenous administration of 50µg/animal.

[0753] In the second study, rFVII-CTP3-HA versus rFVII-CTP5-HA pharmacokinetic parameters, were examined in male Spargue Dawley rats following a single intravenous or subcutaneous administration of 100 µg/animal.

RESULTS

[0754] **Determination of FVII-CTP 3 and FVII-CTP 5 antigen level**

[0755] FVII antigen level was determined using Human FVII ELISA kit (Zymotest HyPhen) (Table 46). T

[0756] **Table 46**. Summarizes the calculated protein concentration which is the average of three independent runs.

	FVII 3 CTP		FVII 5 CTP	
	FVIIS 46 el. Conc. Dial	FVII HA 46 el. Conc. Dial	FVIIS el. Conc. Dial	FVII HA 5 100%B Conc. Dial
AVE (ng\ml)	3.78E+06	1.59E+06	1.88E+06	7.92E+05
SD	1.30E+06	6.03E+05	7.15E+05	3.57E+05
CV (%)	3.43E+01	3.80E+01	3.80E+01	4.51E+01

Western blot analysis of the examined samples

[0757] FVII-CTP$_{3,\ 5}$ samples were loaded on 4-12% bisTrisgel (*NuPage, invitrogene*) using Precision plus dual color protein marker (Bio-Rad). The SDS-PAGE analysis was performed by western immune-blot using polyclonal anti FVII Ab (R&D systems), anti CTP polyclonal Ab (Adar biotech production) or anti Gla Ab (American diagnostica). In summary, FVII fused to three and five CTP migrated at 80 and 100kDa, respectively (see Figure 31).

Comparative assessment of FVII *in vitro* potency

[0758] FVII activity assay, which was performed in Sheba medical center, the national coagulation center, is a PT based assay using immunoadsorbed plasma deficient in factor VII (Siemens). The PT reagent is innovin and the assay is performed in the Sysmex CA 1500 instrument. FVII normal range is within 55-145%. Sample activities are summarized in Table 47.

Table 47: Sample activity

Sample	Concentration (mg/ml) according to (NANODROP)	Concentration in tested sample (µg/ml)	Results (%)	Average-% of plasma
FVII-5CTP FVIIS el. Conc. Dial	2.19	2	**87**	16%
		1	30	
		0.5	10	
FVII-5CTP HA 5 100%B conc. Dial	1	2	97	21%
		1	36	
		0.5	13	
FVIIS 46 el. Conc. Dial	3.17	2	100	18%
		1	35	
		0.5	12	
FVII HA 46 el. Conc.	1.5	2	92	20%

Dial (1)		1	33	
		0.5	10	

[0759] The normal level of circulating FVII in the body is around 0.5µg/ml. Both, FVII-CTP_3 and FVII-CTP_5 exhibit about 5 fold reductions in their coagulation activity versus normal human pool plasma.

Pharmacokinetic study

[0760] Two pharmacokinetic studies were performed in order to determine the FVII-CTP_3 and FVII-CTP_5 (after FVII select and FVII HA column) pharmacokinetics (PK) profile and parameters. In the first study, FVII-CTP_3, and FVII-CTP_5 following FVII select/ HA purification were administered in a single intravenous injection to Spargue Dawley rats (six rats per substance) in a dose of 50 µg/rat.

[0761] Blood samples were drawn retro-orbital from 3 rats alternately at 0.083, 0.5 2, 5, 8, 24, 48, 72, 96 and 120 hours post dosing. Citrated plasma (0.38%) was prepared immediately after sampling and stored at -20 until analysis.

[0762] In the second study, only samples after HA column were tested. These samples were administered in a single intravenous or subcutaneous injection to Spargue Dawley rats (six rats per substance) using a dose of 100 µg/rat. Blood samples were collected at the same time points and conditions as at the first study above.

[0763] **Table 48.** First study design (FVII select vs. FVII HA).

Treated Groups	Test Article	No. of animals/ group/	Dose Route	Dose Level (µg per animal)	Injected Vol. (µl)	Conc. (µg/ml)	Time-Points (hours post-dose)
A	FVII-CTP*3 batch 46 HA	6	IV	50	200	250	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24, 48, 72, 96, 120
B	FVII-CTP*3 batch 46 FVIIS	6	IV	50	200	250	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24, 48, 72, 96, 120
C	FVII-CTP*5batch 5 HA	6	IV	50	200	250	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24, 48, 72, 96,

							120
D	FVII-CTP*5 batch 5 FVIIS	6	IV	50	200	250	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24, 48, 72, 96, 120

[0764] Table 49. Second study design (IV vs. SC)

Treated Groups	**Test Article**	**No. of animals/ group/**	**Dose Route**	**Dose Level (µg per animal)**	**Injected Vol. (µl)**	**Conc. (µg/ml)**	**Time-Points (hours post-dose)**
A	FVII-CTP*3 batch 46 HA	6	**IV**	100	200	500	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24,48,72, 96,120
B	FVII-CTP*3 batch 46 HA	6	**SC**	100	200	500	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24,48,72, 96,120
C	FVII-CTP*5batch 5 HA	6	**IV**	100	200	500	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24,48,72, 96,120
D	FVII-CTP*5 batch 5 HA	6	**SC**	100	200	500	0 (Pre-dose) 0.083 0.5, 2, 5, 8, 24,48,72, 96,120

[0765] The main differences between these two studies are the dosages and the route of administration. In the first study, rats were injected IV with 50 µg\rat, while in the second

study, the rats were injected IV or SC with 100 µg\rat (total 500 µg/kg; rats weigh 200 g). The increase in the dosage is due to the change in the type of administration; SC administration requires higher amounts to achieve effects similar to IV administration.

Analysis of PK study

[0766] FVII concentration in plasma samples were quantified using human FVII Elisa kits (zymutest FVII-Biophen). Pharmacokinetic profiles were calculated and reflect the mean for 3 animals at each time point. Terminal half-live values were calculated using PK solutions 2.0 software. The table below summarizes the calculated FVII concentrations at the different sampling time points. PK profile and a summary of the PK parameters are presented in table below.

[0767] Table 50. First pharmacokinetic study (FVII select vs. FVII HA) -FVII concentrations (ng\ml).

Time (hour)	FVII CTP*3 BATCH 46 HA	FVII CTP*3 BATCH 46 FVII S	FVII CTP*5 BATCH 5 HA	FVII CTP*5 BATCH 5 FVII S
0.083	1816.3	1633.9	2064.3	1853.5
0.5	1523.7	1409.9	1351.4	1418.0
2	1284.9	1041.7	1389.7	834.4
5	607.9	531.6	722.7	737.2
8	524.2	430.0	712.2	614.6
24	115.5	132.9	272.5	201.8
48	21.1	31.6	62.3	90.4
72	9.5	15.8	29.1	31.8
96	BLQ	5.8	7.0	16.9
120	BLQ	BLQ	8.5	13.4

[0768] Table 51. Second pharmacokinetic study (IV vs. SC) -FVII concentrations (ng\ml).

Time (hour)	FVII CTP*3 BATCH 46 HA-IV	FVII CTP*5 BATCH 5 HA-IV	FVII CTP*3 BATCH 46 HA-SC	FVII CTP*5 BATCH 5 HA-SC
0.083	6452.6	6153.3	5.0	BLQ
0.5	3930.7	3660.6	14.5	14.6
2	1992.3	2176.2	113.6	96.2
5	1598.9	2087.3	106.6	70.5

8	781.6	1075.6	188.9	129.7
24	268.5	627.2	155.0	239.2
48	51.9	143.3	43.0	88.6
72	8.8	39.0	7.0	36.7
96	BLQ	10.8	BLQ	10.4
120	BLQ	8.2	BLQ	8.7

[0769] Table 52. PK Analysis- first pharmacokinetic study (FVII S vs. HA).

	FVII CTP*3 BATCH 46 HA	**FVII CTP*3 BATCH 46 FVII S**	**FVII CTP*5 BATCH 5 HA**	**FVII CTP*5 BATCH 5 FVII S**
half-life (0.083-8 hr) (hr)	4.3	4.0	5.51	5.59
half-life (8-72\96\120hr) (hr)	11.1	12.1	16.46	20.29
half-life (8-72) (hr)	11.1	13.4	13.62	15.64
AUC(O-t)(obs area)(8-72/96/120hr)	14566.9	13686.4	21812.7	19307.9
AUC (∞) area(8-72/96/120hr)	14718.2	13788.1	22013.9	19701
Vd(area)/kg (ml/kg)(8-2/96/120hr)	271.1	316.1	269.7	371.5
CL(area)/kg(ml/hr/kg) (8-72/96/120hr)	17.0	18.1	11.356	12.69

[0770] The addition of five CTP elongated FVII half-life compared to 3 CTPs. Both forms of 5 CTP (i.e FVIIS and FVII HA) were detected at the long time points (96 and 120 hr), while FVII-3 CTP HA and FVIIS -3 CTP were detected until 72 hr and 96 hr, respectively. Based on this fact, the half-life of FVII-5 CTPs is longer than 3CTPs variants (see **Figure 32**). Comparing half-life of all examined materials (3 and 5 CTPs) at the same time points (8-72 hr) showed that the half-life are similar, although 5 CTP are quite longer (**Figure 32**).

[0771] Table 53**: PK analysis - second pharmacokinetic study-(IV vs. SC).**

	FVII CTP*3 BATCH 46 HA-IV	**FVII CTP*5 BATCH 5 HA-IV**	**FVII CTP*3 BATCH 46 HA-SC**	**FVII CTP*5 BATCH 5 HA-SC**
half-life (0.083-8 hr) (hr)	3.0	3.9	-1.8	-3.18

half-life (8-72\96\120hr) (hr)	9.9	14.6	13.14	22.94
half-life (8-72) (hr)	9.9	13.0	13.14	29.47
AUC(O-t)(obs area)(8-72/96/120hr)	28866.8	43761.0	6600	9822.7
AUC (∞) area(8-72/96/120hr)	28993.0	43934.4	6733	10110.8
Vd(area)/kg (ml/kg)(8-72/96/120hr)	246.4	240.5	1407.6	1636.8
CL(area)/kg(ml/hr/kg) (8-72/96/120hr)	17.2	11.4	74.261	49.452

[0772] Again, as observed in the first study, the addition of 5 CTPs elongated FVII half-life as compared to adding 3 CTP, both in the initial and terminal half-life and in both administration ways (IV and SC, see **Figure 33**). As expected, following SC administration, FVII was first detected in the blood at a later time point as compared to when it was administered IV.

[0773] In the above, two PK studies were summarized. The main purpose of the first study was to check the difference between FVII-3CTP and FVII-5 CTP after 2 different columns: FVII select and FVII HA. In our previous studies, harvest vs. purified proteins were checked and it was found that the difference between 3 and 5 CTP versions of FVII was greater when harvest was injected to the rats.

[0774] There was no significant difference between the results of FVII 3\5 CTP after both columns, hence it was decided to inject FVII HA 3\5 CTP in the second study (IV vs. SC).

EXAMPLE 8: FVIIa-CTP$_3$ (MOD-5014) SURVIVAL STUDY IN FVIII DEFICIENT MICE FOLLOWING SUBCUTANEOUS INJECTION

Study Objective

[0775] To evaluate the efficacy of NovoSeven®, MOD-5014 (FVIIA-CTP$_3$) and MOD-5019 (FVIIA-CTP$_5$) in a tail vein transection study, following subcutaneous administration.

FVIIa-CTP$_3$ (MOD-5014) and FVIIa-CTP$_5$ (MOD 5019) analytical properties:

Protein determination by A280

[0776] Theoretical extinction coefficient of NovoSeven® was calculated using ProtParam algorithm (http://web.expasy.org/protparam). The calculation is based on amino acid

sequence. The calculated extinction coefficient for NovoSeven® is 1.406, and for MOD-5019 is 1.075 (values represent the absorbance of 1 g/L at 280 nm). Extinction coefficient of MOD-5014 was determined by amino acid analysis at Mscan. The extinction coefficients for MOD-5014 is 1.27.

Clotting assay of FVIIa - STACLOT VIIa-rTF

[0777] FVIIa is derived from intra-chain cleavage of the single-chain FVII. Native tissue factor (TF) is a cofactor of FVIIa, upon binding to TF, FVII mediates the activation of Factor X to Xa, while itself is transformed to FVIIa. The soluble tissue factor is the extra cellular part of native tissue factor. It can no longer activate FVII by auto activation, but the FVIIa bound to tissue factor can activate FX to FXa.

[0778] The recombinant soluble tissue factor (rsTF) used in this assay is utilizing the FVIIa specificity to construct a FVIIa clotting test. Recombinant soluble tissue factor (rsTF), in the presence of FVIIa, calcium and phospholipids, produces coagulation of plasma without activating FVII to FVIIa.

[0779] The observed clotting time in this system has an inverse relationship with the FVIIa content in the tested sample, with no interference of FVII presence in the sample.

[0780] FVIIa activity was evaluated for reconstituted NovoSeven®, and for MOD-5014 and MOD-5019 prior to each study.

[0781] FVIIa specific activity (which is calculated as the activity/ ml divided by protein concentration) was calculated based on A280 and is presented in Table 54. When comparing specific activity of the two molecules, which differ in molecular weight, compensation must be made in order to normalize the activity (i.e. because of the molecular weight difference, the number of active sites in 1 mg of NovoSeven® is 1.185 fold higher than in MOD-5014 and 1.307 fold higher than MOD-5019). Hence, calculation of the conversion factor is presented in the following formula:

$$\text{Normalized_SA} = \frac{\text{SA(FVIa-CTP}_3\text{)}}{\text{MW.(Native_FVII)}} \times \text{MW(FVII CTP}_3\text{)} =$$

$$= \frac{\text{SA(FVIIa CTP}_3\text{)}}{45079.1\text{Da}} \times 53419.5\text{Da} = \text{SA(FVIIa-CTP}_3\text{)} * 1.185$$

Table 54- MOD-5014 Specific activity compared to NovoSeven®

Sample	Protein conc. By A280 (mg/ml)	Specific Activity (U/mg FVIIa)	Fold decrease from ®NovoSeven
®NovoSeven	0.93	52,487	1.0
MOD-5014 batch 73	1.4	25,490	2.05
MOD-5019 batch 9	3.0	11,698	4.48

Study outline

[0782] The most significant measurement is the ability of the protein to induce a clot *in vivo*, after a traumatic event. In order to evaluate the ability of MOD-5014 to stop bleeding, the same FVIII deficient mice model was employed for a bleeding challenge.

[0783] FVIII deficient mice were administrated with a single subcutaneous injection of MOD-5014, MOD-5019 or NovoSeven®. Group A and B were dosed with NovoSeven® and MOD-5014 respectively, in equivalent amounts as FVIIa activity. Group C was dosed with MOD-5019 in equivalent amount FVIIa protein as MOD-5014, in order to evaluate the critical factor (activity or amount of protein). The administrated doses were 4.2 mg/kg of NovoSeven®, and 8.6 mg/kg of MOD-5014 and MOD-5019. The tail vein was transected 2.7cm from tail tip 12 hours post administration, and mice survival was recorded for 24 hours.

[0784] **Table 55 – Group designation**

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Group	Injection date	Test Article	Administered Dose		Injected Volume (µl)	No. of mice per group	Bleeding time, hours post dosing
			mg FVII /Kg	mU/Kg			
A	13.1.13	®NovoSeven	4.23	221,876	100	10	12
B	15.1.13	MOD-5014, batch 73	8.59	218,750	160	10	12
C	27.1.13	MOD-5019, batch 9	8.59	100,496	160	10	12

RESULTS

[0785] The experiment data is summarized in Table 56- and in Figure 34.

[0786] Table 56**.** TVT study results

Time post TVT (h)	No. of surviving mice			% survival		
	NovoSeven®	MOD-5014	MOD-5019	NovoSeven® ®	MOD-5014	MOD-5019
0	9	10	10	100	100	100
1	9	10	10	100	100	100
2	9	10	10	100	100	100
3	8	10	8	89	100	80
4	6	9	8	67	90	80
5	5	9	7	56	90	70
6	4	8	5	44	80	50

7	3	8	5	33	80	50
8	2	7	5	22	70	50
9	1	6	5	11	60	50
10	1	5	5	11	50	50
11	1	3	5	11	30	50
12	1	3	5	11	30	50
24	1	3	4	11	30	40

[0787] 24 hours post TVT, only 11% of NovoSeven® injected mice have survived. 30% of MOD-5014 and 40% of MOD-5019 have survived to this time point. Surprisingly, subcutaneously injected MOD-5014 and MOD-5019 shows improved mice survival in comparison to NovoSeven®.

[0788] Factor VIIa, like other coagulation factors, is normally injected intravenously, in order to be directly available in the blood stream. However, the present invention shows that the compositions provided herein are surprisingly more effectively absorbed into the bloodstream after SC administration. To be able to administer FVIIa subcutaneously serves as an advantage as it can be used for prophylactic applications. Subcutaneous injections are also much easier for patients to self-inject, and are advantage when the patients are very young and their veins are small and difficult to find.

[0789] Hence, the subcutaneous application can be used for prophylactic treatment.

EXAMPLE 9: COMPARATIVE PK-PD STUDY OF RECOMBINANT MOD-5014 Vs. NovoSeven® FOLLOWING SUBCUTANEOUS ADMINISTRATION IN SD RATS

Study Objectives

[0790] To determine the pharmacokinetic and pharmacodynamic parameters of MOD-5014 versus commercial rFVIIa in SD rats following a single SC administration.

[0791] To compare two independent experiments (05010 & 05034) containing MOD-5014 products originated from two different clones (clone no. 28 vs. 61) by their pharmacokinetics parameters.

[0792] **Experimental Methods**

Animals

[0793] 24 males SD rats arrived from Harlan Laboratories Israel, Ltd, at least 4 days before the injections begin. The animals were healthy young adults, at ~200 gr at study initiation. The body weight variation of animals at the time of treatment initiation should not exceed ± 20% of the mean weight of each sex. The health status of the animals used in this study is examined on arrival. Only animals in good health are acclimatized to laboratory conditions

and are used in the study.

Clotting assay of FVIIa - STACLOT VIIa-Rtf

[0794] The recombinant soluble tissue factor (rsTF) used in this assay is utilizing the FVIIa specificity to construct a FVIIa clotting test. rsTF, in the presence of FVIIa, calcium and phospholipids produce coagulation of plasma, without activating FVII to FVIIa.

[0795] The observed clotting time in this system has an inverse relationship with the FVIIa content in the tested sample, with no interference of FVII presence in the sample.

[0796] FVIIa activity was evaluated for both NovoSeven® following reconstitution and MOD-5014 prior to each study. FVIIa specific activity was calculated based on A280. When comparing specific activity of the two molecules, which differ in MW, compensation must be made in order to normalize the activity (i.e. because of the molecular weight difference, the number of active sites in 1 mg of NovoSeven® is 1.185 fold higher than in MOD-5014).

PK solver software

[0797] The pharmacokinetic parameters were calculated using PK solver software. The IV administration curve analyzed as two compartmental CA bolus, and the SC administration as NCA Extravascular- Log linear trapezoidal analysis. Half-life, AUC, clearance and volume distribution specifications were calculated and the output parameters were studied in comparison between groups of experiments.

Experimental materials

[0798] Experiment no. 05010:

A. NovoSeven® RT: (Lot # AU61553 prepared on 31.7.12*) FVIIa concentration by A280: 0.86 mg/ml. FVIIa Staclot activity assay: 56,867 U/mg. Injected dose: **946µg/kg**. *Pool of NovoSeven® aliquots, all from the same Lot no.

B. Clone 28: MOD-5014 RS12-001: 0.77 mg/ml** based on A280. FVIIa Staclot activity assay: 34,162 U/mg. Injected dose: **850µg** FVIIa**/kg**.

[0799] Experiment no. 05034:

A. NovoSeven® RT: (Lot #AU61347 prepared on 1.1.13) FVIIa concentration by A280: 0.82mg/ml, diluted to 0.4 mg/ml with sterile NS buffer. FVIIa Staclot activity assay: 55,688 U/mg. Injected dose: **360µg/kg** and **20,047.7 U/kg.**

B. Clone 61: MOD-5014 Batch 75: 1.9 mg/ml** based on A280, diluted to 0.89 mg/ml with formulation buffer. Injected dose: **20,047.7 U/kg**. FVIIa clotting activity: 25,002* U/mg based on FVIIa Staclot activity assay.

C. Clone 61: MOD-5014 Batch 81A: 2.36 mg/ml based on A280 (filtered on the morning of study day and re-measured at 280nm), diluted to 0.4 mg/ml with formulation buffer. Injected dose: **360µg**FVIIa**/kg.** FVIIa clotting activity: 24943U/mg based on FVIIa Staclot

activity assay.

D. Clone 61: MOD-5014 Batch 81A: 2.36 mg/ml based on A280, diluted to 0.89 mg/ml with formulation buffer. Injected dose: **20,047.7 U/kg.** FVIIa clotting activity: 24,943U/mg based on FVIIa Staclot activity assay.

[0800] **Study outlines**

[0801] **Experiment no. 05010**

[0802] MOD-5014 and NovoSeven® were administered in a single intravenous or subcutaneous injection to SD Rats in a dose of 0.9 mg/kg body weight. Blood samples were drawn from sinus orbital eye from 3 rats alternately at 0.5, 4, 8, 12, 24, 34, 48 and 58 hours post dosing. Citrated plasma (0.32%) was prepared immediately after sampling and stored at -20°c until analysis. The study was performed at "Science in Action," Nes-Ziona. FVIIa clotting activity level was evaluated and detailed PK analysis was performed at Prolor-Biotech.

Table 57: Study design 05010

Treated Groups	Test Article	No. of animals /group	No. of animals/ group/ Time point	Dose Route	Gender	Dose Level (µg/kg)	Injected Vol. (µl)	Time-Points (hours post-dose)
A	rFVIIa (NovoSeven®)	6	3	IV	M	946	220	0, 0.5, 4, 8, 12, 24, 34, 48, 58
B	rFVIIa RS12-001 (clone 28)	6	3	IV	M	850	220	0, 0.5, 4, 8, 12, 24, 34, 48, 58
C	rFVIIa (NovoSeven®)	6	3	SC	M	946	220	0, 0.5, 4, 8, 12, 24, 34, 48, 58
D	rFVIIa RS12-001 (clone 28)	6	3	SC	M	850	220	0, 0.5, 4, 8, 12, 24, 34, 48, 58

[0803] **Experiment no. 05034**

[0804] MOD-5014 and NovoSeven® were administered in a single subcutaneous injection to SD Rats in a dose of 0.9 mg/kg body weight. Blood samples were drawn from sinus orbital eye from 3 rats alternately at 0.5, 2, 4, 6, 8, 12, 24, 34, 48 and 72 hours post dosing. Citrated plasma (0.32%) was prepared immediately after sampling and stored at -20°c until analysis. The study was performed at "Science in Action," Nes-Ziona.

[0805] FVIIa clotting activity level was evaluated and detailed PK analysis was performed at Prolor-Biotech.

Table 58: Study design 05034

Treated. Groups	Test Article	No. of animals/ group/ Time-point ***	Dose Route	Gender	Dose Level Per Animal (µg/kg)	Dose Level Per Animal (U/kg)	Injected Vol. (µl)	Time-Points (hours post-dose)
A	FVIIa (NovoSeven®)	3	SC	M	**360**	20047.7	207	0, 0.5, 2, 4, 6, 8, 12, 24, 34, 48,72
B	FVIIa 75 (clone 61)	3	SC	M	801.84	**20047.7**	207	0, 0.5, 2, 4, 6, 8, 12, 24, 34, 48, 72
C	FVIIa 81A (clone 61)	3	SC	M	**360**	8979.48	207	0, 0.5, 2, 4, 6, 8, 12, 24, 34, 48, 72
D	FVIIa 81A (clone 61)	3	SC	M	803.74	**20047.7**	207	0, 0.5, 2, 4, 6, 8, 12, 24, 34, 48, 72

RESULTS

[0806] FVIIa activity in blood samples was quantitated using STACLOT VIIa-rTF kit (Stago). Pharmacokinetic profile was calculated for each protein and is the mean of 3 animals at each time point.

[0807] **Experiment no. 05010**

[0808] Figure 35 presents the PK profile of FVIIa following IV and SC administration of either NovoSeven® or MOD-5014. Summary of FVIIa activity values for each time point is presented in Table 59. IV and SC administration have different PK pattern as presented in Figure 35 similar to previous results. The Cmax following IV injection is higher than that obtained after SC injection, due to the presence of the drug immediately following administration in the blood (measured at 0.5hr, Table 59 and Table 60). However, after SC administration drug molecules transfer to intracellular matrix and tissues, thus Cmax can be measured only after 2hr from injection. The total recovery of the drug after SC administration is lower than Cmax value after IV injection.

[0809] 8hr after injection, Novoseven® manifested an equal PK pattern when injected by either IV or SC, (**Figure35**). Moreover, clotting activity for the NovoSeven®-treated mice was undetectable at time points later than 12 hours, while **MOD-5014-treated rats** continued

to retain measurable activity at 58 hours post dosing (Table 59 and **Figure 35**).

[0810] Table 59. FVIIa clotting activity of MOD-5014 vs. NovoSeven® following IV or SC administration

Time (hr)	NovoSeven® IV (A)		MOD-5014 IV (B)		NovoSeven® SC (C)		MOD-5014 SC (D)	
	mU/ml	%CV	mU/ml	%CV	mU/ml	%CV	mU/ml	%CV
0.5	304651.7	18.7	232818.3	5.0	11491.7	2.4	3691.7	19.0
4	40068.3	7.8	62085.0	9.5	21385.0	22.6	12018.3	15.8
8	5276.7	2.5	25931.7	6.1	5525.0	32.5	6445.0	2.2
12	255.0	13.8	5633.3	9.3	297.7	41.4	924.7	24.1
24	1.3	7.1	251.3	11.8	1.3	89.2	249.3	60.3
34	0.0		78.3	4.5	0.0		63.7	85.5
48			29.0	9.9	0.0		35.0	47.2
58			10.3	4.6	0.0		13.7	33.5

After background reduction: 15mU/ml.

[0811] Table 60. PK parameters of MOD-5014 vs. NovoSeven® following IV or SC administration

A. IV

PK Parameters	Novoseven RT (A)	MOD-5014 (RS 12-001) (B)
Half-life-α (0.5-4hr)	0.24	1.04
Half-life-β (4-58hr)	1.31	3.17
AUC o-inf mU/ml*h	702467.95	820778.67
Vss [U/Kg/(mU/ml)]	0.13	0.13
CL [(U/Kg)/(mU/ml)/h]	0.08	0.04
MRT (hr)	1.74	3.62

B. SC

PK Parameters	Novoseven RT (B)	MOD-5014 (RS 12-001) (C)
Half-Life (hr)	1.40	7.78

Cmax (mU/ml)	21385.00	12018.33
AUC 0-inf (mU/ml*h)	115099.72	84158.87
MRT 0-inf (hr)	4.32	7.04
Vz/F (U/Kg)/(mU/ml)	0.95	3.88
Cl/F (U/Kg)/(mU/ml)/h	0.47	0.35

Experiment no. 05034

[0812] Figure 36 presents the PK profile of FVII a following SC administration of either NovoSeven® or MOD-5017. Two different batches of clone no. 61 (#75 and #81) were examined in the same concentration or the same activity units, compared to NovoSeven®. Summary of FVIIa activity values for each time point is presented in Table 61.

[0813] The results indicate a similar PK pattern after SC administration corresponding to previous experiments. Moreover, clotting activity for the NovoSeven® treated rats was undetectable at time points later than 12 hours, while MOD-5014 treated rats continued to retain measurable activity at 24 hours post dosing (Table 61_and **Figure 36**; and after background reduction: 56 mU/ml (8, 12 hr) or 32 mU/ml (0.5, 2, 6, 14 hr)).

[0814] Clone no. 61 batch #81 (D) Cmax (1,301mU/ml) was lower than the Cmax values of clone no. 61 batch #75 (B) and NovoSeven® (A) (3,521mU/ml and 5,908mU/ml respectively), although they were all injected by the same unit activity (Table 61). However, batch #75 (B) and #81 (D) have the same activity units (559 mU/ml and 478 mU/ml respectively) measured 8hr after injection (Table 61 and Table 62; and after background reduction: 56 mU/ml (8, 12 hr) or 32 mU/ml (0.5, 2, 6, 14 hr)).

[0815] Table 61**:** FVIIa clotting activity of MOD-5014 (Clone 61 #75, #81) vs. NovoSeven® following single SC administration.

Time (hr)	NovoSeven® (A)		MOD-5014 Clone 61 Batch 75 (B) - equal U/kg		MOD-5014 Clone 61 Batch 81A (C) - equal conc.FVIIa µg/kg		MOD-5014 Clone 61 Batch 81A (D) - equal U/kg	
	mU/ml	%CV	mU/ml	%CV	mU/ml	%CV	mU/ml	%CV
0.5	3271.3	46.5	350.3	26.6	101.3	24.1	208.7	51.2
2	5908.0	18.1	3521.3	70.9	1294.7	7.0	1301.3	31.6
6	1411.7	23.6	1349.7	45.6	425.3	27.6	663.0	13.4
8	1029.0	12.4	559.3	52.7	152.7	19.5	478.0	25.4
12	121.3	9.9	563.0	17.4	148.7	36.3	712.7	16.2
24	1.0	25.0	117.0	41.9	21.3	36.4	99.0	36.7

After background reduction: 56mU/ml (8,12hr) or 32mU/ml (0.5, 2, 6, 14hr).

[0816] Table 62**:** PK parameters of MOD-5014 (Clone 61 #75, #81) vs. NovoSeven® following single SC administration.

PK Parameters	NovoSeven® RT (A)	MOD-5014 Clone 61 Batch 75 (B)- equal U/kg	MOD-5014 Clone 61 Batch 81A (C)- equal conc.FVIIa µg/kg	MOD-5014 Clone 61 Batch 81A (D)- equal U/kg
Half-Life (hr)	1.67	5.70	4.62	6.41
Cmax (mU/ml)	5908.00	3521.33	1294.67	1301.33
AUC 0-inf (mU/ml*h)	24688.18	20456.96	6260.23	13098.16
MRT 0-inf (hr)	3.73	7.86	6.40	10.59
Vz/F (U/Kg)/(mU/ml)	1.96	8.06	9.55	14.15
Cl/F (U/Kg)/(mU/ml)/ h	0.81	0.98	1.43	1.53

[0817] This report summarized two PK studies; 05010 & 05034. The results provide specific insight on the impact of CTP fusion to FVII on protein half-life and clearance in subcutaneous administration and address the paradigm of its specific activity following this modification. In these studies, SD rats were administered with a single SC injection of MOD-5014 originated from two clones, and two different batches, compared to recombinant commercial FVIIa (NovoSeven®). The components were injected at similar FVIIa concentration (µg/Kg) or at the same activity level (U/Kg) and the PK activity based analysis was performed.

[0818] The purpose of the first study was to verify the different PK parameters after IV and SC administration. Based on this study we can conclude that there is a difference between the PK pattern measured after IV or SC administration. A $t^{1/2}$ of 7.78 hr measured after MOD-5014 SC injection, and only 4.2 hr after IV injection. AUC values were the same Table 60.

[0819] The second study however, focused on the differences between two batches of MOD-5014 clone no. 61, which were injected by the same FVIIa concentration or at an equal activity unit, compared to NovoSeven®. In this study we showed that clone 61 batch #75 manifested better PK parameters than batch #81. Batch #81, which was injected by the same unit activity level, had lower Cmax for an unknown reason. Moreover, the same Cmax was measured when injecting clone 61 batch #81 in two different doses (by FVIIa concentration or by unit activity), instead of 2.5 fold between the two activity values. Following analysis of both studies together, we can conclude that clone 28 manifested a prolonged $t^{1/2}$ parameter that clone 61 #75 (the better batch) after SC injection (7.78hr and 5.7hr respectively, Table 62). The results show that dissimilar time point samples create different PK pattern, which lead to variation in the PK curves. The patterns of the curves can teach us more about the drug behavior in the blood. Therefore, we decided to determine the time points similar to

those detected by Baxter (0, 0.5, 2, 6, 8, 12, 24, 34, 48, 72hr). Moreover, the FVIIa concentration in 05010 experiment was too high, and was revised in the following SC experiment (05034). For future PK studies, it was decided to inject the component at 360µg FVIIa/kg for a dose.

EXAMPLE 10: WARFARIN TREATED RATS AS A MODEL FOR EVALUATING FACTOR VIIa in vivo

Materials & Methods

[0820] PT Assessment: SD rats were given orally 10mg/Kg of Warfarin and at a designated time point plasma was collected and prothrombin time (PT) was measured using a standard procedure. In order to assess the long term hemostatic effect Placebo, NovoSeven® or MOD-5014 were injected to the Warfarin treated animals and PT was measured.

[0821] Tail Clip Challenge: Warfarin treated animals were injected with Placebo, NovoSeven® or MOD-5014 at designated time points the animals were challenged by complete cut of the tail tip (0.5 cm from the tip) and bleeding intensity was measured in gr for 30min post transection.

Results

Warfarin administration to SD-rats results in a prolongation of PT and aPTT.

[0822] Warfarin prevent the reduction of vitamin K, and consequently decreases vitamin K dependent coagulation factors concentration in the blood.
Male SD rats received oral treatment of warfarin. The reduction of Vitamin K dependent coagulation factors was accompanied by prolongation of PT and aPTT. Results are presented in Figure 37.

[0823] Due to coagulation-factors wash out from the blood, PT and aPTT values increase gradually in the first 48 hours following warfarin administration. The effect decreases after that.

Warfarin effect can be restored by acute IV treatment with NovoSeven® or MOD-5014.

[0824] SD-rats received a pre-treatment of Warfarin. 24 hours later, MOD-5014, NovoSeven® or buffer were injected intravenous blood samples were drawn 15 minutes post injection. 15min post injection, MOD-5014 as well as NovoSeven® successfully restored PT values to normal (Fig. 38).

The effect of increasing dose of MOD-5014 and NovoSeven® on PT values in warfarin treated rats.

[0825] SD-rats were treated with 10mg/Kg warfarin in parallel to 100-1000 µg/Kg MOD-5014 or NoveSeven IV injection. 24 hours post treatment, PT was determined in plasma samples. NovoSeven® injected 24 hours before PT determination, did not have any significant effect on PT values in all the doses tested. In contrast, MOD-5014 shows a dose-response behavior 24 hours after administration (Figure 39).

[0826] SD-rats were treated with 10mg/Kg warfarin in parallel to 1000 µg/Kg MOD-5014 or NoveSeven IV injection. PT was determined in plasma samples 10, 24, 36 and 48 hours post treatment. MOD-5014 restored PT values to normal up to 48 hours post dosing, while the effect of NovoSeven® no longer exists after 24 hours (Figure 40).

MOD-5014's long lasting effect can be demonstrated by Tail Clip assay in Warfarin injected rats

[0827] SD-rats were treated with Warfarin 24 hours before tail clip. Rats were anesthetized and placed on a warm pad, the tail tip was placed in 37°c saline and a complete amputation of the tail was performed 0.5 cm from tail tip. Blood was collected for 30 minutes and blood loss was determined by weight.

[0828] Vehicle or 500 µg/Kg MOD-5014 or NovoSeven® was administrated 15 min, 24 or 48 hours before tail clip. Results are presented in Figure 41. Rats treated with warfarin lost 5 fold more blood than naïve rats. 15 min post injection, tail clip of MOD-5014 and Novoseven treated rats resulted in reduced bleeding which is comparable to naïve rats. The effect of MOD-5014 is completely preserved 24 hours post injection, and partially preserved after 48 hours.

Sub-cutaneous injection of MOD-5014 is also demonstrating a long lasting effect.

[0829] SD-rats were treated with 10mg/Kg warfarin in parallel to 2000 µg/Kg MOD-5014 or NoveSeven SC injection. PT was determined in plasma samples 10, 24, 36 and 48 hours post treatment.

[0830] MOD-5014 is able to restore PT values to normal up to 48 hours post dosing, while the effect of NovoSeven® no longer exists after 24 hours (Figure 42).

SC injection of MOD-5014 reduces blood loss for at 48 hours.

[0831] SD-rats were treated with Warfarin 24 hours before tail clip. Rats were anesthetized and placed on a warm pad, the tail tip was placed in 37°c saline and a complete amputation of the tail was performed 0.5 cm from tail t

[0832] ip. Blood was collected for 30 minutes and blood loss was determined by weight.

[0833] Vehicle or 1000 µg/Kg MOD-5014 or NovoSeven® was SC administrated 15 min,

24 or 48 hours before tail clip. Results are presented in Figure 43.

EXAMPLE 11: COMPERATIVE ASSESSMENT OF CLOTTING ACTIVITY OF MOD-5014 AND NOVOSEVEN®

[0834] **Study Objectives – (I)** To characterize the *in vitro* clotting activity and FX activation under different conditions of MOD-5014 in comparison with NovoSeven®. **(II)** To compare *ex vivo*, prothrombin time (PT) and activated partial thromboplastic time (aPTT) profiles in human hemophilia and FVII-deficient plasma upon MOD-5014 and NovoSeven® spiking.

[0835] **Materials and Methods**

[0836] **Materials** - MOD-5014 GMP-1: 2.5 mg/ml (based on A280) and NovoSeven® Lot# CU60430: 0.943 mg/ml (based on A280)

[0837] **Method – Clotting Assay**

[0838] The clotting activity of FVIIa was measured using commercially available Staclot VIIa-rTF kit (Ref# 00281, Stago). This method included clotting time measurement of FVII-deficient plasma using the STA Compact MAX or Start4 instruments. Specific amounts of FVIIa were added to the plasma following the addition of phospholipids, Ca^{2+}, and recombinant soluble tissue factor (rsTF). The latter is the extracellular portion of the native tissue factor, which can no longer activate FVII to FVIIa by auto activation. However, it possesses a cofactor function specific for factor VIIa. The FVIIa bound to soluble tissue factor converts factor X to the active factor Xa. The observed clotting time has an inverse relationship with FVIIa level in the plasma, since the soluble tissue factor does not activate FVII to FVIIa. The obtained clotting time was converted to activity (mU/ml) using a FVIIa standard curve and the specific activity was calculated based on FVIIa protein concentration. This method provided the potential *in vitro* activity of FVIIa, with the limitation of using sTF, which only partially mimics the *in vivo* setting.

[0839] **Method – FVII Chromogenic Assay**

[0840] MOD-5014 and NovoSeven® potency were assessed by the commercially available kit BIOPHEN FVII (Ref#221304, HYPHEN BioMed). This is a chromogenic assay intended for testing FVII activity. FVII forms an enzymatic complex with tissue factor and converts factor X into the activated factor Xa in the presence of phospholipids and calcium. Factor X is present in the assay in a constant concentration and in excess. The concentration of activated factor Xa is measured by its activity on a specific chromogenic substrate (SXa-11), which it

cleaves to generate pNA. The amount of pNA is directly proportional to Factor X activity, and there is a direct relationship between the amount of Factor VII and the level of Factor Xa activity, measured by the amount of pNA released and determined by color development at 405nm.

[0841] **Method- prothrombin time (PT) and activated partial thromboplastic time (aPTT)**

[0842] Prothrombin Time (PT) and Activated Partial Prothrombin Time (aPTT) were measured using a Siemens CA-1500 autoanalyzer and validated using routine clinical human plasma diagnostic testing at A.M.L.

[0843] *MOD-5014 GMP-1:* 2.5 mg/ml based on absorption at A280, diluted to 0.5-0.0008 mg/ml with hemophilic human plasma/FVII deficient plasma.

[0844] *FVIIa clotting activity*: 16,720 U/mg based on FVIIa Staclot activity assay.

[0845] *NovoSeven® Lot#CU60430:* 0.943 mg/ml based on absorption at A280, diluted to 0.5-0.0008 mg/ml with hemophilic human plasma/FVII deficient plasma.

[0846] *FVIIa clotting activity:* 50,494 U/mg based on FVIIa Staclot activity assay.

[0847] **Matrix:** Human plasma (FVIII deficient) BIORECLAMATION (Cat# HMPLCIT-FACT8DEF, Lot# BRH779222-BRH779233). FVII Deficient plasma, Cat#HBM-DP030K.

RESULTS

[0848] **Potency Determination:** The specific activities of MOD-5014 and NovoSeven® were evaluated using a qualified Staclot VIIa-rsTF kit. The average specific activity obtained for MOD-5014 (batches RS005, GMP1, ER01) and NovoSeven® as determined by 4 independent assays are presented in **Table 63** below.

[0849] **Table 63 - Specific activities of MOD-5014 and NovoSeven®**

	Run# 1	Run#2	Run#3	Run#4	Avg. (U/mg)	%CV
MOD-5014 batch ER01	17041	14223	16344	14644	15563	8.6
MOD-5014 batch GMP1	15894	17390	17819	15775	16720	6.2
MOD-5014 batch RS005	23300	20471	17790	18323	19971	12.5
NovoSeven®	46889	45900	49522	47644	47489	3.2

[0850] *Conclusion*: MOD-5014 specific activity was 2 to 2.5-fold lower than NovoSeven®. This might be a consequence of reduced molar content of FVIIa in MOD-5014 when spiking

on mass base rather than on molar base, as MOD-5014 consists of 83.4% FVIIa with 3 CTP cassettes attached at the C-terminus.

[0851] **Clotting Activity Inhibition in the Presence of TFPI:** The clotting activity of MOD-5014 and NovoSeven® was evaluated in the presence of tissue factor pathway inhibitor (TFPI), a natural inhibitor if FVIIa. The inhibitor was added at a range of concentrations (3125ng/ml to 0.006ng/ml) following the addition of MOD-5014 or NovoSeven® at a fixed concentration, FVII-deficient plasma, tissue factors and phospholipids, and was incubated 15 min at 37ºC. The observed specific activity was converted to inhibition %. The assay was repeated 3 times and the mean results are presented in **Table 64** and **Figure 44**.

[0852] **Table 64 - Mean Activity Inhibition in the Presence of TFPI**

		NovoSeven		MOD-5014	
TFPI conc. (ng/mL)	**TFPI log conc.**	**Activity (mU/mL)**	**% Inhibition**	**Activity (mU/mL)**	**% Inhibition**
0	NA	72.3	NA	66.8	NA
1250	3	2.9	96	7.3	89
625	3	9.5	87	13.2	80
62.5	2	45	38	47.4	29
6.25	1	55.3	24	56.7	15
0.625	0	54.5	25	56.7	15

[0853] *Conclusion:* TFPI inhibited NovoSeven® and MOD-5014 at a similar dose-dependent manner. The difference in the values might be a consequence of assay variability, as reported in method qualification (%CV≤ 25%).

[0854] **Clotting Activity Inhibition in the Presence of Heparin:** The clotting activity of MOD-5014 and NovoSeven® was measured in the presence of heparin at a wide range of concentrations. Heparin was added following the addition of a fixed concentration of MOD-5014 or NovoSeven®, FVII-deficient plasma, tissue factors and phospholipids, and was incubated 15 min at 37ºC. The results are presented in **Table 65**.

[0855] **Table 65 - Activity Inhibition in the Presence of Heparin**

	NovoSeven		MOD-5014	
Heparin Conc. (U/µl)	**Activity (mU/ml)**	**% Inhibition**	**Activity (mU/ml)**	**% Inhibition**
0	68.8	NA	56.9	NA
1	0	100	0	0
0.5	0	100	0	0
0.25	0	100	0	0
0.1	2.1	97	2.1	96

[0856] *Conclusion:* Heparin possesses high potency in this specific assay, as over 96%

inhibition was observed for both MOD-5014 and NovoSeven® even at an extremely low concentration (0.1U/ µl).

[0857] **Clotting Activity in the Presence of Anti-Thrombin (AT):** The specific activities of MOD-5014 and NovoSeven® were evaluated in the presence of Anti thrombin III (ATIII), which is a mild inhibitor of FVIIa. Anti-thrombin was added at a range of concentrations (525µg/ml to 0.01ng/ml) following the addition of MOD-5014 or NovoSeven®, FVII-deficient plasma, tissue factors and phospholipids, and was incubated 15 min at 37°C. The observed specific activity was converted to inhibition %. The results are presented in **Table 66** and **Figure 45**.

[0858] **Table 66 - Activity Inhibition in the Presence of AT III**

		NovoSeven		MOD-5014	
AT conc. (ng/mL)	**AT log conc.**	**Activity (mU/mL)**	**% Inhibition**	**Activity (mU/mL)**	**% Inhibition**
0	NA	88.1	NA	70.8	NA
525000	6	0	100	0	100
105000	5	30.6	65	27	62
10,500	4	61.5	30	53	25
1,050	3	66.8	24	53.4	25
105	2	70.2	20	53.3	25
10.5	1	68.4	22	50.7	28
1.050	0	69.5	21	52.9	25

[0859] *Conclusion:* MOD-5014 and NovoSeven® were inhibited at a similar manner by AT III.

[0860] **Factor X Activation by NovoSeven® and MOD-5014:** The potency of MOD-5014 and NovoSeven® was evaluated and the average EC_{50} values obtained for MOD-5014 and NovoSeven® were calculated (0.41 and 0.38 ng/ml, respectively). The results are presented in **Table 67** and a representative dose-response curve is presented in **Figure 46**.

[0861] **Table 67 - FX activation by MOD-5014 and NovoSeven**

		O.D	
rFVIIa Conc. (ng/ml)	**Log Conc.**	**MOD-5014**	**NovoSeven**
100.00	2.00	0.385	0.363
20.00	1.30	0.358	0.365
4.00	0.60	0.368	0.352
0.80	-0.10	0.286	0.303
0.160	-0.80	0.17	0.178
0.0320	-1.49	0.127	0.138

0.00640	-2.19	0.117	0.133
0.00128	-2.89	0.111	0.123

[0862] **FX activation in the presence of TFPI:** The potency of MOD-5014 and NovoSeven® was evaluated in the presence of TFPI. The latter was added at a range of concentrations (20µg/ml to 0.002ng/ml) to two concentrations of MOD-5014 and NovoSeven® (EC_{70}) (0.6 and 4ng/ml) and FX activation was measured. The results are presented in **Table 68** and **Figure 47**. The assay performed in the presence of NovoSeven® and MOD-5014 at a concentration of 4 ng/ml is presented in **Table 69** and **Figure 48**.

[0863] **Table 68 - Factor X Activation in the Presence of TFPI (#1)**

		O.D	
TFPI Conc. (ng/ml)	**Log Conc.**	**MOD-5014 (0.69 ng/ml)**	**NovoSeven® (0.64 ng/ml)**
20000.00	4.30	0.346	0.429
4000.00	3.60	0.691	0.83
800.00	2.90	1.006	1.044
160.00	2.20	1.11	1.337
32.00	1.51	1.36	1.384
6.40	0.81	1.384	1.418
1.28	0.11	1.387	1.466
0.2560	-0.59	1.457	1.428
0.051200	-1.29	1.454	1.518
0.010240	-1.99	1.446	1.514
0.002048	-2.69	1.478	1.504
Control		**1.51**	**1.51**

[0864] **Table 69 - Factor X Activation in the Presence of TFPI (#2)**

TFPI Conc. (ng/ml)	**Log Conc.**	**NovoSeven® (4 ng/ml)**	**MOD-5014 (4 ng/ml)**
20000.00	4.30	0.206	0.17
4000.00	3.60	0.406	0.38
800.00	2.90	1.016	0.836
160.00	2.20	1.338	1.143
32.00	1.51	1.496	1.465
6.40	0.81	1.588	1.541
1.28	0.11	1.661	1.565
0.2560	-0.59	1.697	1.616
0.051200	-1.29	1.726	1.604

0.010240	-1.99	1.703	1.638
0.002048	-2.69	1.715	1.653
Control	0	1.656	1.581

[0865] *Conclusion:* MOD-5014 and NovoSeven® demonstrated a very similar inhibition curve of FX activation in the presence of TFPI at both concentrations of the compound.

[0866] **FX Activation in the Presence of TFPI and Heparin -** The potency of MOD-5014 and NovoSeven® was evaluated in the presence of TFPI and heparin. TFPI at different concentrations (20µg/ml to 0.002ng/ml) and 1U/µl heparin were added to a constant concentration of MOD-5014 and NovoSeven® (0.7ng/ml) and FX activation was measured. The results are presented in **Table 70** and **Figure 49**.

[0867] **Table 70 - FX Activation in the Presence of TFPI and Heparin**

TFPI Conc. (ng/ml)	**Log Conc.**	**NovoSeven® (0.7 ng/ml) + 1U/µl heparin**	**MOD-5014 (0.7 ng/ml) + 1U/µl heparin**
20000.00	4.30	0.222	0.15
4000.00	3.60	0.241	0.115
800.00	2.90	0.409	0.32
160.00	2.20	0.764	0.539
32.00	1.51	1.014	0.915
6.40	0.81	1.207	1.164
1.28	0.11	1.309	1.261
0.051200	-1.29	1.373	1.347
0.010240	-1.99	1.305	1.293
0.002048	-2.69	1.335	1.266
Control	0	1.473	1.373

[0868] *Conclusion:* MOD-5014 and NovoSeven® exhibited similar activation of FX in the presence of TFPI. Heparin had no significant influence on the inhibition profile when added with TFPI.

[0869] **FX Activation in the Presence of Anti-Thrombin:** The potency of MOD-5014 and NovoSeven® was evaluated in the presence of anti-thrombin (AT III). Different concentrations of AT III (1.68mg/ml to 0.16µg/ml) were added to a constant concentration of MOD-5014 and NovoSeven® (0.7ng/ml) and FX activation was measured. The results are presented in **Table 71** and **Figure 50**.

[0870] **Table 71 - FX Activation in the Presence of Anti-Thrombin**

	O.D

AT conc. (µg/ml)	Log conc.	NovoSeven	MOD-5014
1680	3.2	0.784	0.656
500	2.7	0.933	0.975
100	2.0	1.138	0.974
20	1.3	1.151	1.112
4	0.6	1.172	1.135
0.8	-0.1	1.2	1.075
0.16	-0.8	1.234	1.182
control		1.283	1.09

[0871] *Conclusion*: MOD-5014 and NovoSeven® exhibited similar activation of FX at the presence of AT III.

[0872] **FX Activation in the Presence of Constant AT III Concentration and Varying Heparin Concentrations:** Anti-thrombin III (AT III) was diluted to a constant concentration (20 µg/ml) and was added to a constant concentration of MOD-5014 and NovoSeven® (0.7ng/ml). Heparin was added at different concentrations (6.25U/µl-0.002U/µl) to the mixture and FX activation was measured. The results are presented in **Table 71** and **Figure 51**.

[0873] **Table 71 - FX Activation in the Presence of Heparin at Different Concentrations**

		O.D.	
Heparin conc. (U/µl)	Log conc.	NovoSeven	MOD-5014
6.25	0.8	1.002	1.024
1.25	0.1	0.98	0.896
0.25	-0.6	1.039	0.917
0.01	-2.0	1.198	1.139
0.002	-2.7	1.242	1.294

[0874] *Conclusion:* MOD-5014 and NovoSeven® exhibited similar and moderate inhibition by heparin at constant AT III concentrations.

[0875] **PT and aPTT measurements**

[0876] PT and aPTT measurements are presented in **Table 72**.

[0877] **Table 72**

	PT (sec)	aPTT (sec)

Test article	Tested concentration (mg/ml)	Required dilution	In Hemophilic Plasma	In FVII Deficient Plasma	In Hemophilic Plasma	In FVII Deficient Plasma
MOD-5014 GMP-1 2.5mg/ml	0.5	5	10.0	10.2	21.0	21.0
	0.1	25	8.7	8.9	22.8	21.0
	0.02	125	8.6	8.8	30.9	<21.0
	0.004	625	8.5	8.8	45.0	24.0
	0.0008	3125	8.8	9.3	62.5	25.9
Novoseven 0.943mg/ml	0.5	1.9	No coagulation		No coagulation	
	0.1	9.3	8.5	8.7	21.0	21.0
	0.02	47	8.3	8.6	26.6	21.0
	0.004	234	8.3	8.6	38.9	22.7
	0.0008	1169	8.5	8.7	55.0	25.4
Control (Only Plasma)	0	0	11.9	No coagulation	87.1	27.5
Normal values			11-13.5		25-35	
			Extrinsic pathway		Intrinsic pathway	

[0878] *Conclusion:* The PT and aPTT measurements were comparable for both MOD-5014 and NovoSeven® when spiked at a similar range of concentrations.

[0879] **FX Activation in the Presence of Constant Heparin Concentration and Varying AT Concentrations**

Study Objective

[0880] To compare the ability of MOD-5014 and NovoSeven to activate FX in the presence anti-thrombin and at constant concentration of heparin.

Study Design and Results

[0881] The potency of MOD-5014 and NovoSeven was evaluated in the presence of anti-thrombin (AT) and heparin. Different concentrations of AT (1.68mg/ml to 0.032µg/ml) and 1U/µl heparin were added to a constant concentration of MOD-5014 and NovoSeven (0.7ng/ml) and FX activation was measured. IC_{50} values obtained for anti-thrombin were 49.65µg/ml for MOD-5014 and 65.70 µg/ml for NovoSeven. The results are presented in Table 121, **Figure 52.**

[0882] **Table 121: FX Activation in the Presence of AT and Heparin**

		O.D.	
AT conc. (µg/ml)	Log conc.	NovoSeven	MOD-5014
1680	3.2	0.141	0.197
500	2.7	0.201	0.153
100	2.0	0.503	0.404
20	1.3	1.037	1.078
4	0.6	1.165	1.259
0.8	-0.1	1.215	1.254
0.16	-0.8	1.186	1.235
0.032	-1.5	1.163	1.244
Control		1.433	1.405

[0883] Conclusion: MOD-5014 and NovoSeven exhibited similar activation of FX in the presence of AT and 1U/µl heparin, suggesting that AT has a significantly more pronounced effect on FVIIa inhibition than heparin.

[0884] **COMPARATIVE PT AND aPTT MEASUREMENTS**

Study Objective

[0885] To compare PT & aPTT profiles in human hemophilia and FVII-deficient plasma upon MOD-5014 and NovoSeven spiking.

Study Design

[0886] PT and aPTT were measured using a Siemens CA-1500 autoanalyzer and validated using routine clinical human plasma diagnostic testing at A.M.L. MOD-5014 GMP-1: 2.5 mg/ml based on absorption at A280, diluted to 0.5-0.0008 mg/ml with hemophilic human plasma/FVII deficient plasma.

[0887] FVIIa clotting activity: 16,720 U/mg based on FVIIa Staclot activity assay.

[0888] NovoSeven Lot#CU60430: 0.943 mg/ml based on absorption at A280, diluted to 0.5-0.0008 mg/ml with hemophilic human plasma/FVII deficient plasma. FVIIa clotting activity: 50,494 U/mg based on FVIIa Staclot activity assay.

[0889] Matrix:

- Human plasma (FVIII deficient) BIORECLAMATION (Cat# HMPLCITFACT8DEF, Lot# BRH779222-BRH779233).
- FVII Deficient plasma, Cat#HBM-DP030K.

[0890] MOD-5014 and NovoSeven were spiked into the above matrixes in concentrations of

[0891] 0.5-0.0008 mg/ml and PT and aPTT were measured. Results are summarized in Table 122.

[0892] **Table 122: PT and aPTT Measurements**

Test article	Tested concentration (mg/ml)	Required dilution	PT (sec)		aPTT (sec)	
			In Hemophilic Plasma	In FVII Deficient Plasma	In Hemophilic Plasma	In FVII Deficient Plasma
MOD-5014 GMP-1 2.5mg/ml	0.5	5	10.0	10.2	21.0	21.0
	0.1	25	8.7	8.9	22.8	21.0
	0.02	125	8.6	8.8	30.9	<21.0
	0.004	625	8.5	8.8	45.0	24.0
	0.0008	3125	8.8	9.3	62.5	25.9
Novoseven 0.943mg/ml	0.5	1.9	No coagulation		No coagulation	
	0.1	9.3	8.5	8.7	21.0	21.0
	0.02	47	8.3	8.6	26.6	21.0
	0.004	234	8.3	8.6	38.9	22.7
	0.0008	1169	8.5	8.7	55.0	25.4
Control (Only Plasma)	0	0	11.9	No coagulation	87.1	27.5
Normal values			11-13.5		25-35	
			Extrinsic pathway		Intrinsic pathway	

[0893] Conclusion: The PT and aPTT measurements were comparable for both MOD-5014 and NovoSeven when spiked at a similar range of concentrations.

SUMMARY

[0894] MOD-5014 activity was evaluated by a variety of *in vitro* and *ex vivo* methods that assessed different aspects of its coagulation activity in comparison to NovoSeven® at equal mass. Initially, MOD-5014 activity was compared to NovoSeven® in the qualified Staclot FVIIa assay. The study demonstrated that MOD-5014 activity is 2 to 2.5-fold lower than that of NovoSeven. A factor X activation test by a chromogenic assay in the presence of TF, phospholipids and calcium also proposed a slightly lower measure of activity of MOD-5014 in comparison to NovoSeven®, as reflected by EC_{50}.

[0895] The variability between methods might be due to differences in assay sensitivity or endpoint (soluble vs. full-chain TF and clotting time vs. OD measurement, respectively). The lower MOD-5014 activity might be due to the fact that 84.4% of MOD-5014 corresponds to FVIIa and 15.6% corresponds to CTP.

[0896] MOD-5014 inactivation in the presence of TFPI, a major FXa-dependent inhibitor of the extrinsic coagulation pathway, was evaluated by the two *in vitro* assays mentioned above (Staclot and factor X activation). Incubating MOD-5014 or NovoSeven® at two fixed concentrations with increasing concentrations of TFPI resulted in a dose-dependent reduction in clotting or FXa enzymatic activity. Both compounds demonstrated a very similar de-

activation pattern, reflected by % clotting inhibition.

[0897] Anti-thrombin III was previously reported to inhibit factor VIIa at a slow rate and also demonstrated augmented inhibition at the present of heparin. Anti-thrombin III demonstrated a similar inhibition pattern of both MOD-5014 and NovoSeven® when both compounds were spiked with increasing concentrations of AT III. This pattern was maintained following the addition of heparin, producing a pronounced inhibitory effect.

EXAMPLE 12: COMPARATIVE IN VITRO ASSESSMENT OF MOD-5014 AND NOVOSEVEN® IN THROMBIN GENERATION AND COAGULATION EFFICIENCY

[0898] **Study Objectives – (I)** Comparative assessment of MOD-5014 and NovoSeven® by thrombin generation (TG) in inhibitory platelets rich plasma with high titer of inhibitory antibodies at low and high phospholipids concentration, and **(II)** comparative assessment of MOD-5014 and NovoSeven® by thromboelastography (TEG) in inhibitory platelets rich plasma with high titer of inhibitory antibodies.

[0899] **Materials and Methods**

[0900] **Materials** - MOD-5014: 2.0 mg/ml and NovoSeven® 1.0 mg/ml, stored frozen (-60 to -80ºC). No dose formulation preparation was required. Materials were thawed only once prior to dosing.

[0901] **Method – Thrombin generation (TG) in low and high phospholipid concentrations**

[0902] Human plasma originated from patients with high titer of anti FVIII inhibitory Abs was spiked with increased concentration of MOD-5014 or NovoSeven®, coagulation was stimulated by relipidtaed rhTissue Factor (TF) and a high concentration of phospholipid micelles (Reagent RChigh) mimicking the in vivo situation.

[0903] **Method – Thromboelastography (TEG)**

[0904] Thromboelastography (TEG) is a method of testing the efficiency of coagulation in the blood and is especially important in surgery and anesthesiology. The patterns of changes in strength and elasticity in the clot provide information about how well the blood can perform hemostasis (the halting of blood flow), and how well or poorly different factors are contributing to clot formation.

[0905] Four values that represent clot formation are determined by this test: the R value (or reaction time), the K value, the angle and the MA (maximum amplitude). The R value represents the time until the first evidence of a clot is detected. The K value is the time from

the end of R until the clot reaches 20mm and this represents the speed of clot formation. The angle is the tangent of the curve made as the K is reached and offers similar information to K. The MA is a reflection of clot strength.

RESULTS

[0906] **Thrombin Generation**: The generation of thrombin is a fundamental part of the clotting cascade and as such an estimation of how well a particular individual can generate thrombin may correlate with either a risk of bleeding or thrombosis. It describes all the phases of thrombin generation process (initiation, amplification and inhibition of thrombin generation as well as the integral amount of generated thrombin). According to the experimental system used, thrombin generation may be influenced by most of the factors playing a role in in vivo blood coagulation.

[0907] The kinetic of thrombin generation was monitored by Technoclone TGA (**Figure 53**).

[0908] A Dose-dependent increase of peak thrombin and decrease of lag phase and peak time was observed following spiking with MOD-5014 or Novoseven.

[0909] In the second experiment MOD-5014 or NovoSeven® were spiked at increasing concentrations at the present of TF and at the present of low concentration of phospholipid micelles (Reagent RClow).

[0910] As anticipated a more pronounced repose was observed when spiking the sample with high PL concentration. Both compounds reached maximal TG response at low concentration of PL concentration further confirming its importance for proper activation of the clotting cascade. The result as presented in **Figure 53** and **Figure 54** demonstrate that MOD-5014 *in–vitro* thrombin generation activity is slightly lower compared to NovoSeven® at high and low PL concentration and are aligned with data obtained in PPP FVIII deficient plasma .

[0911] **Coagulation Efficiency**: MOD-5014 and NovoSeven® were added to high titer human FVIII inhibitor platelet reach plasma (PRP), $CaCl_2$ and rhTF thromboplastin were added to trigger clot formation. The R and angel were evaluated. As observed in **Figure 55**, both MOD-5014 and NovoSeven® decreased the clotting time (R) and increased the rate of clot formation (Angel) of high titer FVIII inhibitor plasma at a similar concentration dependent manner, while MOD-5014 demonstrated a minor reduction in its clotting capability. These finding further strengthen the results obtained by ROTEM that CTP attachment doesn't interfere with clot formation.

SUMMARY

[0912] Based on the TG and TEG results in FVIII high titer plasma it appears that MOD-5014 mechanism of TG and clot formation may be similar to NovoSeven® with a slight reduction in it activity. This might be a consequence of reduced molar content of FVIIa in

MOD-5014 when spiking on mass base rather than on molar base as MOD-5014 consists of 83.4% FVIIa with 3 CTP cassettes attached at the C terminus, and therefore will require slightly higher concentrations of MOD-5014 to maintain in vivo hemostatic. Finally it appears that a mechanism of binding to phospholipid may be maintained following the attachment of CTP to FVIIa.

EXAMPLE 13: COMPARATIVE IN VITRO ACTIVITY OF MOD-5014 AND NOVOSEVEN®

[0913] **Study Objectives** – **(I)** A comparative assessment of MOD-5014 and NovoSeven® by thrombin generation (TG) in citrated platelet poor plasma (PPP). **(II)** A comparative assessment of MOD-5014 and NovoSeven® tissue factor (TF) affinity by thrombin generation in citrated PPP. **(III)** A comparative assessment of MOD-5014 and NovoSeven® by thromboelastography (ROTEM) in PPP citrated plasma.

[0914] **Materials and Methods**

[0915] **Materials** - MOD-5014: 2.6 mg/ml and NovoSeven® 2.6 mg/ml, stored frozen (-60 to -80ºC). No dose formulation preparation was required. Materials were thawed to room temperature prior to administration.

[0916] **Method (Objective (I))– Thrombin generation (TG) in low and high phospholipid concentrations**

[0917] Thrombin generation was measured according to Livnat et al. (2006, J. Thromb Haemost. 4(1):192-200; 2008, Haemophila. 14(4):782-786; and 2011, Thromb Haemost. 105(4): 688-95). Briefly, pooled plasma was spiked with escalating concentration of MOD-5014 or NovoSeven®. PPP-Reagent LOW (containing 1µM tissue factor) or MP-reagent (Diagnostica Stago, Lot PPL 1203/01 and MPR 1202/01, respectively) were used as working buffers. Both reagents contained 4µM phospholipids.

[0918] The assay was carried out using two approaches as follow: (a) Re-Calcification only; and (b) Low TF level(1pM).

[0919] Twenty µl of working buffer were placed in round-bottom 96-well microtiter plates. Eighty µl of FVIII-deficient plasma with different concentrations of NovoSeven® or MOD-5014 were added to the buffer, as described in **Table 73**.

[0920] **Table 73**

NovoSeven®/MOD-5014 spiked concentration (µg/ml)	***Predicated *in vivo* concentration**	**Phospholipids concentration**	**Tissue factor(TF) concentration**

	(µg/kg)		w/wo
0	0	4µM	1pM
1.25	40	4µM	1pM
2.5	80	4µM	1pM
5	160	4µM	1pM
10	320	4µM	1pM
15	640	4µM	1pM

* Based on Livnat et al., 2008.

[0921] TG was initiated by adding 20 µl of fluorogenic substrate/$CaCl_2$ buffer (FluCa-kit Thrombinoscope-BV, Diagnostica Stago, Lot FLB 1303/01). Fluorescence was measured using an excitation filter at 390 nm and an emission filter at 460 nm and a fluorometer (Fluoroskan Ascent, Lab system, Helsinki, Finland). Results were displayed as plots and derived parameters, i.e. lag time, endogenous thrombin potential (ETP) and peak height, and were calculated using specialized computer software (version 3.0.0.29, Thrombinoscope-BV Maastricht, the Netherlands). Each sample was tested independently twice in duplicates (run 1 and 2). The mean value of the duplicates is provided and compared to a thrombin standard (Thrombin calibrator, Diagnostica Stago, Lot TC 1208/01).

[0922] **Method – (Objective (II)) – Thrombin generation (TG)**

[0923] Thrombin generation was measured according to Livnat et al. (2006, 2008, 2011). Briefly, pooled plasma was spiked with 3 fairly low escalating concentration of MOD-5014 or NovoSeven® to enable a more sensitive and dose-dependent response (1.25, 2.5, 5, and 10µg/ml). MP-reagent containing 4µM phospholipids was used as working buffer. Each designated sample was spiked with escalating concentrations of TF as described in **Table 74** below, and TG was assessed.

[0924] **Table 74**

NovoSeven®/MOD-5014 spiked concentration (µg/ml)	***Predicated in –vivo concentration (µg/kg)**	**Phospholipids concentration**	**TF concentration w/wo**
0	0	4µM	0,0.5, 1, 2.5,5 pM
1.25	40	4µM	0,0.5, 1, 2.5,5 pM
2.5	80	4µM	0,1, 2,4,5 pM0,0.5, 1, 2.5,5 pM
5	160	4µM	0,0.5, 1, 2.5,5 pM
10	320	4µM	0, 1, 2,4,5 pM

			0,0.5, 1, 2.5,5 pM

* Based on Livnat et al., 2008

TG of each test article at the designated concentrations of TF was assessed by measurement ETP, lag time and height of thrombin peak. Each sample was tested independently twice in duplicates (run 1 and 2). The mean value of the duplicates is provided.

[0925] **Method – Objective (III) – Rotation thromboelastography (ROTEM)**

[0926] Pooled plasma from severe FVIII-deficient patients were spiked with either NovoSeven® or MOD-5014 at 4 concentrations as described in **Table 75** below, and assessed at the following conditions: (a) addition of Kaolin; (b) with low levels of TF; (c) re-classification.

[0927] **Table 75**

NovoSeven®/MOD-5014 spiked concentration (µg/ml)	*Predicated in –vivo concentration (µg/kg)
0	0
2.5	80
10	320
15	640

[0928] ROTEM measurements were conducted with a ROTEM device (Pentapharm, Munich, Germany) using 300 µL of FVIII-deficient plasma placed into cups, with a subsequent addition of 20 mM $CaCl_2$, (NATEM), allegic acid (contact activation, INTEM), or low concentration of tissue factor (EXTEM reagent diluted 1:1700). ROTEM tests were performed according to the manufacturer's instructions at 37°C and were run for a minimum of 45 minutes. The following variables were used: clotting time (CT, sec), i.e. the time between the introduction of $CaCl_2$ and the beginning of clotting; alpha-angle (α-angle, degrees) reflecting clot propagation; and maximum clot firmness (MCF, mm), which reflects clot strength.

RESULTS

[0929] **Objective (I): Comparative *In Vitro* Assessment of MOD-5014 and NovoSeven® by thrombin generation (TG)**

[0930] Thrombin generation has been frequently used for the assessment of hemostatic effect of FVIIa. It was previously shown that FVIIa mediates changes in thrombin generation (TG) in FVIII inhibitor plasma samples. In plasma samples spiked with recombinant FVIIa (rFVIIa), TG was improved in the absence of tissue factor (TF) while the TG potential of

rFVIIa *in vitro* was increased as a result of added TF. Simple, classic pharmacokinetic assays are not available for FVIIa due to the complexity of its mechanism of action. Since thrombin is the final product generated, a TG assay could be used for the assessment of the pharmacokinetics and potential efficacy of MOD-5014 and NovoSeven®. This assay is suitable for monitoring the pharmacokinetics of inhibitor-bypassing agents during treatment and may be useful for predicting responses to treatment. Therefore real-time measurement of thrombin concentration generated in plasma gives valuable information regarding the homeostasis of the coagulation system.

[0931] The objective of this study was to compare the *in vitro* thrombin generation ability of MOD-5014 and NovoSeven® in severe hemophilia A plasma at a range of concentrations that potentially correlate to the proposed clinical doses in the first-in-human (FIH) study. This could provide a prediction of the minimal effective dose as part of the preparations for first in human (FIH) study.

[0932] **Comparative Thrombin Generation following re-calcification**

[0933] MOD-5014 and NovoSeven® were spiked at a wide range of concentrations to severe hemophilia A pooled plasma. The study was repeated twice, and the results are provided in **Tables 76-79** below, and **Figures 56, 57, 58(A-E), 59(A-D), 60, 61, 62(A-E)** and **63(A-D)**.

[0934] **Table 76 (Run #1) NovoSeven® Derivative Values for Figure 56**

	1.25 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	15 µg/ml
Lag time	29.5	20.67	15.83	12.33	11.5
Lag t ime SD	2.17	0	0.5	0.33	0.17
ETP	141	242	460	727	659.5
ETPSD	13	0	115	84	15.5
Peak	6.2	11.45	20.33	33.45	39.85
Peak SD	0.69	0	2.32	0.03	1.34
tt Peak	44.17	33.67	28.5	22.83	21.33
tt Peak SD	1.83	0	0.5	0.5	0
Start Tail	76	76	73	71.5	74
Start Tail SD	0	0	2	3.5	0
Edited	1	1	0	0	1

[0935] **Table 77 (Run #1) MOD-5014 Derivative Values for Figure 57**

Lag time	37.33	29.33	20.17	16.17	13.67
Lag t ime SD	0.67	0.33	0.5	0.5	0
ETP	108.5	144.5	295	426	623.5
ETPSD	2.5	0.5	39	19	179.5
Peak	4.85	6.85	12.32	20.32	26.57
Peak SD	0.15	0.11	0.34	0.76	2.46
tt Peak	52.5	42.83	34	28	25.33
tt Peak SD	0.83	0.5	0.33	0.67	1
Start Tail	76	76	75	72.5	76
Start Tail SD	0	0	0	0.5	0
Edited	1	1	1	0	1

[0936] **Table 78 (Run #2) NovoSeven® Derivative Values for Figure 60**

	1.25 µg/ml	**2.5 µg/ml**	**5 µg/ml**	**10 µg/ml**	**15 µg/ml**
Lag time	18.7	13.85	12.35	10.35	8.68
Lag t ime SD	0	0.5	0.67	0.33	0.33
ETP	226	269	363.5	554.5	786
ETPSD	0	16	26.5	76.5	31
Peak	8.96	11.45	17.61	29.23	46.22
Peak SD	0	0.8	1.64	2.03	4.13
tt Peak	33.72	29.04	25.04	21.2	18.19
tt Peak SD	0	0.67	0.67	0.17	0.5
Start Tail	75	75	69	57	43.5
Start Tail SD	0	0	2	10	0.5
Edited	1	0	0	1	0

[0937] **Table 79 (Run #2) MOD-5014 Derivative Values for Figure 61**

Lag time	30.05	20.2	15.36	12.19	10.18
Lag time SD	0	0.17	0.33	0.5	0.17
ETP	118	179	300.5	486.5	602.5
ETPSD	0	1	18.5	19.5	111.5
Peak	4.36	7.2	12.86	19.8	28.59
Peak SD	0	0.18	0.42	0.73	3.15
tt Peak	46.74	35.55	28.88	24.54	21.37
tt Peak SD	0	0.83	0.17	0.17	0.33
Start Tail	76	76	74.5	67.5	69.5
Start Tail SD	0	0	0.5	0.5	5.5
Edited	1	1	0	0	0

[0938] A dose-dependent response was observed following the addition of the two compounds. At low concentrations of 1.25-2.5 µg/ml, which presumably mimic 40 and 80 µg/kg, respectively, a poor TG was observed, as reflected by increased lag and reduced ETP (**Figures 56, 57, 60 and 61**). Although the higher concentration provided a pronounce improvement in the TG profile, none of the tested compounds were able to provide a complete restoration of the TG as obtained with FVIII (**Figures 71 and 72; Table 80**).

[0939] **Table 80** (Derivative values for **Figure 72**)

	FVIII + Low TF	FVIII
Lag time	6.17	11.33
Lag time SD	0.17	0.33
ETP	1869	1698
ETP SD	1	48
Peak	323.34	233
Peak SD	13.43	17.69
tt Peak	8.67	13.5
tt Peak SD	0	0.17
StartTail	27.5	33.5
Start Tail SD	0.5	0.5
Edited	0	0

[0940] These results are in line with published studies and suggest that hFVIIa is less effective as a bypassing agent than other replacement therapies for thrombin correction, demonstrated by lower TG peak, ETP, and other parameters. Overlay analysis (**Figures 58 (A-E), 59 (A-D), 62 (A-E) and 63 (A-D)**) suggested a slight reduction in MOD-5014 TG

response as compared to NovoSeven®, reflected mainly as increased lag time, and a lower thrombin peak (estimated as 30-40% lower than in NovoSeven®).This might be a consequence of reduced molar content of FVIIa in MOD-5014 when spiking on mass base rather than on molar base as MOD-5014 consists of 83.4% FVIIa with 3 CTP cassettes attached at the C terminus. The two independent runs were consistent with each other, providing similar results with minor variation.

[0941] **Comparative Thrombin Generation at low TF concentrations**

[0942] When severe hemophilia A pooled plasma was spiked with low levels of TF, a background TG response was observed that increased as MOD-5014 and NovoSeven® concentrations increased in the tested samples. (**Figures 64, 65, 66(A-E), 67, 68, 69(A-E), 70(A-C); Tables 81-84**)

[0943] **Table 81** (NovoSeven® Derivative values for **Figure 64**)

	Control	1.25 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	15 µg/ml
Lag time	7.33	4.33	4.17	4.17	4.17	4.17
Lag time SD	0	0	0.17	0.17	0.17	0.17
ETP	592	880	903	1093.5	1256.5	1350
ETP SD	0	85	34	68.5	77.5	72
Peak	28.6	86.23	91.39	62.16	77.12	92.79
Peak SD	0.81	0.74	0.89	3.23	3.62	3.15
tt Peak	17.33	12.83	12.33	12.33	12	11.5
tt Peak SD	0.67	0.17	0	0	0	0.17
Start Tail	59	64	59	54.5	50	54
Start Tail SD	3	0	2	0.5	2	0
Edited	0	1	1	0	0	1

[0944] **Table 82** (MOD-5014 Derivative values for **Figure 65**)

	1.25 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	15 µg/ml
Lag time	4.33	4.17	4.17	4.33	4.17
Lag time SD	0	0.17	0.17	0	0.17
ETP	699.5	822.5	932	1088	1223.5
ETP SD	6.5	39.5	13	35	26.5
Peak	81.28	89.32	86.83	68.13	72.19
Peak SD	0.41	1.93	1.72	3.65	2.76
tt Peak	12.67	12.33	12.83	12.83	12.17
tt Peak SD	0.33	0	0.17	0.17	0.17
Start Tail	50.5	50.5	47.5	48	46.5
Start Tail SD	3.5	3.5	2.5	3	3.5
Edited	0	1	0	0	0

[0945] **Table 83** (NovoSeven® Derivative values for **Figure 67**)

	Control	1.25 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	15 µg/ml
Lag time	6.17	3.5	3.67	3.67	3.67	3.67
Lag time SD	0.17	0.17	0	0	0	0
ETP	526.5	671.5	790.5	795	928	933
ETP SD	2.5	13.5	18.5	5	27	44
Peak	25.84	40.33	46.66	53.84	61.35	65.60
Peak SD	3.4	0.4	0.47	0.13	0.18	0.66
tt Peak	36	18.83	18.5	18.5	18.67	18.33
tt Peak SD	0.33	0.17	0.17	0.17	0	0
Start Tail	56.5	53.5	56	56.5	61	64
Start Tail SD	1.5	0.5	4	2.5	2	2
Edited	0	0	1	0	0	0

[0946] **Table 84** (MOD-5014 Derivative values for **Figure 68**)

	1.25 µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml	15 µg/ml
Lag time	4	3.83	4	4	3.83
Lag time SD	0	0.17	0.33	0.33	0.5
ETP	687	687.5	723.5	784	833.5
ETP SD	18	21.5	49.5	4	94.5
Peak	33.55	38.62	42.52	49.28	51.34
Peak SD	0.15	1.51	4.87	3.88	2.12
tt Peak	13.83	12.17	13	12.67	12.33
tt Peak SD	0.17	0.17	0.67	1	1
Start Tail	48	52	51	53.5	64
Start Tail SD	1	2	6	7.5	6
Edited	0	0	0	0	1

[0947] A similar pattern of dose-dependent response was observed in this study for both products; however, larger amplitude was obtained due to response enhancement by TF. Again, MOD-5014 demonstrated reduced activity that was more pronounced in comparison to the re-calcifications study, requiring a higher MOD-5014 concentration to provide a suitable overlay between the two (**Figure 66 (A-E) and Figure 70 (A-C)**). To study this further, an *in vitro* study was performed further investigating MOD-5014 and FVIIa affinity to TF and its effect on TG, as described below.

[0948] *Conclusions:* Both products demonstrated a dose-dependent TG response when spiked to severe hemophilia A pooled plasma, with an initial poor response at concentrations mimicking clinical dose of 40-80 µg/kg. These results presumably suggest that doses lower than 40-80µg/kg will not provide an adequate *in vivo* response. Furthermore, MOD-5014 demonstrated a reduced TG performance when spiked at a similar concentration as NovoSeven®, suggesting that a slightly increased concentration (30-40%) might be needed in

the clinical setting to provide proper initial hemostatic effect which is comparable to that of NovoSeven®.

[0949] **Objective (II): Comparative *In Vitro* Assessment of MOD-5014 and NovoSeven® Affinity to TF**

[0950] FVIIa seems to have at least two independent effector mechanisms: the tissue factor (TF)-dependent FVIIa-mediated activation of factor X (FX), which is the classic inducer of the extrinsic pathway of coagulation, and a TF-independent activity of high-dose FVIIa on endogenous phospholipid (PL) surfaces of monocytes or platelets. MOD-5014 demonstrated reduced activity when compared to NovoSeven® that was more pronounced in the presence present of TF. Higher MOD-5014 concentrations were required to provide a suitable overlay between the two compounds (**Figure 66 (A-E) and Figure 70 (A-C)**). Although it was previously reported that MOD-5014 affinity to TF was similar to NovoSeven® as measured by SPR and in vitro activation assays, the objective of this study was to further investigate this *in vitro* affinity of MOD-5014 and FVIIa to TF by TG.

[0951] MOD-5014 and NovoSeven® were spiked at an escalating range of concentrations to severe hemophilia A pooled plasma, in the presence of escalating concentrations of TF. The study was repeated twice, and the results of each run are provided in **Figures 73-88 and Tables 85-90**.

[0952] **Table 85** (Derivative Values for **Figure 73**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	33.67	3.67	2.33	2.33	1.33	36	4.67	2.67	2.33	1.33
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	0	326	869	1938	1934	0	264	884	1817	1908
ETP SD	1	0	0	0	0	1	0	0	0	0
Peak	18.73	12.88	22.47	95.76	233.67	8.23	9.04	27.8	99.11	233
Peak SD	0	0	0	0	0	0	0	0	0	0
t Peak	91.67	19.33	12	7.67	5	86	22.33	10.33	8	6.67
t Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	0	79	58	33	25	0	25	69	33	25
Start Tail SD	1	0	0	0	0	1	0	0	0	0
Edited	0	0	0	0	0	0	0	0	0	0

[0953] **Table 86** (Derivative Values for **Figure 75**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	28.67	5.33	3	2.67	3.67	80	6.67	3	2.67	3.67
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	0	336	801	1934	3632	0	248	448	1038	3356
ETP SD	1	0	0	0	0	1	0	0	0	0
Peak	13.8	14.13	26.41	98.73	252.42	9.39	10.27	22.99	95.69	240.75
Peak SD	0	0	0	0	0	0	0	0	0	0
tt Peak	41	19.67	12.33	8	8.67	56.33	22	12	8.33	5
tt Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	0	72	58	24	28	0	64	36	34	23
Start Tail SD	1	0	0	0	0	1	0	0	0	0
Edited	0	0	0	0	0	0	1	0	0	0

[0954] **Table 87** (Derivative values for **Figure 77**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	18	8	3.33	2.67	3.67	27.67	8.67	3	3	3.67
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	396	418	836	1831	3527	233	348	680	983	3318
ETP SD	0	0	0	0	0	0	0	0	0	0
Peak	16.83	18.67	36.25	132.89	258.79	10.43	13.22	28.28	84.2	263.12
Peak SD	0	0	0	0	0	0	0	0	0	0
tt Peak	31	20	12	7.33	6.67	40.67	21.33	10.67	8.33	6.67
tt Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	25	69	59	29	22	56	25	39	38	23
Start Tail SD	0	0	0	0	0	0	0	0	0	0
Edited	0	0	0	0	0	1	0	0	0	0

[0955] **Table 88** (Derivative values for **Figure 81**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	17	7.33	5.67	3	3.67	20.33	8.67	8	3	3.67
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	737	718	665	1612	3633	387	460	754	1659	3602
ETP SD	0	0	0	0	0	0	0	0	0	0
Peak	23.87	28.93	43.13	90.01	236.54	16.58	19.99	32.2	92.28	236.43
Peak SD	0	0	0	0	0	0	0	0	0	0
tt Peak	29.33	16	13.67	8.33	8	32.67	23	13.67	8.67	8
tt Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	56	64	45	34	23	75	72	52	34	25
Start Tail SD	0	0	0	0	0	0	0	0	0	0
Edited	1	1	0	0	0	0	0	1	0	0

[0956] **Table 89** (Derivative values for **Figure 82**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	25	7.33	3.33	2.67	1.67	32.67	6	3.33	2.67	1.67
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	0	333	734	1238	1629	0	367	760	1307	1603
ETP SD	0	0	0	0	0	0	0	0	0	0
Peak	13.5	13.65	26.25	136.91	252.3	10.75	11.15	30.43	105.24	248.68
Peak SD	0	0	0	0	0	0	0	0	0	0
tt Peak	40.67	22.33	14.33	7.67	4.67	59	23	13	8.33	4.67
tt Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	0	72	78	33	28	0	78	68	31	28
Start Tail SD	0	0	0	0	0	0	0	0	0	0
Edited	0	0	0	0	0	0	0	1	0	0

[0957] **Table 90** (Derivative Values of **Figure 83**)

	NovoSeven					MOD-5014				
	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM	TF 0 pM	TF 0.5 pM	TF 1 pM	TF 2.5 pM	TF 5 pM
Lag time	23.33	7	4	2.67	2	29.33	7	3.67	2.67	2
Lag time SD	0	0	0	0	0	0	0	0	0	0
ETP	388	499	639	1105	1581	302	384	784	1087	1531
ETP SD	0	0	0	0	0	0	0	0	0	0
Peak	16.18	21.07	30.22	169.4	260.86	12.28	14.34	28.88	103.07	254.16
Peak SD	0	0	0	0	0	0	0	0	0	0
tt Peak	34.33	19	14.33	7.67	4.67	51.67	21.33	12.33	7.67	4.67
tt Peak SD	0	0	0	0	0	0	0	0	0	0
StartTail	23	64	58	32	23	34	52	69	36	23
Start Tail SD	0	0	0	0	0	0	0	0	0	0
Edited	1	1	1	0	0	1	0	1	0	0

[0958] Spiking with increasing concentrations of TF with fixed doses of either NovoSeven® or MOD-5014 provided a TF-dependent increase in TG performance, as reflected by reduced lag time and increased ETP and thrombin pick (**Figures 79 (A-C), 80 (A-C), 87 (A-E) and 88 (A-E)**). For both compounds at all doses and TF concentrations below 5pM, poor to moderate TG response was observed as reflected by increased lag time and reduced ETP. A higher concentration of TF (5pM) provided a pronounced improvement in the TG profile (**Figures 79 (A-C), 80 (A-C), 87 (A-E) and 88 (A-E)**). However, none of the tested compounds were able to provide a complete restoration of the TG as obtained with FVIII (**Figures 71** and **72,** and **Table 82**). Interestingly, increasing the concentrations of NovoSeven® or MOD-5014 at constant TF levels did not improve TG performance (**Figures 79 (A-C), 80 (A-C), 87 (A-E) and 88 (A-E)**), further emphasizing the importance of TF for the proper activity of both compounds.

[0959] Comparison of the TG profiles of MOD-5014 and NovoSeven® by overlay analysis

(**Figures 74 (A-E), 76 (A-E), 78 (A-E), 82, 85 (A-C), 86 (A-E); Table 89**) suggested a slight reduction in MOD-5014 response (estimated as 20-30% lower) without TF or at very low TF concentration (0.5pM). As the level of TF increases, a similar response is observed at all MOD-5014 and NovoSeven® concentrations on both repeats (**Figures 74 (A-E), 76 (A-E), 78 (A-E), 82, 85 (A-C), 86 (A-E); Table 89**).

[0960] *Conclusion:* This study further confirms the results *in vitro* by spiking both compounds at fixed concentrations in the presence of escalating concentrations of TF. The amount of TF in the sample was predominantly responsible for the increased TG response, further confirming a biological similarity between MOD-5014 and NovoSeven®.

[0961] **Objective (III) Comparative Assessment of MOD-5014 and NovoSeven® Activity Using Rotation Thromboelastography (ROTEM)**

[0962] Over the last decade, thromboelastography emerged as a valuable tool for monitoring hemostasis in coagulopathy, blood transfusion and clotting factor replacement therapy. In this respect, thromboelastography has been used in hemophilia, factor VIII or IX replacement therapy, and assessment of the effect of aFVIIa in hemophilia patients with inhibitors. ROTEM may be used for assessing of the effects of coagulation and anti-fibrinolytic agents in thrombocytopenia, Glanzmann's thrombasthenia and hemodilution. The objectives of the study were to compare the *in vitro* ROTEM performance of MOD-5014 and NovoSeven® in severe hemophilia A plasma at a range of concentrations which correlate to the proposed clinical doses, and assess the minimal effective dose as part of the preparations for the FIH study.

[0963] The results of the experiments are presented in the **Tables 91 and 92**.

[0964] **Table 91: Spiking FVIII-Deficient Plasma with Factor VIII**

Test	Treatment	Clot time (CT) (sec)	α-angle (degrees)	MCF (mm)
NATEM	Intact	No clot	No clot	No clot
	FVIII	1359	17	15
INTEM	Intact	1123	9	11
	FVIII	335	72	14

[0965] **Table 91** shows plasma clotting without FVIII and following plasma spiking with FVIII (1 U/mL), which was used as control compound in the study. In the NATEM test, no clotting was observed in the intact plasma, while spiking the plasma samples with FVIII was followed by slight clot formation. In contrast, in the INTEM test clot formation occurred in

both intact and treated plasma, whereas in the latter it was much stronger (CT was shorter and the α-angle was 8-fold higher). Maximum clot firmness (MCF) was slightly increased. These results suggest that the intrinsic system (INTEM) may be a useful test for the assessment of FVIII-replacement in hemophilia A.

[0966] **Table 92: The Effect of MOD-5014 and Novo Seven on Clot Formation in FVIII-Deficient Plasma**

Test	Treatment	Conc. (μg/mL)	Clot time (sec)	α-angle (degrees)	MCF (mm)
NATEM	Control	-	No clot	No clot	No clot
	NovoSeven	2.5	No clot	No clot	No clot
		10	nd	nd	nd
		15	1100	20	16
	MOD-5014	2.5	No clot	No clot	No clot
		10	1601	10	12
		15	1244	14	15
INTEM	Control	-	1123	9	11
	NovoSeven	2.5	810	12.5	10
		10	531	25	13
		15	472	26	12
	MOD-5014	2.5	797	14.5	12
		10	611	22.5	14.5
		15	660	27	13
EXTEM	Control	-	404	19.5	10.5
	NovoSeven	2.5	371	20.5	12.5
		10	340	27	13
		15	377	30	14
	MOD-5014	2.5	355	22.5	12.5
		10	324	28.5	14
		15	323	30	13.5

nd – not determined

[0967] **Table 92** shows the effects of FVIIa on clot formation in FVIII-deficient plasma.

[0968] *Recalcification with contact activation of plasma (INTEM test):* CT was shorter and the α-angle increased gradually in plasma treated with both types of FVIIa at 2.5 and 10 µg/mL compared to non-treated plasma. By increasing FVIIa concentration to 15 µg/mL, the changes in clot formation did not differ from that of plasma treated with 10 µg/mL FVIIa. No difference was found between the activities of MOD-5014 and NovoSeven.

[0969] *Recalcification with activation of the extrinsic pathway (EXTEM test):* There was a decrease in CT and increase of MCF in both MOD-5014 and NovoSeven-treated plasma compared to non-treated plasma. An increase in clot propagation (α-angle) was observed to a similar extend in plasma treated with both agents at 2.5 and 10 µg/mL, with no further change at 15 µg/mL.

[0970] *Conclusions:* INTEM appeared to be the most reliable test among the different ROTEM tests utilized to compare the activity of MOD-5014 and NovoSeven. A dose of 2.5µg/ml, which mimics an *in vivo* dose of 80µg/kg, resulted in a low response for both compounds as reflected by slightly decreased clotting time, and increased α-angle compared to non-treated plasma, tested utilizing both INTEM and EXTEM. Increasing concentrations of MOD-5014 and NovoSeven® to 10 and 25 µg/ml was followed by dose-dependent stimulatory effect on clot formation. The results also demonstrate no essential difference in the effects of either agent on clot formation, providing very similar ROTEM values at both agents.

EXAMPLE 14: ASSESSMENT OF MOD-5014 PHARMACOKINETICS, PHARMACODYNAMICS AND CORRECTION OF HEMOPHILIC COAGULOPATHY IN DOGS WITH HEMOPHILIA A (FVIII DEFICIENCY)

[0971] **Study Objective** – The objective of the current study was to evaluate and characterize MOD-5014 pharmacokinetics, pharmacodynamics, and correction of hemophilic coagulopathy in dogs with severe hemophilia A (FVIII deficiency, <0.01% FVIII).

[0972] **Justification of Test System**

[0973] The Chapel Hill hemophilia A dog colony has the advantage of no detectable FVIII activity in FVIII bioassays and chromogenic assays, and little or no FVIII antigen by ELISA (<0.005 U/ml). This model has been used in the past as part of the non-clinical pharmacological characterization of different clotting factors. It has been proposed that this model can provide an accurate recapitulation of human biology within the particular area of

hemostasis, and provide comparable PK/PD parameters to those previously observed in human studies. In addition, TEG, which is used as a screening tool assessing overall hemostatic dysfunction or correction in clinical setting has been shown to be a predictive tool in this model as well suggesting further correlation between human and dog hemostasis.

[0974] **Materials and Methods**

[0975] **Materials** - MOD-5014: 2.6 mg/ml, stored frozen (-60 to -80°C). No dose formulation preparation was required. Materials were thawed to room temperature prior to administration.

[0976] The pre-formulated dosing formulation was handled aseptically and administered as received; no dose formulation preparation was required. Test article (MOD-5014) was thawed only once prior to dosing. The test article was removed from frozen storage and thawed to room temperature prior to administration.

[0977] **Method: *Ex vivo* phase**: The *ex vivo* phase was conducted according to Knudsen *et al* 2011, Haemophilia. 17(6):962-970, in order to establish the minimal effective dose which would support dose selection for the *in vivo* study.

[0978] During the first phase, dose dependent *ex vivo* Whole Blood Clotting Time (WBCT) and Thromboelastography (TEG) assessments of MOD-5014 spiked into fresh blood, from two

individual FVIII deficient dogs, were performed. The lowest dose of MOD-5014, which significantly improved the TEG and WBCT performance, was considered the minimal effective dose. Based on the first phase and the establishment of the minimal effective dose, the doses for the *in vivo* second phase were selected.

[0979] Fresh canine blood was drawn from two individual FVIII hemophilic dogs and analyzed independently in order to confirm reproducibility of results. Briefly, 15 mL of canine blood were drawn into a syringe, transferred to a conical tube and spiked with MOD-5014 to final concentrations of 0.568, 1.136, 2.273, 4.545, and 9.909 µg/mL, which correlates to anticipated *in vivo* Cmax following administration of 50, 100, 200, 400, 800 µg/kg of MOD-5014 in FVIII deficient dogs assuming the following: Canine estimated weight is 20 kg and Blood volume for each animal is 40 mL/lb (88 mL/kg).

[0980] Blood from these spiking was assayed within <5 min of adding the test material as follows:

[0981] Step 1: Whole Blood Clotting Time (WBCT): 1mL of blood spiked with MOD-5014 to designated final concentrations was used for the WBCT. Briefly, the whole blood clotting time (WBCT) is a modification of the Lee-White clotting time using two siliconized glass tubes (VacutainerTM #6431, Becton-Dickinson, Rutherford, NJ) in a 28° C water bath. One

mL of whole blood spiked with MOD-5014 at designated concentrations was split equally between two siliconized tubes. A timer was started. After one minute, one tube was tilted every 30 sec, the other left undisturbed. When a clot was formed in the tilted tube, the second tube was then tilted every 30 sec until a clot formed. The time for formation of a fully gelled clot in the second tube was recorded as the WBCT. In naïve hemophilia A dogs, the WBCT is generally > 40 minutes but can be > 60 min. The test is stopped if the value exceeds 60 min.

[0982] Step 2: Thromboelastography: 1 mL of blood spiked with MOD-5014 to designated final concentrations was mixed with Kaolin (Lot # is provided by Haemoscope), 360 µl of this mixture was placed in the cup for testing. TEG recordings were allowed to proceed for approximately 90 min. A typical range of TEG values is provided in the **Table 93** below.

[0983] **Table 93 - Thromboelastography Typical Range of Values**

TEG (Kaolin activated)	R (min)	K (min)	Angle (degrees)	MA (mm)
Normal (n=2)	7.4 ± 1.1	1.6 ± 0.2	67.2 ± 2.9	61.4 ± 20.6
Hemophilia A (n=2)	> 60	NA	NA	NA

[0984] The lowest dose that demonstrates significant clotting improvement will be used as the initial dose for the *in vivo* study, proposing proper monitoring of blood clotting and hemostasis as measured during the phase II of the study and by utilizing the different assays as described below.

[0985] **Method: *In vivo* phase**: Six naïve mixed breed Chapel Hill hemophilia A dogs (*Canis familiaris*) weighing approximately 20 kg were used in this study. Dogs received IV doses of 50 µg/kg (N=2), 200 µg/kg (N=4), 400 µg/kg (N=2), or 600 µg/kg (N=2) MOD-5014. Doses were given as a 5 minute IV infusion. Blood samples were collected for concentration and activity measures by transcutaneous puncture of the cephalic vein prior to dosing, and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 32, 48, 72 and 96 hours post-dosing. Four dogs were administered twice with MOD-5014 during a period of up to 8 days, and 2 additional dogs were administered once. The study was designed as a dose-staggered escalating study, i.e. following each injection in-time clotting analysis determined the timing of the next dose.

[0986] During the *in vivo* phase, MOD-5014 pharmacokinetics and pharmacodynamics were analyzed, and WBCT, thromboelastography, aPTT, basic clinical pathology, and a clinical observation (based on animal behavior) were assessed. Bioanalytical assessment was conducted for the test article.

[0987] ***In vivo* phase – Part A**

[0988] During the first part, 2 naïve dogs were injected on day 1 with an initial dose of 50 µg/kg MOD-5014 and monitored for WBCT and TEG performance. Once these values

returned to baseline (pre-dose), a second dose of 200 µg/kg MOD-5014 was injected. **Table 94** presents the outline of Part A.

[0989] **Table 94 – Study design Part A**

Animal	Test Article	Injection time point	Dose Route	injection Dose (µg/kg)	Animal Weight (Kg)	injection Dose Volume (mL/animal)
N06	MOD-5014	T=0	IV	50	20	0.384
N06	MOD-5014	T=48hr (post initial dose)	IV	200	20	1.538
P14	MOD-5014	T=0	IV	50	22.6	0.434
P14	MOD-5014	T=TBD (post initial dose)	IV	200	22.6	1.736

[0990] ***In vivo* phase – Part B**

[0991] During the second part and following the completion of the Part A, two additional naïve dogs were injected on day 1. The animals were again monitored for WBCT and TEG performance. Once those values returned to baseline (pre-dose), a second dose was injected. **Table 95** presents the outline of Part B.

[0992] **Table 95 – Study design Part B**

Animal	Test Article	Injection time point	Dose Route	injection Dose (µg/kg)	Animal Weight (Kg)	injection Dose Volume (mL/animal)
Blondie	MOD-5014	T=0	IV	200	20.8	1.6
Blondie	MOD-5014	T=48 (post initial dose)	IV	400	20.8	3.2
Josie	MOD-5014	T=0	IV	200	20.5	1.576
Josie	MOD-5014	T=48 (post initial dose)	IV	400	20.5	3.15

[0993] ***In vivo* phase – Part C**

[0994] During Part B and following he completing of Part A, two additional naïve dogs were injected on day 1 (**Table 96**).

[0995] **Table 96 – Study design of Part C**

Animal	Test Article	Injection time point	Dose Route	injection Dose (µg/kg)	Animal Weight (Kg)	injection Dose Volume (mL/animal
Joanie	MOD-5014	T=0	IV	600	20.6	4.75
N05	MOD-5014	T=0	IV	600	18.4	4.24

[0996] **Justification for Route of Administration**

[0997] The intravenous route is the intended route of administration of this test article in humans.

[0998] **Justification of Dose Levels**

[0999] The dose levels were selected on the basis of the proposed dose range which was previously tested for rhFVIIa and the dose range considered for further assessment in the Phase I study.

[01000] In order to assess the hemostatic potential of MOD-5014 which will enable the prediction of the minimal effective dose in Hemophilia A dogs prior to infusion, a range of doses was evaluated in WBCT and TEG spiking studies at a dose range that approximates the maximal expected level following *in vivo* IV administration. In both assays, spiking of MOD-5014 at the lowest dose (0.568 µg/mL, which corresponds to 50 µg/kg) demonstrated a minor improvement in hemostatic effect that improved as concentrations increased but without a complete normalization of TEG values, in line with Knudsen et al., 2011. Based on the obtained results the lowest dose to be further studied *in vivo* was the lowest assessed dose i.e. 50 µg/kg.

[01001] **Administration**

[01002] Administration was by IV. The test article was administered up to 2 times during 8 days. Approximately 10% of the test article was injected over ~1 min and the dog was observed for any obvious clinical reaction (e.g., urticaria and listlessness). When the dog was considered as tolerant to the injection, the remaining 90% was infused over 1 to 5 minutes. The injection time and volume were recorded.

[01003] Different doses were provided by varying the dose volume. Administration of test article was followed by a saline flush.

[01004] The animals were fasted prior to dosing.

[01005] **Study Evaluations**

[01006] *Physical examinations*: dogs underwent a general examination prior to study entry, and only those with normal general examinations were included.

[01007] Cage side observations: any concerns regarding the animal's behavior or general health noted in the clinical examination of the animal were recorded.

[01008] Detailed clinical observations: During the infusion phase of the study, blood sampling was performed.

[01009] Body weights were recorded at the start of the study and between Days 7 and 14 following study initiation.

[01010] Food consumption: Dogs were fed their usual amount of food on a daily basis and observed for food consumption.

[01011] **Clinical Pathology**

[01012] Clinical pathology evaluations were conducted on study animals at pre-dose. Platelet counts, WBC, HCT, and HGB were performed by FOBRL. Samples were collected for further clinical chemistry tests should any clinical events have occurred.

[01013] **Plasma Analysis**

[01014] **Sample Collection and Handling**

[01015] All blood samples were taken by transcutaneous puncture of the cephalic vein using a 21G or comparable butterfly needle. 10 mL of blood was collected in a 10 mL syringe containing 1 mL anticoagulant (3.2% sodium citrate [0.12M]). The sodium citrate was diluted 1:10 as the blood was collected, according to standard lab protocol. The 10 mL blood sample was transferred from the syringe to a 15 mL polypropylene conical tube and gently inverted to ensure mixing without hemolysis. The blood was then centrifuged at 3000g for fifteen minutes without brake at 4°C. Following centrifugation, the plasma supernatant was transferred into new 15 mL polypropylene conical tube and centrifuged at 3000g without brake at 4° C for an additional 7 minutes, to ensure sufficient separation of plasma from other blood materials. After the second centrifugation, plasma from each sample was transferred by a polypropylene pipette to a polypropylene tube and immediately placed in a -80°C freezer.

[01016] Multiple aliquots of 100 µL of plasma were transferred to Micronics tubes (or comparable) and frozen rapidly (-80°C). Except for WBCT and TEG, all assays (enzyme-linked immunosorbant assay [ELISA], activated partial thromboplastin time [aPTT], and thrombin generation assay [TGA]) were performed in batch for all animals after the infusion and sampling were completed.

[01017] **MOD-5014 ELISA**

[01018] MOD-5014 ELISA was performed utilizing an assay that specifically and selectively detects MOD-5014, utilizing a commercially available anti-FVIIa antibody (Ab) and an in-house polyclonal anti-CTP Ab. Citrated plasma samples were collected at pre-dosing (i.e. baseline), 15 min, 30 min, 1, 2, 4, 6, 8, 12, 24, 32, 48, 72, and 96 hours.

[01019] **FVIIa Clotting Activity**

[01020] FVIIa clotting activity was measured utilizing the commercially available STACLOT VIIa-rTF kit (Ref #00281, Stago) adjusted to quantitate FVIIa activity in hemophilic dog matrix.

[01021] **WBCT**

[01022] The WBCT assays were performed by a two-tube procedure at 28°C, and tested after collection. One mL of whole blood was collected with 1 mL syringe and was divided equally between two siliconized tubes (Vacutainer™, #6431, Becton-Dickinson, Rutherford, NJ). The

first tube was tilted every 30 sec. Following clot formation, the second tube was tilted and observed every 30 sec. The endpoint was the clotting time of the second tube. In this study, WBCT was performed by FOBRL at pre-dosing (i.e. baseline), 15 min, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours, or until baseline values were reached.

[01023] **TEG**

[01024] Blood for TEG was drawn at each sampling point and tested at FOBRL/UNC within 2 min post-collection using the Haemoscope TEG 5000 Thromboelastography Analyzer according to the manufacturer's instructions. Briefly, the first 3 mL of blood were discarded, then 1 mL of blood was drawn and mixed with Kaolin (Lot #A-30-05, provide by Haemoscope). 360 µL of this premixed blood/initiator was placed in the instrument and analyzed. TEG recordings were allowed to proceed for approximately 60-90 min. The test was performed at pre-dosing (i.e. baseline), 15 min, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours, or until baseline values were reached.

[01025] **aPTT**

[01026] aPTT was determined at FOBRL/UNC using the ST4 coagulation instrument (Diagnostica Stago, Asnieres, France). The test mixture consisted of equal portions of partial thromboplastin reagent (Triniclot, Diagnostica Stago), 0.025 M $CaCl_2$ and citrated test plasma. Samples were tested at pre-dosing (i.e. baseline), 15 min, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours.

[01027] **Data Analysis**

[01028] Non-compartmental analysis and pharmacokinetic modeling were performed on individual animal data with Phoenix WinNonlin version 6.3 (Pharsight Corporation, Sunnyvale, CA.). Analysis was performed on MOD-5014 plasma concentration data as well as on activity data assuming an initial specific activity of 15,563 units/mg.

[01029] For non-compartmental analysis a program for IV infusion was used. The area under the curve from time zero to the last measureable time point (AUC_{0-t}) was estimated using the trapezoidal method. Log-linear regression over the last three or more time points was used to estimate the elimination constant (λ) which was used to estimate the terminal half-life ($t_{1/2}$) and AUC from zero to infinity ($AUC_{0-\infty}$) from the following equations:

$$t_{1/2} = \ln(2)/\lambda$$

$$AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda$$

where Ct is the last measureable concentration. Plasma clearance (CL) was calculated from dose divided by $AUC_{0-\infty}$. The maximum concentration (Cmax) and the time it was observed (Tmax) were determined directly from the data. Since the analysis was based on an IV

infusion, the concentration at the start of infusion was set to 0 and an initial volume of distribution was not calculated.

[01030] For pharmacokinetic modeling, various models and fitting strategies were investigated including evaluation of one-, two-, and three-compartment models and different weighting schemes.

[01031] Models were evaluated based on the correlation of observed and predicted concentrations, the relative values of parameter estimates and error estimates, and the Akaike Inclusion Criterion, a mathematical evaluation for comparing one model to another.

[01032] Both plasma concentration and activity versus time data were described best by a two-compartment model. Concentration data were weighted by the inverse of the square of the

predicted concentration and activity data were weighted by the inverse of the predicted concentration.. The weighting schemes prevent the early, high concentrations from exerting too

much influence over the curve fit. The model is illustrated in **Figure 90**.

[01033] The model generates estimates for the volume of distribution of the central compartment (V1), the elimination rate constant k10 and the inter-compartmental rate constants, k12 and k21. The central compartment is the compartment into which drug is administered and from which samples are collected, in this case, vascular space. The rate constants associated with the distribution and elimination phases of the curve, α and β, are calculated from te intercompartmental rate constants. Other parameters calculated from the primary parameters include AUC, Cmax, Tmax, CL, MRT and the half-lives associated with the distribution and elimination phases of the curve ($t_{1/2\alpha}$, $t_{1/2\beta}$).

RESULTS

[01034] **Ex vivo Study Results**:

[01035] **WBCT**

[01036] WBCT was measured following spiking of MOD-5014 to the blood of two individual hemophilic A dogs, at final concentrations ranging from 0-9.09 μg/mL, which are calculated to be equivalent to *in vivo* Cmax following administration of 50, 100, 200, 400, 800 μg/kg of MOD-5014, respectively. The baseline (pre) WBCT value was different between the two dogs, but within the acceptable range for Hemophilia A animals (see **Table 97**).

[01037] **Table 97 - WBCT Following MOD-5014 Spiking Canine Blood**

Dog ID	Conc. (µg/mL)	WBCT (min)		Dog ID	Conc. (µg/mL)	WBCT (min)
P22	0 (Pre)	41.5		O93	0 (Pre)	52
P22	0.568	37		O93	0.568	42.5
P22	1.136	34.5		O93	1.136	45.5

Dog ID	Conc. (µg/mL)	WBCT (min)		Dog ID	Conc. (µg/mL)	WBCT (min)
P22	2.273	33.5		O93	2.273	41.5
P22	4.545	35.5		O93	4.545	40
P22	9.09	32.5		O93	9.09	34

[01038] Significant reduction in WBCT was observed as MOD-5014 concentration increased with optimal correction (~30 min, based on internal laboratory assessment) at the highest range of MOD-5014 concentrations, as presented in **Table 97 and Figure 89**.

[01039] For Dog P22, a lower WBCT baseline was observed and lower MOD-5014 concentrations were effective, while Dog O93 required higher MOD-5014 concentrations to reach the target WBCT value due to its higher baseline value.

[01040] **Thromboelastography**

[01041] MOD-5014 at a range of doses was spiked independently into whole blood from two Hemophilia A dogs and was used to simulate a relevant concentration in blood shortly after IV injection of doses ranging from 50-800 µg/kg. Kaolin–TEG parameters were improved at a dose dependent manner with some fluctuation and variability between the two individuals due to assay variability or technical error (**Table 98**).

[01042] **Table 98 - Effect of MOD-5014 spiked in canine hemophilia blood assessed by Kaolin–activated TEG**

Thromboelastography (Kaolin)												
Dog ID	Conc. (µg/mL)	R (min)	K (min)	Angle	MA (mm)		Dog ID	Conc. (µg/mL)	R (min)	K (min)	Angle	MA (mm)
P22	0 (Pre)	>60.0	---	---	---		O93	0 (Pre)	>60.0	---	---	---
P22	0.568	43.4	23.2	9.3	38		O93	0.568	40.8	13.2	15.1	46.1
P22	1.136	37.2	12	17.7	48.4		O93	1.136	38.1	10.1	19.6	36.4
P22	2.273	41.9	24	9.6	49.4		O93	2.273	32.4	14.5	15.3	40.1

Thromboelastography (Kaolin)												
Dog ID	Conc. (µg/mL)	R (min)	K (min)	Angle	MA (mm)		Dog ID	Conc. (µg/mL)	R (min)	K (min)	Angle	MA (mm)
P22	4.545	32.8	12.2	17.7	49.3		O93	4.545	36.4	14.3	14.8	50.3
P22	9.09	26.8	n/a	12.6	n/a		O93	9.09	31.4	9.4	16.7	43.7

[01043] The obtained values were comparable to the ones reported at Knudsen et al 2011, for recombinant rhFVIIa, taking to account the reduced specific activity of MOD-5014 (2.3-2.5 fold reduction in FVIIa specific activity) and lower FVIIa content (72.3%).

[01044] *Conclusion:* In both assays, the lowest dose of 0.568 µg/mL, which corresponds to 50

μg/kg, demonstrated an improvement in hemostatic effect which improved as concentrations increased without a complete normalization of TEG values, aligned with Knudsen *et al* 2011.

[01045] **In vivo Study Results:**

[01046] **In-life Clinical Examinations**

[01047] All infusions were well-tolerated. No injection site reactions were noted. No significant changes in hemoglobin, hematocrit, white blood cell count, or platelet counts was noted. No changes in appetite or other behaviors were noted during the study period. Since no clinical events occurred during the study period, additional clinical chemistry parameters (e.g. liver enzymes and renal function) were not performed.

[01048] **Plasma, Pharmacokinetic and Clotting Analysis**

[01049] The pharmacokinetics data is presented in **Tables 99** and **100** below.

[01050] **Figure 91** and **Figure 92** present the mean MOD-5014 plasma concentration and activity over time following IV infusion. Plasma concentrations show an initial, relatively fast decline over the first 8 to 12 hours followed by a slower decline. Activity declines over time falling below the lower limit of the assay (12.6 mU/mL) after about 32 hours in the 50 μg/kg group and beyond 48 hours in the 200 and 400 μg/kg groups. Activity data from the 600 μg/kg dose group at 72 hours and beyond are excluded from the analysis because the results were increasing with time and not consistent between the two animals.

[01051] **Figures 93(A-B) through Figures 96(A-B)** plot the concentration and activity together for each dog.

[01052] In the 50 and 200 μg/kg dose groups (**Figures 93(A-B)** and **94(A-D)**), concentration and activity followed similar patterns through 8 to 12 hours after infusion and then concentrations tended to level off while activity continued to decline until it dropped below the level of the assay. In the 400 and 600 μg/kg groups (**Figures 95(A-B)** and **96(A-B)**), activity appeared to drop somewhat faster than concentration starting immediately after infusion. Concentration curves tended to level off while activity curves declined faster until they dropped below the assay limit or, in the case of the 600 μg/kg group, were excluded from analysis.

[01053] Results of non-compartmental analysis are shown in **Table 99** and **Table 100** for concentration and activity data, respectively. Results of non-compartmental analysis indicated that AUC_{0-t} was nearly as large as $AUC_{0-\infty}$ in all cases indicating that the duration of sampling was adequate to describe the pharmacokinetic and pharmacodynamic profiles. **Figures 97(A-J)** and **Figures 98(A-J)** show the concentration and activity versus time for each dog. The estimated terminal slope is represented on these graphs by a solid line. Each graph also contains information related to the estimation of the terminal slope including the coefficient

of determination (Rsq and Rsq_adjusted), the number of points used to estimate the slope, and the terminal $t_{1/2}$ (HL_Lambda_z).

[01054] With one exception (in the 200 µg/kg group) plasma concentration and activity levels both were highest at the first time point measured (0.25 hr). Both concentration and activity were dose related and approximately dose proportional as indicated by the C_{max}/Dose and CL parameters estimates across dose groups. By both concentration and activity measures, CL appeared to be somewhat faster at the lowest dose. At the lowest dose, concentrations and activity dropped below the assay limits after 32 hours, possibly limiting the full characterization of the pharmacokinetics at this dose. CL was similar based on concentration measurements compared to activity measurements. The $t_{1/2}$ estimated based on concentrations were much longer after the 400 and 600 µg/kg doses (>40 hours) compared to the 200 µg/kg group (19.3 hours) and the 50 µg/kg group (7.76 hours). Terminal $t_{1/2}$ based on activity was approximately 3 to 5 hours and similar across dose groups.

[01055] Estimates of AUC and CL based on the models agreed very well with those derived from non-compartmental analysis. The apparent volume of distribution derived from the models was approximately 90 to 120 mL/kg (larger in the low dose group) based on plasma concentrations and 40 to 70 mL/kg based on activity. The $t_{1/2\beta}$ estimated by the models for both plasma and activity measurements were very similar to the terminal $t_{1/2}$ estimated by non-compartmental analysis.

[01056] MRT is an estimate of the time an individual drug molecule is in circulation. MRT estimates were consistent across dose groups based on activity, approximately 4 to 7 hours. Based on concentration, MRT was longer at the higher doses (approximately 25 hours) compared to the 200 and 50 µg/kg groups (16.6 and 11.7 hours, respectively).

[01057] Parameters calculated for concentration and activity data by non-compartmental analysis are summarized in the tables below. With the exception of the 50 µg/kg dose group in which the characterization of the pharmacokinetics may have been limited by assay sensitivity, plasma clearance was similar across dose groups when measured both by plasma concentration and by activity.

[01058] **Table 99 - Mean MOD-5014 Pharmacokinetic Parameters Based on Concentration Measurements Following IV Infusion in Dogs Estimated by Non-compartmental Analysis**

Dose (µg/kg)	C_{max} (ng/mL)	T_{max} (hr)	$AUC_{0-\infty}$ (hr*ng/mL)	CL (mL/hr/kg)	$T_{1/2}$ (hr)
50	371	0.250	2230	22.8	7.76
200	2210	0.313	15000	13.5	19.3

400	4380	0.250	38100	10.7	47.6
600	5280	0.250	47000	12.8	45.8

[01059] **Table 100 – Mean MOD-5014 Pharmacokinetic Parameters Based on Activity Following IV Infusion in Dogs Estimated by Non-compartmental Analysis**

Dose (µg/kg)	**Dose Units/kg**	**C_{max} (mU/mL)**	**T_{max} (hr)**	**$AUC_{0-\infty}$ (hr*mU/mL)**	**CL (mL/hr/kg)**	**$T_{1/2}$ (hr)**
50	778.15	10800	0.250	29900	26.2	3.34
200	3,112.6	52100	0.250	172000	18.2	4.72
400	6,225.2	104000	0.250	404000	15.6	4.72
600	9,337.8	155000	0.250	700000	13.4	5.22

[01060] It was previously reported that, following administration of rhFVIIa to hemophilic dogs, its half-life based on antigen FVIIa level was 3hr, and based on activity 1.8hr (Knudsen et al., 2011, summarized below in **Table 101**).

[01061] **Table 101**

NovoSeven® (270µg/kg) Knudsen et al. 2011	**Concentration Measurements**		**Activity Measurements**	
	CL (ml/hr/kg)	$T_{1/2}$ (hr)	CL (ml/hr/kg)	$T_{1/2}$ (hr)
	24.5	3	46.1	1.8

[01062] The attachment of CTP to FVIIa significantly increased the half-life to 19.3-45 hr and 4.72-5.22 based on antigen and activity level, respectively, thus extending product half-life by an average of 3-5-fold compared to reported data. Clearance was affected in a similar manner, and was significantly reduced by 2-3-fold as compared to Knudsen et al. 2011.

[01063] **Whole Blood Clotting Time (WBCT) Analysis**

[01064] WBCTs were tested after collection by FOBRL at pre-dosing (i.e. baseline), 15 min, 1, 2, 4, 6, 8, 12, 24, 48, with the aim to monitor it until baseline values were achieved. As presented in **Table 102**, the average pre-dose WBCT time of all animals was actually at the lower range of hemophilic dogs anticipated WBCT (50-35min).

[01065] **Table 102 –WBCT Analysis**

Time (hr)	Blondie 200ug/kg	Blondie 400ug/kg	Joanie 600ug/kg	Josie 200ug/kg	Josie 400ug/kg	N05 600ug/kg	N06 50ug/kg	N06 200ug/kg	P14 50ug/kg	P14 200ug/kg
0	31	28	30	32.5	39.5	39.5	37	30.5	45	40.5
0.25	24.5	24	27	30	31	29.5	31	30	35.5	38
1	15.5	25	29.5	29	28	30.5	31.5	26.5	35.5	19
2	24	26	30.5	34	26.5	31	35	28	38.5	38
4	27	28	31	36	32	29.5	35.5	28	36	42
6	27	26	32	31.5	34	31	33.5	31	49	38.5
8	32.5	26.5	31.5	35.5	31	39.5	30.5	29.5	40	38
12	31	29	37.5	38.5	37	30	18	31	34.5	33
24	28.5	34	41	34	42	39.5	30.5	32.5	38	47
48	28	34	31	39.5	42	29	30.5	27.5	40.5	27.5
72	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
96	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
168	n/a	31	28	n/a	30	30.5	n/a	32	n/a	31
336	n/a	31.5	n/a	n/a	29	n/a	n/a	33.5	n/a	40

[01066] **MOD-5014 Characterization in Hemophilic Dogs by whole blood Kaolin-Thromboelastography (WB Kaolin-TEG)**

[01067] Kaolin-TEG parameters were monitored at designated time points post MOD-5014 administration for all dogs and at all doses. The objectives of this specific analysis were to characterize the kinetics of TEG with time, to assess the minimal dose in which normalization of the different parameters (R, K, Angle, MA) occurred across different animals, the dose-dependent response within individuals and to finally compare the overall TEG profile following MOD-5014 administration to published data for rhFVIIa at a similar dose range as well as to normal values obtained in healthy dogs (Knudsen et al., 2011). The individual data is provided in **Figures 101 (A-D), 102 (A-D), 103 (A-D), 104 (A-D), 105 (A-D) and 106 (A-D).**

[01068] **MOD-5014 whole blood kaolin-TEG performance following administration at different doses**

[01069] The initial dose of 50µg/kg administered to 2 dogs was unable to significantly improve TEG performance, as reflected by poor R, K, angle and MA values post-administration (animal P14; **Figures 102 (A-D)**), but the majority of the parameters were improved when the dose was increased to 200µg/kg. In the second animal (N06) TEG values were also not improved following administration of 50 µg/kg MOD-5014; this might be a consequence of the partially normalized TEG values at pre-dose, which potentially had a direct effect on the poor post-administration response, although no further improvement was observed post-dosing. Based on the above it was proposed that the 50µg/kg dose is not adequate to support and correct the hemostatic defect in the tested dogs.

[01070] Administration of 200 µg/kg MOD-5014 in the majority of the animals corrected the TEG parameters to values which are still below normal range, yet provided an effect which is significantly more pronounced than the 50ug/kg dose. At a dose of 400µg/kg a slight improvement in TEG values and a prolonged response was obtained (**Figures 102 (A-D); 103 (A-D) and 104 (A-D)**). When dosing two different animals with 600µg/kg, the response in one animal (Joanie; **Figure 105 (A-D)**) was comparable to animals dosed with 400µg/kg. While the K time, Angle and MA values in animal N05 (**Figure 106 (A-D)**) reached the normal range, this might also be a consequence of improved pre-dose values and inter-animal variability. It appears that administration of MOD-5014 at a range of doses enabled partial correction of TEG values, while the optimal minimal effective dose was at the range of 200-400µg/kg of MOD-5014.

[01071] **Intra-animal variability of MOD-5014 whole blood kaolin-TEG dose-dependent response**

[01072] An intra-animal dose-dependent improvement was observed when a higher dose was injected to the same animal. Some variation at different time points was observed, that could reflect normal biological variation (**Figures 101 (A-D), 102 (A-D), 103 (A-D), 104 (A-D), 105 (A-D) and 106 (A-D)**). The improvement was most pronounced when dosing at 200 and 400 µg/kg (Blondie and Josie), as presented in **Figures 101 (A-D), 102 (A-D), 103 (A-D), 104 (A-D), 105 (A-D) and 106 (A-D)**.

[01073] **Reproducibility of MOD-5014 whole blood kaolin-TEG results between individuals (inter-animal variability)**

[01074] As observed in **Figures 101 (A-D), 102 (A-D), 103 (A-D), 104 (A-D), 105 (A-D) and 106 (A-D)**, although animals corrected TEG values following administration of MOD-5014, they demonstrated different profiles through the time of measurement. This might be a result of biological variability, considering the different genetic background of each animal and the slight differences in pre-dose values. The observed variation actually mimics the clinical setting, in which individual TEG values may vary between individuals receiving similar dosing, and may eventually translate to different efficacy and bleeding control capability of FVIIa.

[01075] **MOD-5014 as a long-acting product**

[01076] Comparison of the correction capability of MOD-5014 and rhFVIIa suggests that MOD-5014 maximum and minimum values are very similar to the results reported for hFVIIa. However, a marked improvement in the duration of the reaction was observed with MOD-5014. Following hFVIIa administration the majority of the hFVIIa analyzed parameters dropped back to baseline 4 hours post-dosing (**Figures 107(A-D)**), while MOD-5014 enables

an extended effect reaching baseline values 24 hours post-dosing and provides a more sustained and prolonged response (**Figures 108(A-D)**; marked with black arrows). The data suggests that the duration of the TEG response correlates to FVIIa short PK-PD profile in hemophilic dogs, which was further investigated, subsequently confirming the extended response following MOD-5014 administration.

[01077] **Activated Partial Thromboplastin Time (aPTT) performance**

[01078] Using the Triniclot reagent as activator and a 60 second incubation, the aPTT values for canine hemophilia A plasma were 65.6 seconds (data not shown). Following MOD-5014 administration, aPTT was reduced in a dose-dependent manner down to 40 sec. When comparing this information to previously reported data, it was found to be very similar to the values obtained pre- and post-administration of 193µg/kg rhFVIIa to a FVIII-deficient dog (Brinkhous et al., 1989), while a retrospective analysis suggests a prolonged effect when administrating 200µg/kg MOD-5014, with a subsequent return to baseline aPTT values 24 hours post-dosing.

[01079] *Conclusions:* In this study, the safety, PK, PD and hemophilic coagulopathy correction ability of a novel, long-acting FVIIa (MOD-5014) was assessed in FVIII-deficient dogs. MOD-5014 was administered in relevant clinical doses, while the potential minimal effective dose in the dogs was initially established based on an *ex vivo* study.

[01080] MOD-5014 was well tolerated by all dogs and no adverse events were observed. Dose-dependent response was observed in both the PK and PD (activation measurement) analyses, further confirming the long-acting properties of MOD-5014 in comparison to published parameters (Knudsen et al. 2011).

[01081] Recombinant human FVIIa has been shown previously to be efficacious in treating hemophilic canine bleeds. A marked improvement of TEG coagulation profile was observed following administration of MOD-5014 at doses of 200-400µg/kg, while a lower dose of 50µg/kg was unable to correct TEG values, suggesting that it is below the minimal effective dose in dogs. A dose-dependent response was observed following MOD-5014 administration, while inter-individual variation was higher than expected and might be a consequence of biological variability. When comparing MOD-5014 performance to published data, MOD-5014 had a pronounced and prolonged effect at lower doses (270 and 200 µg/kg, respectively). MOD-5014 *in vivo* activity was also reflected by a reduction in aPTT values, further confirming a sustained activation of the coagulation system.

[01082] In general, testing MOD-5014 in hemophilic dogs offers several advantages over rodent models such as mice and rats. Hemophilic dogs exhibit a disease phenotype closely recapitulating that of humans; moreover, dogs are more comparable with humans in regard to

body weight as well as to FVIIa dosing requirements and to pharmacokinetic characteristics.

[01083] Overall, this study further confirmed the longevity of MOD-5014 in a relevant, well-established model previously shown to provide an accurate recapitulation of human biology, specifically with respect to hemostasis. The data obtained in this study have significant value and provide the first evidence that MOD-5014 is a safe and effective long-acting FVIIa in large animals that can be used potentially as an agent for both prophylactic and on-demand treatment of hemophilic patients with inhibitors. Thus, MOD-5014 has a tremendous potential to significantly benefit patients by reducing the frequency of administration and enabling prophylactic use.

EXAMPLE 15: COMPARITIVE EVALUATION OF MOD-5014 AND RECOMBINANT FVIIa THROMBOGENIC POTENTIAL FOLLOWING SINGLE ADMINSTRATION IN THE WESSLER RABBIT MODEL

[01084] **Objective:** The objective of this study was to evaluate MOD-5014, which is intended for the treatment of patients with Hemophilia A or B with inhibitors to FVIII or FIX, respectively. Another aim of this study was to evaluate MOD-5014 for on demand treatment of spontaneous bleedings (e.g. joint bleeding) with the aim of less frequent administrations, as well as for prophylactic use with an anticipated dosing regimen of two to three times a week which may significantly improve patients' clinical condition and quality of life.

[01085] A further objective of this study was to evaluate the thrombogenic potential of the test item MOD-5014 using a semi-quantitative method described by Wessler *et al* (1959) Serum-induced thrombosis. Studies of its induction, and evolution under controlled conditions in vivo. *Circulation* Nov; 20:864-74; and Wesslet et al (1959) Biologic assay of a thrombosis inducing activity in human serum. *Journal of Applied Physiology* 14, 943-946. Comparisons were made with a negative control group and a positive control group of recombinant FVIIa NovoSeven® at a dose level of 0.1 and 0.3 mg/kg.

[01086] This study was conducted in anaesthetised rabbits.

[01087] **Justification:** The purpose of this study was to assess the thrombogenic potential of MOD-5014. The Wessler rabbit model is a classical model for assessing venous thrombosis. The sensitivity of this model is based on 1) the high endogenous rabbit haemostasis potential with a higher coagulation factor level than humans and 2) that venous stasis provokes a tremendous tissue damage providing a generally massive predisposition for thrombus formation, if exposed to activated factors (Wessler *et* al (1959), Karges *et* al (1994) Activity of coagulation and fibrinolysis parameters in animals. Arzneimittelforschung. Jun;

44(6):793-7.) Novoseven was used as a positive control based on a previous study which explored the thrombogenicity of recombinant human FVIIa in a rabbit stasis model (Diness *et* al (1992) Recombinant human factor VIIa (rFVIIa) in a rabbit stasis model. *Thromb Res* 67(2), 233-41).

[01088] **Study Outline:** The negative control, positive control and MOD-5014 were administered once intravenously; this being the clinical route of administration.

[01089] **Dose Selection Rational:** The dose levels were selected on the basis of available data from a rabbit stasis study that was conducted on Novoseven (Diness et al (1992) Recombinant human factor VIIa (rFVIIa) in a rabbit stasis model. *Thromb Res* 67(2), 233-41) and in consideration of MOD-5014 anticipated doses in the Phase 1-2a clinical study. The low dose is the formal low clinical dose on a µg/kg basis and was approved by the FDA to use as one of the low doses (50 µg/kg) in the Phase 1-2a study (See Example 16 below). The higher dose is 400 µg/kg, the anticipated highest clinical dose on a µg/kg basis in the Phase 1-2a clinical study.

Materials and Methods

[01090] **Test Items: Description, Identification and Storage**

[01091] The test item was identified as MOD-5014, ER batch 1017295 (expiry date: April 2015) When not in use the test article was stored in a sealed container.

[01092] Storage Conditions: Frozen (-60 to -90°C).

[01093] The negative control was the vehicle for the test article which was identified as 20mM Citrate 150mM NaCl 13.3mM Glycine pH 6.4.

[01094] Storage Conditions: Refrigerated (2 to 8°C).

[01095] The positive control item was identified as NovoSeven.

[01096] Name: NovoSeven (refereed as MOD-5000)

[01097] Batch No.: 050115

[01098] Description/Appearance: Frozen, clear colourless liquid

[01099] Expiry date of powder vial: August 2016

[01100] Storage conditions: Frozen (-60 to -90°C)

[01101] The anaesthetic agent was identified as Ketamine and Xylazine.

[01102] When not in use the anaesthetic agent was stored in a sealed container, at room temperature (nominally 15 to 25°C) and protected from light.

[01103] **TEST ITEM (MOD-5014) AND POSITIVE CONTROL FORMULATION**

[01104] **Preparation**

[01105] The test article was thawed for 20-30 minutes on ice. On each day, prior to

use, 4.8 ml of formulation buffer was added to 3ml of the test article (2.6 mg/ml) to final concentration of 1 mg/ml. Different doses were achieved by altering the dose volume.

[01106] NovoSeven was thawed for 20-30 minutes on ice, and was used as received. Different doses were achieved by altering the dose volume.

[01107] **Anaesthetic agents**

[01108] The anesthetic agents were identified as Ketamine and Xylazine. When not in use the anesthetic agent were stored in a sealed container, at room temperature (nominally 15 to 25°C) and protected from light.

[01109] **Test animals**

[01110] A total of 40 male Hsdlf:(NZW) (New Zealand White) rabbits were obtained from Harlan UK The rabbits weighed between 2.45 to 3.40 kg on the day of dosing.

[01111] **EXPERIMENTAL DESIGN**

[01112] Food and water were freely available, except when the animals were removed from the home cage for the study procedures.

[01113] Anaesthesia was induced and maintained by an intramuscular injection of Ketamine (40 mg/kg) plus Xylazine (5 mg/kg). Following induction of anaesthesia, the rabbits were placed on a heated blanket. Temperature was monitored with a rectal probe and maintained within acceptable limits (nominally 35 to 39°C).

[01114] The neck was shaved ventrally to facilitate bilateral dissection of the jugular veins. Once the veins had been located, a distal and a proximal ligature were placed at a distance of between 1 and 1.5 cm, but not tightened.

[01115] Test item, positive control or vehicle were injected into the contralateral (to the first jugular being ligated) ear vein using a volume adapted to the dose (see Table below).

[01116] Treatments employed for the study are shown in **Table 103** below:

[01117] **Table 103**

Group	Treatment	Dose level (mg/kg)	Dose volume (mL/kg)	Dose concentration (mg/ml)	Animal Numbers
1	Negative Control	-	0.15	-	5
2	MOD-5014	0.05	0.05	1.0	5
3	MOD-5014	0.1	0.10	1.0	5
4	MOD-5014	0.2	0.20	1.0	5
5	MOD-5014	0.3	0.30	1.0	5
6	MOD-5014	0.4	0.40	1.0	5
7	NovoSeven	0.1	0.11	0.943	5
8	NovoSeven	0.3	0.32	0.943	5

[01118] Immediately following dose administration the proximal end of the appropriate jugular vein was isolated from the circulation by tightening the ligature. This was then repeated on the remaining jugular vein. After 25 seconds both distal ligatures were tightened. Ten minutes after tightening one vein segment (this was kept the same for all animals) was carefully removed from the animal, transferred to a Petri dish containing 3.8 % sodium citrate solution and cut open. The formation of thrombi was evaluated as described below in Table 104. This procedure was then repeated on the remaining jugular vein 30 minutes after tightening.

[01119] **Table 104**

Degree of thrombus formation	Score
Liquid blood without thrombi	0
Few small thrombi	0.5 to 1
Several medium-sized thrombi or many small thrombi	2
A greater number of medium-sized thrombi	3
Few larger thrombi	3.5
One larger thrombus	4

[01120] **STATISTICAL ANALYSIS**

[01121] Thrombus formation scores after 10 and 30 minutes were provided for analysis. Data at each time-point were analysed separately.

The following comparisons of interest were made using a Wilcoxon Rank Sum test:

Group 1 vs. Groups 2 to 8
Group 3 vs. Group 7
Group 5 vs. Group 8

Results

[01122] The results are summarised in **Table 105**, individual animal data are presented in **Table 106**.

[01123] **Table 105:** Summary of the thrombogenicity assessment following intravenous administration of MOD-5014 and NovoSeven

Group	Treatment and dose level (mg/kg)	Group mean score(± sd)	
		10 minutes	30 minutes
1	Negative Control	0.0 ±0.00	0.2 ±0.27

2	MOD-5014 (0.05)	0.1 ±0.22	3.4 ±0.82
3	MOD-5014 (0.1)	0.1 ±0.22	3.5 ±0.87
4	MOD-5014 (0.2)	0.2 ±0.45	3.8 ±0.45
5	MOD-5014 (0.3)	0.6 ±0.89	4.0 ±0.00
6	MOD-5014 (0.4)	0.3 ±0.45	3.6 ±0.89
7	NovoSeven (0.1)	0.1 ±0.22	3.5 ±0.87
8	NovoSeven (0.3)	0.5 ±0.87	3.8 ±0.45

sd – standard deviation

n = 5

[01124] **Table 106:** Thrombogenicity assessment following intravenous administration of MOD-5014 and NovoSeven – individual animal data

Group	Treatment and dose level (mg/kg)	Animal ID	Thrombus formation score after 10 minutes	30 minutes
1	Negative Control	1	0	0.5
		2	0	0
		3	0	0
		4	0	0.5
		5	0	0
2	MOD-5014 (0.05)	6	0	3.5
		7	0	2
		8	0	3.5^
		9	0.5	4
		10	0	4
3	MOD-5014 (0.1)	11	0	3.5
		12	0	4
		13	0	4
		14	0	4
		15	0.5	2
4	MOD-5014 (0.2)	16	0	4
		17	1	3
		18	0	4
		19	0	4
		20	0	4

5	MOD-5014 (0.3)	21	1	4
		22	0	4
		23	2	4
		24	0	4
		25	0	4
6	MOD-5014 (0.4)	26	0	4
		27	0	2
		28	1	4
		29	0.5	4*
		30	0	4
7	NovoSeven (0.1)	31	0.5	2
		32	0	4
		33	0	3.5
		34	0	4
		35	0	4
8	NovoSeven (0.3)	36	0	4
		37	0.5	3
		38	2	4
		39	0	4
		40	0	4

Vein segment removed after ^23 minutes and *26 minutes due to light anaesthesia

[01125] **Wessler score**

[01126] Intravenous administration of the negative control produced a Wessler score of 0.0 after 10 minutes. The score ranged from 0.0 to 0.5 with a mean score of 0.2 ± 0.27 after 30 minutes.

[01127] Intravenous administration of MOD-5014 at a dose level of 0.05 mg/kg produced Wessler scores in the range of 0.0 to 0.5 with a group mean score of 0.1 ± 0.22 after 10 minutes. The score ranged from 2.0 to 3.5 with a mean score of 3.4 ± 0.82 after 30 minutes.

[01128] Intravenous administration of MOD-5014 at a dose level of 0.1 mg/kg produced Wessler scores in the range of 0.0 to 0.5 with a group mean score of 0.1 ± 0.22 after 10 minutes. The score ranged from 2.0 to 4.0 with a mean score of 3.5 ± 0.87 after 30 minutes.

[01129] Intravenous administration of MOD-5014 at a dose level of 0.2 mg/kg produced Wessler scores in the range of 0.0 to 1.0 with a group mean score of 0.2 ± 0.45 after 10 minutes. The score ranged from 3.0 to 4.0 with a mean score of 3.8 ± 0.45 after 30 minutes.

[01130] Intravenous administration of MOD-5014 at a dose level of 0.3 mg/kg produced Wessler scores in the range of 0.0 to 2.0 with a group mean score of 0.6 ± 0.89 after 10 minutes. After 30 minutes all scores were 4.0.

[01131] Intravenous administration of MOD-5014 at a dose level of 0.4 mg/kg produced Wessler scores in the range of 0.0 to 1.0 with a group mean score of 0.3 ± 0.45 after 10 minutes. The score ranged from 2.0 to 4.0 with a mean score of 3.6 ± 0.89 after 30 minutes.

[01132] Intravenous administration of NovoSeven at a dose level of 0.1 mg/kg produced Wessler scores in the range of 0.0 to 0.5 with a group mean score of 0.1 ± 0.22 after 10 minutes. The score ranged from 2.0 to 4.0 with a mean score of 3.5 ± 0.87 after 30 minutes.

[01133] Intravenous administration of NovoSeven at a dose level of 0.3 mg/kg produced Wessler scores in the range of 0.0 to 2.0 with a group mean score of 0.5 ± 0.87 after 10 minutes. The score ranged from 3.0 to 4.0 with a mean score of 3.8 ± 0.45 after 30 minutes.

[01134] Statistical Analysis Results, **Table 107**

Table 107

Variable	Pairwise comparison	Method	P-value
Thrombus formation score after 10 minutes	Group 1M vs. Group 2M	W	1.0000
	Group 1M vs. Group 3M	W	1.0000
	Group 1M vs. Group 4M	W	1.0000
	Group 1M vs. Group 5M	W	0.4444
	Group 1M vs. Group 6M	W	0.4444
	Group 1M vs. Group 7M	W	1.0000
	Group 1M vs. Group 8M	W	0.4444
	Group 3M vs. Group 7M	W	1.0000
	Group 5M vs. Group 8M	W	1.0000
Thrombus formation score after 30 minutes	Group 1M vs. Group 2M	W	0.0079**
	Group 1M vs. Group 3M	W	0.0079**
	Group 1M vs. Group 4M	W	0.0079**
	Group 1M vs. Group 5M	W	0.0079**
	Group 1M vs. Group 6M	W	0.0079**
	Group 1M vs. Group 7M	W	0.0079**
	Group 1M vs. Group 8M	W	0.0079**
	Group 3M vs. Group 7M	W	1.0000
	Group 5M vs. Group 8M	W	1.0000

* P≤0.05
** P≤0.01
*** P≤0.001

W = Wilcoxon Rank Sum test

[01135] The statistical analysis shows that thrombus formation after 10 minutes is comparable for all groups. However, the analysis shows that after 30 minutes administration of MOD-5014 at all dose levels produced a statistical significant effect on thrombus

formation when compared to the negative control.

[01136] Administration of MOD-5014 at a dose level of 0.1 and 0.3 mg/kg did not produce a statistically significant difference on thrombus formation when compared to NovoSeven at the same dose levels.

[01137] **Conclusion**

[01138] Administration of MOD-5014 at a dose level of 0.1 and 0.3 mg/kg and NovoSeven at the same dose range in the anaesthetised rabbit produced Wessler scores that were comparable and thus similar thrombogenic potential.

EXAMPLE 16: A PHASE 1/2A, OPEN-LABEL, MULTICENTER, DOSE ESCALATION STUDY TO ASSESS THE SAFETY, PHARMACOKINETICS AND PHARMACODYNAMICS PROFILE OF A LONG-ACTING RECOMBINANT FACTOR VIIA (MOD-5014) IN ADULT MEN WITH HEMOPHILIA A OR B

[01139] **Objectives:**

[01140] Primary: To assess the acute safety and tolerability of single intravenous (IV) administration of escalating MOD-5014 doses in hemophilic subjects with and without inhibitors.

[01141] Secondary: To evaluate the pharmacokinetic profile of single IV administration of escalating MOD-5014 doses in hemophilic subjects with and without inhibitors.

[01142] Exploratory: To evaluate the pharmacodynamic response of single IV administration of escalating MOD-5014 doses in hemophilic subjects with and without inhibitors.

[01143] **Study Design and Procedures**

[01144] This will be a single-dose, open label, dose-escalating study. The study will be performed in two stages, each stage will be similar in design (visits and assessments):

[01145] **Stage 1:** This stage will include three escalating dose groups, with four subjects in each dose group. The initial MOD-5014 dose will be 25 μg/kg followed by doses of 50 and 100, μg/kg. The first subject in each dose cohort will receive a single IV injection followed by a 72 hr safety observation period before dosing the remaining three subjects in that cohort. Dosing of the last subject in each cohort will be followed by a 7, 14 and 30-day safety observation period. The Stage 1 data will be submitted to the FDA prior to initiation of Stage 2. Only upon approval to continue from the DSMB and notification to the IRB, will enrolment into Stage 2 be initiated.

[01146] **Stage 2:** This stage will be similar in design to Stage 1 and will include three escalating dose groups to evaluate doses higher than those evaluated in Stage1. The doses to

be evaluated in Stage 2 include 200, 300 and 400 µg/kg of MOD-5014, with 4 subjects in each dose group.

[01147] For each study stage, the decision to proceed to the higher dose level will be made by DSMB after review of relevant safety data (including adverse events, clinical laboratory and vital signs), collected up to and including 7 days post dose of the last subject of the previous dose group.

[01148] Common Terminology Grades for Adverse Events (CTCAE) guidelines will be used to determine maximum tolerated dose (MTD) and dose limiting toxicity (DLT) (**Table 108**). Dose escalation will be permitted if the prior dose is well tolerated, and there are no safety or tolerability concerns over 7 days post-dosing.

[01149] **Stopping Rules and Dose Limiting Toxicities (DLT)**

[01150] DLT is defined as a clinically significant adverse event or abnormal laboratory value assessed as unrelated to disease progression, intercurrent illness or concomitant medications and occurring up to 7 days after dosing day. For example, grade 3 or 4, which is definitely or possibly drug-related. In case a thromoembolic event is reported, the study will be stopped and the DSMB will be requested to further investigate the event and its relationship to MOD-5014 single dose administration. Table 108 Summarizes dose escalation criteria and decision tree.

[01151] **Table 108:** MOD-5014 Related Toxicity and Dose Escalation Criteria Scheme

CTCAE grade	Number of subjects with CTCAE grade out of total number of subjects per dose group	Action
Grade 1 or Grade 2 Toxicity	4/4	Escalate to next dose level
Grade 3 Toxicity	0/4	Escalate to next dose level
	1/4 or 2/4	Add 4 subjects to current dose level
	1/8 to 3/8	Escalate to next dose level
	3/4 or 4/8	DLT; previous dose level is MTD
Grade 4 Toxicity	1/4	DLT; previous dose level is MTD
Throughout the study any thromboembolic events	Adverse thromboembolic complications	Stop study and investigate with the DSMB

[01152] **Study Procedures**

[01153] Each study stage will be comprised of a screening period (28 ± 1 days), a treatment day and a follow-up period (7, 14 and 30 days). For each study stage and dose group, visit scheduling and assessments will be similar.

[01154] **Screening Period (Day -28 to -1) – Visit 1**

[01155] After signing informed consent, adult male subjects aged 18-65 years old, who have been diagnosed with moderate or severe congenital Hemophilia A or B, will be screened for study eligibility by assessment of inclusion and exclusion criteria. Screening procedures will include a collection of demographic data, complete and disease-related medical history, physical examination (including neurological assessments, height and weight), vital signs (blood pressure, pulse rate, oral temperature and respiration rate), ECG, adverse events, concomitant medications and safety laboratory evaluations including hematology, biochemistry, urinalysis, coagulation (prothrombin time (PT) and activated partial thromboplastin time (aPTTa)).

[01156] **Pre-Dosing Day (Day -1)**

[01157] Eligible subjects will be contacted by phone the night before dosing to confirm that: Subjects have not received replacement therapy as described below (**Table 109**). Time of phone call with respect to the time restrictions described below, will be taken into consideration. If they will have a bleeding episode during that night and will administer replacement therapy, they should not come to the clinic the next morning, but call their study contact person.

[01158] **Table 109:** Prohibited Replacement Therapy prior to Dosing

Replacement Therapy	Time prior to study
Factor VII or FVIIa containing product (either plasma derived or recombinant)	within 48 hours prior to study drug administration (when calling ask about last day and a half)
FVIII	within 72 hours prior to study drug administration (when calling ask about last two and a half days)
FIX	within 96 hours prior to study drug administration (when calling ask about last three and a half days)
APCC containing product	within 48 hours prior to study drug administration (when calling ask about last day and a half)

[01159] **Treatment Day (Day 0) – Visit 2**

[01160] ***Pre-Dose***

[01161] At the clinic, the following assessments and procedures will be done prior to

dosing: physical examination (including neurological assessments), vital signs, electrocardiogram (ECG), safety laboratory (hematology, biochemistry and urinalysis), PT, aPTT within 1 hour pre-dosea, immunogenicity, pharmacokinetics (within 1 hour pre-dose)**b**, pharmacodynamics (FVIIa activity via STAClot assay), thrombolastography (within 1 hour pre-dose), thrombin generation (within 1 hour pre-dose), adverse events and concomitant medications.

[01162] ***Dosing***

[01163] Subjects will receive a single intravenous injection based on weight and MOD-5014 dose (defined by dose cohort).

[01164] ***Post-Dose***

[01165] The following assessments and procedures will be performed on Day 0, at post-dosing time points as indicated:

- Vital signs (blood pressure, pulse, respiratory rate, temperature) at 1 ± 0.5 hour, 4 ± 0.5 hours and 8 hours ± 0.5 hours
- ECG at 1 ± 0.5 hour, 4 ± 0.5 hours and 8 ± 0.5 hours
- Safety laboratory (hematology, biochemistry and urinalysis) assessments at 4 ± 0.5 hours
- Coagulation panel at 30 ± 5 minutes, 2 hours ±20 minutes, 4 ± 0.5 hours and 8 ± 1 hours
- Pharmacokinetics at 30 ± 5minutes, 2 hours ± 20 minutes, 4 ± 0.5 hours and 8 ± 1 hours
- Pharmacodynamics at 30 ± 5minutes, 2 hours ± 20 minutes, 4 ± 0.5 hours and 8 ± 1 hours. Assays include:
    - FVIIa activity via STAClot assay
    - Thrombolastography (optional)
    - Thrombin generation
- Concomitant medications
- Local skin reaction at administration site at 10 minutes and 2 hours ± 20 minutes following the end of the IV administration
- Adverse events

[01166] **Follow-Up (FU) Period (Visits 3 to 8, Day 1 to 30)**

[01167] The follow-up period will start the day after dosing. Subjects are followed for 30 days post-dose and undergo six follow-up visits as outlined below. At FU Visit 3 / Day 1 (24 hours), FU Visit 4 / Day 2 (48 hours) and FU Visit 5/ Day 3 (72 hours) the following are performed: physical examination (including neurological assessments) (only visit 3 and 5),

vital signs, ECG, safety laboratory (hematology, biochemistry and urinalysis), coagulation panel (single blood draw), pharmacokinetics (single blood draw), pharmacodynamics (single blood draw), thrombolastography, thrombin generation, adverse events, local skin reactions and concomitant medications. If rescue medication was administered within the first 72 hours, only FU Visit 5 is performed.

[01168] The following study procedures will be done at FU Visit 6 / Day 7 if no rescue treatment for bleeding episodes was administered: physical examination (including neurological assessments), vital signs, ECG, safety laboratory (hematology, biochemistry and urinalysis), coagulation panel, pharmacokinetics, pharmacodynamics, thrombolastography, thrombin generation, adverse events and concomitant medications. In the event that a bleeding episode occurred after the first 72 hours and rescue treatment was administered, a telephone questionnaire on patient clinical well being is completed.

[01169] ***FU Visits 7-8, Days 14 and 30 (Termination Visit)***

[01170] The following study procedures will be done on these visits: immunogenicity, anti HCP antibodies (only visit 8), adverse events and concomitant medications.

[01171] **Study Duration**

[01172] Study duration for each participating subject is up to 58 days as follows:

- Screening period: -28 to -1 days before drug administration
- Treatment: 1 day single-dose administration
- Follow up: at least 72 hours acute safety, 7 days safety, and 14, and 30 days immunogenicity

[01173] **Sample Size and Target Population**

[01174] Twenty-four (24) adult male subjects (age 18-65 years old), 12 subjects in each study phase, diagnosed with moderate or severe congenital Hemophilia A or B. Each dose group include 4 subjects.

[01175] **Inclusion Criteria**

[01176] Subjects must meet all inclusion criteria to be eligible for the study:

1. Men, 18-65 years of age, inclusive, at the screening visit.
2. Diagnosis of moderate or severe congenital Hemophilia A or B with or without inhibitors, defined as less than or equal to 3% normal FVIII or FIX levels, respectively.
3. Body Mass Index (BMI) less than 35.0 kg/m2.
4. Adequate venous access.
5. Fertile men must agree to use a barrier contraceptive (condom) for 90 days post-dosing and are restricted from donating sperm during the study and for 90 days after dosing.

[01177] **Investigational Product Route and Dosage Form**

[01178] MOD-5014, a long-acting modified recombinant Factor VIIa, are provided in glass vials containing a clear solution of 1 mg/mL MOD-5014 in citrate buffer.

[01179] The frozen vials will be thawed on the day of administration at room temperature 2 hours prior to dose preparation. The thawed vials are pulled into one or more syringes (depending on IP quantity for injection). Detailed instructions for handling and administration are provided in an "Instructions for use" document.

[01180] Six dose groups are planned (three in each stage); subjects in each stage and dose group receive a single administration of MOD-5014 as an IV injection based on subject body weight (see Table 110 for an example of doses to be administered)

[01181] **Table 110: Example MOD-5014 Doses and Calculated Injection Duration**

Stage	Dose group	Dose of MOD-5014	Vol injection based on Subject weight[a]				Duration of injection
			70 kg	80 kg	90 kg	100 kg	
1	1	25 µg/kg	1.8 ml	2 ml	2.3 ml	2.5 ml	Up to 1 min
	2	50 µg/kg	3.5 ml	4 ml	4.5 ml	5 ml	Up to 1 min
	3	100 µg/kg	7 ml	8 ml	9 ml	10 ml	Up to 2 min
2	4	200 µg/kg	14 ml	16 ml	18 ml	20 ml	Up to 4 min
	5	300 µg/kg	21 ml	24 ml	27 ml	30 ml	Up to 6 min
	6	400 µg/kg	28 ml	32 ml	36 ml	40ml	Up to 8 min

[a]Maximal injection rate is 10 mL/min

[01182] **Rescue Medication**

[01183] In the event a subject experiences a bleeding episode during the study period (within the 30 days post dose assessments), he is treated according to hospital common practices and his clinical condition.

[01184] **Safety Endpoints (Primary)**

[01185] The primary safety endpoint will consist of monitoring and recording:

1. Adverse events and concomitant medication use throughout the study
2. Immunogenicity, no occurrence of neutralizing antibodies
3. ECG, vital signs, physical examination (including neurological assessments).
4. Laboratory parameters, including serum chemistry profile, liver enzymes, hematology, coagulation panel (PT time, aPTT, thrombin& anti-thrombin complex, prothrombin fragment 1+2, D-dimer), and urinalysis
5. Local tolerability (Injection site reaction)

[01186] **Pharmacokinetic (PK) Endpoints (Secondary)**

[01187] PK response are evaluated following a single injection by measuring MOD-5014 plasma concentration, as measured by a specific ELISA assay. The following

parameters are calculated: Cmax, AUC, terminal half-life, Vss, clearance, recovery and Tmax.

Cmax – maximum MOD-5014 plasma concentration

Tmax – time to Cmax

Tlag – absorption lag-time for MOD-5014

AUC – area under the curve to the final concentration ≥ limit of quantitation(LOQ), AUC(0-t) and to infinity AUCinf

λz – elimination rate constant

$t_{1/2}$ – half-life

Vss – volume steady state, clearance and recovery

[01188] PK sampling times are on Day 0 (within 1 hour pre-dose). All post-injection measurements will be initiated at the end of the IV administration: 30 ± 5 mins, 2 hours ± 20 minutes, 4 ± 0.5 hours 8 ± 1 hours, 24 ± 2 hours (Day 1), 48 ± 3 hours (Day 2), 72 ± 3 hours (Day 3) and at Day 7 post-dose.

[01189] **Pharmacodynamic (PD) Endpoints (Exploratory)**

[01190] Pharmacodynamics are assessed by FVIIa activity via STAClot assay. PD is measured at the following time points: Day 0 (within 1 hour pre-dose). All post-injection measurements are initiated at the end of the IV administration: 30 ± 5 mins, 2 hours ± 20 minutes, 4 ± 0.5 hours 8 ± 1 hours, 24 ± 2 hours (Day 1), 48 ± 3 hours (Day 2), 72 ± 3 hours (Day 3) and at Day 7 post-dose. Bioanalysis is performed.

[01191] **Statistical Methods**

[01192] Descriptive statistics are used to summarize demographics, baseline characteristics, safety and PK/PD data, including sample size (n), mean and standard deviation.

[01193] A data review for PK, PD and safety data for each dose group in Stage 1 is conducted.

EXAMPLE 17: TOXICOLOGY STUDIES
COMPREHENSIVE ASSESSMENT OF THE SAFETY AND EFFICACY OF FACTOR VIIA-CTP SUPPORTING PHASE 1/2A STUDY

[01194] **Objective**

[01195] The objective of these studies was to assess the safety, PK and PD of FVIIa-CTP following administration to rats and monkeys as part of toxicological studies supporting

the ongoing phase 1/2a study in hemophilic patients.

[01196] **Methods**

[01197] FVII-CTP was expressed in CHO cells, purified and activated utilizing a CTP specific purification process.

[01198] GMP batch was used in the toxicological studies assessed in male rats and monkeys supported by toxicokinetic analysis confirming proper margins above the initial clinical dose in the FIH study.

[01199] **Studies** (A summary of the studies performed is presented below in **Table 111**)

[01200] **Acute Toxicokinetic *GLP study* of MOD-5014: A Single Intravenous Dose PK/PD and Toxicity Study in Male Cynomolgus Monkeys:**

[01201] The study in male Cynomolgus monkeys was a GLP-compliant single-dose toxicology study to evaluate and characterize the acute toxicity, estimate the maximum tolerated dose (MTD), and evaluate the toxicokinetics of the MOD-5014 in male cynomolgus monkeys.

[01202] *Study Design*

[01203] Twenty-six male cynomolgus monkeys (2 to 4 kg), approximately2 to 4 years of age, were obtained from Covance Reseach Products, Inc. Twenty-four were assigned to treatment groups according to Table 1.

[01204] *Methods*

[01205] Animals received a single, IV slow bolus (2 to 3 minutes) saphenous vein injection of MOD-5014 (Lot No. ER 1017295, 2.6 mg/mL). Blood samples were collected from the femoral vein in tubes containing citrate buffer. Samples were collected from all animals prior to dosing and at 0.25, 0.5, 2, 4, 6, 8, 12, and 24. Additional samples were collected from recovery animals at 48, 60 and 72 hours after dosing. All samples were collected from control group animals (Group 1) but only 0.5 hr samples were assayed. Plasma MOD-5014 concentrations were determined by a validated ELISA (Bioanalytical validation report AR5437) at Intertek Pharmaceutical Services (San Diego, CA). Activity of MOD-5014 was determined in a qualified clotting assay (STAGO) (Qualification report CD-05-0276) at OPKO Biologics, Israel.

[01206] *Data Analysis*

[01207] Non-compartmental analysis was performed on data from individual animals with Phoenix WinNonlin version 6.3 (Pharsight Corporation, Sunnyvale, CA.). Analysis was performed on MOD-5014 plasma concentration data as well as on clotting activity data assuming an initial specific activity of 15,563 units/mg (MOD-5014 ER#1017295 Release

SUM). A program for IV bolus administration was used.

[01208] For plasma concentration analysis, the area under the curve from time zero to 24 hours (AUC_{0-24h}) was estimated using the trapezoidal method. The maximum concentration (C_{max}) and the time it was observed (T_{max}) were determined directly from the data. The plasma concentration at time zero (C_0) was estimated by back extrapolation through the first two time points. The initial apparent volume of distribution (Vi) was calculated from the following equation:

[01209] $Vi = Dose / C_0$

[01210] For animals in the recovery groups, additional data were available for 48, 60 and 72 hr time points. The profile of these additional time points demonstrated a flatter terminal phase that was not seen in the profiles of animals terminated at 24 hours. Therefore, terminal slopes and related parameters were only estimated from recovery animals and not from those terminated at 24 hours. Even though this means that terminal slopes and related parameters were estimated from only 2 animals per group, the resulting estimates still describe the PK profiles better than estimates based on just 24 hour data.

[01211] For recovery animals, log-linear regression over the last three time points was used to estimate the elimination rate constant (λ) which was used to estimate terminal half-life ($t_{1/2}$) and AUC from zero to infinity ($AUC_{0-\infty}$) from the following equations:

[01212] $t_{1/2} = \ln(2)/\lambda$

[01213] $AUC_{0-\infty} = AUC_{0-24h} + C_{24h}/\lambda$

[01214] where C_{24h} is the concentration at 24 hr. Serum clearance (CL) was calculated from dose divided by $AUC_{0-\infty}$. The volume in the terminal phase (Vz) and at steady state (Vss) were calculated from the following equations:

[01215] $Vss = Dose * AUMC/AUC^2$

[01216] $Vz = CL/\lambda$

[01217] Where AUMC is the area under the first moment curve.

[01218] For clotting activity data analysis, the flatter terminal slope in the later time points is not apparent. Therefore, the terminal slopes were estimated from all animals by log-linear regression through the last three or more time points and related parameters were calculated as described above.

[01219] A summary of all of the studies completed is described in **Table 111** below:

[01220] **Table 111**

Study	Species	Route of Admin.	Frequency of Admin.	Dose/ Compliance	End Points	Study Status
Acute Toxicokinetic	Male Rat	IV	Single dose	0, 1,3,9, 21 mg/kg **Non-GLP**	Clinical observation, bodyweight, food consumption, PK-PD, Clinical pathology (Hematology, Coagulation, Clinical Chemistry), thrombus formation.	Completed
Acute Toxicokinetic	Male Cyn Monkey	IV	Single dose	0,1,3,9 mg/kg **Non-GLP**	Clinical condition, bodyweight, food consumption, PK-PD. Clinical pathology (Hematology, Coagulation, Clinical Chemistry)	Completed
Acute Toxicokinetic GLP study	Male Rat	IV	Single dose	0,1,3,9 mg/kg **GLP**	Clinical condition, bodyweight, food consumption, blood chemistry, expanded coagulation panel, TK, organ weight, histology, pathology, intravascular coagulation, thrombus formation.	Completed
Acute Toxicokinetic GLP study	Male Cyn Monkey	IV	Single dose	0,1,7.5,15mg/kg **GLP**	Clinical condition, bodyweight, food consumption, blood chemistry, TK, organ weight, histology, pathology, intravascular coagulation, thrombus formation.	Completed
Wessler Model	NZW Rabbit	IV	Single dose	0.05,0.1,0.2,0.3,0.4mg/kg	To evaluate the thrombogenic potential of the MOD-5014 using a semi-quantitative method described by Wessler et al (1959).	Completed

[01221] **Results**

[01222] ***Sprague Dawley Rats***: All animals were in good clinical condition. The only detailed clinical observation observed within the main study animals that was considered test article-related (MOD-5014-related) was noted in one 9 mg/kg animal with purple or red discoloration of the tail . Reversible, test article-related changes in clinical pathology were observed in the main study animals which included mild reductions in prothrombin time in all test article groups. (**Figure 110**, **Tables 112** and **113**)

[01223] **Table 112:** *Rat* MOD-5014 Pharmacokinetic Parameters Based on Plasma Concentration Following IV Bolus Injection Estimated by Non-compartmental Analysis

Dose (mg/kg)	**Cmax (ng/mL)**	**Tmax (hr)**	**AUC0-t (hr*ng/mL)**	**T1/2 (hr)**
1	10,700	0.50	72,200	7.8
3	24,700	0.50	154,000	15.8
9	102,000	0.50	529,000	7.9
21	190,000	0.50	1,110,000	7.5

[01224] **Table 113:** *Rat* MOD-5014 Pharmacodynamics Parameters Based on Plasma Concentration Following IV Bolus Injection Estimated by Non-compartmental Analysis

Dose (mg/kg)	**Cmax (mu/mL)**	**Tmax (hr)**	**AUC0-t (hr*mu/mL)**	**t1/2 (hr)**
1	155,000	0.50	825,000	5.3
3	606,000	0.50	2,200,000	6.7
9	1,660,000	0.50	5,330,000	5.17
21	3,200,000	0.50	13,400,000	5.8

[01225] There were no test article-related macroscopic findings or organ weight changes in the main study animals at the terminal or recovery necropsies.

[01226] **Cynomolgus Monkeys Non-GLP and GLP Studies:**

[01227] All animals were in good clinical condition, administration of a single dose of MOD-5014 intravenously to male cynomolgus monkeys produced decreases in prothrombin time and anti-thrombin III, and an increase in D-dimer at doses of 7.5 and 15 mg/kg.(**Figure 111**, **Tables 114**, **115, 116, 117**) These changes were indicative of a prothrombotic state, which is consistent with the intended mechanism of action of the drug.

[01228] **Table 114:** *Monkey* MOD-5014 Pharmacokinetic Parameters Based on Plasma Concentration Following IV Bolus Injection Estimated by Non-compartmental Analysis (the non-GLP study)

Dose (mg/kg)	**Cmax (ng/mL)**	**$AUC_{0-\infty}$ (hr*ng/mL)**	**$T_{1/2}$ (hr)**
1	14300	48800	29.0
3	41200	150000	29.5
9	153000	536000	15.3

[01229] **Table 115:** *Monkey* MOD-5014 Pharmacodynamics Parameters Based on Plasma Concentration Following IV Bolus Injection Estimated by Non-compartmental Analysis (the non-GLP study)

Dose (mg/kg)	**(U/kg)**	**Cmax (mU/mL)**	**$AUC_{0-\infty}$ (hr*mu/mL)**	**$t_{1/2}$ (hr)**
1	21,600	158000	471000	5.6
3	64,800	383000	1360000	5.9
9	194,400	1900000	5750000	8.98

[01230] **Table 116:** Summary of MOD-5014 Pharmacokinetics in Male Cynomolgus Monkeys (GLP study)

		Plasma Concentrations			**Clotting Activity**		
Parameter	**Units**	**1 mg/kg**	**7.5 mg/kg**	**15 mg/kg**	**1 mg/kg (15,563)**	**7.5 mg/kg (116,722.5)**	**15 mg/kg (233,445)**

C_{max}	(ng/mL)	14,700	118,000	279,000	268,000	2,240,000	4,700,000
C_0	(ng/mL)	18,300	146,000	428,000	243,000	2,600,000	6,970,000
C_{max}/D	(ng/mL)/ (mg/kg)	14,700	15,700	18,600	0.0172	0.0192	0.0201
T_{max}	(hr)	0.25	0.25	0.25	0.42	0.96	0.25
AUC_{0-24h}	(hr*ng/mL)	86,900	841,000	1,660,000	1,290,000	13,600,000	26,200,000
$AUC_{0-\infty}$	(hr*ng/mL)	99,300	1,020,000	2,170,000	1,330,000	14,800,000	29,700,000
$AUC_{0-\infty}/D$	(hr*ng/mL)/ (mg/kg)	99,300	136,000	145,000	0.0851	0.127	0.127
Vi	(mL/kg)	56.0	52.0	38.0	91.5	86.4	39.7
Vz	(mL/kg)	411	126	213	82.3	75.6	83.8
Vss	(mL/kg)	150	90.0	92.5	72.0	67.5	82.1
CL	(mL/hr/kg)	10.1	7.38	6.94	11.8	7.96	7.95
$t_{1/2}$	(hr)	28.3	11.9	21.5	4.86	6.61	7.25

Activity units are in mU rather than ng

[01231] The plasma concentration and clotting activity data from individual animals used in the GLP study are presented in Tables 117 and 118, respectively. Activity data presented the mean of duplicate measurements for each animal at each time point.

[01232] **Table 117:** Plasma Concentration Data from Individual Animals

[01233] Pre-dose activity measures were BLQ and set to 'missing' for estimation of PK parameters.

		Time (hr)											
		0.00	0.25	0.50	2.0	4.0	6.0	8.0	12	24	48	60	72
Group	Animal	Final_Conc (ng/mL)											
1	101			BLQ									
	102			BLQ									
	103			BLQ									
	104			BLQ									
	105			BLQ									
	106			BLQ									
2	201	BLQ	13800	11590	9340	6495	4450	3550	2396	433			
	202	BLQ	15790	13540	9550	7315	2960	3078	2658	526			
	203	BLQ	18390	14630	11690	6800	3680	3604	2610	571			
	204	BLQ	12540	9810	7800	6365	4760	3620	2356	674			
	205	BLQ	15940	12410	9420	7430	5404	3146	2060	394	203	136	117
	206	BLQ	11690	9000	6610	5795	4660	3500	2318	627	181	129	97
	N	0	6	6	6	6	6	6	6	6	2	2	2

		Time (hr)											
		0.00	0.25	0.50	2.0	4.0	6.0	8.0	12	24	48	60	72
Group	Animal	Final_Conc (ng/mL)											
	Mean		**14692**	**11830**	**9068**	**6700**	**4319**	**3416**	**2400**	**537**	**192**	**132**	**107**
	SD		**2485**	**2156**	**1729**	**616**	**867**	**240**	**217**	**109**	**16**	**5**	**14**
3	301	BLQ	106700	86600	65450	46700	38000	26510	19370	7040			
	302	BLQ	115000	94900	73550	51900	44850	38000	20890	8425			
	303	BLQ	121300	91500	76400	54800	48750	43200	21430	8545			
	304	BLQ	130900	99300	98000	59800	60400	54200	38400	9620			
	305	BLQ	118450	110950	75140	55700	45100	29300	24250	8305	2018	1178	488
	306	BLQ	115700	91200	67550	56450	42800	28070	34140	11850	2009	591	500
	N	**0**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**2**	**2**	**2**
	Mean		**118008**	**95742**	**76015**	**54225**	**46650**	**36547**	**26413**	**8964**	**2014**	**885**	**494**
	SD		**7994**	**8566**	**11607**	**4485**	**7599**	**10799**	**7912**	**1635**	**6**	**415**	**9**
4	401	BLQ	297200	168200	111000	107300	88050	71150	47450	19730			
	402	BLQ	300000	215200	138100	103600	88800	76850	51700	17020			
	403	BLQ	249400	139800	143600	110200	81200	77900	43700	17350			
	404	BLQ	317800	170200	164300	102100	77550	62950	50800	16990			
	405	BLQ	287000	246400	197200	131800	85160	76900	57700	22890	2722	1930	1405
	406	BLQ	219600	191600	125000	96900	86750	82500	41050	25900	3390	1574	1334
	N	**0**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**2**	**2**	**2**
	Mean		**278500**	**188567**	**146533**	**108650**	**84585**	**74708**	**48733**	**19980**	**3056**	**1752**	**1370**
	SD		**36728**	**37930**	**30623**	**12220**	**4376**	**6800**	**5991**	**3693**	**472**	**252**	**50**

[01234] **Table 118:** Clotting Activity Data from Individual Animals

[01235] Mean of two determinations per animal (mU/mL), pre-dose activity measures were BLQ and set to 'missing' for estimation of PK parameters, pre-dose value for #404 was considered negligible and also set to missing.

		Time (hr)											
		0.0	0.25	0.50	2.0	4.0	6.0	8.0	12	24	48	60	72
Group	Animal	Activity_mu_ml (mIU/mL)											
1	101			BLQ									
	102			BLQ									
	103			BLQ									
	104			BLQ									
	105			BLQ									
	106			BLQ									
2	201	BLQ	357500	240000	189500	80000	50000	32300	21100	3725			
	202	BLQ	325000	275000	174000	83000	60500	25900	31800	6375			
	203	BLQ	172500	240000	172500	111750	66250	37200	32100	4100			
	204	BLQ	137500	292500	132000	93750	62500	73100	34100	6675			
	205	BLQ	137500	185000	135000	121750	61500	61400	31800	5175	BLQ		
	206	BLQ	95000	210000	127500	80500	55500	70500	33400	5750	BLQ		
	N	**0**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**0**	**0**	**0**
	Mean		**204167**	**240417**	**155083**	**95125**	**59375**	**50067**	**30717**	**5300**			
	SD		**109472**	**39762**	**26618**	**17759**	**5757**	**20697**	**4804**	**1199**			
3	301	BLQ	1800000	1725000	2100000	960000	706500	583000	331000	96750			
	302	BLQ	2325000	625000	1455000	1070000	616500	567500	242500	118000			
	303	BLQ	775000	1150000	1537500	850000	627000	582500	310000	99750			
	304	BLQ	1925000	3100000	1455000	1025000	695000	777500	322000	173500			
	305	BLQ	725000	2200000	1140000	1000000	601500	667500	387000	100250	8650	1900	475
	306	BLQ	1650000	2200000	1477500	1085000	675000	695000	436000	96500	13200	3375	1000
	N	**0**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**2**	**2**	**2**
	Mean		**1533333**	**1833333**	**1527500**	**998333**	**653583**	**645500**	**338083**	**114125**	**10925**	**2638**	**738**
	SD		**647045**	**873165**	**313377**	**85829**	**44201**	**82846**	**66655**	**30173**	**3217**	**1043**	**371**
4	401	BLQ	3300000	3250000	2175000	1430000	1125000	905000	572500	240500			
	402	BL	5100	3550	2100	1635	1110	1040	9525	345			

		Time (hr)											
		0.0	0.25	0.50	2.0	4.0	6.0	8.0	12	24	48	60	72
Group	Animal	Activity_mu_ml (mIU/mL)											
		Q	000	000	000	000	000	000	00	000			
	403	BLQ	3950000	2550000	1825000	1200000	1145000	1200000	1170000	283000			
	404	350	3800000	2950000	2400000	1365000	755000	1085000	902500	243500			
	405	BLQ	6300000	5550000	2125000	1290000	1202500	1135000	1185000	240000	29000	5550	1000
	406	BLQ	5750000	2550000	1725000	1435000	1167500	735000	1147500	328000	20000	6850	1525
	N	**0**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**2**	**2**	**2**
	Mean		**4700000**	**3400000**	**2058333**	**1392500**	**1084167**	**1016667**	**988333**	**280000**	**24500**	**6200**	**1263**
	SD		**1196244**	**1123833**	**245798**	**148551**	**164520**	**170078**	**236044**	**46969**	**6364**	**919**	**371**

[01236] The Plasma Concentration PK Parameters from Individual Animals is presented in **Table 119.**

Group	Animal	C0 (ng/mL)	Cmax (ng/mL)	Cmax/Dose (ng/mL)/ (mg/kg)	Tmax (hr)	Vi (mL/kg)	AUC0-24h (hr*ng/mL)	AUC0-24h/Dose (hr*ng/mL)/ (mg/kg
2	201	16400	13800	13800	0.25	60.98	86290	86290
	202	18400	15800	15800	0.25	54.35	89010	89010
	203	23100	18400	18400	0.25	43.29	96820	96820
	204	16000	12500	12500	0.25	62.50	83370	83370
	205	20500	15900	15900	0.25	48.78	87830	87830
	206	15200	11700	11700	0.25	65.79	77980	77980
	Mean	**18300**	**14700**	**14700**	**0.25**	**55.95**	**86900**	**86883**
	SD	**3040**	**2490**	**2490**	**0.00**	**8.71**	**6270**	**6263**
3	301	131000	107000	14200	0.25	57.25	679600	90613
	302	139000	115000	15300	0.25	53.96	783100	104413
	303	161000	121000	16200	0.25	46.58	823600	109813
	304	173000	131000	17500	0.25	43.35	1081000	144133
	305	126000	118000	15800	0.25	59.52	807300	107640
	306	147000	116000	15400	0.25	51.02	872200	116293
	Mean	**146000**	**118000**	**15700**	**0.25**	**51.95**	**841000**	**112151**
	SD	**17700**	**7990**	**1070**	**0.00**	**6.21**	**134000**	**17830**
4	401	525000	297000	19800	0.25	28.57	1583000	105533
	402	418000	300000	20000	0.25	35.89	1688000	112533

Group	Animal	C0 (ng/mL)	Cmax (ng/mL)	Cmax/Dose (ng/mL)/ (mg/kg)	Tmax (hr)	Vi (mL/kg)	AUC0-24h (hr*ng/mL)	AUC0-24h/Dose (hr*ng/mL)/ (mg/kg
	403	445000	249000	16600	0.25	33.71	1562000	104133
	404	593000	318000	21200	0.25	25.30	1647000	109800
	405	334000	287000	19100	0.25	44.91	1938000	129200
	406	252000	220000	14600	0.25	59.52	1571000	104733
	Mean	**428000**	**279000**	**18600**	**0.25**	**37.98**	**1660000**	**110989**
	SD	**124000**	**36700**	**2450**	**0.00**	**12.52**	**142000**	**9501**

[01237] The clotting activity PK parameters from individual animals is presented in Table 120:

Group	Animal	C0 (mIU/ mL)	Cmax (mIU/mL)	Cmax_D (mIU/mL(/mIU/ kg)	Tmax (hr)	Vi (mL/kg)	AUC0-24h (hr*mIU/ mL)	AUC0-24h/Dose (hr*mIU/ mL)/ Dose
2	201	533000	358000	0.0230	0.25	29.20	1246000	0.0801
	202	384000	325000	0.0209	0.25	40.53	1332000	0.0856
	203	173000	240000	0.0154	0.50	89.96	1326000	0.0852
	204	138000	293000	0.0188	0.50	112.78	1340000	0.0861
	205	138000	185000	0.0119	0.50	112.78	1260000	0.0810
	206	95000	210000	0.0135	0.50	163.82	1210000	0.0777
	Mean	**243000**	**268000**	**0.0172**	**0.42**	91.51	**1290000**	0.0826
	SD	**175000**	**67700**	**0.00435**	**0.129**	50.23	**53600**	0.0035
3	301	1880000	2100000	0.0180	2.0	62.09	13800000	0.1182
	302	8650000	2330000	0.0199	0.25	13.49	12480000	0.1069
	303	775000	1540000	0.0132	2.0	150.61	11770000	0.1008
	304	1930000	3100000	0.0266	0.50	60.48	15370000	0.1317
	305	725000	2200000	0.0188	0.50	161.00	13090000	0.1121
	306	1650000	2200000	0.0188	0.50	70.74	14800000	0.1268
	Mean	**2600000**	**2240000**	**0.0192**	**0.96**	86.40	**13600000**	0.1161
	SD	**3010000**	**503000**	**0.00431**	**0.813**	57.46	**1380000**	0.0118
4	401	3350000	3300000	0.0141	0.25	69.69	21740000	0.0931
	402	7330000	5100000	0.0218	0.25	31.85	27270000	0.1168
	403	6120000	3950000	0.0169	0.25	38.14	26530000	0.1136
	404	4890000	3800000	0.0163	0.25	47.74	24520000	0.1050
	405	715000	6300000	0.0270	0.25	32.65	30350000	0.1300

	406	0 13000000	5750000	0.0246	0.25	17.96	26870000	0.1151
	Mean	**6970000**	**4700000**	**0.0201**	**0.25**	39.67	**26200000**	0.1123
	SD	**3290000**	**1200000**	**0.00512**	**0.00**	17.61	**2890000**	0.0124

Group	Animal	t1/2 (hr)	AUC0-∞ (hr*mIU/mL)	AUC0-∞/Dose (hr*mIU/mL)/(mIU/kg)	Vz (mL/kg)	CL (mL/hr/kg)	Vss (mL/kg)
2	201	4.92	1270000	0.0817	86.9	12.2	59.5
	202	5.79	1390000	0.0890	93.9	11.2	68.6
	203	4.11	1350000	0.0867	68.3	11.5	65.8
	204	5.05	1390000	0.0895	81.5	11.2	74.6
	205	4.69	1300000	0.0835	81.2	12.0	77.9
	206	4.59	1250000	0.0805	82.2	12.4	85.7
	Mean	**4.86**	**1330000**	**0.0851**	**82.3**	**11.8**	**72.0**
	SD	**0.562**	**59400**	**0.00382**	**8.39**	**0.528**	**9.34**
3	301	7.05	14800000	0.127	80.3	7.89	61.2
	302	6.13	13500000	0.116	76.4	8.63	70.0
	303	6.50	12700000	0.109	86.1	9.19	79.3
	304	7.32	17200000	0.147	71.7	6.79	63.5
	305	6.21	14500000	0.124	72.3	8.06	70.2
	306	6.45	16300000	0.139	66.8	7.18	61.1
	Mean	**6.61**	**14800000**	**0.127**	**75.6**	**7.96**	**67.5**
	SD	**0.473**	**1670000**	**0.0143**	**6.89**	**0.890**	**7.04**
4	401	8.23	24600000	0.105	113	9.49	95.7
	402	9.70	32100000	0.138	102	7.27	87.3
	403	7.16	29500000	0.126	81.9	7.93	84.4
	404	7.14	27000000	0.116	89.0	8.64	84.3
	405	4.94	33800000	0.145	49.2	6.90	63.2
	406	6.31	31300000	0.134	67.9	7.47	78.0
	Mean	**7.25**	**29700000**	**0.127**	**83.8**	**7.95**	**82.1**
	SD	**1.63**	**3420000**	**0.0147**	**23.0**	**0.964**	**10.9**

[01238] **Figure 112** and **Figure 113** show the mean MOD-5014 plasma concentration and clotting activity following IV injection. **Figure 114** through **Figure 116** plot the mean concentration and clotting activity together for each dose level. Individual plots of plasma concentration and clotting activity are presented in **Figures 117A-R**. These figures illustrate that over the first 24 hours after dosing plasma concentrations and clotting activity decline at similar rates. Beyond 24 hours, plasma concentrations tended to level off and activity levels continued to decline. The post-24 hour data are only available from the recovery animals, 2 per group.

[01239] Results of non-compartmental analysis are shown in **Table 119** and **Table 120**

for plasma concentration and clotting activity data, respectively. Plasma concentrations were highest at the first time point measured (0.25 hr). Clotting activity Tmax was observed at 0.25 hr or 0.50 hr in most animals. Both concentration and activity increased with increasing dosebut not completely dose proportionally. There was a trend for $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ to increase 30 to 50% more than the increase in dose. Terminal half-life was longer based on plasma concentrations than based on clotting activity: approximately 12 to 28 hours versus approximately 5 to 7 hours. CL appeared to be about 50% faster at the lowest dose of 1 mg/kg compared to the 7.5 mg/kg and 15 mg/kg doses. CL based on plasma concentrations and based on clotting activity were similar.

[01240] **Table 119**: MOD-5014 Pharmacokinetic Parameters Based on Plasma Concentrations Following IV Bolus Injection in Male Cynomolgus Monkeys

		Dose mg/kg		
Parameter	**Units**	**1**	**7.5**	**15**
C_{max}	(ng/mL)	14,700	118,000	279,000
C_0	(ng/mL)	18,300	146,000	428,000
C_{max}/D	(kg*ng/mL/mg)	14,700	15,700	18,600
T_{max}	(hr)	0.25	0.25	0.25
$AUC_{0\text{-}24h}$	(hr*ng/mL)	86,900	841,000	1,660,000
$AUC_{0\text{-}\infty}$	(hr*ng/mL)	99,300	1,020,000	2,170,000
$AUC_{0\text{-}\infty}/D$	(hr*kg*ng/mL/mg)	99,300	136,000	145,000
Vi	(mL/kg)	56.0	52.0	38.0
Vz	(mL/kg)	411	126	213
Vss	(mL/kg)	150	90.0	92.5
CL	(mL/hr/kg)	10.1	7.38	6.94
$t_{1/2}$	(hr)	28.3	11.9	21.5

[01241] **Table 120:** MOD-5014 Pharmacodynamic Parameters Based on Activity Measurements Following IV Bolus Injection in Male Cynomolgus Monkeys

		Dose mg/kg (units/kg)		
		1	**7.5**	**15**
Parameter	**Units**	**(15,563)**	**(116,722.5)**	**(233,445)**
C_{max}	(mIU/mL)	268,000	2,240,000	4,700,000
C_0	(mIU/mL)	243,000	2,600,000	6,970,000
C_{max}/D	(kg*mIU/mL/IU)	0.0172	0.0192	0.0201
T_{max}	(hr)	0.42	0.96	0.25
$AUC_{0\text{-}24h}$	(hr*mIU/mL)	1,290,000	13,600,000	26,200,000
$AUC_{0\text{-}\infty}$	(hr*mIU/mL)	1,330,000	14,800,000	29,700,000
$AUC_{0\text{-}\infty}/D$	(hr*kg*mIU/mL/IU)	0.0851	0.127	0.127
Vi	(mL/kg)	91.5	86.4	39.7
Vz	(mL/kg)	82.3	75.6	83.8

Vss	(mL/kg)	72.0	67.5	82.1
CL	(mL/hr/kg)	11.7	7.93	7.95
$t_{1/2}$	(hr)	4.86	6.61	7.25

Activity based on 15,563 units/mg

[01242] No consistent trends were apparent from the estimates of the volume of distribution. The volume at steady state (Vss), when theoretically plasma and tissue concentrations are at equilibrium, and in the terminal phase (Vz), when concentrations are declining, were higher based on estimates from plasma concentrations than on clotting activity. Initial volume of distribution (Vi) was somewhat higher at the 1.0 mg/kg and 7.5 mg/kg dose groups based on clotting activity than based on plasma concentrations. Vi was also higher at the two lower doses than at the high dose. This observation could be related to the fact that clotting activity measures did not all peak at the earliest time point and then sharply decline. Instead, some of the clotting activity curves peaked at a later time producing a flatter initial phase of the curve. This pattern led to a lower estimate of C_0 and thus a higher estimate of Vi based on clotting activity.

[01243] **Summary for the GLP study**

[01244] Plasma concentrations were highest at the first time point measured (0.25 hr). Clotting activity Tmax was observed at 0.25 hr or 0.50 hr in most animals. Both concentration and activity parameters increased with increasing dose, but not completely dose proportionally. There was a trend for $AUC_{0\text{-}24h}$ and $AUC_{0\text{-}\infty}$ to increase 30 to 50% more than the increase in dose. Terminal half-life was longer based on plasma concentrations than based on clotting activity: approximately 12 to 28 hours versus approximately 5 to 7 hours. CL appeared to be about 50% faster at the lowest dose of 1 mg/kg compared to the 7.5 mg/kg and 15 mg/kg doses. CL based on plasma concentrations and based on clotting activity were similar.

[01245] No consistent trends were apparent from the estimates of the volume of distribution. The apparent volume at steady state (Vss), when theoretically plasma and tissue concentrations are at equilibrium, and in the terminal phase (Vz), when concentrations are declining, were higher based on estimates from plasma concentrations than those based on clotting activity. Initial volume of distribution (Vi) was somewhat higher at the 1.0 mg/kg and 7.5 mg/kg dose groups based on clotting activity than that based on plasma concentrations. Vi was also higher at the two lower doses than at the high dose. This observation could be related to the fact that clotting activity measures did not all peak at the earliest time point and then sharply decline. Some of the clotting activity curves peaked at a later time producing a flatter initial phase of the curve. This pattern led to a lower estimate of

C_0 and thus a higher estimate of Vi based on clotting activity.

[01246] Following a slow IV bolus injection in male cynomolgus monkeys, MOD-5014 exposure based on plasma concentrations and based on clotting activity increased with increasing dose, but somewhat more than the increase in dose. Thus, MOD-5014 exposure based on plasma concentrations and based on clotting activity was dose related but not dose proportional. CL measured by concentrations and activity were similar and both were about 50% higher following a dose of 1 mg/kg compared to CL following doses of 7.5 mg or 15 mg/kg. Terminal half-life was longer based on plasma concentrations than based on clotting activity: approximately 12 to 28 hours versus approximately 5 to 7 hours.

[01247] NZW Rabbit results are presented above in Example 15.

[01248] **Conclusions:**

[01249] **Safety Assessment Summary:**

[01250] All the findings noted in the toxicological studies, are consistent with and anticipated based on the mechanism of action of this drug. There were no new or unique findings associated with MOD-5014 that have not also been seen for commercial rhFVII or other coagulation factor drugs**.** The thrombogenic effects were tested in Wessler model and was shown to be comparable to rhFVIIa.

[01251] **Pharmacokinetic Advantages:**

[01252] The results showed that recovery was enhanced two-fold, and that MOD-5014 provided a superior half-life, four-times that of commercial rhFVII. In addition, there was improved exposure to the MOD-5014 (AUC 3-4X). Analysis of in vitro clotting activity showed comparable in vitro clotting activity compared with the commercial product.

[01253] Overall, MOD-5014 demonstrated excellent safety profiles, providing adequate exposure margins above the proposed administered clinical doses in the First in Human (FIH) phase 1-2A clinical trial study.

EXAMPLE 18: Comparative Assessment of MOD-5014 and hFVIIa In vitro Platelet Binding

[01254] **1. PROJECT RATIONALE AND SUMMARY**

[01255] 1.1. Objective

[01256] These studies were designed to assess *in vitro* platelet binding of MOD-5014 as compared to wild type recombinant human FVIIa. This is an important characteristic of FVIIa-like molecules as bypassing agents in hemophilia, since platelet surface activity is thought to be an important component of FVIIa mechanism of action in this clinical setting. In addition to simple phospholipids, platelets have numerous surface features that

contribute to binding and activity of coagulation proteases. Thus, assays of phospholipid binding and activity alone may be misleading in predicting biological effects of coagulation factors such as FVIIa.

[01257] 1.2. Methods

[01258] The studies examined:

- Platelet binding as assessed by flow cytometry
- Platelet binding as assessed by platelet surface thrombin generation

[01259] 1.3. Results and Conclusions

[01260] The flow cytometry studies did not provide useful data. These assays allowed FVIIa and MOD-5014 to bind to activated human platelets. Binding was detected by an affinity-purified polyclonal antibody against human FVII. While the antibody preparation that used by OPKO for flow cytometry bound equally well to FVIIa and MOD-5014 on western blots, it did not do so in ELISA or in the flow cytometry assays. Thus, this approach was not useful for comparing FVIIa and MOD-5014 binding to platelets.

[01261] As an alternative approach, the ability of MOD-5014 binding of platelets to support thrombin generation on the platelet surface was assessed. This approach is based on the fact that FVIIa has little ability to catalyze activation of FXa in solution. However, when FVIIa and modified FVIIa molecules bind to activated platelets, they are much more efficient in activating FX. The FXa produced on the activated platelets can then be measured by its ability to activate prothrombin to thrombin on the platelet surface.

[01262] It was found that at equimolar concentrations, MOD-5014 supported rates of platelet surface thrombin generation that were slightly less than FVIIa. However, the lag before onset of thrombin generation was about 40% longer with MOD-5014 than with FVIIa. This is consistent with the biochemical studies showing that MOD-5014 has a slightly lower maximal rate of FX activation than wild type FVIIa. The longer time to maximal thrombin generation could be overcome by a higher level of MOD-5014. Many factors enter into dosing considerations. However, these *in vitro* data suggest that MOD-5014 may require slightly different dosing than FVIIa.

[01263] **2. EVALUATION OF PLATELET BINDING BY FLOW CYTOMETRY**

[01264] 2.1. Method

[01265] Platelets were isolated from healthy human donor blood and activated with thrombin and convulxin (a collagen receptor agonist). The activated platelets were incubated with 10, 50, 100, and 250 nM of MOD-5014 or FVIIa, and subsequently fixed with 1% paraformaldehyde. The fixed platelets were stained for FVIIa using a polyclonal

anti-human FVII antibody and a FITC-linked secondary antibody. The stained platelets were analyzed by flow cytometry. The mean fluorescence intensity (brightness) of the platelet population at each FVIIa concentration was determined.

[01266] 2.2. Results

[01267] **Figure 118** shows the mean fluorescence intensity of the platelet populations at each concentration of MOD-5014 or FVIIa. Despite the low overall binding detected for MOD-5014, MOD-5014 and FVIIa had similarly shaped binding curves. If MOD-5014 does not binding well to the platelets, this would result in a very flat curve that does not appear to saturate. Thus, it seems most likely that the antibody used to detect FVIIa/MOD-5014 might not detect MOD-5014 well. It was observed that the same polyclonal antibody used to detect binding in the flow cytometry studies gave an equally strong signal with SDS denatured samples of MOD-5014 and wild type FVIIa.

[01268] The ability of the polyclonal antibody to detect FVIIa and MOD-5014 was then tested using an ELISA assay. In this assay, microtiter wells were coated with the antibody to capture the protein samples. Binding of the protein to the capture antibody was detected with the same polyclonal antibody. As FVIIa and MOD-5014 remain in their native conformations, MOD-5014 was detected considerably less well than FVIIa.

[01269] This prompted us to seek a different technique to detect binding of FVIIa and MOD-5014.

[01270] 3. **EVALUATION OF PLATELET BINDING BY THROMBIN GENERATION**

[01271] 3.1. Method

[01272] In these experiments, platelets were again activated with thrombin and convulxin, incubated with different concentrations of FVII or MOD-5014, and subsequently added to plasma concentrations of FX (135 nM) and prothrombin (1.2 μM) in the presence of the chromogenic substrate Gly-Pro-Arg-paranitroanilide (500 μM). Under these conditions, the FVIIa or MOD-5014 that has bound to the platelet activates FX. The FXa thus formed on the platelet surface combines with platelet-derived FVa, and the resulting complex activates prothrombin to thrombin, which is detected by cleavage of the chromogenic substrate.

[01273] 3.2. Results

[01274] Substrate cleavage in the absence of platelets was minimal. In **Figure 119**, the maximal rate of substrate cleavage at each concentration is plotted as a function of FVIIa or MOD-5014 concentration. The rate of thrombin generation is somewhat lower for MOD-5014 than for FVIIa. This is consistent with activity assays performed on

phospholipid vesicles.

EXAMPLE 19: Biochemical properties of MOD -5014 relative to commercial recombinant hFVIIa - effect of a carboxy-terminal peptide (CTP) on factor VIIa activity

PROJECT RATIONALE AND SUMMARY

[01275] These studies were designed to assess the biochemical properties of MOD -5014 relative to commercial recombinant hFVIIa, referred to herein as MOD-5000.

[01276] The studies examined:

- Synthetic substrate cleavage of MOD-5014
- Tissue factor (TF) binding of MOD-5014 as measured by synthetic substrate cleavage
- TF binding of MOD-5014 as measured by factor X (FX) activation
- Kinetics of FX activation by TF-bound MOD-5014
- Lipid binding of MOD-5014 as measured by FX activation
- Kinetics of factor activation by lipid-bound MOD-5014
- Inactivation of MOD-5014 by anti-thrombin (AT)
- Inactivation of MOD-5014 by TFPI

[01277] Overall the data suggests that, relative to MOD-5000, MOD-5014 has similar mechanism of action with a slightly reduced catalytic activity. These results demonstrated slightly reduced activity of TF-bound MOD-5014 and somewhat more reduced activity, independent of TF.

[01278] These effects were reflected mainly by reaction rates rather than the extent of reactions, and reactions for which the entire time course can be measured do go to completion.

[01279] The slightly reduced rate of AT inhibition suggests an extension of MOD-5014 half-life in vivo with a proper inhibition response.

[01280] **EXPERIMENTAL MATERIALS**

- MOD-5014 GMP-1: 2.5 mg/ml (based on A280)
- NovoSeven Lot# CU60430: 0.943 mg/ml (based on A280), referred to as MOD-5000.

SYNTHETIC SUBSTRATE CLEAVAGE OF MOD-5014

[01281] Rationale: Cleavage of synthetic substrate should depend exclusively on the availability of a functional active site.

[01282] Methods: MOD-5000 and MOD-5014 were diluted to equal concentrations on a molar basis. The same concentration was then added to a fixed concentration of the

substrate Pefachrome FVIIa (methylsul fonyl-D-cyclohexylalanyl-2-aminobutyryl-arginine-p-nitroanilide) and cleavage of the substrate was monitored by the appearance of a yellow color.

[01283] Results

[01284] Concentration: FVIIa 360 nM; Substrate 500 µM

[01285] Analysis: Absorbance at 405 nm was converted to concentration of p-nitroaniline using the known extinction coefficient. Concentration of p-nitroaniline was plotted vs. time to determine a rate of substrate

[01286] cleavage. Data was fitted to:

$$rate = k_1[VIIa]$$

[01287] k_1= 27.5 mol pNA/min/mol VIIa

[01288] Conclusion: On a molar basis, MOD-5000 and MOD-5014 have the same rate of substrate cleavage (**Figure 120**). For subsequent studies, measurement of substrate cleavage is used as a control for dilutions and pipetting.

TF BINDING OF MOD-5014 AS MEASURED BY SYNTHETIC SUBSTRATE CLEAVAGE

[01289] Rationale: When factor VIIa binds TF, a conformational change occurs in factor VIIa that results in an increase in the rate of substrate cleavage. This means that increased substrate cleavage can be used to monitor factor VIIa binding to TF.

[01290] Methods: Varying concentrations of MOD-5000 and MOD-5014 were added to a fixed concentration of

[01291] TF and incubated for 5 minutes. Substrate (Pefachrome FVIIa) was added. Substrate cleavage was monitored at 405 nm by the appearance of yellow color.

Results:

[01292] Concentration: FVIIa 0-25 nM; TF 8.7 nM; Substrate 500 µM

[01293] Analysis: As the concentration of TF is well above the expected Kd, at low concentrations all of FVIIa should be bound to TF. The rate of substrate cleavage will be that of the VIIa/TF complex. Once the concentration of FVIIa exceeds that of TF, the rate of substrate cleavage should drop to that of free FVIIa. Since FVIIa and TF form a 1:1 molar complex, the concentration of FVIIa at which the change in rate of substrate cleavage occurs is a check on the estimated concentration of FVIIa.

[01294] Data was fitted to:

[01295] $$rate = k_1[VIIa] + k_2[VIIa/TF]$$

	MOD-5000	MOD-5014	
k_1	27.5	27.5	mol pNA/min/mol VIIa
k_2	365	357	mol pNA/min/mol VIIa/TF

[01296]

[01297] Conclusion: MOD-5000 (Novoseven) and MOD-5014 show the same inflection point at the expected concentration of TF (8.7 nM) (**Figure 121**). This confirms that the molar concentrations of MOD-5000 and MOD-5014 as predicted by substrate cleavage are correct. MOD-5014 had a very slightly lower rate of substrate cleavage when bound to TF (98%) relative to MOD-5000 (**Figure 121**).

TF BINDING OF MOD-5014 AS MEASURED BY FACTOR X ACTIVATION

[01298] Rationale: Cleavage of FX by FVIIa is slow relative to cleavage by the FVIIa/TF complex. Therefore, binding of FVIIa to TF can be assessed by measuring the rate of FX activation.

[01299] Methods: Varying concentrations of MOD-5000 and MOD-5014 were added to a fixed concentration of TF and the rate of FX activation was measured. Factor X activation was assessed by cleavage of the synthetic substrate Pefachrome FXa (methoxycarbonyl-D-cyclohexylalanyl-glycyl-arginine paranitroanilide). Cleavage of synthetic substrate is converted to FXa concentration by a standard curve. Neither FVIIa nor FVIIa/TF cleaves the FX substrate at an appreciable rate.

[01300] Results

[01301] Concentration: FVIIa 0-2 nM; TF 10 pM; FX 135 nM; Substrate 500 µM

[01302] Factor X concentration in plasma is 8 µg/mL (~ 135 nM).

[01303] Analysis: The rate of FX activation should increase as FVIIa binds to TF. Once all of the TF is saturated with FVIIa, the rate of FX activation will have reached a maximum value (**Figure 122A**).

[01304] Data was fitted to:

[01305]
$$v = V_{max}\frac{[VIIa]^n+[TF]+K_d^n-\sqrt{([VIIa]^n+[TF]+K_d^n)^2-4[VIIa]^n[TF]}}{2[TF]}$$

	MOD-5000	MOD-5014	
V_{max}	1.40	1.30	nM FXa/min
K_d	3.3	3.0	pM
Hill value	0.93	0.91	

[01306]

[01307] Conclusion: There is a very slight negative cooperativity (Hill value <1) in the binding of FVIIa to TF. This is the same for MOD-5000 and MOD-5014. When bound to TF, MOD-5014 has a slightly reduced rate of FX activation (93%) relative to MOD-5000. The

affinity of MOD-5014 for TF is equivalent to the affinity of MOD-5000 (**Figure 122A**).

RATE OF FX ACTIVATION AS A FUNCTION OF FX CONCENTRATION

[01308] Rationale: The slightly reduced rate of MOD-5014 activation when bound to TF could be a consequence of reduced affinity for FXa or reduced turnover of FX once it is bound to the complex. Measuring the rate of FX activation as a function of FX concentration established the kinetic parameters of the complex.

[01309] Methods: Varying concentrations of FX were incubated with a fixed concentration of FVIIa/TF complex.

[01310] Factor X activation was assessed by cleavage of a synthetic substrate (Pefachrome FXa). Cleavage of the synthetic substrate is converted to FXa concentration by a standard curve.

Results

[01311] Concentration: FVIIa 1 nM; TF 5 pM; FX 0-1500 nM; Substrate 500 µM

[01312] Analysis: As more FX was added, more of the FVIIa/TF complex should have FX bound up to the point where all FVIIa/TF complexes have bound FX. At that point the reaction was limited by the rate at which FX was activated. Therefore, the rate of FX activation should have increased with an increasing concentration of FX, with the shape of the curve asymptotically approaching a maximum rate (**Figure 123**).

[01313] Data was fitted to:

[01314] $$v = V_{max}\left(\frac{[S]}{K_m + [S]}\right)$$

[01315]

	MOD-5000	MOD-5014	
V_{max}	1.78	1.64	nM FXa/min
K_m	140	120	nM

[01316] Conclusion: When bound to TF, MOD-5014 had a slightly reduced turnover of FX (92%) relative to MOD-5000. The binding of FX to the MOD-5014/TF complex was the same as its binding to the MOD-5000/TF complex (**Figure 123**).

LIPID BINDING OF MOD-5014 AS MEASURED BY FX ACTIVATION

[01317] Rationale: Factor X activation on platelets is thought to contribute to the hemostatic effect of FVIIa. This platelet activity is thought to occur in a low-TF environment, or in the absence of TF. Factor X activation without TF can be studied on lipid vesicles.

[01318] Methods: Factor X activation by FVIIa on lipids is a function of binding of both enzyme (FVIIa) and protein substrate (FX). The lipid ratio was PC:PE:PS 41:44:14,

designed to mimic the composition of highly activated platelets. The lipids were prepared as large unilamellar vesicles (200 nm). Increasing concentrations of vesicles were added to FVIIa and FX. Factor X activation was assessed by the cleavage of a synthetic substrate (Pefachrome FXa). Cleavage of the synthetic substrate was converted to FXa concentration by a standard curve.

Results

[01319] Concentration: FVIIa 20 nM; FX 500 nM; Lipids 0-1000 µM; Substrate 500 µM.

[01320] Analysis: The rate of FXa generation was plotted against the concentration of lipid vesicles (**Figure 124A**). As expected, FXa generation increased with the increasing concentrations of lipid since more surface area was available for the reaction. At a sufficiently high concentration of lipids, the rate of the reaction decreased as FVIIa and FX were segregated onto different lipid vesicles. This template response is expected for this system. Data was not fitted to an equation and the lines shown are for visual reference only. The difference in the rate of FXa generation between MOD-5000 and MOD-5014 were not due to differences in affinity for the lipids. This is shown in **Figure 124B**, where the rate of FXa generation relative to the maximum for each is plotted against lipid concentration.

[01321] Conclusion: The rate of FX activation in the absence of TF is lower for MOD-5014 (~60%) relative to MOD-5000. The affinity of MOD-5014 for lipids is the same as for MOD-5000.

KINETICS OF FX ACTIVATION BY LIPID-BOUND MOD-5014

[01322] Rationale: The reduced rate of FX activation in the absence of TF for MOD-5014 relative to MOD-5000 could be a consequence of reduced affinity for FXa or reduced turnover of FX once it is bound to the enzyme on the lipid surface.

[01323] Methods: Varying FX concentrations were incubated with a fixed concentration of FVIIa and lipid vesicles. Factor X activation was assessed by cleavage of a synthetic substrate (Pefachrome FXa). Cleavage of synthetic substrate was converted to FXa concentration by a standard curve.

Results

[01324] Concentration: FVIIa 20 nM; FX 0-2500 nM; Lipids 100 µM; Substrate 500 µM.

[01325] Analysis: As more FX is added, more of the FVIIa on the lipid surface should have FX bound up to the point where all FVIIa is bound to FX. At that point the reaction is limited by the rate at which FX is activated. Therefore, the rate of FX activation should increase with an increasing concentration of FX, with the shape of the curve asymptotically

approaching a maximum rate. As expected, the affinity for FX of FVIIa is reduced (higher Km) in the absence of TF and the rate of FXa generation is reduced in the absence of TF (**Figure 125**).

[01326] Data was fitted to:

$$v = V_{max}\left(\frac{[S]}{K_m + [S]}\right)$$

	MOD-5000	MOD-5014	
V_{max}	0.253	0.115	nM FXa/min
K_m	878	848	nM FXa/min

[01327] Conclusion: The rate of FX activation on a lipid surface in the absence of TF is lower for MOD-5014 (45%) relative to MOD-5000. The binding of FX to MOD-5014 on the lipid surface was the same as its binding to MOD-5000 (**Figure 125**).

INACTIVATION OF MOD-5014 BY AT

[01328] Rationale: A significant part of FVIIa clearance in vivo is thought to be through formation of a FVIIa complex with AT. The rate of this reaction is measurable in vitroonly when FVIIa is bound to TF. To proceed at a measurable rate, the in vitro reaction also requires high concentrations of heparin, which is thought to mimic the effects of a naturally occurring glycosaminoglycan.

[01329] Methods: Factor VIIa was incubated with TF to allow formation of the complex. The complex was incubated with AT and heparin. The reaction was stopped at timed intervals by the addition of polybrene (hexadimethrine bromide) to neutralize the heparin. The residual FVIIa/TF activity was measured by the cleavage of a synthetic substrate (Pefachrome FVIIa). At the concentrations used in the assay, polybrene did not alter substrate cleavage.

Results

[01330] Concentration: FVIIa 10 nM; TF 11 nM; AT 1 µM; Heparin 5 U/mL; FVIIa/TF 8.2 nM; Polybrene 100 µg/mL; Substrate 500 µM.

[01331] Analysis: The concentration of FVIIa/TF, measured as a rate of substrate cleavage, was plotted against the time in minutes (**Figure 126**). As expected, AT/heparin inhibited FVIIa, leading to a loss of FVIIa/TF activity.

[01332] Data was fitted to:

$$v = V_{t=0} e^{-k*time}$$

	MOD-5000	MOD-5014	
V_0	11.89	11.93	
k	0.354	0.217	min^{-1}

[01333]

[01334] Conclusion: Similar values for the activity at T=0 indicate that equal amounts of MOD-5000 and MOD-5014 were present in the reaction. MOD-5014 was inhibited slightly more slowly (62%) than MOD-5000 (**Figure 126**). Both reactions proceeded to complete inhibition.

INACTIVATION OF MOD-5014 BY TFPI

[01335] Rationale: TFPI is the physiologic inhibitor of the FVIIa/TF complex. The K2 domain of TFPI forms an initial complex with FXa. This complex binds FVIIa/TPI, where the K1 domain of TFPI interacts with FVIIa. Therefore, FX activation by FVIIa/TF should lead to inhibited complexes and to shutdown of FVIIa-TFPI.

[01336] Methods: Factor VIIa and TF were incubated together to form a complex. The complex was added to TFPI/FX/FXa substrate. Factor X activation was assessed by cleavage of a synthetic substrate (Pefachrome FXa). Cleavage of the synthetic substrate was converted to FXa concentration by a standard curve.

Results

[01337] Concentration – inhibition: FVIIa 1 nM; TF 20 pM; FX 135 nM; TFPI 0-5 nM; Substrate 500 µM.

[01338] Analysis: As expected, initial FXa generation occurred at the same rate in all reactions (**Figure 127 A-C**). In the presence of TFPI, the rate of FXa generation slowed as the FVIIa/TFPI complex was inhibited by TFPI/FXa (lower two panels). Shutdown of the TFPI complexes occurred more rapidly at higher concentrations of TFPI (lower two panels). The amount of FXa formed before FVIIa/TFPI was shut down is a measure of TFPI interaction with FVIIa/TF. Since MOD-5014 has a slightly reduced rate of FXa generation which would therefore slow formation of FXa/TFPI complexes, the reaction took longer to reach a plateau with MOD-5014 than with MOD-5000.

[01339] Conclusion: As shown in the upper panel, the concentration dependence for TFPI inhibition of MOD-5014/TF generation of FXa is very similar to that of MOD-5000. MOD-5014 might be slightly more sensitive to TFPI inhibition (124%); alternatively, this might be an artifact of the slightly slower rate of FXa generation.

[01340] While certain features of the disclosure have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the disclosure.

CLAIMS

What is claimed is:

1. A CTP-modified polypeptide consisting of a Factor VII coagulation factor and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein said polypeptide does not include a signal peptide.
2. The CTP-modified polypeptide of claim 1, wherein the sequence of at least one CTP is selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.
3. The CTP-modified polypeptide of any one of claims 1-2, wherein at least one CTP is glycosylated.
4. The CTP-modified polypeptide of any one of claims 1-3, wherein at least one CTP is truncated.
5. The CTP-modified polypeptide of claim 1, wherein at least one CTP is attached to said coagulation factor via a linker.
6. The CTP-modified polypeptide of claim 5, wherein said linker is a peptide bond.
7. The CTP-modified polypeptide of any one of claims 1-6, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.
8. A pharmaceutical formulation comprising a buffer, a tonicity agent, and a CTP-modified polypeptide consisting of a Factor VII coagulation factor and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein said polypeptide does not include a signal peptide.
9. The pharmaceutical formulation of claim 8, wherein said buffer comprises 20 mM citrate.
10. The pharmaceutical formulation of any one of claims 8-9, wherein said tonicity agent is 150 mM sodium chloride.
11. The pharmaceutical formulation of any one of claims 8-10, wherein said formulation is a liquid formulation.
12. The pharmaceutical formulation of any one of claims 8-11, wherein said formulation is at a pH of about 6.4.
13. The pharmaceutical formulation of any one of claims 8-12, wherein said formulation comprises 20 mM citrate, 13.3 mM Glycine, 150 mM sodium chloride, at a pH of about

6.4.

14. The pharmaceutical formulation of any one claims 8-13, wherein the sequence of at least one CTP consists of an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.
15. The pharmaceutical formulation of any one of claims 8-14, wherein said at least one CTP is glycosylated.
16. The pharmaceutical formulation of any one of claims 8-15, wherein said at least one CTP is truncated.
17. The pharmaceutical formulation of any one of claims 8-16, wherein at least one CTP is optionally attached to said polypeptide sequence of interest via a linker.
18. The pharmaceutical formulation of claim 17, wherein the linker is a peptide bond.
19. The pharmaceutical formulation of any one of claims 8-18, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.
20. The pharmaceutical formulation of any one of claims 8-19, wherein said polypeptide is administered daily, every other day, every three days, once weekly, twice weekly, or once every other week, or any combination thereof.
21. The pharmaceutical formulation of any one of claims 8-20, wherein said subject is a human adult or a human child.
22. The pharmaceutical formulation of any one of claims 8-19 and 21, for a daily, every other day, once a week, twice a week, or once every other week administration to a subject in need thereof.
23. The pharmaceutical formulation of claim 22, wherein said administration is intravenous or subcutaneous administration.
24. A pharmaceutical composition comprising the CTP-modified polypeptide of any one of claims 1-7, and a pharmaceutically acceptable carrier.
25. A pharmaceutical composition comprising the pharmaceutical formulation of any one of claims 8-24.
26. A method of extending the biological half-life of a Factor VII (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby extending the biological half-life of said coagulation factor, wherein said coagulation factor does not include a signal peptide.
27. The method of claim 26, wherein at least one CTP is encoded by an amino acid

sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.

28. The method of any one of claims 26-27, wherein at least one CTP is glycosylated.

29. The method of any one of claims 26-28, wherein at least one CTP is truncated.

30. The method of any one of claims 26-29, wherein at least one CTP is attached to said coagulation factor via a linker.

31. The method of claim 30, wherein said linker is a peptide bond.

32. The method of claim 31, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

33. A method of improving the area under the curve (AUC) of a Factor VII (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby improving the AUC of said coagulation factor, wherein said coagulation factor does not include a signal peptide.

34. The method of claim 33, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.

35. The method of any one of claims 33-34, wherein at least one CTP is glycosylated.

36. The method of any one of claims 33-35, wherein at least one CTP is truncated.

37. The method of any one of claims 33-36, wherein at least one CTP is attached to said coagulation factor via a linker.

38. The method of claim 35, wherein said linker is a peptide bond.

39. The method of claim 38, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

40. A method of reducing the dosing frequency of a Factor VIIa (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby reducing the dosing frequency of said coagulation factor, wherein said coagulation factor does not include a signal peptide.

41. The method of claim 40, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.

42. The method of any one of claims 40-41, wherein at least one CTP is glycosylated.

43. The method of any one of claims 40-42, wherein at least one CTP is truncated.

44. The method of any one of claims 40-43, wherein at least one CTP is attached to said coagulation factor via a linker.

45. The method of claim 44, wherein said linker is a peptide bond.

46. The method of claim 45, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

47. A method of preventing or treating a blood clotting or coagulation disorder in a subject, the method comprising the step of administering a CTP-modified coagulation factor polypeptide of any one of claims 1-7 to said subject, thereby preventing or treating a blood clotting or coagulation disorder in said subject.

48. The method of claim 47, wherein said disorder is hemophilia.

49. The method of claim 48, wherein said hemophilia comprises hemophilia A or hemophilia B with inhibitors.

50. The method of any one of claims 47-49, wherein said subject is a human adult.

51. The method of any one of claims 47-49, wherein said subject is a human child.

52. The method of any one of claims 47-49, wherein said administering is via a subcutaneous route.

53. The method of any one of claims 47-49, wherein said administering is via the intravenous route.

54. A CTP-modified polypeptide consisting of a Factor VII coagulation factor and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein said coagulation factor is activated and does not include a signal peptide.

55. The CTP-modified polypeptide of claim 54, wherein the sequence of at least one CTP is selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

56. The CTP-modified polypeptide of any one of claims 54-55, wherein at least one CTP is glycosylated.

57. The CTP-modified polypeptide of any one of claims 54-56, wherein at least one CTP is truncated.

58. The CTP-modified polypeptide of claim 57, wherein at least one CTP is attached to said

coagulation factor via a linker.

59. The CTP-modified polypeptide of claim 58, wherein said linker is a peptide bond.

60. The CTP-modified polypeptide of any one of claims 54-59, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

61. The CTP-modified polypeptide of any one of claims 54-60, wherein said activated FVIIa-CTP_3 comprises a light chain and a heavy chain linked by a disulfide bond.

62. The CTP-modified polypeptide of claim 61, wherein separation of said light chain and said heavy chain on an SDS-PAGE gel occurs under denaturing conditions, and wherein said light chain migrates at about 25 kDa molecular weight and said heavy chain migrates at about 60 kDa molecular weight.

63. A pharmaceutical formulation comprising a buffer, a tonicity agent, and a CTP-modified polypeptide consisting of an activated Factor VII coagulation factor and three chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said coagulation factor, wherein said polypeptide does not include a signal peptide.

64. The pharmaceutical formulation of claim 63, wherein said buffer comprises 20 mM citrate.

65. The pharmaceutical formulation of any one of claims 63-64, wherein said tonicity agent is 150 mM sodium chloride.

66. The pharmaceutical formulation of any one of claims 63-65, wherein said formulation is a liquid formulation.

67. The pharmaceutical formulation of any one of claims 63-66, wherein said formulation is at a pH of about 6.4.

68. The pharmaceutical formulation of any one of claims 63-67, wherein said formulation comprises 20 mM citrate, 13.3 mM Glycine, 150 mM sodium chloride, at a pH of about 6.4.

69. The pharmaceutical formulation of any one claims 63-68, wherein the sequence of at least one CTP consists of an amino acid sequence selected from the group consisting of: SEQ ID NO: 1 and SEQ ID NO: 2.

70. The pharmaceutical formulation of any one of claims 63-69, wherein said at least one CTP is glycosylated.

71. The pharmaceutical formulation of any one of claims 63-70, wherein said at least one CTP is truncated.

72. The pharmaceutical formulation of any one of claims 63-71, wherein at least one CTP is optionally attached to said polypeptide sequence of interest via a linker.
73. The pharmaceutical formulation of claim 72, wherein the linker is a peptide bond.
74. The pharmaceutical formulation of any one of claims 63-73, wherein the sequence of said CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.
75. The pharmaceutical formulation of any one of claims 63-74, wherein said polypeptide is administered daily, every other day, every three days, once weekly, twice weekly, or once every other week, or any combination thereof.
76. The pharmaceutical formulation of any one of claims 63-75, wherein said subject is a human adult or a human child.
77. The pharmaceutical formulation of any one of claims 63-74 and 76, for a daily, every other day, once a week, twice a week, or once every other week administration to a subject in need thereof.
78. The pharmaceutical formulation of claim 77, wherein said administration is intravenous or subcutaneous administration.
79. A pharmaceutical composition comprising the CTP-modified polypeptide of any one of claims 54-62, and a pharmaceutically acceptable carrier.
80. A pharmaceutical composition comprising the pharmaceutical formulation of any one of claims 63-78.
81. A method of extending the biological half-life of a Factor VII (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby extending the biological half-life of said coagulation factor, wherein said coagulation factor is activated and does not include a signal peptide.
82. The method of claim 81, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.
83. The method of any one of claims 81-82, wherein at least one CTP is glycosylated.
84. The method of any one of claims 81-83, wherein at least one CTP is truncated.
85. The method of any one of claims 81-84, wherein at least one CTP is attached to said coagulation factor via a linker.
86. The method of claim 85, wherein said linker is a peptide bond.

87. The method of claim 86, wherein the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

88. A method of improving the area under the curve (AUC) of a Factor VII (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby improving the AUC of said coagulation factor, wherein said coagulation factor is activated and does not include a signal peptide.

89. The method of claim 88, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.

90. The method of any one of claims 88-89, wherein at least one CTP is glycosylated.

91. The method of any one of claims 88-90, wherein at least one CTP is truncated.

92. The method of any one of claims 88-91, wherein at least one CTP is attached to said coagulation factor via a linker.

93. The method of claim 92, wherein said linker is a peptide bond.

94. The method of any one of claims 88-93, wherein the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

95. A method of reducing the dosing frequency of a Factor VIIa (FVII) coagulation factor, comprising the step of attaching three chorionic gonadotropin carboxy terminal peptides (CTP) to the carboxy terminus of said FVII coagulation factor, thereby reducing the dosing frequency of said coagulation factor, wherein said activated form of the FVII coagulation factor does not include a signal peptide.

96. The method of claim 95, wherein at least one CTP is encoded by an amino acid sequence selected from the group consisting of: SEQ ID NO:1 and SEQ ID NO: 2.

97. The method of any one of claims 95-96, wherein at least one CTP is glycosylated.

98. The method of any one of claims 95-97, wherein at least one CTP is truncated.

99. The method of any one of claims 95-98, wherein at least one CTP is attached to said coagulation factor via a linker.

100. The method of claim 99, wherein said linker is a peptide bond.

101. The method of any one of claims 95-100, wherein the sequence of said activated CTP-modified coagulation factor is as set forth in SEQ ID NO: 46.

102. A method of preventing or treating a blood clotting or coagulation disorder in a subject, the method comprising the step of administering an activated CTP-modified coagulation factor polypeptide of any one of claims 54-62 to said subject, thereby preventing or treating a blood clotting or coagulation disorder in said subject.

103. The method of claim 102, wherein said disorder is hemophilia.

104. The method of claim 103, wherein said hemophilia comprises hemophilia A or hemophilia B with inhibitors.

105. The method of any one of claims 102-104, wherein said subject is a human adult.

106. The method of any one of claims 102-105, wherein said subject is a human child.

107. The method of any one of claims 102-106, wherein said administering is via a subcutaneous route.

108. The method of any one of claims 102-107, wherein said administering is via the intravenous route.

109. Use of a CTP-modified coagulation factor polypeptide of any one of claims 1-7 for preventing or treating a blood clotting or coagulation disorder in a subject, the use comprising the step of administering a CTP-modified coagulation factor polypeptide of to said subject, thereby preventing or treating a blood clotting or coagulation disorder in said subject.

110. Use of claim 109, wherein said disorder is hemophilia.

111. Use of claim 109, wherein said hemophilia comprises hemophilia A or hemophilia B with inhibitors.

112. Use of any one of claims 47-49, wherein said subject is a human adult.

113. Use of any one of claims 47-49, wherein said subject is a human child.

114. Use of any one of claims 47-49, wherein said administering is via a subcutaneous route.

115. Use of any one of claims 47-49, wherein said administering is via the intravenous route.

116. Use of a CTP-modified coagulation factor polypeptide of any one of claims 54-62 for preventing or treating a blood clotting or coagulation disorder in a subject, the use comprising the step of administering an activated CTP-modified coagulation factor polypeptide, thereby preventing or treating a blood clotting or coagulation disorder in said subject.

117. The use of claim 116, wherein said disorder is hemophilia.

118. The use of claim 117, wherein said hemophilia comprises hemophilia A or hemophilia B with inhibitors.

119. The use of any one of claims 116-118, wherein said subject is a human adult.

120. The use of any one of claims 116-119, wherein said subject is a human child.

121. The use of any one of claims 116-120, wherein said administering is via a subcutaneous route.

122. The use of any one of claims 116-121, wherein said administering is via the intravenous route.

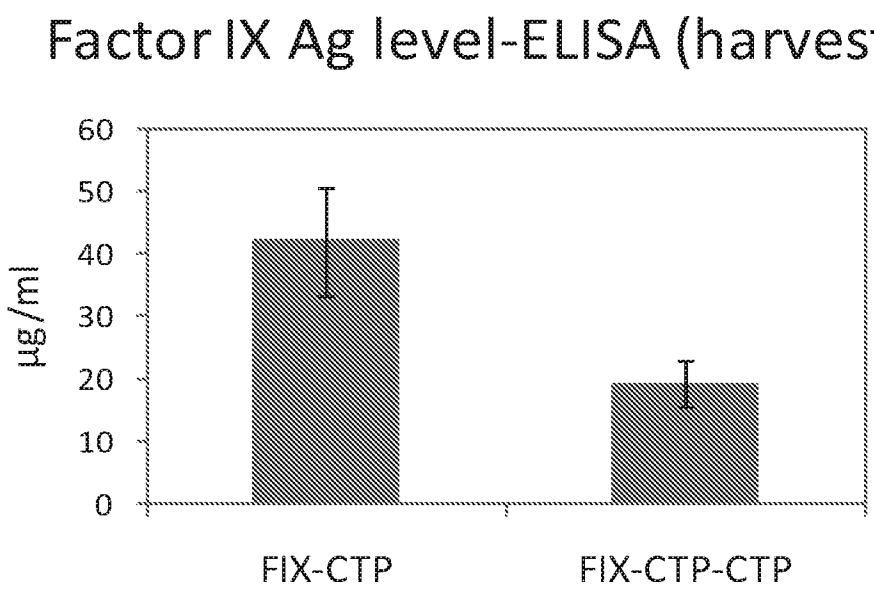


FIGURE 1A

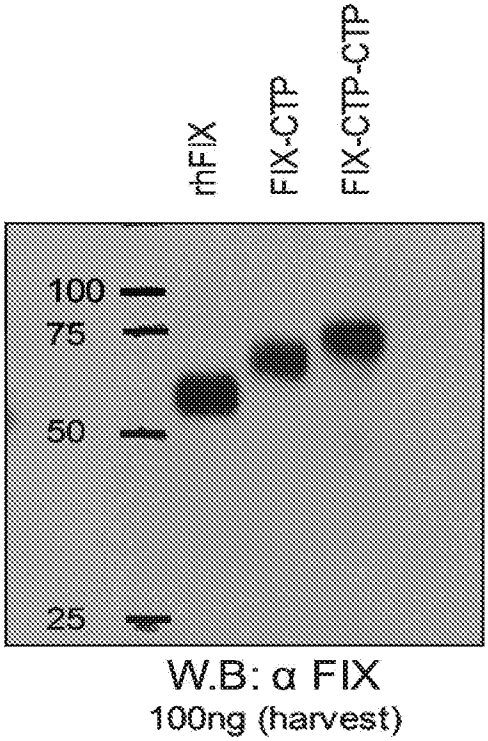


FIGURE 1B

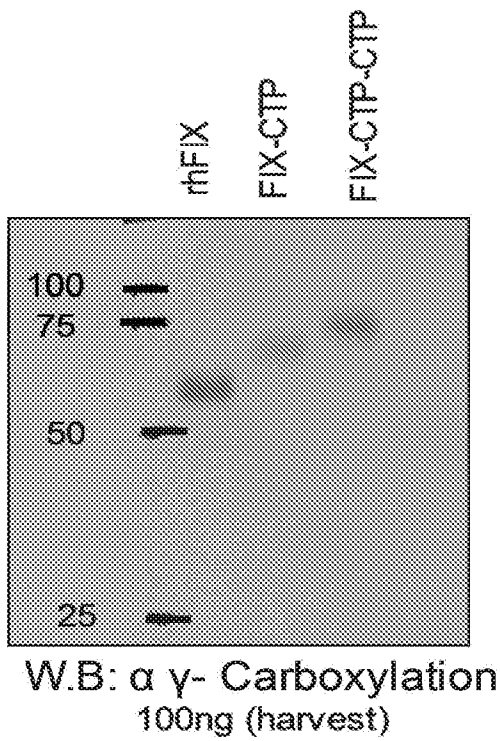


FIGURE 1C

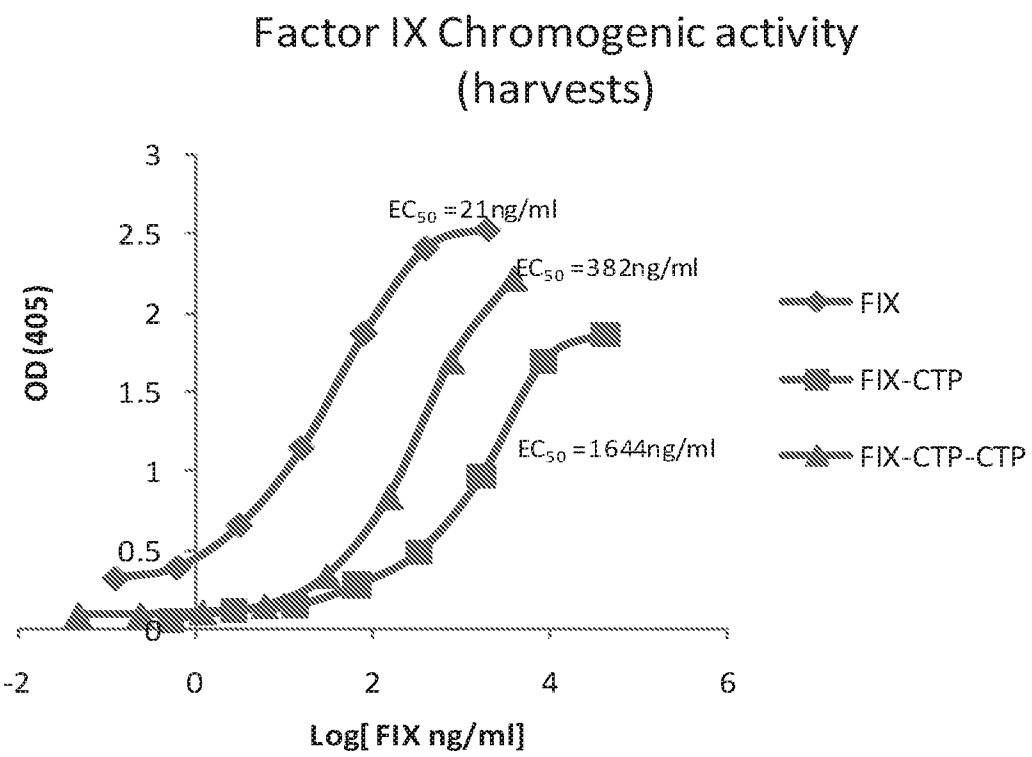


FIGURE 2

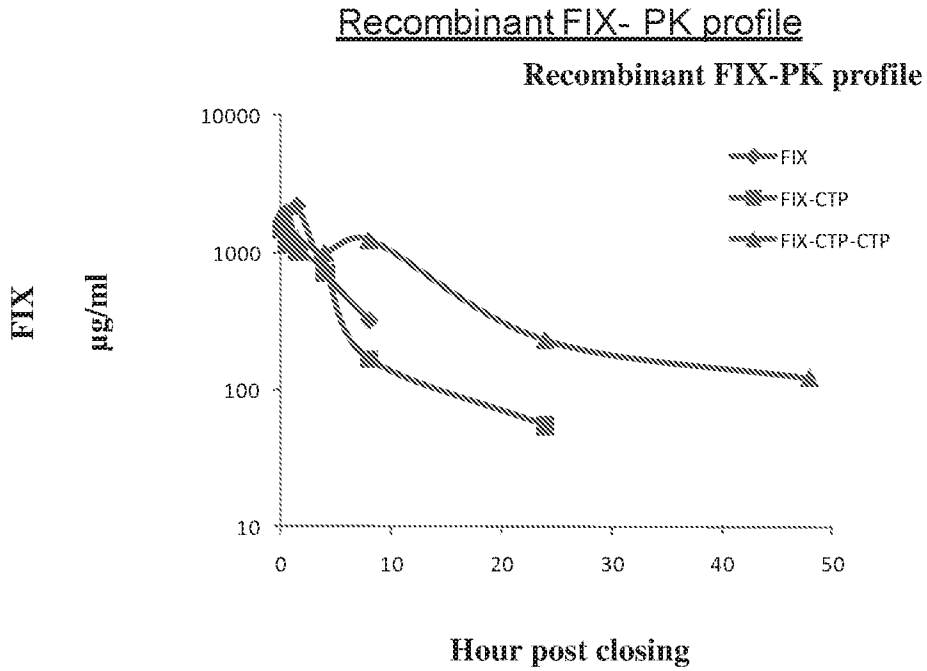


FIGURE 3

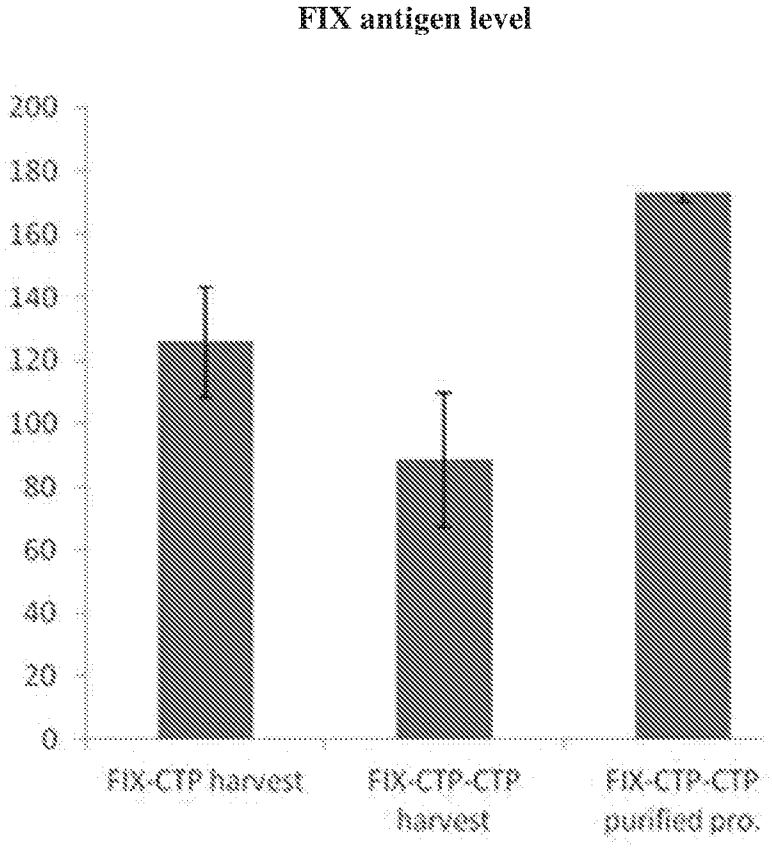
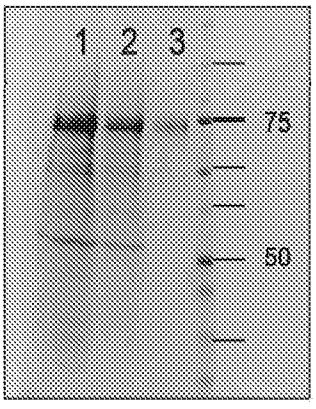
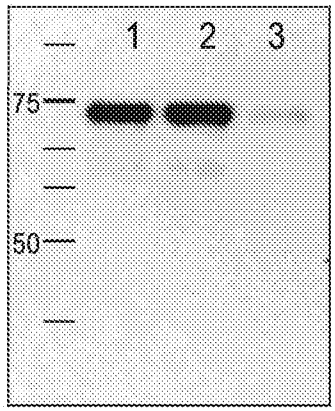


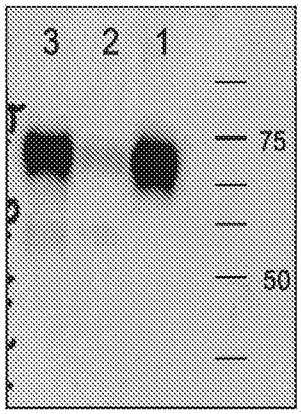
FIGURE 4



Coomassie SDS-PAGE
FIGURE 5A



W.B: anti FIX
FIGURE 5B



W.
FIGURE 5C

1-FIX-$(CTP)_2$ Harvest
2-Unbound
3-Conc. elution (MOD3012)

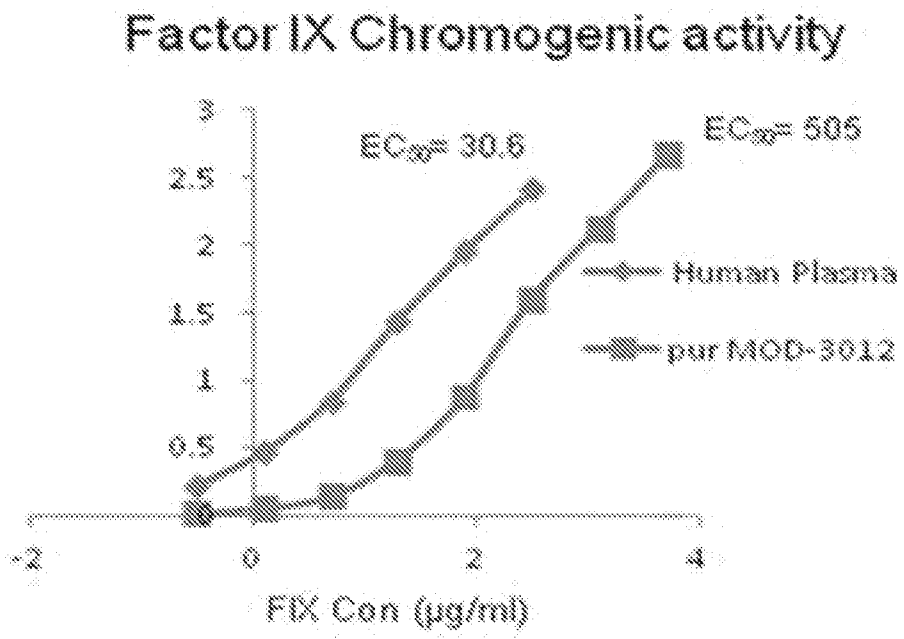


FIGURE 6

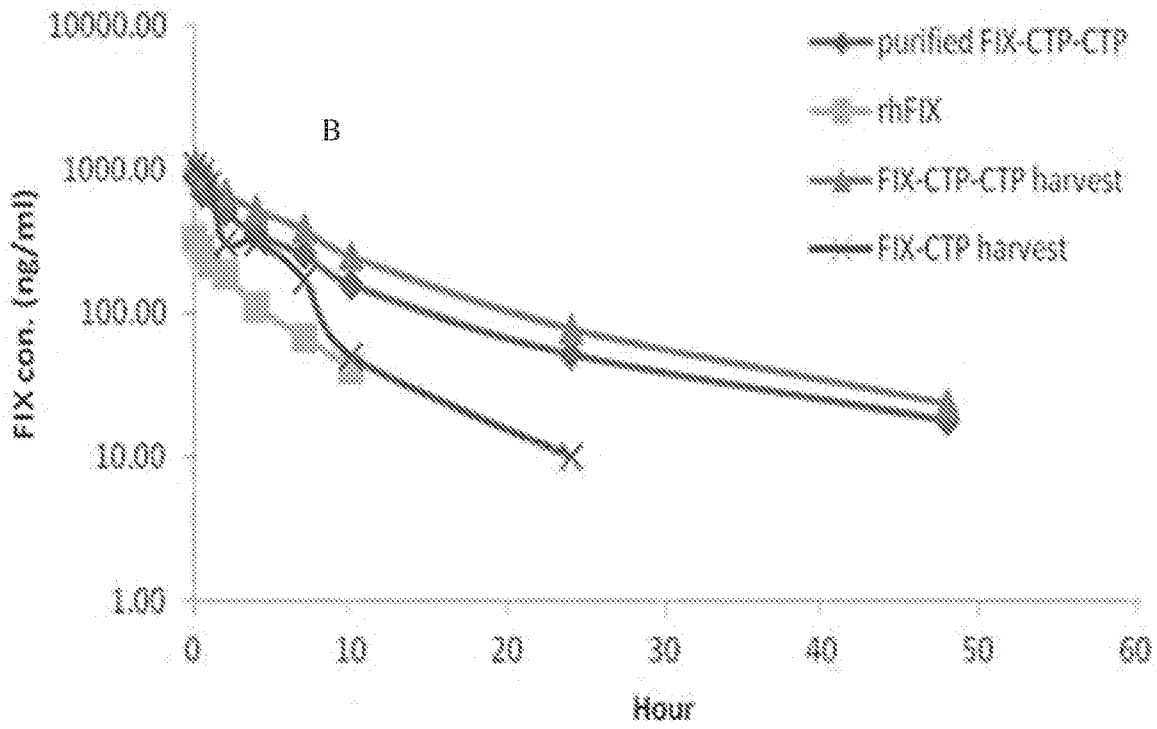


FIGURE 7

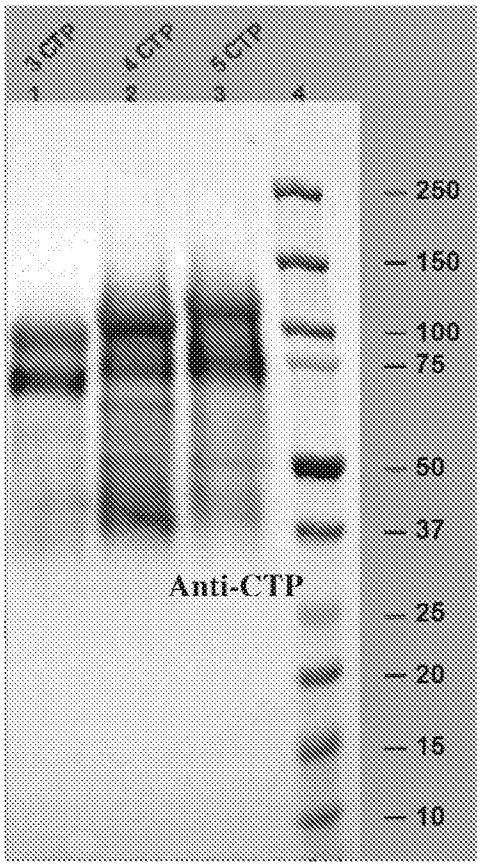


FIGURE 8A

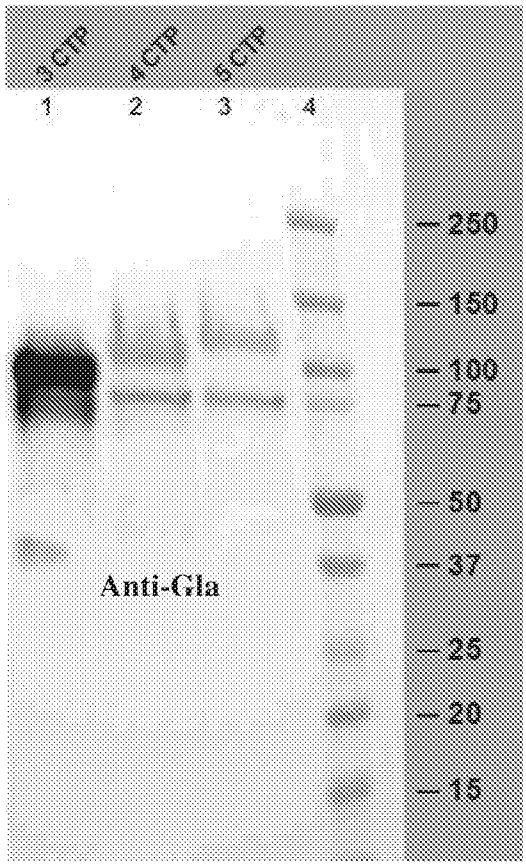


FIGURE 8B

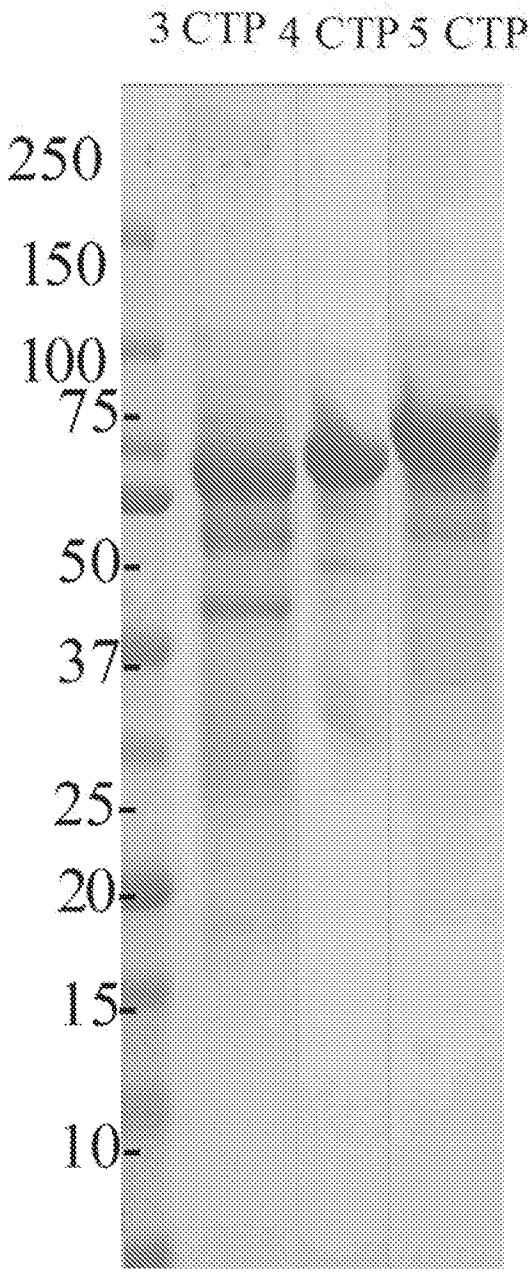


FIGURE 9

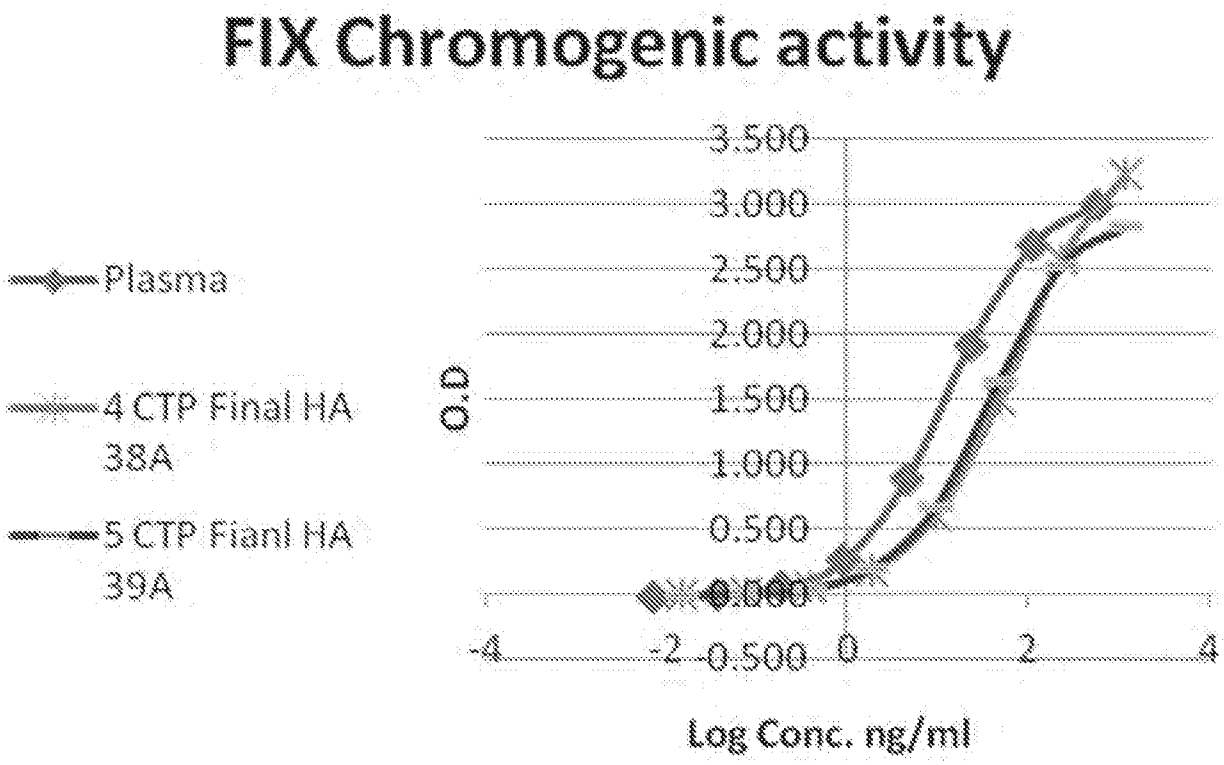

FIX Chromogenic activity
Plasma
4 CTP Final HA 38A
5 CTP Fianl HA 39A
O.D.
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
-4
-2
0
2
4
Log Conc. ng/ml

FIGURE 10

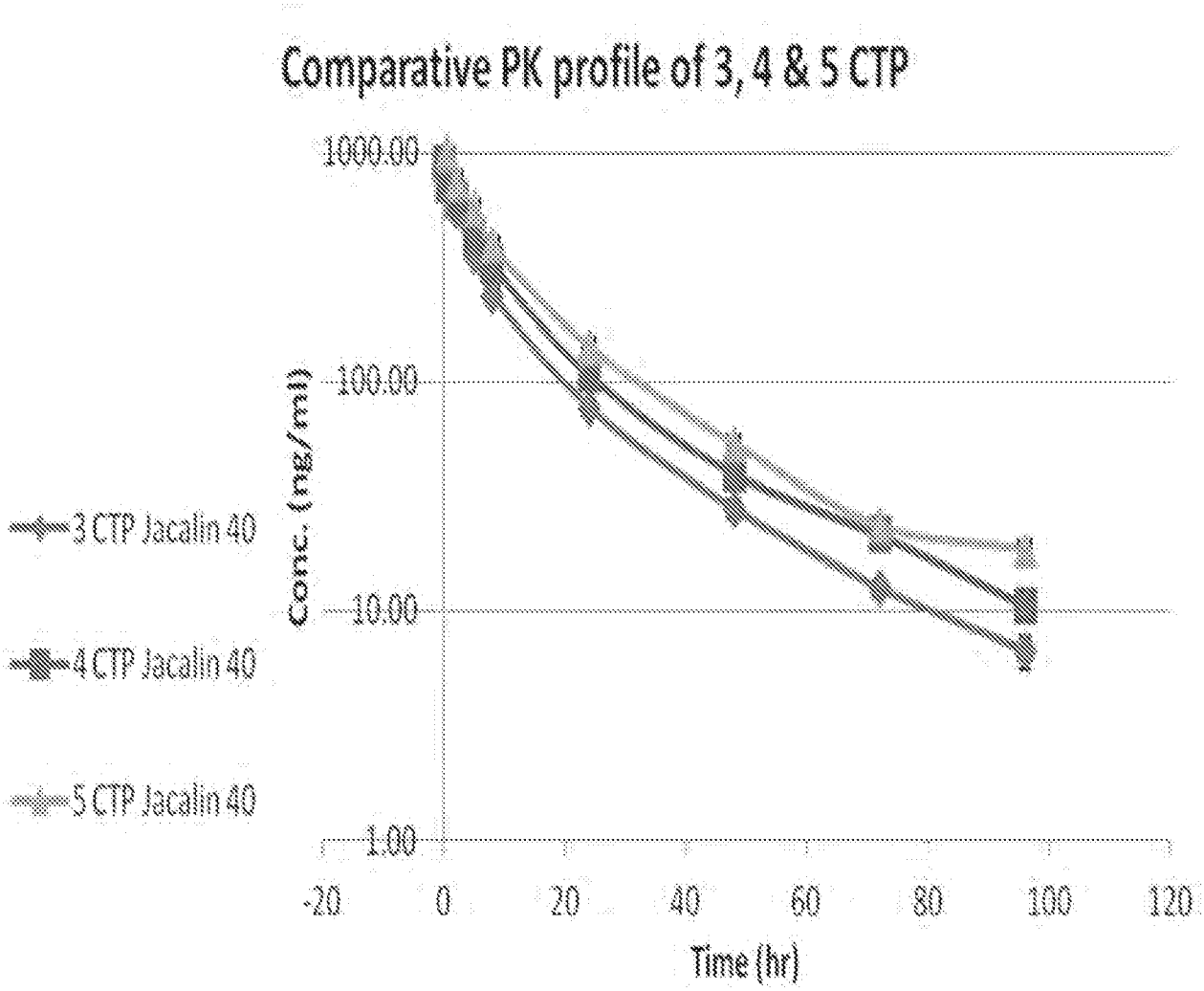

Comparative PK profile of 3, 4 & 5 CTP
1000.00
100.00
10.00
1.00
Conc. (mg/ml)
3 CTP Jacalin 40
4 CTP Jacalin 40
5 CTP Jacalin 40
-20
0
20
40
60
80
100
120
Time (hr)

FIGURE 11

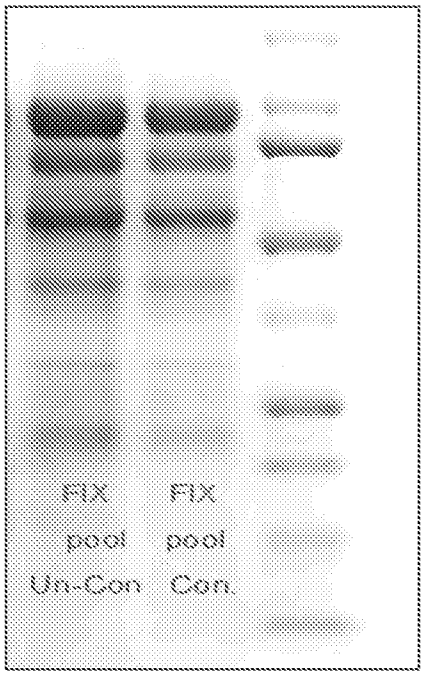


FIGURE 12A

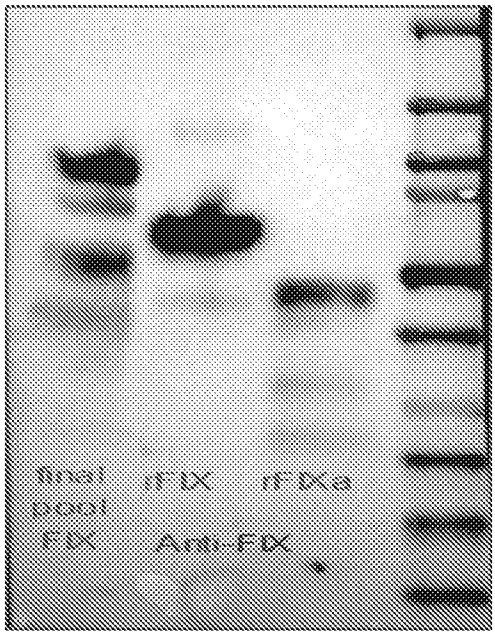


FIGURE 12B

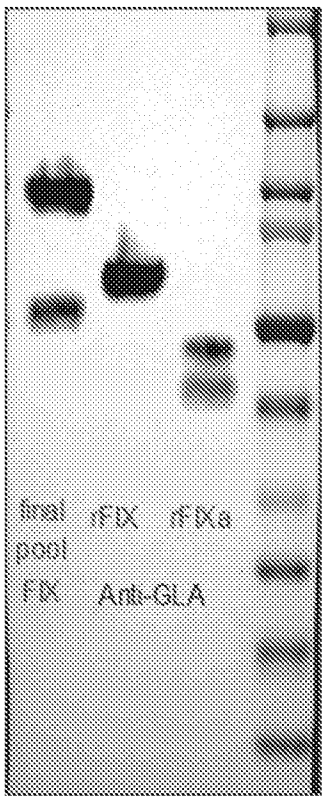


FIGURE 12C

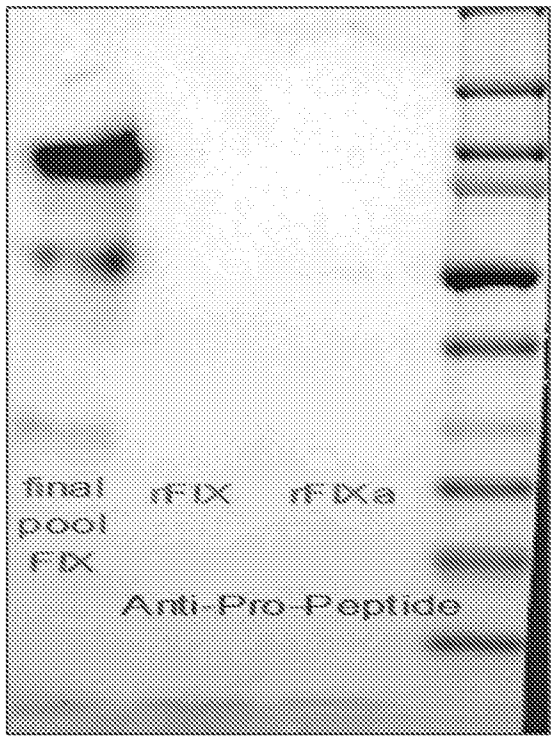


FIGURE 12D

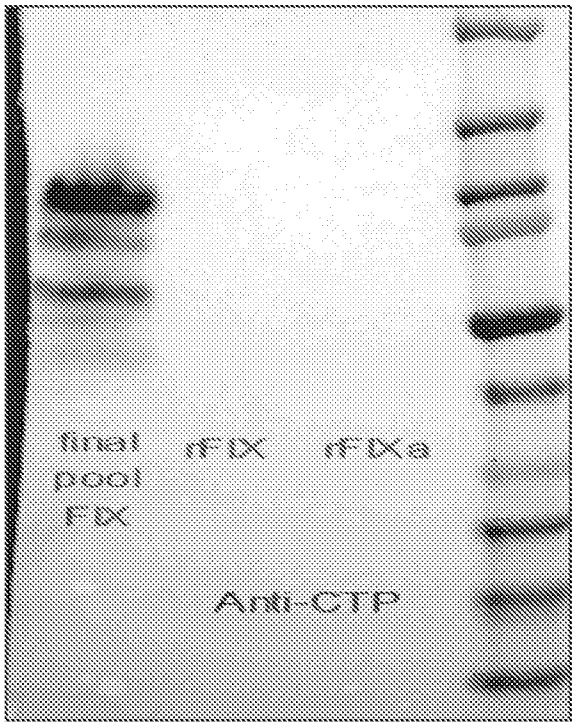


FIGURE 12E

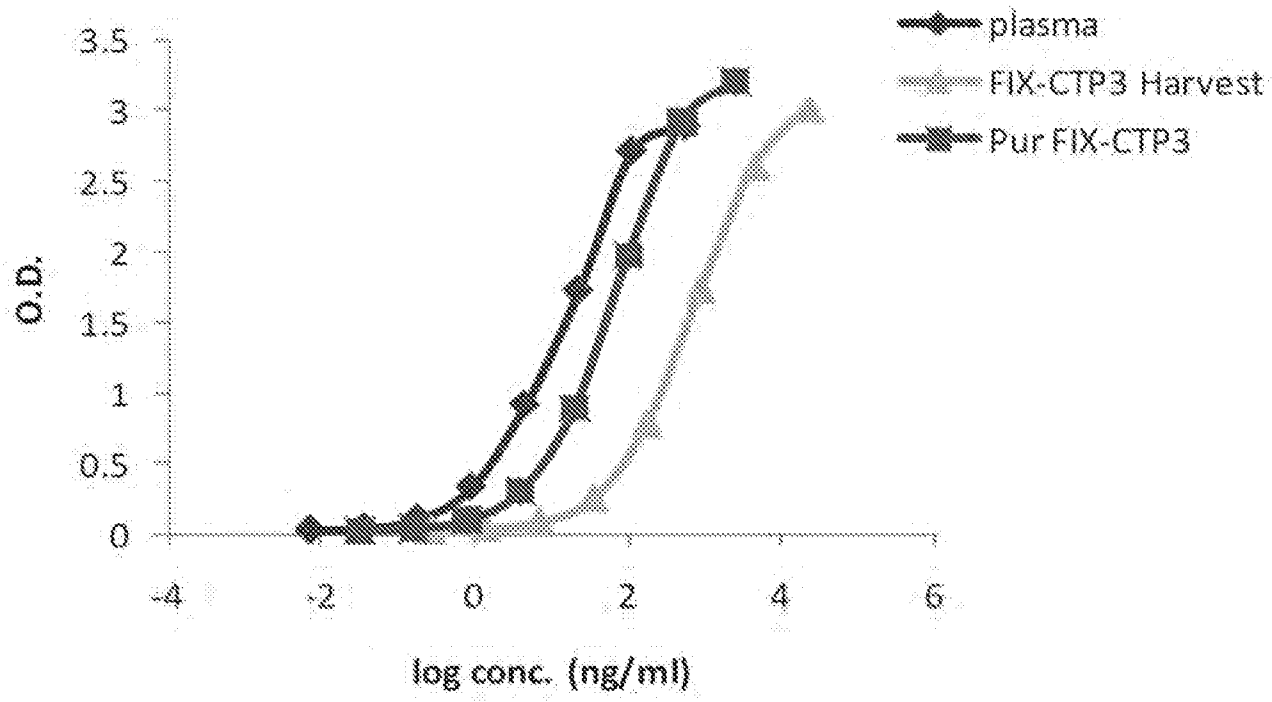

plasma
FIX-CTP3 Harvest
Pur FIX-CTP3
O.D.
3.5
3
2.5
2
1.5
1
0.5
0
-4
-2
0
2
4
6
log conc. (ng/ml)

FIGURE 13

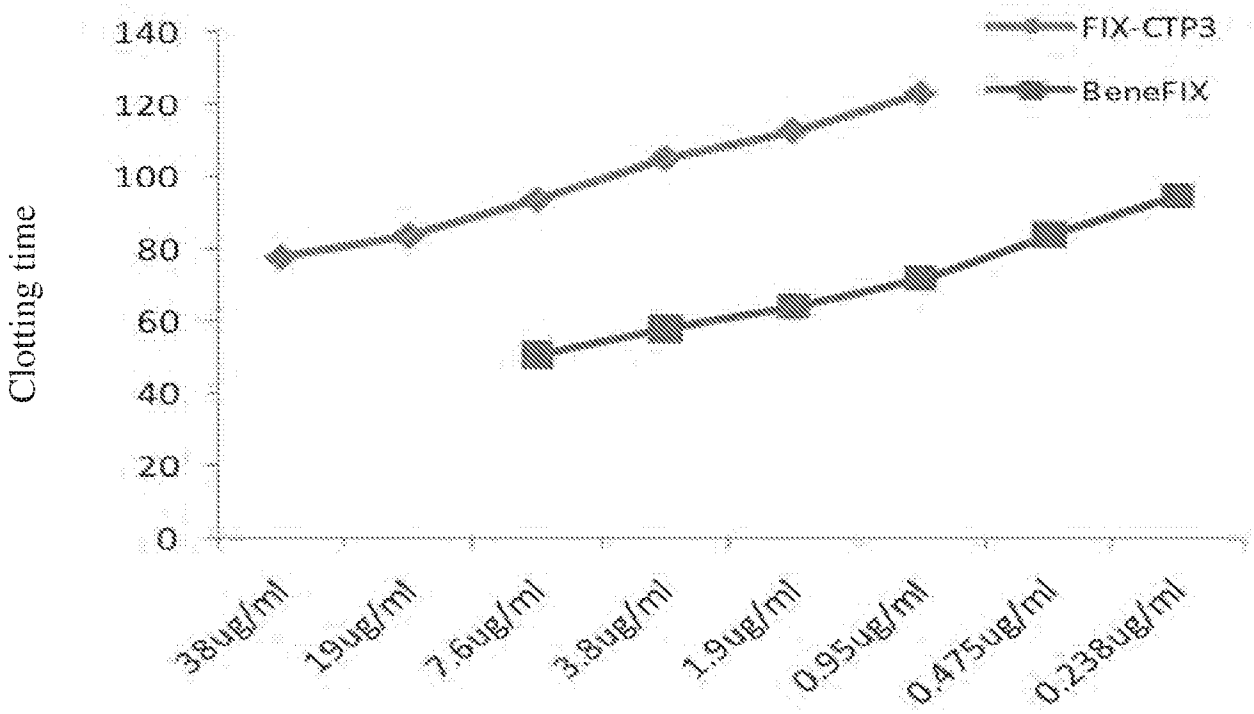

140
120
100
80
60
40
20
0
Clotting time
FIX-CTP3
BeneFIX
38ug/ml
19ug/ml
7.6ug/ml
3.8ug/ml
1.9ug/ml
0.95ug/ml
0.475ug/ml
0.238ug/ml

FIGURE 14

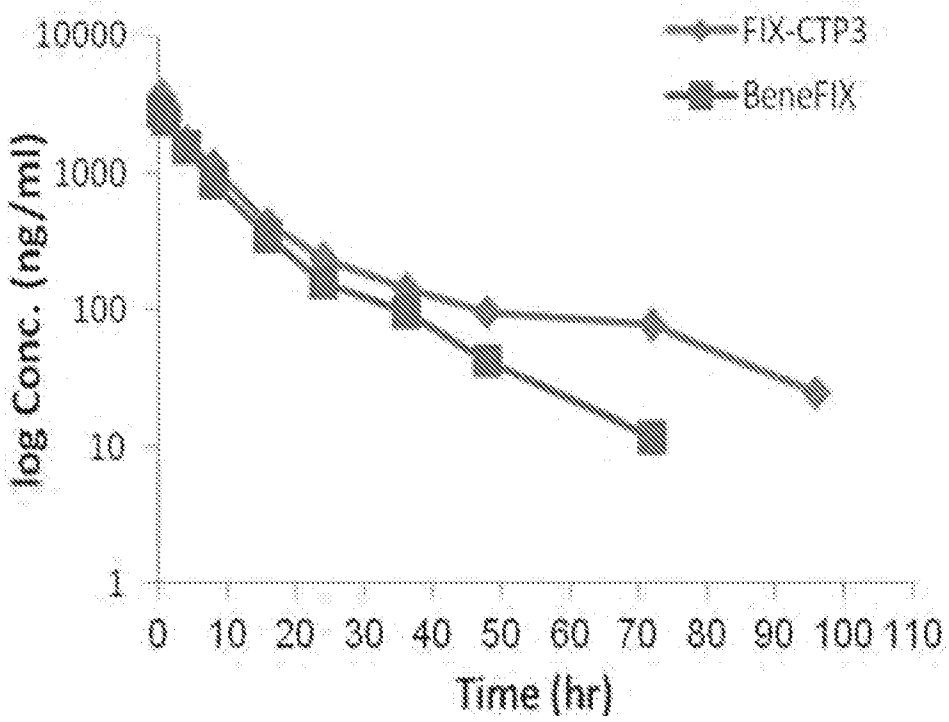

10000
1000
100
10
1
log Conc. (ng/ml)
0 10 20 30 40 50 60 70 80 90 100 110
Time (hr)
FIX-CTP3
BeneFIX

FIGURE 15

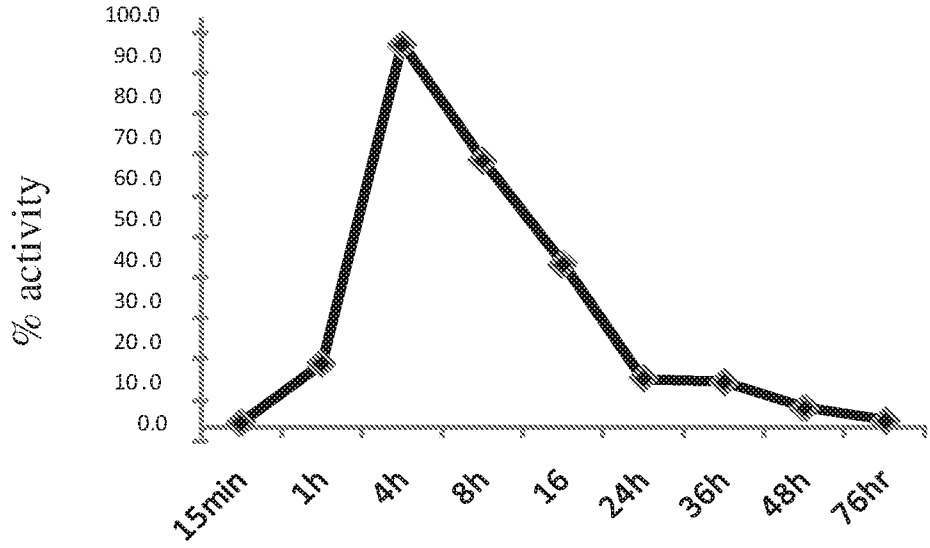


FIGURE 16A

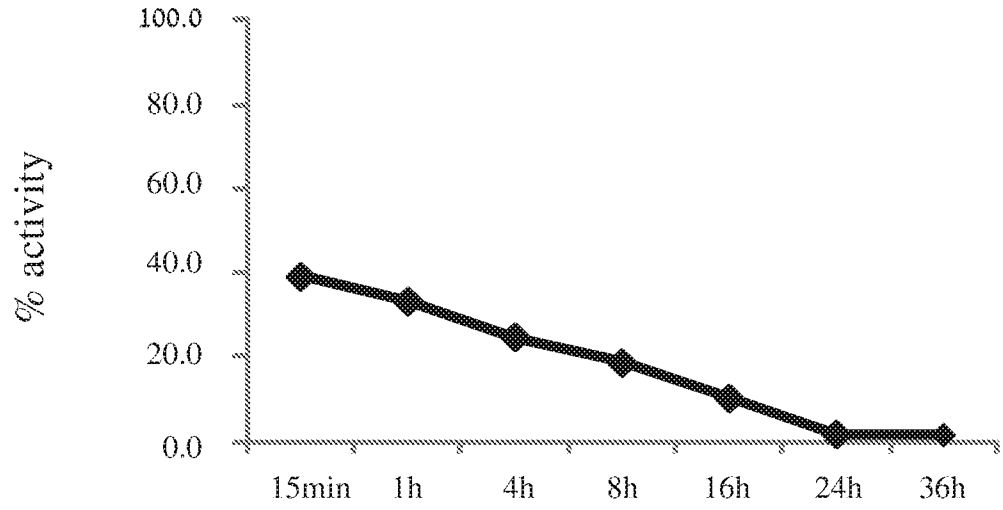


FIGURE 16B

First bleeding challenge :
Hemoglobin OD value

	FIX- CTP_3	BeneFIX	FIXKO
#1	6.84	10.26	19.92
#2	0.72	10.14	13.32
#3	4.68	11.16	16.38
#4	N	9.18	7.92
#5	7.86	7.77	9.72
#6	4.14	9.15	14.7

FIGURE 17A

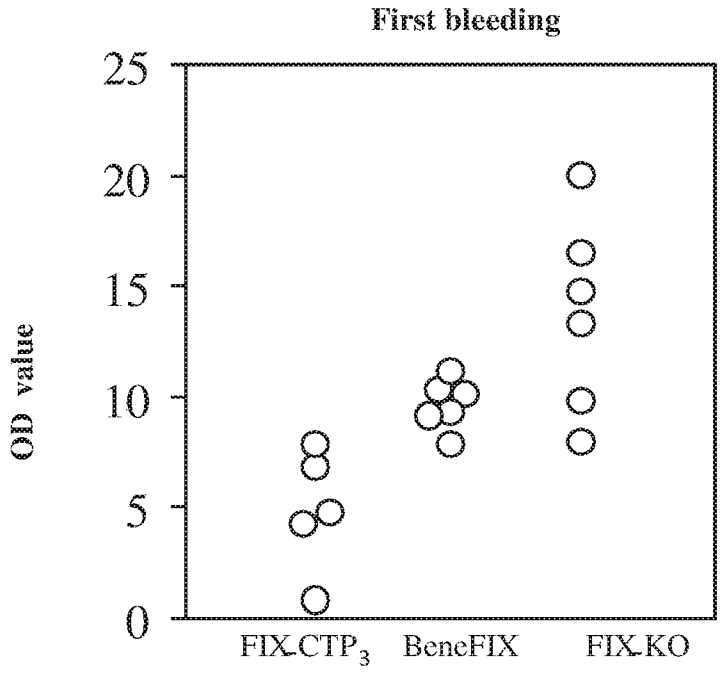


FIGURE 17B

First bleeding time (min)

	cohort# 1	cohort #3	FIXKO
#1	10	10	10
#2	5.12	10	8.17
#3	10	10	10
#4		10	10
#5	10	7	10
#6	10	10	10

FIGURE 17C

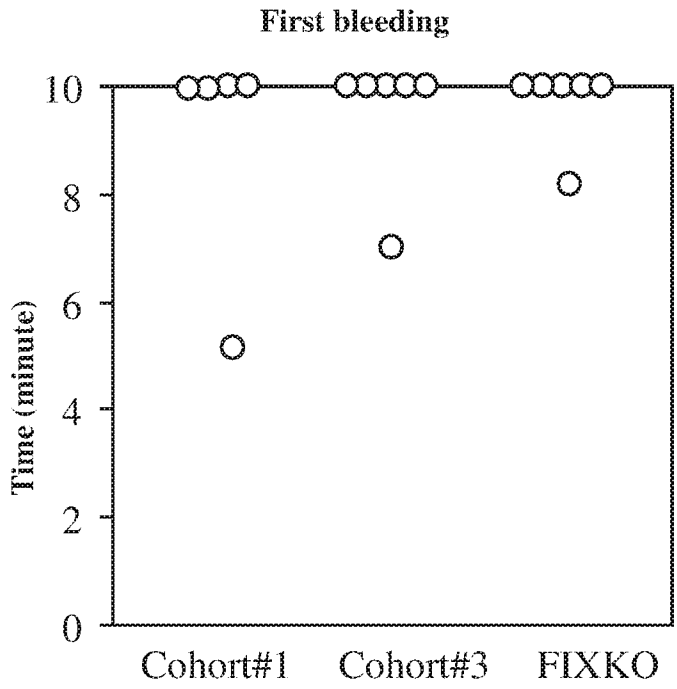


FIGURE 17D

Second bleeding OD value

	FIX-CTP$_3$	BeneFIX	FIXKO
#1	0.324	1.368	1.32
#2	0.358	0.516	0.43
#3	0.006	0.548	0.6
#4			1.26
#5	0.064	0.158	0.46
#6	0.045	0.992	0.384

FIGURE 18A

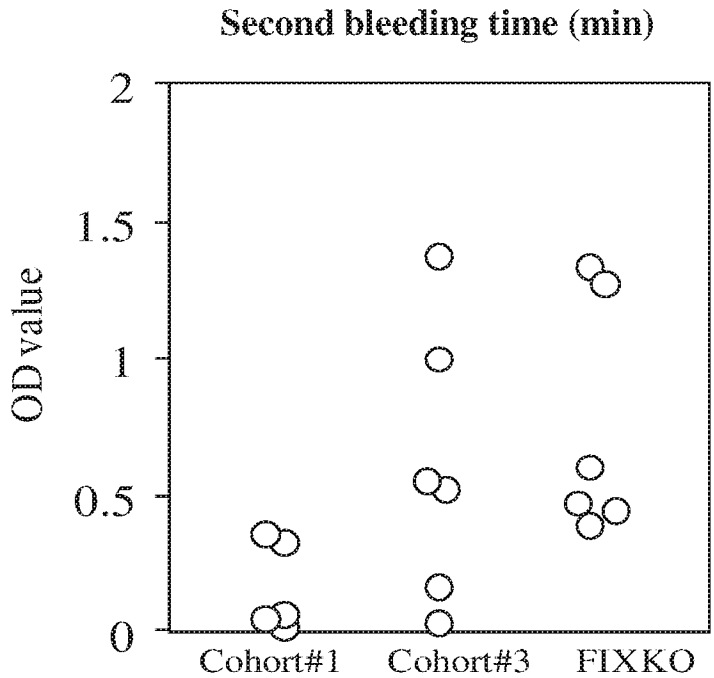


FIGURE 18B

	cohort#1	cohort #3	FIXKO
#1	4.63	10	10
#2	2.5	10	8.7
#3	1.2	10	7.13
#4		5	10
#5	3.87	7.4	10
#6	1.83	10	6.5

FIGURE 18C

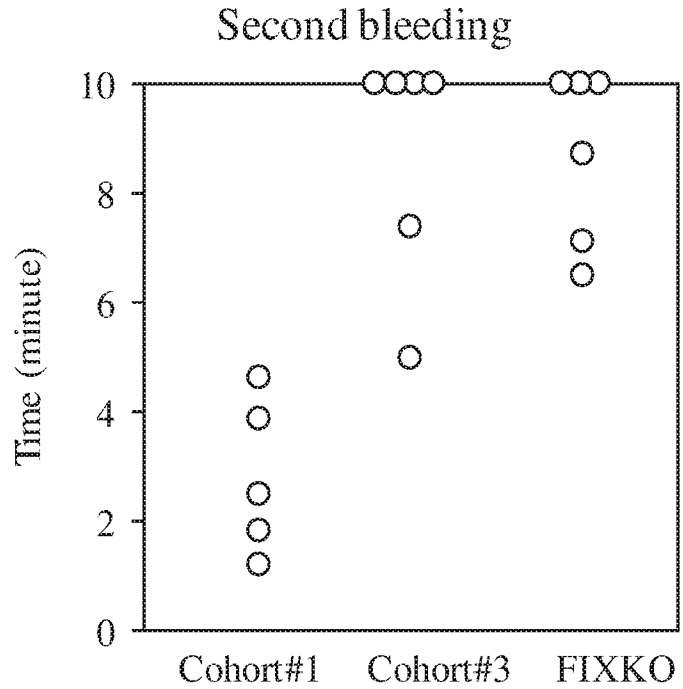


FIGURE 18D

FIGURE 19A

FIGURE 19B

FIGURE 19C

FIGURE 19D

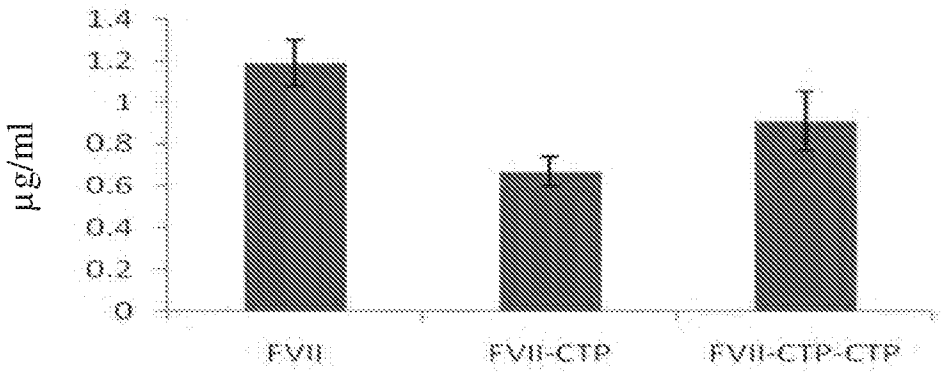


FIGURE 20A

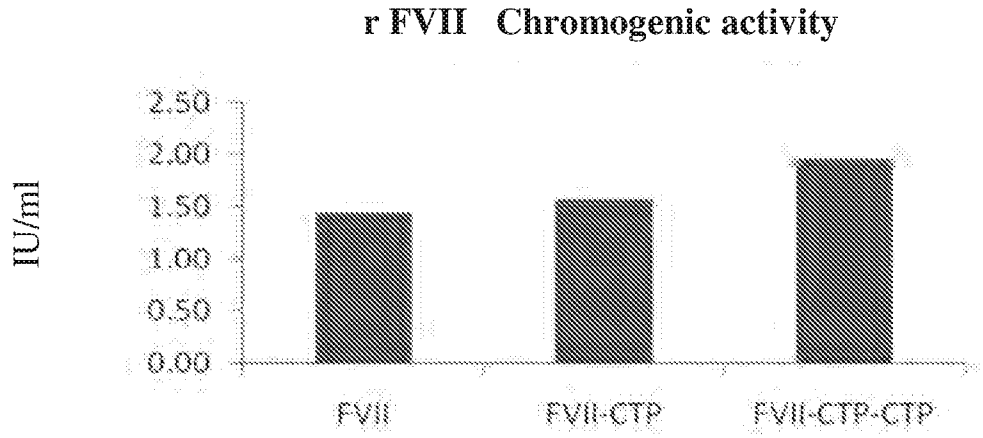


FIGURE 20B

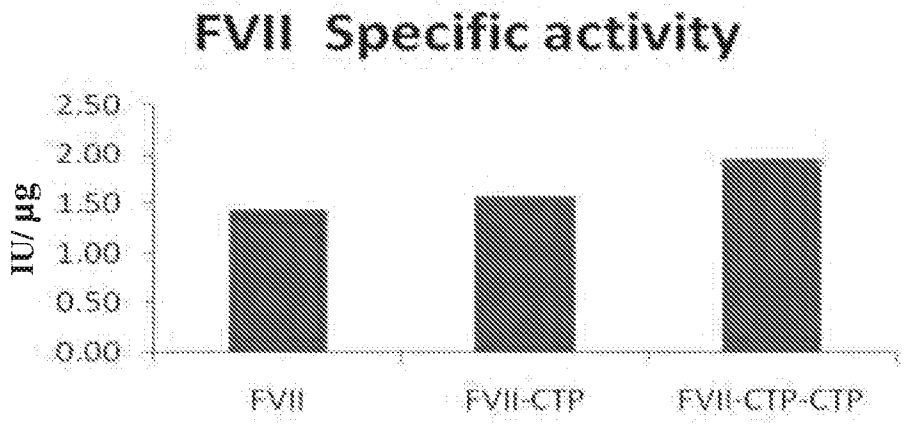


FIGURE 20C

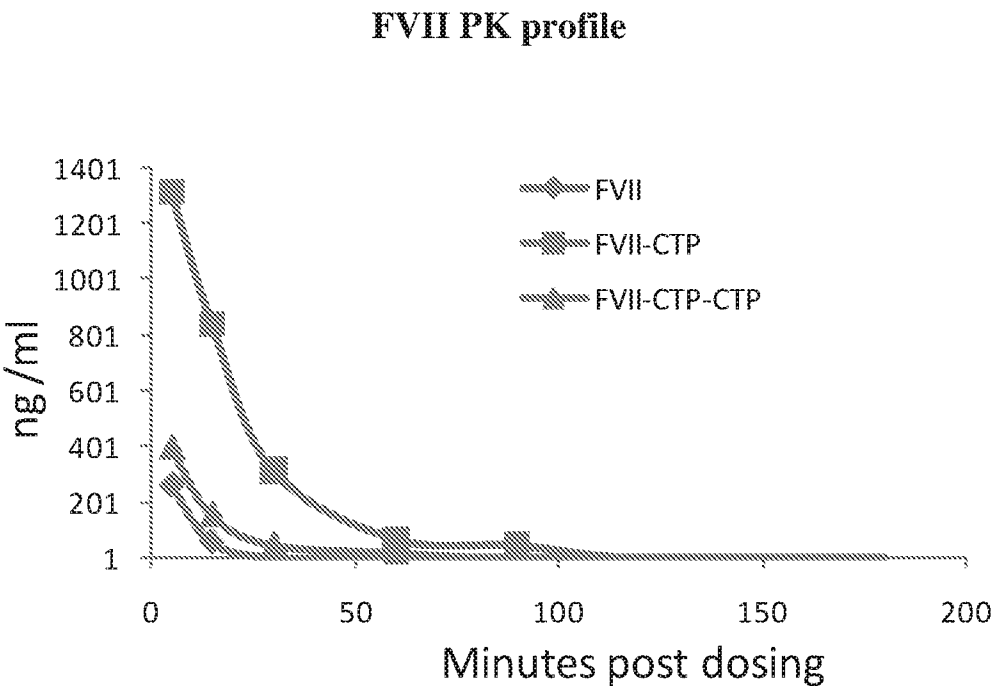


FIGURE 20D

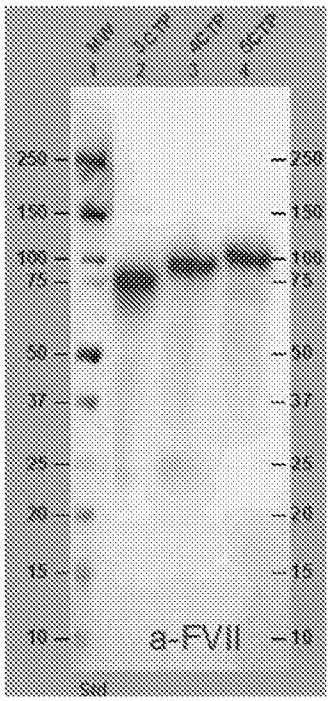


FIGURE 21A

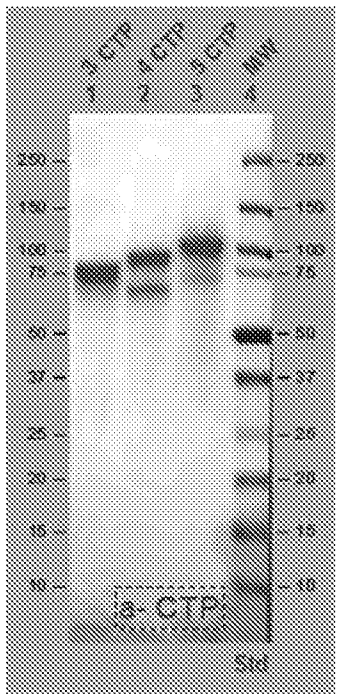


FIGURE 21B

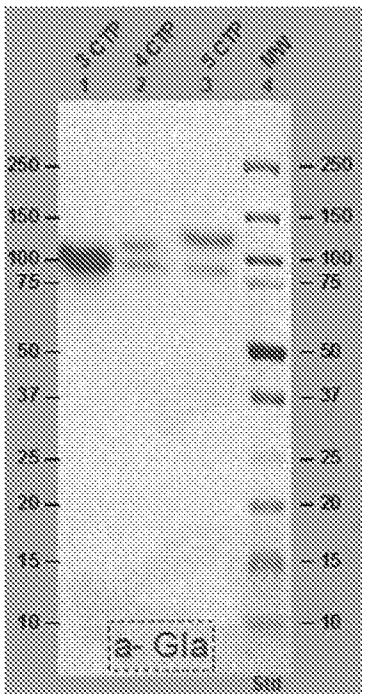


FIGURE 21C

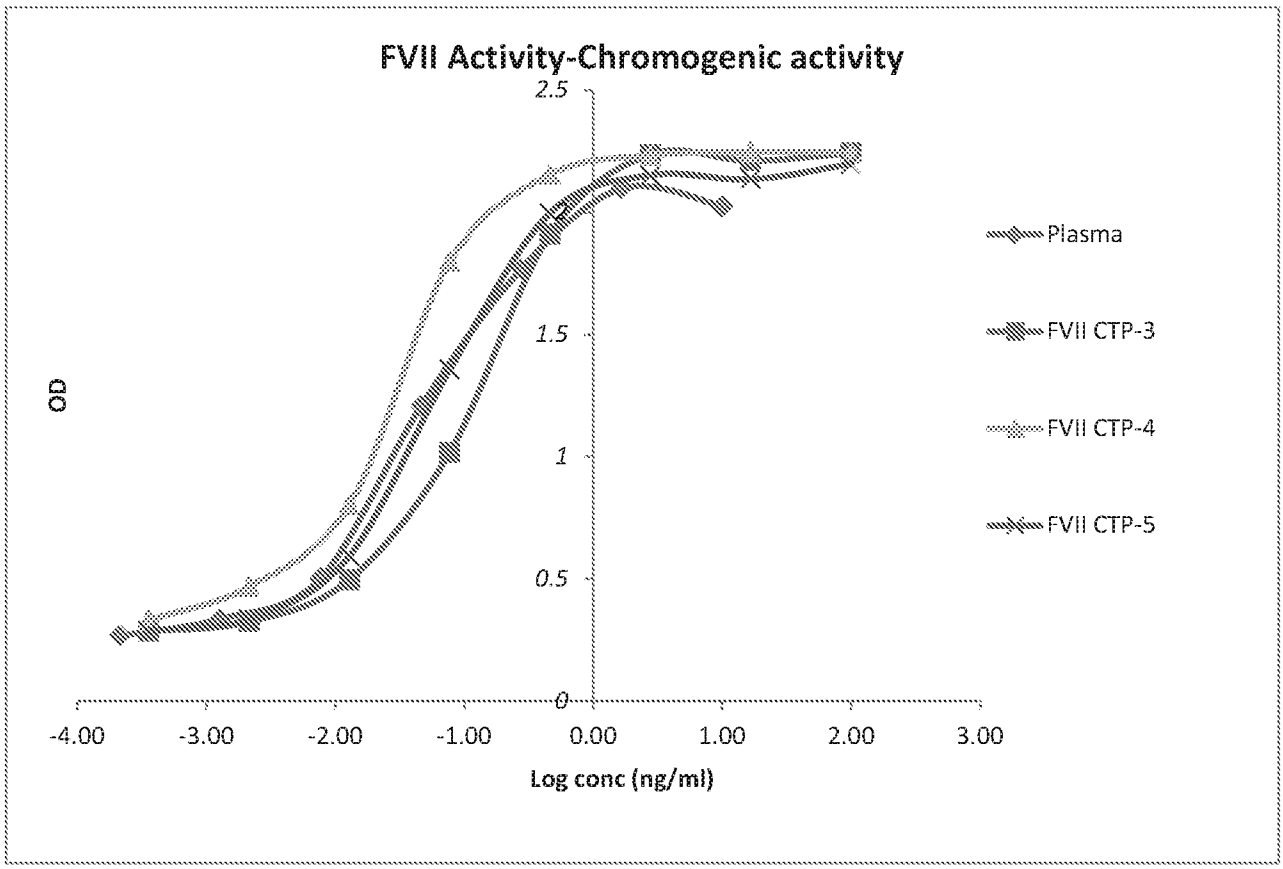

FVII Activity-Chromogenic activity
2.5
1.5
1
0.5
0
OD
-4.00
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
Log conc (ng/ml)
Plasma
FVII CTP-3
FVII CTP-4
FVII CTP-5

FIGURE 22

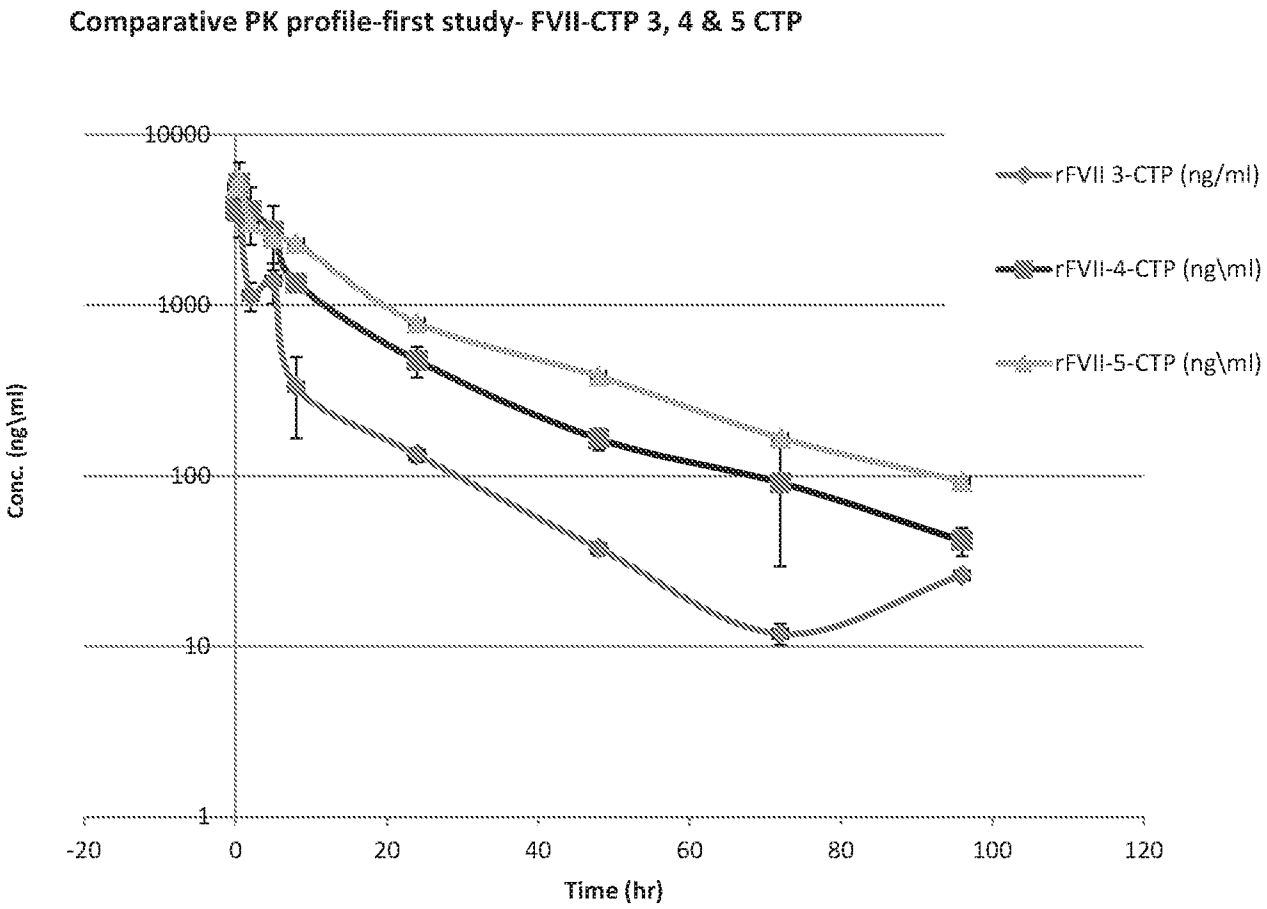

Comparative PK profile-first study- FVII-CTP 3, 4 & 5 CTP
10000
1000
100
10
1
Conc. (ng\ml)
-20
0
20
40
60
80
100
120
Time (hr)
rFVII 3-CTP (ng/ml)
rFVII-4-CTP (ng\ml)
rFVII-5-CTP (ng\ml)

FIGURE 23

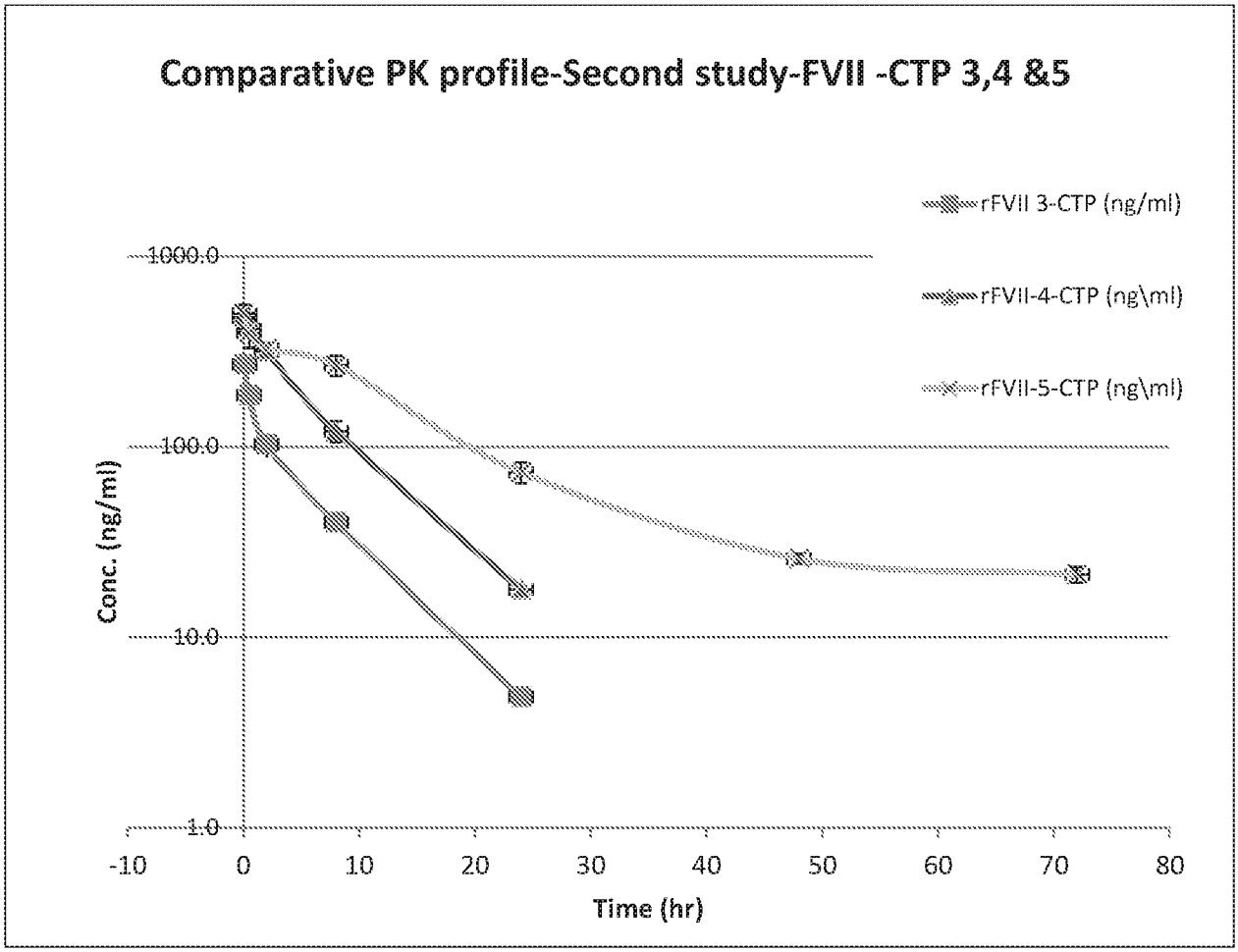

Comparative PK profile-Second study-FVII -CTP 3,4 &5
rFVII 3-CTP (ng/ml)
rFVII-4-CTP (ng\ml)
rFVII-5-CTP (ng\ml)
1000.0
100.0
10.0
1.0
Conc. (ng/ml)
-10
0
10
20
30
40
50
60
70
80
Time (hr)

FIGURE 24

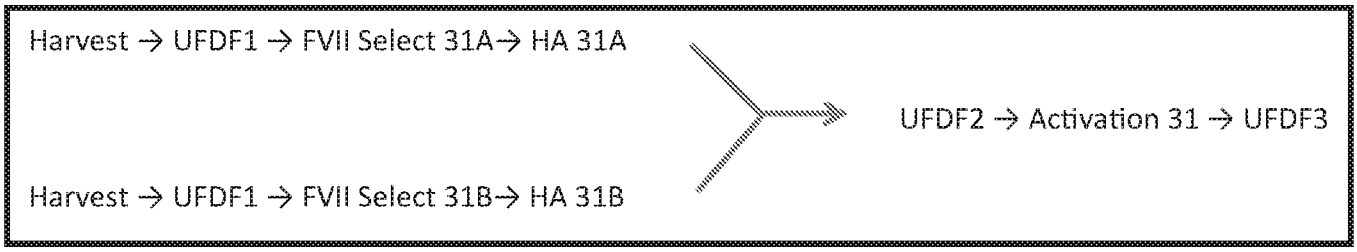


FIGURE 25A

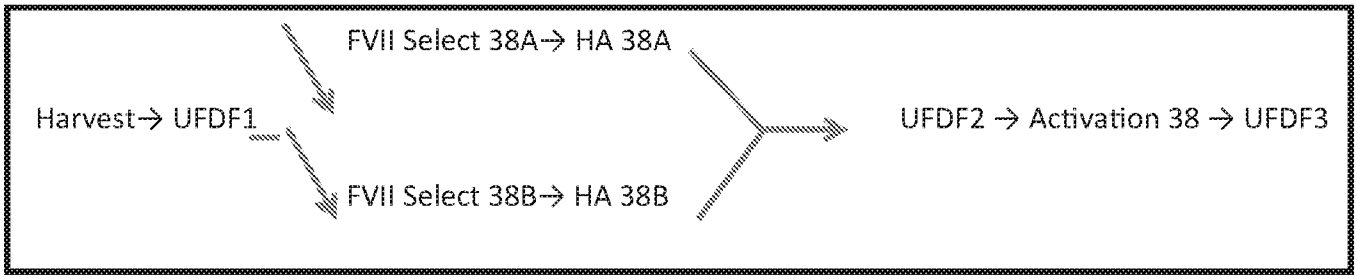


FIGURE 25B

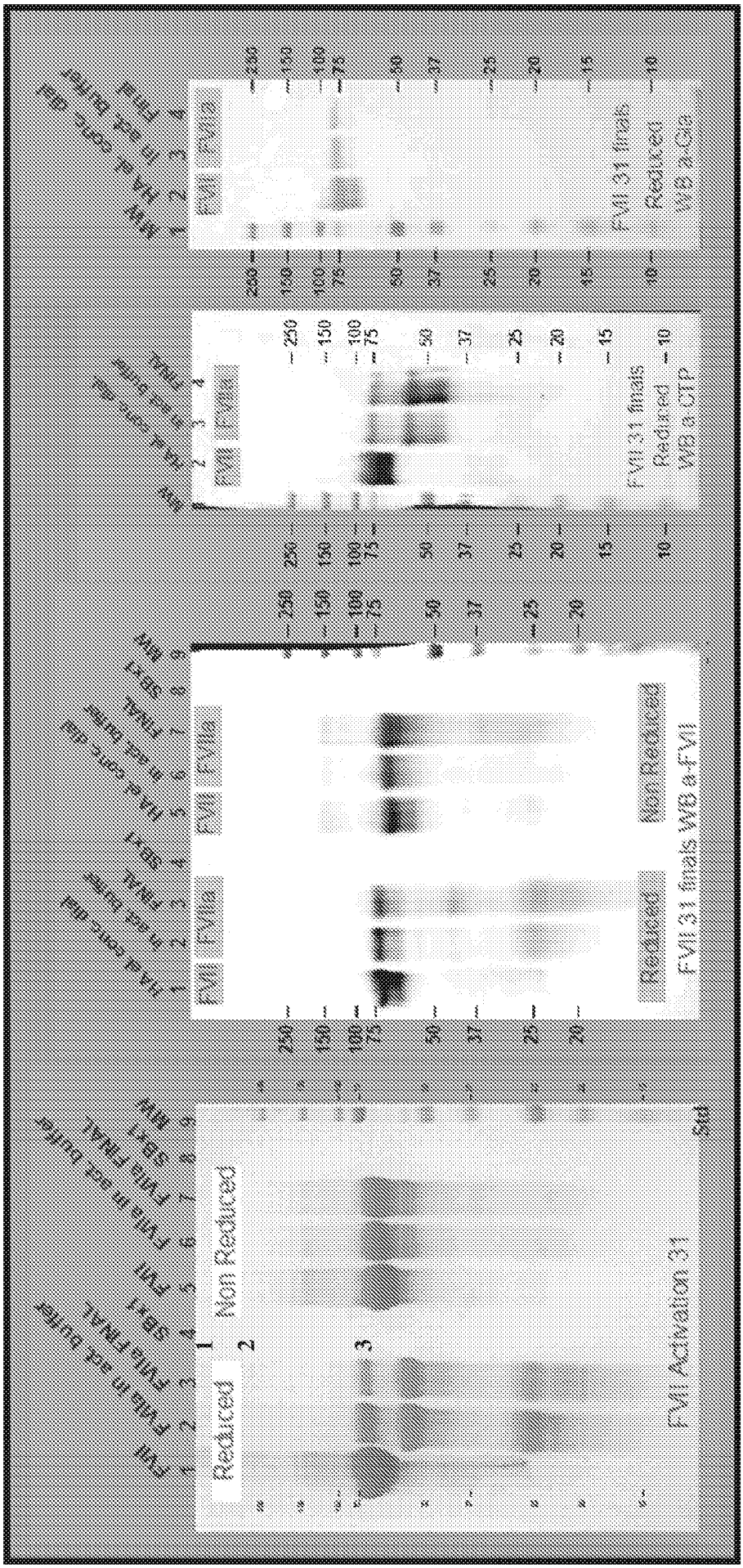


FIGURE 26A FIGURE 26B FIGURE 26C FIGURE 26D

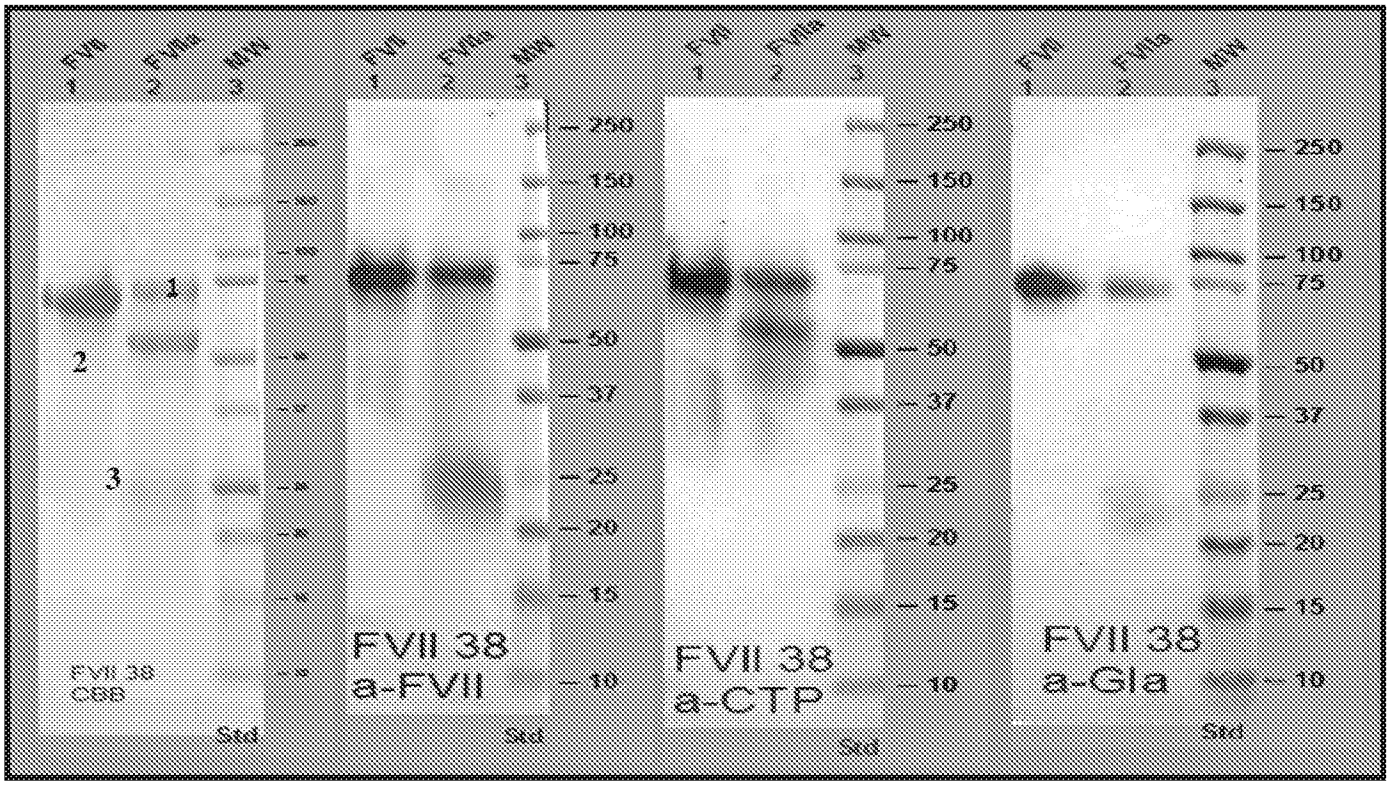


FIGURE 26E FIGURE 26F FIGURE 26G FIGURE 26H

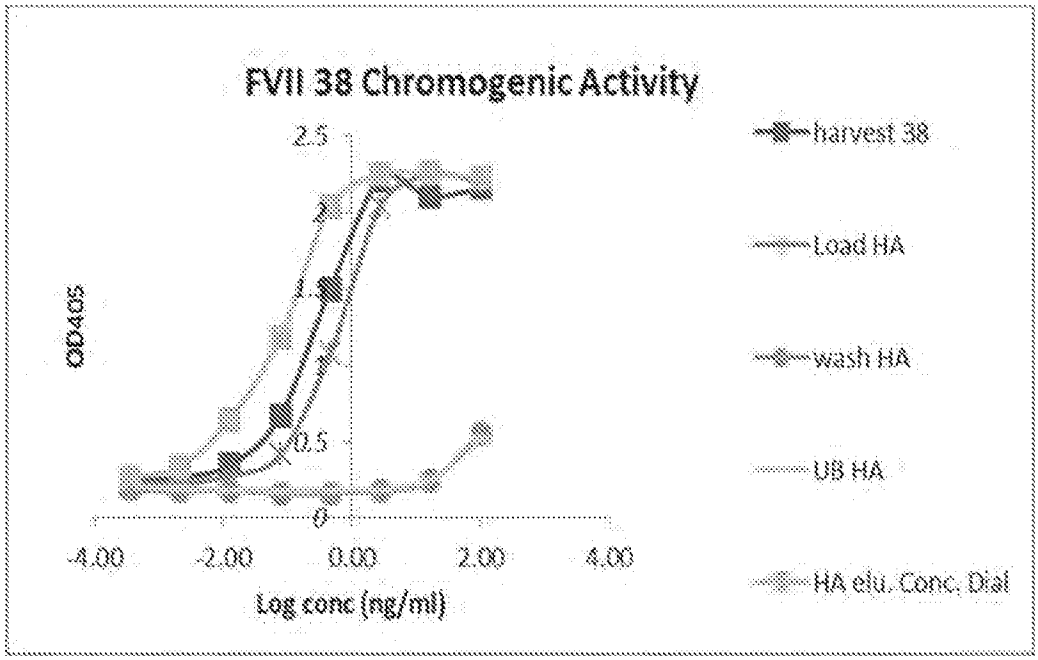


FIGURE 27

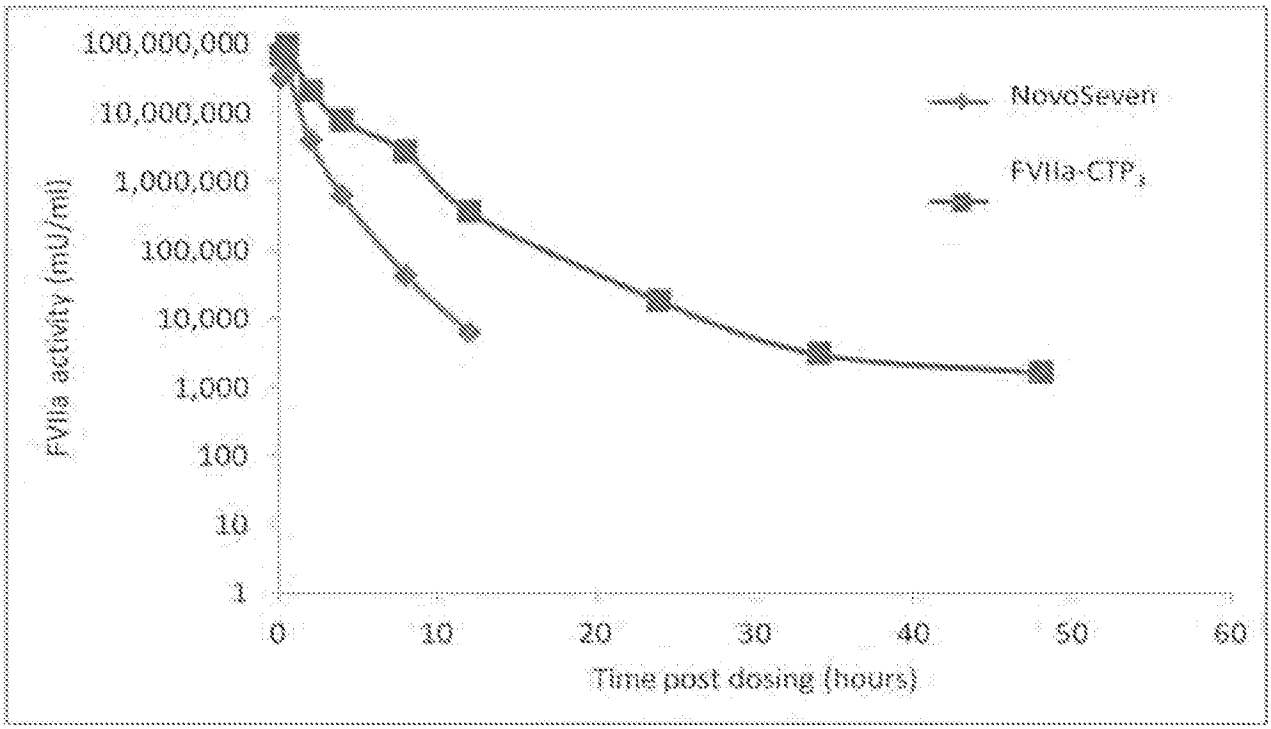

100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
FVIIa activity (mU/ml)
0
10
20
30
40
50
60
Time post dosing (hours)
NovoSeven
FVIIa-CTP$_3$

FIGURE 28

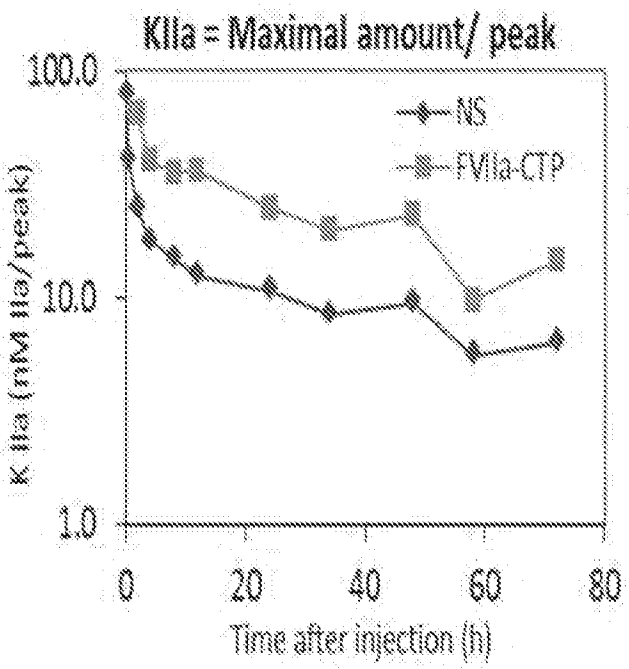


FIGURE 29A

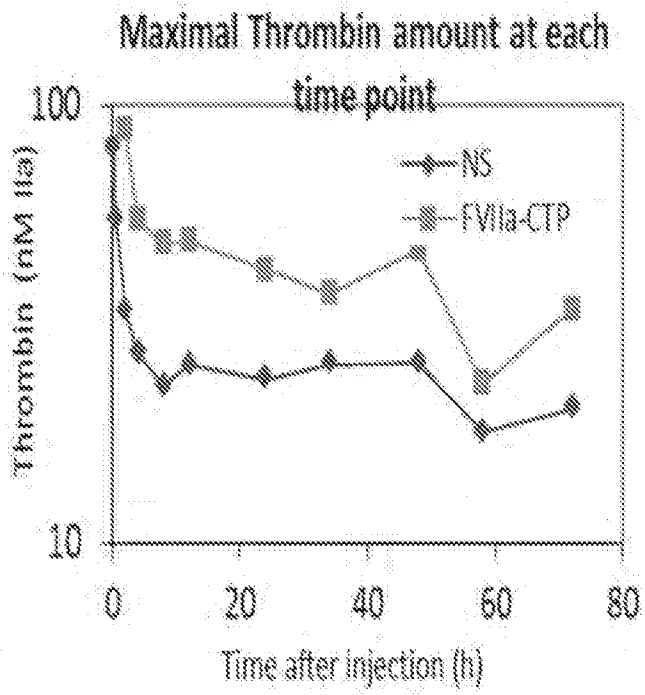


FIGURE 29B

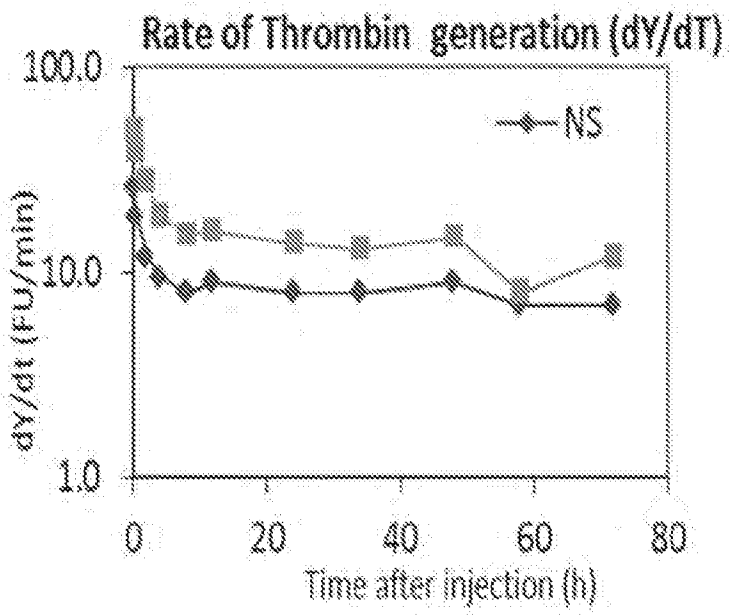


FIGURE 29C

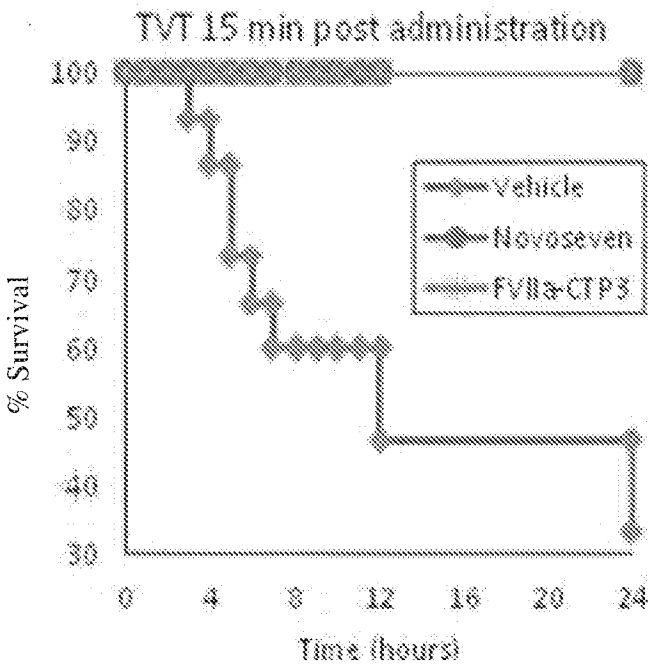


FIGURE 30A

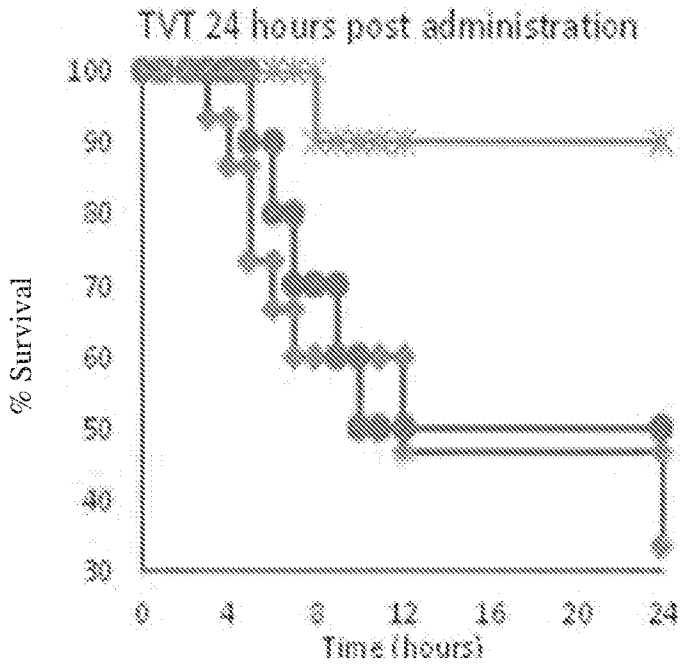


FIGURE 30B

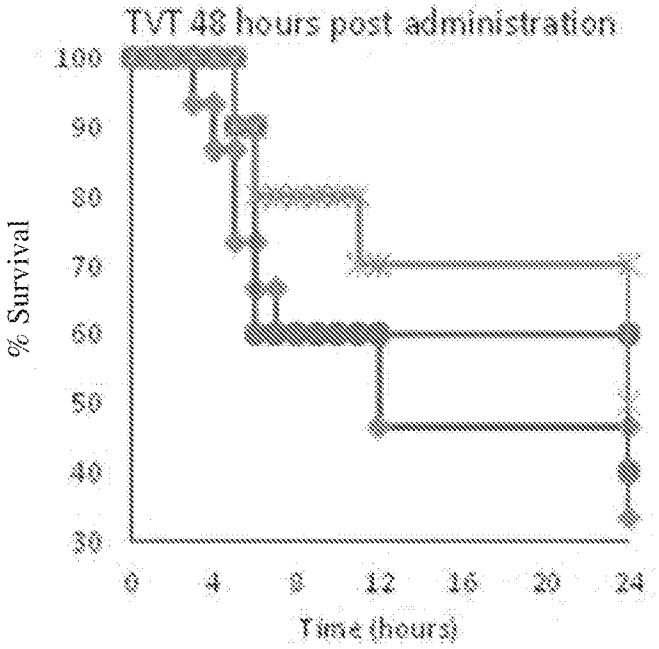


FIGURE 30C

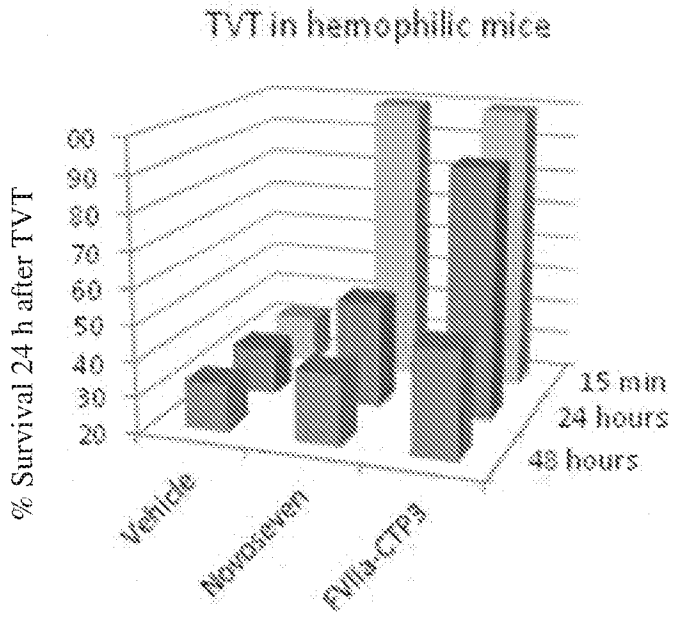


FIGURE 30D

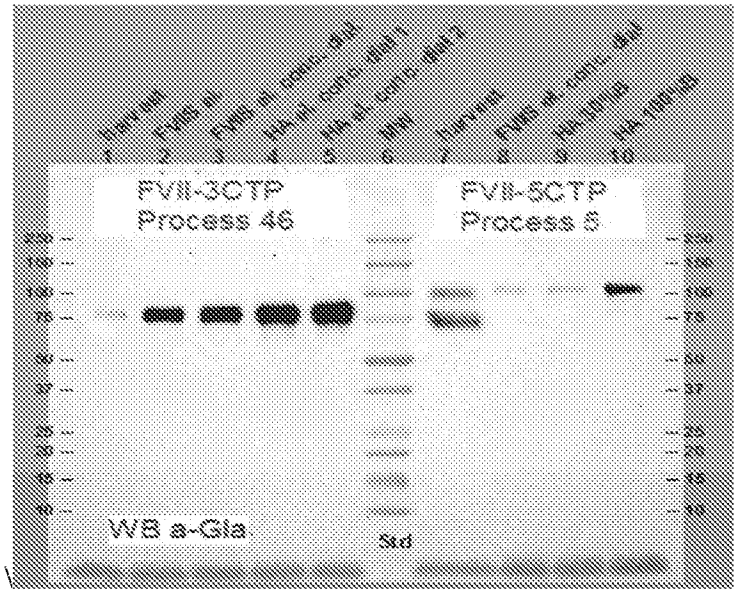


FIGURE 31A

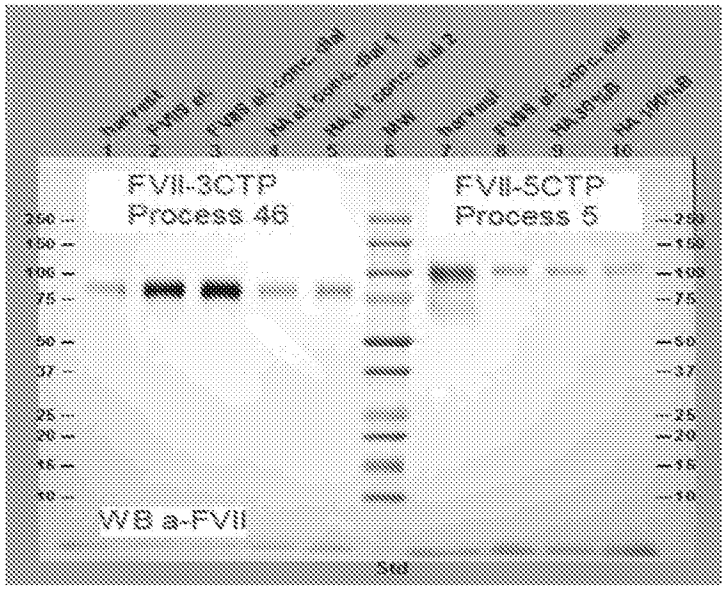


FIGURE 31B

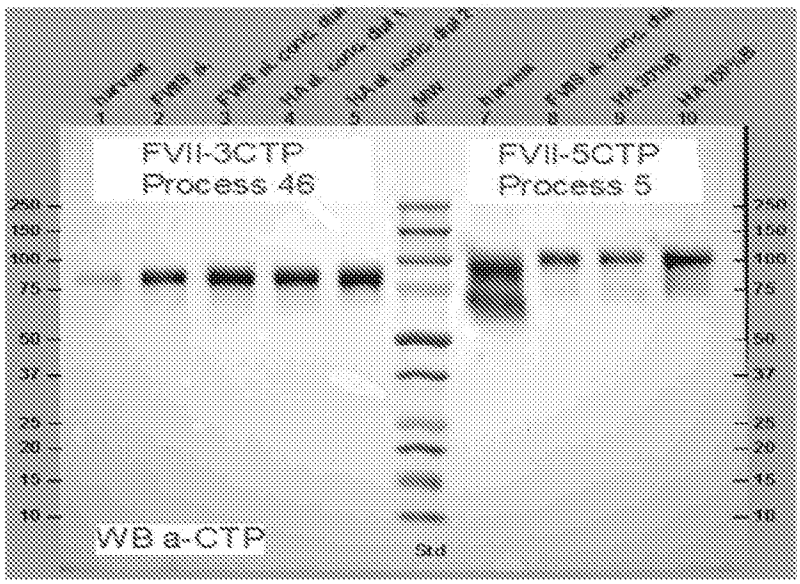


FIGURE 31C

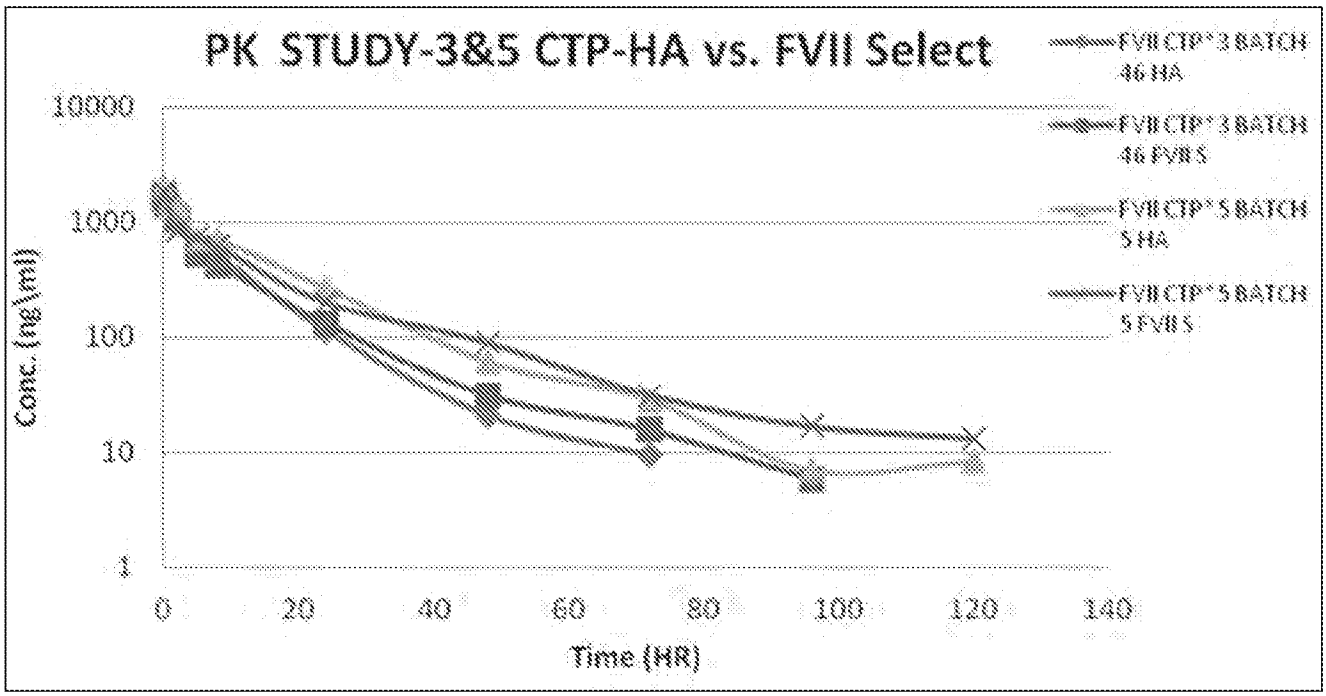


FIGURE 32

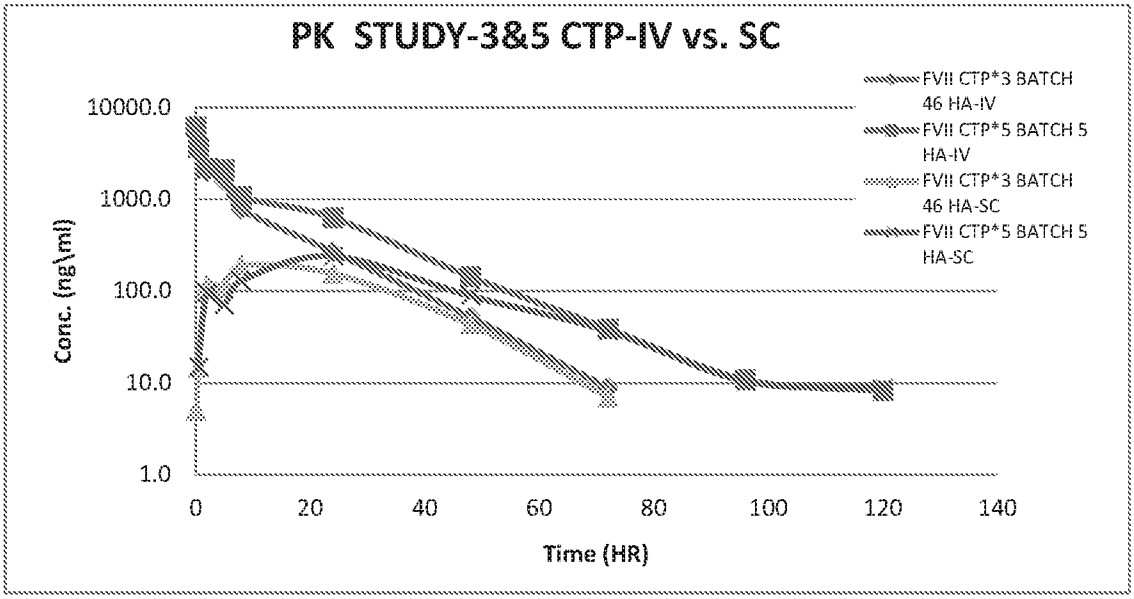

PK STUDY-3&5 CTP-IV vs. SC
FVII CTP*3 BATCH 46 HA-IV
FVII CTP*5 BATCH 5 HA-IV
FVII CTP*3 BATCH 46 HA-SC
FVII CTP*5 BATCH 5 HA-SC
10000.0
1000.0
100.0
10.0
1.0
Conc. (ng\ml)
0
20
40
60
80
100
120
140
Time (HR)

FIGURE 33

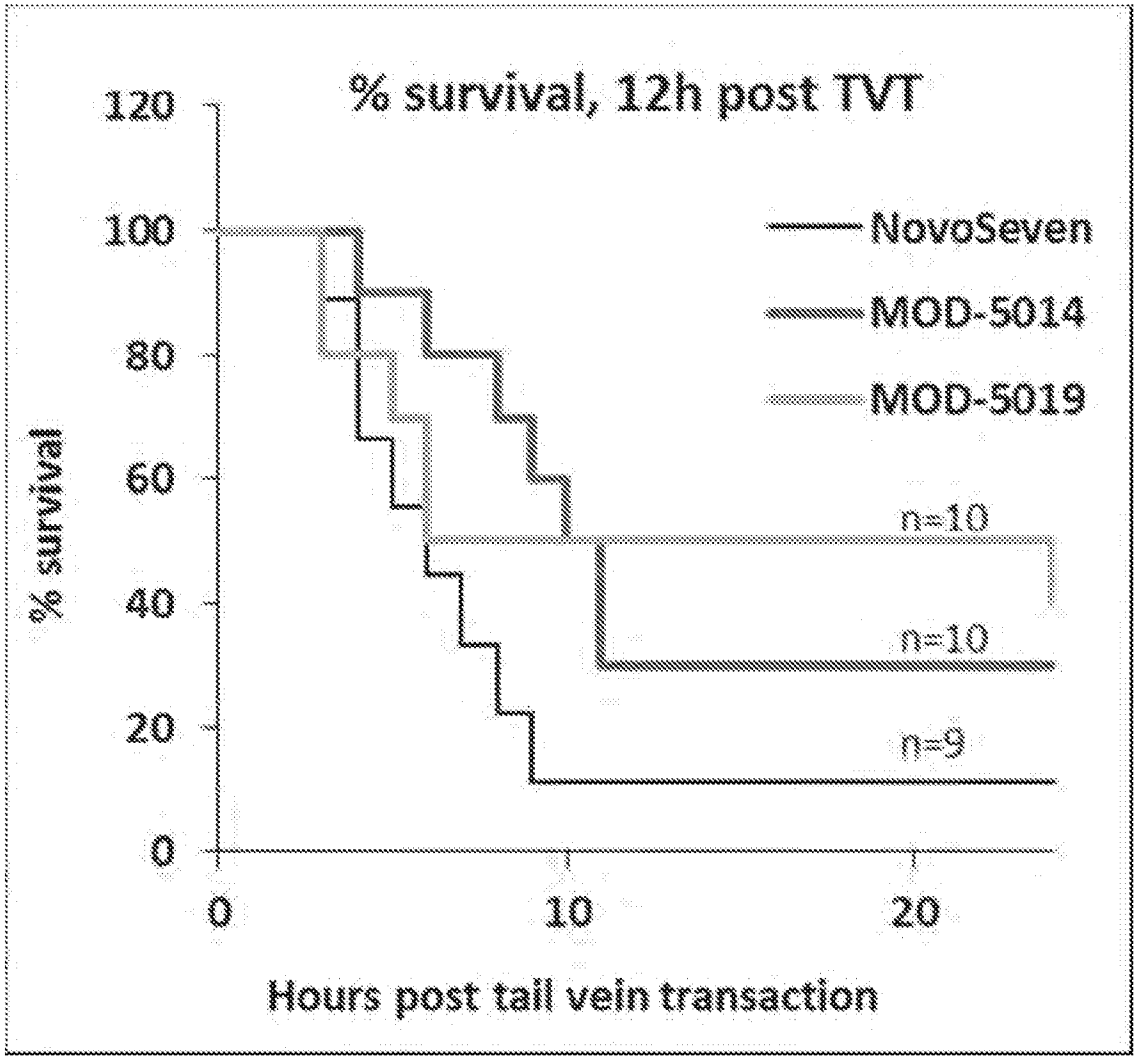

% survival, 12h post TVT
120
100
80
60
40
20
0
% survival
0
10
20
Hours post tail vein transaction
NovoSeven
MOD-5014
MOD-5019
n=10
n=10
n=9

FIGURE 34

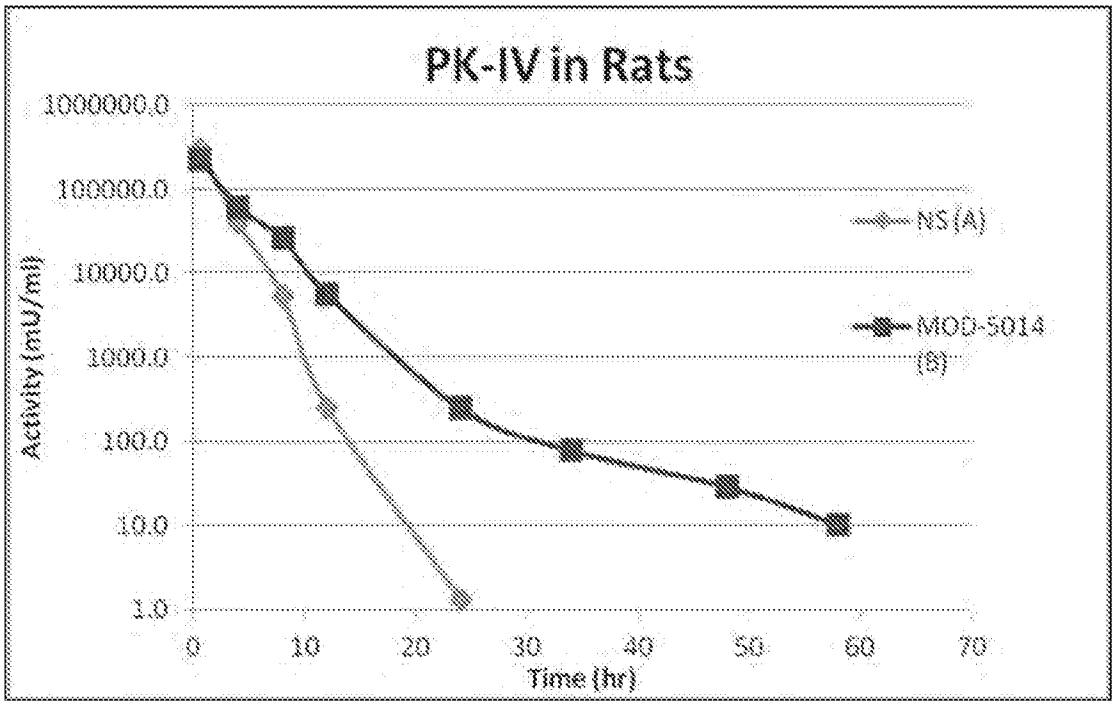


FIGURE 35A

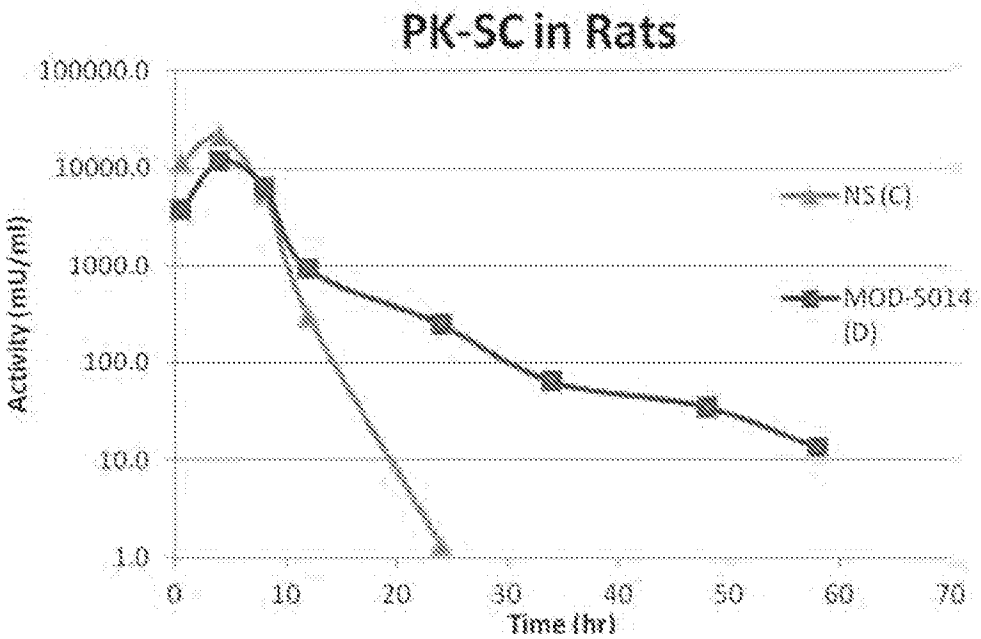


FIGURE 35B

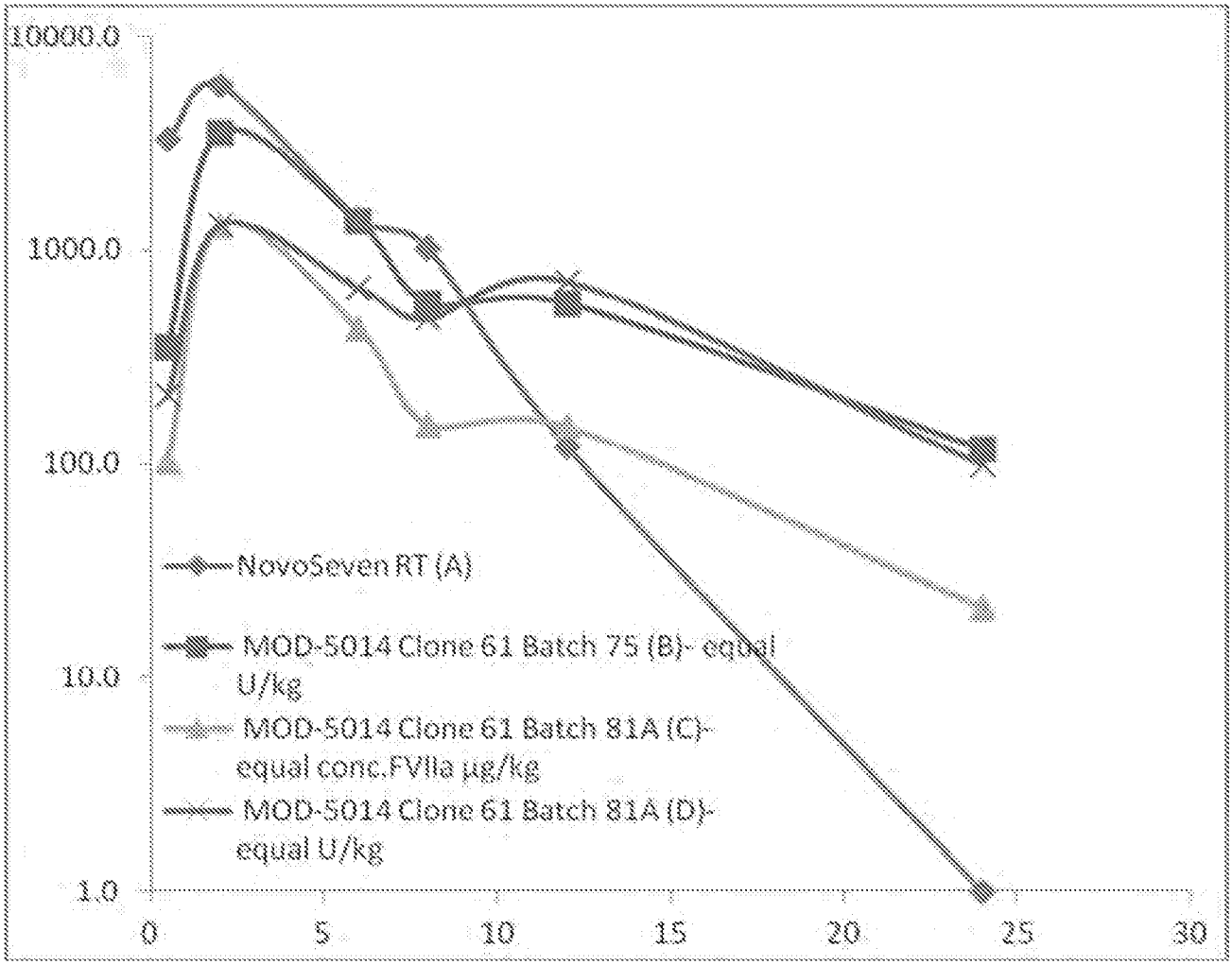

10000.0
1000.0
100.0
10.0
1.0
0
5
10
15
20
25
30
NovoSeven RT (A)
MOD-5014 Clone 61 Batch 75 (B)- equal U/kg
MOD-5014 Clone 61 Batch 81A (C)- equal conc.FVIIa µg/kg
MOD-5014 Clone 61 Batch 81A (D)- equal U/kg

FIGURE 36

Time	PT	aPTT
0	10.71	16.54
10	21.37	12.90
24	40.48	48.60
36	79.05	58.35
48	80.50	100.10
60	49.75	85.48
72	27.50	63.50

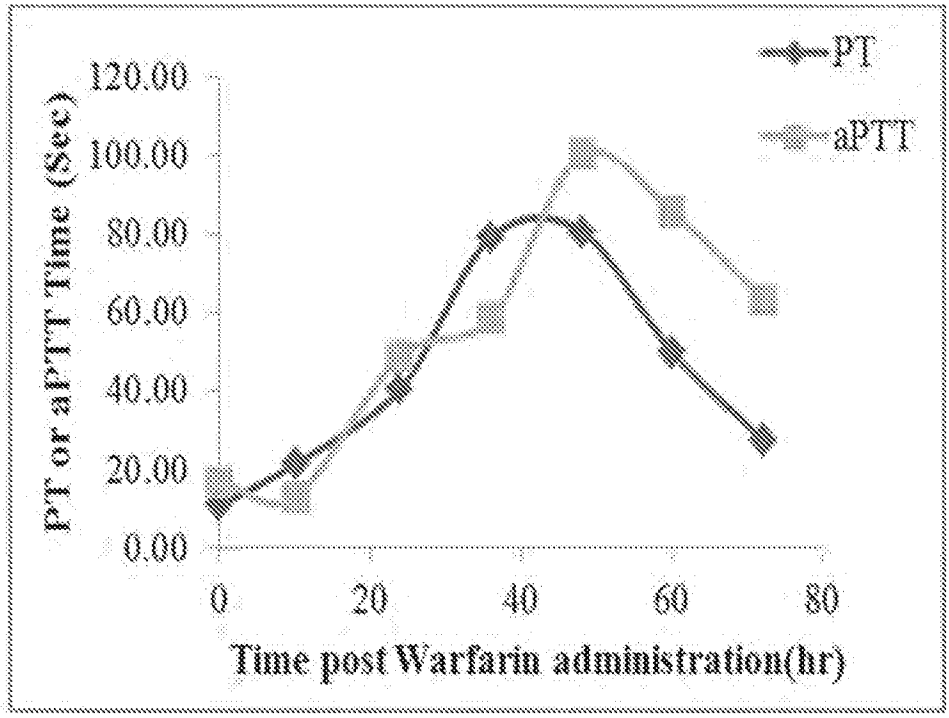


FIGURE 37

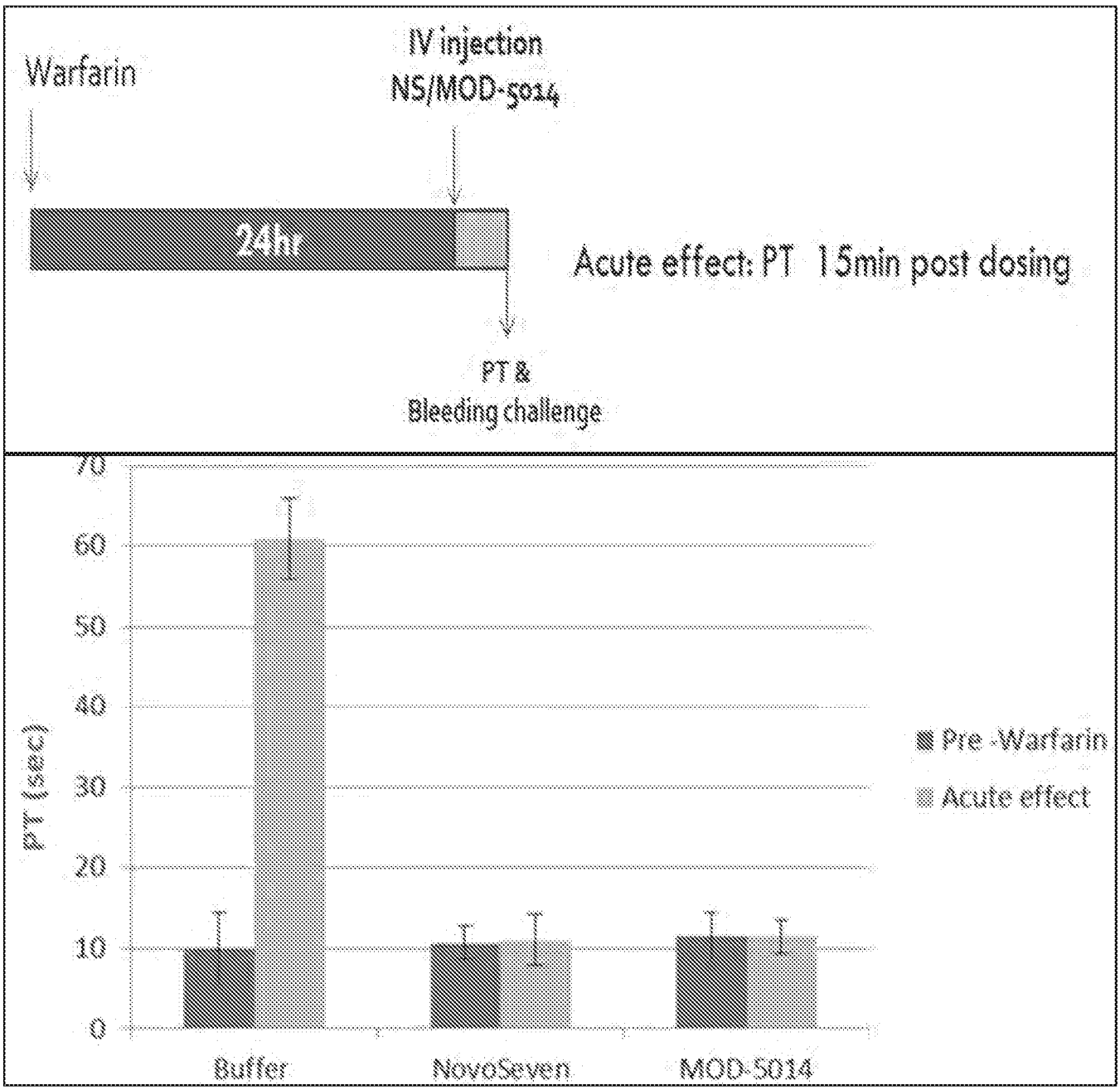
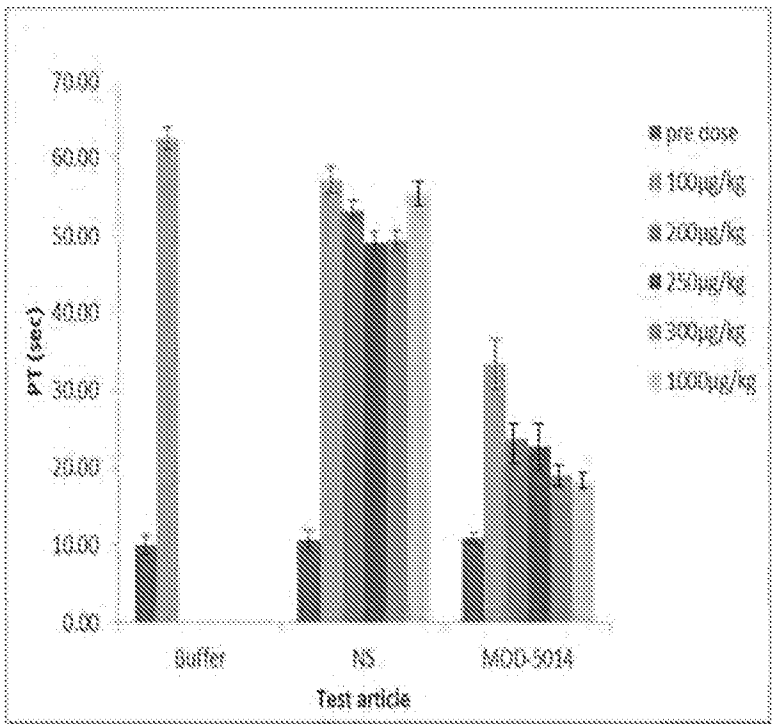
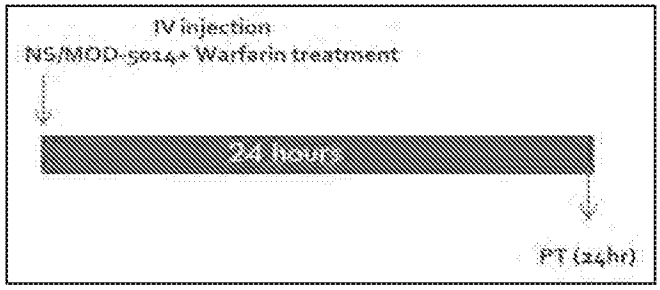

Warfarin
IV injection
NS/MOD-5014
24hr
Acute effect: PT 15min post dosing
PT &
Bleeding challenge
70
60
50
40
30
20
10
0
PT (sec)
Buffer
NovoSeven
MOD-5014
Pre -Warfarin
Acute effect

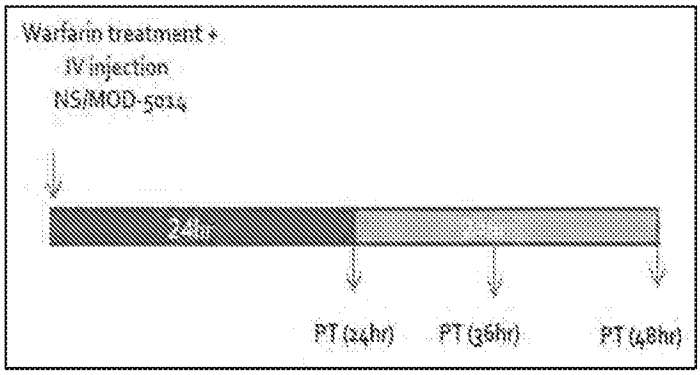
FIGURE 38



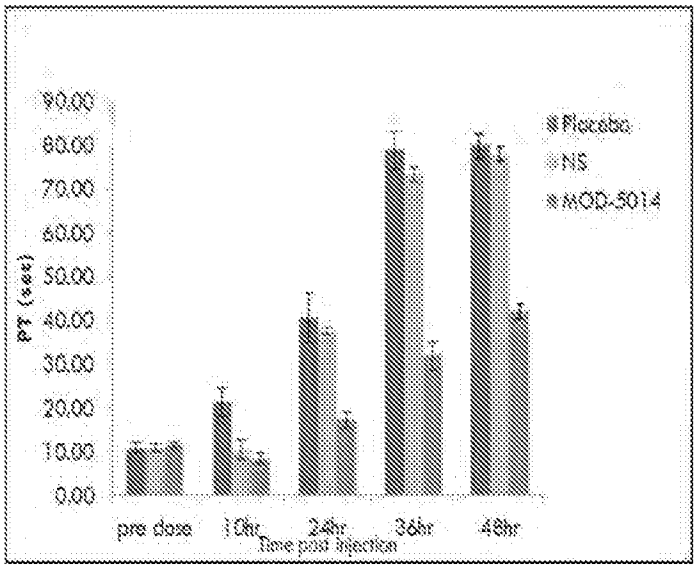


	Placebo	NS	MOD-5014
pre dose	10.03	10.67	10.80
100µg/kg	62.58	57.35	33.48
200µg/kg		53.35	23.75
250µg/kg		49.08	22.72
300µg/kg		49.38	19.00
1000µg/kg		55.60	18.20

FIGURE 39



	Placebo	NS	MOD-5014
pre dose	10.87	10.93	11.60
10hr	21.37	10.50	8.45
24hr	40.48	37.60	17.37
36hr	79.05	73.30	32.00
48hr	80.5	78.00	42.00



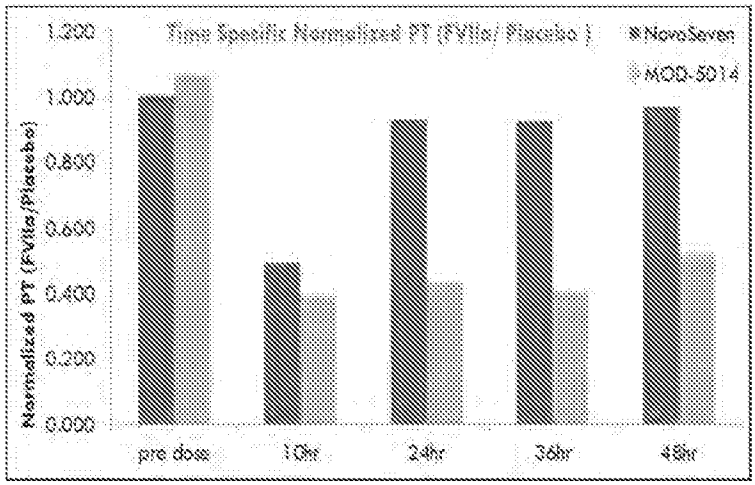


FIGURE 40

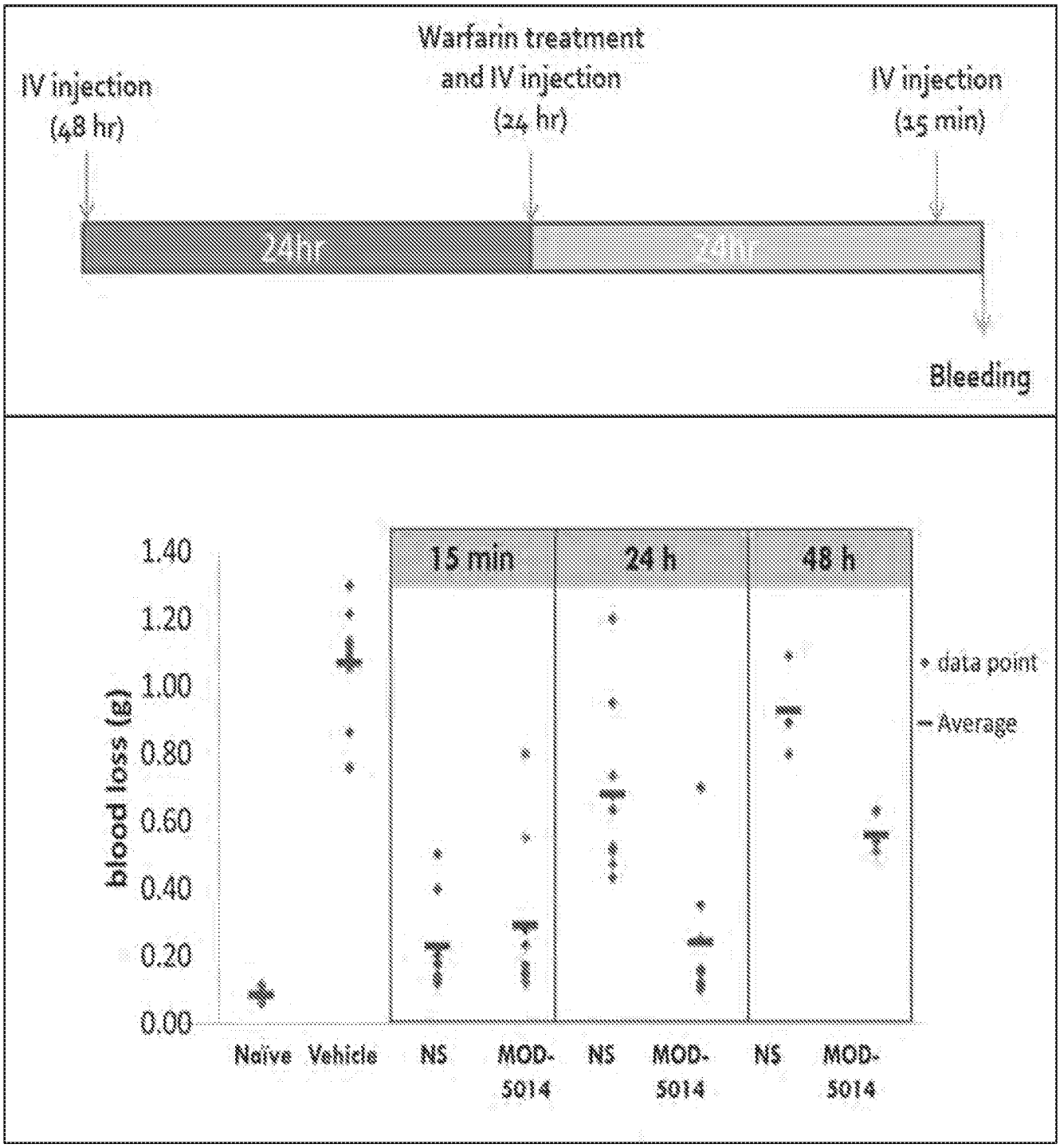
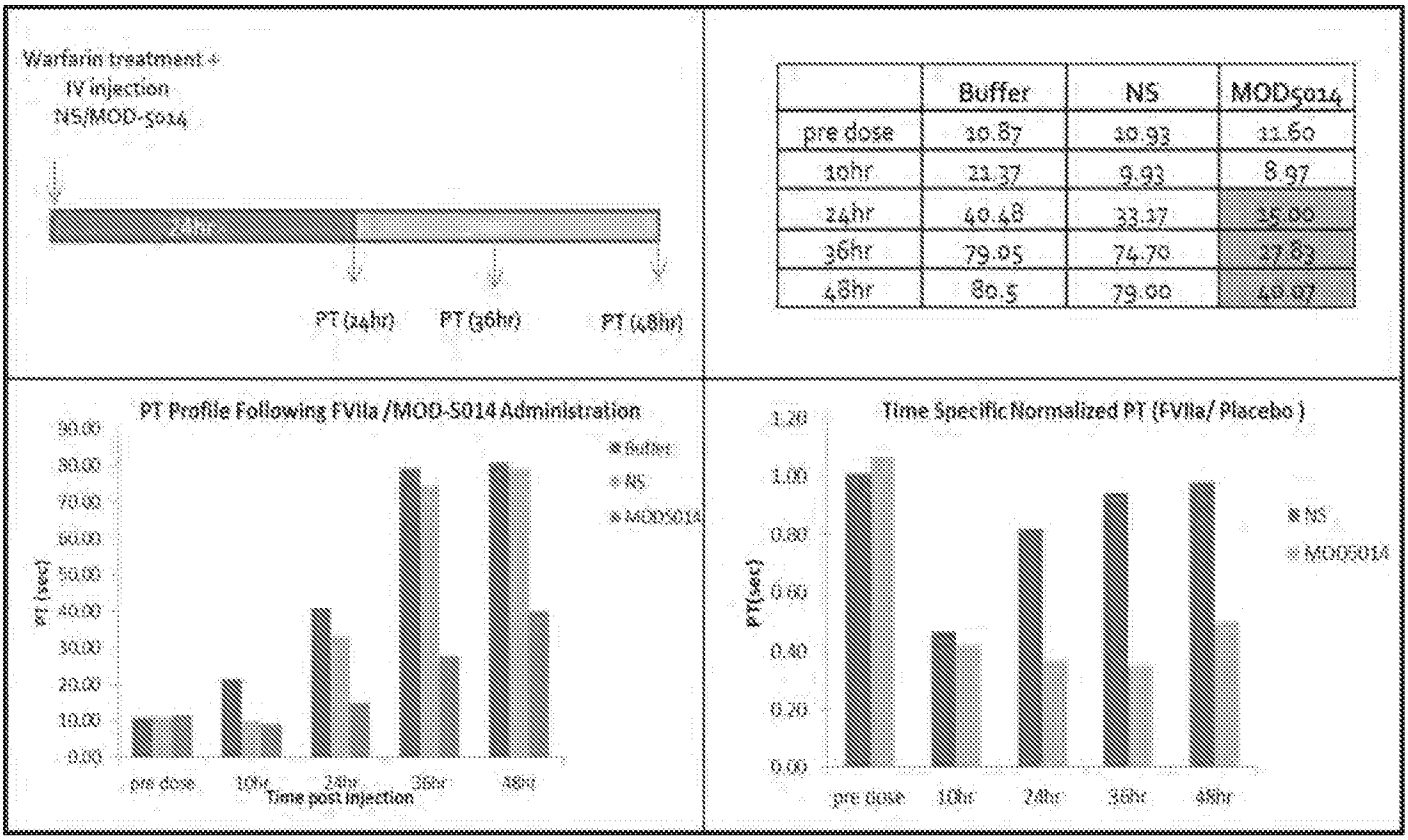

Warfarin treatment
and IV injection
(24 hr)
IV injection
(48 hr)
IV injection
(15 min)
24hr
24hr
Bleeding
15 min
24 h
48 h
blood loss (g)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
data point
Average
Naive
Vehicle
NS
MOD-5014
NS
MOD-5014
NS
MOD-5014

FIGURE 41



	Buffer	NS	MOD5014
pre dose	10.67	10.93	11.60
10hr	21.37	9.93	8.97
24hr	40.48	33.37	15.00
36hr	79.05	74.70	27.83
48hr	80.5	79.00	41.07

FIGURE 42

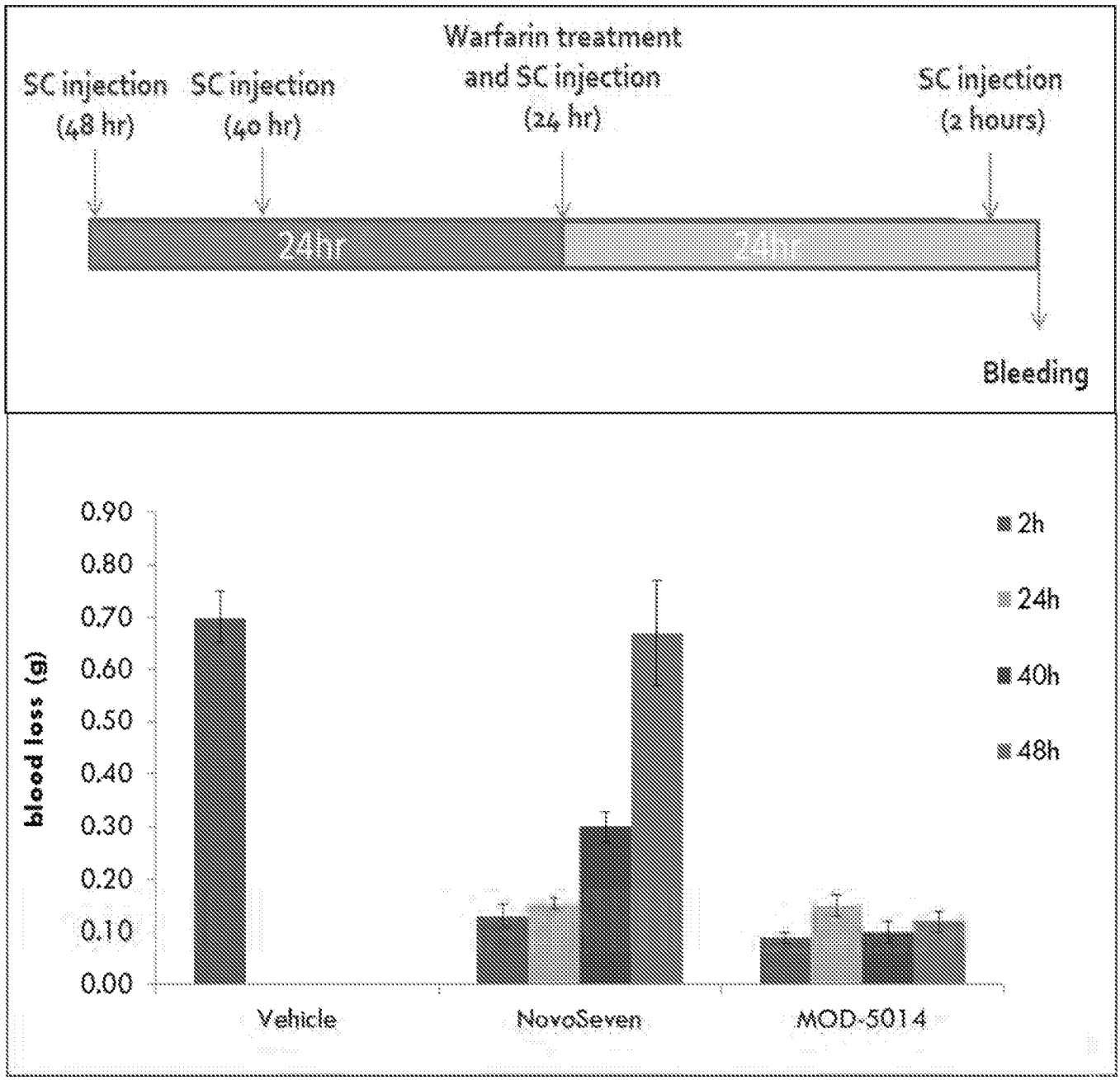

SC injection
(48 hr)
SC injection
(40 hr)
Warfarin treatment
and SC injection
(24 hr)
SC injection
(2 hours)
24hr
24hr
Bleeding
blood loss (g)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
2h
24h
40h
48h
Vehicle
NovoSeven
MOD-5014

FIGURE 43

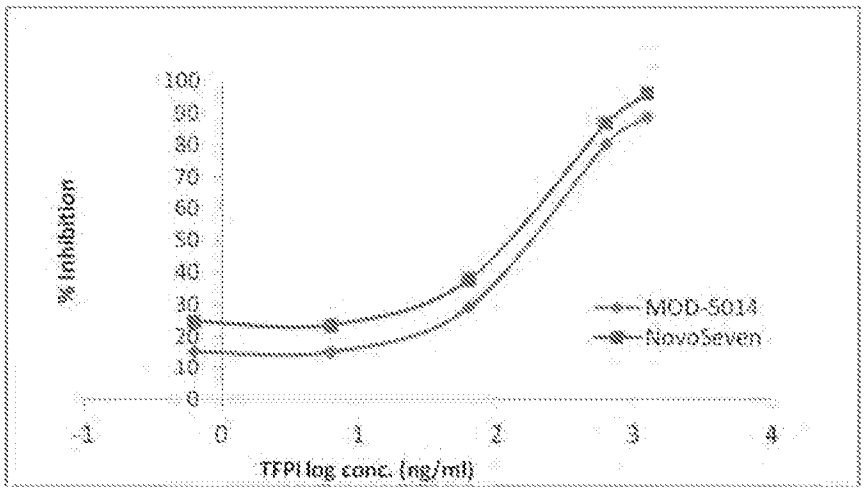


FIGURE 44

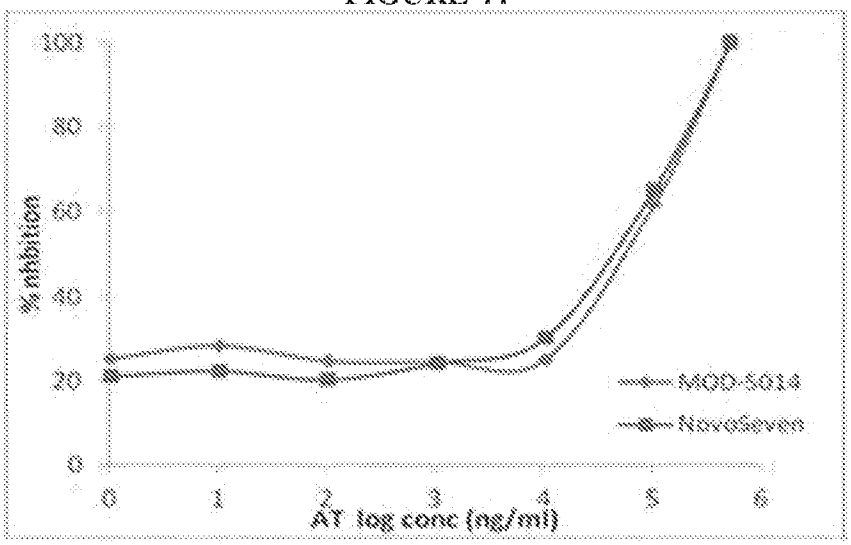


FIGURE 45

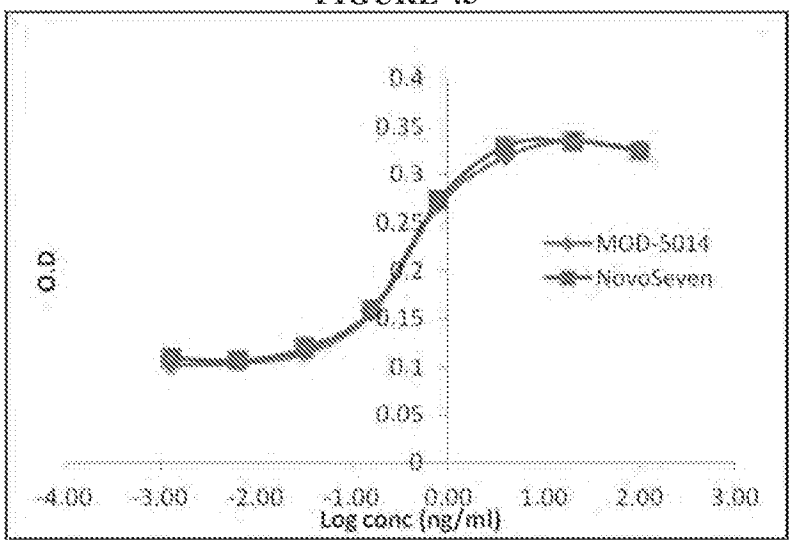


FIGURE 46

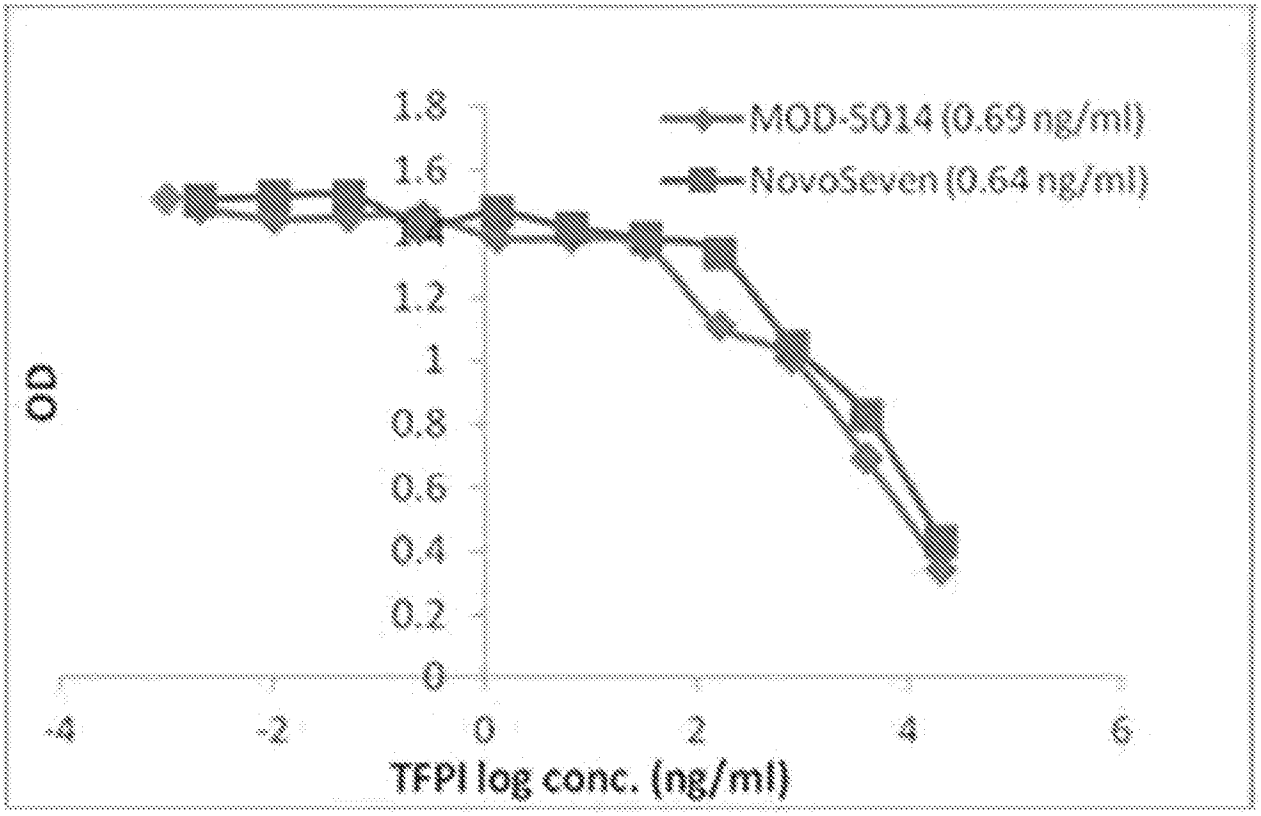


FIGURE 47

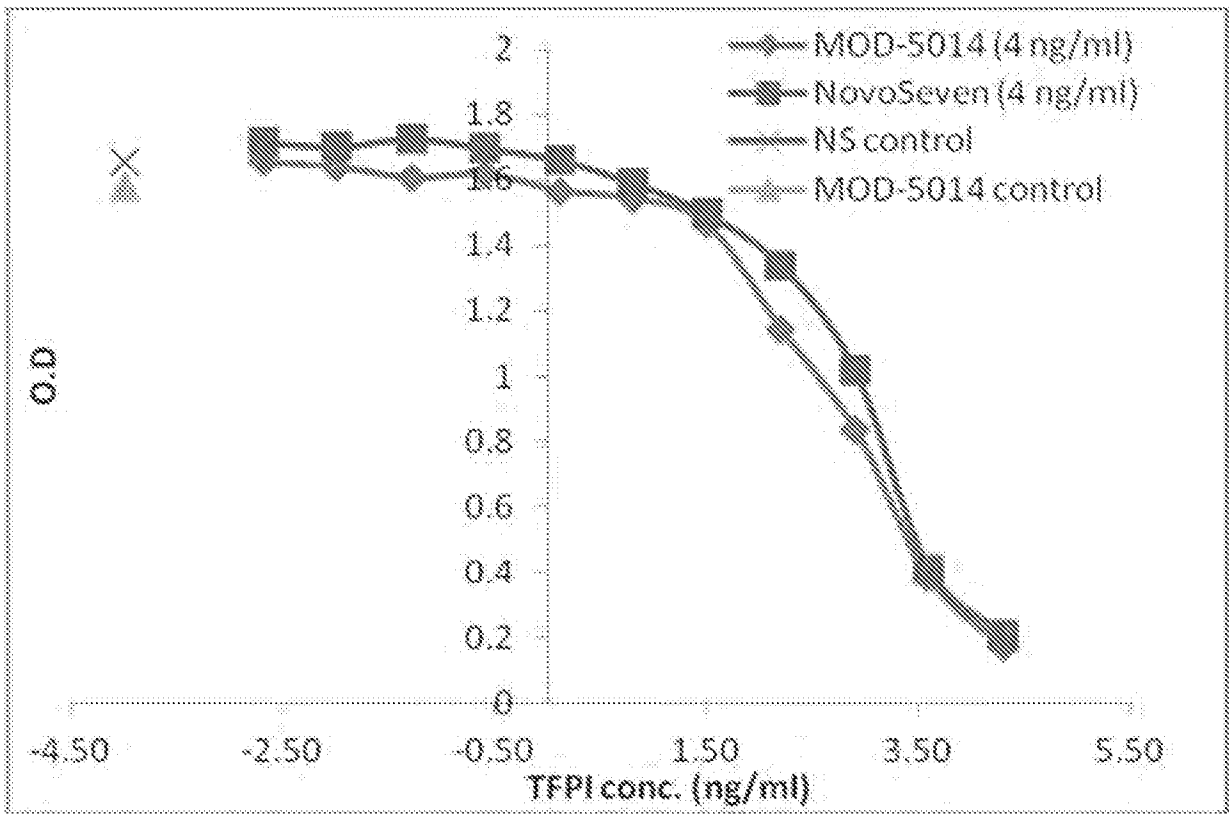


FIGURE 48

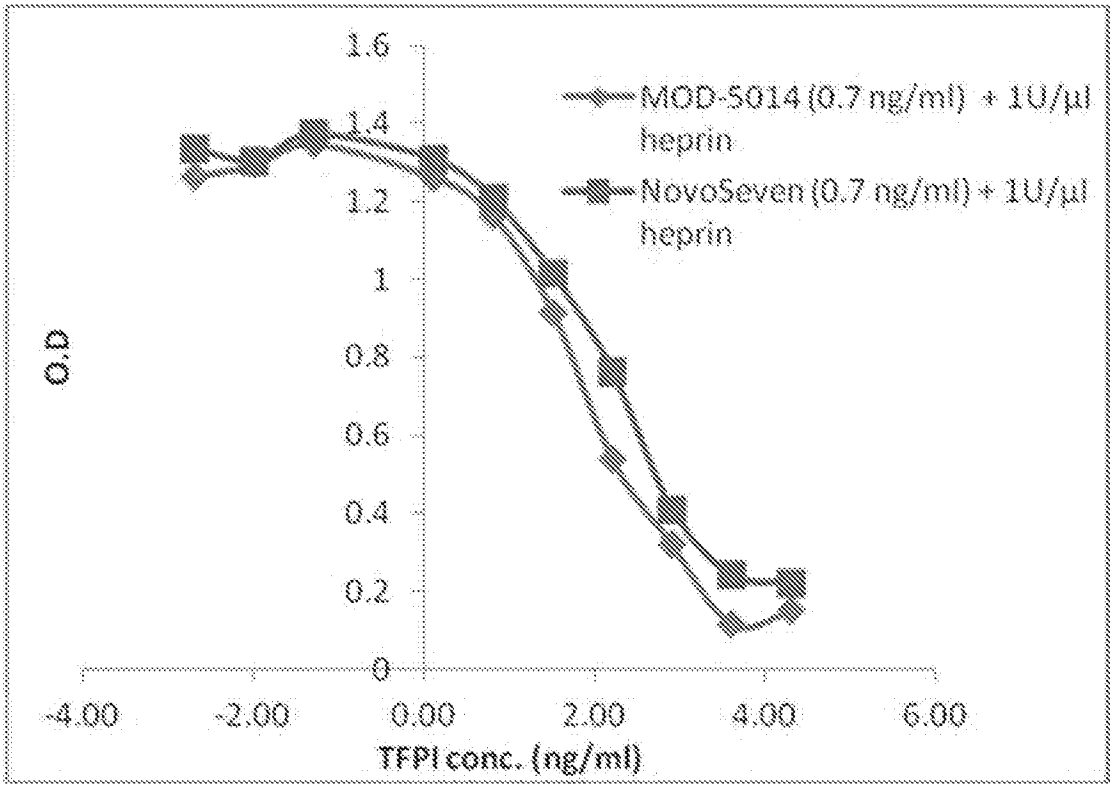


FIGURE 49

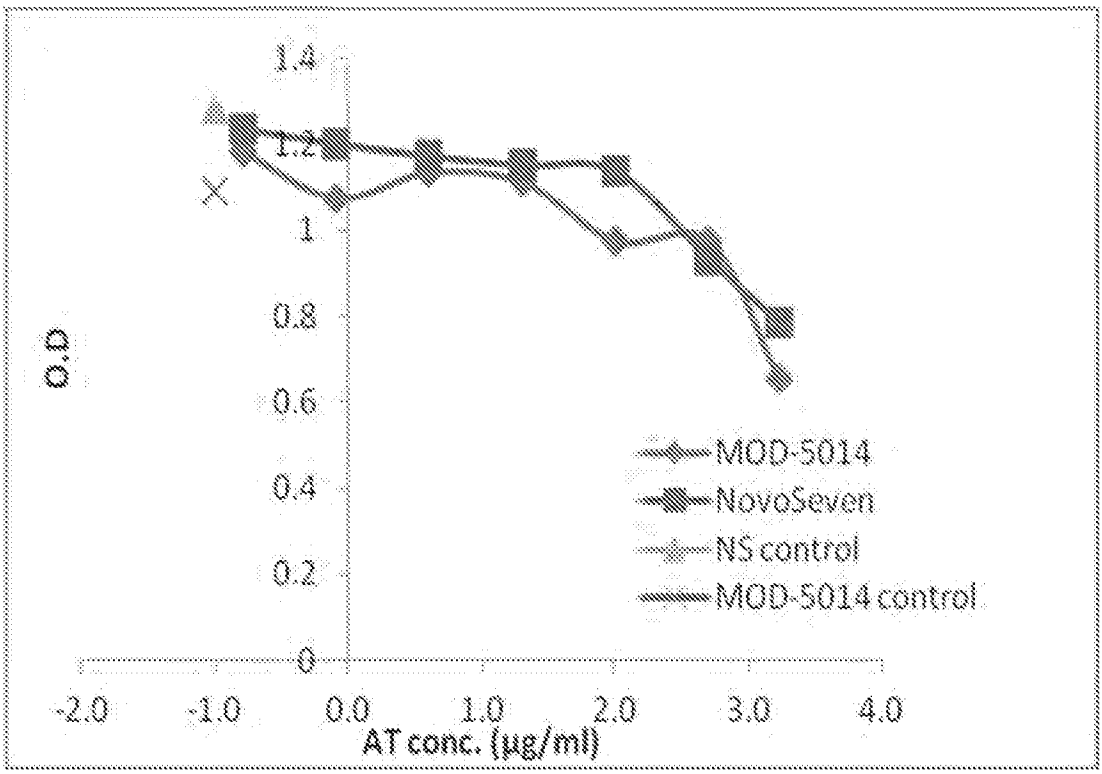


FIGURE 50

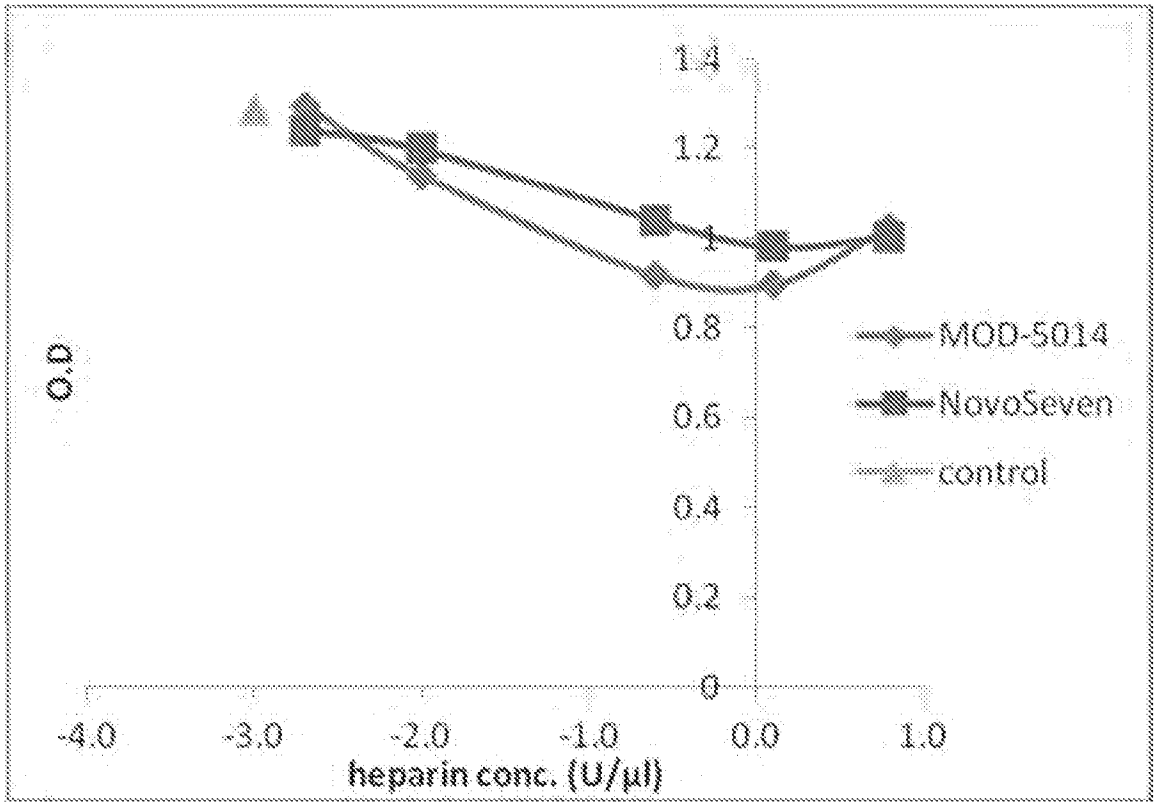


FIGURE 51

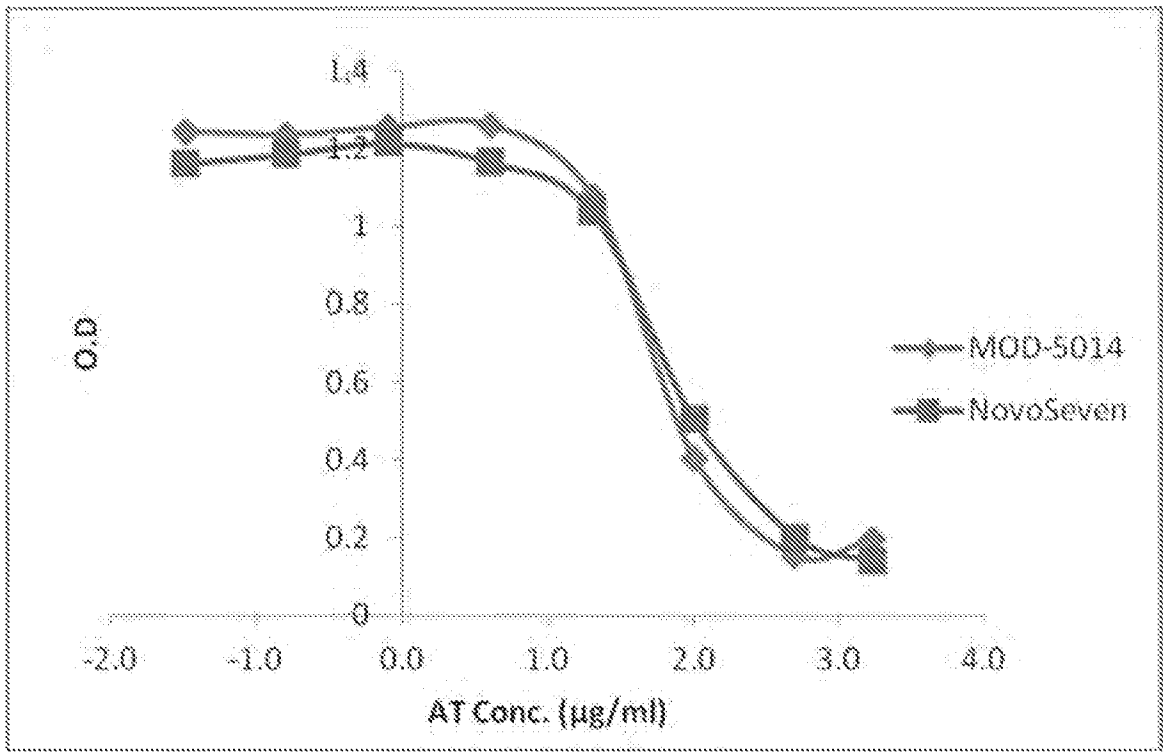


FIGURE 52

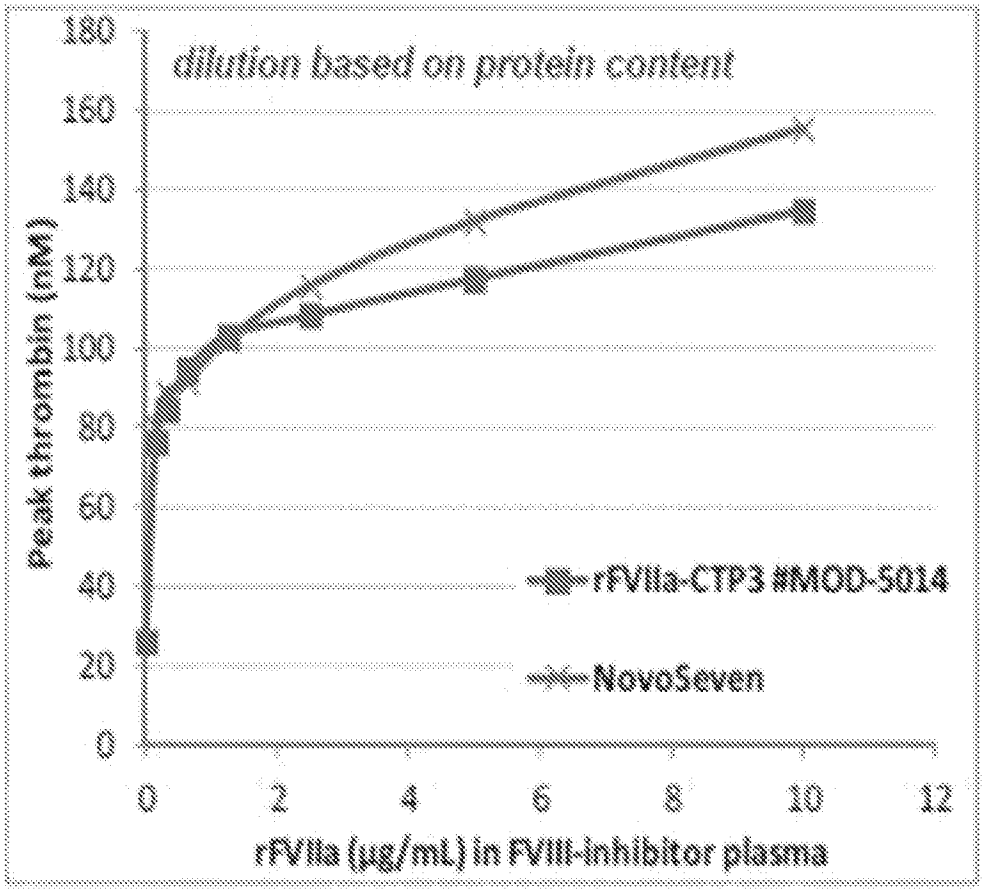


FIGURE 53

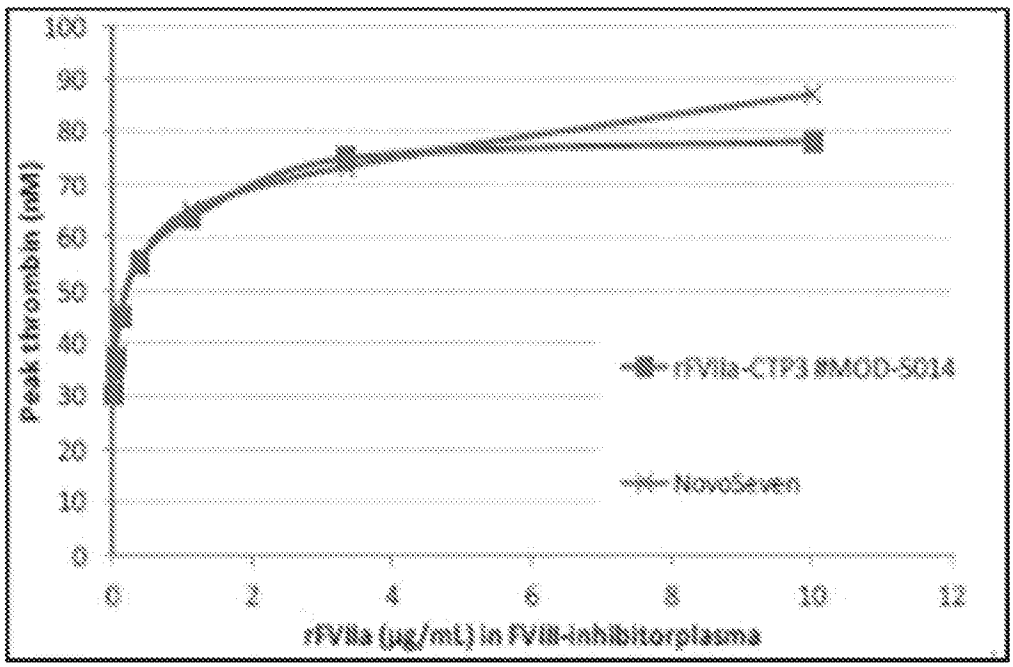


FIGURE 54

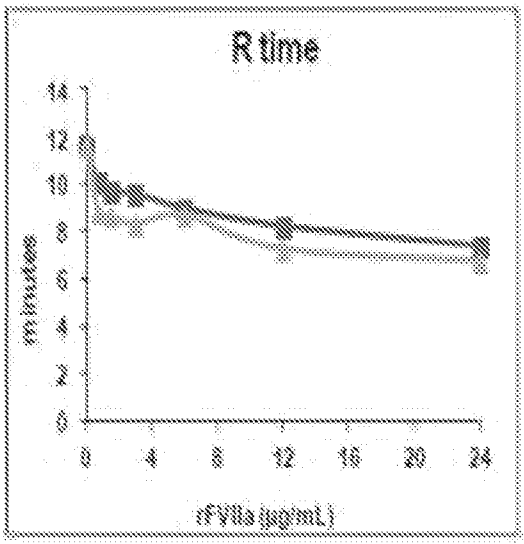


FIGURE 55A

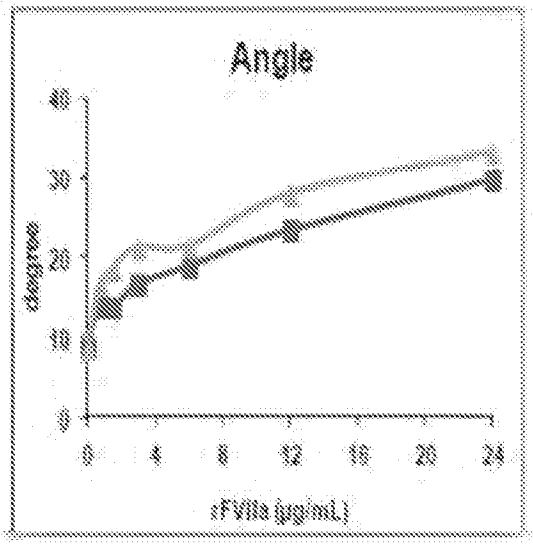


FIGURE 55B

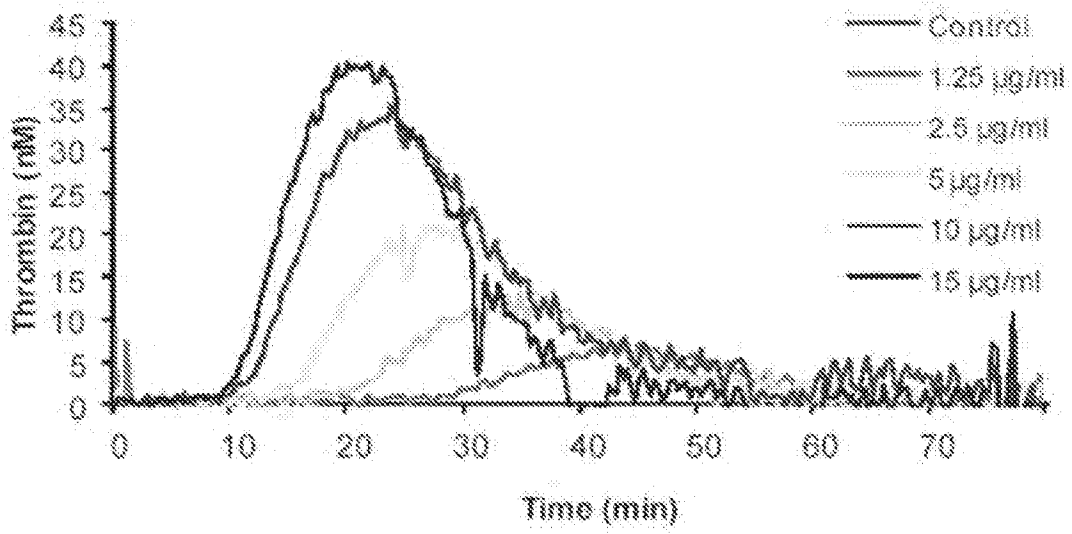


FIGURE 56

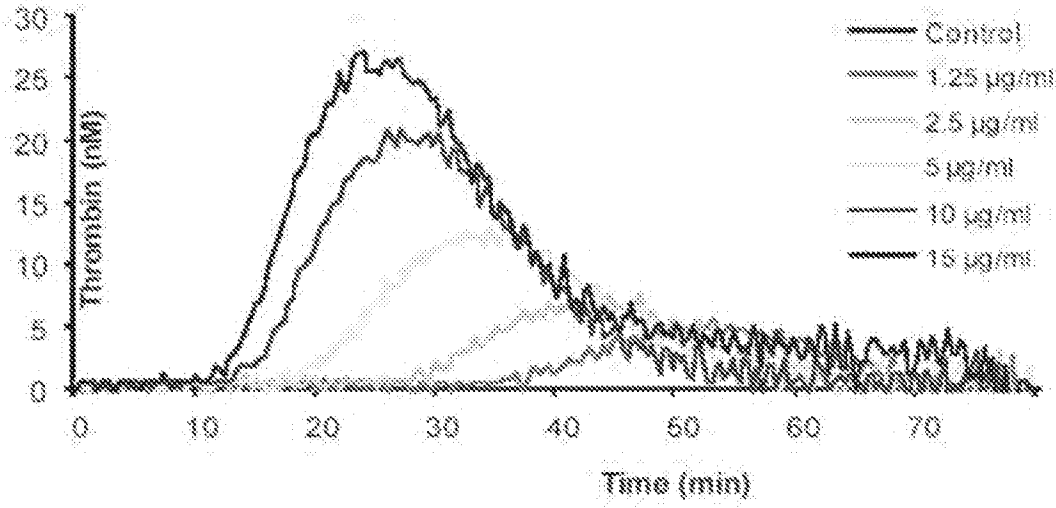


FIGURE 57

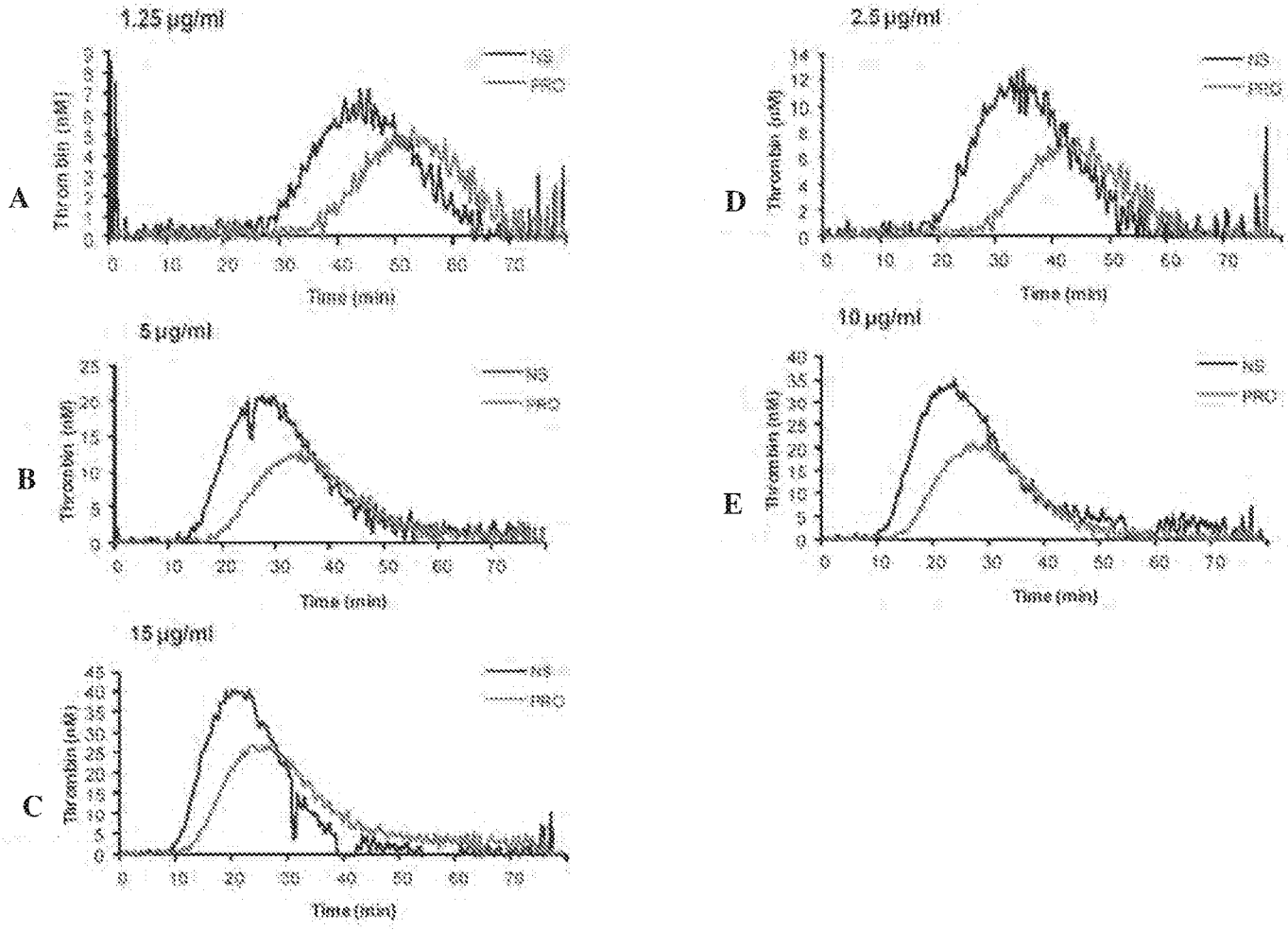


FIGURE 58 (A-E)

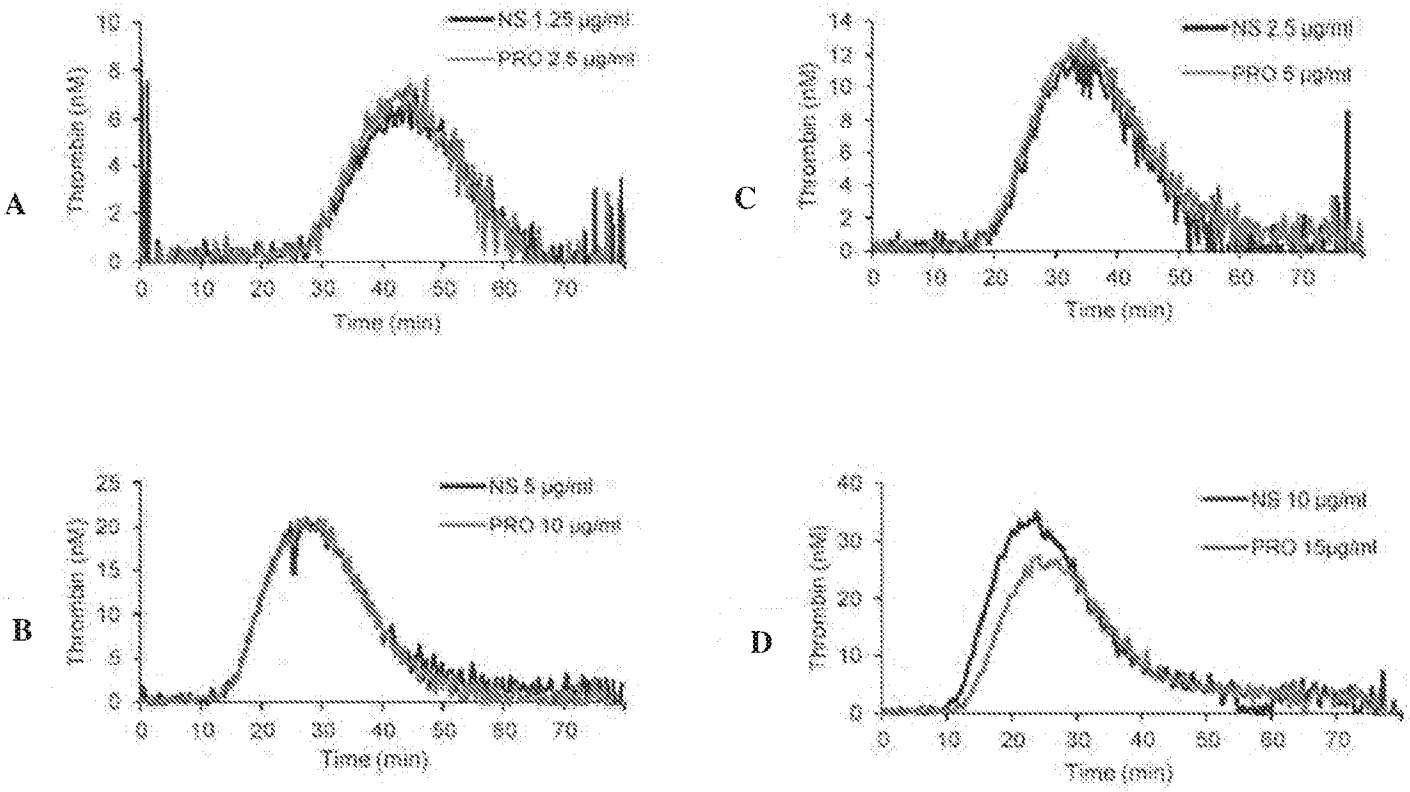


FIGURE 59 (A-D)

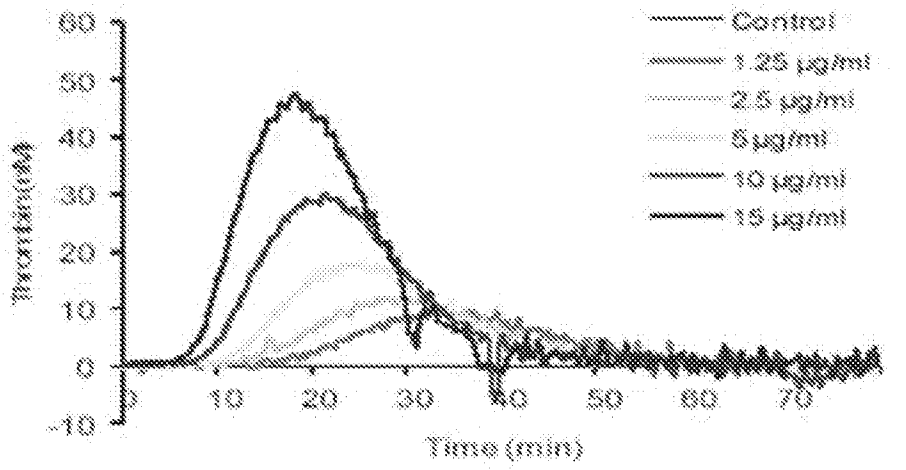


FIGURE 60

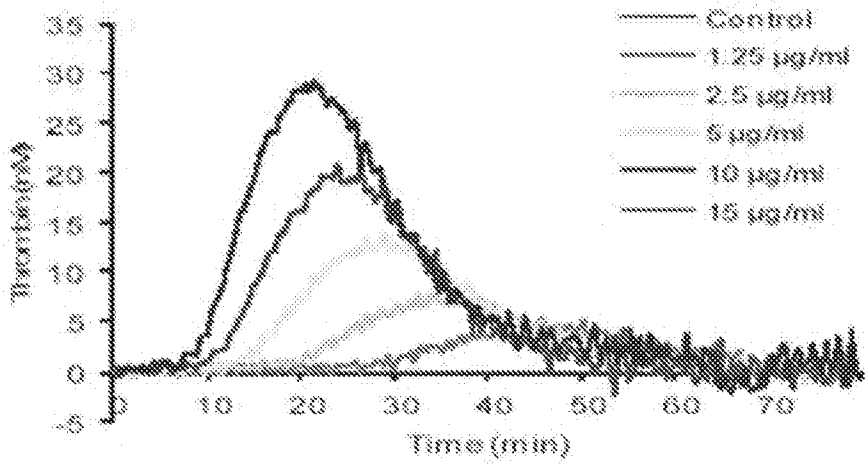


FIGURE 61

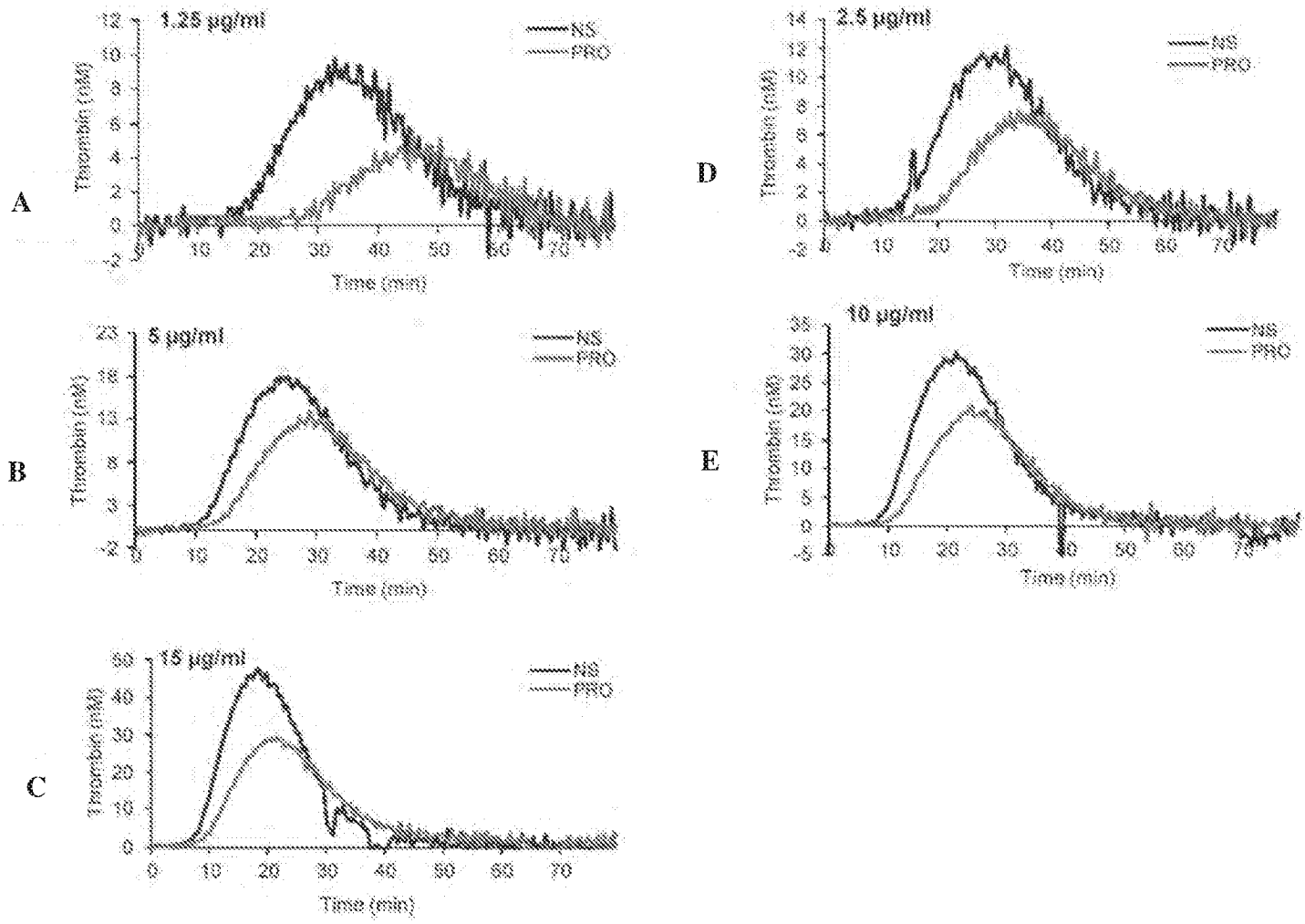


FIGURE 62 (A-E)

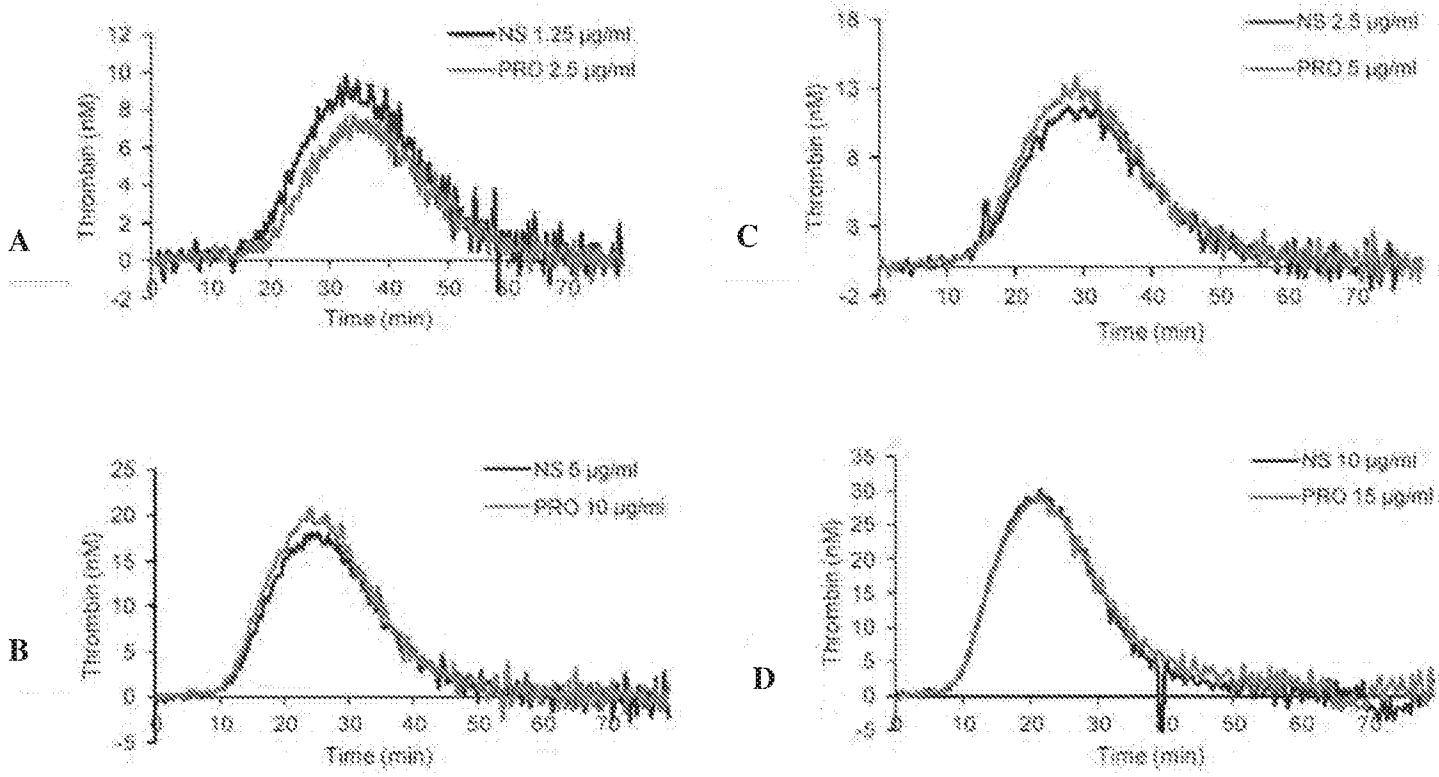


FIGURE 63 (A-D)

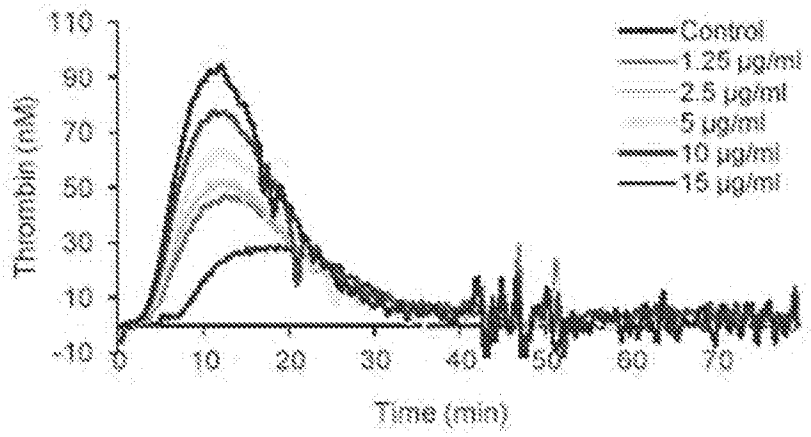


FIGURE 64

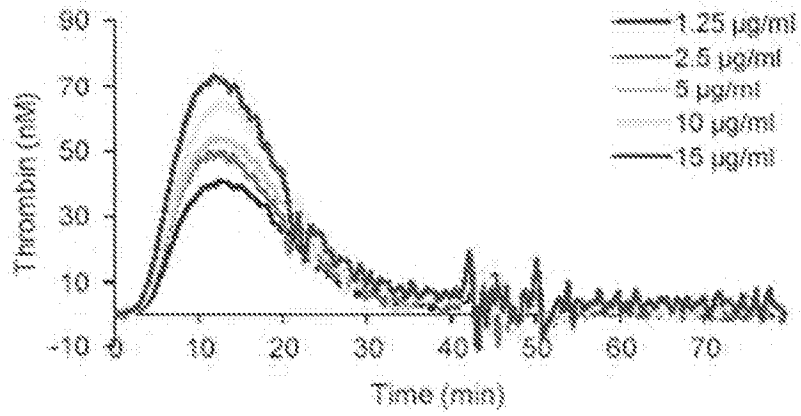


FIGURE 65

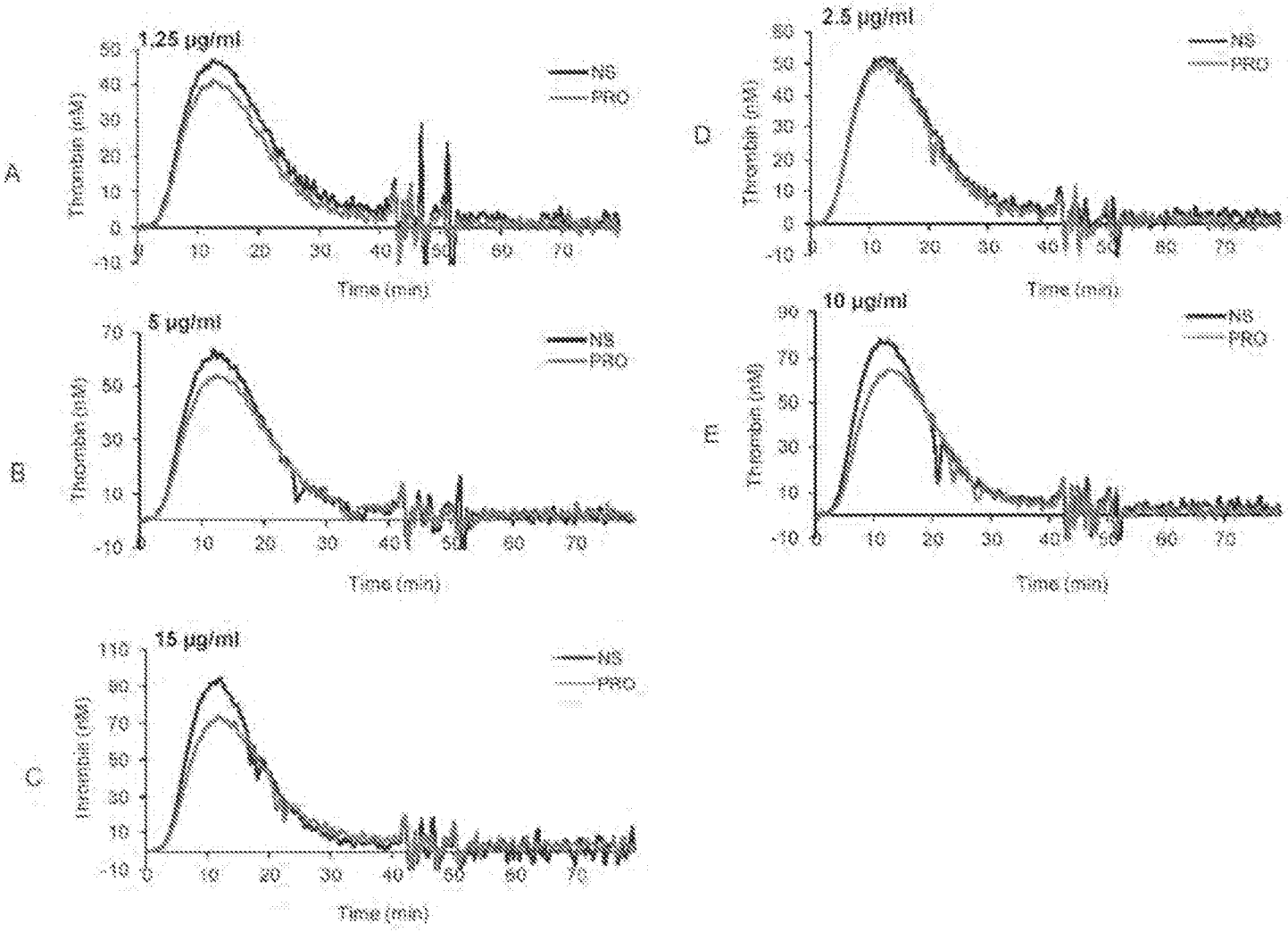


FIGURE 66 (A-E)

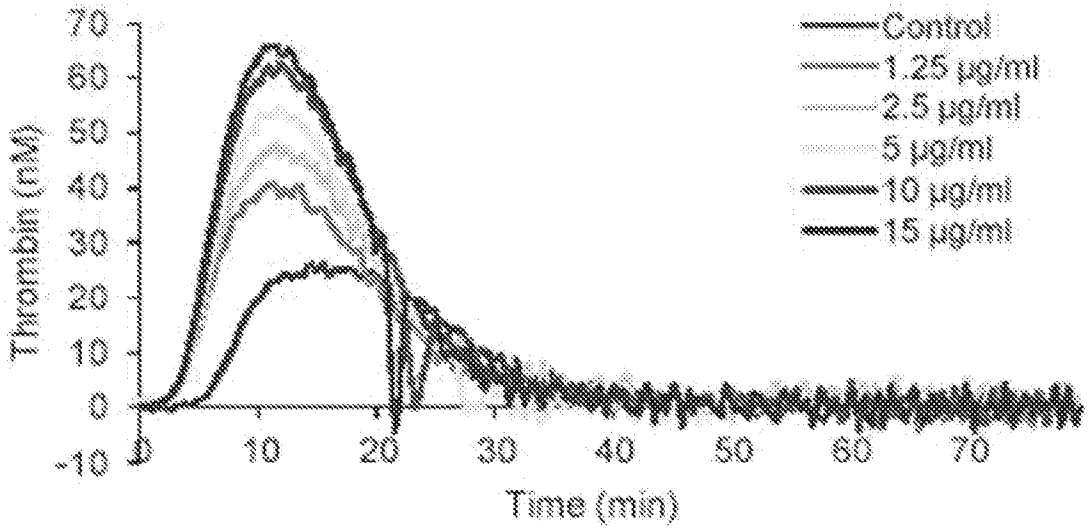


FIGURE 67

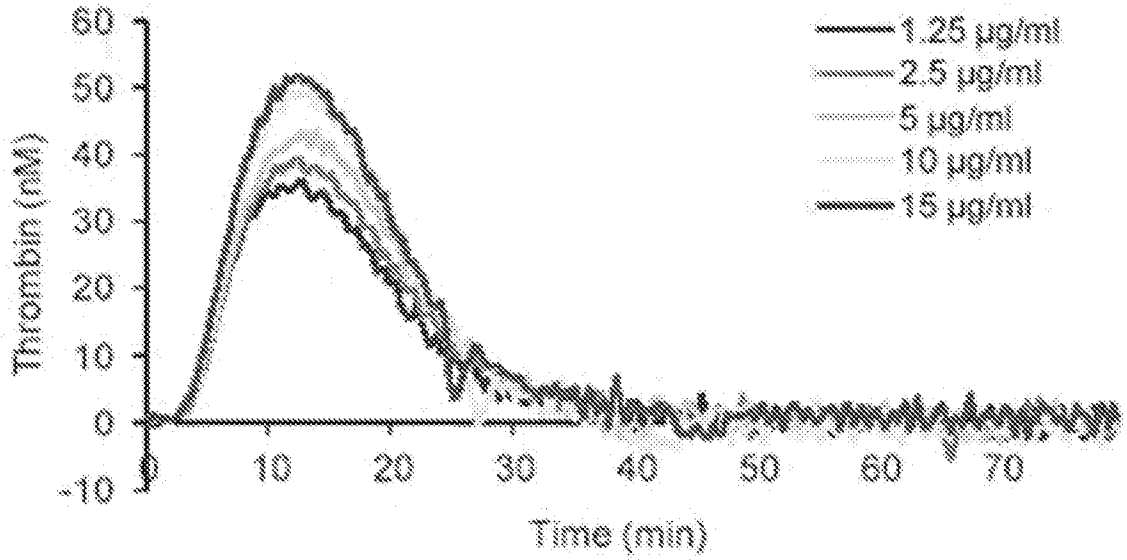


FIGURE 68

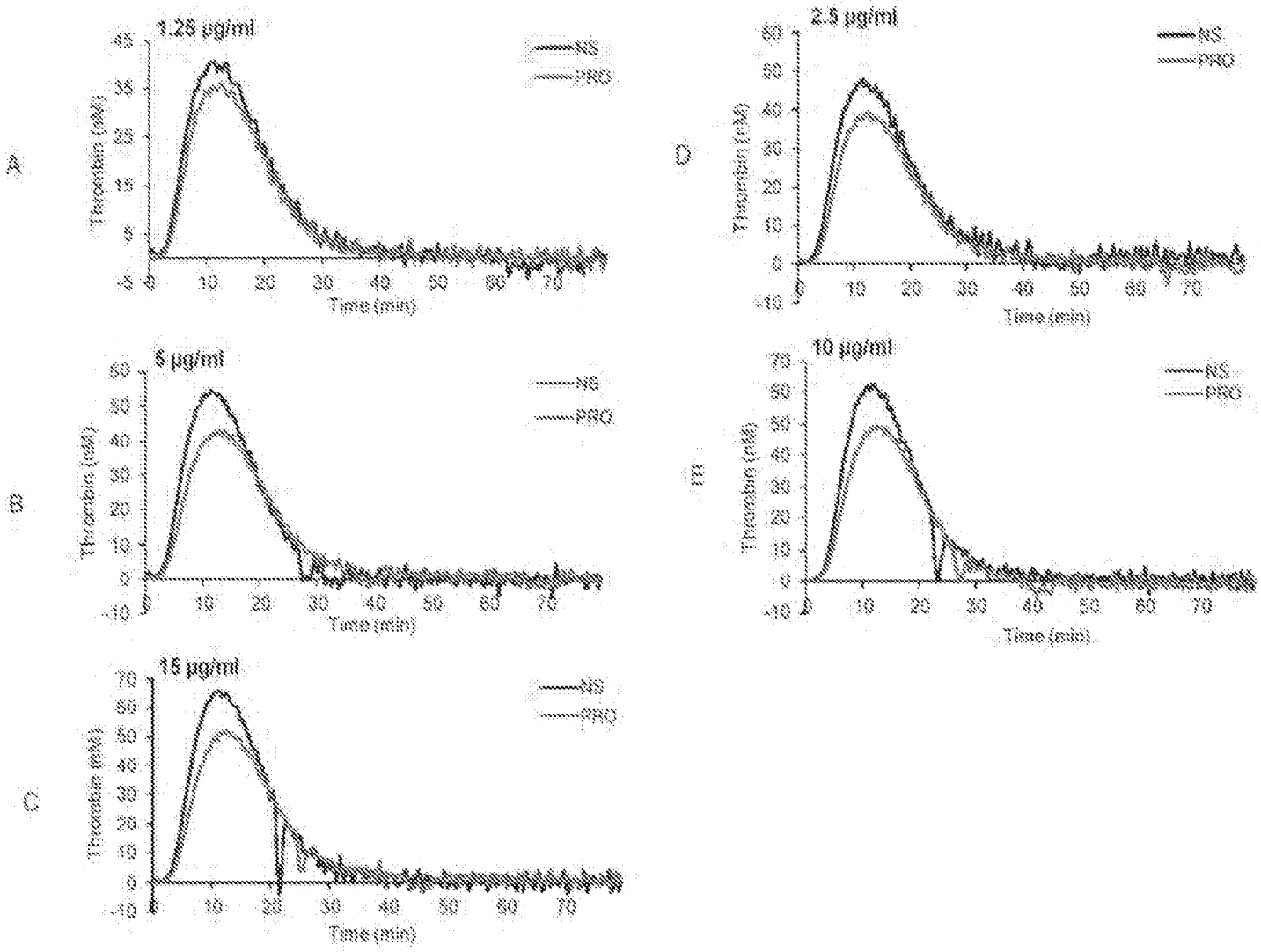

A
1.25 µg/ml
NS
PRO
Thrombin (nM)
Time (min)
D
2.5 µg/ml
NS
PRO
Thrombin (nM)
Time (min)
B
5 µg/ml
NS
PRO
Thrombin (nM)
Time (min)
E
10 µg/ml
NS
PRO
Thrombin (nM)
Time (min)
C
15 µg/ml
NS
PRO
Thrombin (nM)
Time (min)

FIGURE 69 (A-E)

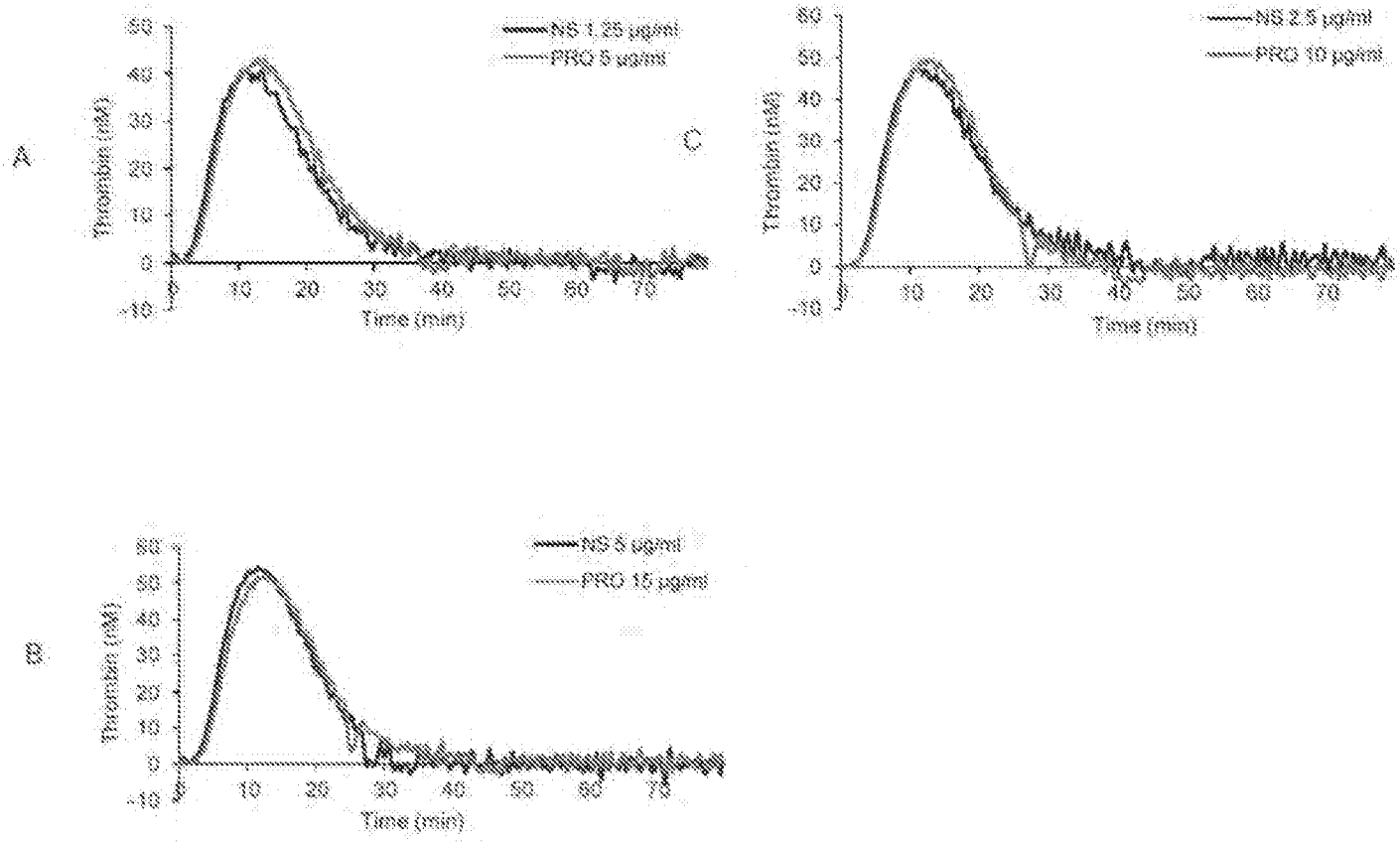


FIGURE 70 (A-C)

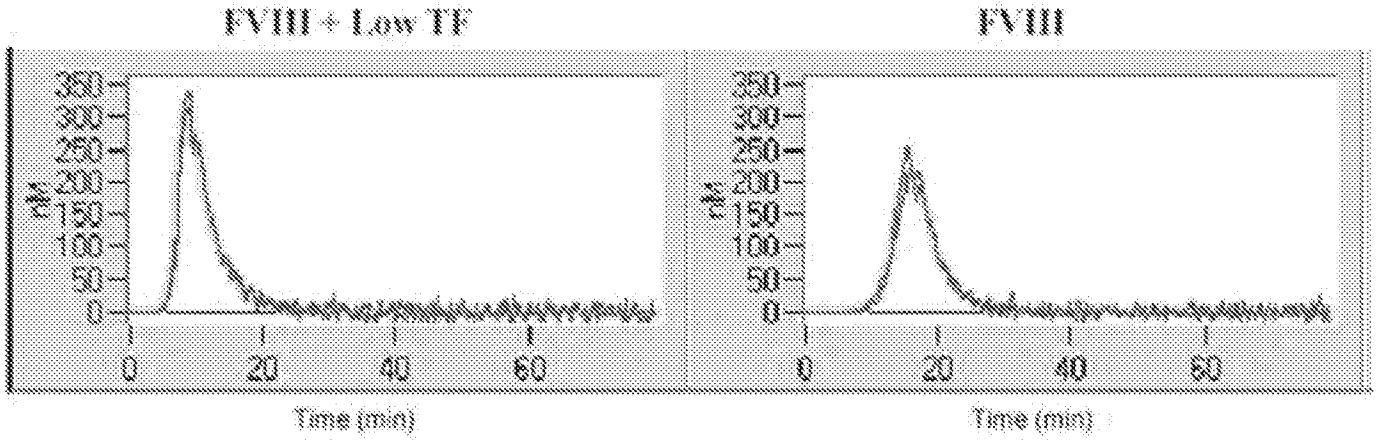


FIGURE 71

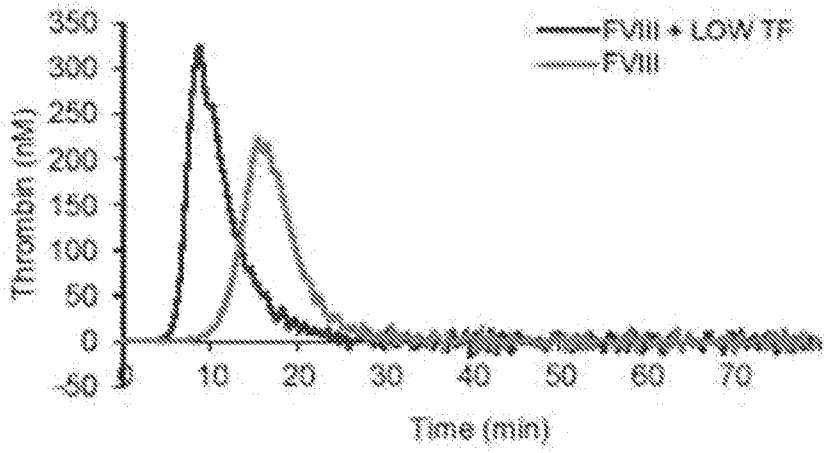


FIGURE 72

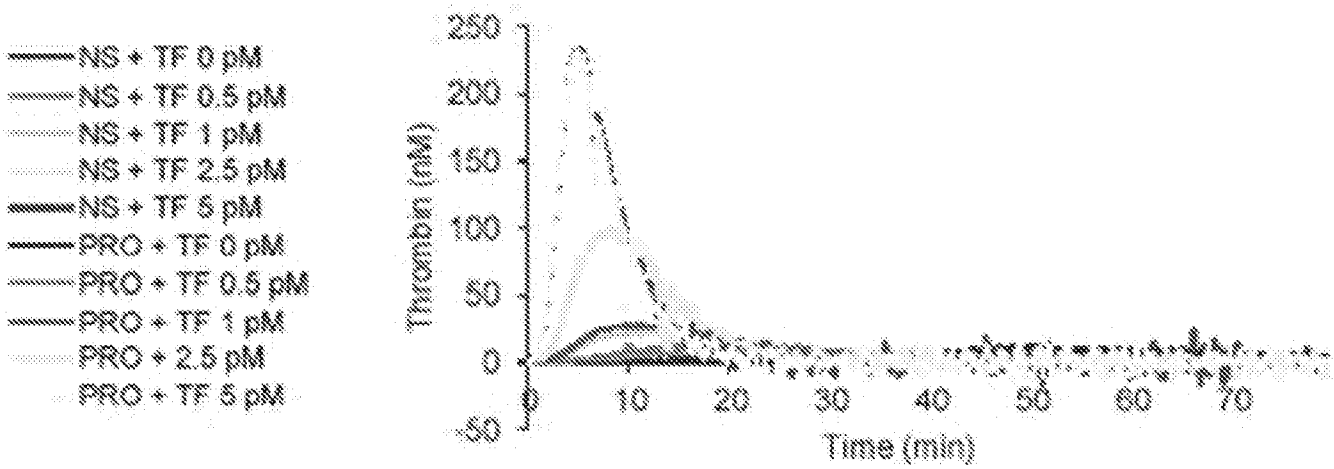


FIGURE 73

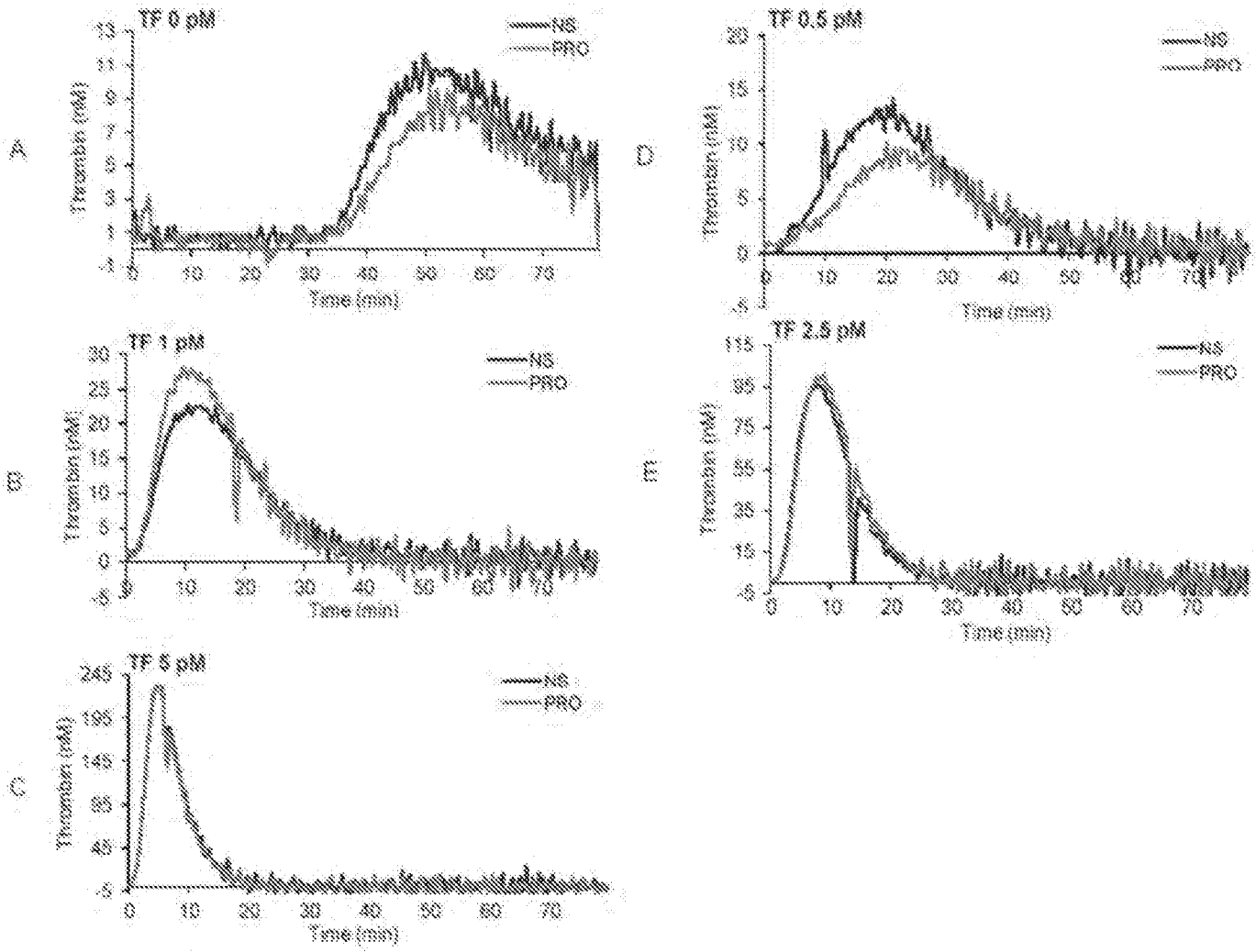


FIGURE 74 (A-E)

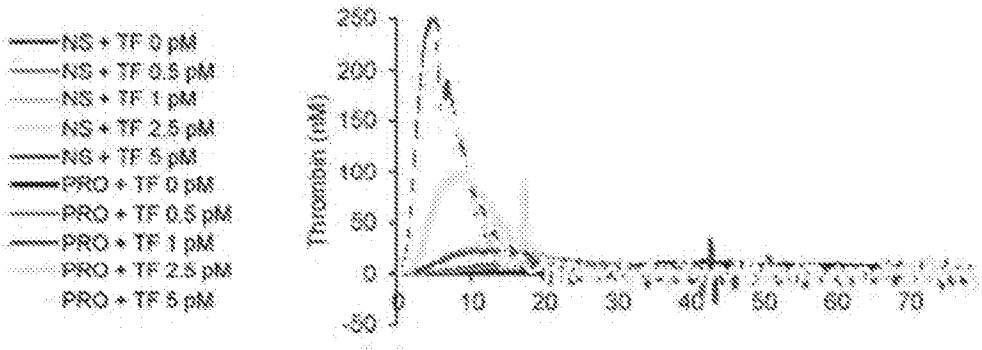


FIGURE 75

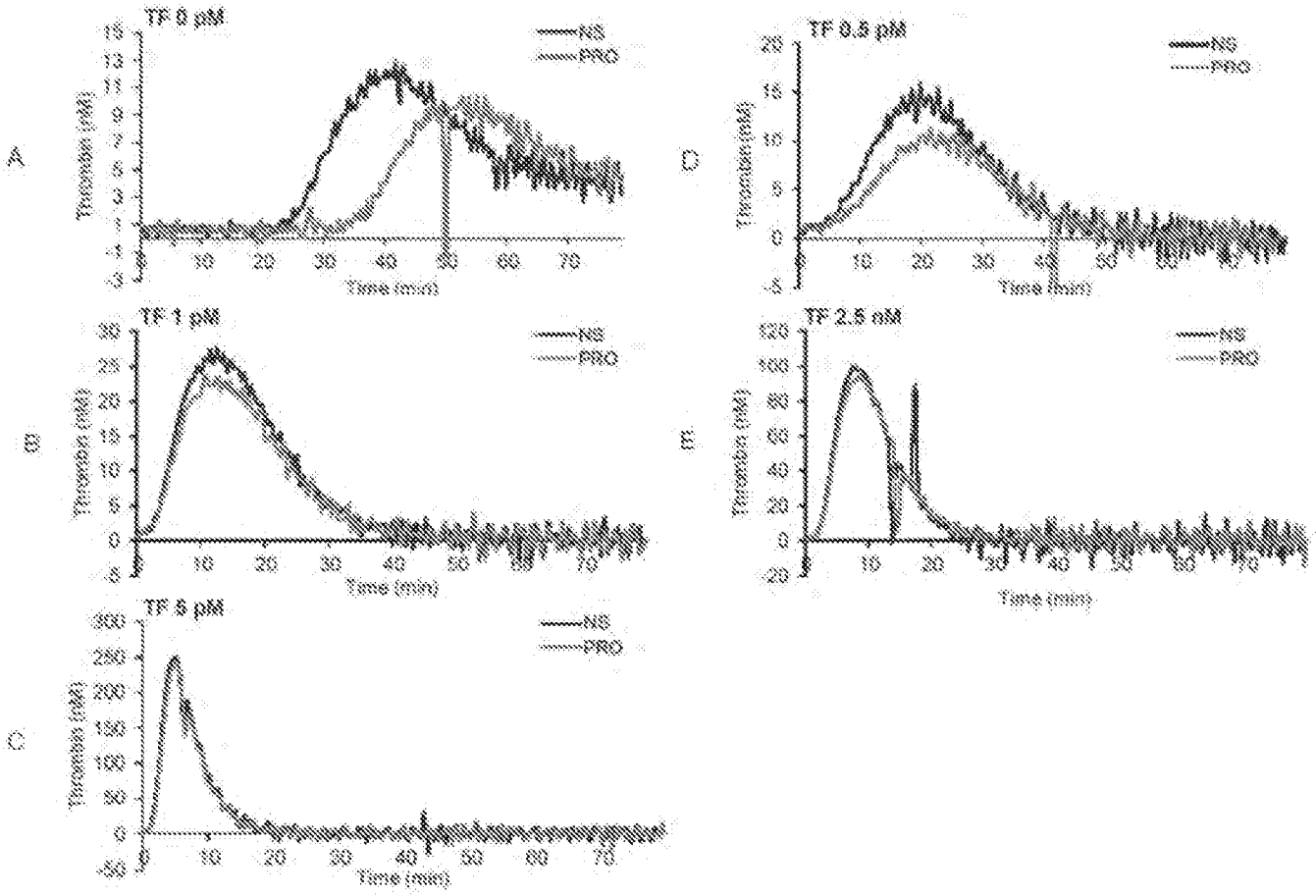


FIGURE 76 (A-E)

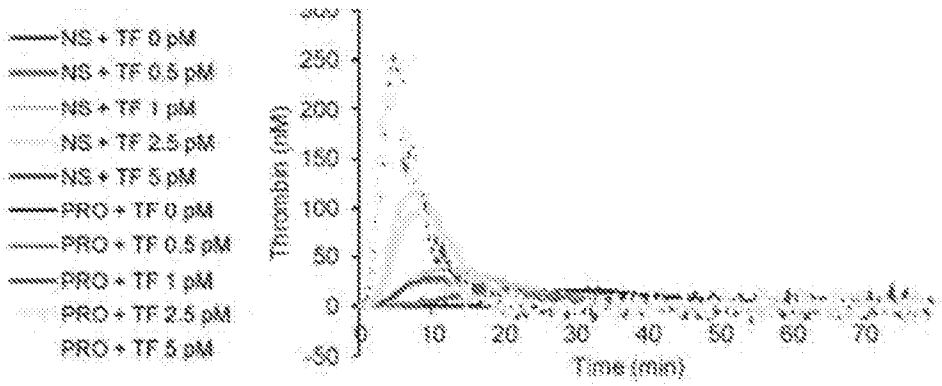


FIGURE 77

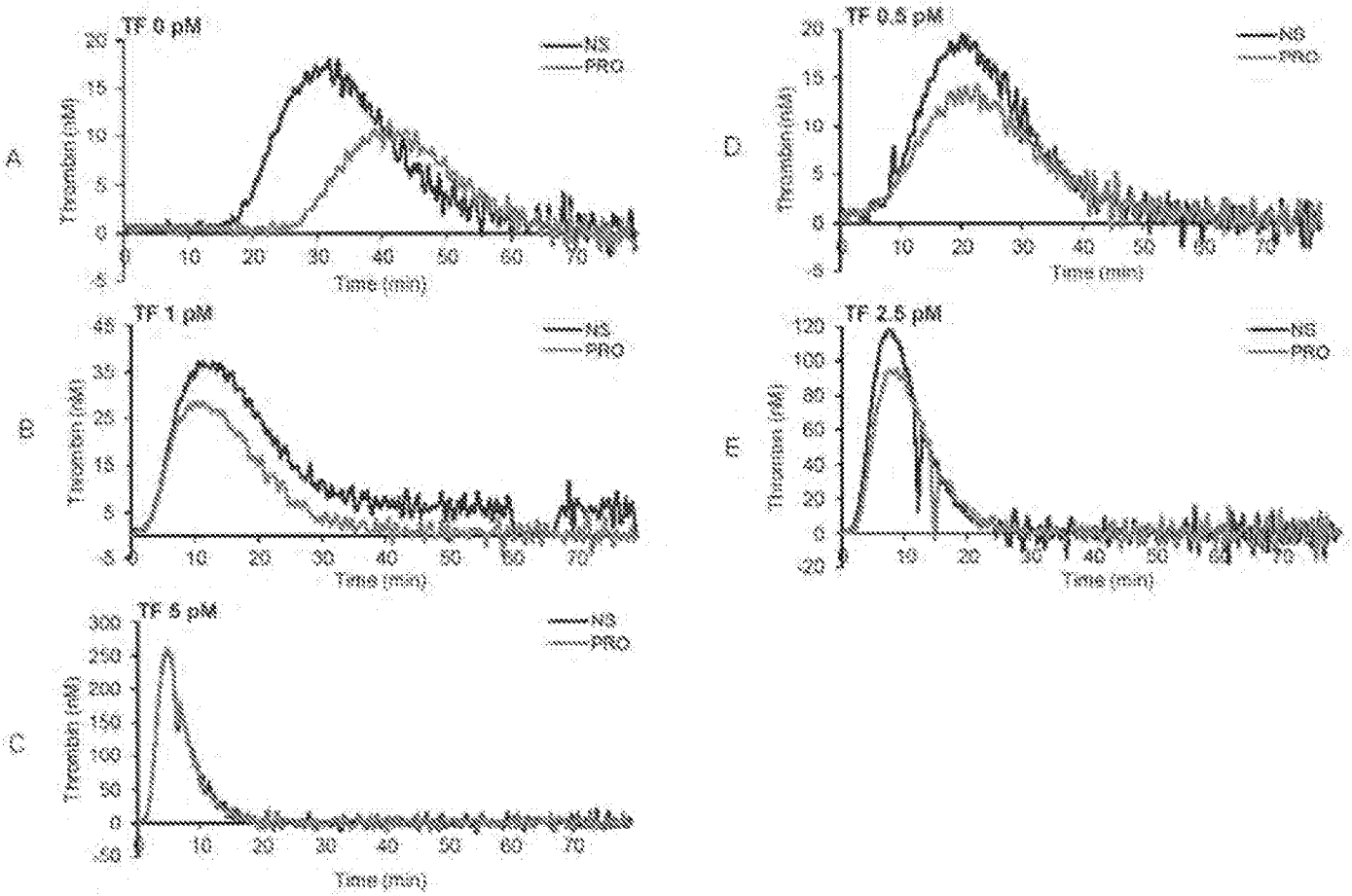


FIGURE 78 (A-E)

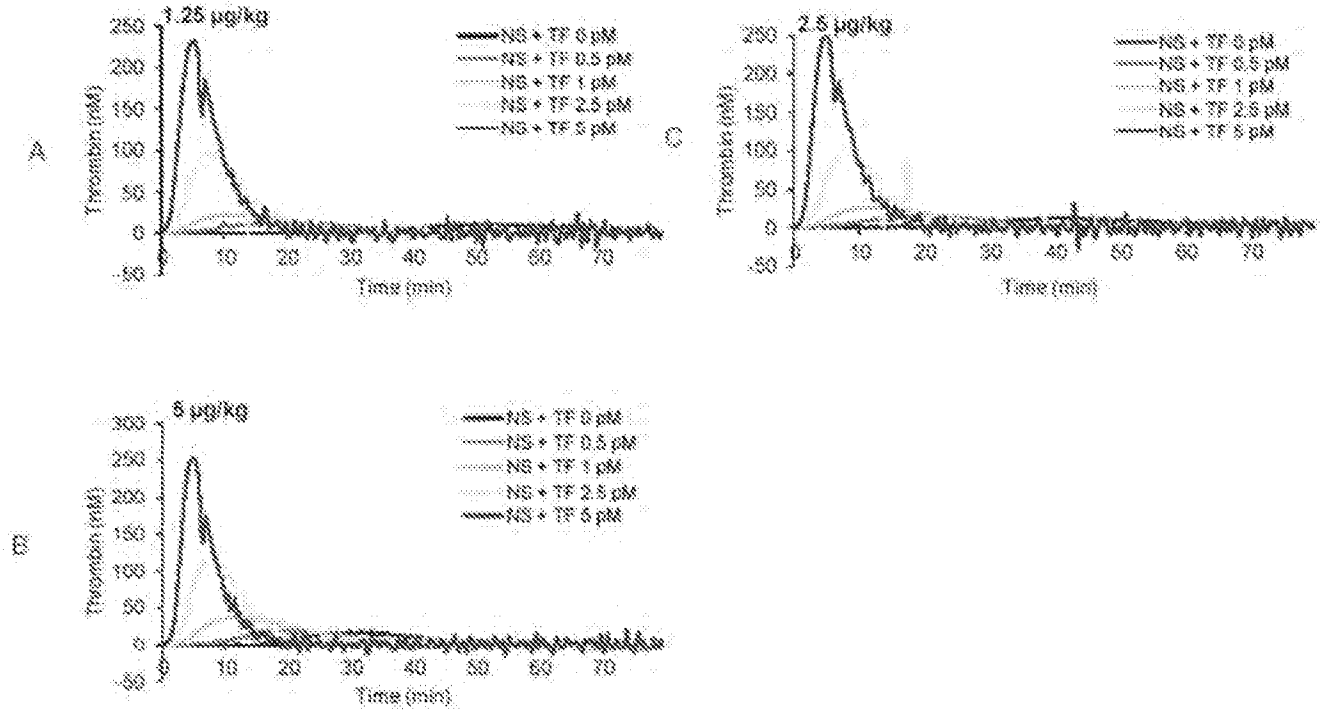


FIGURE 79 (A-C)

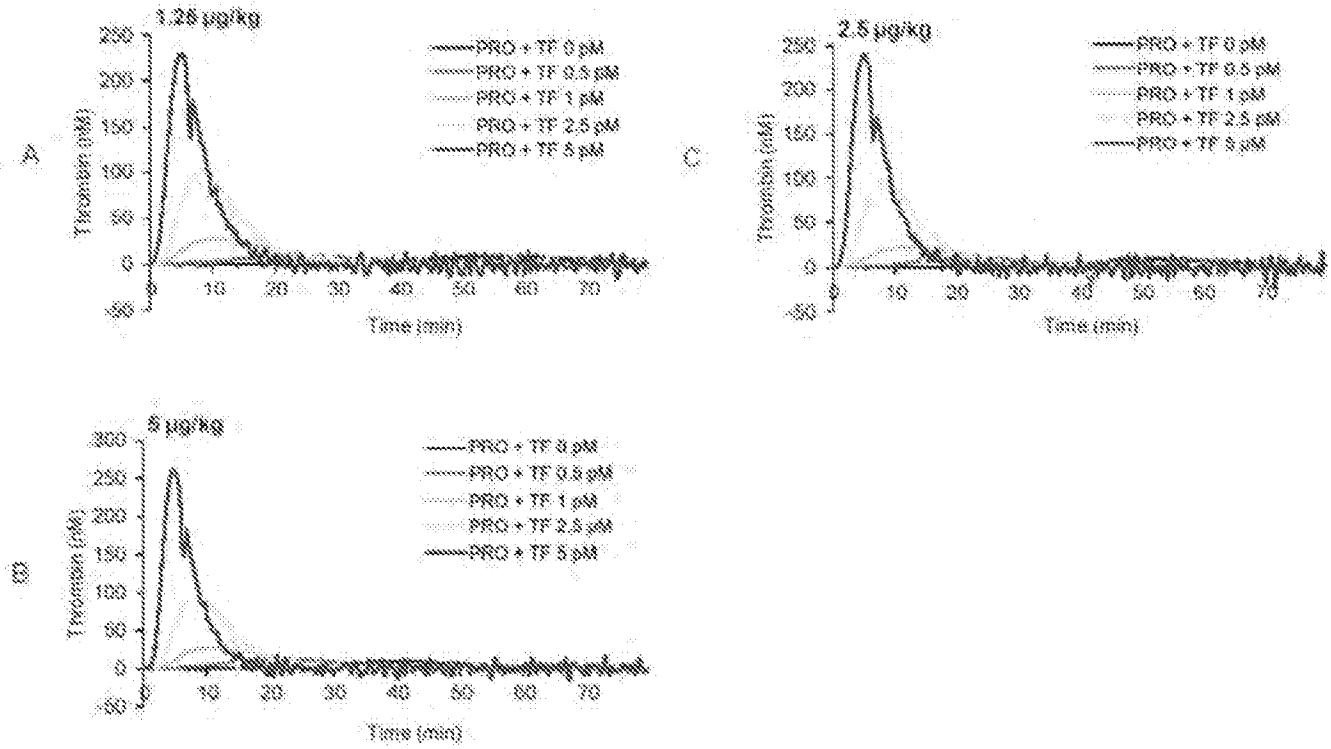


FIGURE 80 (A-C)

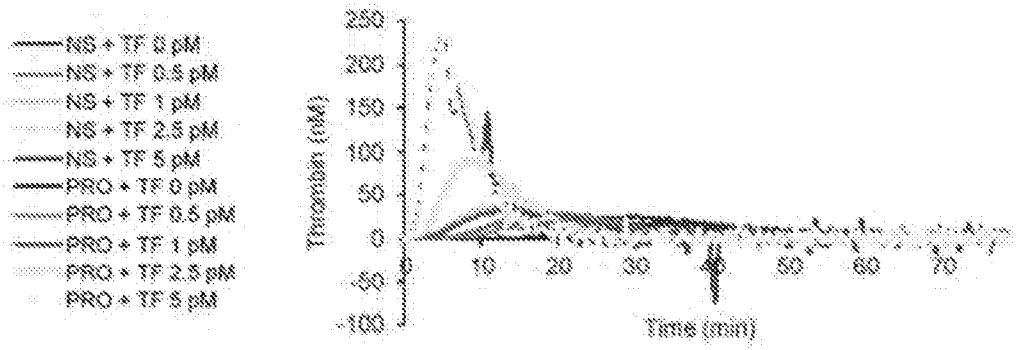


FIGURE 81

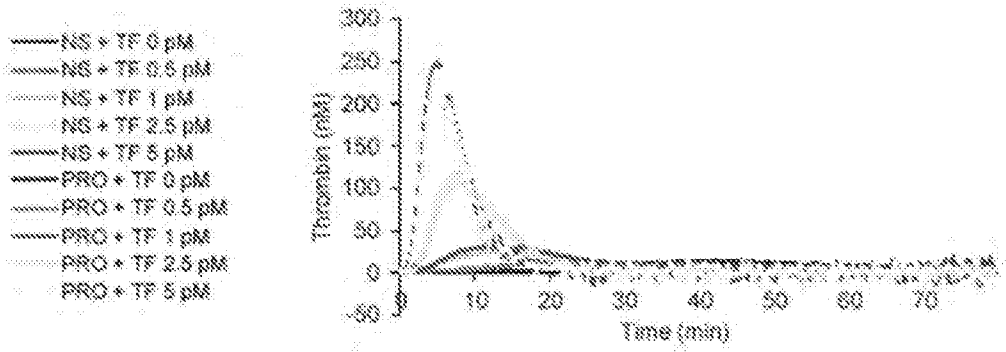


FIGURE 82

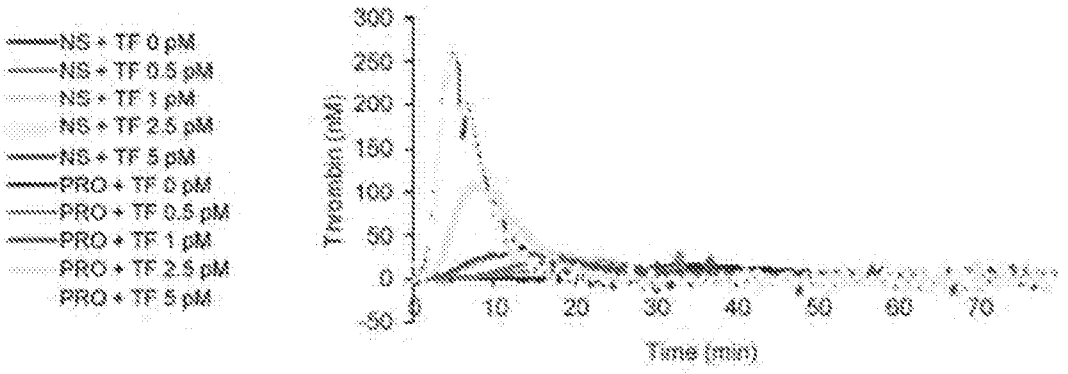


FIGURE 83

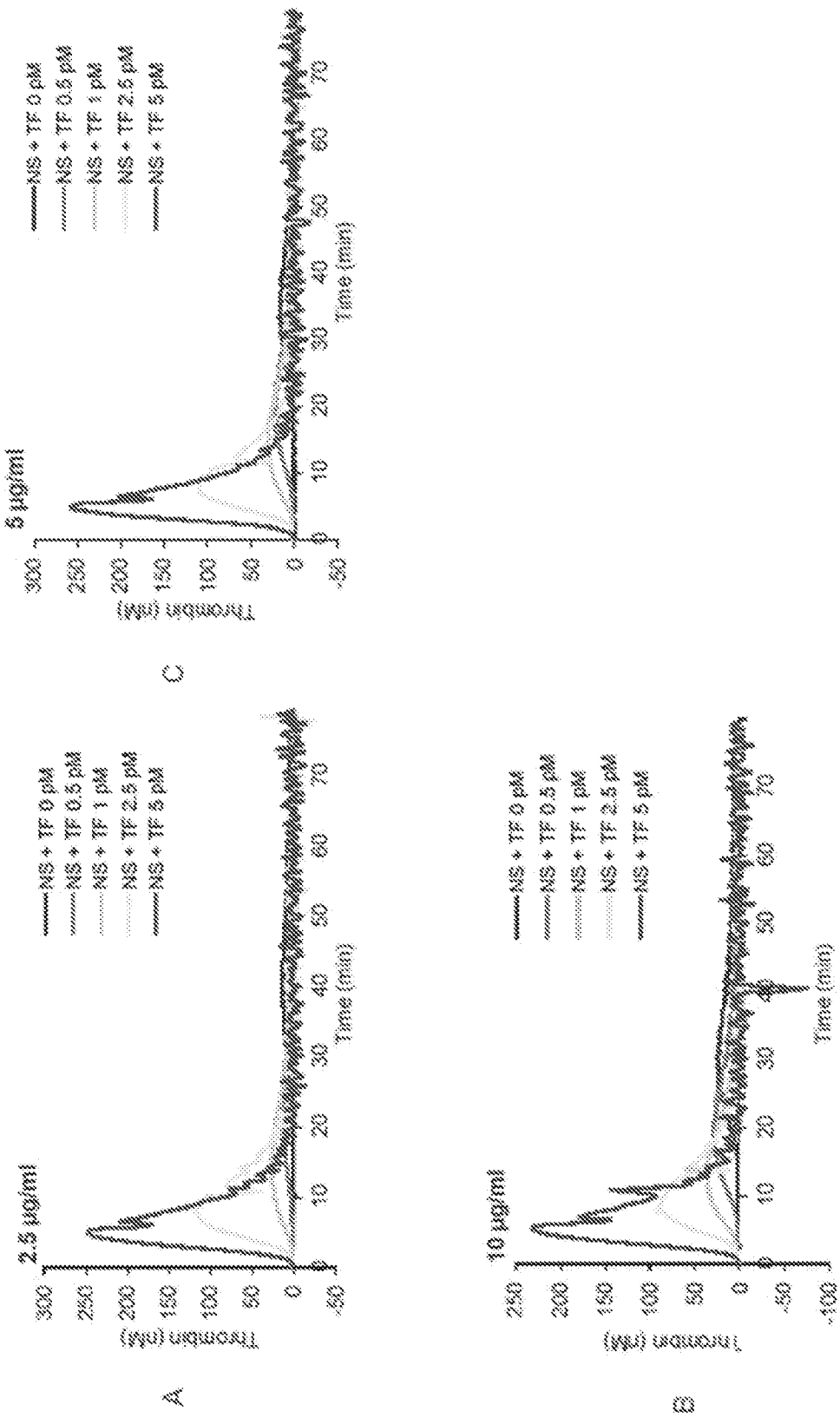

2.5 µg/ml
Thrombin (nM)
Time (min)
NS + TF 0 pM
NS + TF 0.5 pM
NS + TF 1 pM
NS + TF 2.5 pM
NS + TF 5 pM
A
5 µg/ml
Thrombin (nM)
Time (min)
NS + TF 0 pM
NS + TF 0.5 pM
NS + TF 1 pM
NS + TF 2.5 pM
NS + TF 5 pM
C
10 µg/ml
Thrombin (nM)
Time (min)
NS + TF 0 pM
NS + TF 0.5 pM
NS + TF 1 pM
NS + TF 2.5 pM
NS + TF 5 pM
B

FIGURE 84 (A-C)

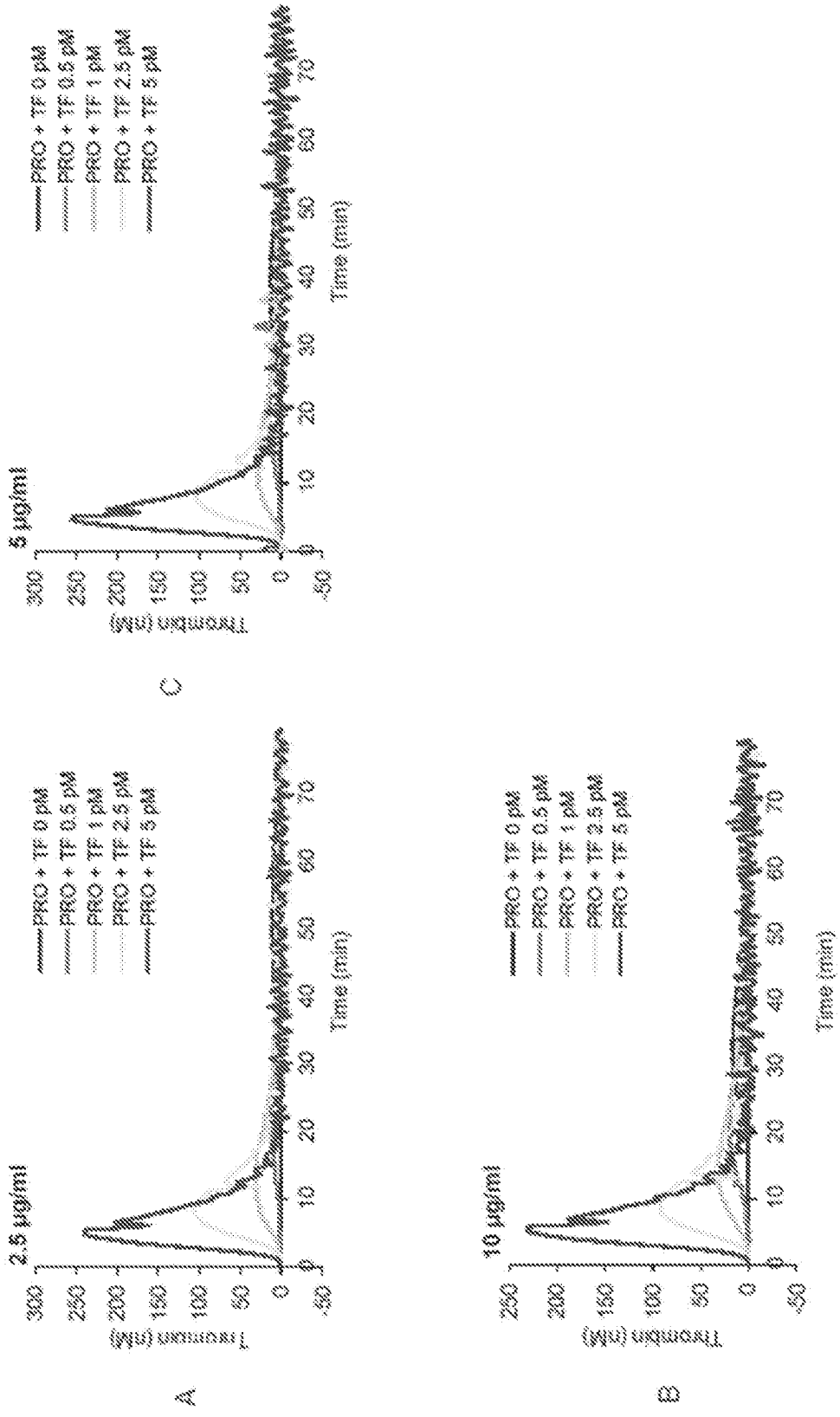

A
2.5 µg/ml
Thrombin (nM)
Time (min)
PRO + TF 0 pM
PRO + TF 0.5 pM
PRO + TF 1 pM
PRO + TF 2.5 pM
PRO + TF 5 pM
B
10 µg/ml
Thrombin (nM)
Time (min)
PRO + TF 0 pM
PRO + TF 0.5 pM
PRO + TF 1 pM
PRO + TF 2.5 pM
PRO + TF 5 pM
C
5 µg/ml
Thrombin (nM)
Time (min)
PRO + TF 0 pM
PRO + TF 0.5 pM
PRO + TF 1 pM
PRO + TF 2.5 pM
PRO + TF 5 pM

FIGURE 85 (A-C)

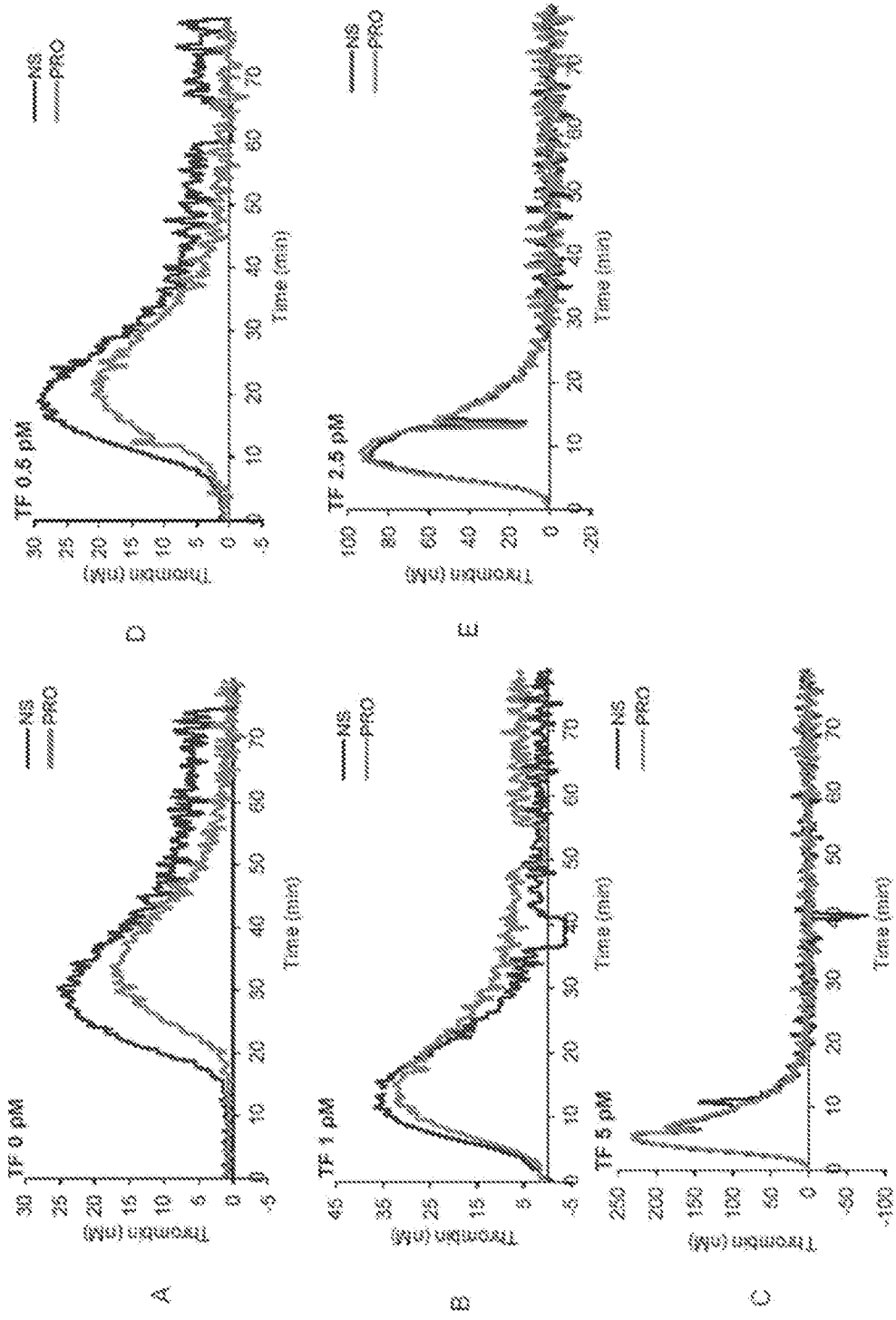

A
TF 0 pM
B
TF 1 pM
C
TF 5 pM
D
TF 0.5 pM
E
TF 2.5 pM
NS
PRO
Thrombin (nM)
Time (min)

FIGURE 86 (A-E)

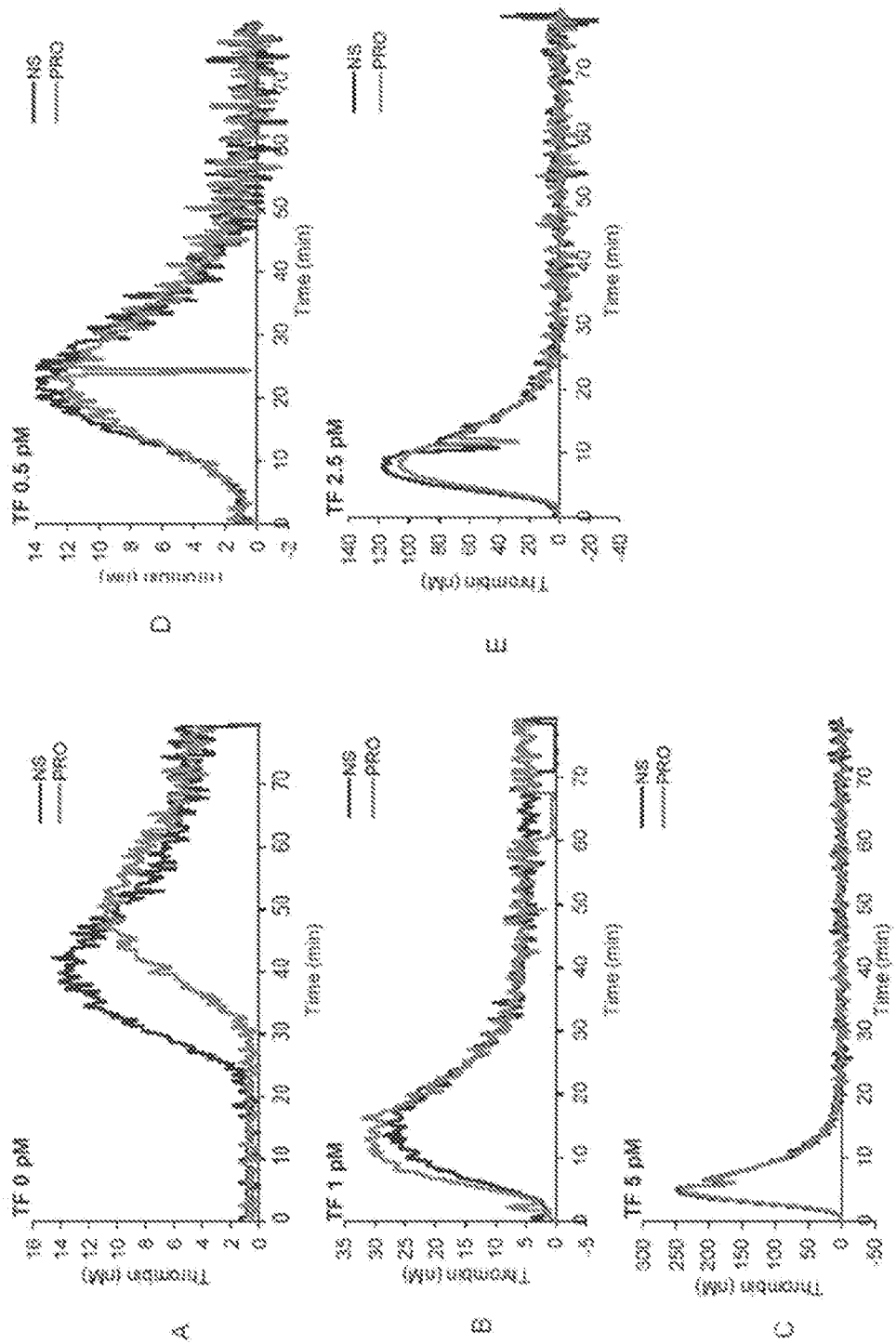

A
TF 0 pM
NS
PRO
Thrombin (nM)
Time (min)
B
TF 1 pM
C
TF 5 pM
D
TF 0.5 pM
E
TF 2.5 pM

FIGURE 87 (A-E)

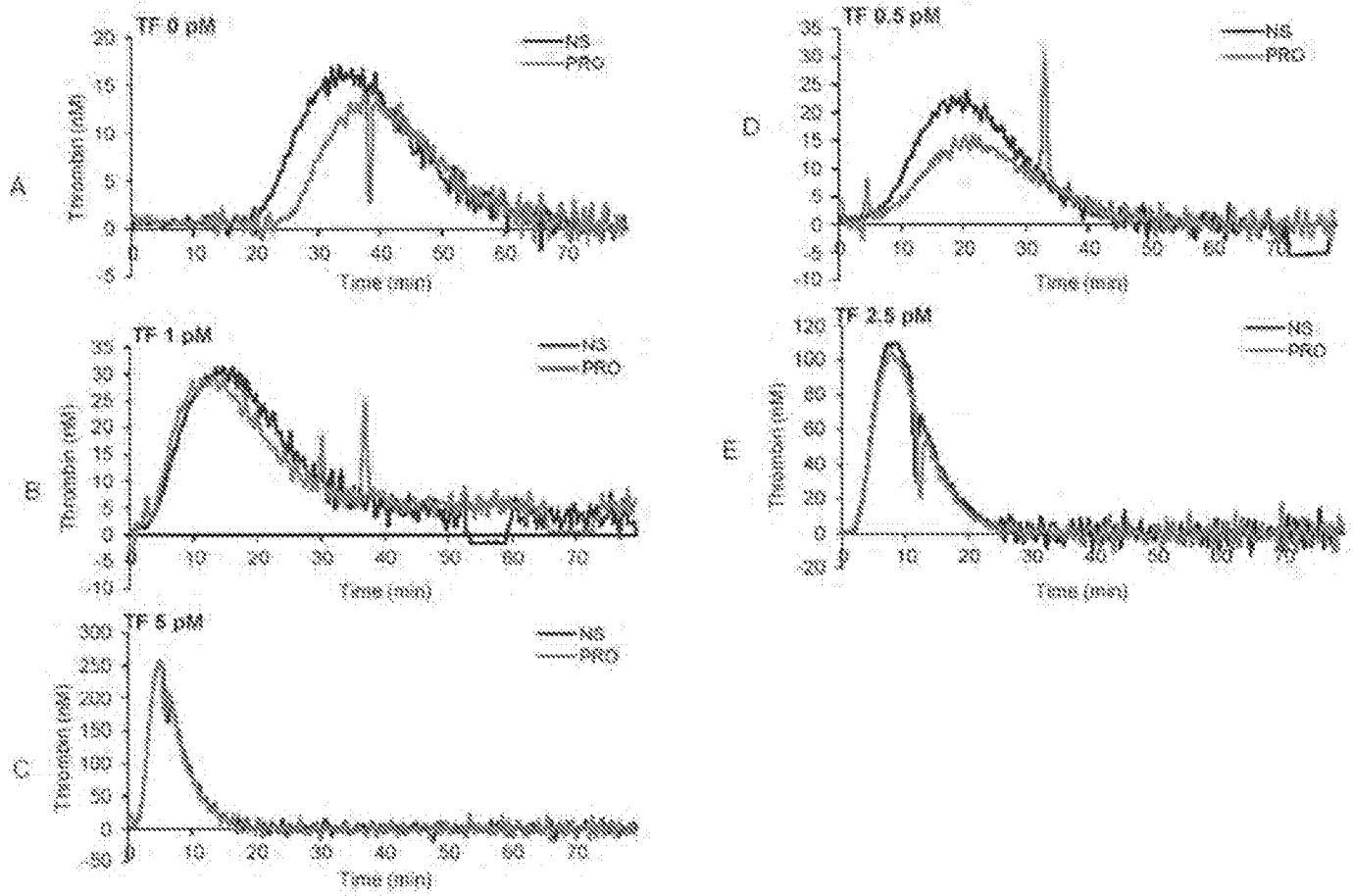


FIGURE 88 (A-E)

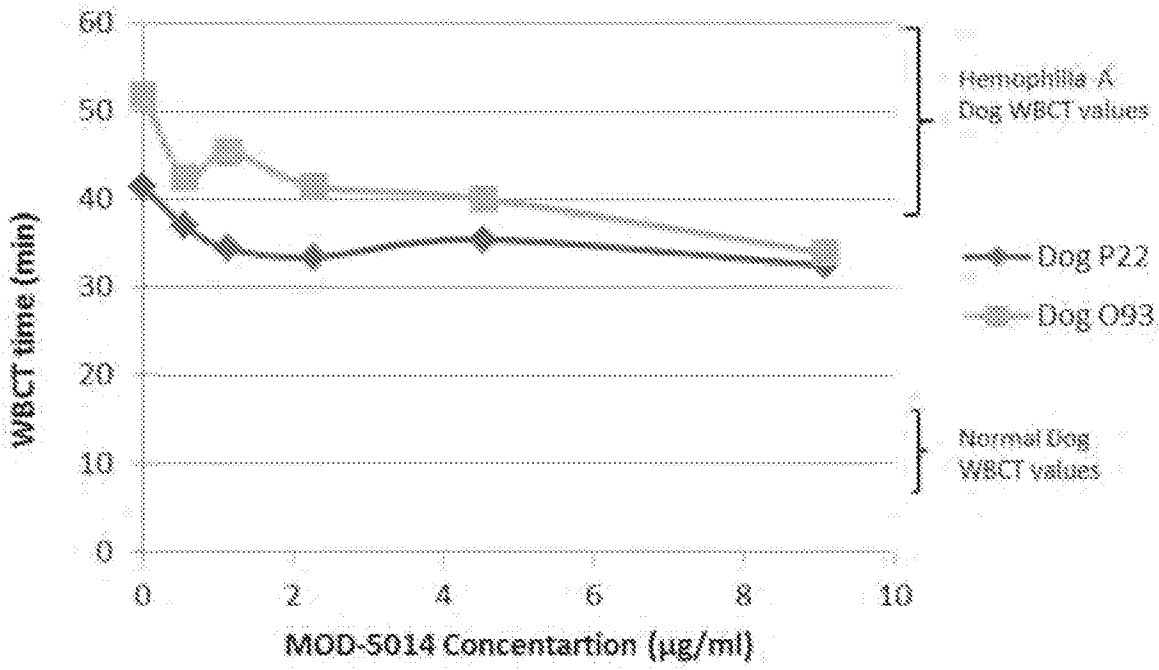


FIGURE 89

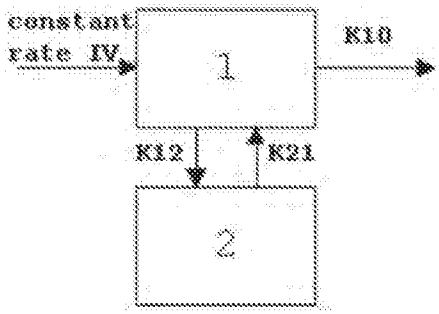


FIGURE 90

Plasma_Concentration

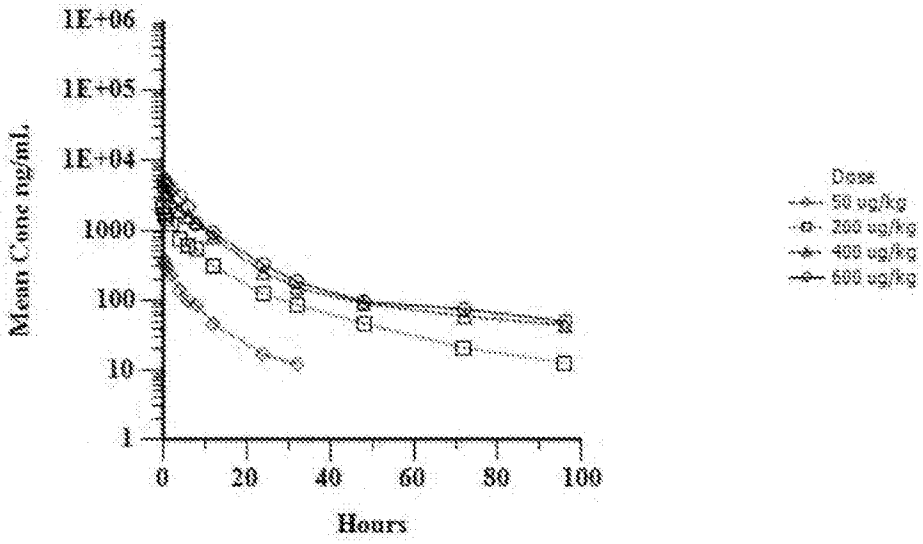


FIGURE 91

Activity

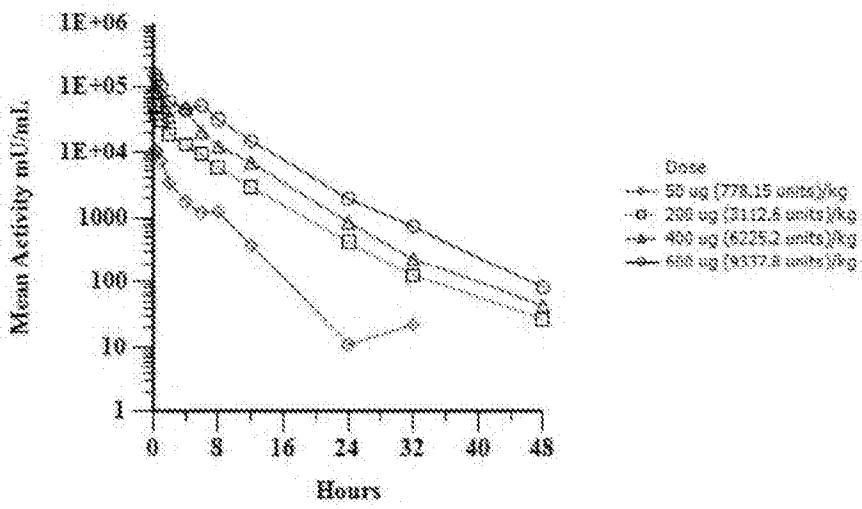


FIGURE 92

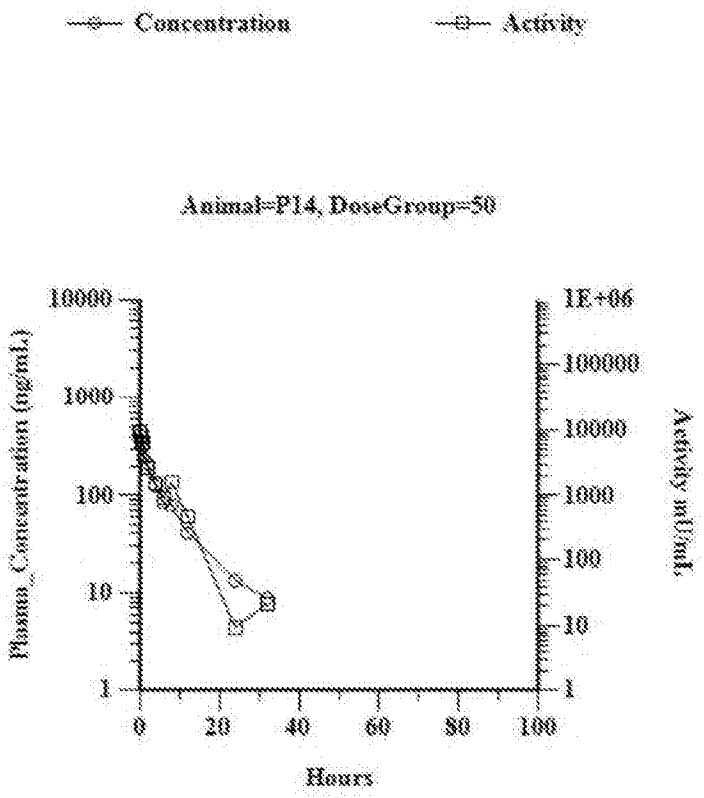


FIGURE 93A

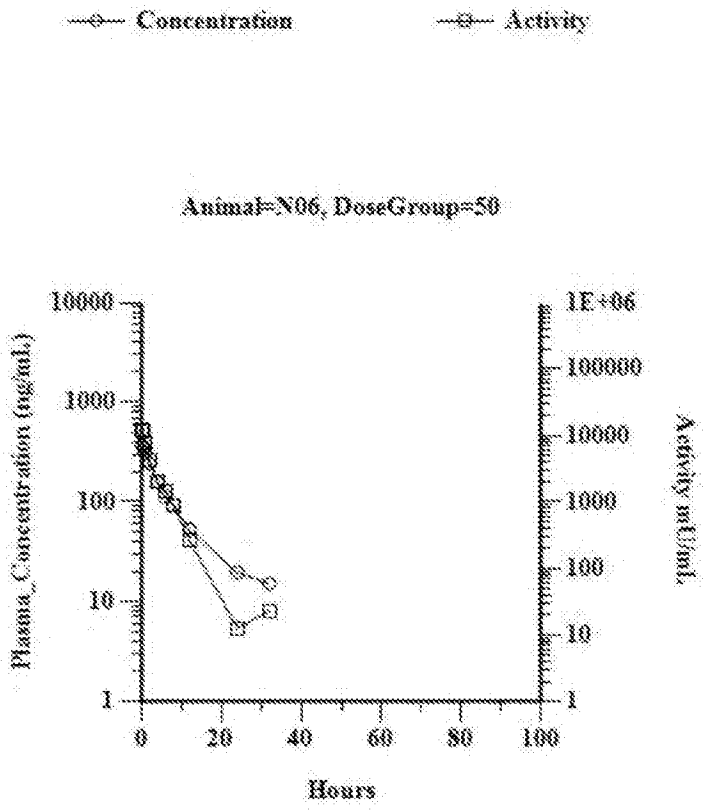


FIGURE 93B

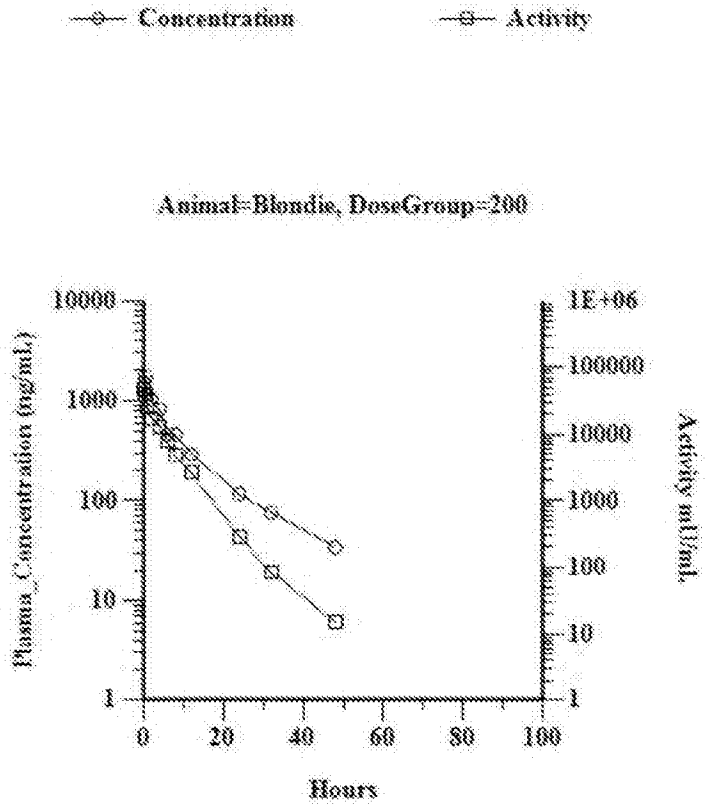


FIGURE 94A

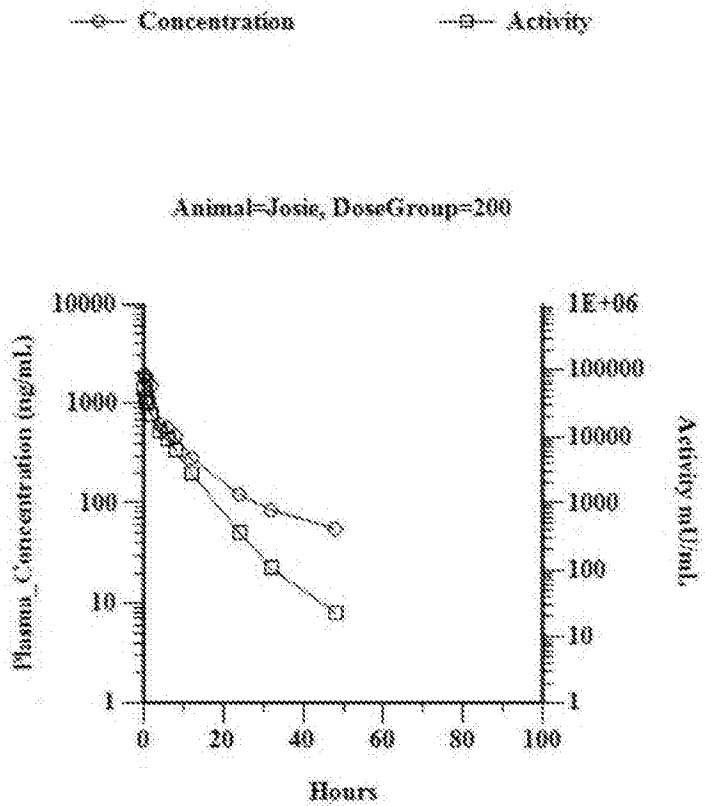


FIGURE 94B

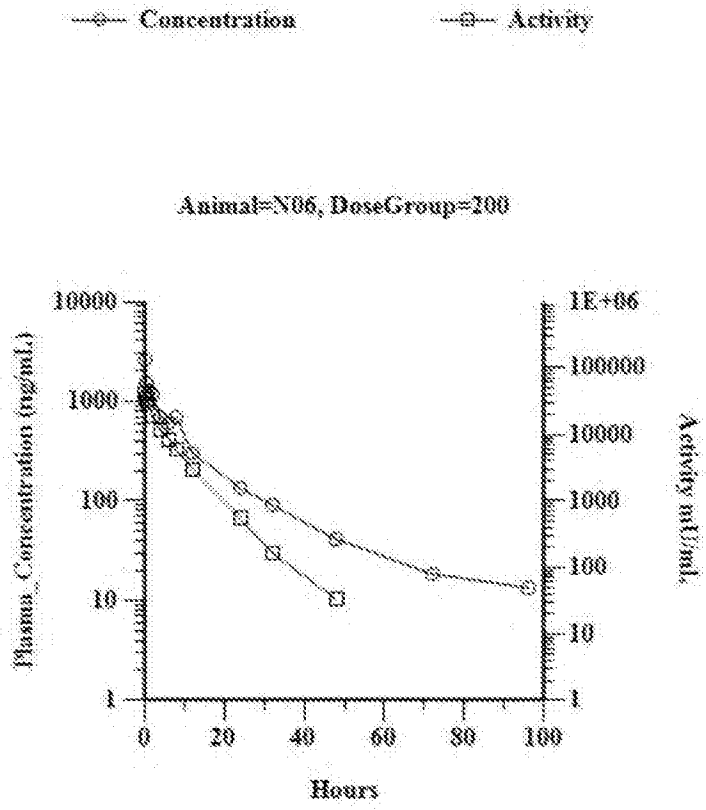


FIGURE 94C

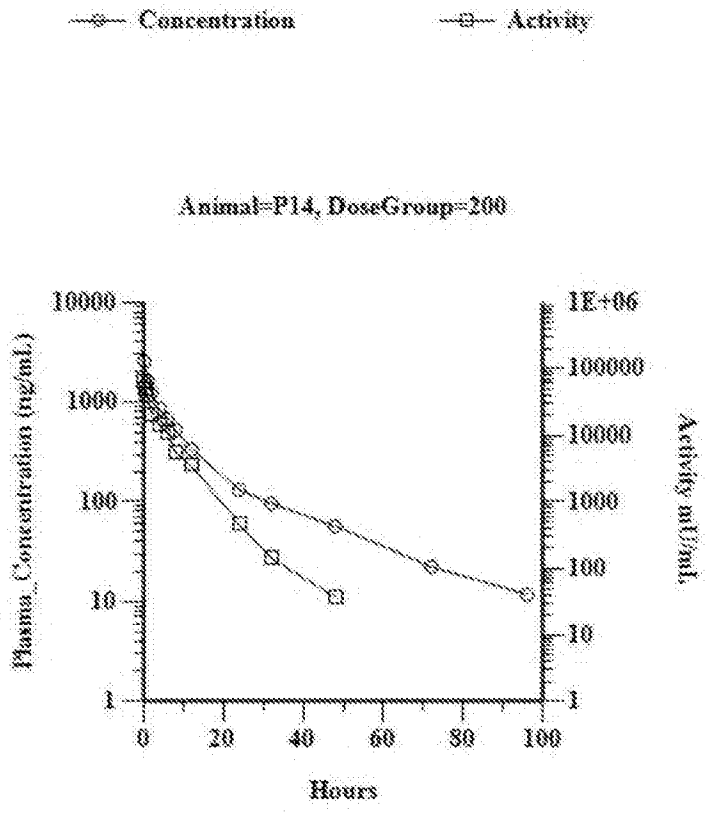


FIGURE 94D

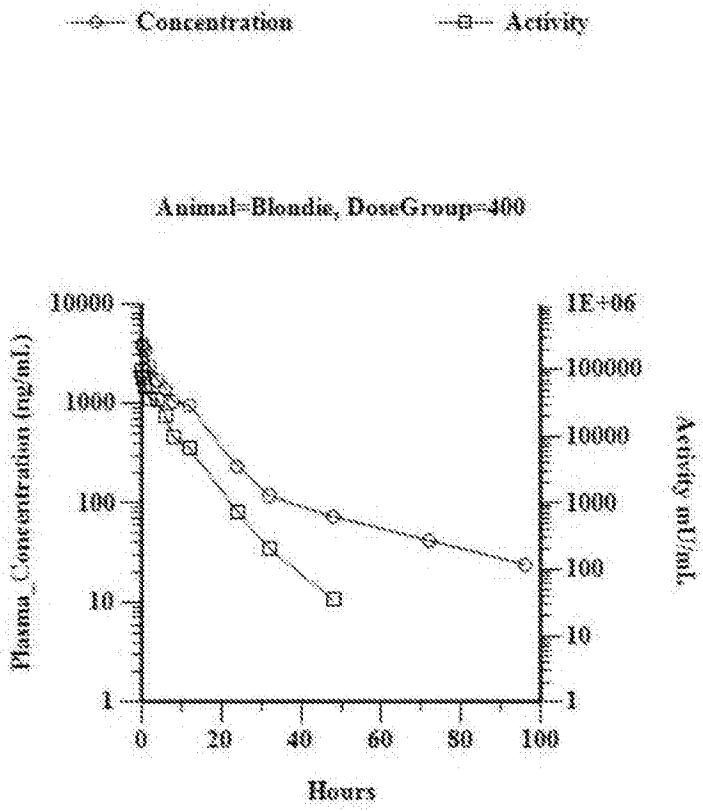


FIGURE 95A

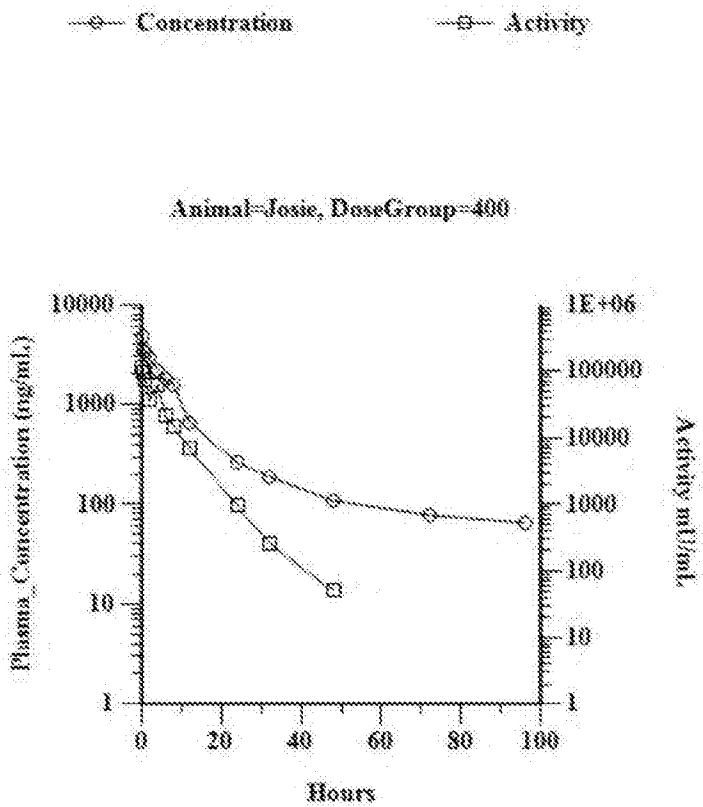


FIGURE 95B

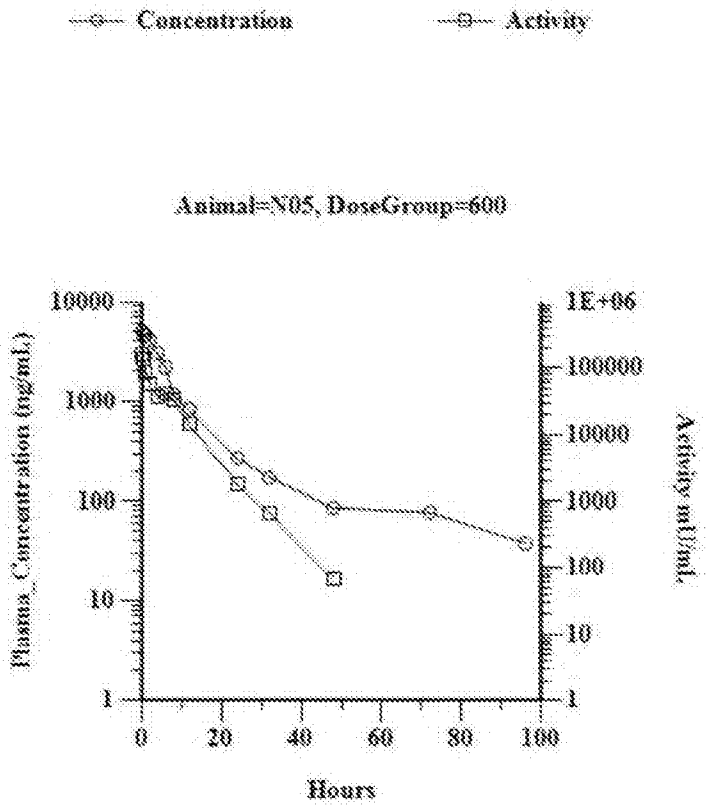


FIGURE 96A

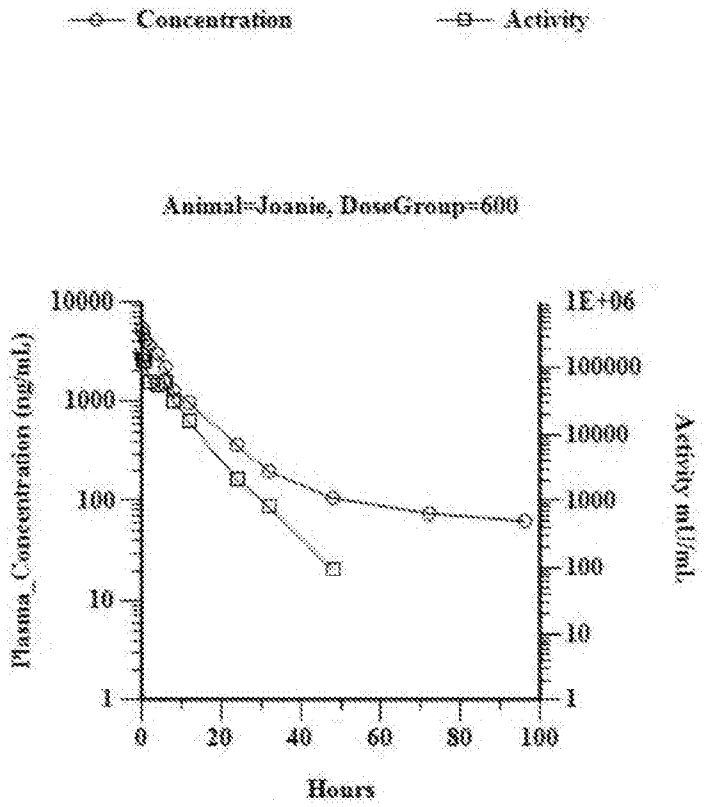


FIGURE 96B

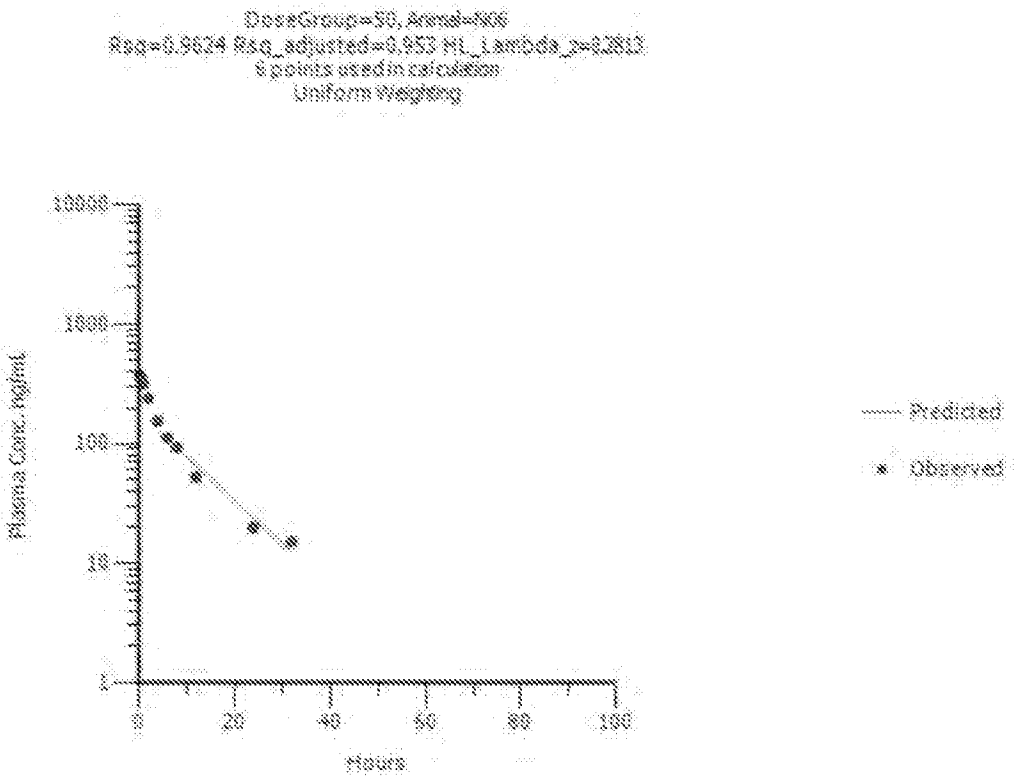


FIGURE 97A

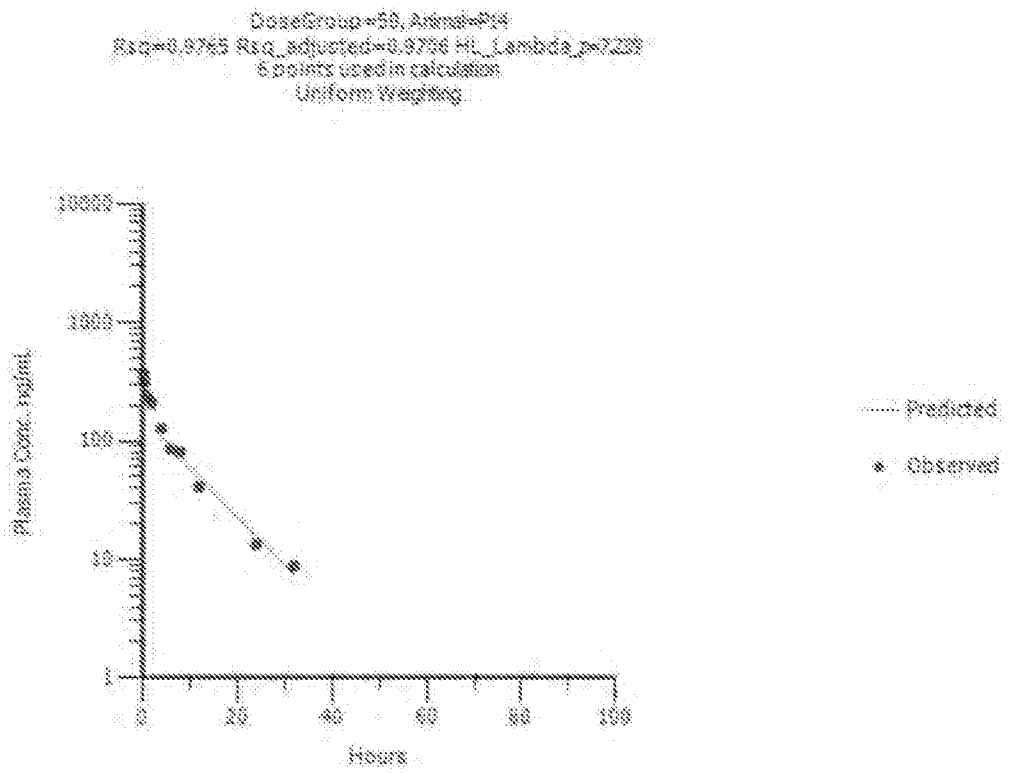


FIGURE 97B

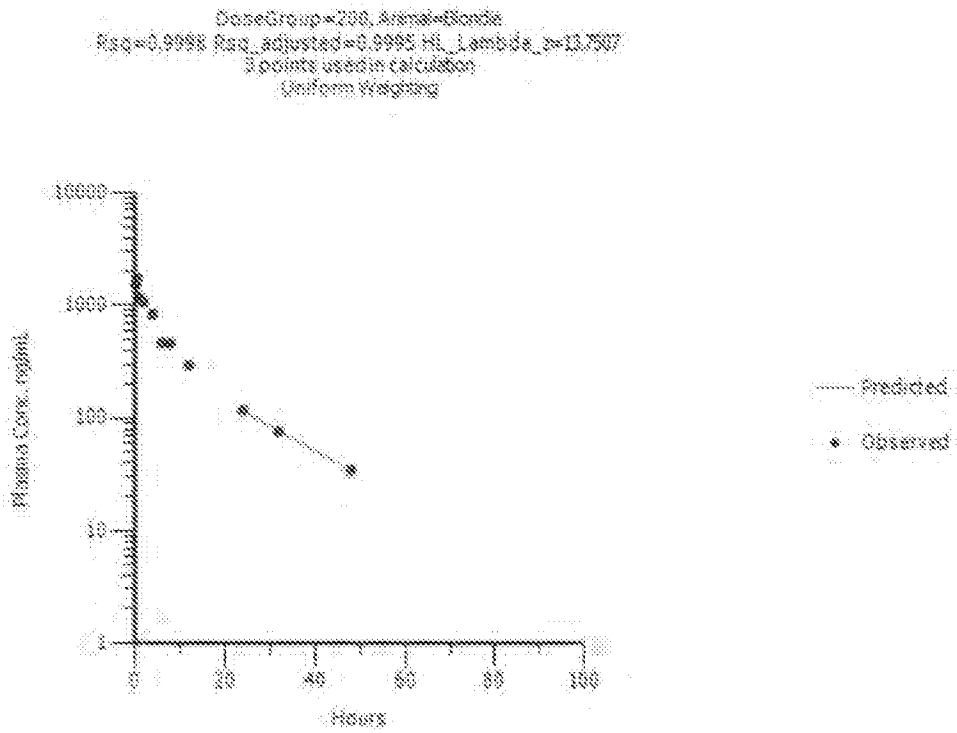


FIGURE 97C

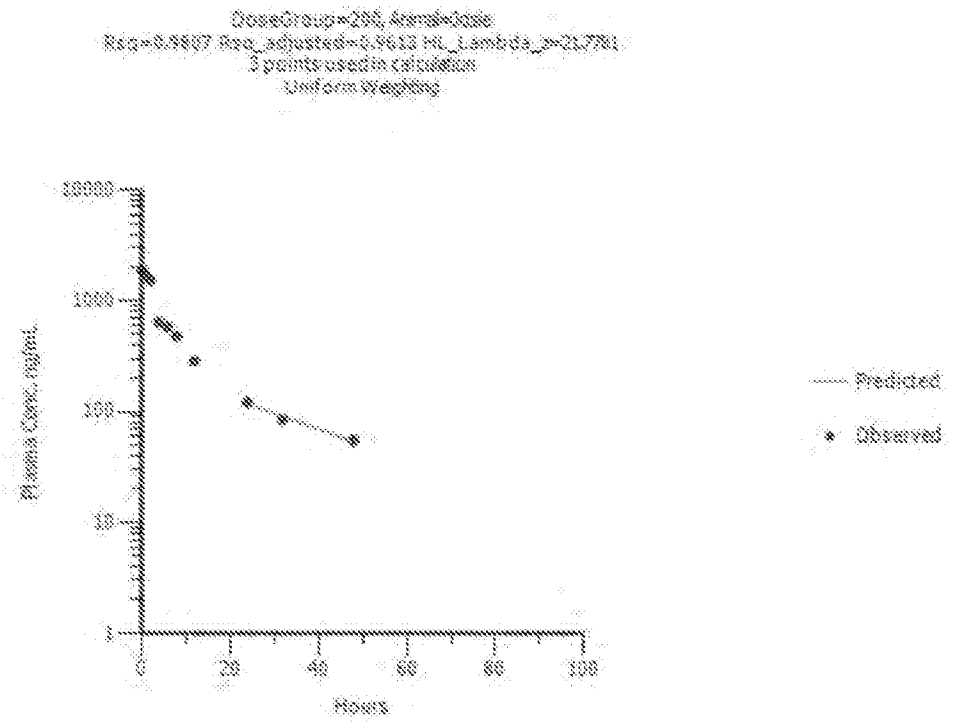


FIGURE 97D

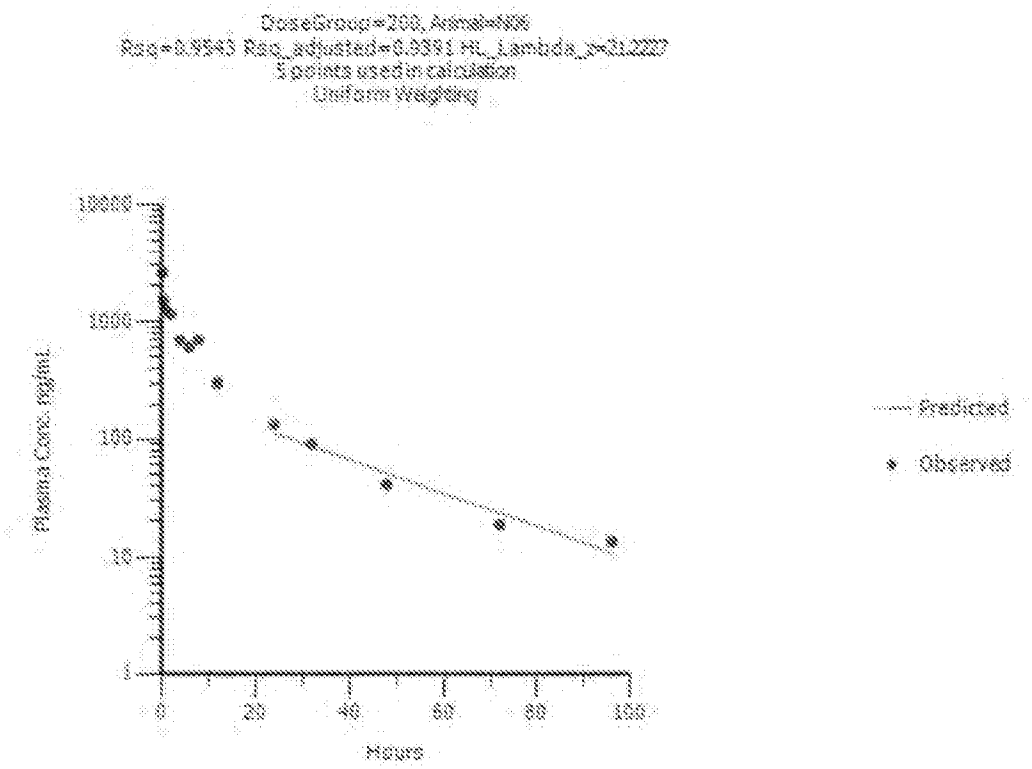


FIGURE 97E

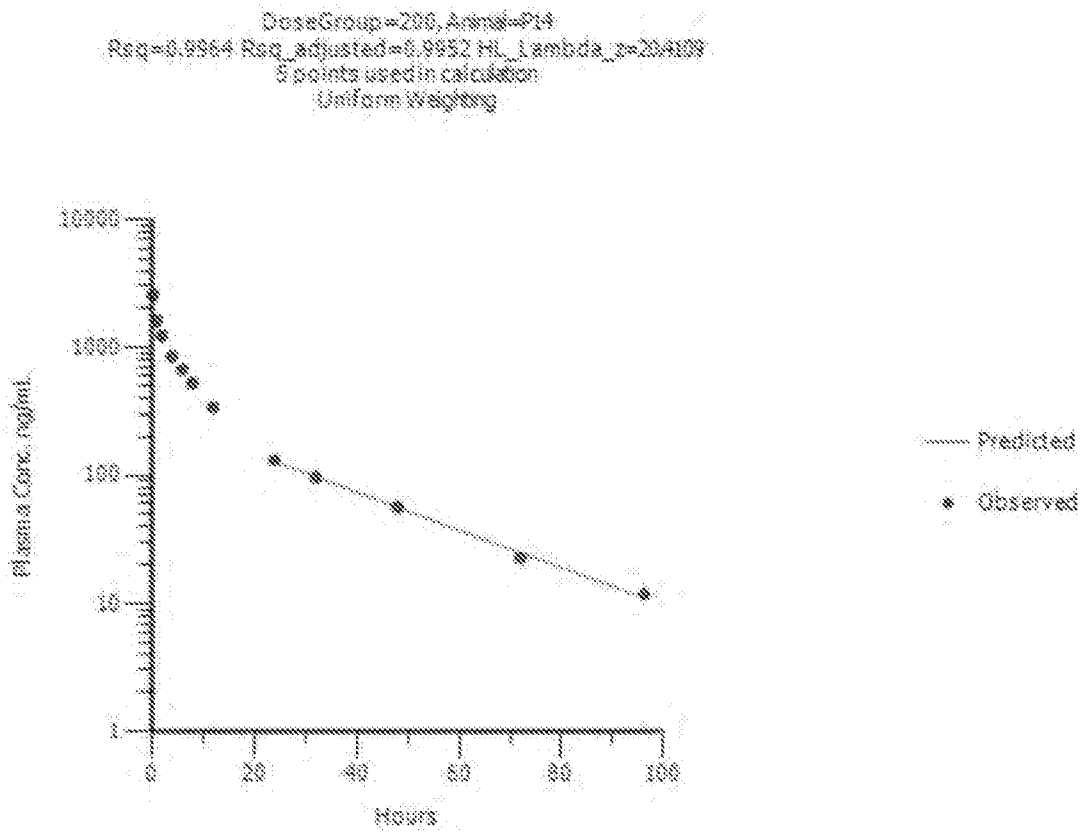


FIGURE 97F

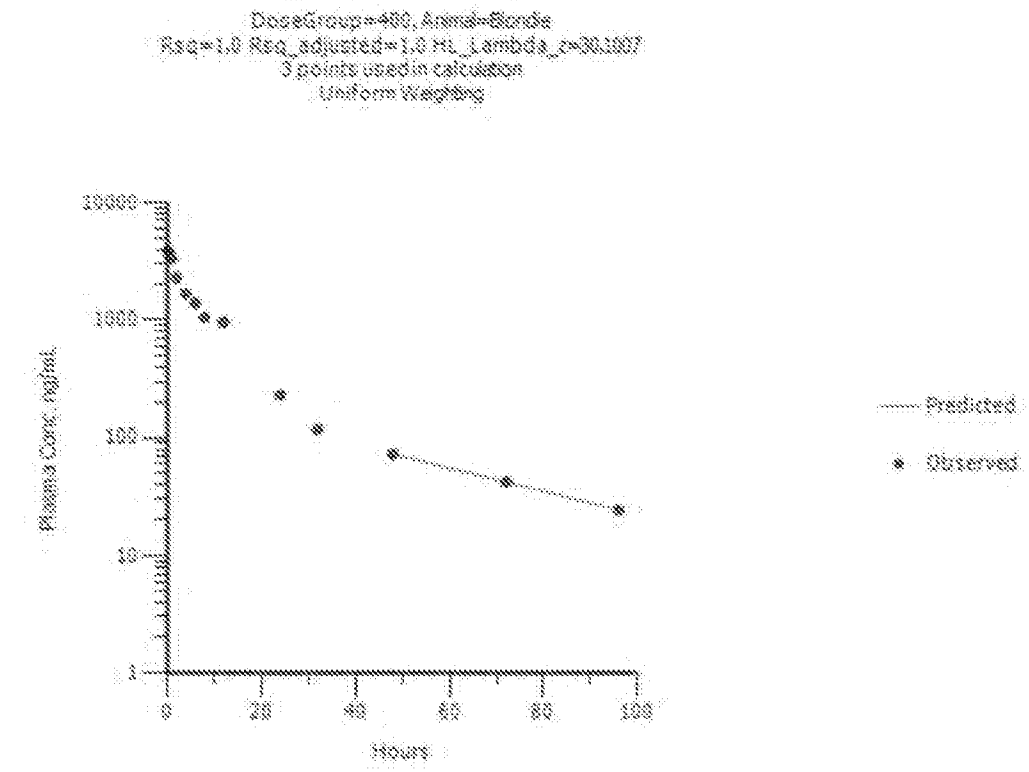


FIGURE 97G

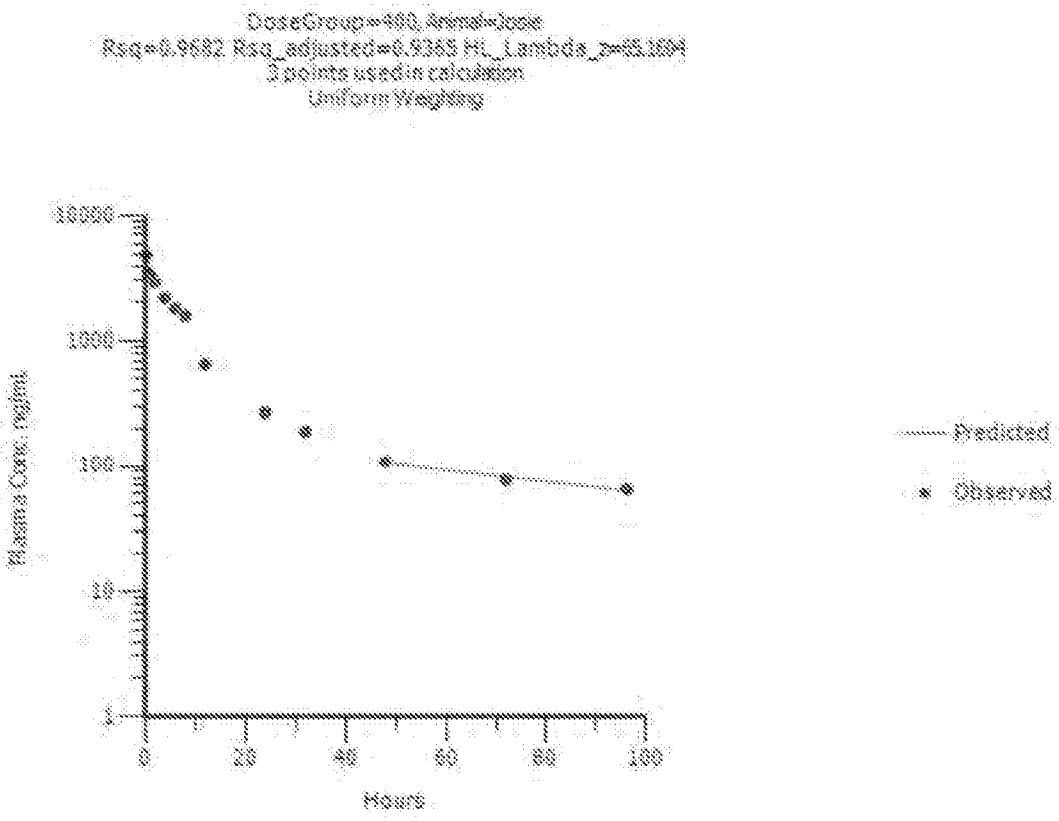


FIGURE 97H

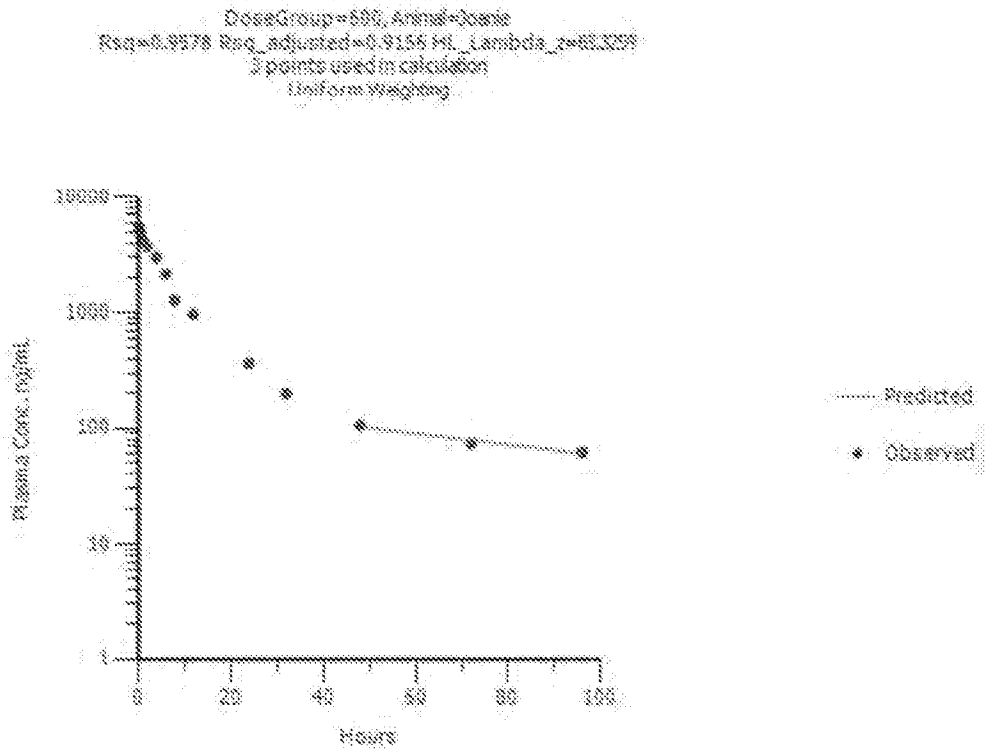


FIGURE 97I

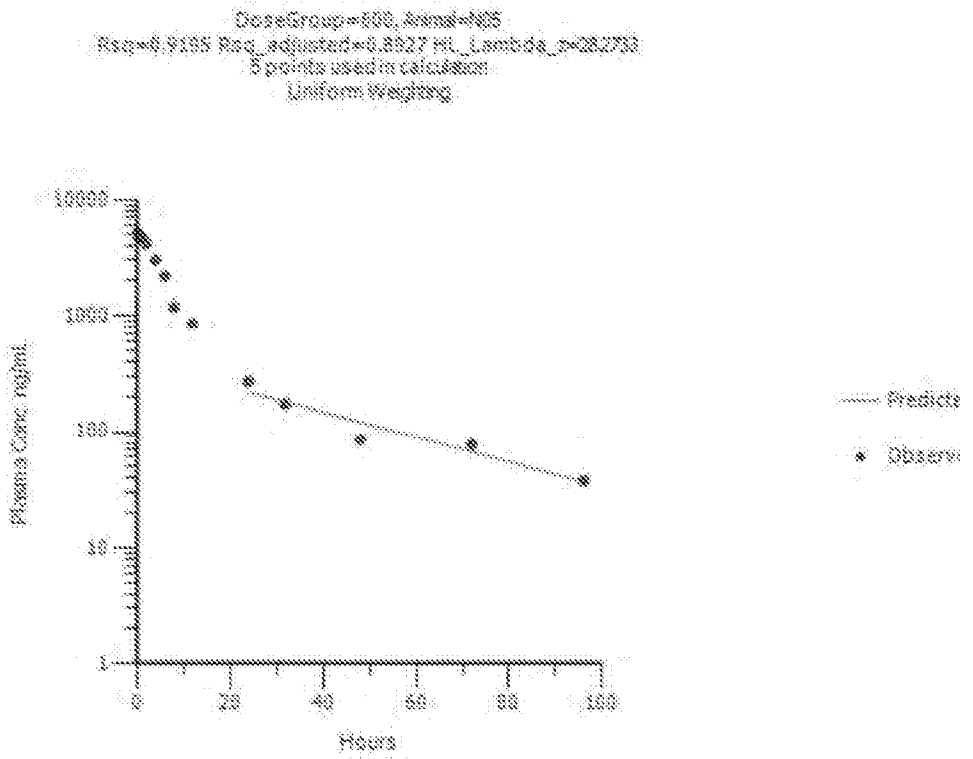


FIGURE 97J

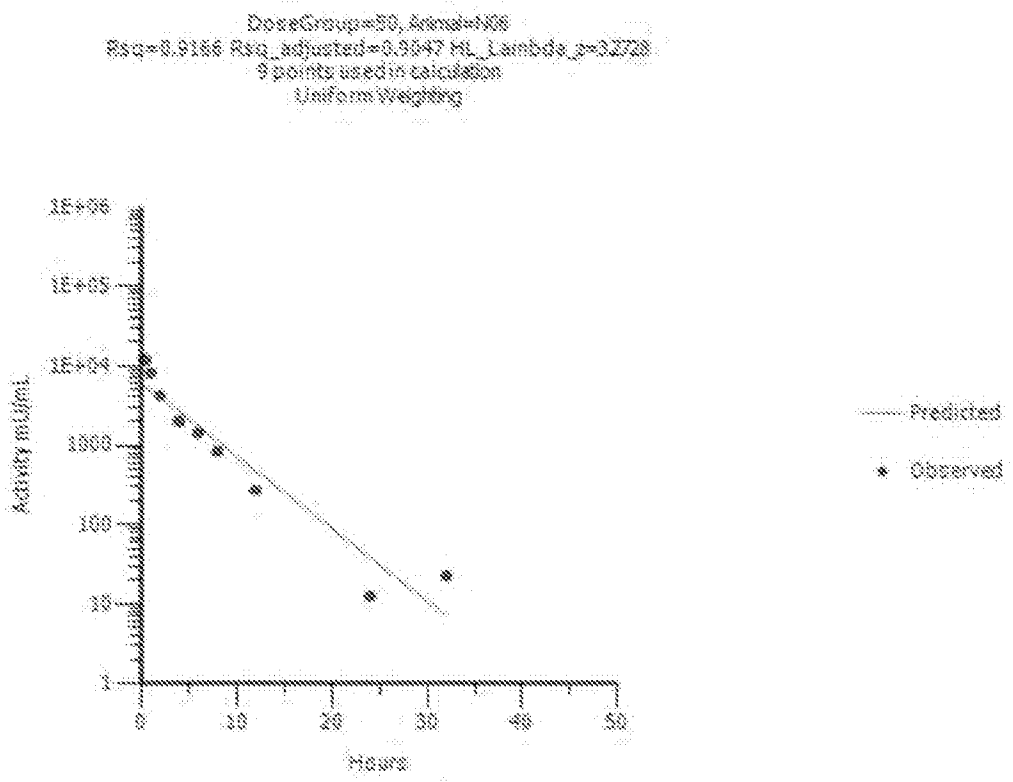


FIGURE 98A

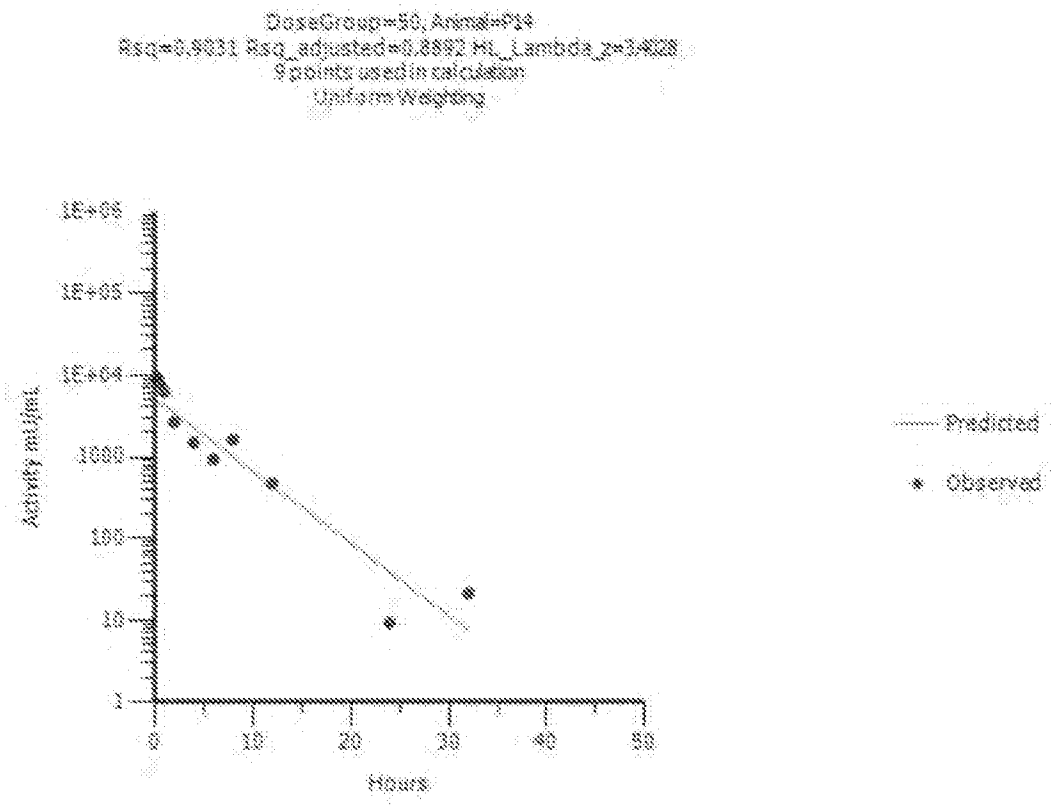


FIGURE 98B

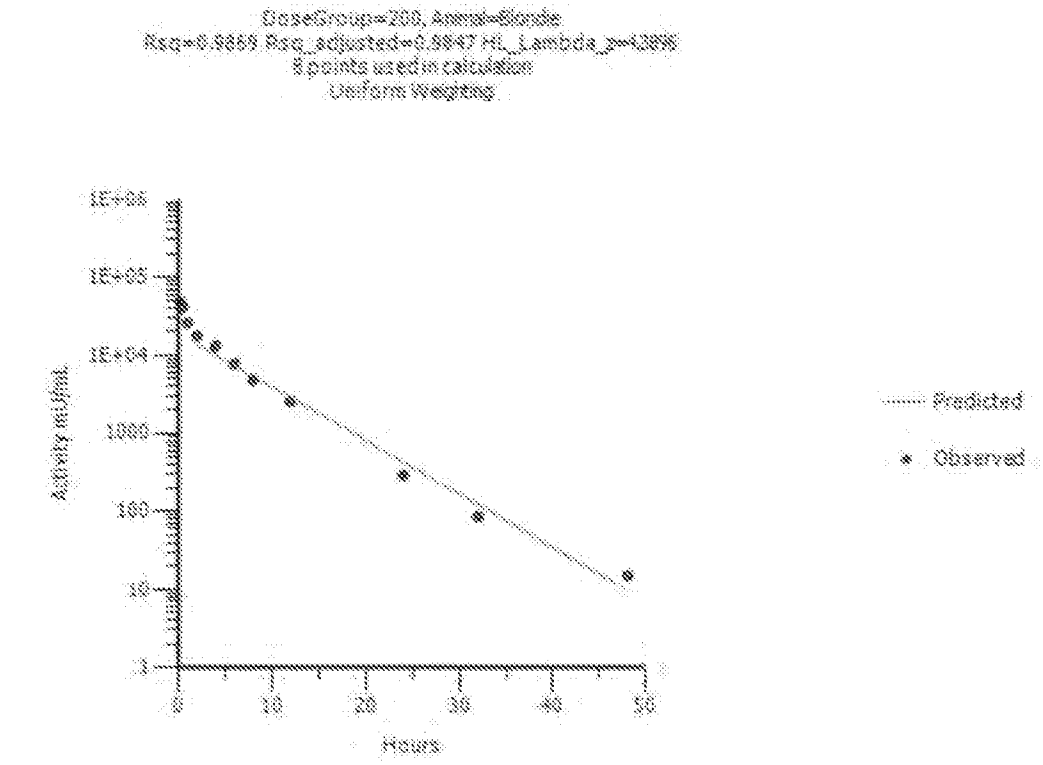


FIGURE 98C

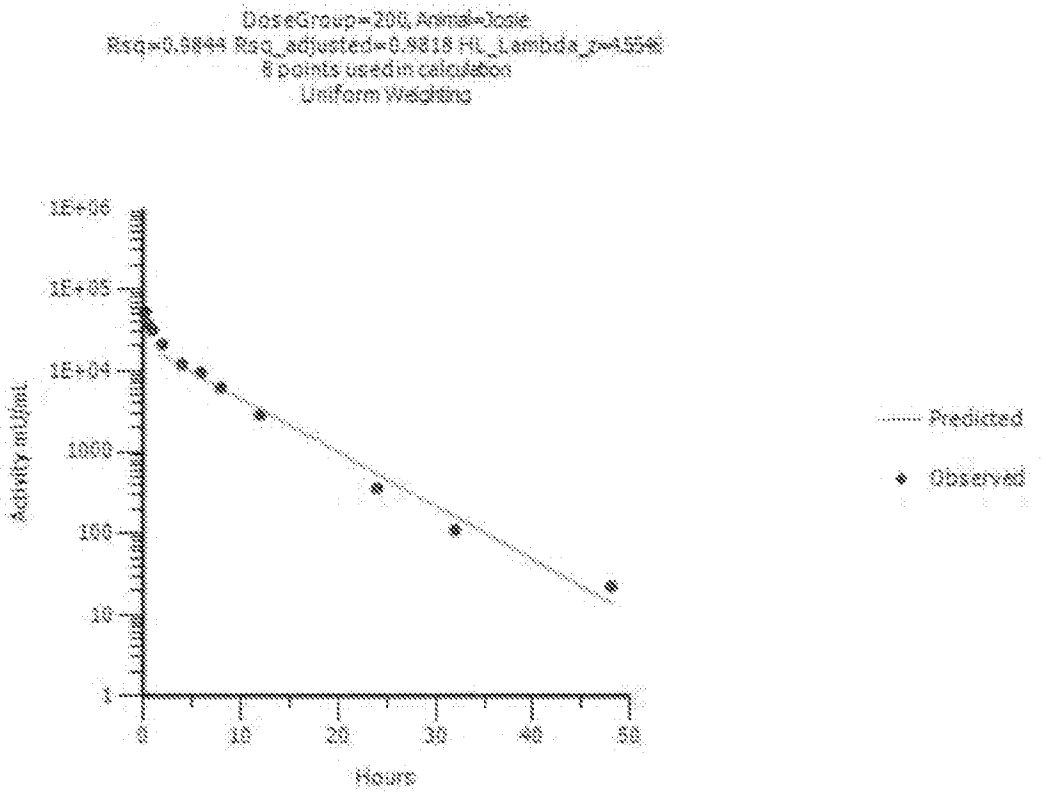


FIGURE 98D

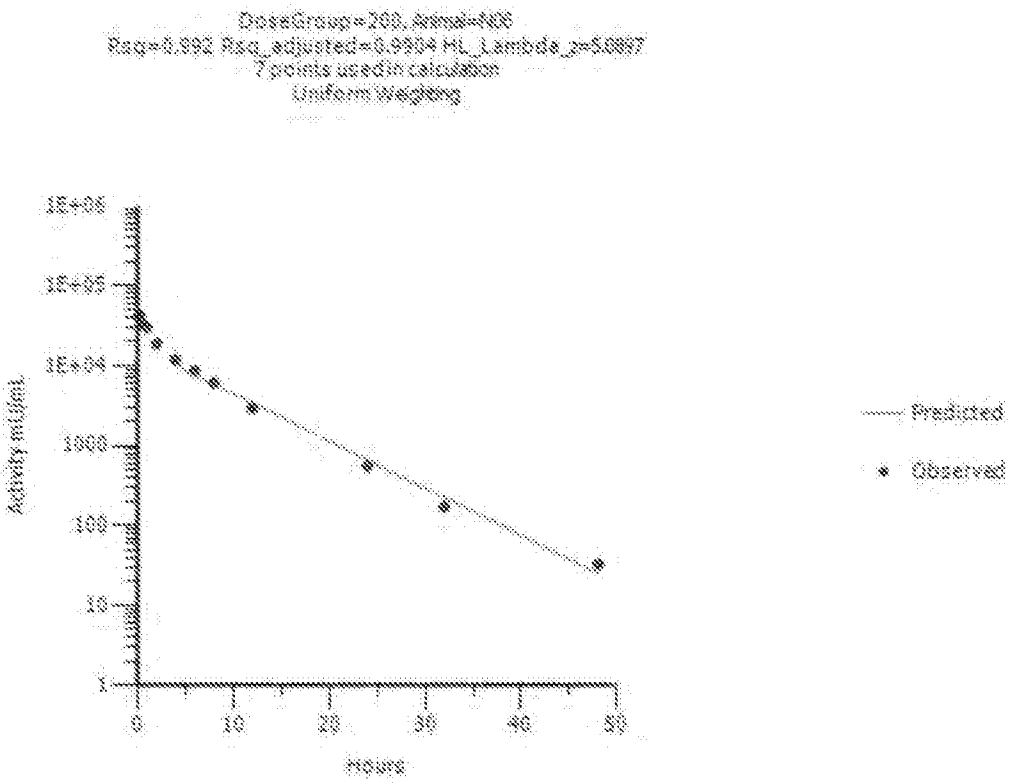


FIGURE 98E

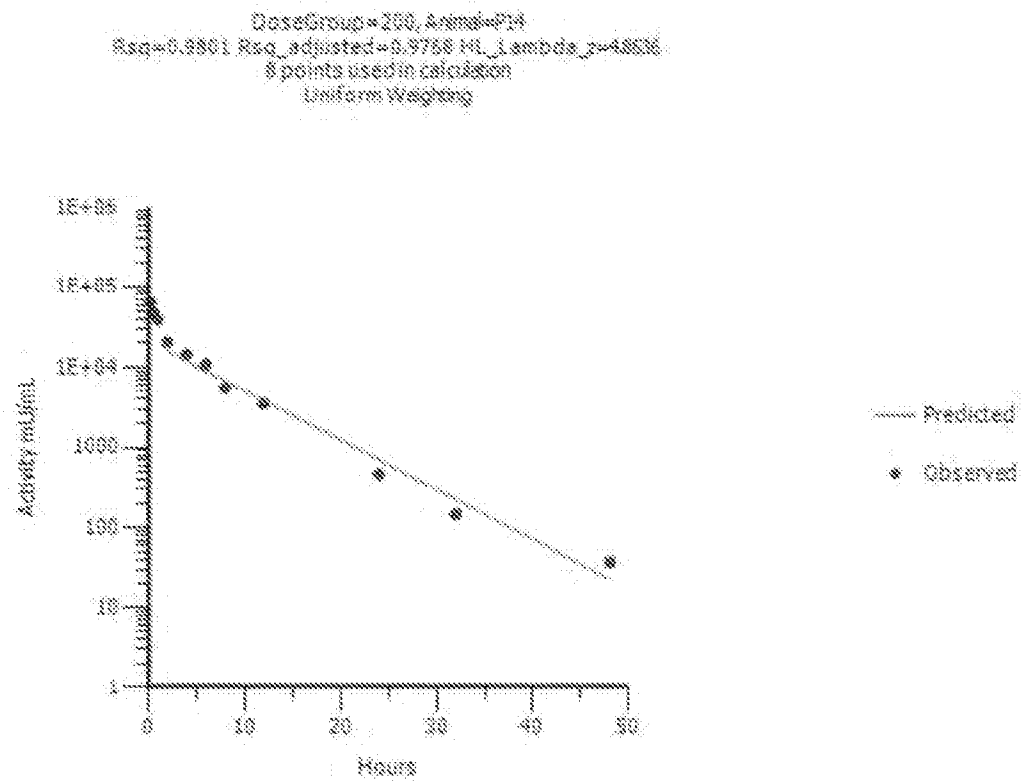


FIGURE 98F

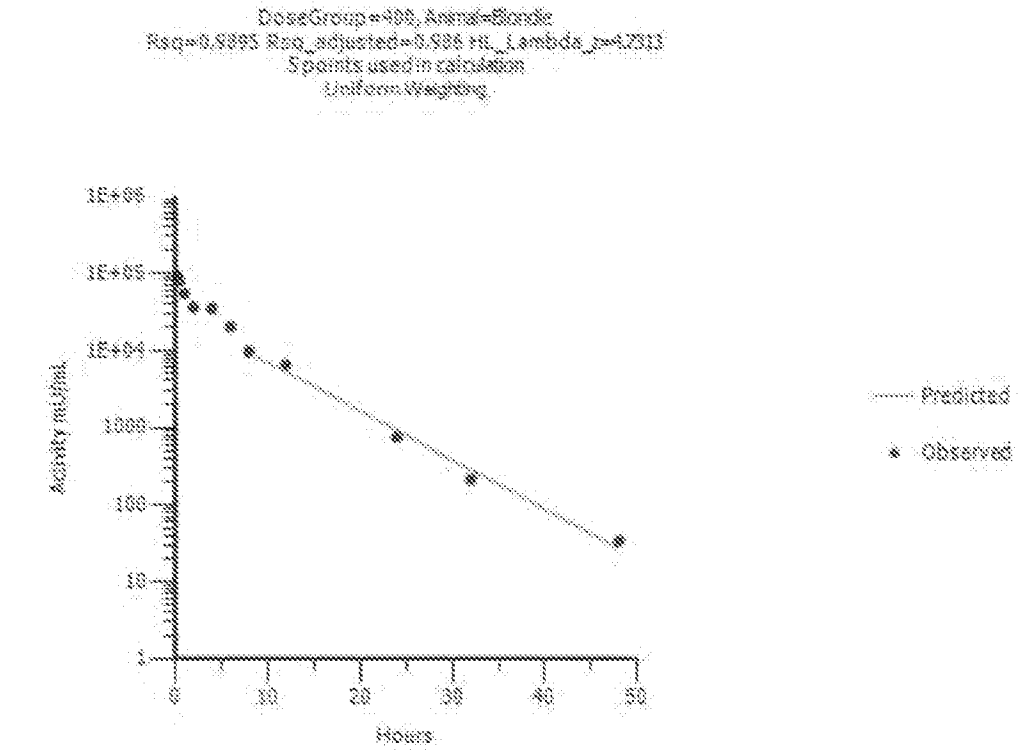


FIGURE 98G

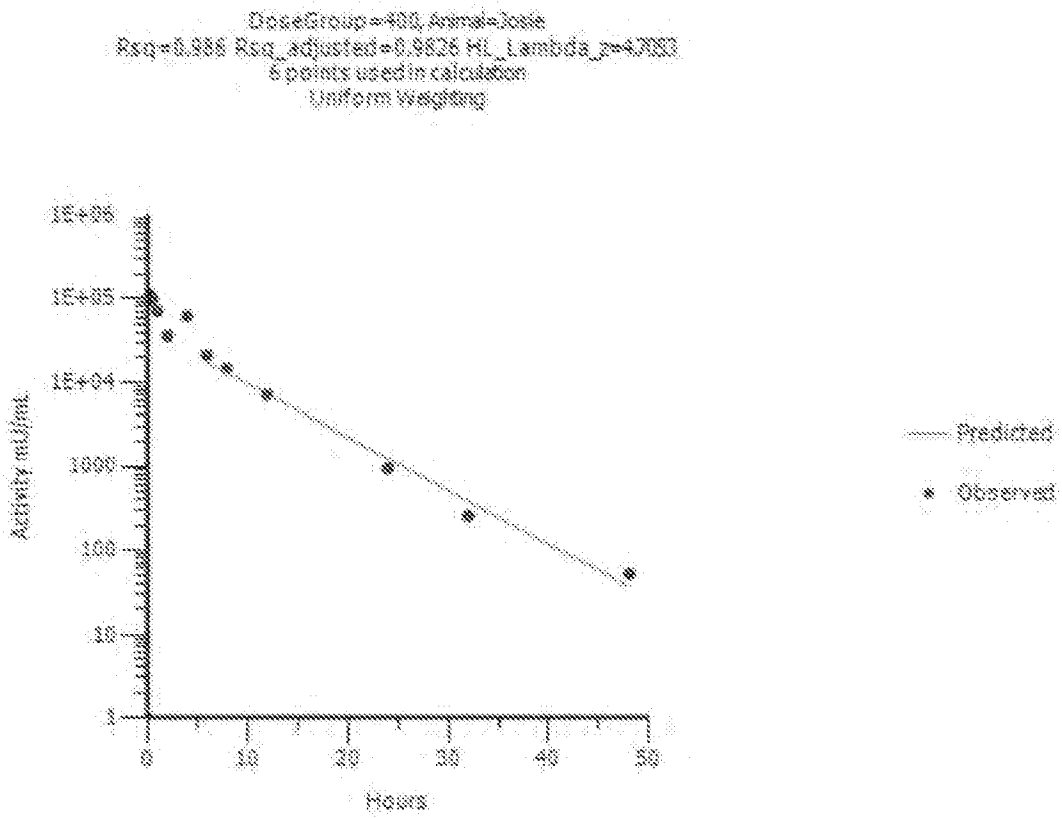


FIGURE 98H

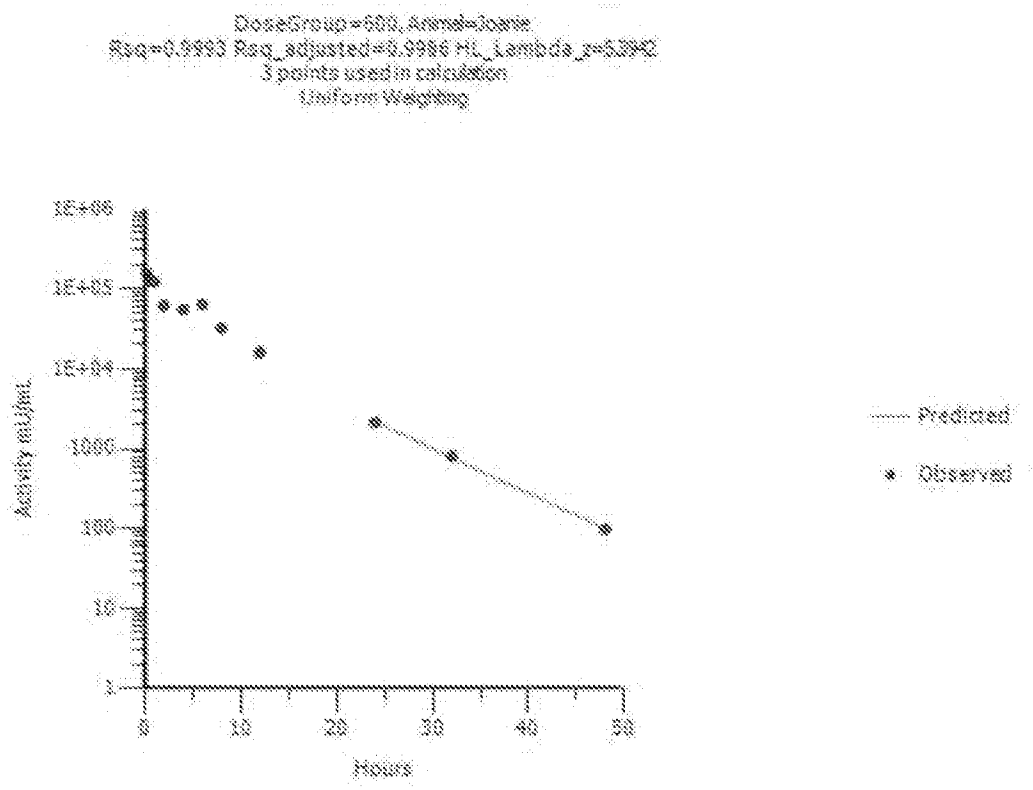


FIGURE 98I

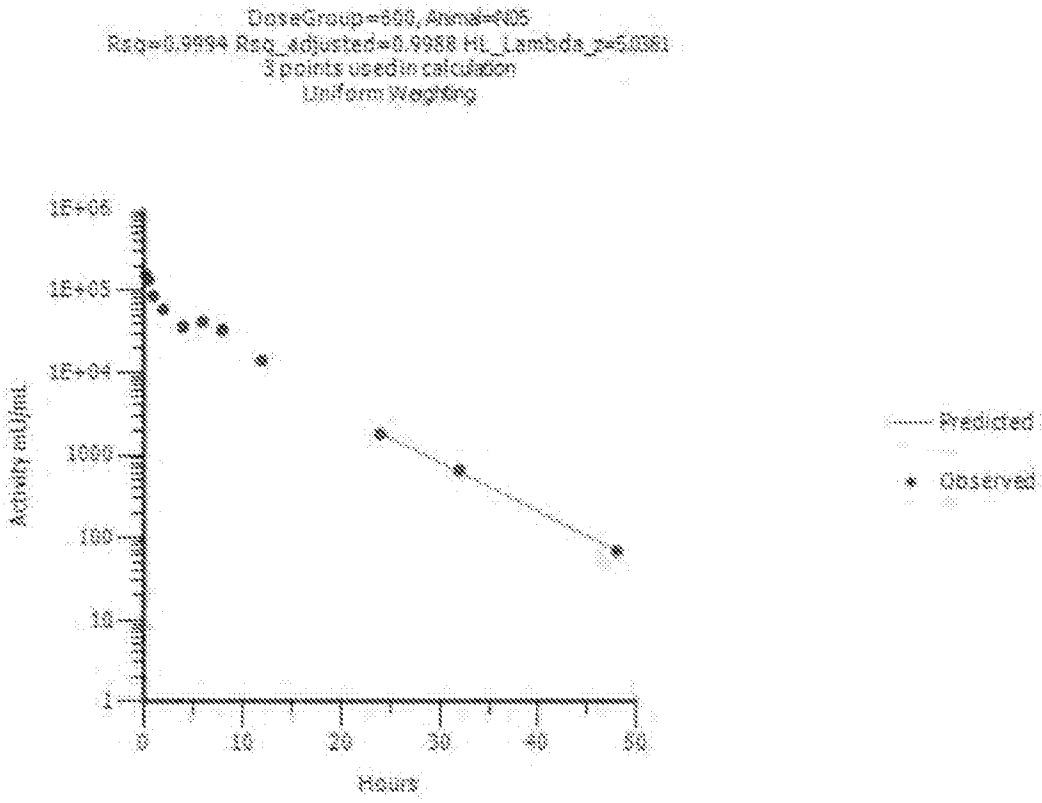


FIGURE 98J

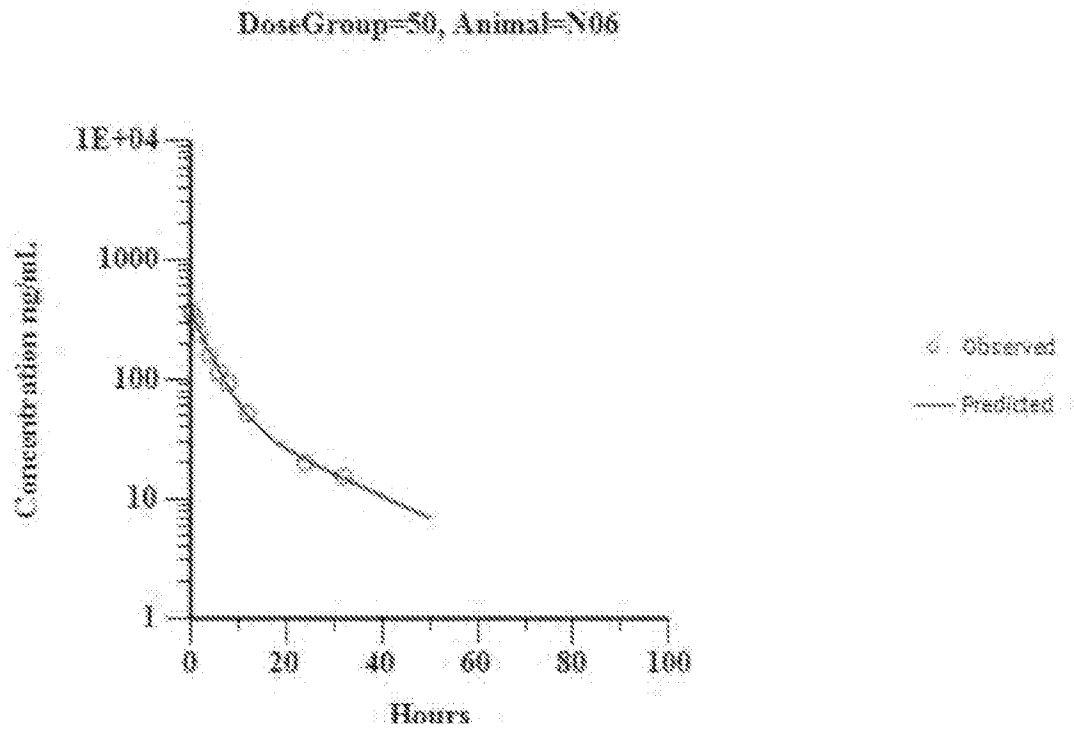


FIGURE 99A

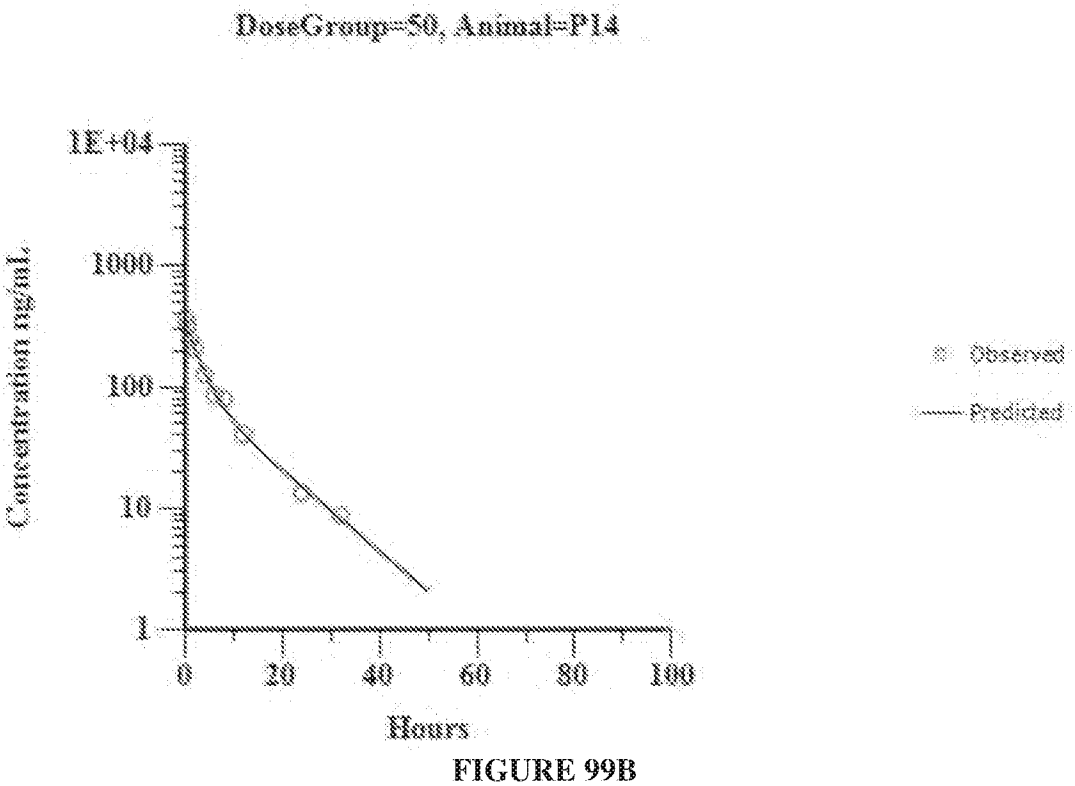


FIGURE 99B

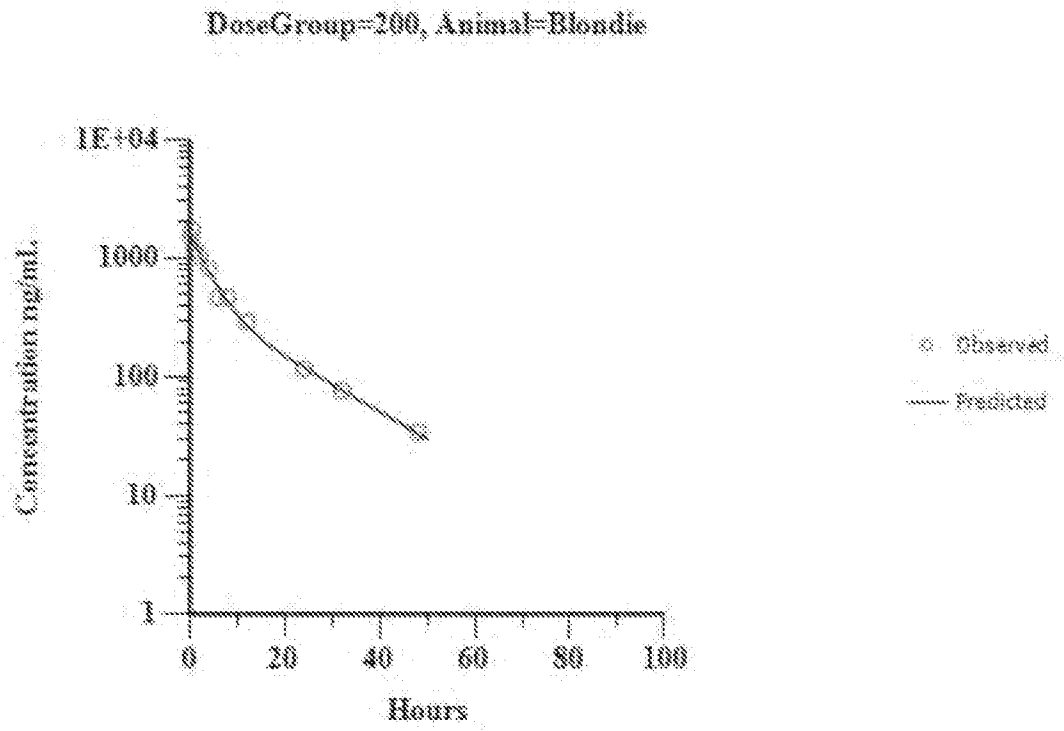


FIGURE 99C

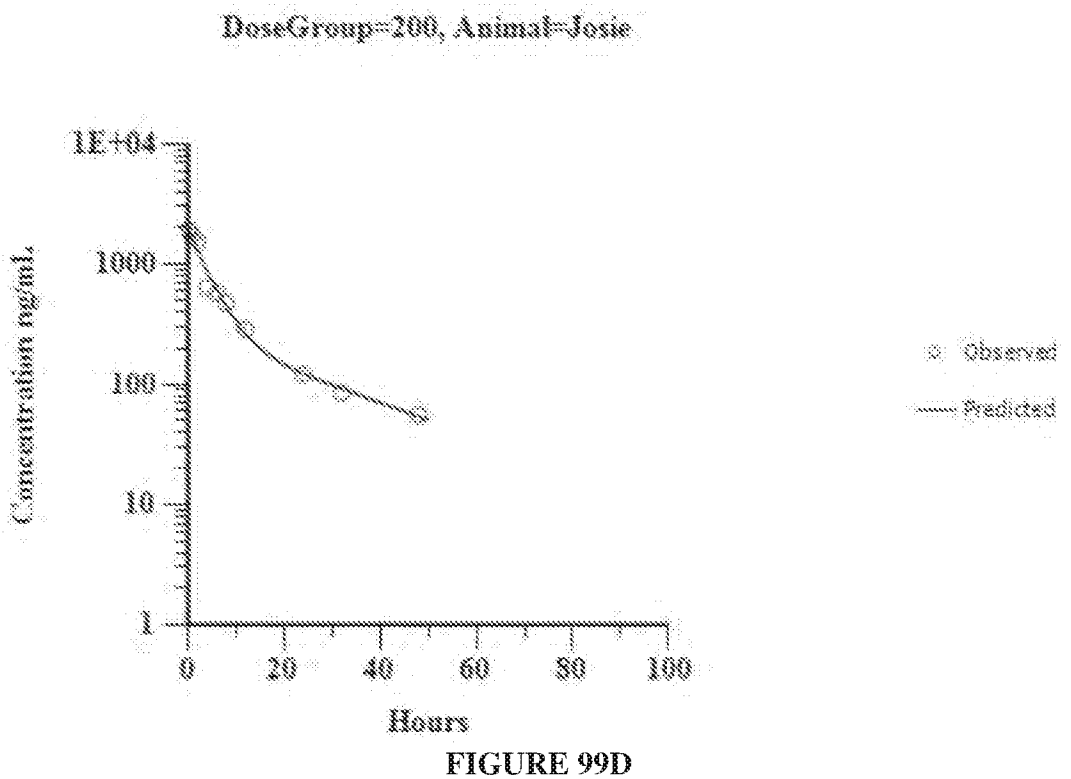


FIGURE 99D

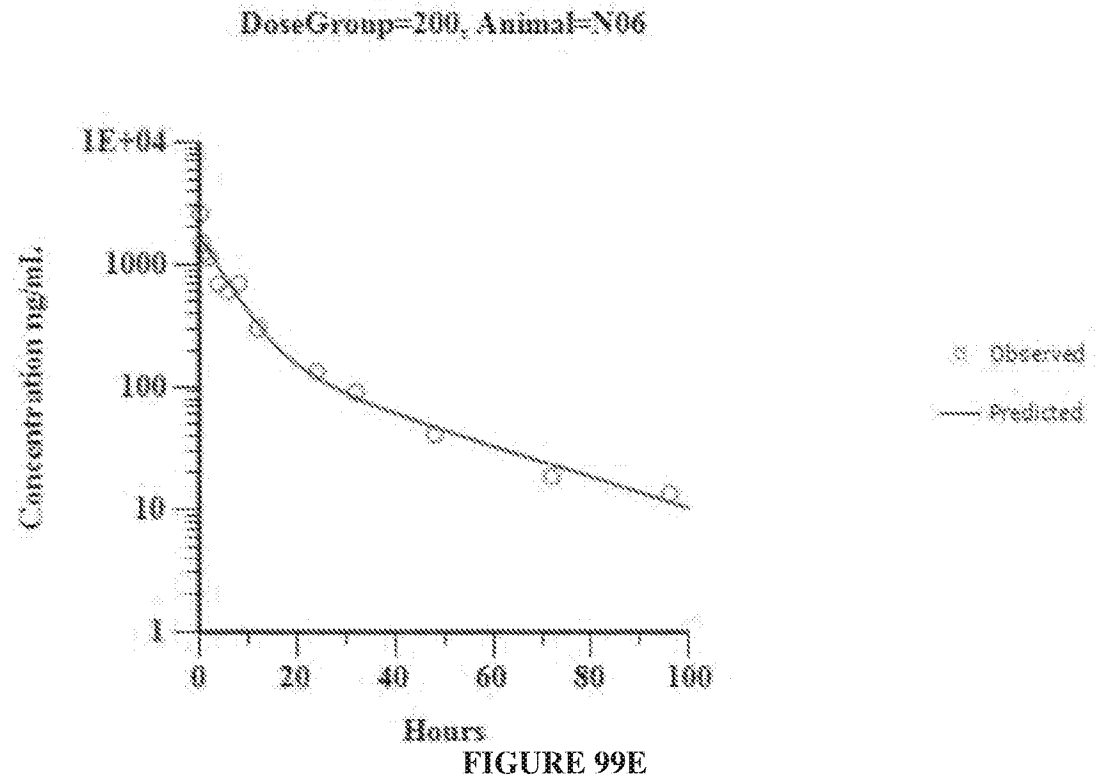


FIGURE 99E

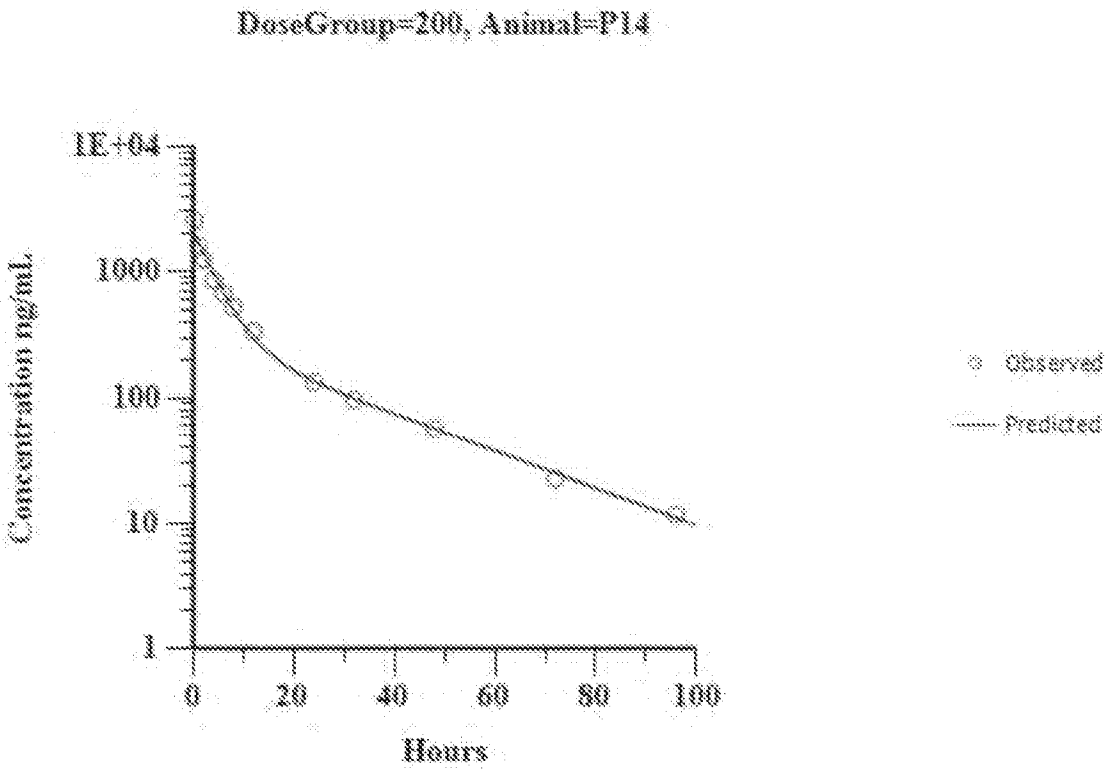


FIGURE 99F

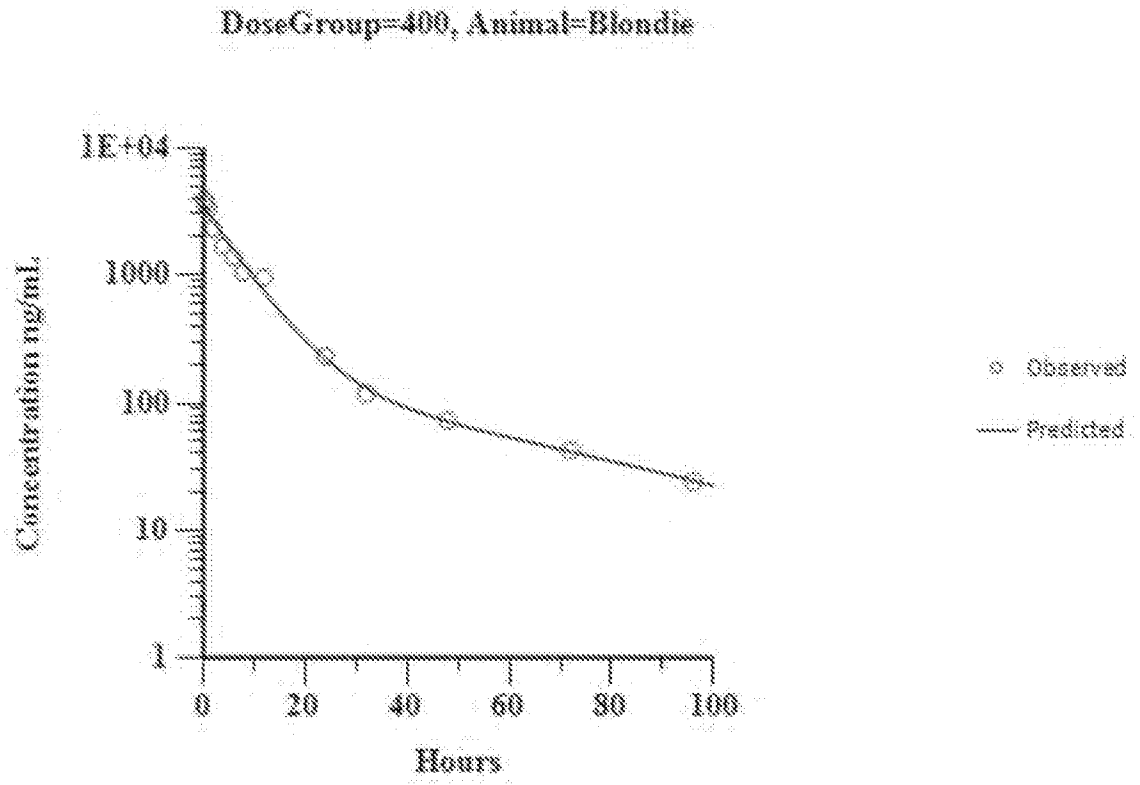


FIGURE 99G

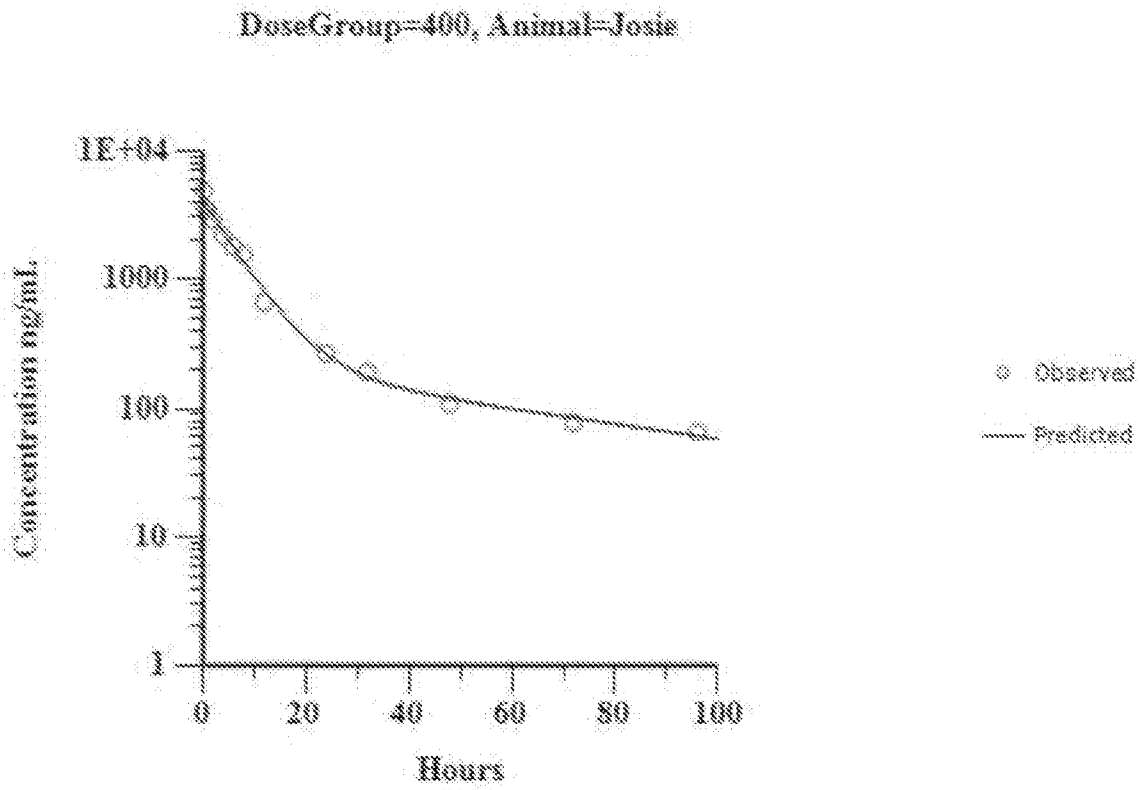


FIGURE 99H

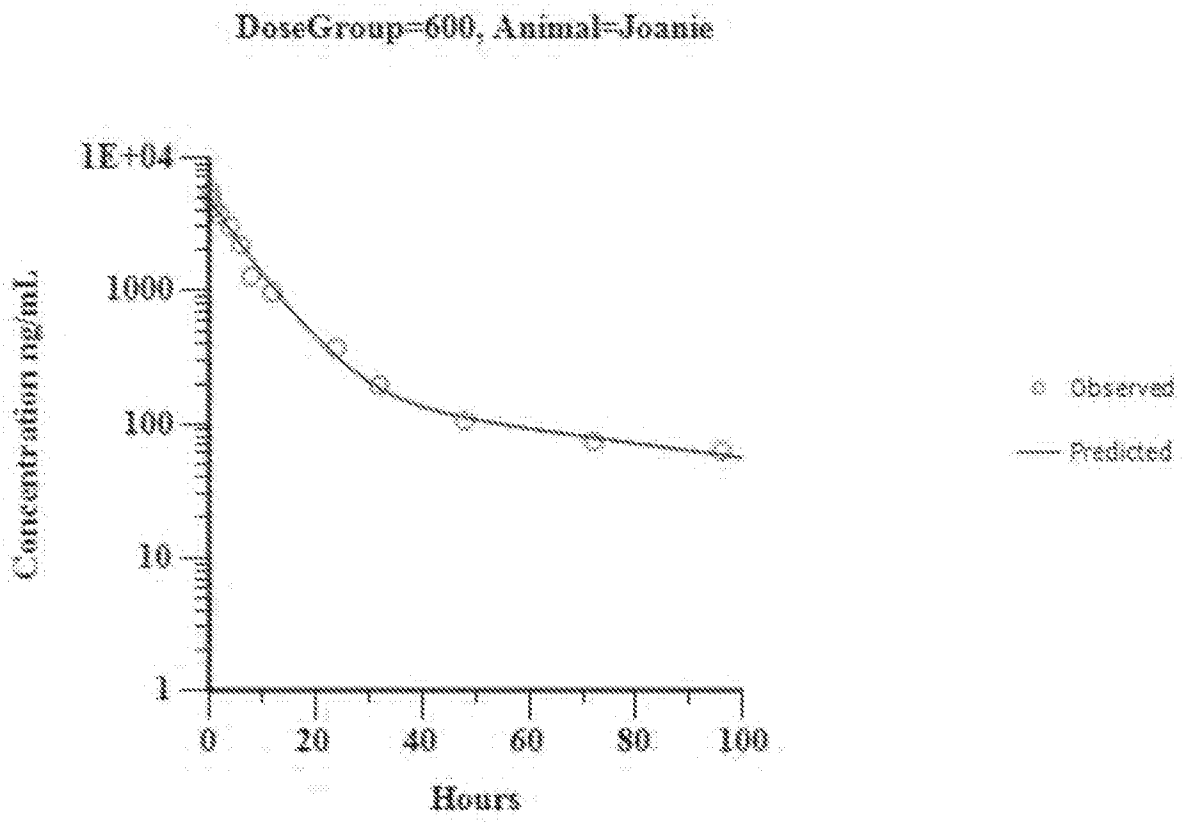


FIGURE 99I

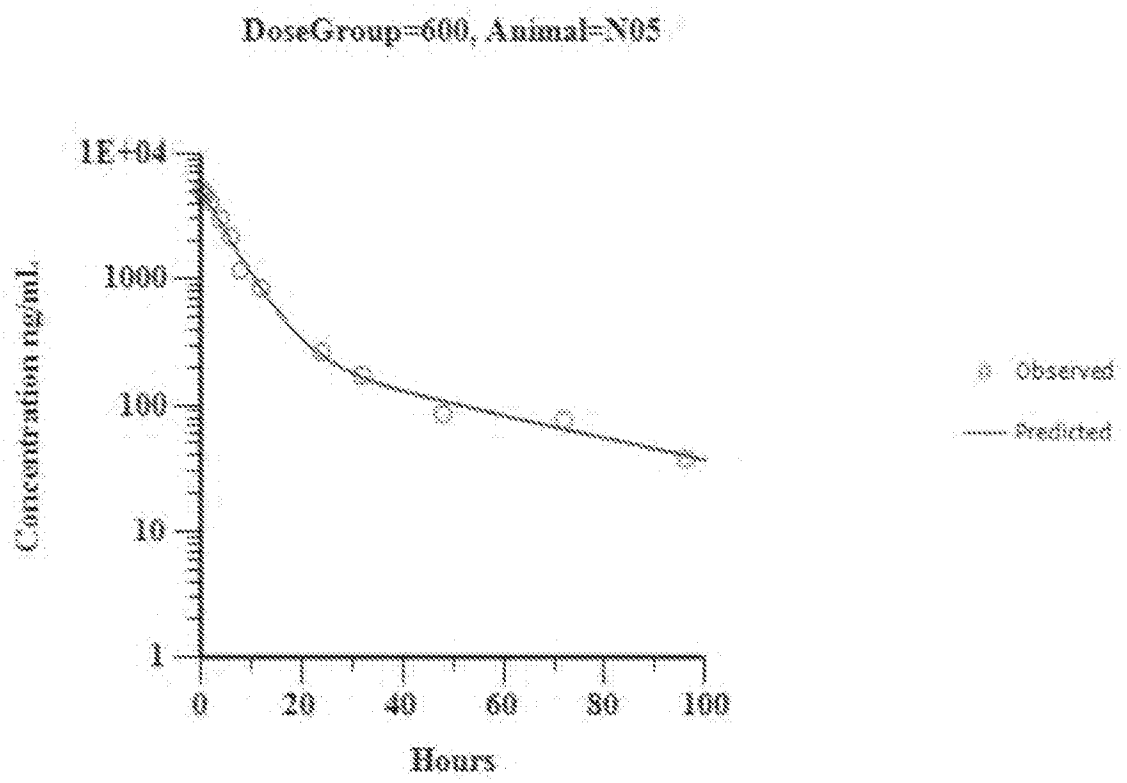


FIGURE 99J

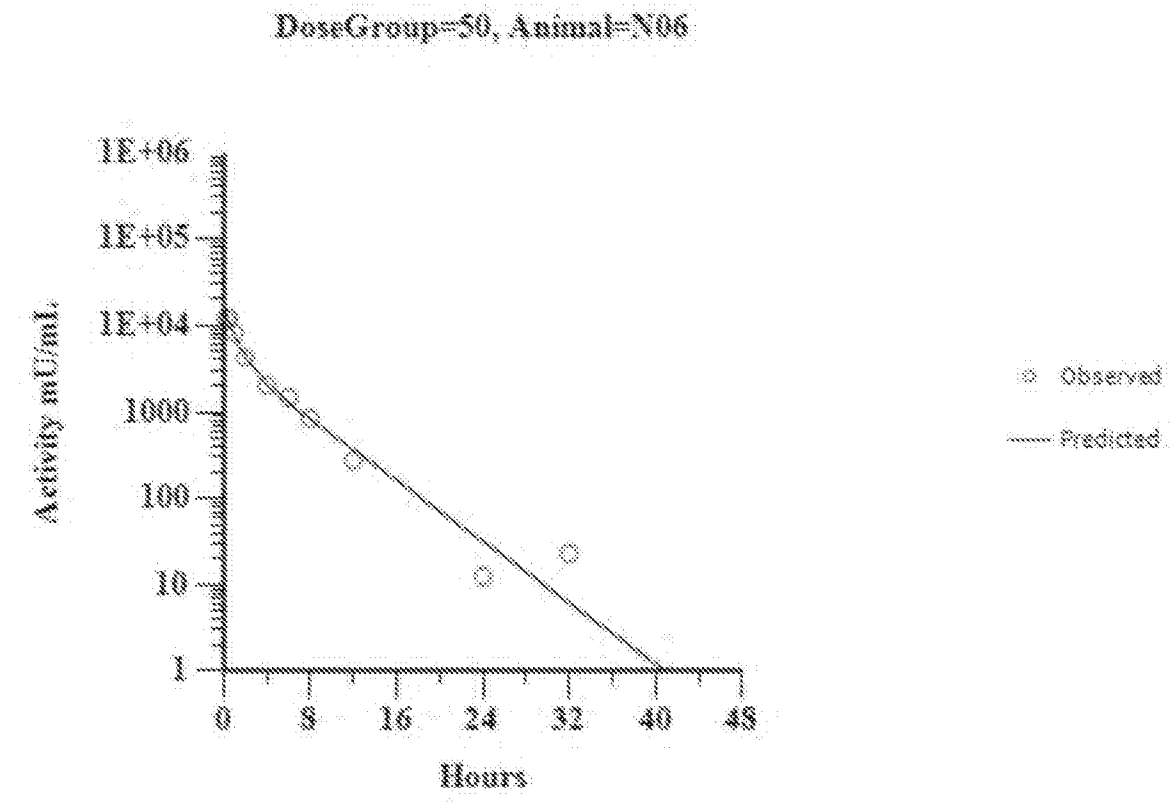


FIGURE 100A

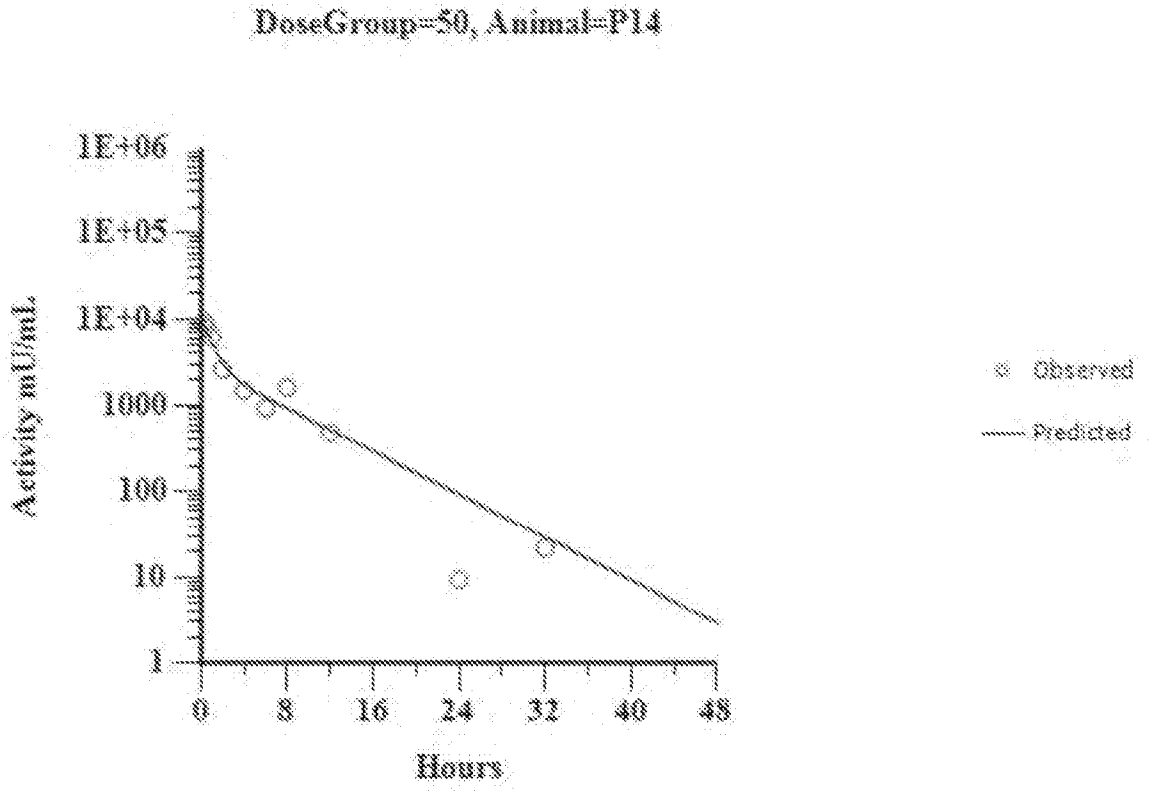


FIGURE 100B

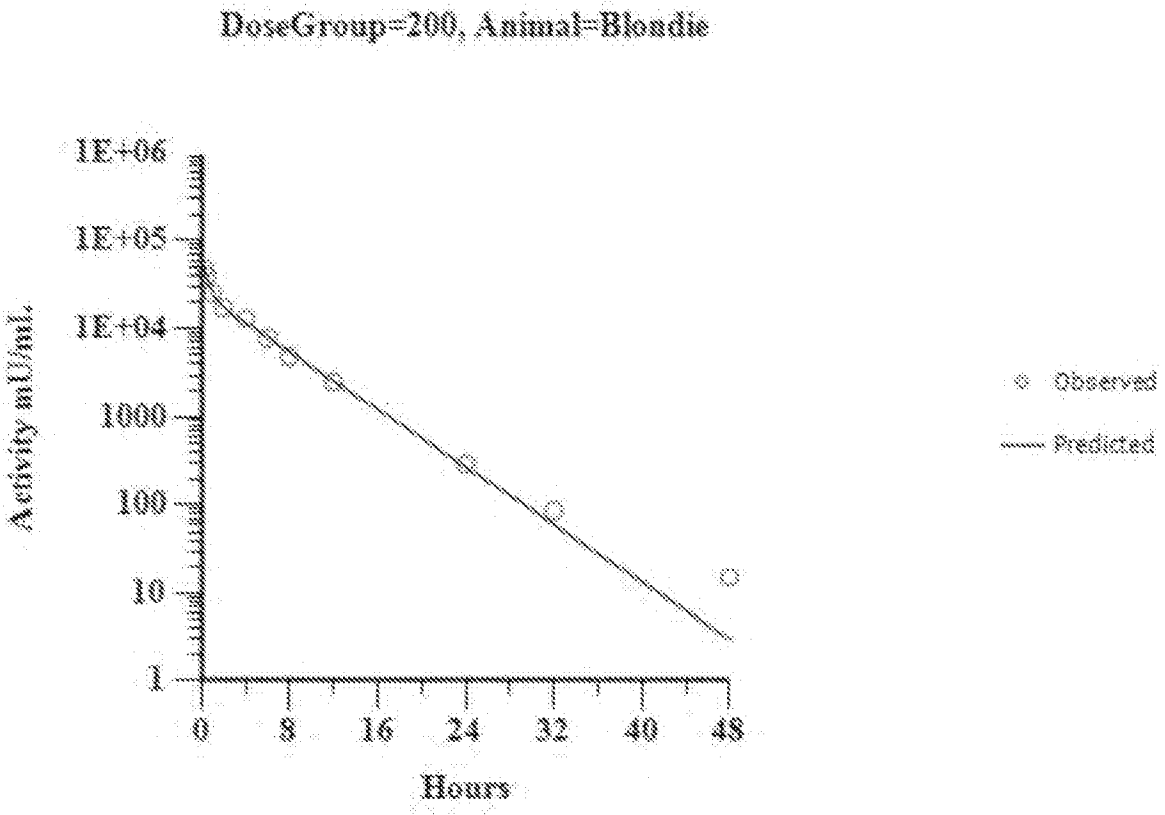


FIGURE 100C

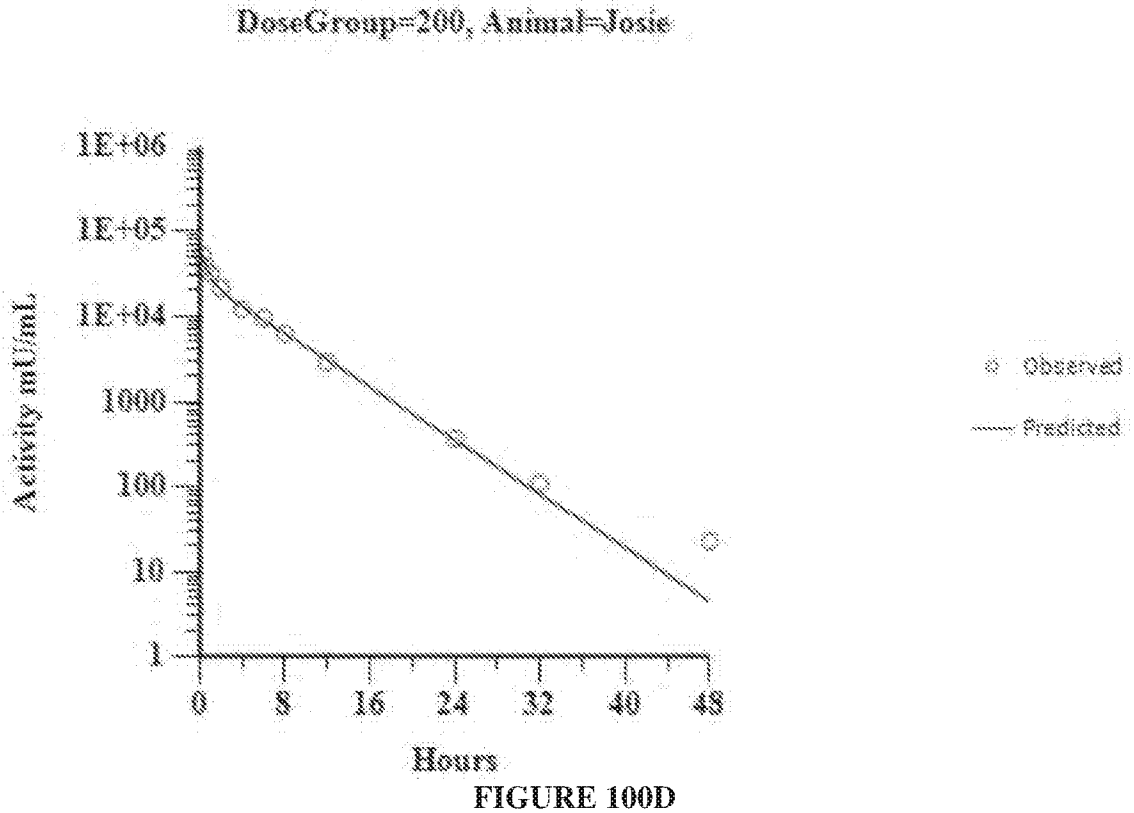


FIGURE 100D

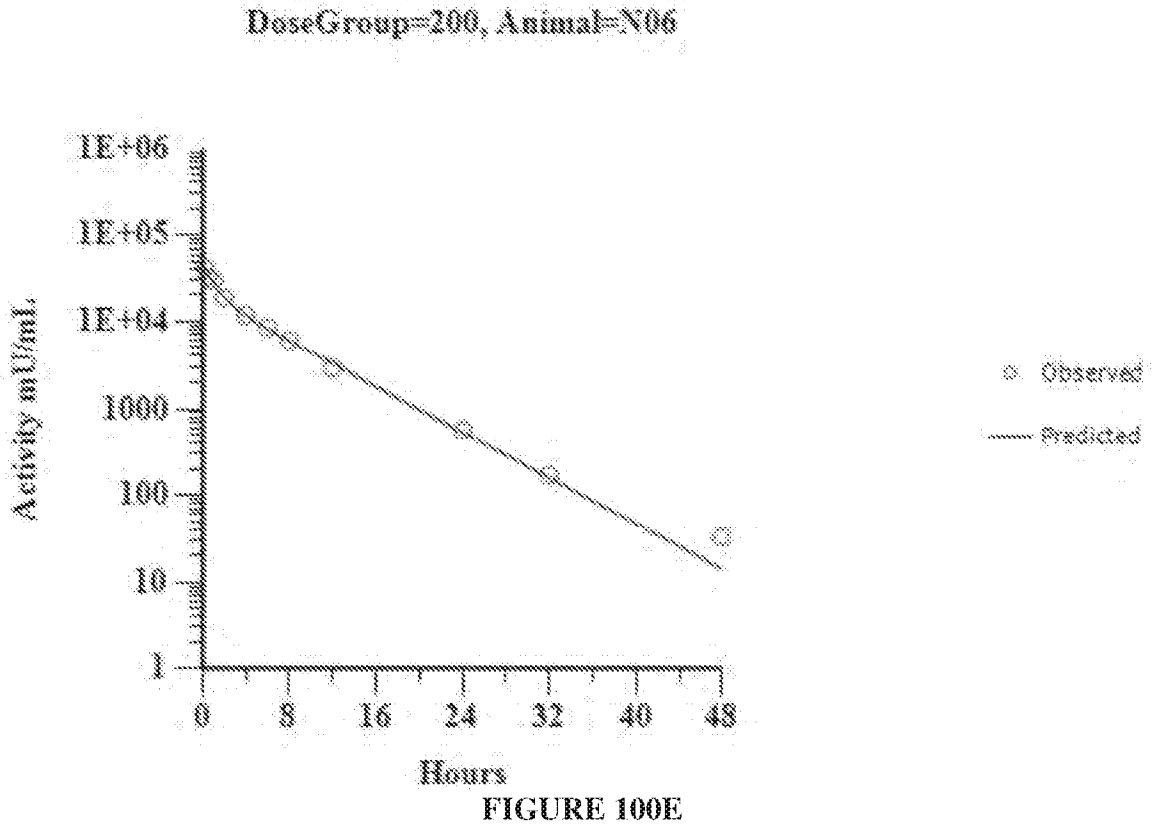


FIGURE 100E

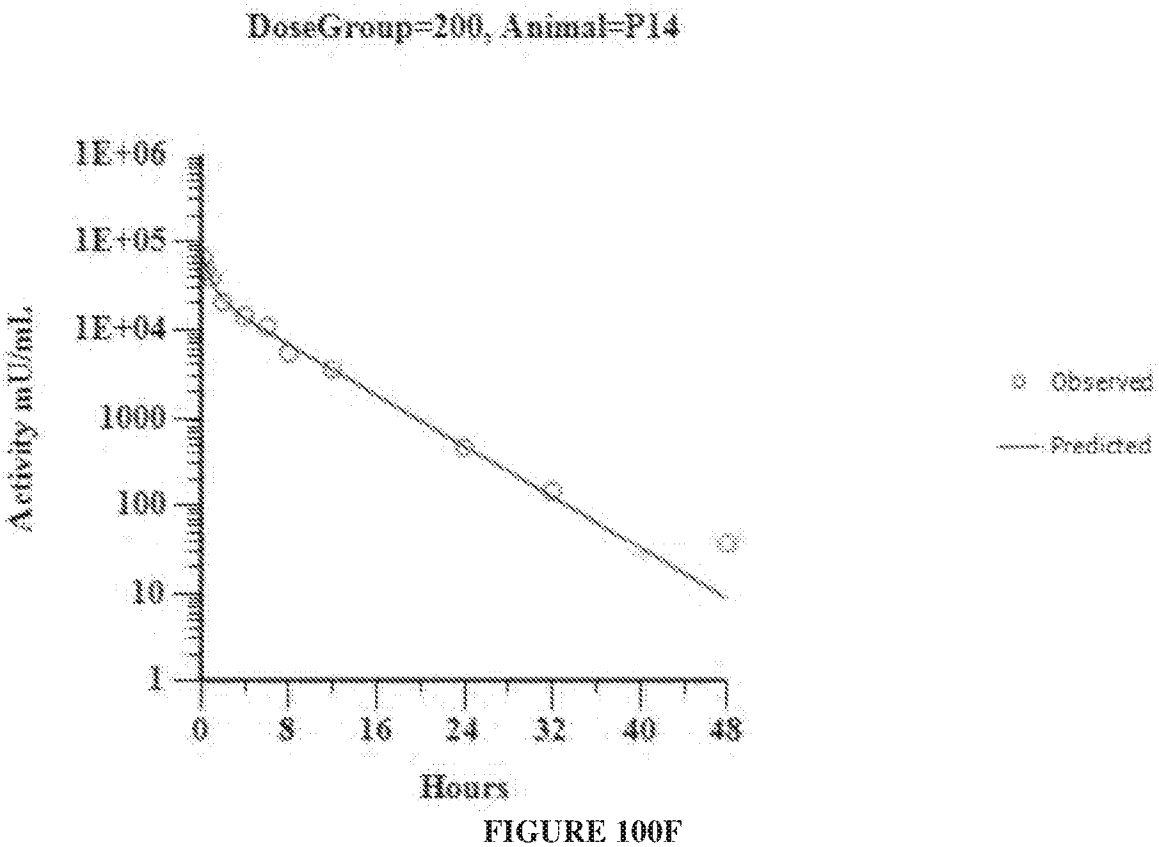


FIGURE 100F

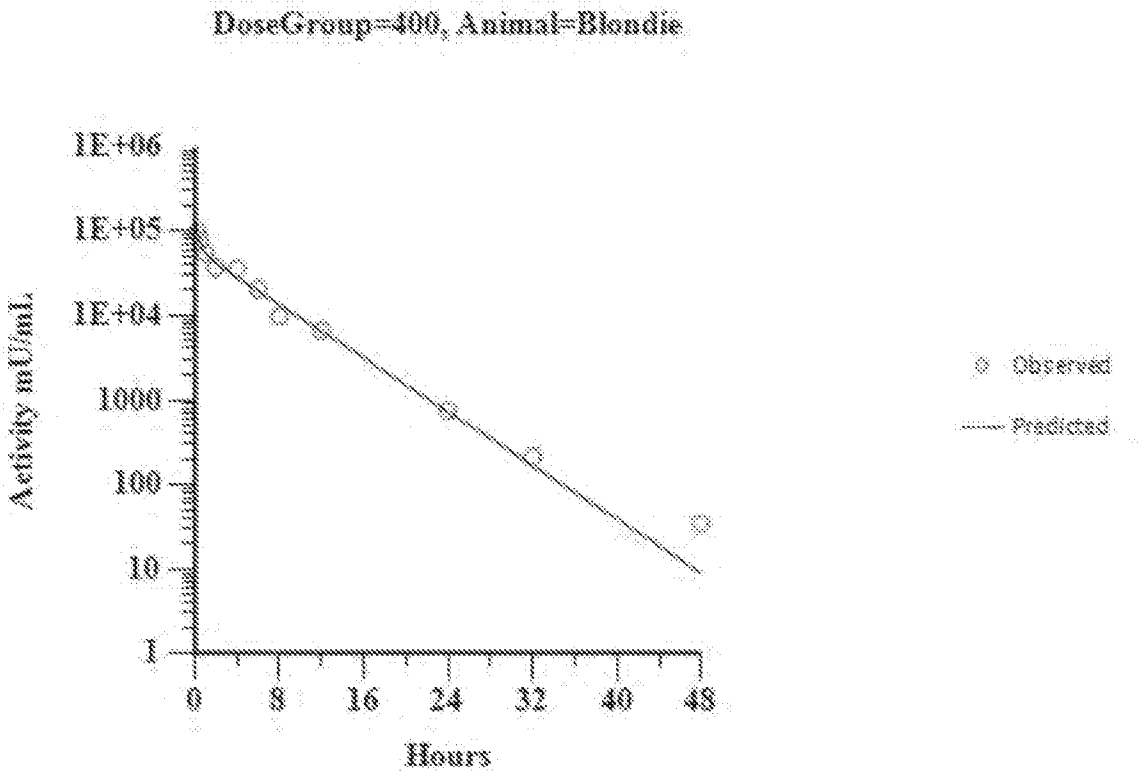


FIGURE 100G

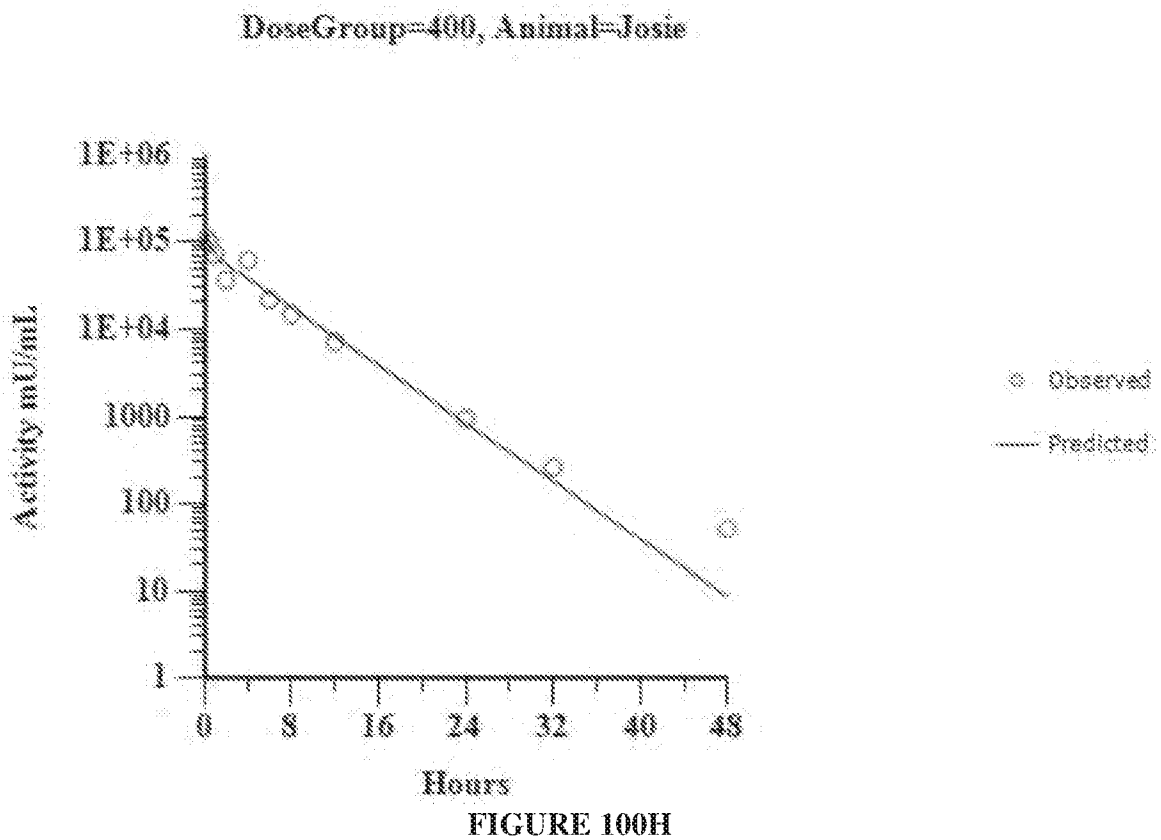


FIGURE 100H

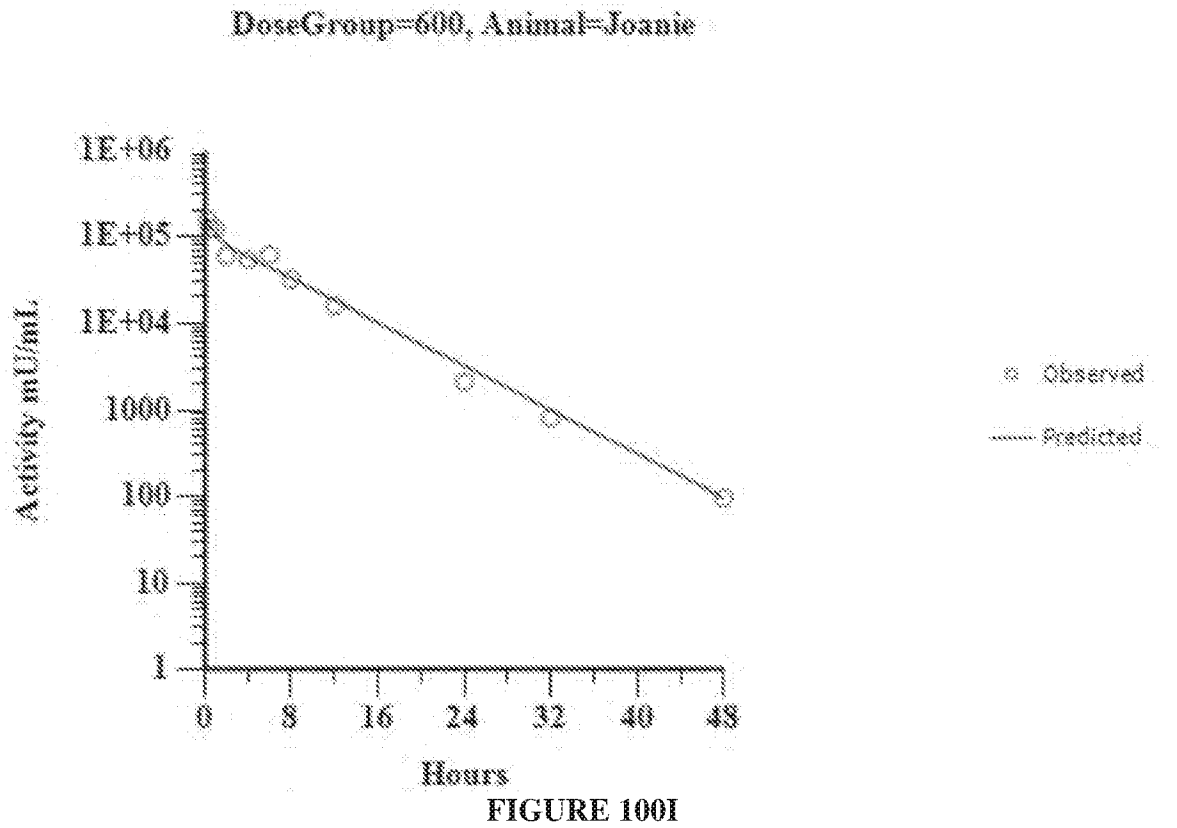


FIGURE 100I

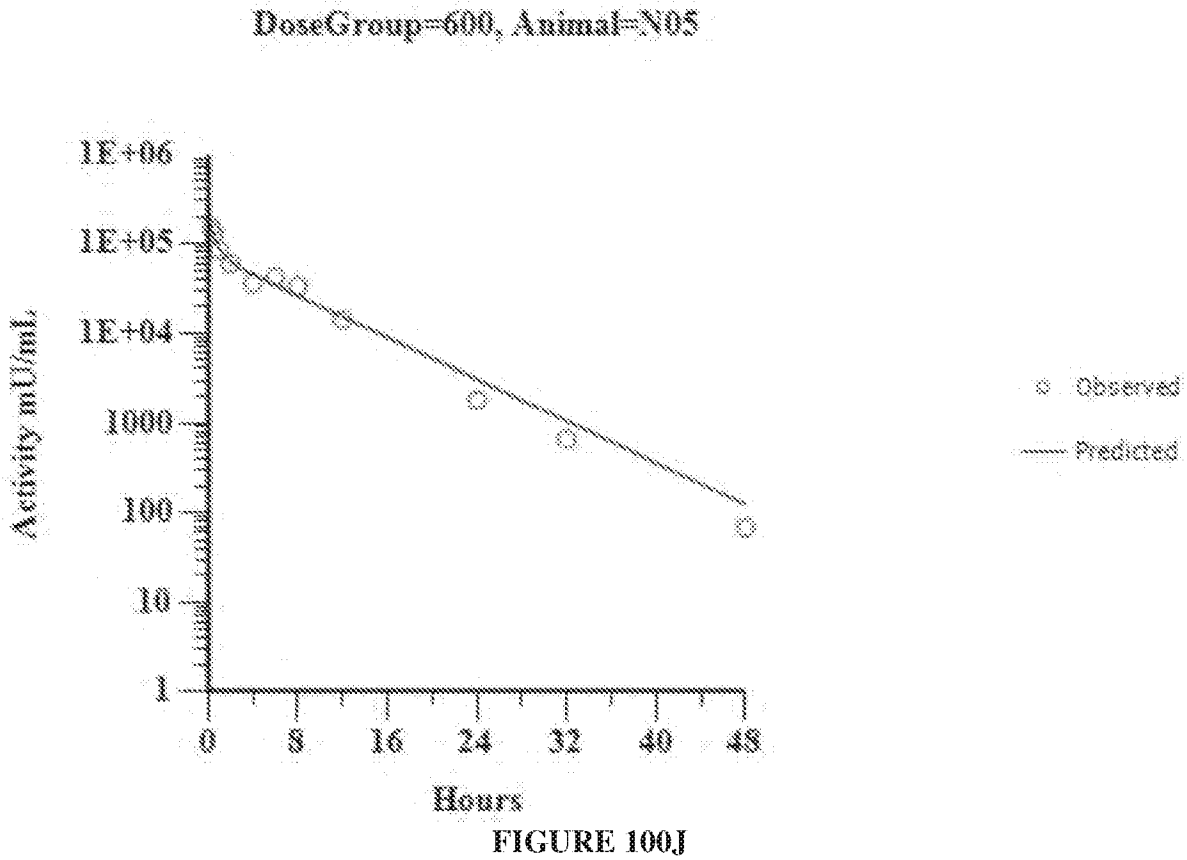


FIGURE 100J

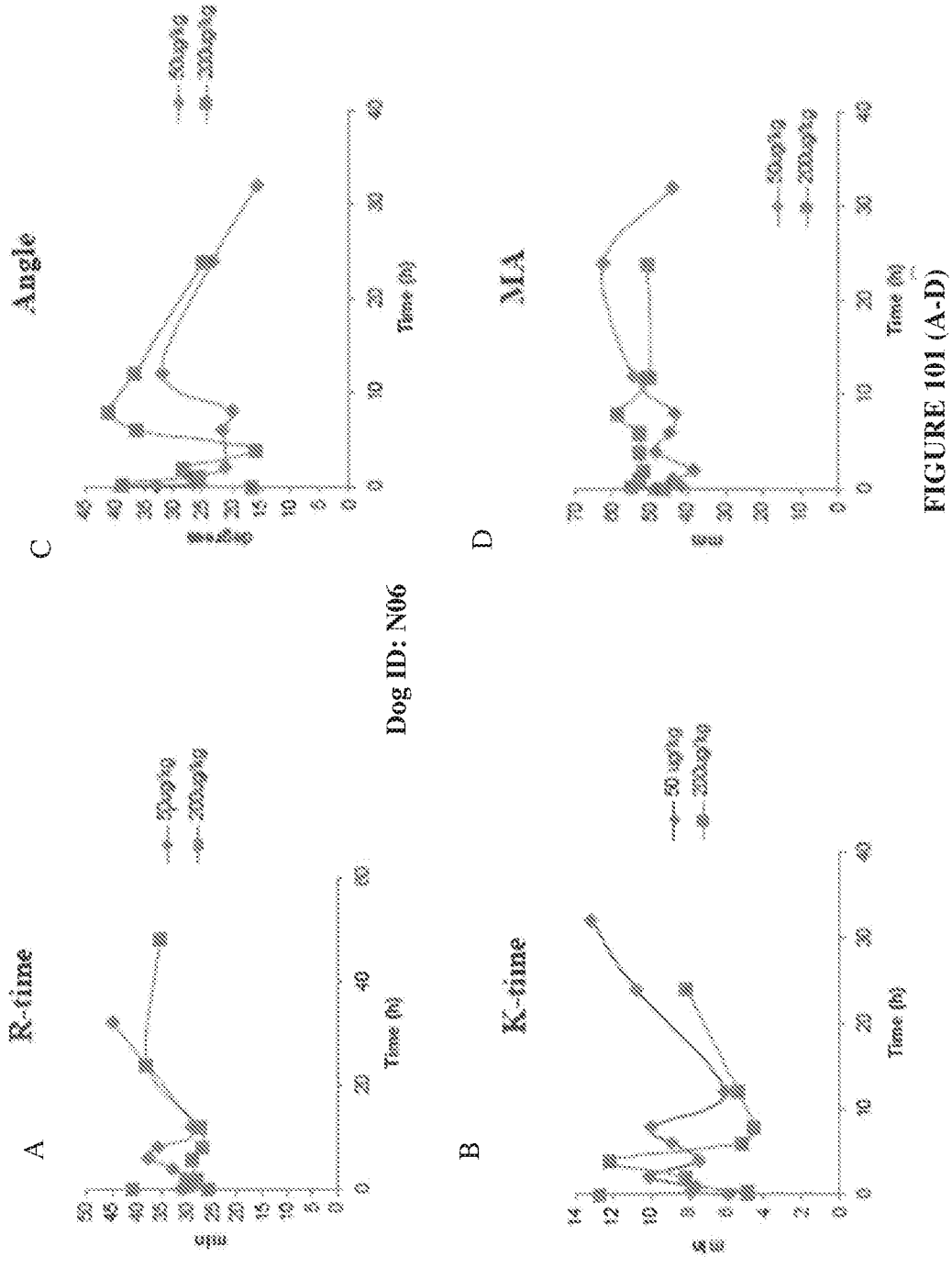


FIGURE 101 (A-D)

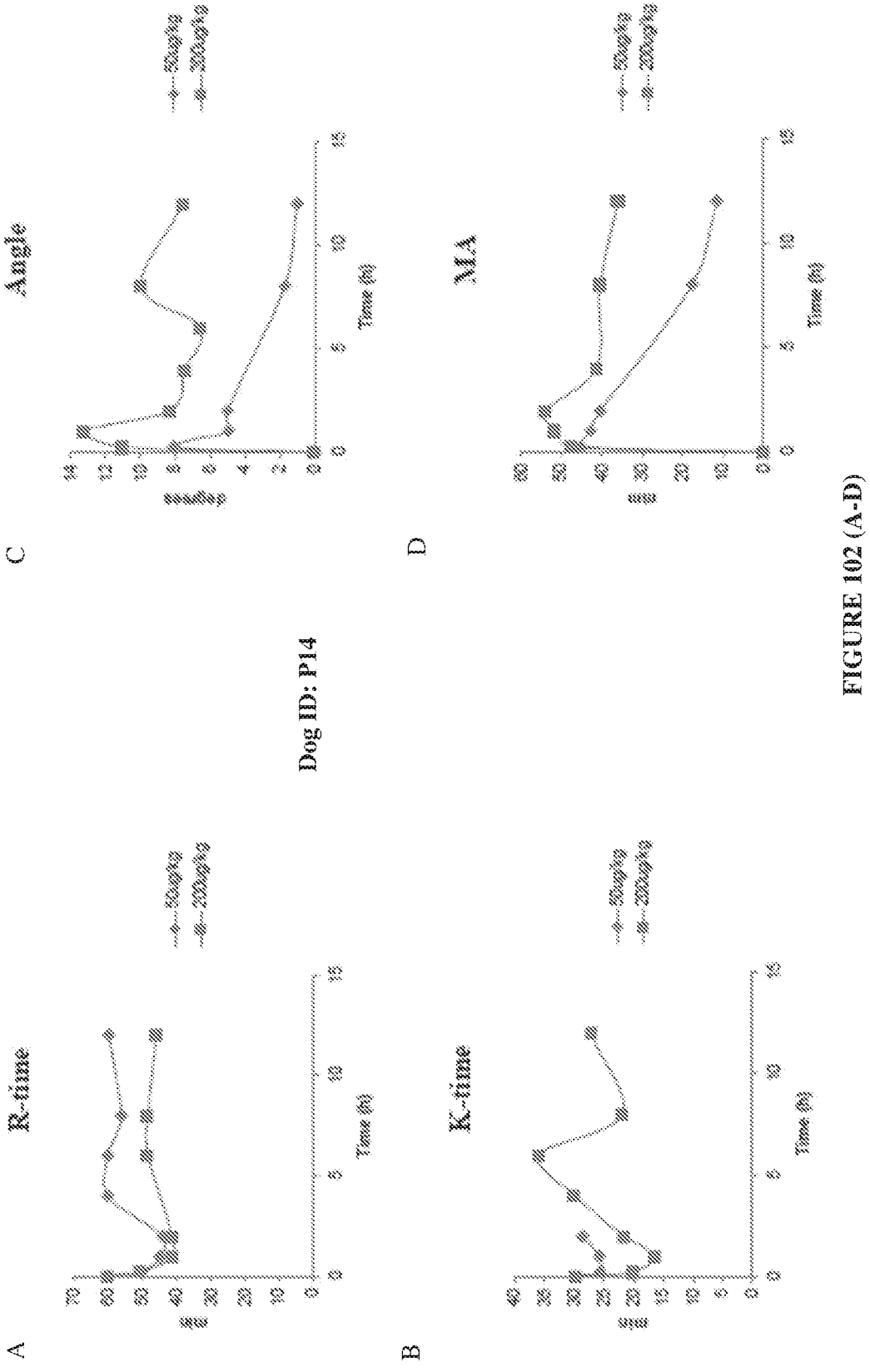


FIGURE 102 (A-D)

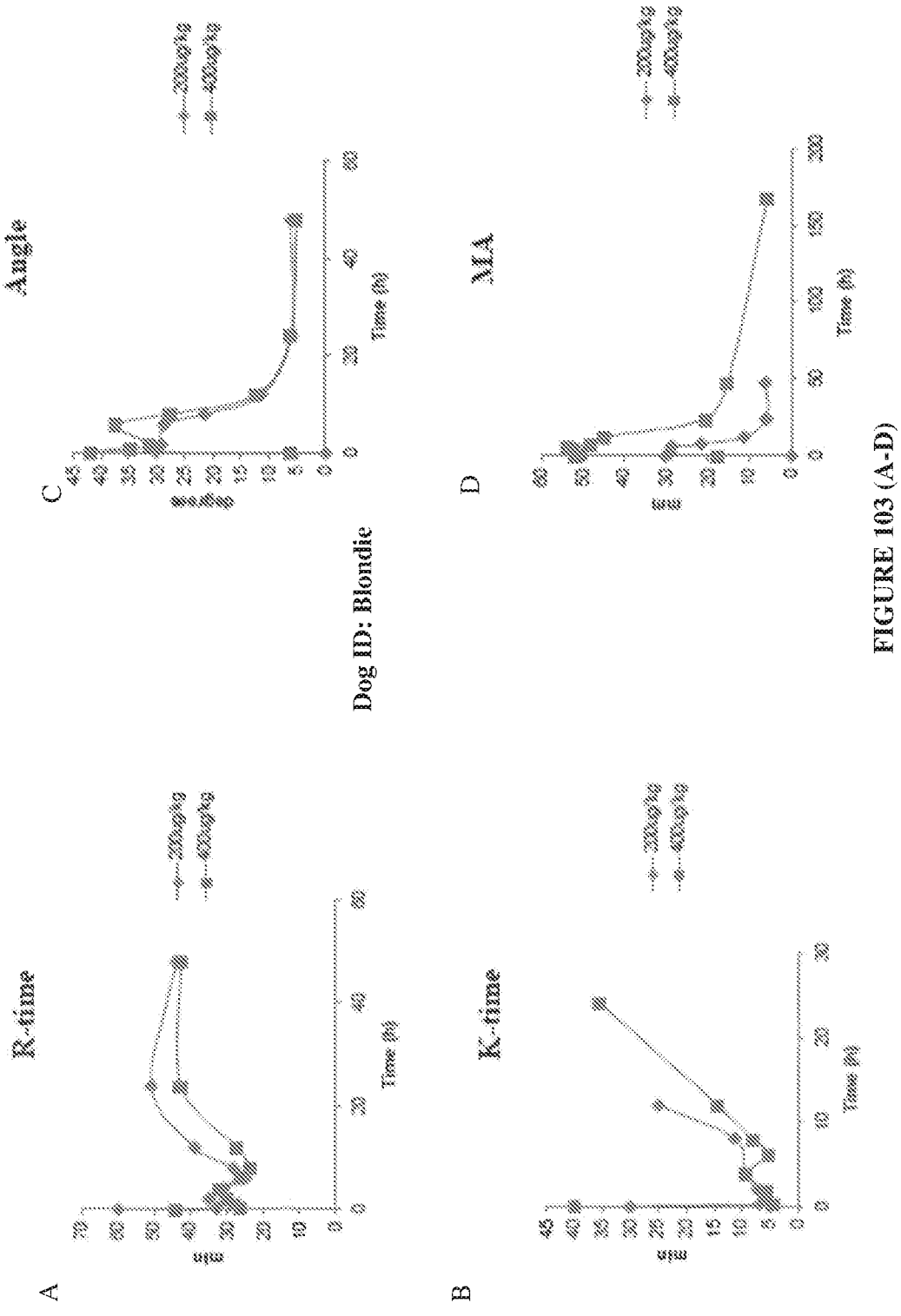


FIGURE 103 (A-D)

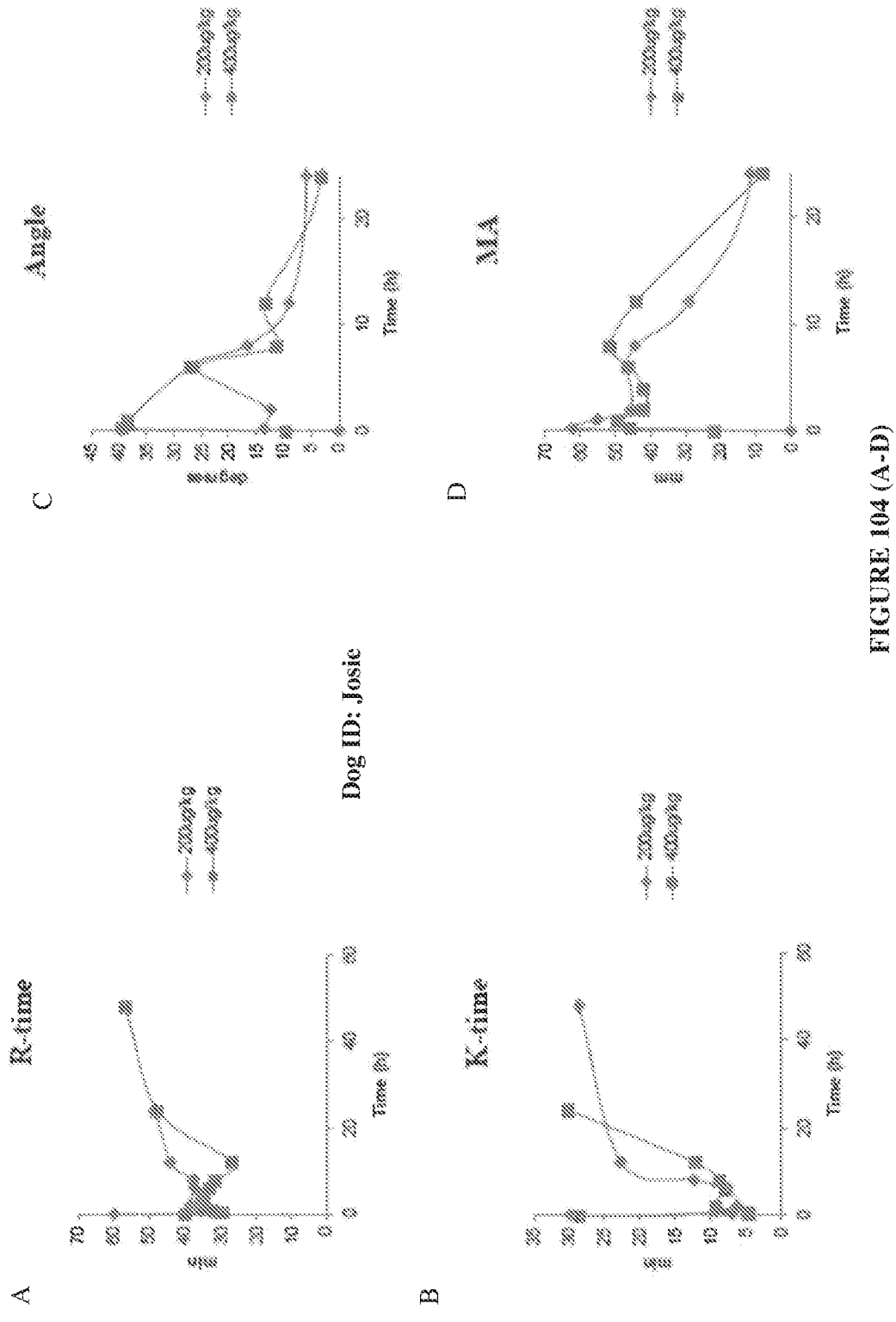


FIGURE 104 (A-D)

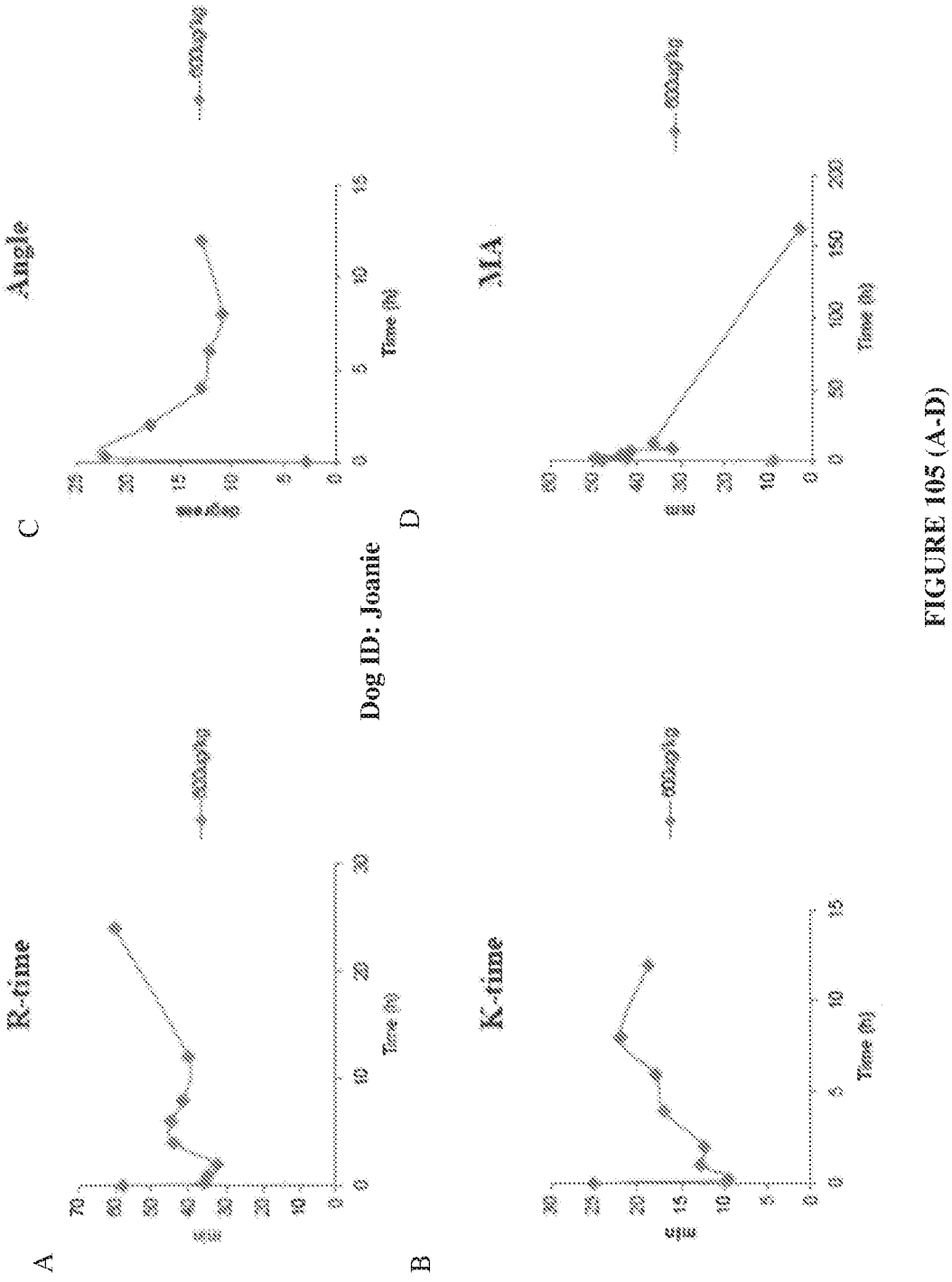


FIGURE 105 (A-D)

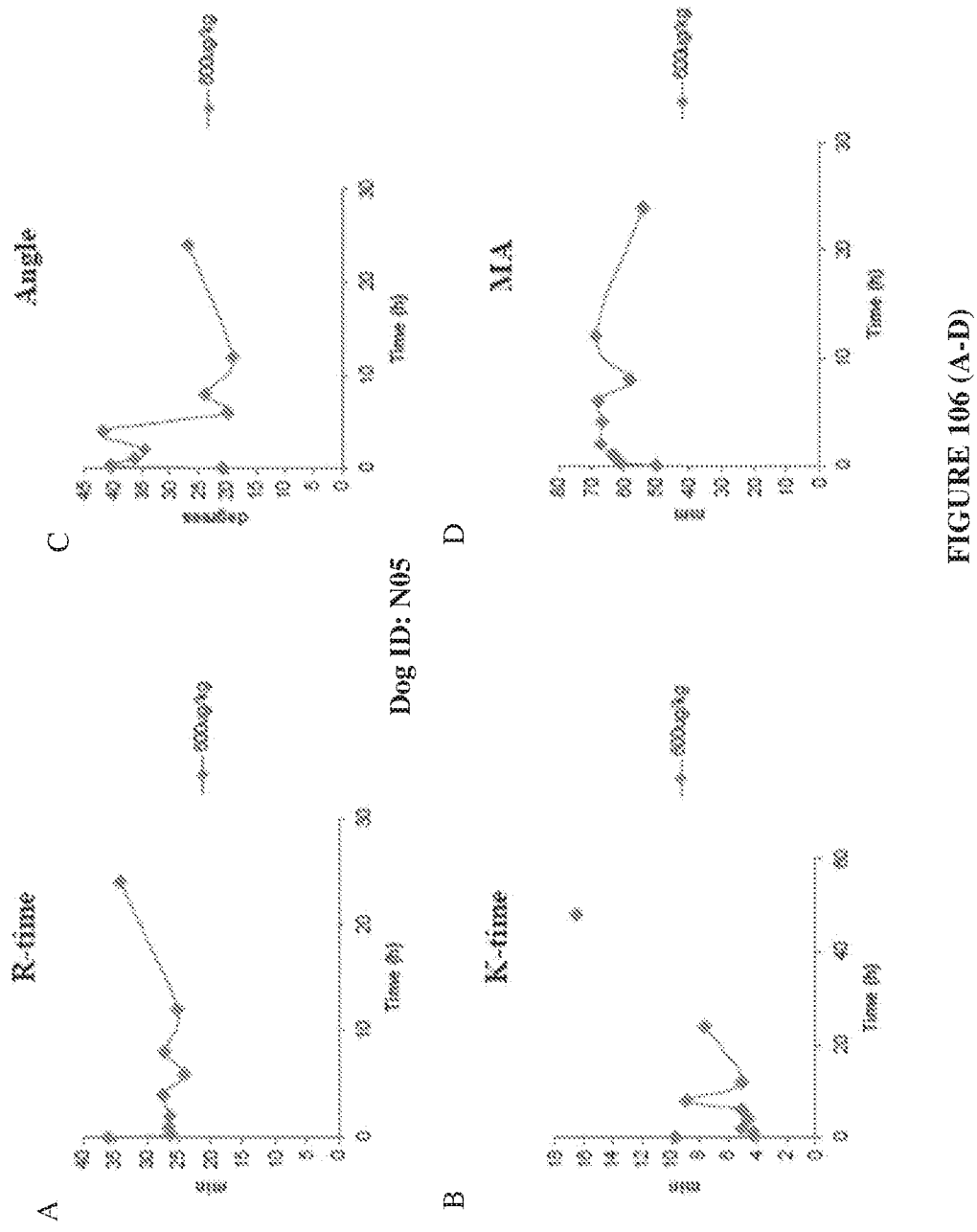
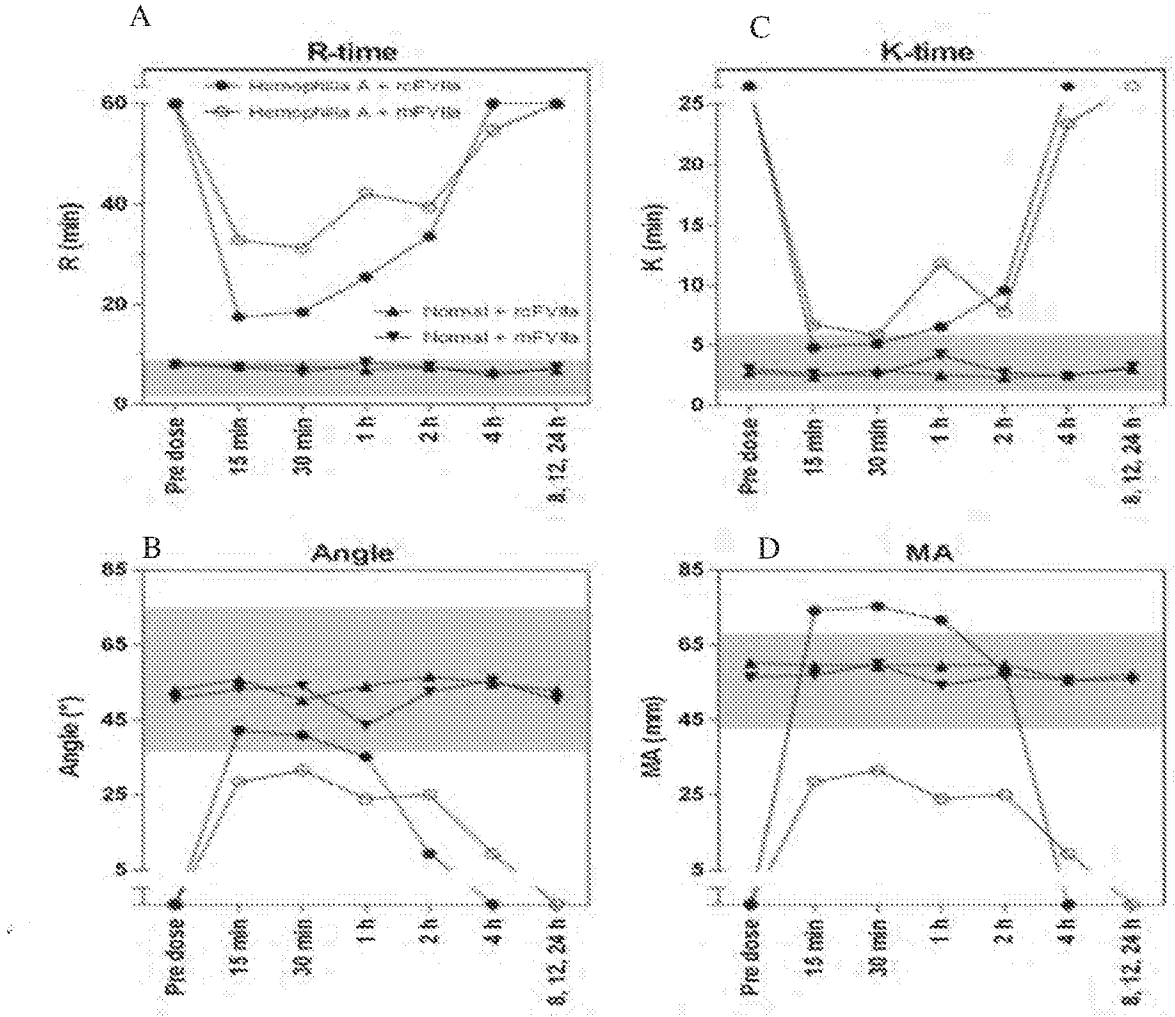
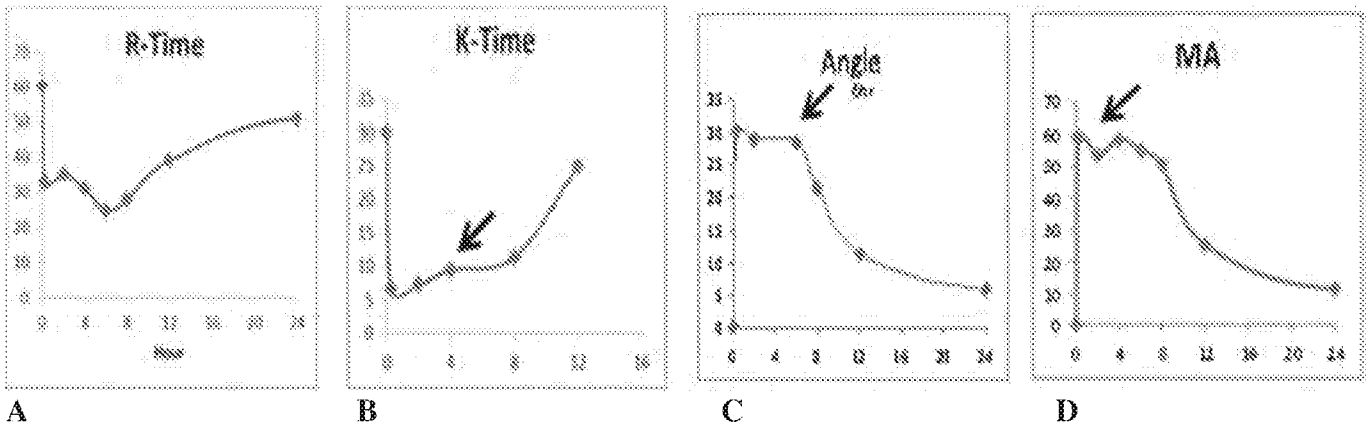


FIGURE 106 (A-D)



FIGURES 107 (A-D)



FIGURES 108 (A-D)

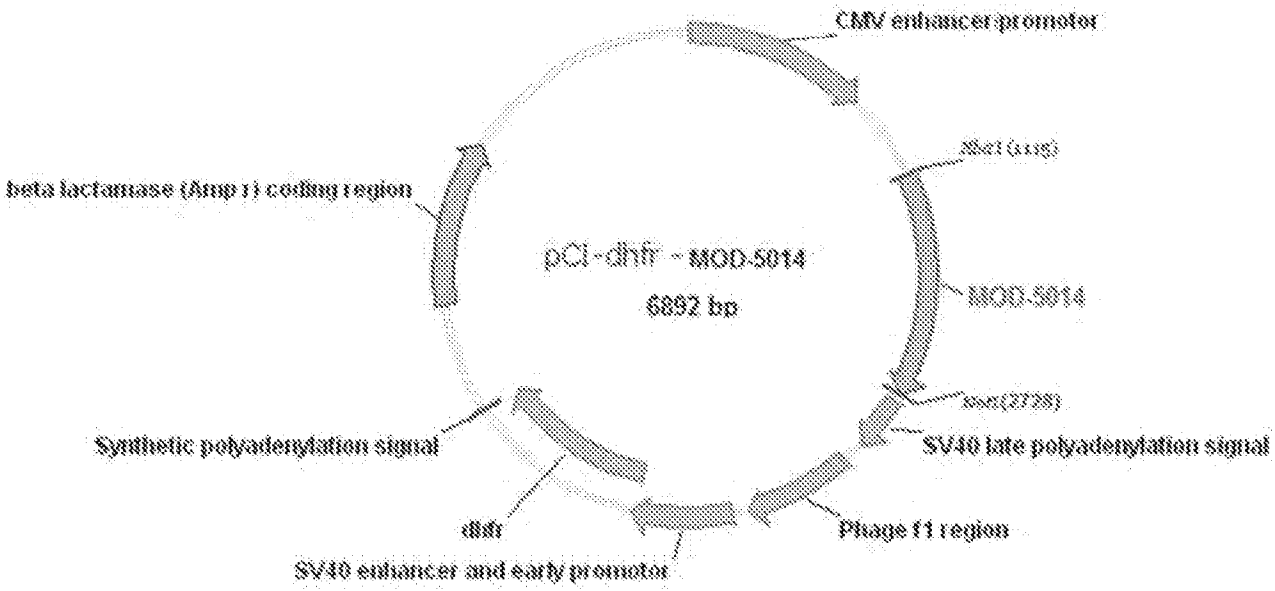


FIGURE 109

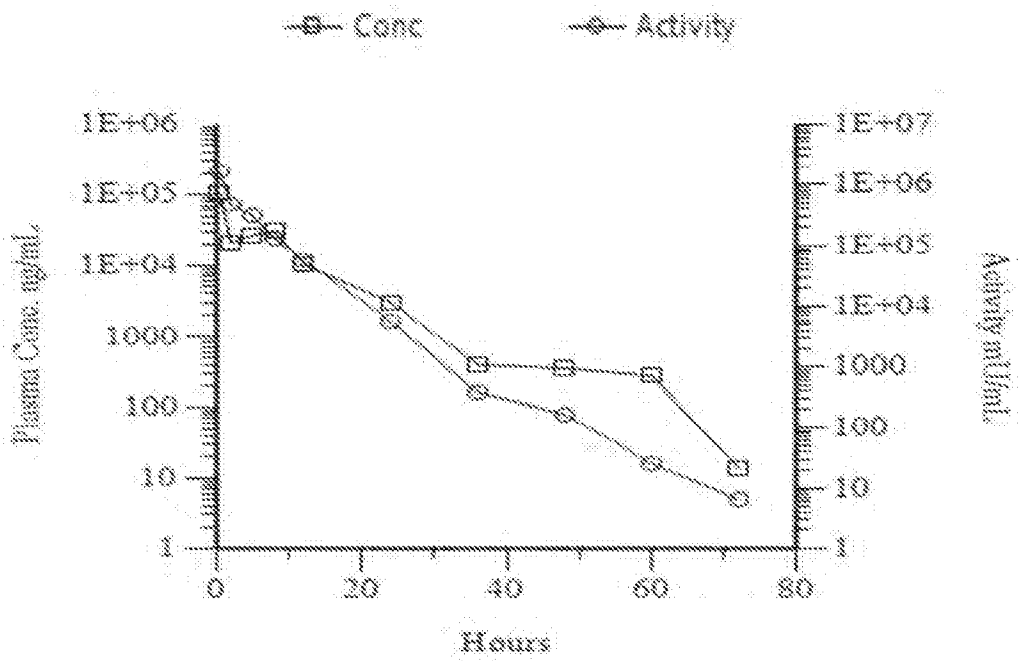


FIGURE 110

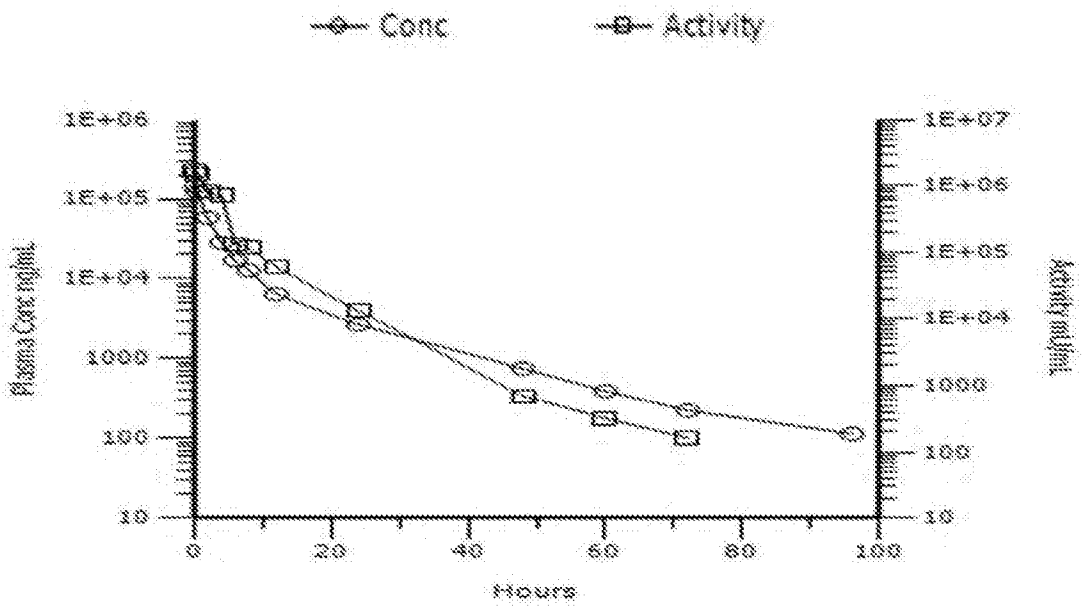


FIGURE 111

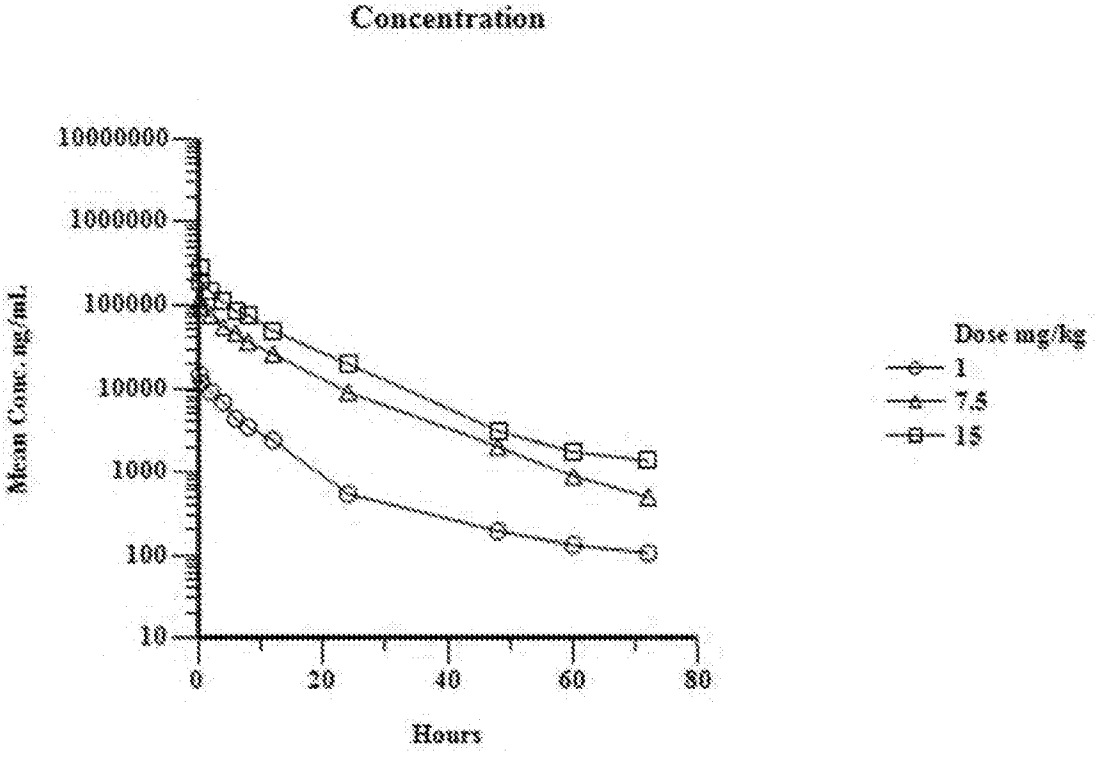


FIGURE 112

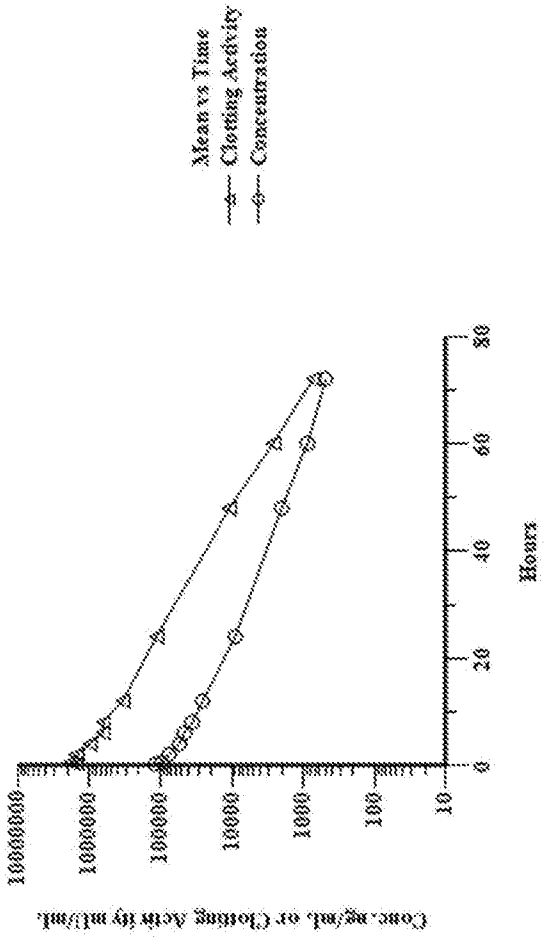


FIGURE 115

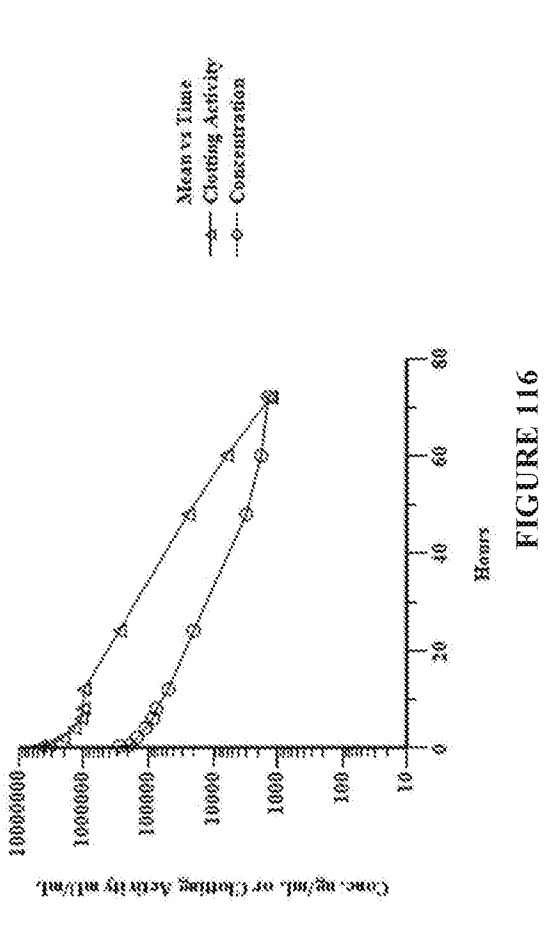


FIGURE 116

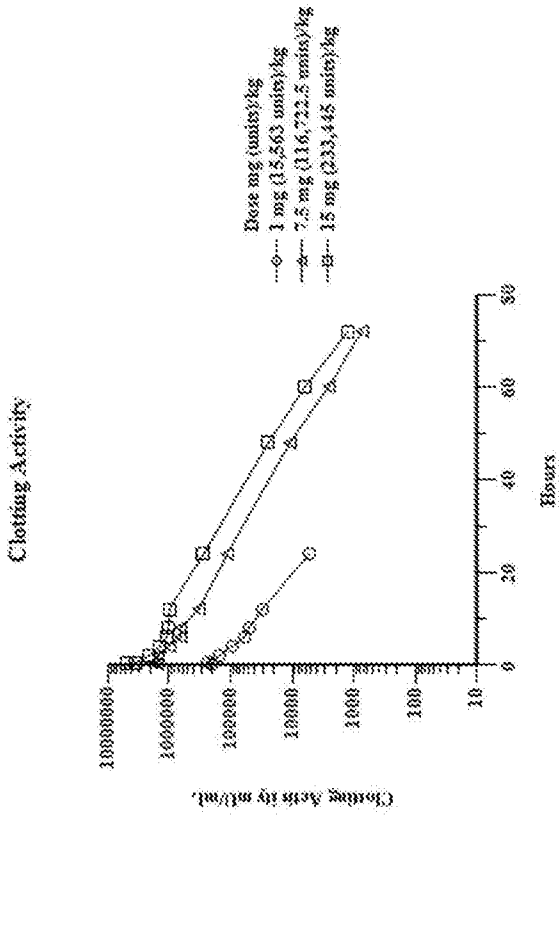


FIGURE 113

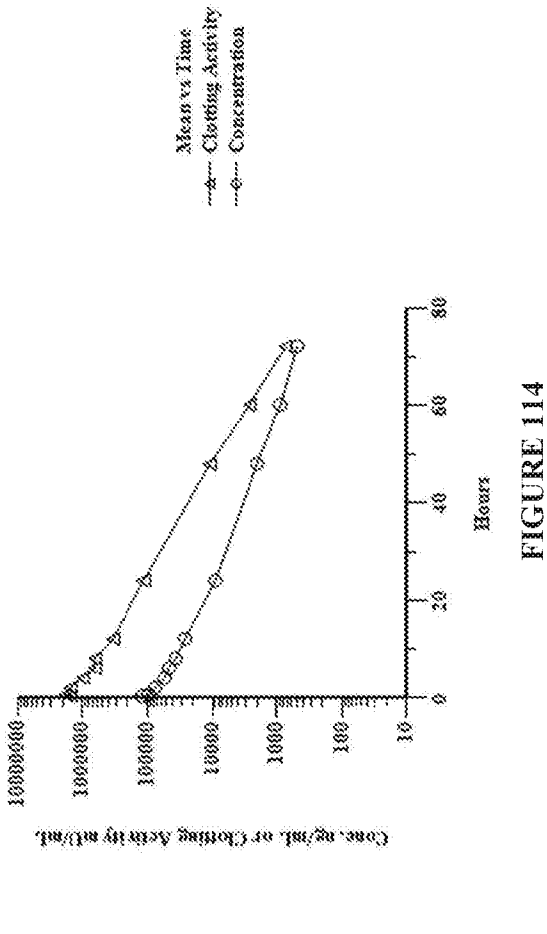


FIGURE 114

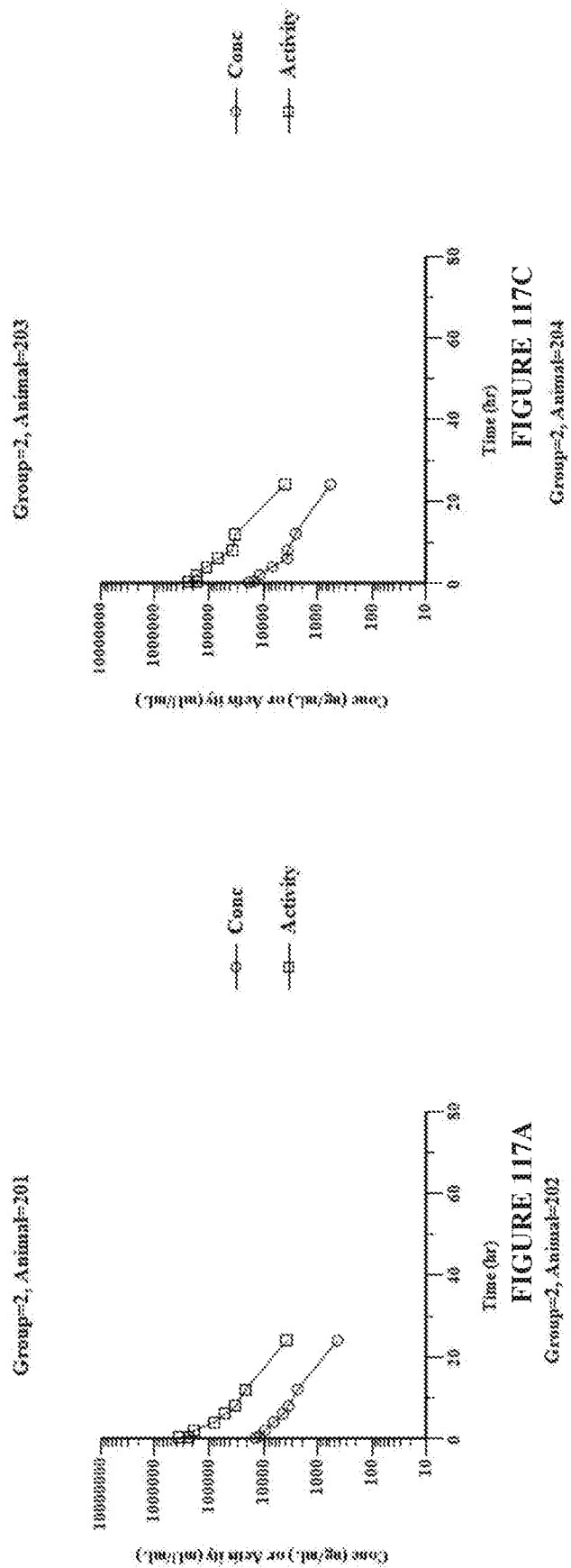


FIGURE 117A

FIGURE 117B

FIGURE 117C

FIGURE 117D

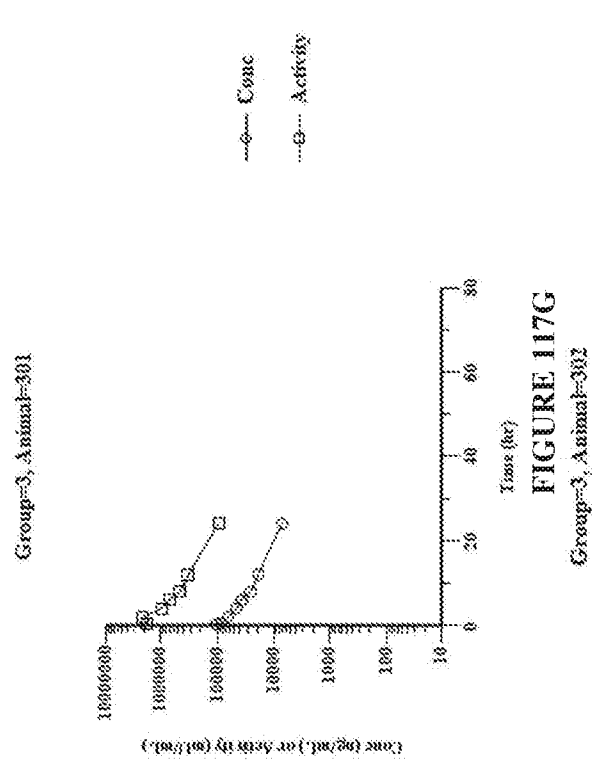


FIGURE 117G

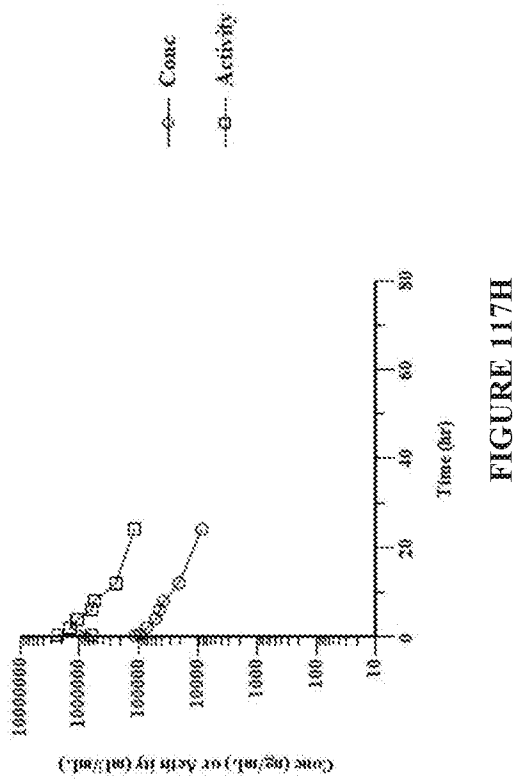


FIGURE 117H

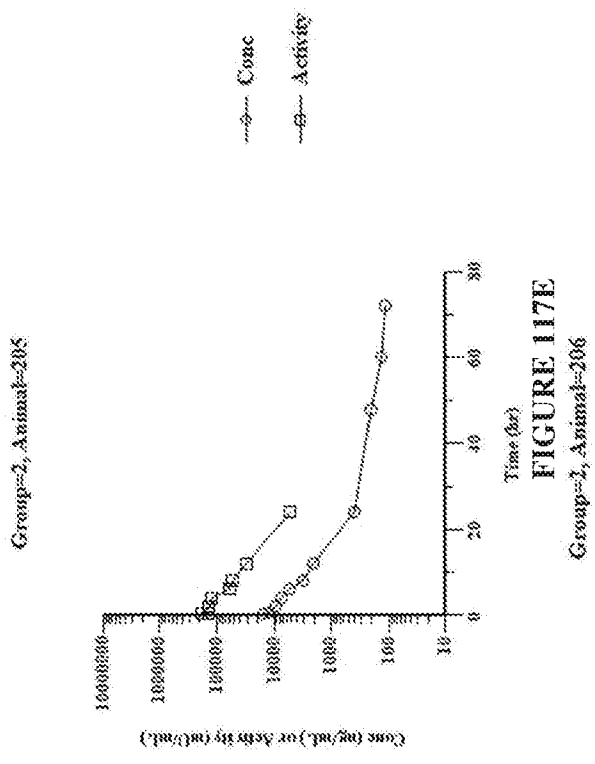


FIGURE 117E

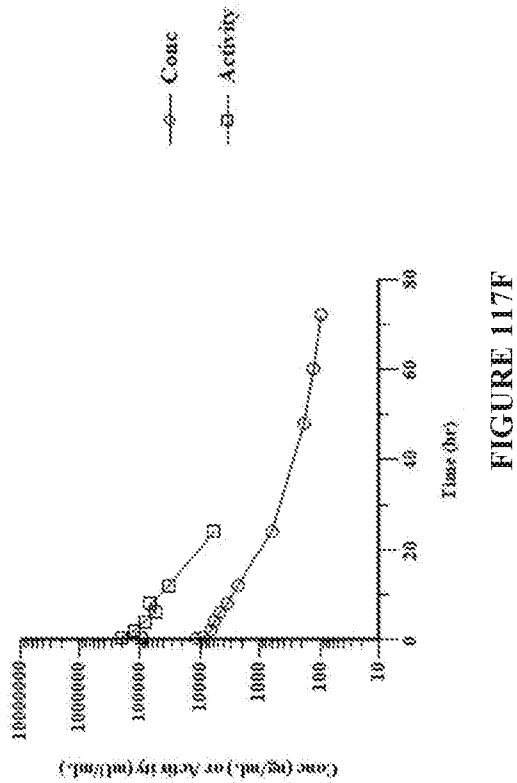


FIGURE 117F

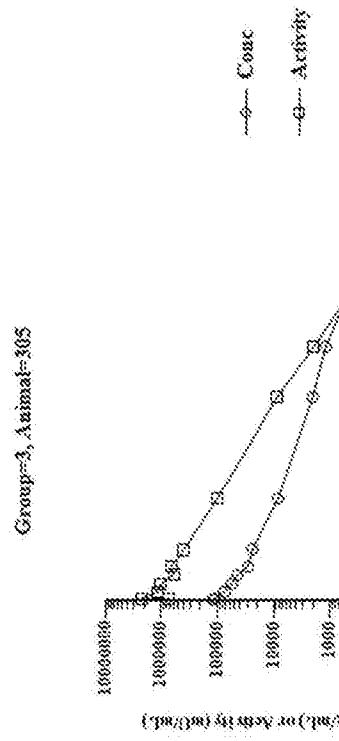


FIGURE 117K

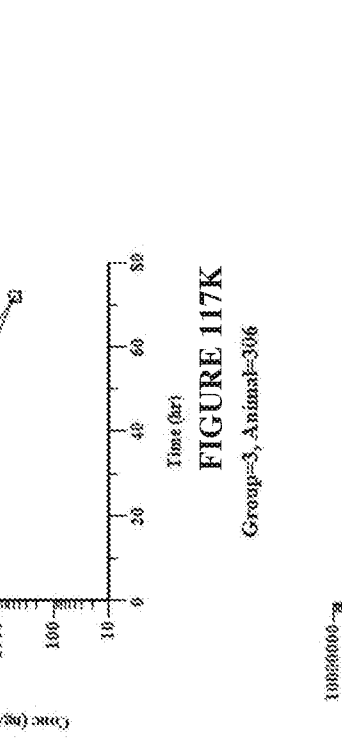


FIGURE 117L

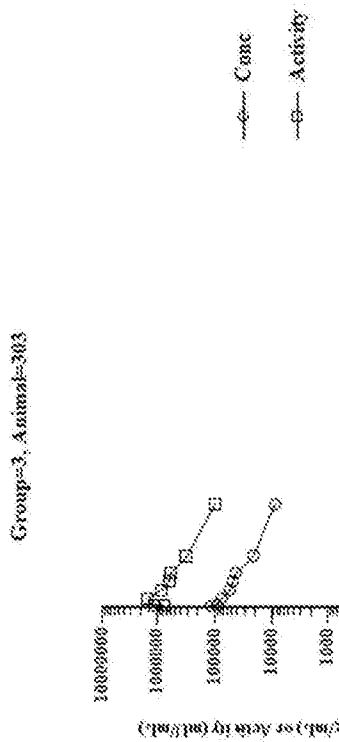


FIGURE 117I

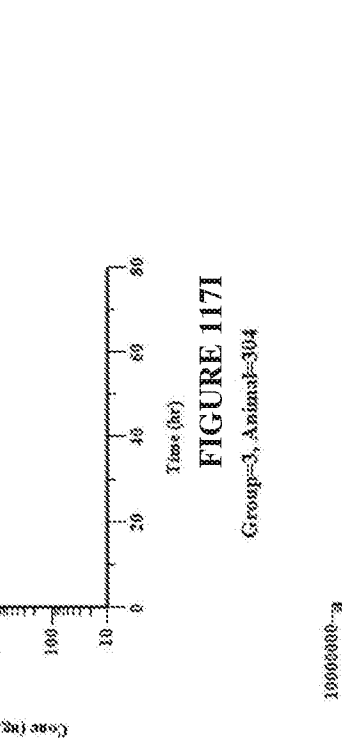


FIGURE 117J

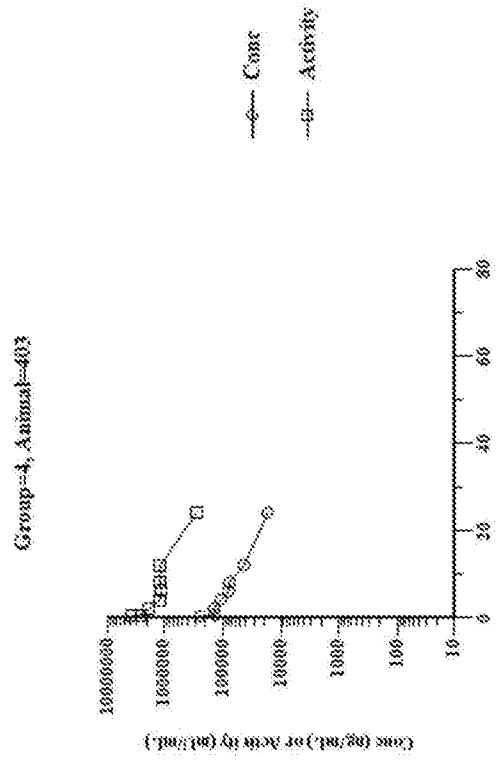


FIGURE 117M

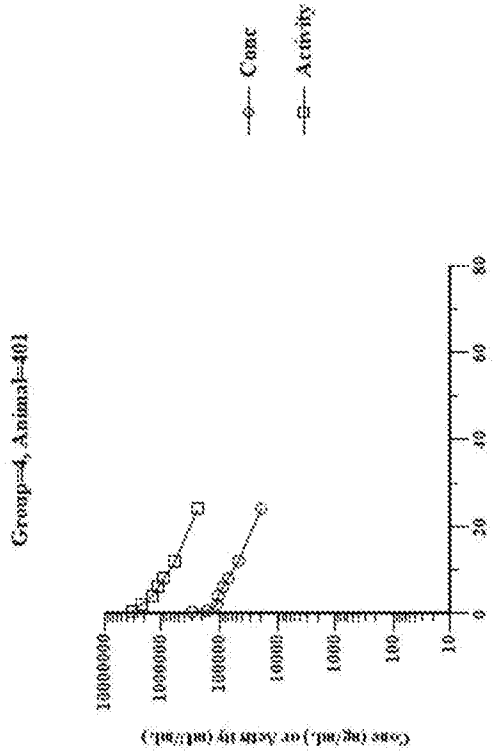


FIGURE 117N

FIGURE 117O

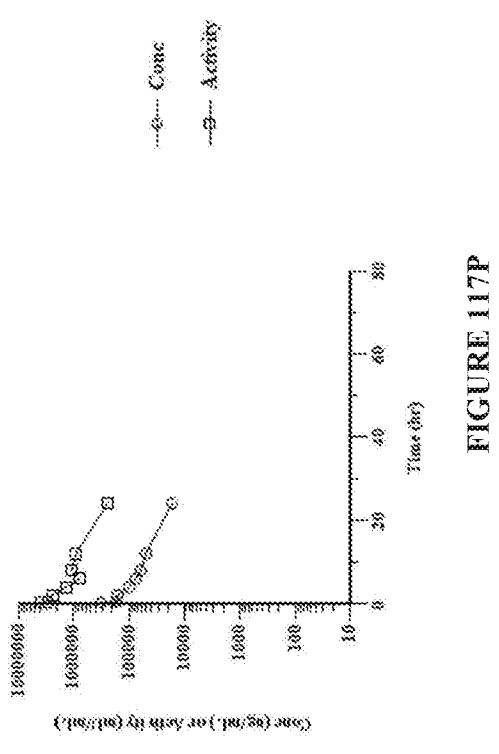


FIGURE 117P

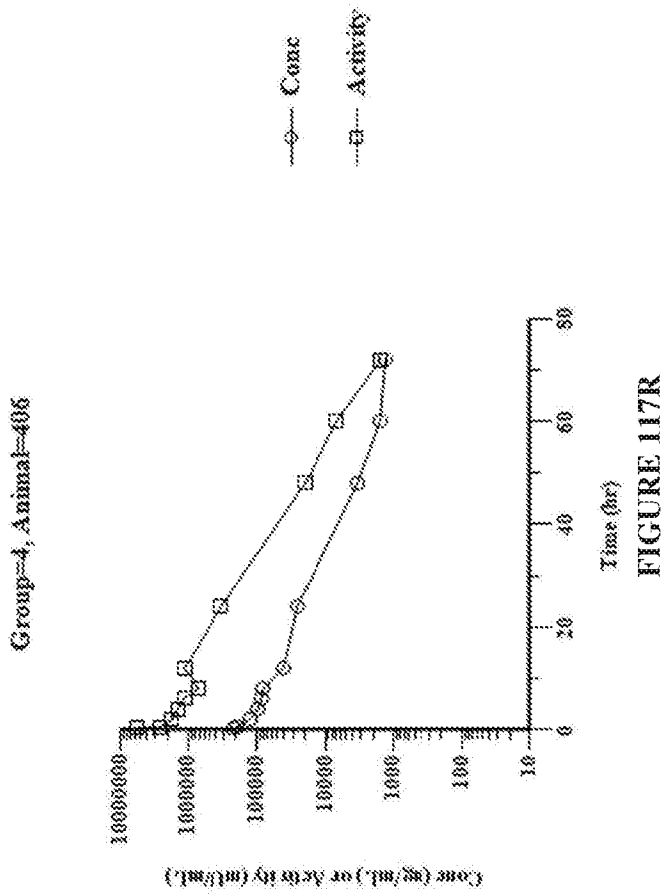


FIGURE 117R

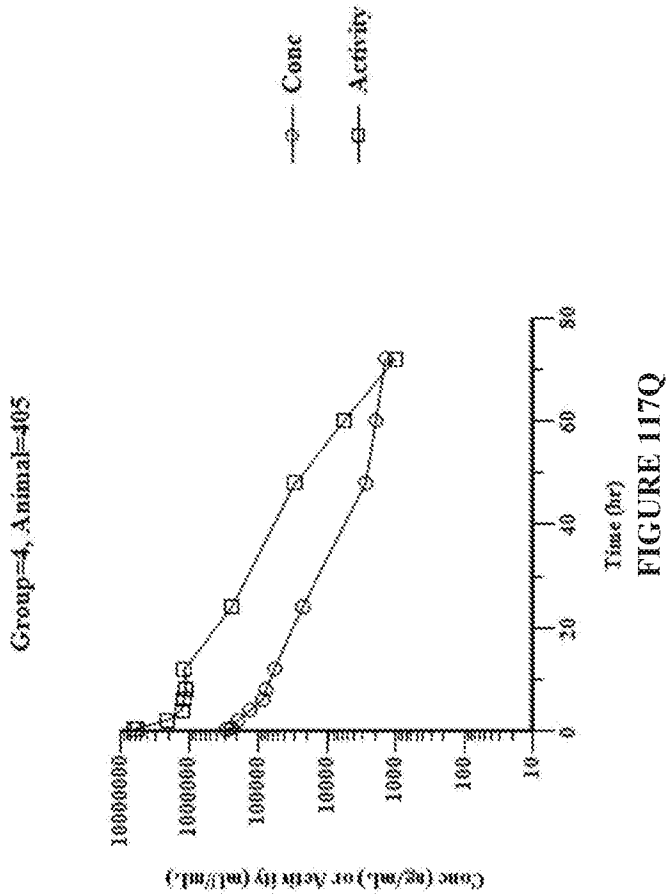


FIGURE 117Q

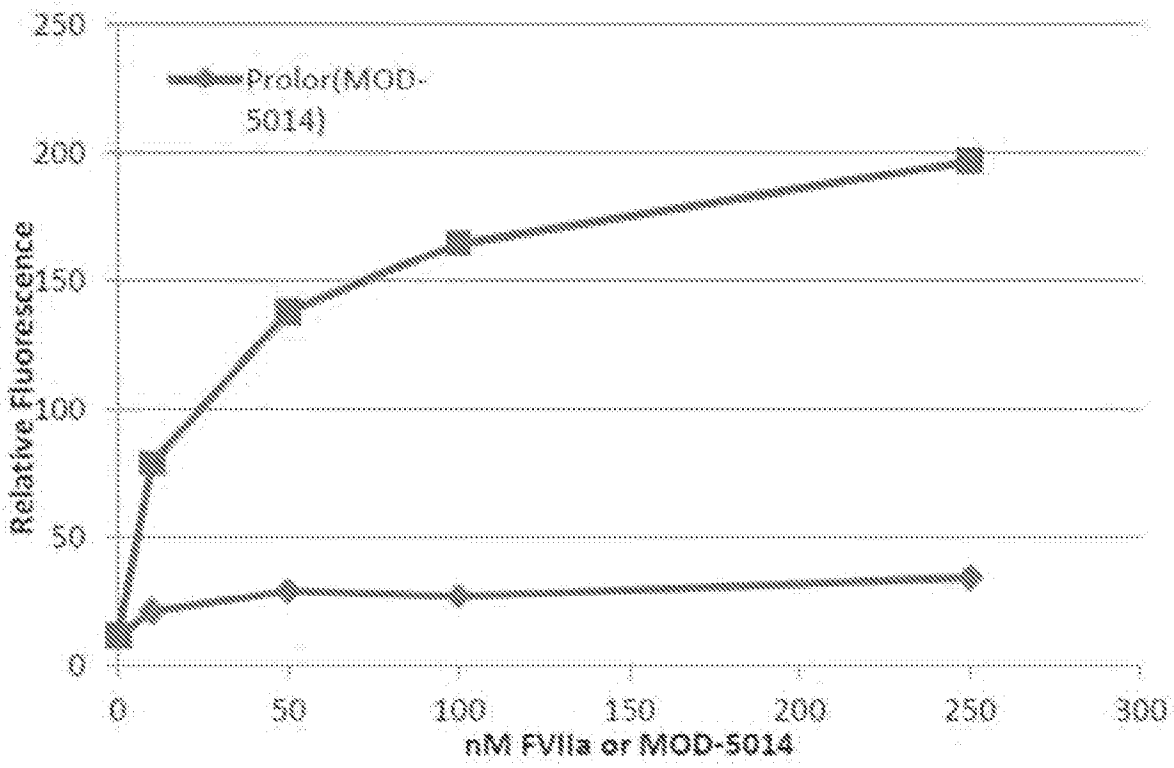


FIGURE 118

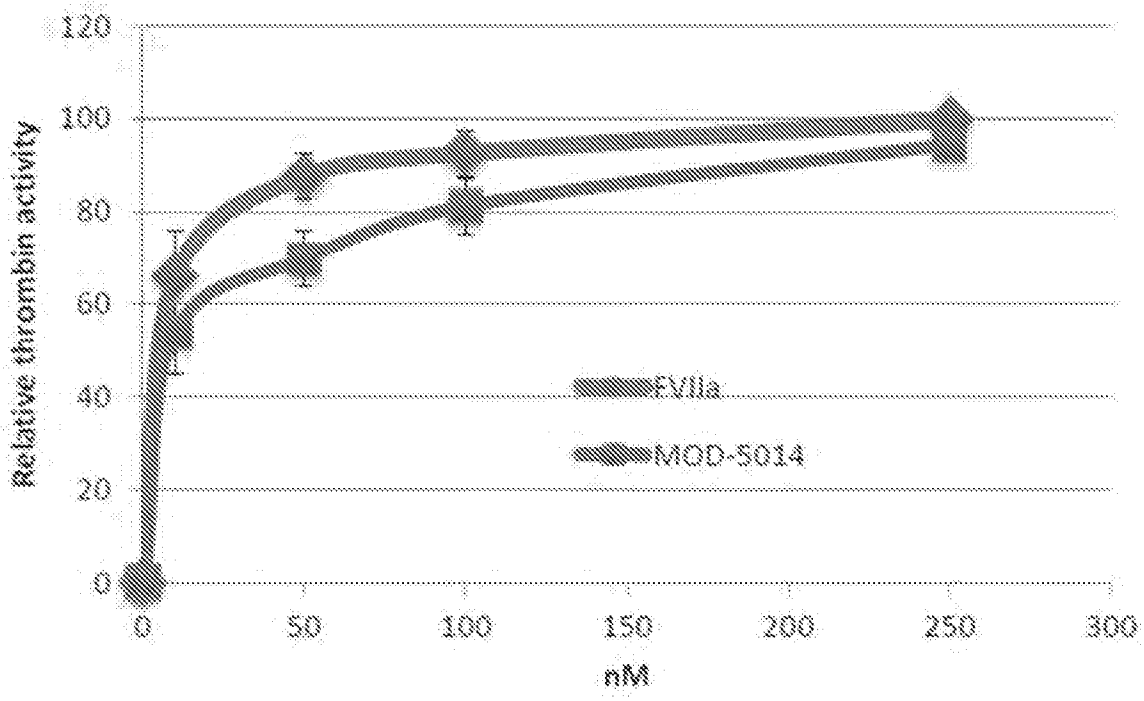


FIGURE 119

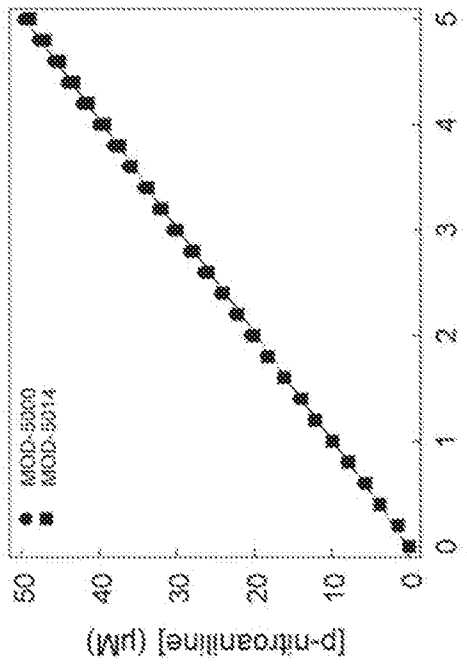


FIGURE 120

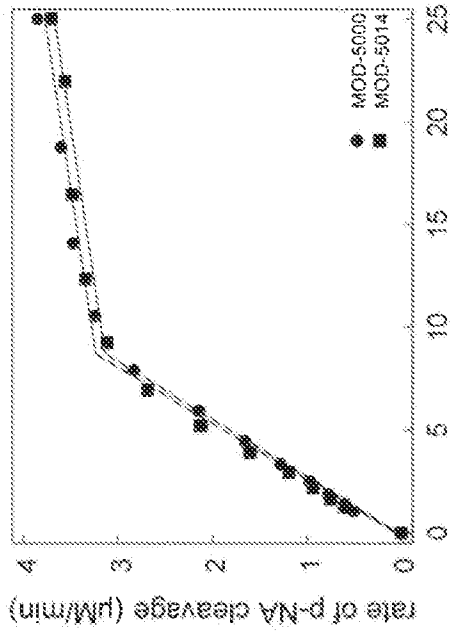


FIGURE 121

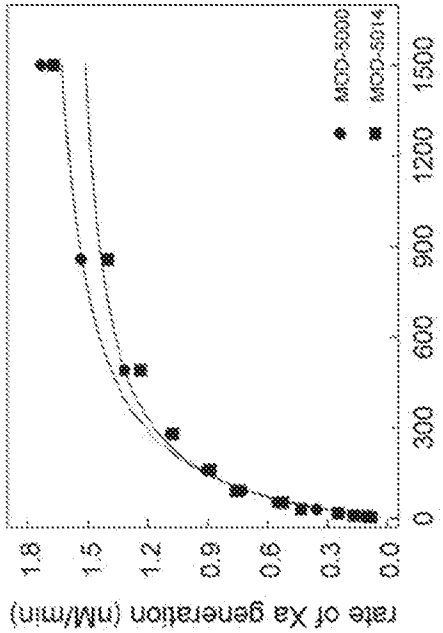


FIGURE 123

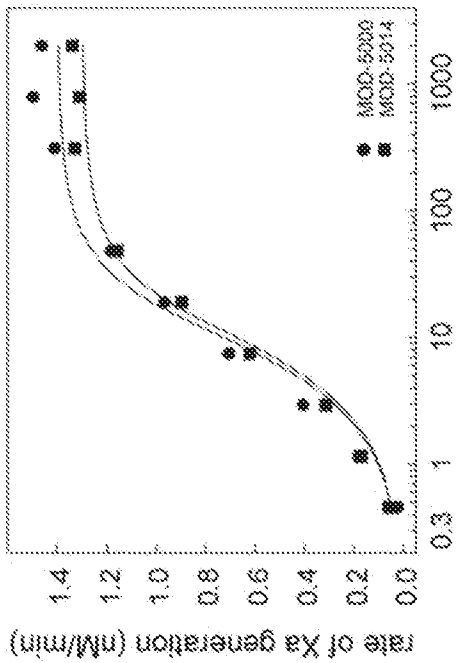


FIGURE 122

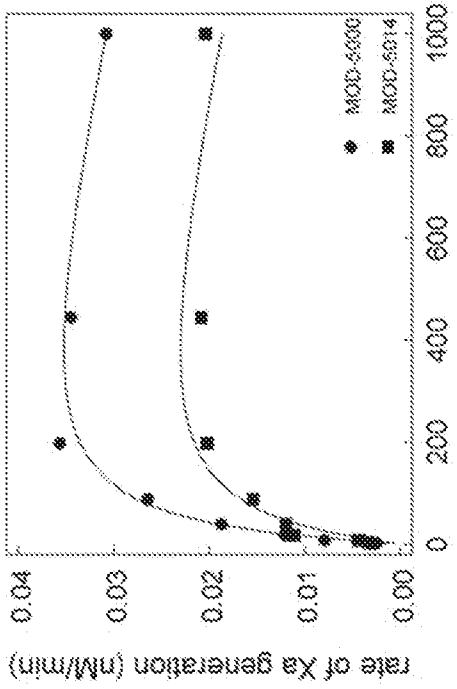


FIGURE 124A

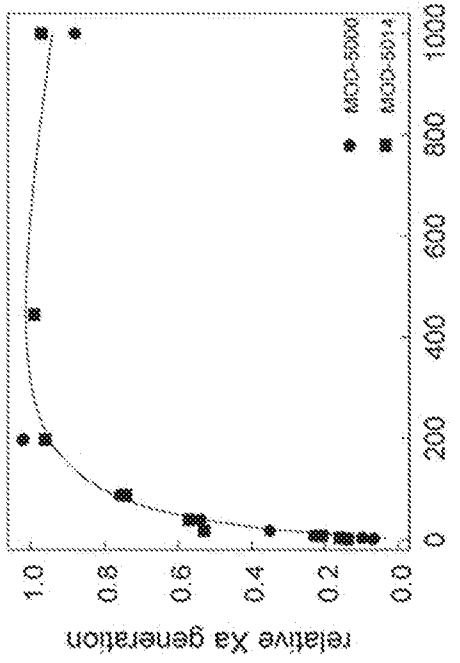


FIGURE 124B

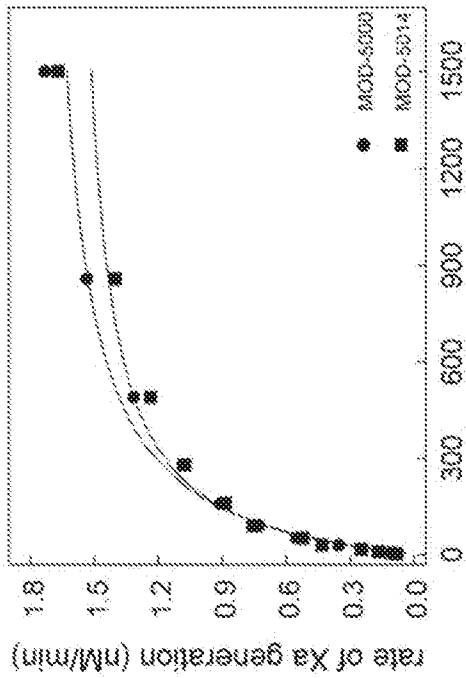


FIGURE 125

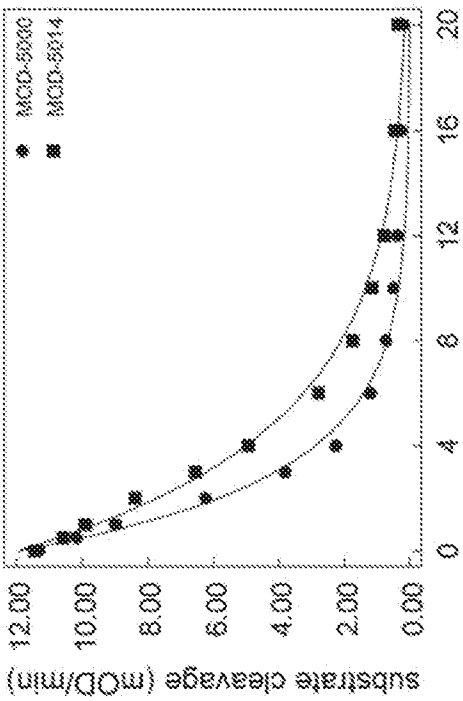


FIGURE 126

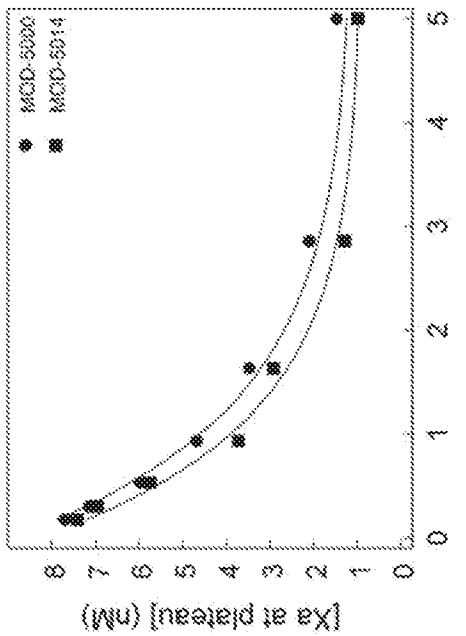


FIGURE 127A

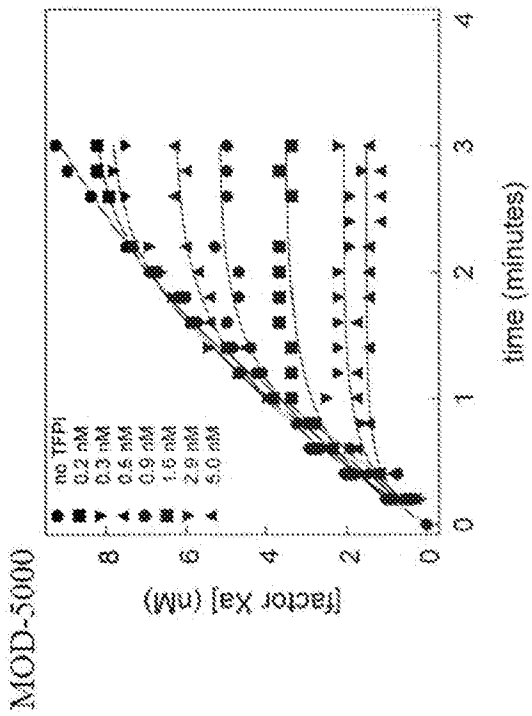


FIGURE 127B

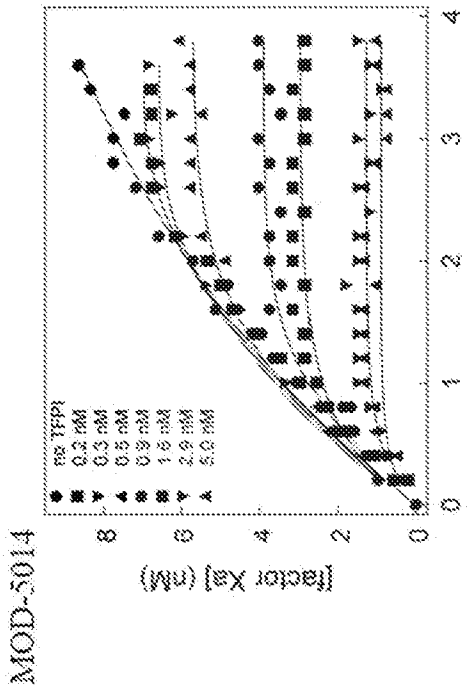


FIGURE 127C