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(54) Title: RECOMBINANT FACTOR VIII FORMULATIONS

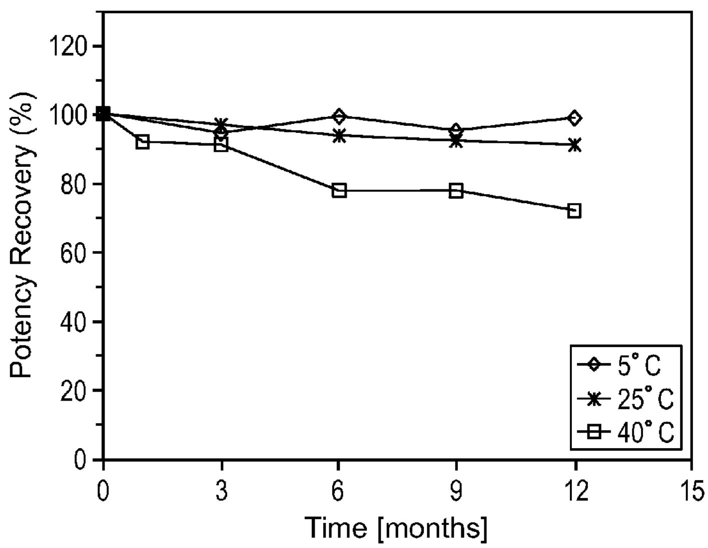

FIG. 18

(57) Abstract: Provided are liquid and lyophilized recombinant Factor VIII formulations, including formulations for polymer-conjugated FVIII such as PEGylated Factor VIII.

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RECOMBINANT FACTOR VIII FORMULATIONS

BACKGROUND

[0001] This application claims the benefit of U.S. Provisional Application No. 61/779,495, filed March 15, 2013 and U.S. Provisional Application No. 61/869,191, filed August 23, 2013, both of which are hereby incorporated herein by reference in their entireties.

[0002] Hemophilia A is caused by deficiencies in coagulation factor VIII ("FVIII") and is the most common hereditary coagulation disorder, with an estimated incidence of 1 per 5000 males. The current treatment for hemophilia A involves intravenous injection of recombinant or plasma-derived human FVIII. Injections of FVIII are either given on demand in response to a bleeding event or as a prophylactic therapy that is administered 2 to 4 times a week. Although numerous studies have shown that prophylactic therapy decreases the complications of hemophilia A, the need for frequent intravenous injections creates barriers to patient compliance and affects patient quality of life. The requirement for frequent injections is primarily due to the short circulating FVIII half-life of 12 to 14 hours in patients.

[0003] Covalent addition of long-chain polymers, such as polyethyleneglycol ("PEG"), has been shown to increase the half-life of protein therapeutics. PEGylation is the covalent attachment of PEG molecules to proteins.

[0004] U.S. Patent No. 5,763,401 (Nayar) discloses stable, albumin-free, lyophilized formulations of full-length recombinant FVIII ("FL-rFVIII"). U.S. Patent No. 7,632,921 (Pan et al.) and Mei *et al., Rational design of a fully active, long-acting PEGylated factor VIII for hemophilia A treatment,* 116 BLOOD 270-279 (2010) disclose cysteine enhanced FVIII mutants that are covalently bound to one or more biocompatible polymers such as PEG. U.S. Patent No. 7,087,723 (Besman et al.) pertains to albumin-free FVIII formulations. Österberg et al., *Development of a freeze-dried albumin-free formulation of recombinant factor VIII SQ,* Pharmaceutical Research, vol. 14, No. 7 (1997), pp. 892-898 and Österberg et al., *B-domain deleted recombinant factor VIII formulation and stability,* Seminars in Hematology, vol. 38, No. 2, suppl. 4 (April 2001), pp. 40-43 discuss formulations of B-domain deleted FVIII for lyophilization, including formulations containing sodium chloride, sucrose, histidine, calcium chloride dehydrate and polysorbate 80. Fatouros et al., *Recombinant factor VIII SQ – influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution,* Int. J. of Pharmaceutics 155 (1997) 121-131

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discloses the properties of rFVIII SQ on storage in solutions without albumin. WO2011/027152 (Jezek et al.) discloses formulations of FVIII.

SUMMARY

[0004a] In a first aspect the present invention provides a rFVIII formulation comprising:

(a) a range of from about 1 mM to about 5 mM divalent cation;

(b) a range of from about 150 mM to about 250 mM sodium chloride or potassium chloride;

(c) a range of from about 50 ppm to about 200 ppm of a non-ionic surfactant; and

(d) a range of from about 100 IU/ml to about 5000 IU/ml of a rFVIII, wherein the rFVIII comprises an amino acid sequence that has one or more non-cysteine residues in the amino acid sequence of SEQ ID NO: 3 replaced with cysteine residues such that at least one pair of cysteine residues creates a disulphide bond not found in wild type FVIII;

wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5.

[0004b] In a second aspect the present invention provides a rFVIII formulation comprising:

(a) about 0 mM, or a range of from about 1 mM to about 20 mM histidine;

(b) a range of from 0.5% to 20% of sucrose or trehalose;

(c) a range of from about 1 mM to about 5 mM divalent cation;

(d) a range of from about 10 mM to about 50 mM sodium chloride;

(e) about 0 mM, or a range of from about 20 ppm to about 80 ppm of a non-ionic surfactant;

(f) about 0%, or a range of from about 1.0% to about 5.0% glycine; and

(g) a range of from about 100 IU/ml to about 5000 IU/ml of conjugated rFVIII;

wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5.

[0004c] In a third aspect the present invention provides a method of treating hemophilia A comprising administering a therapeutically effective amount of a rFVIII formulation of the first or second aspects to a patient in need thereof.

[0004d] In a fourth aspect the present invention provides use of a rFVIII formulation of the first or second aspects in the manufacture of a medicament for treating hemophilia A.

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[0005] In one embodiment, the invention concerns a rFVIII formulation comprising: (a) a range of from about 1 mM to about 5 mM divalent cation; (b) a range of from about 150 mM to about 250 mM sodium chloride or potassium chloride; (c) a range of from about 50 ppm to about 200 ppm of a non-ionic surfactant; and (d) a range of from about 100 IU/ml to about 5000 IU/ml of a rFVIII, wherein the rFVIII comprises an amino acid sequence that has one or more non-cysteine residues in the amino acid sequence of SEQ ID NO: 3 replaced with cysteine residues; wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5. In another embodiment, the invention concerns a rFVIII formulation comprising: (a) a range of from 10 mM to 100 mM MOPS; (b) a range of from 0.5% to 10% by weight of a sugar or a sugar alcohol; (c) a range of from 0.5 mM to 20 mM of a divalent cation; (d) a range of from 10 mM to 100 mM sodium chloride or potassium chloride; (e) a range of from 50 to 150 ppm of a non-ionic surfactant; and (f) a range of from about 100 IU/mI to about 1500 IU/mil of rFVIII; wherein the rFVIII formulation contains less than 5.0 % by weight of components other than rFVIII having primary or secondary amine groups.

[0006] In yet another embodiment, the invention concerns a rFVIII formulation comprising: (a) a range of from 10 mM to 100 mM MOPS; (b) a range of from 150 mM to 300 mM NaCl; (c) a range of from 1 mM to 20 mM divalent cation; and (d) a range of from about 100 IU/ml to about 5000 IU/ml of nonconjugated rFVIII. In a further embodiment, the invention concerns a rFVIII formulation comprising: (a) a range of from 10 mM to 100 mM MOPS or histidine; (b) a range of from 25 mM to 200 mM NaCl; (c) a range of from 1 mM to 20 mM divalent cation; and (d) a range of from about 100 IU/ml to about 5000 IU/ml of conjugated rFVIII. In yet a further embodiment, the invention concerns a rFVIII formulation comprising: (a) about 0 mM, or a range of from about 1 mM to about 20 mM histidine; (b) a range of from 0.5% to 20% of sucrose or trehalose; (c) a range of from about 1 mM to about 5 mM divalent cation; (d) about 0 mM, or a range of from about 10 mM to about 50 mM sodium chloride; (e) about 0 mM, or a range of from about 20 ppm to about 80 ppm of a nonionic surfactant; (f) about 0%, or a range of from about 1.0% to about 5.0%, glycine and (g) a range of from about 100 IU/ml to about 5000 IU/mil of conjugated rFVIII; wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The skilled artisan will understand that the drawings, described below, are for illustration purposes only. The drawings are not intended to limit the scope of the disclosure provided herein or the scope of the claims in any way.

[0008] FIG. 1 shows schematically the domains of full-length human factor VIII and BDD-rFVIII.

[0009] FIG. 2 is a graph showing the relative turbidity of BDD-rFVIII mutants having domains linked by disulfide bonds. The turbidity was measured in buffer comprising increasing concentration of sodium chloride. Turbidity was measured by A_{340nm}. In addition to sodium chloride, the buffer comprised 20 mM histidine, 2.5 mM calcium chloride, 29 mM sucrose, 293 mM glycine and 80 ppm polysorbate 80.

[0010] FIG. 3 is a graph showing the relative turbidity of BDD-rFVIII mutants having domains linked by disulfide bonds. The turbidity was measured in buffer comprising increasing concentration of polysorbate 80. Turbidity was measured by A_{340nm}. In addition to polysorbate 80, the buffer comprised 20 mM histidine, 30 mM sodium chloride, 2.5 mM calcium chloride, 29 mM sucrose and 293 mM glycine.

[0011] FIG. 4 is a graph showing the relative turbidity of BDD-rFVIII mutants having domains linked by disulfide bonds. The turbidity was measured in buffer comprising increasing concentration of human serum albumin ("HSA"). Turbidity was measured by A_{340nm}. The buffer comprised 20 mM histidine, 30 mM sodium chloride, 2.5 mM calcium chloride, 29 mM sucrose, 293 mM glycine and 80 ppm polysorbate 80.

[0012] FIG. 5 shows the relative turbidity of BDD-rFVIII mutants having domains linked by disulfide bonds. The turbidity was measured in a buffer comprising increasing concentration of sodium chloride in combination with polysorbate 80 and HSA. Turbidity was measured by A_{340nm}. In addition to sodium chloride, HSA and polysorbate 80, the buffer comprised 20 mM histidine, 2.5 mM calcium chloride, 29 mM sucrose and 293 mM glycine.

[0013] FIG. 6 shows clarity changes for BDD-rFVIII mutants with disulfide bonds linking domains in solution before and after addition of excipients. From left to right: (1) combination of excipients (HSA, sodium chloride and polysorbate 80), (2) HSA, (3) sodium chloride, (4) polysorbate 80, and (5) before addition of HSA, polysorbate 80 and sodium chloride.

[0014] FIG. 7 is a graph showing liquid stability of full-length FVIII in histidine, MOPS and TEA buffers during 7 days storage at 40°C.

[0015] FIG. 8 is a graph showing rFVIII stability in MOPS and histidine buffer at 25°C.

[0016] FIG. 9 is a diagram showing the structure of PEGylated BDD-rFVIII. The chains protruding from the A3 region represent the PEG molecule.

[0017] FIG. 10 is a graph showing the effect of sodium chloride on the potency recovery of PEGylated BDD-rFVIII during 6 days storage at 23°C.

[0018] FIG. 11 is a graph showing the effect of sodium chloride on the potency recovery of unPEGylated BDD-rFVIII during 6 days storage at 23°C.

[0019] FIG. 12 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation A after 26 weeks. Formulation A contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 293 mM glycine, 29 mM sucrose and 80 ppm polysorbate 80.

[0020] FIG. 13 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation B after 26 weeks. Formulation B contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 346 mM glycine, 38 mM sucrose and 80 ppm polysorbate 80.

[0021] FIG. 14 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation C after 26 weeks. Formulation C contains 2.5 mM calcium chloride, 20 mM histidine, 234 mM sucrose and 80 ppm polysorbate 80.

[0022] FIG. 15 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation D after 26 weeks. Formulation D contains 2.5 mM calcium chloride, 20 mM histidine, 211 mM trehalose and 80 ppm polysorbate 80.

[0023] FIG. 16 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation A up to 30 months. Formulation A contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 293 mM glycine, 29 mM sucrose and 80 ppm polysorbate 80.

[0024] FIG. 17 is a graph showing normalized potency trends for PEGylated BDD-rFVIII in lyophilized Formulation B up to 13 weeks. Formulation B contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 346 mM glycine, 38 mM sucrose and 80 ppm polysorbate 80.

[0025] FIG. 18 is a graph showing the normalized potency trends for PEGylated BDD-rFVIII (200 IU/mL) in lyophilized Formulation A up to 12 months. Formulation A contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 293 mM glycine, 29 mM sucrose and 80 ppm polysorbate 80.

[0026] FIG. 19 is a graph showing the normalized potency trends for PEGylated BDD-rFVIII (1200 IU/mL) in lyophilized Formulation A up to 9 months. Formulation A contains 2.5 mM calcium chloride, 30 mM sodium chloride, 20 mM histidine, 293 mM glycine, 29 mM sucrose and 80 ppm polysorbate 80.

[0027] FIG. 20 is the amino acid sequence of BDD-rFVIII SQ (SEQ ID NO: 3).

[0028] FIG. 21 is the amino acid sequence of FL-rFVIII (SEQ ID NO: 1).

DETAILED DESCRIPTION

[0029] For the purposes of interpreting this specification, the following definitions will apply, unless otherwise indicated. All references cited herein are incorporated by reference herein in their entireties.

[0030] Factor VIII: Factor VIII ("FVIII") is a coagulation factor that circulates as a heterodimer composed of a heavy chain of approximately 200 kDa and a light chain of 80 kDa. The heavy chain contains structurally related A1 and A2 domains, as well as a unique B domain, and light chain comprises the A3, C1, and C2 domains. *See, e.g.,* Mei et al., 116 BLOOD 270-279 (2010). *See also* FIG.1, showing the domains of FVIII. The term "Factor VIII" or "FVIII" as used herein refers to all Factor VIII molecules, whether derived from blood plasma or produced through the use of recombinant DNA techniques, that have some procoagulant activity characteristic of wild type human FVIII. As used herein, FVIII includes modified or truncated forms of wild type or recombinant Factor VIII that retain some or all of the procoagulant activity of wild type Factor VIII or activated wild type Factor VIII, including variants or truncated forms that have procoagulant activity exceeding the activity of wild type Factor VIII or activated wild type Factor VIII. FVIII also includes fusion products containing active Factor VIII, such as fusions with an immunoglobulin fragment or domain. Commercially available examples of therapeutic preparations containing FVIII include those sold under the trade name KOGENATE FS (available from Bayer Healthcare LLC, Berkeley, CA, U.S.A.).

[0031] Recombinant Factor VIII: Recombinant Factor VIII ("rFVIII") as used herein refers to FVIII that is produced using recombinant technology, or a biologically active derivative thereof, and does not include FVIII obtained from mammalian plasma.

[0032] Full-length, native human Factor VIII ("FL-FVIII") is a 2,351 amino acid, single chain glycoprotein. The expressed 2,351 amino acid sequence is provided as SEQ. ID. NO: 1. When the expressed polypeptide is translocated into the lumen of the endoplasmic reticulum, however, a 19-amino acid signal sequence is cleaved, resulting in a second sequence. This second sequence, herein provided as SEQ. ID. NO: 2, lacks the leading 19 amino acids and is the sequence conventionally used by researchers to assign a numeric location (e.g., Arg^{372}) to a given amino acid residue of VIII. Thus, unless specifically noted, all assignments of a numeric location of an amino acid residue as provided herein are based on SEQ. ID. NO: 2. For example, as is conventional and as used herein, when referring to mutated amino acids in BDD rFVIII, the mutated amino acid is designated by its position in the sequence of full-length FVIII. For example, a BDD rFVIII mutant can include a K1808C amino acid substitution wherein the lysine (K) at the position analogous to 1808 in the full-length sequence (here, SEQ ID NO: 2) is substituted to cysteine (C).

[0033] B-domain deleted ("BDD") Factor VIII: As used herein, BDD or BDD-rFVIII is characterized by having an amino acid sequence with a deletion of all or part of the B-domain. In one embodiment, BDD is the molecule known as BDD-SQ, which contains a deletion of all but 14 amino acids of the B-domain of Factor VIII. In BDD-SQ, the first 4 amino acids of the B-domain (SEQ ID NO: 4) are linked to the 10 last residues of the B-domain (SEQ ID NO: 5). *See, e.g.,* Lind *et al., Novel forms of B-domain-deleted recombinant factor VIII molecules,* 232 EUROPEAN JOURNAL OF BIOCHEMISTRY 19-27 (1995). *See also* FIG. 1 showing BDD by domain organization. BDD-SQ as used herein comprises the amino acid sequence of SEQ ID NO: 3. The B-domain of Factor VIII seems to be dispensable as a BDD molecule having a 90 kD A1-A2 heavy chain plus 80 kD light chain has been shown to be effective as a replacement therapy for hemophilia A. FVIII molecules having other portions of the B-domain deleted or all of the B-domain deleted are also included in the formulations and methods of the present invention.

[0034] BDD mutant or BDD-rFVIII mutant: BDD mutant or BDD-rFVIII mutant is a variant of BDD-SQ that maintains at least some of the FVIII procoagulant activity and differs from BDD-SQ by at least one amino acid residue. BDD mutant or BDD-rFVIII mutant includes variants that differ in amino acid sequence from BDD-SQ, for example, without limitation, by site-directed mutation of one or more amino acid residues. Without limitation, BDD mutant or BDD-rFVIII mutant includes the FVIII polypeptides with introduced cysteine residues disclosed in U.S. Patent No. 7,928,199 (Griffin et al.).

[0035] PEGylation: PEGylation is the covalent attachment of long-chain polyethylene glycol (PEG) molecules to proteins, such as by attaching a PEG that has an active functionality that binds to a site present on FVIII. One method used for PEGylation is the attachment of a functionalized PEG moiety to lysine residues or N-terminal amines that are present in the native protein. Because FVIII contains many amine residues, amine-functionalized polymers are randomly conjugated to different sites on FVIII.

[0036] Site-directed PEGylation allows targeting of the PEG molecules to specific sites. These specific sites can include introduced surface-exposed cysteines to which the PEG polymer can be conjugated. *See* U.S. Patent No. 7,632,921 (Pan et al.). PEG may also be attached to FVIII by covalent linkage to a saccharide on FVIII. *See, e.g.*, U.S. Pat. App. Pub. 20110112028 (Turecek et al.). PEG may be attached to FVIII by enzymatic coupling of PEG to a glycan on FVIII, such as an O-glycan. Stennicke et al. disclose selective coupling of PEG to a unique O-glycan in the FVIII B-domain by incubating full-length FVIII with sialidase and excess CMP-SA-glycerol-PEG reagent in a buffer. Stennicke et al., "*A novel B-domain O-glycoPEGylated FVIII (N8-GP) demonstrates full efficacy and prolonged effect in hemophilic mice models,*" 121 (11) BLOOD 2108-16 (2013). U.S. Pat. App. Pub. 20130137638 (Bolt) discloses PEG attachment to a FVIII variant with a truncated B-domain. The FVIII molecule is covalently conjugated with a hydrophilic polymer via an O-linked oligosaccharide in the truncated B domain. U.S. Pat. App. Pub. 20120322738 (Behrens) discloses methods of conjugating polymers to FVIII, including covalently conjugating PEG to FVIII via an O-linked saccharide in the B-domain. As used herein, a PEGylated FVIII includes PEGylation by any method, including the various methods known in the art discussed above.

[0037] International Unit, IU: International Unit, or IU, is a unit of measurement of the blood coagulation activity (potency) of FVIII as measured by a standard assay. Standard assays include the one stage assay, as described in the art. *See, e.g.*, Lee *et al.*, *An effect of predilution on potency assays of Factor VIII concentrates,* 30 THROMBOSIS RESEARCH 511-519 (1983). The one-stage assay is based upon the activated partial thromboplastin time (aPTT). FVIII acts as a cofactor in the presence of Factor IXa, calcium, and phospholipid in the enzymatic conversion of Factor X to Xa. In this assay, the diluted test samples are incubated at 37°C with a mixture of FVIII deficient plasma substrate and aPTT reagent. Calcium chloride is added to the incubated mixture and clotting is initiated. An inverse relationship exists between the time (seconds) it takes for a clot to form and logarithm of the concentration of FVIII:C. Activity levels for unknown samples are interpolated by comparing the clotting times of various dilutions of test material with a curve constructed from a series of dilutions of standard material of known activity and are reported in International Units per mL (IU/mL). Also useful are chromogenic assays, which may be purchased commercially, including the assay under the trade name COATEST SP FVIII (available from Chromogenix

AB, Molndal, Sweden). The chromogenic assay method consists of two consecutive steps where the intensity of color is proportional to the FVIII activity. In the first step, Factor X is activated to FXa by FIXa with its cofactor, FVIIIa, in the presence of optimal amounts of calcium ions and phospholipids. Excess amounts of Factor X are present such that the rate of activation of Factor X is solely dependent on the amount of FVIII. In the second step, Factor Xa hydrolyzes the chromogenic substrate to yield a chromophore and the color intensity is read photometrically at 405 nm. Potency of an unknown is calculated and the validity of the assay is checked with the slope-ratio statistical method. Activity is reported in International Units per mL (IU/mL).

[0038] Freeze-drying, freezing, lyophilizing: "Freeze-drying," unless otherwise indicated by the context in which it appears, shall be used to denote the portion of a lyophilization process in which the temperature of a pharmaceutical preparation is raised in the primary and secondary drying phases in order to drive water out of the preparation. The "freezing" steps of a lyophilization process are those steps which occur prior to the primary and secondary drying stages. "Lyophilizing," unless otherwise indicated, shall refer to the entire process of lyophilization, including both the freezing steps and the freeze-drying steps.

[0039] Within certain aspects of the present disclosure, formulations comprising rFVIII and BDD-rFVIII, including formulations comprising PEGylated FVIII and BDD-rFVIII, can be lyophilized according to methodology known in the art. For example, U.S. Patent Nos. 5,399,670 and 5,763,401 describe methodology for producing lyophilized FVIII formulations of enhanced solubility, which methodology may be employed to lyophilize the formulations described herein. The lyophilization process has a freezing phase, a primary drying phase, and a secondary drying phase. In the freezing phase, there is an annealing step. The freezing phase is performed at temperature not higher than -40°C, the annealing step occurs at temperature not higher than -15°C, the primary drying is performed at temperature not higher than 0°C, and the secondary drying is done at temperature not higher than 30°C. Once the set temperature is reached for the freezing temperature, annealing temperature, final freezing temperature, primary drying temperature, and secondary drying temperature, such temperature can be held for a reasonable time period as would be readily understood by one of skill in the art considering the particular protein sample involved, such as for one hour, two hours, three hours, or greater than three hours.

[0040] Anneal: The term "anneal" shall be used to indicate a step in the lyophilization process of a pharmaceutical preparation undergoing lyophilization, prior to the freeze-drying of the preparation, in which the temperature of the preparation is raised from a lower temperature to a higher temperature and then cooled again after a period of time.

[0041] Bulking agent: For the purposes of this application, bulking agents are those chemical entities which provide structure to the "cake" or residual solid mass of a pharmaceutical preparation after it has been lyophilized and which protect it against collapse. A crystallizable bulking agent shall mean a bulking agent as described herein which can be crystallized during lyophilization.

[0042] Surfactant: As used herein, the term "surfactant" includes "non-ionic surfactants" such as polysorbates including polysorbate 20 and polysorbate 80, polyoxamers including poloxamer 184 or 188, pluronic polyols (sold under the trade name PLURONIC, manufactured by the BASF Wyandotte Corporation), and other ethylene/polypropylene block polymers. Non-ionic surfactants stabilize the rFVIII during processing and storage by reducing interfacial interaction and prevent protein from adsorption. The use of non-ionic surfactants permits the formulations to be exposed to shear and surface stresses without causing denaturation of the rFVIII. The formulations disclosed herein include formulations having one or more non-ionic surfactant(s), exemplified herein are formulations having a polysorbate, such as polysorbate 20 (sold under the trade name TWEEN 20) or polysorbate 80 (sold under the trade name TWEEN 80).

[0043] Osmolality: As used herein, the term "osmolality" refers to a measure of solute concentration, defined as the number of osmoles of solute per kg of solvent. A desired level of osmolality can be achieved by the addition of one or more stabilizer such as a sugar or a sugar alcohol including mannitol, dextrose, glucose, trehalose, and/or sucrose. Additional stabilizers that are suitable for providing osmolality are described in references such as the handbook of Pharmaceutical Excipients (Fourth Edition, Royal Pharmaceutical Society of Great Britain, Science & Practice Publishers) or Remingtons: The Science and Practice of Pharmacy (Nineteenth Edition, Mack Publishing Company). Formulations described herein have an osmolality ranging from about 240 mOsm/kg to about 450 mOsm/kg, or about 750 mOsm/kg, or about 1000 mOsm/kg, or from about 270 mOsm/kg to about 425 mOsm/kg, or from about 300 mOsm/kg to about 410 mOsm/kg.

[0044] Whenever appropriate, terms used in the singular also will include the plural and vice versa. The use of "a" herein means "one or more" unless stated otherwise or where the use of "one or more" is clearly inappropriate. The use of "or" means "and/or" unless stated otherwise. The use of "comprise," "comprises," "comprising," "include," "includes," and "including" are interchangeable and not intended to be limiting. The term "such as" also is not intended to be limiting. For example, the term "including" shall mean "including, but not limited to." Furthermore, where the description of one or more embodiments uses the term "comprising," those skilled in the art would understand that, in some specific instances, the embodiment or embodiments can be alternatively described using the language "consisting essentially of" and/or "consisting of."

[0045] As used herein, the term "about" refers to +/- 10% of the unit value provided. As used herein, the term "substantially" refers to the qualitative condition of exhibiting a total or approximate degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, achieve or avoid an absolute result because of the many variables that affect testing, production, and storage of biological and chemical compositions and materials, and because of the inherent error in the instruments and equipment used in the testing, production, and storage of biological and chemical compositions and materials. The term substantially is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0046] The formulations of the invention described herein may be described in terms of the component concentrations by weight, such as by weight percent, or by molarity. It is to be understood that the invention also encompasses lyophilized preparations of these formulations that when reconstituted in suitable diluent, such as saline or water, for administration or storage have the concentrations reported. Ranges herein include the endpoints of the range.

[0047] Unless otherwise noted, percentage terms express weight/volume percentages and temperatures are in the Celsius scale.

COMPOSITION COMPONENTS

[0048] The FVIII compositions of the present invention may include stabilizing agents, buffering agents, sodium chloride, calcium salts, and, advantageously, other excipients. These excipients have been chosen in order to maximize the stability of FVIII in lyophilized preparations and/or in liquid preparations.

[0049] The bulking agents used in the present compositions are preferably selected from the group consisting of mannitol, glycine, and alanine. Mannitol, glycine, or alanine may be present in an amount of 1-5%, 2-3%, and 2.2-2.6%. Glycine may be the chosen bulking agent. Compositions are envisioned that do not contain a bulking agent.

[0050] The stabilizing agents used in the present compositions are selected from the group consisting of sugars or sugar alcohols, including without limitation sucrose, mannitol, dextrose, glucose and trehalose. These agents are present in the compositions in an amount of between 0.5-10%, 1-8%, 2-7%, 3-6%, 4-5%, 1-5%, 1-4%, 1-3%, or 1-2%. In compositions containing a bulking agent, sucrose is the preferred stabilizing agent in an amount of between 1-3%. In compositions lacking a bulking agent, sucrose or trehalose may be chosen as the stabilizing agent in an amount of about 8%. These sugars or sugar alcohols also function as cryo-protective agents.

[0051] In addition, buffers are present in certain of the inventive compositions. Buffers may be useful, for example, in FVIII formulations that are undergoing lyophilization, because it is believed that FVIII can be adversely affected by pH shifts during lyophilization. The buffering agents can be any physiologically acceptable chemical entity or combination of chemical entities which have the capacity to act as buffers, including histidine and MOPS (3-(N-morpholino) propanesulfonic acid). Histidine may be the chosen buffering agent in an amount of about 20 mM.

[0052] In order to preserve the activity of FVIII, the compositions of the present invention may also include calcium or another divalent cation able to interact with FVIII and maintain its activity, presumably by maintaining the association of the heavy and light chains of FVIII. Between 1 mM and 5 mM of a calcium salt can be used. The calcium salt can be calcium chloride, but can also be other calcium salts such as calcium gluconate, calcium glubionate, or calcium gluceptate. The FVIII compositions of the present invention also may include a surfactant, particularly a nonionic surfactant chosen from the group consisting of polysorbate 20 and polysorbate 80, polyoxamers including poloxamer 184 or 188, pluronic polyols (sold under the trade name PLURONIC, manufactured by the BASF Wyandotte Corporation), and other ethylene/polypropylene block polymers. The surfactant can be polysorbate 80 in an amount of about 80 ppm.

[0053] The FVIII used in the present compositions may be covalently attached to a biocompatible polymer, such as PEG. As used herein, the terms “polyethylene glycol” or “PEG” are interchangeable and include any water-soluble poly(ethylene oxide). PEG includes the following structure "--$(OCH_2CH_2)_n$--" where (n) is 2 to 4000. As used herein, PEG also includes "--CH_2CH_2--$O(CH_2CH_2O)_n$--CH_2CH_2--" and "--$(OCH_2CH_2)_nO$--," depending upon whether or not the terminal oxygens have been displaced. The term "PEG" includes structures having various terminal or "end capping" groups, such as without limitation a hydroxyl or a C_{1-20} alkoxy group such as methoxy. The term "PEG" also means a polymer that comprises a majority, that is to say, greater than 50%, of $--OCH_2CH_2--$repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as branched, linear, forked, and multifunctional. As used herein, the term "PEGylation" refers to a process whereby a polyethylene glycol (PEG) is covalently attached to a molecule such as a protein. When a functional group such as a biocompatible polymer is described as activated, the functional group reacts readily with an electrophile or a nucleophile on another molecule.

[0054] The biocompatible polymer used in the conjugates disclosed herein may be any of the polymers discussed herein or known in the art. The biocompatible polymer is selected to provide the desired improvement in pharmacokinetics. For example, the identity, size and structure of the polymer is selected so as to improve the circulation half-life of FVIII or decrease the antigenicity of FVIII without an unacceptable decrease in activity. The polymer can include PEG. For example, the polymer can be a polyethylene glycol terminally capped with an end-capping moiety such as hydroxyl, alkoxy, substituted alkoxy, alkenoxy, substituted alkenoxy, alkynoxy, substituted alkynoxy, aryloxy and substituted aryloxy. In some embodiments, the polymer can include methoxypolyethylene glycol such as methoxypolyethylene glycol having a size range from 3 kD to 200 kD.

[0055] The polymer can have a reactive moiety. For example, the polymer can have a sulfhydryl reactive moiety that can react with a free cysteine on a functional FVIII polypeptide to form a covalent linkage. Such sulfhydryl reactive moieties include thiol, triflate, tresylate, aziridine, oxirane, S-pyridyl, or maleimide moieties. The polymer can be linear and include a "cap" at one terminus that is not strongly reactive towards sulfhydryls (such as methoxy) and a sulfhydryl reactive moiety at the other terminus. The conjugate can include PEG-maleimide having a size range from 5 kD to 64 kD. Alternatively, the polymer can have an amine reactive moiety such as succinimidyl propionate, succinimidyl butanoate, benzotriazole carbonate, hydroxysuccinimide, aldehyde such as propionaldehyde, butryaldehyde, acetal, piperidone, methylketone, etc. (*see, e.g.* U.S. Patent 7,199,223 (Bossard)).

[0056] The FVIII molecule may be conjugated to a biocompatible polymer via conjugation of the polymer to the carbohydrate moieties of FVIII. *See* US Pat. App. Pub. 20110112028 (Turecek et al.). A FVIII molecule may be conjugated to a water-soluble polymer by conjugating a water soluble polymer to an oxidized carbohydrate moiety of FVIII. The water soluble polymer in some embodiments is selected from the group consisting of PEG, polysialic acid ("PSA") and dextran. In still another aspect, the activated water soluble polymer is selected from the group consisting of PEG-hydrazide, PSA-hydrazine and aldehyde-activated dextran. In another aspect of the invention, the carbohydrate moiety is oxidized by incubation in a buffer comprising $NaIO_4$.

[0057] Suitable FVIII proteins to be used in the present invention have homology to specific known amino acid sequences. For example, suitable FVIII variants for use in the present invention are variants that have at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to known FVIII amino acid sequences, for example to the amino acid sequence of full-length FVIII (SEQ ID NO: 1) or that of BDD-FVIII (SEQ ID NO: 3). Also useful in the invention are genetic variants having defined sequence differences from a known FVIII sequence, such as FVIII molecules that comprise an amino acid sequence having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 1-10, 1-5, 2-6 or 3-8 differences in amino acid sequence when compared to a native, known, or control sequence, such as the amino acid sequences of full-length FVIII (SEQ ID NO: 1) or BDD-SQ (SEQ ID NO: 3). Allelic variants are also useful in the present invention. Examples of allelic variants of FVIII are those disclosed in U.S. Patent Application Pub. No. 2010/0256062 (Howard et al.); Howard et al., "*African-Americans Express Multiple Haplotypic Forms of the Wildtype Factor VIII (FVIII) Protein: A Possible Role for Pharmacogenetics in FVIII Inhibitor Development?*" Blood, Vol. 104, 2004, Abstract 384; and Viel, K.R. et al., "*Inhibitors of Factor VIII in Black Patients with Hemophilia,*" The New England Journal of Medicine, Vol. 360, 2009, pp. 1618-27. Allelic variants include those with amino acid substitutions such as histidine for arginine at position 484 (R484H), glycine for arginine at position 776 (R776G), glutamic acid for aspartic acid at position 1241 (D1241E), and valine for methionine at position 2238 (M2238V). The numbering systems used to designate the amino acid substitutions are based on SEQ ID NO: 2 herein.

[0058] Methods of alignment of nucleotide and amino acid sequences for comparison are well known in the art. Alignments for the present invention may be measured using a suitable method, including by using the local homology algorithm (BESTFIT) of Smith and Waterman, Adv. Appl. Math 2:482 (1981), which may conduct optimal alignment of sequences for comparison; by using the homology alignment algorithm (GAP) of Needleman and Wunsch, J. Mol. Biol. 48:443-53 (1970); or by using the search for similarity method (Tfasta and Fasta) of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988). The alignments may be performed by using computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by

Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG® programs (Accelrys, Inc., San Diego, CA).). The CLUSTAL program is well described by Higgins and Sharp, Gene 73:237-44 (1988); Higgins and Sharp, CABIOS 5:151-3 (1989); Corpet et al., Nucleic Acids Res. 16:10881-90 (1988); Huang et al., Computer Applications in the Biosciences 8:155-65 (1992), and Pearson et al., Meth. Mol. Biol. 24:307-31 (1994). One program to use for optimal global alignment of multiple sequences is PileUp (Feng and Doolittle, J. Mol. Evol., 25:351-60 (1987)) which is similar to the method described by Higgins and Sharp, CABIOS 5:151-53 (1989). The BLAST family of programs can be used for database similarity searches, such as for identifying other suitable FVIII molecules. See CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Chapter 19, Ausubel et al., eds., Greene Publishing and Wiley-Interscience, New York (1995).

[0059] It is believed that the B-domain of FVIII is dispensable for activity, as discussed above. In certain embodiments, the FVIII used in the invention may have all or some of the B-domain deleted. Accordingly, the present invention applies to FVIII variants or nucleotide sequences encoding such variants that comprise an amino acid sequence or encode an amino acid sequence having at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to amino acids 1-740 of the full-length FVIII (SEQ ID NO: 1) and at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to amino acids 1689-2351 of the full-length FVIII (SEQ ID NO: 1). Alternatively, the present invention applies to FVIII variants or nucleotide sequences encoding such variants that comprise an amino acid sequence or encode an amino acid sequence having at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the full-length of amino acid sequence SEQ ID NO: 1.

[0060] In certain embodiments of the invention, the FVIII may be the result of site-directed mutation, such as to create a binding site on FVIII to covalently attach a biocompatible polymer such as PEG. Site-directed mutation of a nucleotide sequence encoding polypeptide having FVIII activity may occur by any method known in the art. Methods include mutagenesis to introduce a cysteine codon at the site chosen for covalent attachment of the polymer. This may be accomplished using a commercially available site-directed mutagenesis kit such as the STRATAGENE CQUICKCHANGE II site-directed mutagenesis kit, the CLONETECH TRANSFORMER site-directed mutagenesis kit no. K1600-1, the INVITROGEN GENTAYLOR site-directed mutagenesis system no. 12397014, the PROMEGA ALTERED SITES II in vitro mutagenesis system kit no. Q6210, or the TAKARA MIRUS BIO LA PCR mutagenesis kit no. TAK RR016. Conjugates described herein may be prepared by first replacing the codon for one or more amino acids on the surface of the functional FVIII polypeptide with a codon for cysteine, producing the cysteine mutant in a recombinant expression system, reacting the mutant with a cysteine-specific polymer reagent, and purifying the mutein. In this system, the addition of a polymer at the cysteine site can be accomplished through a maleimide active functionality on the polymer. *See, e.g.,* U.S. Patent 7,632,921 (Pan et al.).

[0061] The amount of sulfhydryl reactive polymer used should be at least equimolar to the molar amount of cysteines to be derivatized and can be present in excess. A 5-fold or a 10-fold molar excess of sulfhydryl reactive polymer can be used. Other conditions useful for covalent attachment are within the skill of those in the art.

[0062] The predefined site for covalent binding of the polymer, *e.g.*, PEG, can be selected from sites exposed on the surface of the rFVIII or BDD rFVIII polypeptide that are not involved in FVIII activity or involved in other mechanisms that stabilize FVIII *in vivo*, such as binding to vWF. Such sites are also best selected from those sites known to be involved in mechanisms by which FVIII is deactivated or cleared from circulation. Sites for substituting an amino acid with a cysteine include an amino acid residue in or near a binding site for (a) low density lipoprotein receptor related protein, (b) a heparin sulphate proteoglycan, (c) low density lipoprotein receptor and/or (d) FVIII inhibitory antibodies. By "in or near a binding site" means a residue that is sufficiently close to a binding site such that covalent attachment of a biocompatible polymer to the site would result in steric hindrance of the binding site. Such a site is expected to be within 20 Á of a binding site, for example.

[0063] The biocompatible polymer can be covalently attached to the rFVIII or BDD rFVIII polypeptide, or mutant variant thereof, at one or more of the FVIII amino acid positions 81, 129, 377, 378, 468, 487, 491, 504, 556, 570, 711, 1648, 1795, 1796, 1803, 1804, 1808, 1810, 1864, 1903, 1911, 2091, 2118 and 2284. One or more sites, such as one or two, on the functional FVIII polypeptide may be the predefined sites for polymer attachment. In particular embodiments, the polypeptide is mono-PEGylated or diPEGylated, meaning one PEG or two PEG molecules are attached to each FVIII, respectively.

[0064] Site directed PEGylation of a FVIII mutant can also be achieved by: (a) expressing a site-directed FVIII mutant wherein the mutant has a cysteine replacement for an amino acid residue on the exposed surface of the FVIII mutant and that cysteine is capped; (b) contacting the cysteine mutant with a reductant under conditions to mildly reduce the cysteine mutant and to release the cap; (c) removing the cap and the reductant from the cysteine mutant; and (d) after the removal of the reductant, treating the cysteine mutant with PEG comprising a sulfhydryl coupling moiety under conditions such that PEGylated FVIII mutein is produced. The sulfhydryl coupling moiety of the PEG is selected from the group consisting of thiol, triflate, tresylate, aziridine, oxirane, S-pyridyl and maleimide moieties, and can be maleimide.

[0065] In another embodiment a biocompatible polymer such as, e.g., PEG, is covalently attached through use of a polymer functionalized with an amine-specific functional group. The polymer may be functionalized with, for example, mPEG tresylate or mPEG succinimidyl succinate such that it is reactive at lysines on FVIII. The coupling can occur at random lysines on FVIII by adding activated mPEG in a solid state to a solution of FVIII and rotating at room temperature. The degree of modification may be loosely controlled by the level of excess activated mPEG used. Rostin et al., "*B-Domain Deleted Recombinant Coagulation Factor VIII Modified with Monomethoxy Polyethylene Glycol*," 11 Bioconjug. Chem., 2000, pp. 387-396. Further examples of PEGylation conditions and reagents are provided in U.S. Patent 7,199,223 (Bossard) and U.S. Patent 4,970,300 (Fulton). The present invention is also directed to methods for covalently attaching a biocompatible polymer to FVIII in which one of the liquid formulations of the invention is the solution in which the reaction occurs.

[0066] The present disclosure also provides methods for the treatment of hemophilia A in a patient, comprising the administration to the patient in need thereof a therapeutically effective amount of one or more formulations described herein. These formulations may be administrated to a patient via intravenous injection, subcutaneous injection, or through continuous infusion.

[0067] As used herein, the term "therapeutically effective amount" of a rFVIII formulation or a PEGylated rFVIII formulation refers to an amount of the formulation that provides therapeutic effect in an administration regimen to a patient in need thereof. For example, for replacement therapy for hemophilia A, an amount of between 10-30 IU/ kg body weight of recombinant full-length FVIII for intravenous injection is recommended. For prophylaxis in a child with hemophilia A, 25 IU/kg body weight of recombinant full-length FVIII for intravenous injection is recommended. Prior to surgery, 15-30 IU/kg (minor surgery) or 50 IU/kg (major surgery) of recombinant full-length FVIII for intravenous injection is recommended for a child with hemophilia A. Corresponding dosages for the various FVIII molecules used in the formulations of the invention can be determined by those of skill in the art. Preferably the therapeutic FVIII formulations of the invention are provided in single use dosages of 100, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, 3600, 3800, 4000, or 5000 IU, or in a range between any two of these dosages, i.e., in a range of from 100 to 250 IU, from 100 to 500 IU, from 1000 to 2000 IU, etc., inclusive of the endpoints. Because of their low viscosity, the presently disclosed rFVIII and PEG-rFVIII formulations can be conveniently processed via, for example, ultrafiltration and sterile filtration and can be administered to a patient via injection, including intravenous injection, subcutaneous injection, and continuous infusion.

[0068] The FVIII compositions described in this application can be lyophilized and reconstituted in the indicated concentrations. These FVIII compositions can also be reconstituted in more dilute form. For example, a preparation according the present invention which is lyophilized and/or normally reconstituted in 2 ml of solution can also be reconstituted in a larger volume of diluent, such as 5 ml. This is particularly appropriate when the FVIII preparation is being injected into a patient immediately, since in this case the FVIII is less likely to lose activity, which may occur more rapidly in more dilute solutions of FVIII.

EMBODIMENT 1

[0069] Recombinant FVIII is produced in the absence of plasma proteins that stabilize plasma-derived FVIII, such as von Willebrand factor (vWF). The absence of such stabilizing proteins makes rFVIII extremely labile. In addition, rFVIII is present at very low concentrations in therapeutic solutions (0.02 mg protein per ml for a therapeutic dose of 1000 IU BDD-SQ), which makes surface adsorption a cause for loss of activity.

[0070] One embodiment of the invention is a formulation of rFVIII, particularly BDD-rFVIII, and even more particularly BDD-rFVIII mutants with cross-linking between the domains, such as between the A1 and A2 or A3 domains. In one embodiment the formulation is of a FVIII having double cysteine mutations which cross-link the A2 and the A1 or the A3 domains, preferably the A2 and the A3 domains, such as through disulfide bridges as described in U.S. Patent 7,928,199 to Griffin et al. (issued Apr. 19, 2011), including without limitation mutants of FVIII, including mutants of BDD SQ (SEQ ID NO: 3), in which one or more cysteines have been introduced at one or more sites; such that at least one pair of cysteines creates a disulfide bond not found in wild type FVIII. In one embodiment, the mutant FVIII comprises at least one pair of recombinantly introduced cysteines, wherein the pair of cysteines replaces a pair of residues selected from the group consisting of Met 662 and Asp 1828, Ser 268 and Phe 673, Ile 312 and Pro 672, Ser 313 and Ala 644, Met 662 and Lys 1827, Tyr 664 and Thr 1826, Pro 264 and Gln 645, Arg 282 and Thr 522, Ser 285 and Phe 673, His 311 and Phe 673, Ser 314 and Ala 644, Ser 314 and Gln 645, Val 663 and Glu 1829, Asn 694 and Pro 1980, and Ser 695 and Glu 1844. Suitable FVIII molecules for the formulations of the present embodiment suffer the disadvantage of aggregating in solution and/or show a high propensity for precipitation. These disadvantages create problems preparing a stable therapeutic dosage. Also, if the FVIII molecules are to be further processed, such as by covalent attachment of a biocompatible polymer such as PEG, the FVIII molecules are preferably in solution to provide good processing, such as good yields upon PEGylation, which requires that the FVIII be in suspension or solution and not be aggregated. In one embodiment, the FVIII formulations of the present application contain sodium chloride or potassium chloride in an amount sufficient to reduce or abolish precipitation and/or aggregation and to provide stability.

[0071] Formulations of Embodiment 1 may be as follows. A rFVIII formulation comprising:

(a) a range of from about 0 mM to about 20 mM, from about 1mM to about 20mM, from about 1 mM to about 50 mM, from about 10 mM to about 50 mM, from about 10 mM to about 20mM, from about 10 mM to about 30mM, or from about 20 mM to about 50 mM histidine;

(b) a range of from about 0 mM to about 29 mM, from about 1 mM to about 29 mM, from about 1 mM to about 300 mM, from about 10 mM to about 30 mM, from about 10 mM to about 100 mM, from about 10 mM to about 200 mM, from about 10 mM to about 50 mM, from about 29 mM to about 58 mM, from about 34 mM to about 58 mM, from about 58 mM to about 100 mM, or from about 100 mM to about 300 mM, or an amount of about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, or about 300 mM of a sugar or sugar alcohol;

(c) a range of from about 1 mM to about 2 mM, from about 1 mM to about 2.5 mM, from about 1.5 mM to about 3.5 mM, or from about 1 mM to about 5 mM divalent cation such as a divalent calcium salt, including calcium chloride;

(d) a range of from about 150 mM to about 250 mM, from about 150 mM to about 220 mM, from about 150 mM to about 200 mM, from about 150 mM to about 190 mM, from about 170 mM to about 250 mM; from about 200 mM to about 220 mM, from about 170 mM to about 200 mM, from about 200 mM to about 250 mM, from about 170 mM to about 220 mM, from about 190 mM to about 220 mM, from about 210 mM to about 220 mM, from about 150 mM to about 180 mM, from about 150 mM to about 160 mM, or from about 220 mM to about 250 mM sodium chloride or potassium chloride;

(e) a range of from about 20 ppm to about 200 ppm, from about 20 ppm to about 50 ppm, from about 20 ppm to about 80 ppm, from about 50 ppm to about 80 ppm, from about 80 ppm to about 100 ppm, from about 80 ppm to about 200 ppm, from about 50 ppm to about 100 ppm, or from about 50 ppm to about 200 ppm of a non-ionic surfactant, or about 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 160, 170, 180, 190, 200, or 210 ppm of a non-ionic surfactant;

(f) a range of from about 0 mM to about 50 mM, from about 1 mM to about 50 mM, from about 50 mM to about 100 mM, from about 100 mM to about 150 mM, from about 150 mM to about 293 mM, from about 150 mM to about 400 mM, from about 200 mM to about 300 mM; from about 250 mM to about 300 mM, or from about 200 mM to about 400 mM glycine, or about 100, 200, 210, 230, 240, 250, 260, 270, 280, 290, 293, 295, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, or about 400 mM glycine; and

(g) a range of from about 100 IU/ml to about 5000 IU/ml, from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 100 IU/ml to about 4000 IU/ml, from about 100 IU/ml to about 1200 IU/ml, from about 250 IU/ml to about 5000 IU/ml, from about 250 IU/ml to about 1000 IU/ml, from about 250 IU/ml to about 2000 IU/ml, from about 250 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, from about 1000 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 4000 IU/ml, or from about 1000 IU/ml to about 5000 IU/ml, or about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3500, 3800, 4000, 4200, 4500, 4800, 5000, 5500, or 6000 IU/ml of a rFVIII selected from rFVIII, BDD-rFVIII, BDD-rFVIII mutants, and BDD-rFVIII mutants with cross-linking between FVIII domains,

wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 6.5, from about pH 6.0 to about pH 7.0, from about pH 6.0 to about pH 7.5, from about pH 6.5 to about pH 7.5, or from about pH 7.0 to about pH 7.5, or a pH of about pH 6.0, 6.5, 7.0, 7.1, 7.2, 7.3, 7.4 or about pH 7.5.

[0072] In another version of Embodiment 1, the invention pertains to a rFVIII formulation comprising:

(a) a range of from about 0 mM to about 20 mM, from about 10 mM to about 50 mM, or from about 10 mM to about 30mM histidine;

(b) a range of from about 1 mM to about 29 mM, from about 10 mM to about 30 mM, from about 10 mM to about 100 mM, from about 10 mM to about 200 mM, from about 10 mM to about 50 mM, from about 29 mM to about 58 mM, or from about 34 mM to about 58 mM, or an amount of about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 mM of a sugar or sugar alcohol;

(c) a range of from about 1 mM to about 2 mM, from about 1 mM to about 2.5 mM, from about 1.5 mM to about 3.5 mM, or from about 1 mM to about 5 mM divalent cation such as a divalent calcium salt, including calcium chloride;

(d) a range of from about 150 mM to about 200 mM, from about 150 mM to about 220 mM, from about 170 mM to about 250 mM sodium chloride or potassium chloride;

(e) a range of from about 20 ppm to about 80 ppm, from about 80 ppm to about 100 ppm, from about 50 ppm to about 100 ppm, or from about 50 ppm to about 200 ppm of a non-ionic surfactant, or about 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, or 120 ppm of a non-ionic surfactant;

(f) a range of from about 1 mM to about 50 mM, from about 150 mM to about 300 mM, from about 150 mM to about 400 mM, from about 200 mM to about 300 mM; or from about 250 mM to about 300 mM, or about 250, 260, 270, 280, 290, 293, 295, 300, 310, or about 320 mM glycine; and

(g) a range of from about 100 IU/ml to about 5000 IU/ml, from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 100 IU/ml to about 4000 IU/ml, from about 100 IU/ml to about 1200 IU/ml, from about 250 IU/ml to about 5000 IU/ml, from about 250 IU/ml to about 1000 IU/ml, from about 250 IU/ml to about 2000 IU/ml, from about 250 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, from about 1000 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 4000 IU/ml, or from about 1000 IU/ml to about 5000 IU/ml, or about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3500, 3800, 4000, 4200, 4500, 4600, 4800, 5000, 5500, or 6000 IU/ml of a rFVIII selected from rFVIII, BDD-rFVIII, BDD-rFVIII mutants, and BDD-rFVIII mutants with cross-linking between FVIII domains, wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5, from about pH 6.5 to about pH 7.5, or from about pH 7.0 to about pH 7.5, or a pH of about pH 6.0, 6.5, 7.0, 7.1, 7.2, 7.3, 7.4 or about pH 7.5.

[0073] In certain embodiments the sugar or sugar alcohol is sucrose and sodium chloride is present.

[0074] In another version of Embodiment 1, the invention pertains to a rFVIII formulation comprising:

(a) a range of from about 10 mM to about 30 mM histidine;

(b) a range of from about 10 mM to about 30 mM, from about 10 mM to about 100 mM, from about 10 mM to about 200 mM, from about 10 mM to about 50 mM, from about 29 mM to about 58 mM, or from about 34 mM to about 58 mM of a sugar or sugar alcohol;

(c) a range of from about 1 mM to about 2 mM, from about 1 mM to about 2.5 mM, from about 1.5 mM to about 3.5 mM, or from about 1 mM to about 5 mM of a divalent calcium salt, including calcium chloride;

(d) a range of from about 150 mM to about 220 mM, from about 170 mM to about 250 mM sodium chloride or potassium chloride;

(e) a range of from about 50 ppm to about 200 ppm of a non-ionic surfactant;

(f) a range of from about 1 mM to about 50 mM, from about 150 mM to about 300 mM, from about 150 mM to about 400 mM, from about 200 mM to about 300 mM; or from about 250 mM to about 300 mM glycine; and

(g) a range of from about 100 IU/ml to about 5000 IU/ml, from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 100 IU/ml to about 4000 IU/ml, from about 100 IU/ml to about 1200 IU/ml, from about 250 IU/ml to about 5000 IU/ml, from about 250 IU/ml to about 1000 IU/ml, from about 250 IU/ml to about 2000 IU/ml, from about 250 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, from about 1000 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 4000 IU/ml, or from about 1000 IU/ml to about 5000 IU/ml, or about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3500, 3800, 4000, 4200, 4500, 4600, 4800, 5000, 5500, or 6000 IU/ml of a rFVIII selected from rFVIII, BDD-rFVIII, BDD-rFVIII mutants, and BDD-rFVIII mutants with cross-linking between FVIII domains, wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5, from about pH 6.5

to about pH 7.5, or from about pH 7.0 to about pH 7.5, or a pH of about pH 6.0, 6.5, 7.0, 7.1, 7.2, 7.3, 7.4 or about pH 7.5.

[0075] In yet another version of Embodiment 1, the invention pertains to a rFVIII formulation comprising:

(a) a range of from about 10 mM to about 30 mM histidine;

(b) a range of from about 10 mM to about 50 mM of sucrose;

(c) a range of from about 1.5 mM to about 3.5 mM calcium chloride;

(d) a range of from about 150 mM to about 220 mM or from about 170 mM to about 220 mM sodium chloride;

(e) a range of from about 70 ppm to about 90 ppm of a non-ionic surfactant;

(f) a range of from about 200 mM to about 300 mM or from about 250 mM to about 300 mM glycine; and

(g) a range of from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 250 IU/ml to about 1000 IU/ml, from about 250 IU/ml to about 2000 IU/ml, from about 250 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, or from about 1000 IU/ml to about 3000 IU/ml of a rFVIII selected from BDD-rFVIII, BDD-rFVIII mutants, and BDD-rFVIII mutants with cross-linking between FVIII domains, wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5, from about pH 6.5 to about pH 7.5, or from about pH 7.0 to about pH 7.5, or a pH of about pH 6.0, 6.5, 7.0, 7.1, 7.2, 7.3, 7.4 or about pH 7.5.

[0076] The rFVIII formulations of embodiment 1 may optionally contain albumin, such as HSA. In certain embodiments, HSA is present at a range of from about 10 to about 50 mg/mL, from about 15 to about 30 mg/mL, from about 20 to about 30 mg/mL or from about 25 to about 30 mg/mL.

EMBODIMENT 2

[0077] During covalent addition of a biocompatible polymer to FVIII it was observed that buffer components may interfere with the covalent addition. For example, when FVIII was covalently coupled to PEG using PEG functionalized to have an amine-reactive group that would add at lysine residues, amine-containing components in the reaction buffer were observed to interfere with the reaction. Accordingly, the present invention includes improved liquid FVIII formulations or buffers in which the polymer addition reaction to FVIII may occur. In one version of Embodiment 2, the liquid FVIII formulations do not comprise, or comprises less than 10% by weight, or less than 5% by weight, or less than 1% by weight, or less than 0.5% by weight or only a trace amount of components with primary or secondary amine groups, other than FVIII. The inventive FVIII formulations of this embodiment include formulations that avoid the use of histidine and glycine. Histidine and glycine contain amines that may interfere with the PEGylation process.

[0078] One version of Embodiment 2 of the invention is a formulation of rFVIII having buffer capacity at pH 6-7 that does not form an insoluble complex or chelate with calcium chloride (an important rFVIII stabilizer) and does not contain components with primary or secondary amine groups, or contains such components at a weight percent of 10% or less, 5% or less, 1% or less, or in trace amounts. This formulation may include MOPS in a range of from 10 mM to 100 mM, in a range of from 10 mM to 70 mM, in a range of from 10 mM to 50 mM, in a range of from 10 mM to 40 mM, in a range of from 10 mM to 30 mM, in a range of from 12 mM to 30 mM, in a range of from 14 mM to 30 mM, in a range of from 16 mM to 30 mM, in a range of from 18 mM to 30 mM, in a range of from 20 mM to 28 mM, in a range of from 12 mM to 28 mM, in a range of from 12 mM to 26 mM, in a range of from 12 mM to 24 mM, in a range of from 12 mM to 22 mM, in a range of from 14 mM to 22 mM, or in a range of from 18 mM to 22 mM, or may contain about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mM MOPS. This formulation includes rFVIII in a range of from about 100 IU/ml to about 1000 IU/ml, from about 100 IU/ml to about 500 IU/ml, from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 2000 IU/ml, from about 500 IU/ml to about 2500 IU/ml, from about 500 IU/ml to about 1200 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 1500 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, from about 1000 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2500 IU/ml, from about 1000 IU/ml to about 1500 IU/ml, from about 1000 IU/ml to about 6000 IU/ml, or from about 1000 IU/ml to about 5000 IU/ml or about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3500, 3800, 4000, 4200, 4500, 4800, 5000, 5500, or 6000 IU/ml of rFVIII. It is also possible that the invention may be used with rFVIII formulations having higher activity than 6000 IU/ml.

[0079] In one version of Embodiment 2, the rFVIII formulation comprises FVIII or BDD that is recombinantly produced. In another version of Embodiment 2, the formulation comprises recombinantly produced full-length FVIII, such as FVIII comprising the amino acid sequence of SEQ ID NO: 1 or an allelic variant thereof. In another version of Embodiment 2, the formulation comprises a mutant of BDD or a mutant of FL-FVIII.

[0080] This formulation may also include a sugar or a sugar alcohol such as sucrose in a range of from 0.5% to 10%, in a range of from 0.6% to 10%, in a range of from 0.7% to 10%, in a range of from 0.8% to 10%, in a range of from 0.9% to 10%, in a range of from 1.0% to 10%, in a range of from 0.6% to 5%, in a range of from 0.6% to 2.5%, in a range of from 0.6% to 2.0%, in a range of from 0.6% to 1.5%, in a range of from 0.6% to 1.2%, in a range of from 0.8% to 1.2%, in a range of from 0.9% to 1.2%, or in a range of from 0.9% to 1.1% by weight, or at about 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0% by weight. This formulation may also include a divalent cation such as a calcium salt, such as calcium chloride, in a range of from 0.5 mM to 20 mM, in a range of from 1 mM to 10 mM, in a range of from 1 mM to 5 mM, in a range of from 1.5 mM to 5 mM, in a range of from 2 mM to 5 mM, in a range of from 2.5 mM to 5 mM, in a range of from 3 mM to 5 mM, in a range of from 3.5 mM to 5 mM, in a range of from 4 mM to 5 mM, in a range of from 1.5 mM to 4.5 mM, in a range of from 1.5 mM to 4 mM, in a range of from 1.5 mM to 3.5 mM, in a range of from 1.5 mM to 3 mM, in a range of from 1.5 mM to 2.5 mM, in a range of from 2 mM to 3 mM, in a range of from 2.2 mM to 2.8 mM, or in a range of from 2.4 mM to 2.6 mM. This formulation may also include sodium chloride or potassium chloride in a range of from 10 mM to 100 mM, in a range of from 10 mM to 70 mM, in a range of from 10 mM to 50 mM, in a range of from 15 mM to 50 mM, in a range of from 20 mM to 50 mM, in a range of from 25 mM to 50 mM, in a range of from 30 mM to 50 mM, in a range of from 15 mM to 45 mM, in a range of from 15 mM to 40 mM, in a range of from 15 mM to 35 mM, in a range of from 20 mM to 45 mM, in a range of from 20 mM to 40 mM, in a range of from 25 mM to 40 mM, in a range of from 25 mM to 35 mM, in a range of from 25 mM to 30 mM, or in a range of from 30 mM to 35 mM. This formulation may also include a non-ionic surfactant such as polysorbate 20 or polysorbate 80 in a range of from 50 to 150 ppm, in a range of from 60 ppm to 150 ppm, in a range of from 70 ppm to 150 ppm, in a range of from 80 ppm to 150 ppm, in a range of from 60 ppm to 140 ppm, in a range of from 60 ppm to 130 ppm, in a range of from 60 ppm to 120 ppm, in a range of from 60 ppm to 110 ppm, in a range of from 60 ppm to 100 ppm, in a range of from 60 ppm to 90 ppm, in a range of from 70 ppm to 90 ppm, in a range of from 70 ppm to 80 ppm, and in a range of from 80 ppm to 90 ppm. This composition provides acceptable stability to rFVIII in solution, and can be used as a reaction buffer during the conjugation of a polymer to FVIII using a polymer functionalized to be active at amine residues.

[0081] In one version of Embodiment 2, the invention is related to a rFVIII formulation comprising

(a) MOPS in a range of from 12 mM to 28 mM, in a range of from 12 mM to 22 mM, or in a range of from 18 mM to 22 mM;

(b) FVIII in a range of from 100 IU/ml to 3000 IU/ml, or in a range of from 1000-1500 IU/ml;

(c) sucrose in a range of from 0.5% to 5%, in a range of from 0.6% to 2.5%, or in a range of from 0.9% to 1.1%;

(d) sodium chloride or potassium chloride in a range of from 10 mM to 50 mM, in a range of from 15 mM to 35 mM, or in a range of from 25 mM to 35 mM;

(e) a divalent calcium salt, such as calcium chloride, in a range of from 1 mM to 5 mM, in a range of from 1.5 mM to 3.5 mM, or in a range of from 2.4 mM to 2.6 mM; and

(f) non-ionic surfactant such as polysorbate 20 or polysorbate 80 in a range of from 60 ppm to 100 ppm, or in a range of from 70 ppm to 90 ppm;

wherein the rFVIII formulation contains less than 10%, less than 5%, less than 1%, less than 0.5%, or less than a trace level, or is essentially free, of a component having a primary or secondary amine group.

[0082] The invention also is directed to a method of conjugating an amine-reactive biocompatible polymer, such as an amine-reactive PEG, to FVIII comprising suspending or dissolving the FVIII in a rFVIII formulation of Embodiment 2, adding the amine-reactive polymer, and incubating the resulting mixture under conditions of time and temperature such that conjugation occurs. Such conditions preferably are at about ambient temperature. The polymer may be added at excess molar amounts (1-100-fold excess) over the FVIII. The polymer and FVIII may be conjugated by incubation together for several hours with rotation or stirring.

[0083] Although the above formulations of Embodiment 2 have been shown to be useful as reaction buffers during polymer addition involving amine-reactive functional groups, it is envisioned that the formulations are also useful in other contexts outside of such reactions and therefore that the formulations may be used when stable FVIII formulations are required.

EMBODIMENT 3

[0084] In Embodiment 3, the rFVIII formulations comprise NaCl, MOPS, a divalent calcium ion or another divalent cation, and optionally a nonionic surfactant and/or optionally a sugar or a sugar alcohol. The formulations of Embodiment 3 in particular are shown to provide storage without aggregation of FVIII molecules that are not conjugated to a biocompatible polymer, such as FVIII not covalently attached to PEG and not covalently attached to any polymer other than glycans present in wild-type FVIII. The formulations of Embodiment 3 are particularly suitable for non-PEGylated BDD. As used herein, "nonconjugated FVIII" refers to FVIII that is not conjugated to a polymer other than to a glycan associated with a native mammalian glycosylation pattern resulting from the host cell in which the FVIII is produced. For example, "nonconjugated FVIII" includes wild type human FVIII that is recombinantly produced in a mammalian host cell such as a BHK cell or a CHO cell such as the marketed products KOGENATE ® and RECOMBINATE® FVIII.

[0085] One version of Embodiment 3 of the compositions described herein is a composition that provides stability for FVIII and contains sodium chloride in a range of from 150 mM to 300 mM, from 150 mM to 275 mM, from 150 mM to 250 mM, from 150 mM to 225 mM, from 150 mM to 200 mM, from 150 mM to 175 mM, from 175 mM to 300 mM, from 175 mM to 275 mM, from 175 mM to 250 mM, from 175 mM to 225 mM, from 175 mM to 200 mM, from 175 mM to 190 mM; from 200 mM to 300 mM, from 200 mM to 275 mM, from 200 mM to 250 mM, from 200 mM to 225 mM, from 200 mM to 210 mM, from 250 mM to 300 mM; or about 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, or 300 mM. The compositions also include MOPS buffer in a range of from 10 mM to 100 mM, in a range of from 10 mM to 60 mM, in a range of from 10 mM to 50 mM, in a range of from 10 mM to 40 mM, in a range of from 10 mM to 30 mM, in a range of from 12 mM to 30 mM, in a range of from 14 mM to 30 mM, in a range of from 16 mM to 30 mM, in a range of from 18 mM to 30 mM, in a range of from 12 mM to 28 mM, in a range of from 12 mM to 26 mM, in a range of from 12 mM to 24 mM, in a range of from 16 mM to 24 mM, in a range of from 18 mM to 24 mM, in a range of from 20 mM to 24 mM, or in a range of from 18 mM to 22 mM. The compositions also include a divalent cation such as calcium chloride in a range of from 1 mM to 20 mM, in a range of from 5 mM to 10 mM, in a range of from 1 mM to 30 mM, in a range of from 6 mM to 30 mM, in a range of from 7 mM to 30 mM, in a range of from 8 mM to 30 mM, in a range of from 5 mM to 20 mM, in a range of from 5 mM to 25 mM, or in a range of from 9 mM to 12 mM. The amount of rFVIII present in the formulations of Embodiment 3 may be the same as the amount provided in Embodiment 1.

[0086] The compositions may also include a sugar or sugar alcohol such as sucrose in a range of from 0.5% to 10%, in a range of from 0.6% to 10%, in a range of from 0.7% to

10%, in a range of from 0.8% to 10%, in a range of from 0.9% to 10%, in a range of from 1.0% to 10%, in a range of from 0.5% to 5%, in a range of from 0.6% to 5%, in a range of from 0.7% to 5%, in a range of from 0.8% to 5%, in a range of from 0.9% to 5%, in a range of from 1.0% to 5%, in a range of from 0.5% to 2.5%, in a range of from 0.6% to 2.5%, in a range of from 0.5% to 2.0%, in a range of from 0.5% to 1.5%, in a range of from 0.6% to 1.2%, in a range of from 0.8% to 1.2%, in a range of from 0.9% to 1.2%, or in a range of from 0.9% to 1.1%. The compositions may also include a non-ionic surfactant such as polysorbate 80 in a range of from 20 ppm to 250 ppm, in a range of from 50 ppm to 250 ppm, in a range of from 50 ppm to 150 ppm, in a range of from 60 ppm to 150 ppm, in a range of from 70 ppm to 150 ppm, in a range of from 80 ppm to 150 ppm, in a range of from 60 ppm to 140 ppm, in a range of from 60 ppm to 130 ppm, in a range of from 60 ppm to 120 ppm, in a range of from 60 ppm to 110 ppm, in a range of from 60 ppm to 100 ppm, in a range of from 70 ppm to 110 ppm, in a range of from 70 ppm to 105 ppm, in a range of from 70 ppm to 100 ppm, in a range of from 80 ppm to 100 ppm, or in a range of from 90 ppm to 110 ppm.

[0087] In certain versions of Embodiment 3 the FVIII formulation is free of histidine and/or (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) ("HEPES") and/or albumin, or contains less than 0.1%, less than 0.5%, less than 0.8%, less than 1.0%, or less than 5.0% by weight of histidine, and/or HEPES, and/or albumin. One version of Embodiment 3 is a FVIII formulation essentially free of histidine, HEPES and albumin.

EMBODIMENT 4

[0088] Polymer-conjugated FVIII, such as PEGylated FVIII, may be more hydrophilic than the corresponding unconjugated FVIII. Accordingly, formulations for conjugated FVIII such as PEGylated FVIII may require different components than those identified for unconjugated FVIII. Applicants prepared a PEGylated FVIII in a buffer that contained elevated levels of NaCl (200 mM). Such elevated levels of sodium chloride were observed to impose difficulties during lyophilization. Applicants discovered compositions of the present invention for polymer-conjugated FVIII that avoid undesirably high levels of NaCl, avoid the formation of aggregates and substantially retain potency of FVIII when stored over six days at ambient temperature. The present application provides the unexpected result that sodium chloride concentration can be reduced from 200 mM to 50 mM and still achieve potency of the rFVIII after storage at ambient temperature. In Embodiment 4, the rFVIII formulations comprise a buffer such as histidine or MOPS, NaCl, a divalent calcium ion or another divalent cation, and optionally a nonionic surfactant and/or optionally a sugar or a sugar alcohol. The formulations of Embodiment 4 in particular are shown to provide storage without aggregation of FVIII molecules that are conjugated to a biocompatible polymer, particularly a hydrophilic biocompatible polymer such as PEG. As used herein, "conjugated FVIII" refers to FVIII that is conjugated to a polymer other than to a glycan associated with a native mammalian glycosylation pattern resulting from the host cell in which the FVIII is produced.

[0089] One version of Embodiment 4 described herein is a rFVIII composition that contains sodium chloride in a range of from 25 mM to 200 mM, in a range of from 25 mM to 175 mM, in a range of from 25 mM to 150 mM, in a range of from 25 mM to 125 mM, in a range of from 25 mM to 100 mM, in a range of from 25 mM to 75 mM, in a range of from 25 mM to 50 mM, in a range of from 40 mM to 55 mM, in a range of from 25 mM to 35 mM, in a range of from 25 mM to 30 mM, in a range of from 30 mM to 60 mM, in a range of from 50 mM to 200 mM, in a range of from 50 mM to 175 mM, in a range of from 50 mM to 150 mM, in a range of from 50 mM to 125 mM, in a range of from 50 mM to 100 mM, or in a range of from 50 mM to 75 mM. If the formulation is to be subjected to lyophilization, then lower levels of NaCl from those provided above are preferred. The amount of rFVIII present in the formulations of Embodiment 4 may be the same as the amount provided in Embodiment 1.

[0090] The compositions also include a buffering agent such as histidine or MOPS buffer in a range of from 10 mM to 100 mM, in a range of from 10 mM to 60 mM, in a range of from 10 mM to 50 mM, in a range of from 10 mM to 40 mM, in a range of from 10 mM to 30 mM, in a range of from 12 mM to 30 mM, in a range of from 14 mM to 30 mM, in a range of from 16 mM to 30 mM, in a range of from 18 mM to 30 mM, in a range of from 12 mM to 28 mM, in a range of from 12 mM to 26 mM, in a range of from 12 mM to 24 mM, in a range of from 16 mM to 24 mM, in a range of from 18 mM to 24 mM, in a range of from 20 mM to 24 mM, or in a range of from 18 mM to 22 mM. The compositions also include a divalent cation such as calcium chloride in a range of from 1 mM to 20 mM, in a range of from 5 mM to 10 mM, in a range of from 1 mM to 30 mM, in a range of from 6 mM to 30 mM, in a range of from 7 mM to 30 mM, in a range of from 8 mM to 30 mM, in a range of from 5 mM to 20 mM, in a range of from 5 mM to 25 mM, or in a range of from 9 mM to 12 mM.

[0091] The compositions may also include a sugar or sugar alcohol such as sucrose or trehalose in a range of from 0.5% to 10%, in a range of from 0.6% to 10%, in a range of from 0.7% to 10%, in a range of from 0.8% to 10%, in a range of from 0.9% to 10%, in a range of from 1.0% to 10%, in a range of from 0.5% to 5%, in a range of from 0.6% to 5%, in a range of from 0.7% to 5%, in a range of from 0.8% to 5%, in a range of from 0.9% to 5%, in a range of from 1.0% to 5%, in a range of from 0.5% to 2.5%, in a range of from 0.6% to 2.5%, in a range of from 0.5% to 2.0%, in a range of from 0.5% to 1.5%, in a range of from 0.6% to 1.2%, in a range of from 0.8% to 1.2%, in a range of from 0.9% to 1.2%, or in a range of from 0.9% to 1.1%. The compositions may also include a non-ionic surfactant such as polysorbate 80 in a range of from 20 ppm to 250 ppm, in a range of from 50 ppm to 250 ppm, in a range of from 50 ppm to 150 ppm, in a range of from 60 ppm to 150 ppm, in a range of from 70 ppm to 150 ppm, in a range of from 80 ppm to 150 ppm, in a range of from 60 ppm to 140 ppm, in a range of from 60 ppm to 130 ppm, in a range of from 60 ppm to 120 ppm, in a range of from 60 ppm to 110 ppm, in a range of from 60 ppm to 100 ppm, in a range of from 70 ppm to 110 ppm, in a range of from 70 ppm to 105 ppm, in a range of from 70 ppm to 100 ppm, in a range of from 80 ppm to 100 ppm, or in a range of from 90 ppm to 110 ppm.

[0092] In certain versions of Embodiment 4 the FVIII formulation is free of histidine and/or HEPES and/or albumin, or contains less than 0.1%, less than 0.5%, less than 0.8%, less than 1.0%, or less than 5.0% by weight of histidine, and/or HEPES, and/or albumin. One version of Embodiment 3 is a FVIII formulation essentially free of histidine, HEPES and albumin.

EMBODIMENT 5

[0093] The invention also includes rFVIII formulations suitable for lyophilization. In certain versions of this embodiment, the FVIII formulations are particularly suitable for lyophilization of conjugated FVIII, PEGylated FVIII, PEGylated BDD, or PEGylated BDD mutants. The rFVIII formulations of this embodiment comprise (1) sodium chloride, and/or sucrose, and/or trehalose, (2) glycine and/or sucrose and/or trehalose; and (3) a divalent cation such as calcium chloride, and optionally contain (1) a nonionic surfactant, and/or (2) histidine, and if NaCl is present, then optionally also a sugar or a sugar alcohol, including without limitation sucrose and/or trehalose.

[0094] The invention includes formulations of Embodiment 5 as follows. A rFVIII formulation comprising:

(a) about 0mM, or a range of from about 1mM to about 20mM, from about 1 mM to about 50 mM, from about 10 mM to about 50 mM, from about 10 mM to about 20mM, from about 10 mM to about 30mM, or from about 20 mM to about 50 mM histidine;

(b) a range of from 0.5% to 20%, a range of from 1.0% to 20%, a range of from 0.6% to 10%, a range of from 0.7% to 10%, a range of from 0.8% to 10%, a range of from 0.9% to 10%, a range of from 1.0% to 10%, a range of from 0.5% to 5%, a range of from 0.6% to 5%, a range of from 0.7% to 5%, a range of from 0.8% to 5%, a range of from 0.9% to 5%, a range of from 1.0% to 5%, a range of from 0.5% to 2.5%, a range of from 0.6% to 2.5%, a range of from 0.5% to 2.0%, a range of from 0.5% to 1.5%, a range of from 0.6% to 1.4%, a range of from 0.8% to 1.4%, a range of from 0.9% to 1.2%, a range of from 3.0% to 9.0%, a range of from 5.0% to 9.0%, a range of from 6.0% to 8.0%, a range of from 7.0% to 9.0%, or a range of from 0.9% to 1.1%, or about 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5%, 10.0%, 12.0%, or15.0% of sucrose or trehalose;

(c) a range of from about 1 mM to about 5 mM, from about 1 mM to about 3 mM, from about 1.5 mM to about 3.5 mM, or from about 1 mM to about 2.5 mM divalent cation such as a divalent calcium salt, including calcium chloride;

(d) about 0 mM, or a range of from about 10 mM to about 50 mM, from about 10 mM to about 40 mM, from about 10 mM to about 35 mM, from about 10 mM to about 30 mM; from about 10 mM to about 20 mM, from about 20 mM to about 50 mM, from about 20 mM to about 40 mM, or from about 20 mM to about 80 mM sodium chloride;

(e) about 0 mM, or a range of from about 20 ppm to about 50 ppm, from about 20 ppm to about 80 ppm, from about 50 ppm to about 80 ppm, from about 80 ppm to about 100 ppm, from about 80 ppm to about 200 ppm, or from about 50 ppm to about 100 ppm of a non-ionic surfactant, or about 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 160, 170, 180, 190, or 200 ppm of a non-ionic surfactant;

(f) about 0%, or a range of from about 1.0% to about 5.0%, a range of from about 1.0% to about 4.0%, a range of from about 1.0% to about 3.0%, a range of from about 1.0% to about 2.0%, a range of from about 1.0% to about 1.5%, a range of from about 1.0% to about 1.4%, a range of from about 0.5% to about 5.0%, a range of from about 0.5% to about 4.0%, a range of from about 0.5% to about 3.0%, a range of from about 0.5% to about 2.0%, a range of from about 0.5% to about 1.5% glycine, or about 1.5%, 1.8%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, 3.0%, 3.3%, 3.5%, or 4.0% glycine and

(g) a range of from about 100 IU/ml to about 5000 IU/ml, from about 100 IU/ml to about 2000 IU/ml, from about 100 IU/ml to about 3000 IU/ml, from about 100 IU/ml to about 4000 IU/ml, from about 100 IU/ml to about 1200 IU/ml, from about 250 IU/ml to about 5000 IU/ml, from about 250 IU/ml to about 1000 IU/ml, from about 250 IU/ml to about 2000 IU/ml, from about 250 IU/ml to about 3000 IU/ml, from about 500 IU/ml to about 1000 IU/ml, from about 500 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 2000 IU/ml, from about 1000 IU/ml to about 3000 IU/ml, from about 1000 IU/ml to about 4000 IU/ml, or from about 1000 IU/ml to about 5000 IU/ml, or about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3500, 3800, 4000, 4200, 4500, 4600, 4800, 5000, 5500, or 6000 IU/ml of rFVIII;

wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 6.5, from about pH 6.0 to about pH 7.0, from about pH 6.0 to about pH 7.5, from about pH 6.5 to about pH 7.5, or from about pH 7.0 to about pH 7.5, or a pH of about pH 6.0, 6.5, 7.0, 7.1, 7.2, 7.3, 7.4 or about pH 7.5.

[0095] In one version of Embodiment 5, the rFVIII formulation comprises sodium chloride and contains less than 2.0% sucrose or sucrose in a range of from 0.5% to 2.0%, and contains less than 1.0%, less than 0.5%, less than 0.1% or no trehalose. In this version, NaCl may be present at a range of from about 10 mM to about 50 mM, from about 10 mM to about 40 mM, from about 10 mM to about 35 mM, from about 10 mM to about 30 mM; from about 10 mM to about 20 mM, from about 20 mM to about 50 mM, from about 20 mM to about 40 mM, or from about 20 mM to about 80 mM sodium chloride. In this version of Embodiment 5, glycine is present at a range of from about 1.0% to about 5.0%, a range of from about 1.0% to about 4.0%, a range of from about 1.0% to about 3.0%, a range of from about 1.0% to about 2.0%, a range of from about 1.0% to about 1.5%, a range of from about 1.0% to about 1.4%, a range of from about 0.5% to about 5.0%, a range of from about 0.5% to about 4.0%, a range of from about 0.5% to about 3.0%, a range of from about 0.5% to about 2.0%, a range of from about 0.5% to about 1.5%, or at about 1.5%, 1.8%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, 2.5%, 2.6%, 2.7%, 2.8%, 2.9%, 3.0%, 3.3%, 3.5%, or 4.0% and sucrose is present at a range of from 0.5% to 5%, a range of from 0.6% to 5%, a range of from 0.7% to 5%, a range of from 0.8% to 5%, a range of from 0.9% to 5%, a range of from 1.0% to 5%, a range of from 0.5% to 2.5%, a range of from 0.6% to 2.5%, a range of from 0.5% to 2.0%, a range of from 0.5% to 1.5%, a range of from 0.6% to 1.4%, a range of from 0.8% to 1.4%, a range of from

0.9% to 1.2%, or a range of from 0.9% to 1.1%, or about 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 3.0%, 4.0% sucrose. In this version of Embodiment 5, histidine is present at a range of from about 1 mM to about 20mM, from about 1 mM to about 50 mM, from about 10 mM to about 50 mM, from about 10 mM to about 20mM, from about 10 mM to about 30mM, or from about 20 mM to about 50 mM and a non-ionic surfactant such as polysorbate 20 or polysorbate 80 is present at a range of from about 20 ppm to about 50 ppm, from about 20 ppm to about 80 ppm, from about 50 ppm to about 80 ppm, from about 80 ppm to about 100 ppm, from about 80 ppm to about 200 ppm, or from about 50 ppm to about 100 ppm of a non-ionic surfactant, or about 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 160, 170, 180, 190, or 200 ppm. In this version of Embodiment 5, trehalose is present at less than 1.0%, less than 0.5%, less than 0.1% by weight or is not present.

[0096] In another version of Embodiment 5, sodium chloride is present at less than 1.0%, less than 0.5%, less than 0.1% by weight or is not present. In this version, sucrose or trehalose is present a range of from 0.5% to 20%, a range of from 1.0% to 20%, a range of from 0.6% to 10%, a range of from 0.7% to 10%, a range of from 0.8% to 10%, a range of from 0.9% to 10%, a range of from 1.0% to 10%, a range of from 3.0% to 9.0%, %, a range of from 5.0% to 9.0%, a range of from 6.0% to 8.0%, %, or a range of from 7.0% to 9.0%, or about 5.0%, 6.0%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5%, 10.0%, or 12.0%. In this version of Embodiment 5, glycine is present at less than 1.0%, less than 0.5%, less than 0.1% by weight or is not present.

[0097] Aspects of the present disclosure may be further understood in light of the following examples, which should not be construed as limiting the scope of the present teachings in any way.

EXAMPLES

Example 1: *Effect of Sodium Chloride, Polysorbate 80, and Human Serum Albumin on BDD-rFVIII Protein Solubility and Stability*

Effect of Sodium Chloride

[0098] Studies were performed on BDD mutants having introduced cysteine residues that permit the stabilization of FVIII by formation of at least one disulfide bond between different domains of FVIII. In particular, BDD-SQ (SEQ ID NO: 3) was mutated at Tyr664Cys:Thr1826Cys to create the C664-BDD mutant used in this example. For methods of preparation, see U.S. Patent 7,928,199 (Griffin et al.). When the C664-BDD mutant was formulated in a buffer containing histidine, unacceptable levels of precipitation were observed.

[0099] A study was performed to determine whether the precipitation observed when the C664-BDD mutant was placed in histidine buffer could be reversed. The buffer solution in which precipitation was observed contained 20 mM histidine, 30 mM sodium chloride, 2.5 mM calcium chloride, 29 mM sucrose, 293 mM glycine and 80 ppm polysorbate 80. The C664 BDD mutant was present at 145 IU/ml. The aim of the study was to develop a formulation that stabilizes BDD-rFVIII mutants. Solubilizers and stabilizers, such as sodium chloride, Polysorbate 80, and human serum albumin (HSA) were tested to either increase the solubility of the mutants or to improve the stability by reducing protein aggregation. Results are shown in FIGs. 2-6. The experiments shown in FIGs. 2 and 5 both involved modification of the NaCl concentration, and the results in each instance showed remarkable turbidity decline from a solution containing 30 mM NaCl when compared to a solution containing about 120 mM NaCl. The study established that as the sodium chloride concentration increased, the turbidity of the solution comprising the mutants decreased, suggesting that sodium chloride reversed the precipitation process. When the sodium chloride concentration was 176 mM or higher, the cloudy solution turned to a clear solution and the turbidity dropped from 0.169 to 0.029, which is more than 80% based on $A_{340\ nm}$ measurements (FIG. 2). These results demonstrated that sodium chloride was an effective solubilizer for the BDD-rFVIII mutants and can reverse their precipitation. In summary, higher sodium chloride concentrations improved the solubility of the BDD-rFVIII mutants. Table 2 shows preferred formulations. "BDD-rFVIII mutants" in Table 2 refers to a formulation of BDD-SQ mutated at Tyr664Cys:Thr1826Cys. "Full-length rFVIII" in Table 2 refers to a formulation of FVIII that has the amino acid sequence of SEQ ID NO: 2 (full-length FVIII).

Table 2

Formulation Composition for full-length rFVIII and BDD-rFVIII mutants

Composition	**BDD-rFVIII mutants**	**Full-length rFVIII**
Sodium chloride (mM)	220	30
Sucrose (mM)	29	29
Histidine (mM)	20	20

Glycine (mM)	293	293
Calcium chloride (mM)	2.5	2.5
Polysorbate 80 (ppm)	80	80

Example 2: *Formulation Development for rFVIII PEGylation through Random Lysine Coupling*

[0100] PEG polymer was conjugated to the full-length rFVIII of SEQ ID NO: 1 using random lysine coupling. In this type of coupling, the reactive groups are primarily the N-terminal amine or the ε-amino group of lysine in a protein. Other primary or secondary amine groups in the formulation could interfere with the reaction. Because many full-length and BDD-rFVIII formulations comprise amino acids, such as glycine and histidine, new formulations were developed for PEGylation of these molecules. While glycine was used as a bulking agent in the full-length rFVIII formulation and could be eliminated during PEGylation, histidine served as a buffer component and needed to be replaced with another buffer.

[0101] A suitable buffer system meets the following criteria: (1) it provides buffer capacity at pH 6-7; (2) it does not form insoluble complex or chelate with calcium chloride, an important rFVIII stabilizer; and (3) it does not comprise primary or secondary amine groups.

[0102] Several commonly used buffers were considered for random PEGylation of rFVIII. As shown in Table 3, only two buffer systems, tri-ethanolamine ("TEA") and MOPS were selected for further investigation.

Table 3

Buffers Considered for Random PEGylation of rFVIII

Buffer at pH 7	Ca^{2+} ppt.	Ca^{2+} chelating	Amine group	pH change during freezing
Citrate		X		
Phosphate	X			X

Buffer at pH 7	Ca^{2+} ppt.	Ca^{2+} chelating	Amine group	pH change during freezing
Histidine			X	
TRIS			X	
Carbonate	X			
Triethanolamine (TEA)				
MOPS or MOPSO				
HEPES			X	

[0103] For this study, full-length rFVIII was dialyzed against the formulations listed in Table 4. The dialyzed rFVIII in the three formulations was placed at 40°C (Figure 7) or 25°C (Figure 8) to establish stability at accelerated conditions and the results are shown in Figures 7 and 8.

Table 4

Buffers Evaluated for Random PEGylation of rFVIII

	NaCl (mM)	*$CaCl_2$ (mM)*	*Tween 80 (ppm)*	*Glycine (mM)*	*Sucrose (mM)*	*Sodium Azide (%)*	*Buffer Agent (20mM)*
1	30	2.5	80	--	29	0.05	TEA
2	30	2.5	80	--	29	0.05	MOPS
3	30	2.5	80	293	29	0.05	Histidine

Example 3: *PEGylation for BDD-rFVIII*

[0104] BDD-rFVIII encounters formulation challenges due to its propensity for aggregation. Therefore, one of the objectives with designing a formulation for PEGylated rFVIII was to ensure its stability in solution. The working formulation for the PEGylated BDD-rFVIII comprised 200 mM sodium chloride, 20 mM MOPS, 10 mM $CaCl_2$, 100 ppm polysorbate 80 and 29 mM sucrose. 200 mM sodium chloride will impose difficulties during freeze-drying. Accordingly, the solubility and potency of the PEGylated BDD-rFVIII were evaluated as a function of sodium chloride concentration in the range of 50 and 250 mM.

[0105] The buffer composition used for the study is shown in Table 5 and the data are summarized in Figures 10 and 11. The PEGylated BDD used in this example comprised the amino acid sequence of SEQ ID NO: 3 with one amino acid mutation to create a free cysteine at which PEG was added. This is shown graphically in FIG. 9. The PEGylated BDD-rFVIII retained more than 87% potency in the formulation comprising 50-150 mM sodium chloride during 6 days storage at 23°C. UnPEGylated BDD-rFVIII retained 70% potency in the same formulation during 6 days storage at 23°C. Both molecules remained soluble during the study with no visual detection of precipitates or opalescence. These and earlier data suggest that 100 mM sodium chloride can be used for further formulation development.

Table 5

Composition of the Formulation Used for Evaluating the Effect of Sodium Chloride

MOPS (mM)	**NaCl (mM)**	**CaCl2 (mM)**	**Polysorbate 80 (ppm)**	**Sucrose (mM)**
20	250	10	100	29
20	200	10	100	29
20	150	10	100	29
20	100	10	100	29
20	50	10	100	29
20	25	10	100	29
20	0	10	100	29

[0106] The effect of sodium chloride on the solubility and aggregation of PEGylated and unPEGylated BDD-rFVIII was investigated.

[0107] UV absorbance of PEGylated BDD-rFVIII in MOPS buffer comprising 25 mM, 55 mM, 75 mM, 125 mM and 200 mM sodium chloride showed no scattering of the PEGylated BDD-rFVIII at all sodium chloride concentration tested, suggesting lack of aggregation. In contrast, the unPEGylated-rFVIII showed considerable scattering at 25 mM, 55 mM and 75 mM sodium chloride most likely due to formation of soluble aggregates. When sodium chloride concentration was increased to 125 mM and 200 mM, no scattering was observed. It was concluded, therefore, that higher salt concentrations prevented aggregate formation.

Example 4: *Development of Freeze-Drying Formulation for PEGylated BDD-rFVIII*

[0108] Four candidate formulations were screened for lyophilization of PEGylated BDD-rFVIII. The PEGylated BDD used in this example comprised the amino acid sequence of SEQ ID NO: 3 with one amino acid mutation to create a free cysteine at which PEG was added. The aim was to evaluate the stability of the lyophilized drug product in these formulations and to select a formulation for the leading stability study. The formulations that were screened were (1) Formulation A, which had been successful for unPEGylated full-length rFVIII, (2) Formulation B, comprising increased solids content compared to Formulation A, (3) Formulation C with sucrose instead of the NaCl used in Formulation A, and (4) Formulation D with trehalose instead of the NaCl used in Formulation A. The last two formulations provided an amorphous matrix for the lyophilized drug product.

[0109] Stability was evaluated at three storage temperatures (5°C, 25°C and 40°C). Table 6 shows the formulation composition for PEGylated BDD-rFVIII used for stability evaluation.

[0110] The concentrations of sucrose and glycine were increased from 29 mM and 293 mM in Formulation A to 38 mM and 346 mM in Formulation B. The additional solids were added to enhance the mechanical strength of the freeze-dried cake and improve the appearance of the final drug product.

Table 6:

Formulation Composition for PEGylated BDD-rFVIII Used in Stability Evaluation

Component	Formulation A	Formulation B	Formulation C	Formulation D
Calcium Chloride	2.5 mM	2.5 mM	2.5 mM	2.5 mM
Sodium Chloride	30 mM	30 mM	X	X
Histidine	20 mM	20 mM	20 mM	20 mM
Glycine	293 mM	346 mM	X	X
Polysorbate 80	80 ppm	80 ppm	80 ppm	80 ppm
Sucrose	29 mM	38 mM	234 mM	X
Trehalose	X	X	X	211 mM
PEGylated BDD-rFVIII concentration (IU/mL)	100 IU/mL	100 IU/mL	100 IU/mL	100 IU/mL

1: pH = 6.8 for all formulations

[0111] Formulations C and D were designed to provide an alternate matrix compared to the other two formulations. Formulations A and B formed a crystalline matrix upon freeze-drying due to the presence of sodium chloride and glycine as structural stability and bulking agents. The concentrations of sucrose and trehalose were increased to 234 mM and 211 mM, respectively, in lieu of including sodium chloride and glycine. This resulted in an amorphous matrix for the freeze-dried drug product.

[0112] The stability program for each of the four candidate formulations was set up for a 26 week time period. Stability was evaluated by potency, moisture content, percent high molecular weight (HMW) impurities and total product related impurities by SEC-HPLC. The potency recovery data for the four formulations are summarized in Figures 12-15.

[0113] The data of potency recovery, moisture content by Karl Fischer, and percent aggregates and product related impurities by SEC-HPLC (tested at 26 weeks) for the four formulations demonstrate that rFVIII is stable in the four formulations.

[0114] Stability for PEGylated BDD-rFVIII was further evaluated with Formulations A and B (see Table 6 for formulation composition). Two drug product lots were prepared at lab-scale and were placed on stability at 5°C and 25°C and 40°C. Potency by the chromogenic assay, percent high molecular weight impurities and total product related impurities by SEC-HPLC, and moisture by Karl Fischer were employed for drug product stability evaluation. Target concentrations and ranges of the components used in Formulation A are presented in Table 7.

Table 7: Target Concentrations and Ranges of the Components Used in the Formulation A

Component	**Formulation A Target Concentrations**	**Low and High Concentration Range**
Calcium Chloride	2.5 mM	1.5 mM to 3.5 mM
Sodium Chloride	30 mM	21 mM to 43 mM
Histidine	20 mM	15 mM to 27 mM
Glycine	293 mM	240 mM to 386 mμM
Polysorbate 80	80 ppm	57 ppm to 103 ppm
Sucrose	29 mM	20 mM to 41 mM
PEGylated BDD-rFVIII concentration (IU/mL)	200 IU/mL 400 IU/mL 1200 IU/mL	188 IU/mL to 250 IU/mL 376 IU/mL to 500 IU/mL 1128 IU/mL to 1500 IU/mL

[0115] These data demonstrated comparable drug product stability in the two formulations. The study with Formulation A was continued up to 30 months, whereas the study with Formulation B was terminated at 3 months (Figures 16 and 17, respectively). rFVIII concentration in Figure 18 and 19 was 400 IU/mL.

[0116] Formulation A was selected for further development and was tested with PEGylated rFVIII at concentrations of 200 IU IU/mL and 1200 IU/mL. The potency profiles at 200 IU/mL and 1200 IU/mL are shown in Figures 18 and 19, respectively. The data demonstrate that Formulation A provides continuous stability for the PEGylated rFVIII.

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[0117] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0118] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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The claims defining the invention are as follows:

1. A rFVIII formulation comprising:
   (a) a range of from about 1 mM to about 5 mM divalent cation;
   (b) a range of from about 150 mM to about 250 mM sodium chloride or potassium chloride;
   (c) a range of from about 50 ppm to about 200 ppm of a non-ionic surfactant; and
   (d) a range of from about 100 IU/ml to about 5000 IU/ml of a rFVIII, wherein the rFVIII comprises an amino acid sequence that has one or more non-cysteine residues in the amino acid sequence of SEQ ID NO: 3 replaced with cysteine residues such that at least one pair of cysteine residues creates a disulphide bond not found in wild type FVIII;
   wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5.

2. The rFVIII formulation of claim 1 further comprising:
   (a) a range of from about 10 mM to about 50 mM histidine;
   (b) a range of from about 10 mM to about 100 mM of a sugar or sugar alcohol; and
   (c) a range of from about 150 mM to about 400 mM glycine.

3. A rFVIII formulation comprising:
   (a) about 0 mM, or a range of from about 1 mM to about 20 mM histidine;
   (b) a range of from 0.5% to 20% of sucrose or trehalose;
   (c) a range of from about 1 mM to about 5 mM divalent cation;
   (d) a range of from about 10 mM to about 50 mM sodium chloride;
   (e) about 0 mM, or a range of from about 20 ppm to about 80 ppm of a non-ionic surfactant;
   (f) about 0%, or a range of from about 1.0% to about 5.0% glycine; and
   (g) a range of from about 100 IU/ml to about 5000 IU/ml of conjugated rFVIII;
   wherein the rFVIII formulation has a pH in a range of from about pH 6.0 to about pH 7.5.

4. The rFVIII formulation of claim 3, wherein
   (a) sodium chloride is present in a range of from about 10 mM to about 50 mM;
   (b) sucrose is present in a range of from 0.5% to 2.0%;

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(c) glycine is present in a range of from about 1.0% to about 5.0%;

(d) histidine is present in a range of from about 1mM to about 20mM; and

(e) a non-ionic surfactant is present in a range of from about 20 ppm to about 80 ppm.

5. The rFVIII formulation of claim 3, wherein

(a) sodium chloride is present at less than 1.0% by weight;

(b) sucrose or trehalose is present in a range of from 0.5% to 20%; and

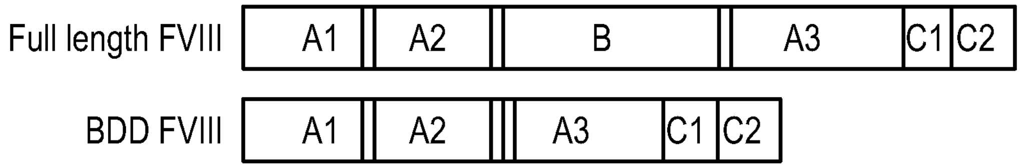
(c) glycine is present at less than 1.0% by weight or is not present.

6. The rFVIII formulation of claim 5, wherein sucrose or trehalose is present in a range of from 1.0% to 10.0%.

7. A method of treating hemophilia A comprising administering a therapeutically effective amount of a rFVIII formulation of any one of claims 1 to 6 to a patient in need thereof.

8. Use of a rFVIII formulation of any one of claims 1 to 6 in the manufacture of a medicament for treating hemophilia A.

FIG. 1



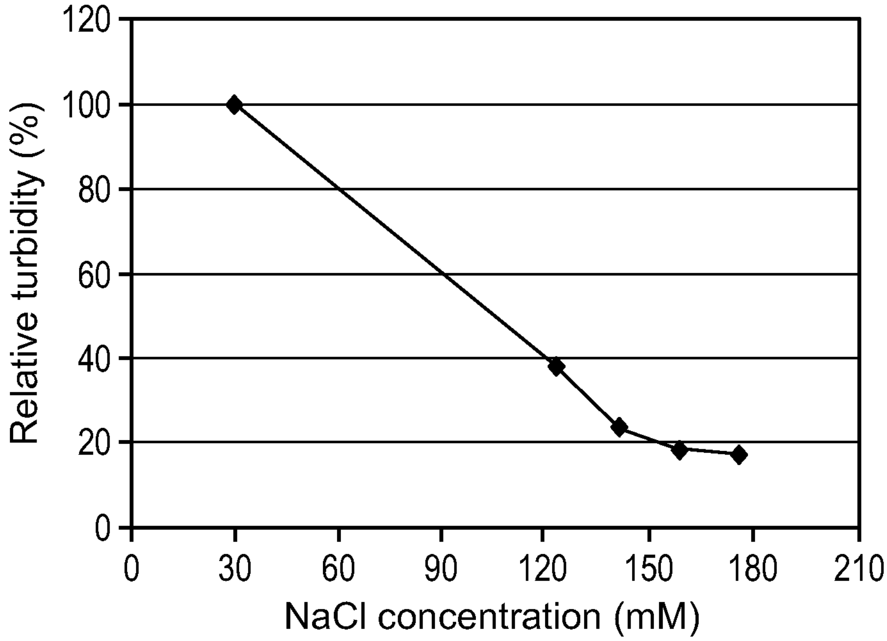

Relative turbidity (%)
0
20
40
60
80
100
120
NaCl concentration (mM)
0
30
60
90
120
150
180
210

FIG. 2

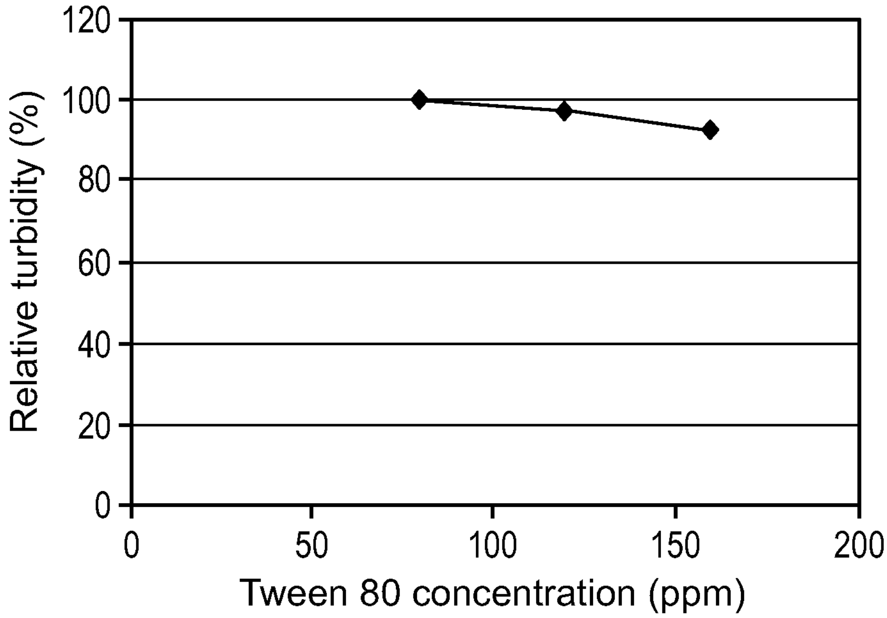

Relative turbidity (%)
0
20
40
60
80
100
120
Tween 80 concentration (ppm)
0
50
100
150
200

FIG. 3

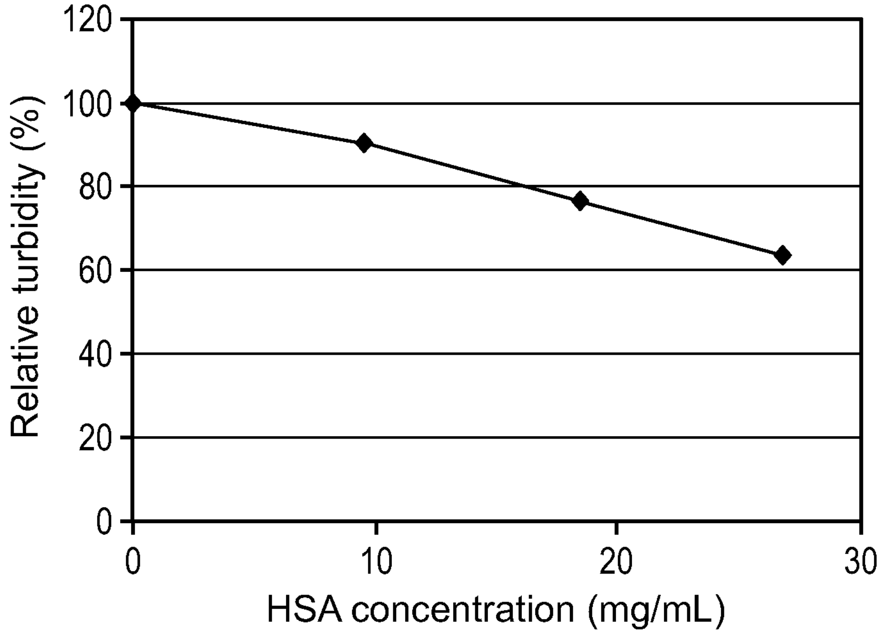

120
100
80
60
40
20
0
Relative turbidity (%)
0
10
20
30
HSA concentration (mg/mL)

FIG. 4

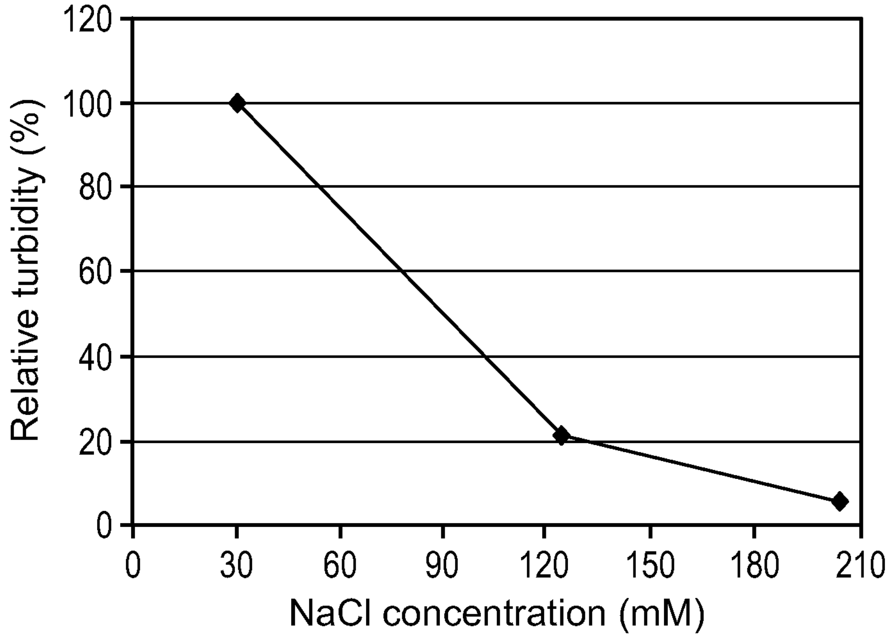

120
100
80
60
40
20
0
Relative turbidity (%)
0
30
60
90
120
150
180
210
NaCl concentration (mM)

FIG. 5

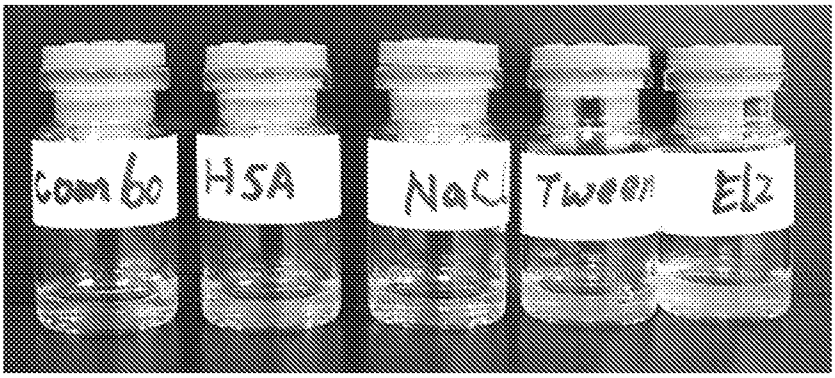


FIG. 6

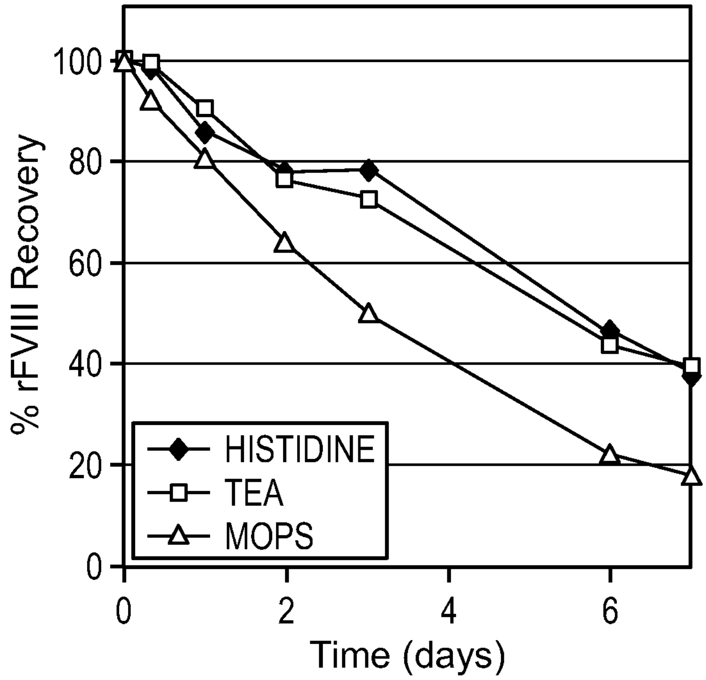

% rFVIII Recovery
100
80
60
40
20
0
0
2
4
6
Time (days)
HISTIDINE
TEA
MOPS

FIG. 7

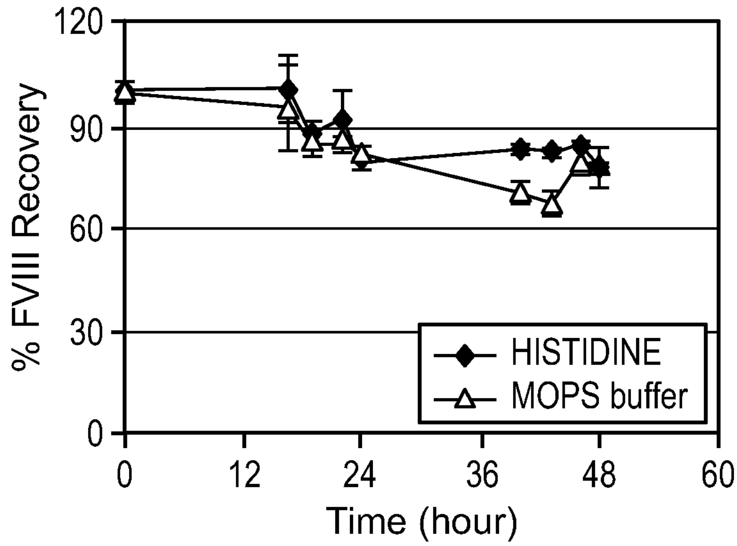
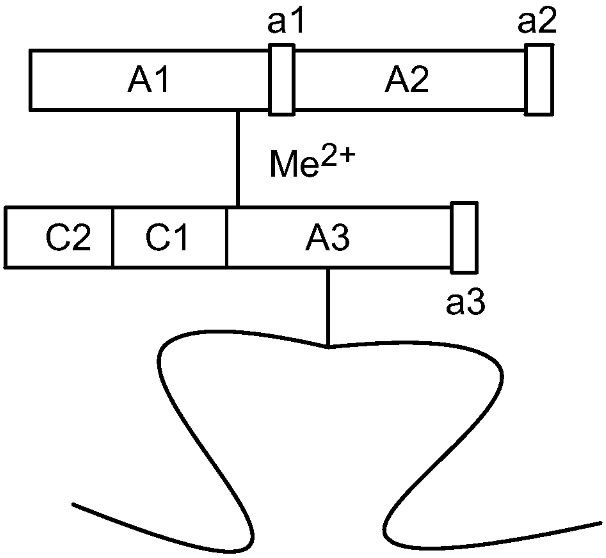

% FVIII Recovery
120
90
60
30
0
0
12
24
36
48
60
Time (hour)
HISTIDINE
MOPS buffer

FIG. 8

FIG. 9



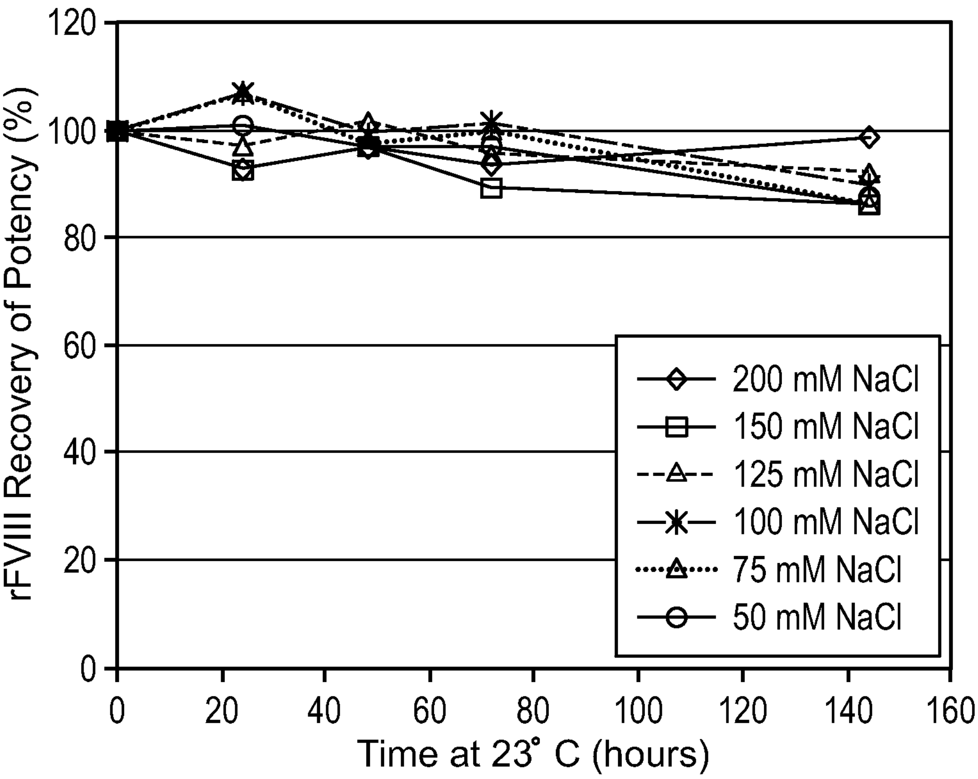

120
100
80
60
40
20
0
rFVIII Recovery of Potency (%)
0 20 40 60 80 100 120 140 160
Time at 23° C (hours)
200 mM NaCl
150 mM NaCl
125 mM NaCl
100 mM NaCl
75 mM NaCl
50 mM NaCl

FIG. 10

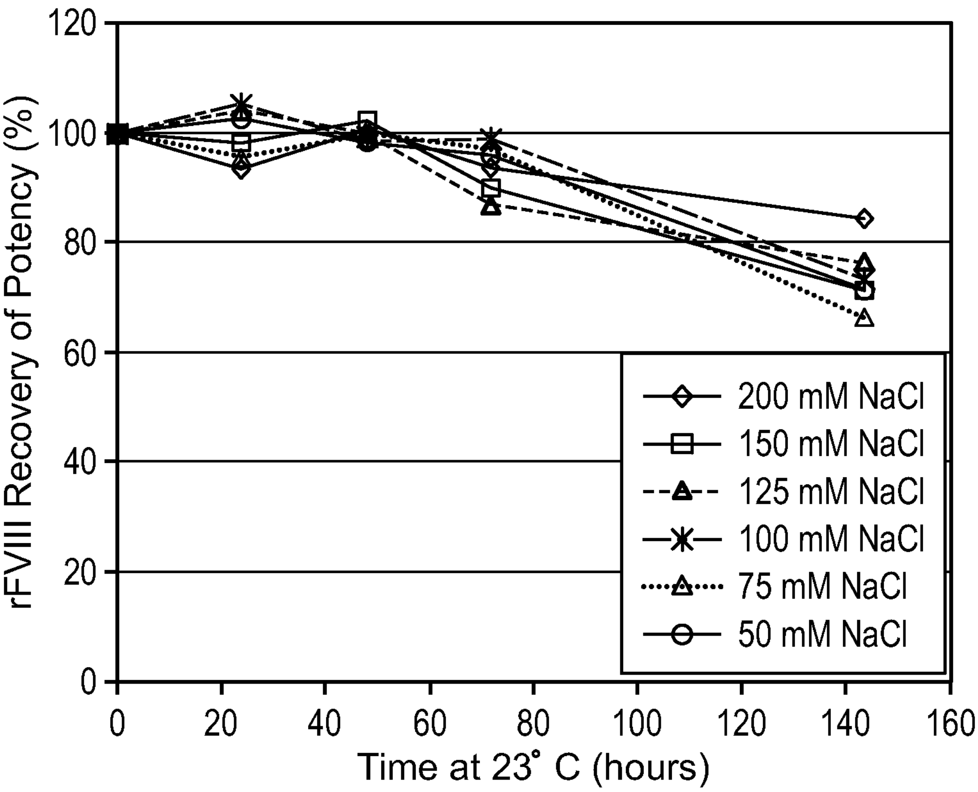

120
100
80
60
40
20
0
rFVIII Recovery of Potency (%)
0 20 40 60 80 100 120 140 160
Time at 23° C (hours)
200 mM NaCl
150 mM NaCl
125 mM NaCl
100 mM NaCl
75 mM NaCl
50 mM NaCl

FIG. 11

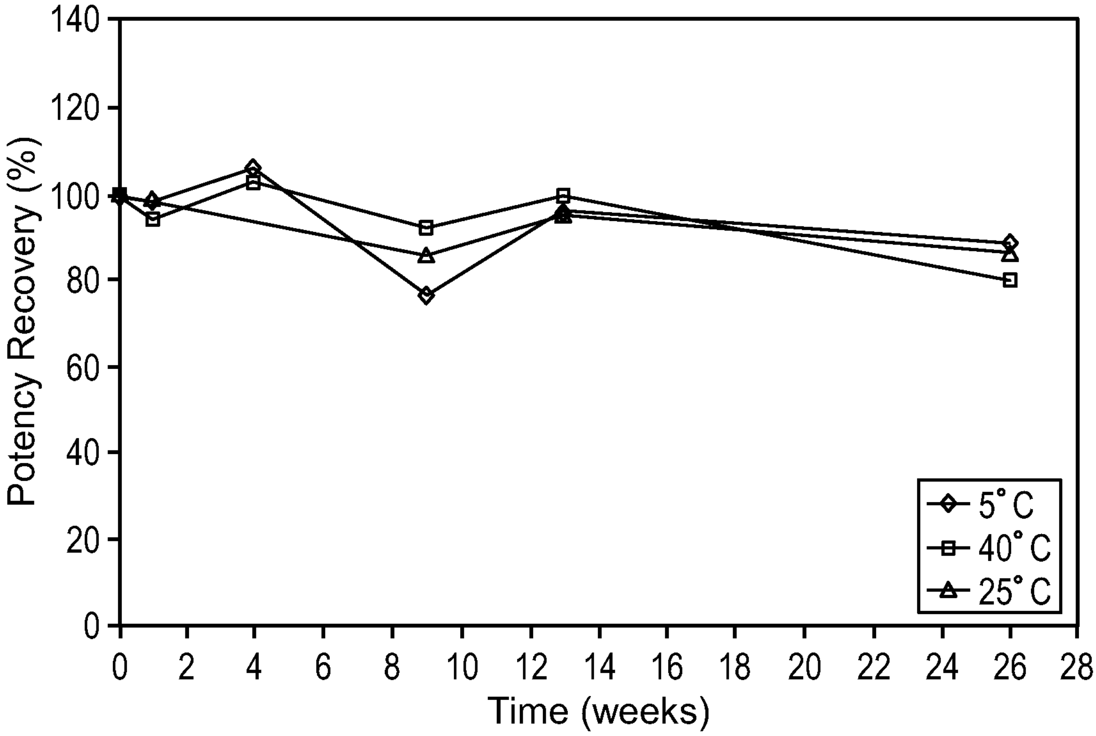

140
120
100
80
60
40
20
0
Potency Recovery (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (weeks)
5° C
40° C
25° C

FIG. 12

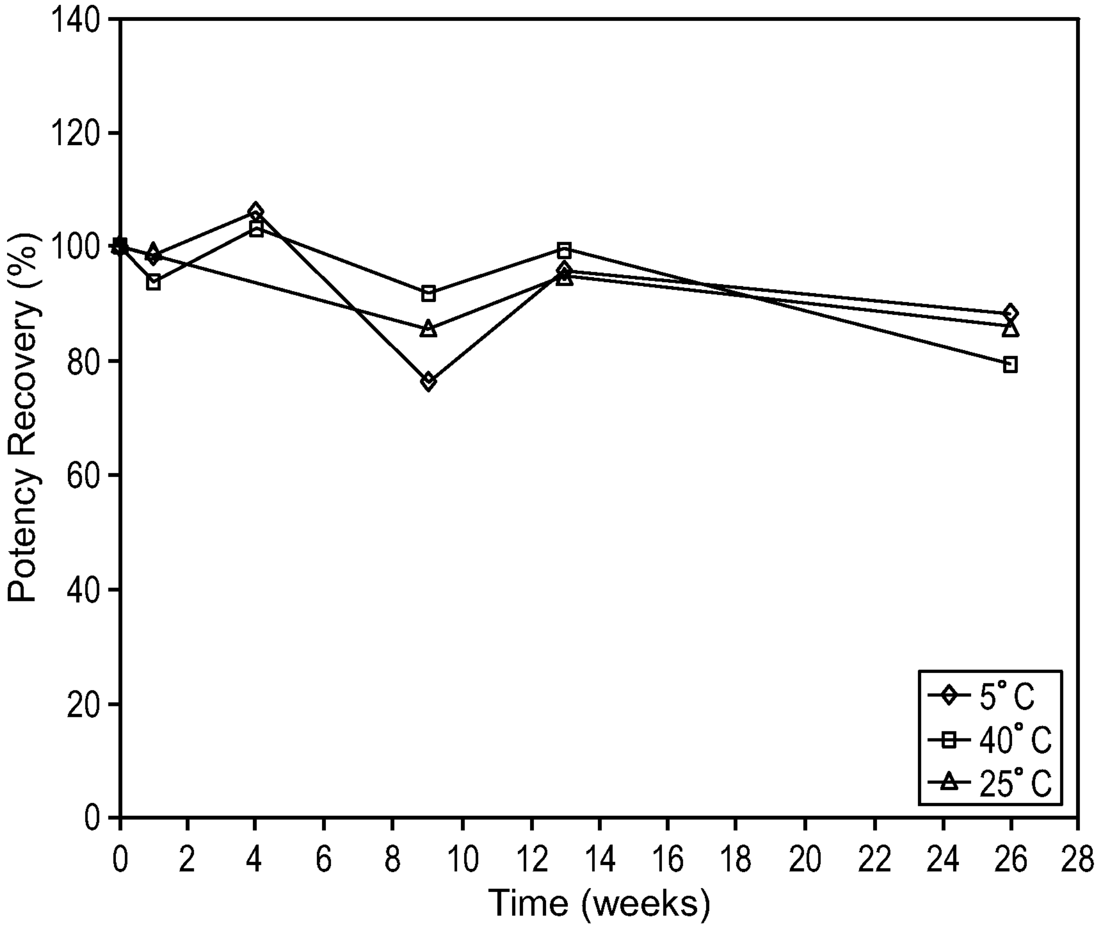

140
120
100
80
60
40
20
0
Potency Recovery (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (weeks)
5° C
40° C
25° C

FIG. 13

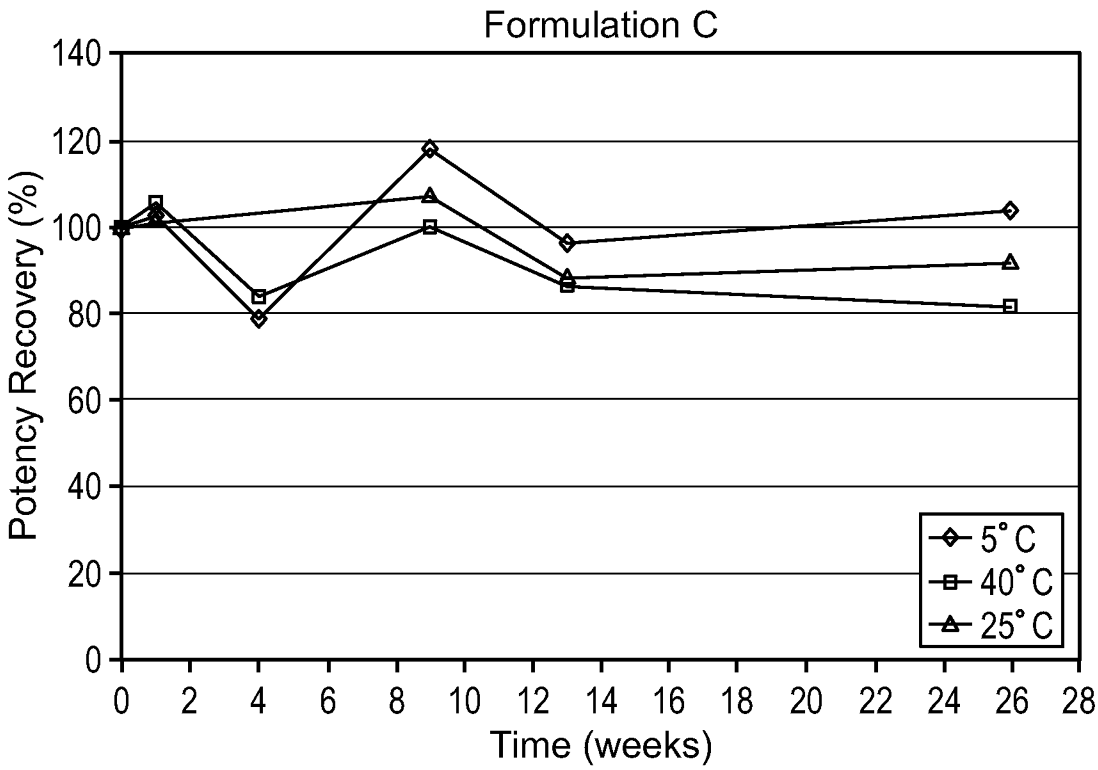

Formulation C
140
120
100
80
60
40
20
0
Potency Recovery (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (weeks)
5° C
40° C
25° C

FIG. 14

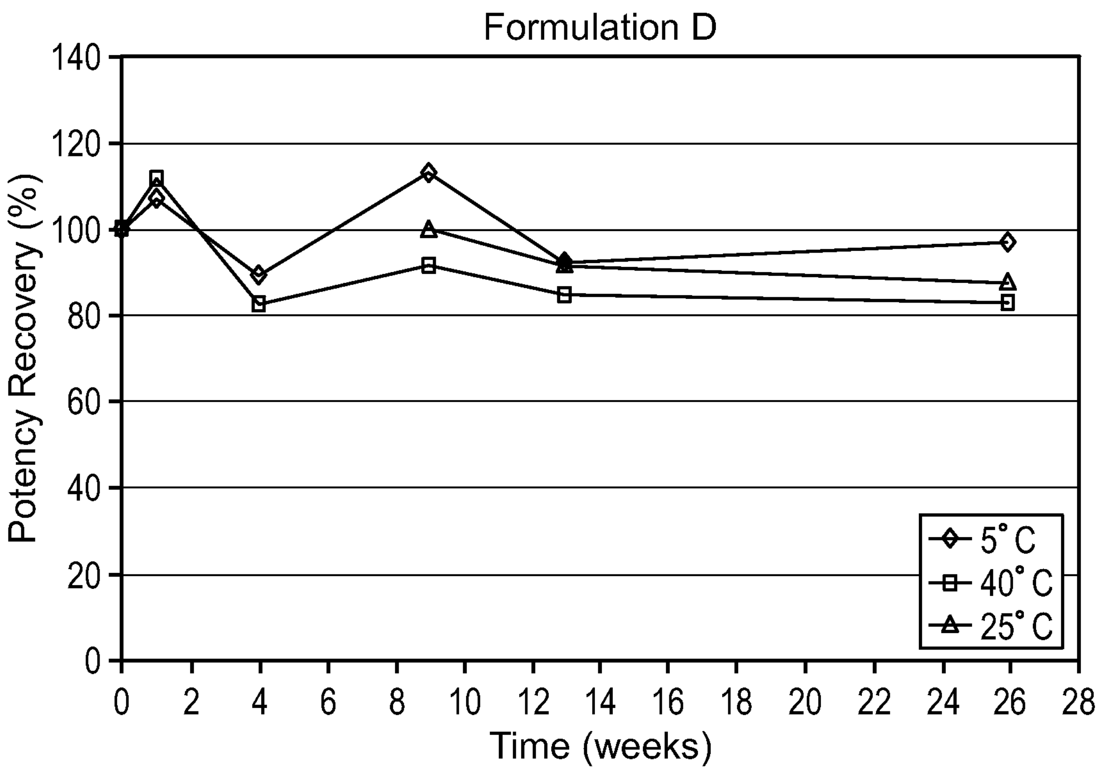

Formulation D
140
120
100
80
60
40
20
0
Potency Recovery (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (weeks)
5° C
40° C
25° C

FIG. 15

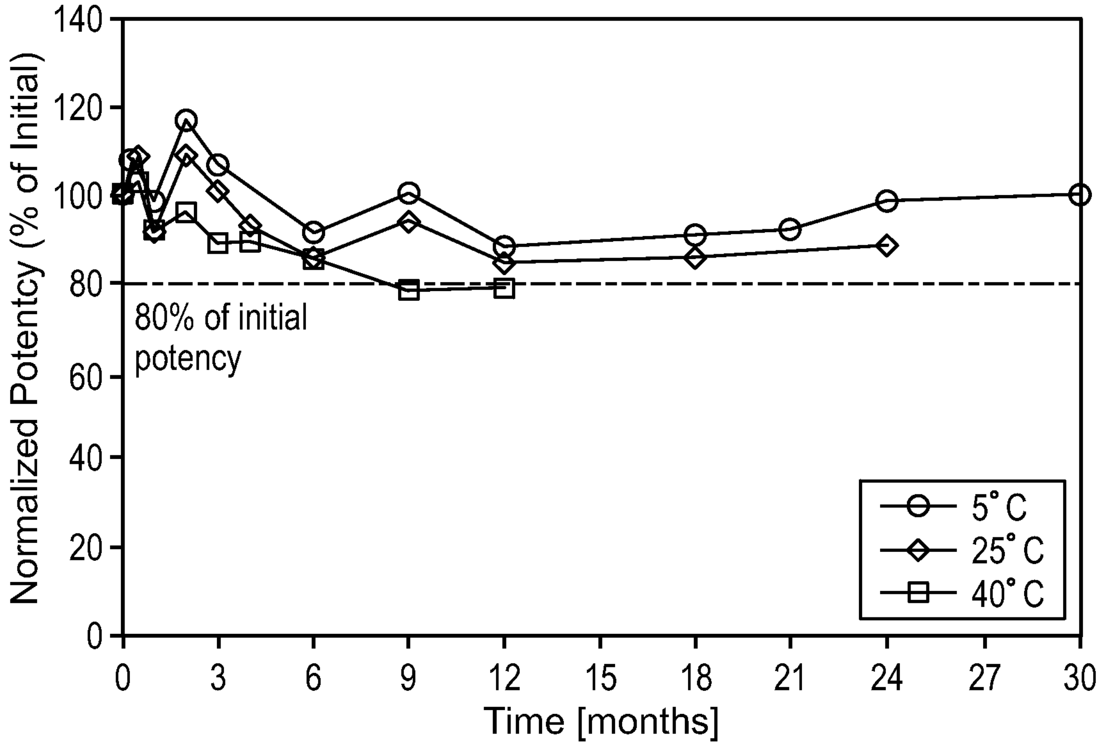

140
120
100
80
60
40
20
0
Normalized Potentcy (% of Initial)
80% of initial
potency
5° C
25° C
40° C
0
3
6
9
12
15
18
21
24
27
30
Time [months]

FIG. 16

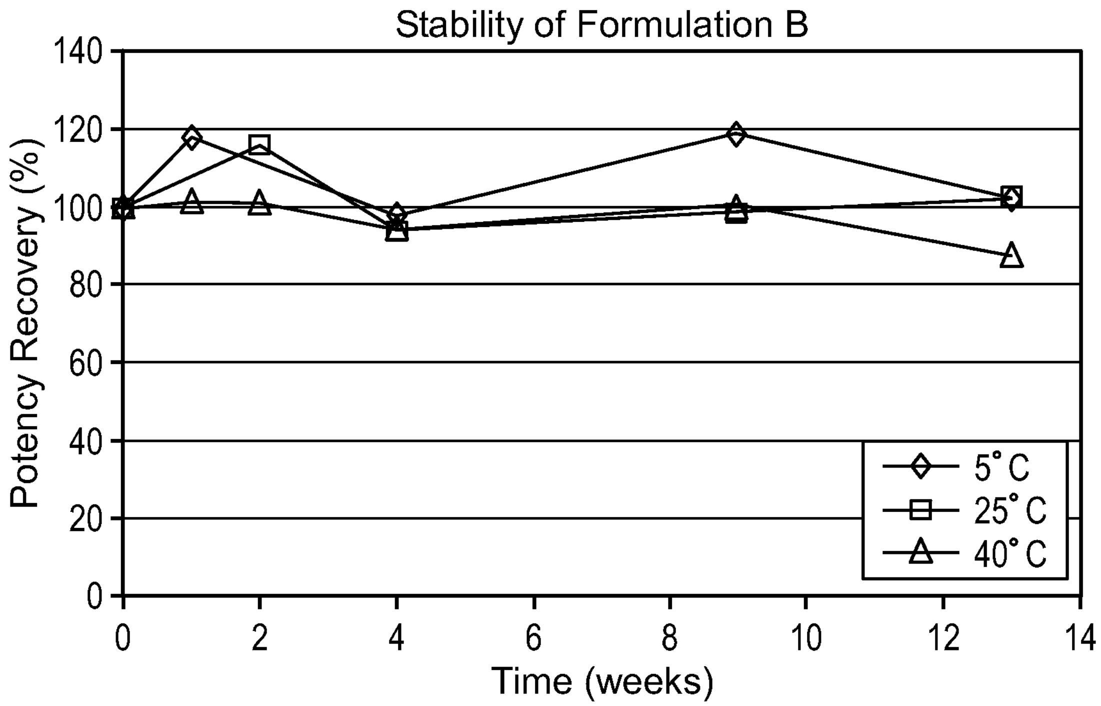

Stability of Formulation B
140
120
100
80
60
40
20
0
Potency Recovery (%)
5° C
25° C
40° C
0
2
4
6
8
10
12
14
Time (weeks)

FIG. 17

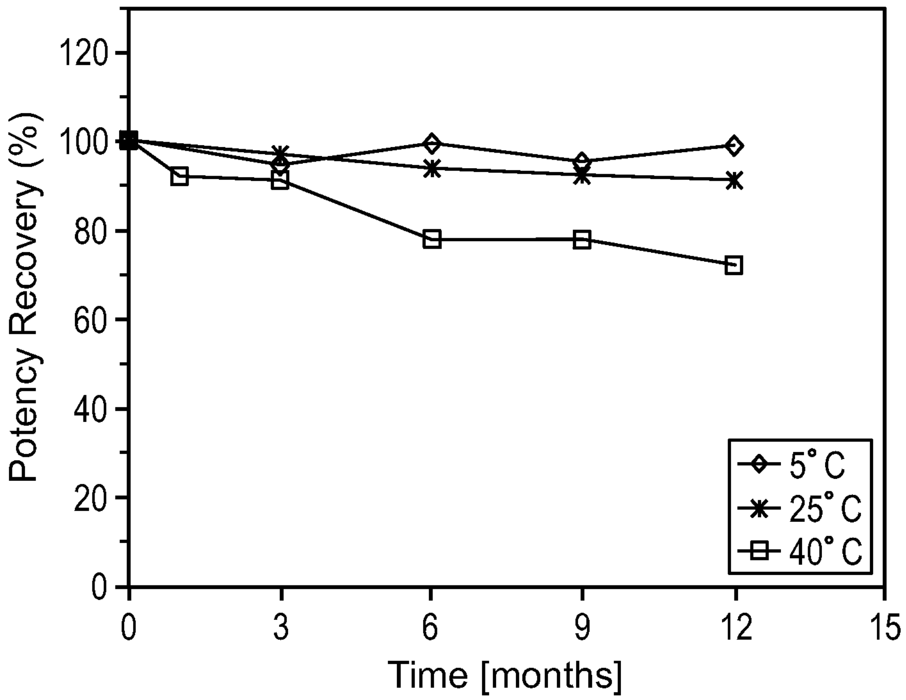

120
100
80
60
40
20
0
Potency Recovery (%)
0
3
6
9
12
15
Time [months]
5° C
25° C
40° C

FIG. 18

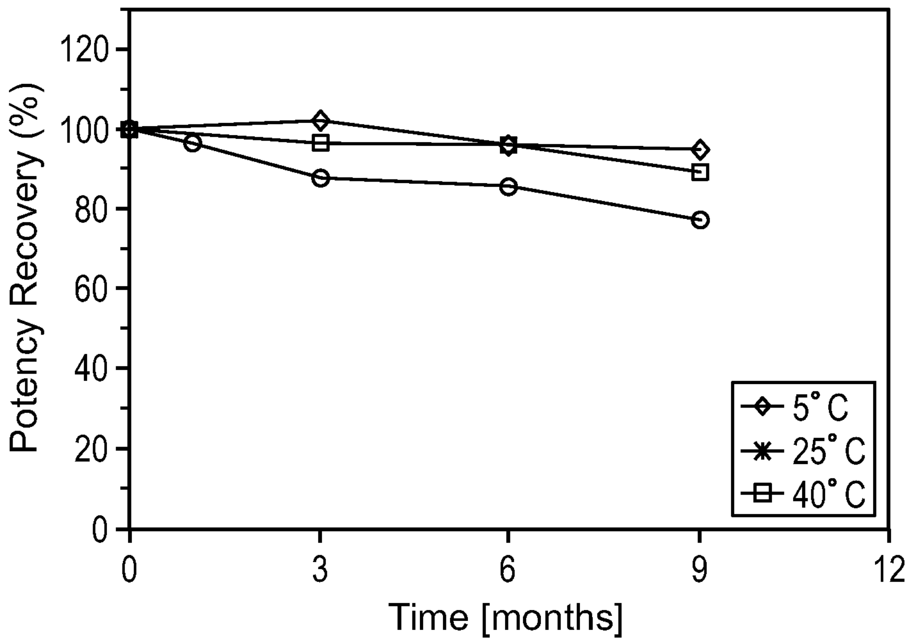

120
100
80
60
40
20
0
Potency Recovery (%)
0
3
6
9
12
Time [months]
5° C
25° C
40° C

FIG. 19

FIG. 20

B-Domain Deleted SQ Human Factor VIII
Amino Acid Sequence SEQ ID NO: 3

```
   1 mqielstcff lcllrfcfsa trryylgave lswdymqsdl gelpvdarfp prvpksfpfn
  61 tsvvykktlf veftdhlfni akprppwmgl lgptiqaevy dtvvitlknm ashpvslhav
 121 gvsywkaseg aeyddqtsqr ekeddkvfpg gshtyvwqvl kengpmasdp lcltysylsh
 181 vdlvkdlnsg ligallvcre gslakektqt lhkfillfav fdegkswhse tknslmqdrd
 241 aasarawpkm htvngyvnrs lpgligchrk svywhvigmg ttpevhsifl eghtflvrnh
 301 rqasleispi tfltaqtllm dlgqfllfch isshqhdgme ayvkvdscpe epqlrmknne
 361 eaedydddlt dsemdvvrfd ddnspsfiqi rsvakkhpkt wvhyiaaeee dwdyaplvla
 421 pddrsyksqy lnngpqrigr kykkvrfmay tdetfktrea iqhesgilgp llygevgdtl
 481 liifknqasr pyniyphgit dvrplysrrl pkgvkhlkdf pilpgeifky kwtvtvedgp
 541 tksdprcltr yyssfvnmer dlasgligpl licykesvdq rgnqimsdkr nvilfsvfde
 601 nrswylteni qrflpnpagv qledpefqas nimhsingyv fdslqlsvcl hevaywyils
 661 igaqtdflsv ffsgytfkhk mvyedtltlf pfsgetvfms menpglwilg chnsdfrnrg
 721 mtallkvssc dkntgdyyed syedisayll sknnaieprs fsqnppvlkr hqreitrttl
 781 qsdqeeidyd dtisvemkke dfdiydeden qsprsfqkkt rhyfiaaver lwdygmsssp
 841 hvlrnraqsg svpqfkkvvf qeftdgsftq plyrgelneh lgllgpyira evednimvtf
 901 rnqasrpysf ysslisyeed qrqgaeprkn fvkpnetkty fwkvqhhmap tkdefdckaw
 961 ayfsdvdlek dvhsgligpl lvchtntlnp ahgrqvtvqe falfftifde tkswyftenm
1021 erncrapcni qmedptfken yrfhaingyi mdtlpglvma qdqrirwyll smgsnenihs
1081 ihfsghvftv rkkeeykmal ynlypgvfet vemlpskagi wrveciggeh lhagmstlfl
1141 vysnkcqtpl gmasghirdf qitasgqygq wapklarlhy sgsinawstk epfswikvdl
1201 lapmiihgik tqgarqkfss lyisqfiimy sldgkkwqty rgnstgtlmv ffgnvdssgi
1261 khnifnppii aryirlhpth ysirstlrme lmgcdlnscs mplgmeskai sdaqitassy
1321 ftnmfatwsp skarlhlqgr snawrpqvnn pkewlqvdfq ktmkvtgvtt qgvkslltsm
1381 yvkeflisss qdghqwtlff qngkvkvfqg nqdsftpvvn sldpplltry lrihpqswvh
1441 qialrmevlg ceaqdly
```

FIG. 21A

Human Factor VIII Amino Acid Sequence
SEQ ID NO: 1

```
   1 mqielstcff lcllrfcfsa trryylgave lswdymqsdl gelpvdarfp prvpksfpfn
  61 tsvvykktlf veftvhlfni akprppwmgl lgptiqaevy dtvvitlknm ashpvslhav
 121 gvsywkaseg aeyddqtsqr ekeddkvfpg gshtyvwqvl kengpmasdp lcltysylsh
 181 vdlvkdlnsg ligallvcre gslakektqt lhkfillfav fdegkswhse tknslmqdrd
 241 aasarawpkm htvngyvnrs lpgligchrk svywhvigmg ttpevhsifl eghtflvrnh
 301 rqasleispi tfltaqtllm dlgqfllfch isshqhdgme ayvkvdscpe epqlrmknne
 361 eaedydddlt dsemdvvrfd ddnspsfiqi rsvakkhpkt wvhyiaaeee dwdyaplvla
 421 pddrsyksqy lnngpqrigr kykkvrfmay tdetfktrea iqhesgilgp llygevgdtl
 481 liifknqasr pyniyphgit dvrplysrrl pkgvkhlkdf pilpgeifky kwtvtvedgp
 541 tksdprcltr yyssfvnmer dlasgligpl licykesvdq rgnqimsdkr nvilfsvfde
 601 nrswylteni qrflpnpagv qledpefqas nimhsingyv fdslqlsvcl hevaywyils
 661 igaqtdflsv ffsgytfkhk mvyedtltlf pfsgetvfms menpglwilg chnsdfrnrg
 721 mtallkvssc dkntgdyyed syedisayll sknnaieprs fsqnsrhpst rqkqfnatti
 781 pendiektdp wfahrtpmpk iqnvsssdll mllrqsptph glslsdlqea kyetfsddps
 841 pgaidsnnsl semthfrpql hhsgdmvftp esglqlrlne klgttaatel kkldfkvsst
 901 snnlistips dnlaagtdnt sslgppsmpv hydsqldttl fgkkssplte sggplslsee
 961 nndskllesg lmnsqesswg knvsstesgr lfkgkrahgp alltkdnalf kvsisllktn
1021 ktsnnsatnr kthidgpsll ienspsvwqn ilesdtefkk vtplihdrml mdknatalrl
1081 nhmsnkttss knmemvqqkk egpippdaqn pdmsffkmlf lpesarwiqr thgknslnsg
```

FIG. 21B

```
1141 qgpspkqlvs lgpeksvegq nflseknkvv vgkgeftkdv glkemvfpss rnlfltnldn
1201 lhennthnqe kkiqeeiekk etliqenvvl pqihtvtgtk nfmknlflls trqnvegsye
1261 gayapvlqdf rslndstnrt kkhtahfskk geeenleglg nqtkqiveky acttrispnt
1321 sqqnfvtqrs kralkqfrlp leetelekri ivddtstqws knmkhltpst ltqidyneke
1381 kgaitqspls dcltrshsip qanrsplpia kvssfpsirp iyltrvlfqd nsshlpaasy
1441 rkkdsgvqes shflqgakkn nlslailtle mtgdqrevgs lgtsatnsvt ykkventvlp
1501 kpdlpktsgk vellpkvhiy qkdlfptets ngspghldlv egsllqgteg aikwneanrp
1561 gkvpflrvat essaktpskl ldplawdnhy gtqipkeewk sqekspekta fkkkdtilsl
1621 nacesnhaia ainegqnkpe ievtwakqgr terlcsqnpp vlkrhqreit rttlqsdqee
1681 idyddtisve mkkedfdiyd edenqsprsf qkktrhyfia averlwdygm sssphvlrnr
1741 aqsgsvpqfk kvvfqeftdg sftqplyrge lnehlgllgp yiraevedni mvtfrnqasr
1801 pysfysslis yeedqrqgae prknfvkpne tktyfwkvqh hmaptkdefd ckawayfsdv
1861 dlekdvhsgl igpllvchtn tlnpahgrqv tvqefalfft ifdetkswyf tenmerncra
1921 pcniqmedpt fkenyrfhai ngyimdtlpg lvmaqdqrir wyllsmgsne nihsihfsgh
1981 vftvrkkeey kmalynlypg vfetvemlps kagiwrvecl igehlhagms tlflvysnkc
2041 qtplgmasgh irdfqitasg qygqwapkla rlhysgsina wstkepfswi kvdllapmii
2101 hgiktqgarq kfsslyisqf iimysldgkk wqtyrgnstg tlmvffgnvd ssgikhnifn
2161 ppiiaryirl hpthysirst lrmelmgcdl nscsmplgme skaisdaqit assyftnmfa
2221 twspskarlh lqgrsnawrp qvnnpkewlq vdfqktmkvt gvttqgvksl ltsmyvkefl
2281 isssqdghqw tlffqngkvk vfqgnqdsft pvvnsldppl ltrylrihpq swvhqialrm
2341 evlgceaqdl y
```

SEQUENCE LISTING

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<120> RECOMBINANT FACTOR VIII FORMULATIONS

<130> BHC 135014 PCT (0081563-000121)

<150> US 61/869,191
<151> 2013-08-23

<150> US 61/779,495
<151> 2013-03-15

<160> 5

<170> PatentIn version 3.5

<210> 1
<211> 2351
<212> PRT
<213> Homo sapiens

<400> 1

Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe
1   5   10   15

Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser
            20   25   30

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
        35   40   45

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
    50   55   60

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Val His Leu Phe Asn Ile
65   70   75   80

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
                85   90   95

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
            100   105   110

His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
        115   120   125

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
    130   135   140

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
145   150   155   160

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
165 170 175

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
180 185 190

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
195 200 205

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
210 215 220

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
225 230 235 240

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
245 250 255

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
260 265 270

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
275 280 285

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
290 295 300

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
305 310 315 320

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
325 330 335

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
340 345 350

Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
355 360 365

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
370 375 380

Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
385 390 395 400

Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro
405 410 415

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
420 425 430

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
        435   440   445

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
    450   455   460

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
465   470   475   480

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
                485   490   495

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
            500   505   510

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
        515   520   525

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
    530   535   540

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
545   550   555   560

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
                565   570   575

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
            580   585   590

Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
        595   600   605

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
    610   615   620

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
625   630   635   640

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
                645   650   655

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
            660   665   670

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
        675   680   685

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
    690   695   700

```
Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
705                 710                 715                 720

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
                725                 730                 735

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
            740                 745                 750

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro
        755                 760                 765

Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp
    770                 775                 780

Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys
785                 790                 795                 800

Ile Gln Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser
                805                 810                 815

Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr
            820                 825                 830

Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn
        835                 840                 845

Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly
    850                 855                 860

Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu
865                 870                 875                 880

Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys
                885                 890                 895

Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn
            900                 905                 910

Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met
        915                 920                 925

Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys
    930                 935                 940

Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu
945                 950                 955                 960

Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu
                965                 970                 975
```

Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
980 985 990

Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala
995 1000 1005

Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser
1010 1015 1020

Asn Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser
1025 1030 1035

Leu Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu
1040 1045 1050

Ser Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg
1055 1060 1065

Met Leu Met Asp Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met
1070 1075 1080

Ser Asn Lys Thr Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln
1085 1090 1095

Lys Lys Glu Gly Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met
1100 1105 1110

Ser Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile
1115 1120 1125

Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro
1130 1135 1140

Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu
1145 1150 1155

Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys
1160 1165 1170

Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro
1175 1180 1185

Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu
1190 1195 1200

Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu
1205 1210 1215

Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile
1220 1225 1230

His Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu
1235 1240 1245

Leu Ser Thr Arg Gln Asn Val Glu Gly Ser Tyr Glu Gly Ala Tyr
1250 1255 1260

Ala Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn
1265 1270 1275

Arg Thr Lys Lys His Thr Ala His Phe Ser Lys Lys Gly Glu Glu
1280 1285 1290

Glu Asn Leu Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu
1295 1300 1305

Lys Tyr Ala Cys Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln
1310 1315 1320

Asn Phe Val Thr Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg
1325 1330 1335

Leu Pro Leu Glu Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp
1340 1345 1350

Asp Thr Ser Thr Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro
1355 1360 1365

Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala
1370 1375 1380

Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser
1385 1390 1395

Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser
1400 1405 1410

Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe
1415 1420 1425

Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys
1430 1435 1440

Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys
1445 1450 1455

Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly
1460 1465 1470

Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser
1475 1480 1485

Val Thr Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp
1490 1495 1500

Leu Pro Lys Thr Ser Gly Lys Val Glu Leu Leu Pro Lys Val His
1505 1510 1515

Ile Tyr Gln Lys Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser
1520 1525 1530

Pro Gly His Leu Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr
1535 1540 1545

Glu Gly Ala Ile Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val
1550 1555 1560

Pro Phe Leu Arg Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser
1565 1570 1575

Lys Leu Leu Asp Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln
1580 1585 1590

Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys
1595 1600 1605

Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser Leu Asn Ala Cys
1610 1615 1620

Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys
1625 1630 1635

Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg
1640 1645 1650

Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu
1655 1660 1665

Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
1670 1675 1680

Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile
1685 1690 1695

Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys
1700 1705 1710

Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr
1715 1720 1725

Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser
1730 1735 1740

Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr
1745 1750 1755

Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu
1760 1765 1770

His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
1775 1780 1785

Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
1790 1795 1800

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly
1805 1810 1815

Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr
1820 1825 1830

Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu
1835 1840 1845

Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu
1850 1855 1860

Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His
1865 1870 1875

Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln
1880 1885 1890

Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp
1895 1900 1905

Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
1910 1915 1920

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His
1925 1930 1935

Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met
1940 1945 1950

Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser
1955 1960 1965

Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr
1970 1975 1980

Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr
1985 1990 1995

Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly
2000 2005 2010

Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
2015 2020 2025

Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro
2030 2035 2040

Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala
2045 2050 2055

Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His
2060 2065 2070

Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser
2075 2080 2085

Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile
2090 2095 2100

Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser
2105 2110 2115

Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr
2120 2125 2130

Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn
2135 2140 2145

Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
2150 2155 2160

Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg
2165 2170 2175

Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys
2180 2185 2190

Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln
2195 2200 2205

Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser
2210 2215 2220

Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
2225 2230 2235

Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe
2240 2245 2250

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Gln Lys  Thr Met Lys Val Thr  Gly Val Thr Thr Gln  Gly Val Lys
    2255                 2260                 2265

Ser Leu  Leu Thr Ser Met Tyr  Val Lys Glu Phe Leu  Ile Ser Ser
    2270                 2275                 2280

Ser Gln  Asp Gly His Gln Trp  Thr Leu Phe Phe Gln  Asn Gly Lys
    2285                 2290                 2295

Val Lys  Val Phe Gln Gly Asn  Gln Asp Ser Phe Thr  Pro Val Val
    2300                 2305                 2310

Asn Ser  Leu Asp Pro Pro Leu  Leu Thr Arg Tyr Leu  Arg Ile His
    2315                 2320                 2325

Pro Gln  Ser Trp Val His Gln  Ile Ala Leu Arg Met  Glu Val Leu
    2330                 2335                 2340

Gly Cys  Glu Ala Gln Asp Leu  Tyr
    2345                 2350

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<213>  Homo sapiens

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Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1               5                   10                  15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
            20                  25                  30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
        35                  40                  45

Thr Leu Phe Val Glu Phe Thr Val His Leu Phe Asn Ile Ala Lys Pro
    50                  55                  60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65                  70                  75                  80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85                  90                  95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
```

130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro

405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp

675 680 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
725 730 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
740 745 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
755 760 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
770 775 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785 790 795 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
805 810 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
820 825 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
835 840 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
850 855 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865 870 875 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
885 890 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
900 905 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
915 920 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
930 935 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp

945 950 955 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
965 970 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
980 985 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
995 1000 1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu
1010 1015 1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu
1025 1030 1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
1040 1045 1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr
1055 1060 1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly
1070 1075 1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys
1085 1090 1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His
1100 1105 1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln
1115 1120 1125

Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe
1130 1135 1140

Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr
1145 1150 1155

Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn
1160 1165 1170

Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His
1175 1180 1185

Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr
1190 1195 1200

Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr

1205 1210 1215

Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg
1220 1225 1230

Gln Asn Val Glu Gly Ser Tyr Glu Gly Ala Tyr Ala Pro Val Leu
1235 1240 1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys
1250 1255 1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu
1265 1270 1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys
1280 1285 1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr
1295 1300 1305

Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu
1310 1315 1320

Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr
1325 1330 1335

Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr
1340 1345 1350

Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser
1355 1360 1365

Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala
1370 1375 1380

Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser
1385 1390 1395

Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser
1400 1405 1410

Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val
1415 1420 1425

Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys Asn Asn Leu
1430 1435 1440

Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln Arg Glu
1445 1450 1455

Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr Lys

1460 1465 1470

Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr
1475 1480 1485

Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys
1490 1495 1500

Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu
1505 1510 1515

Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile
1520 1525 1530

Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg
1535 1540 1545

Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp
1550 1555 1560

Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu
1565 1570 1575

Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys
1580 1585 1590

Lys Lys Asp Thr Ile Leu Ser Leu Asn Ala Cys Glu Ser Asn His
1595 1600 1605

Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu
1610 1615 1620

Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg Leu Cys Ser Gln
1625 1630 1635

Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr
1640 1645 1650

Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile
1655 1660 1665

Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp
1670 1675 1680

Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr
1685 1690 1695

Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser
1700 1705 1710

Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro

1715 1720 1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe
1730 1735 1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu
1745 1750 1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
1760 1765 1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
1775 1780 1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
1790 1795 1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys
1805 1810 1815

Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
1820 1825 1830

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
1835 1840 1845

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu
1850 1855 1860

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu
1865 1870 1875

Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu
1880 1885 1890

Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu
1895 1900 1905

Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly
1910 1915 1920

Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln
1925 1930 1935

Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile
1940 1945 1950

His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
1955 1960 1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe

1970 1975 1980

Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val
1985 1990 1995

Glu Cys Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu
2000 2005 2010

Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala
2015 2020 2025

Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr
2030 2035 2040

Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser
2045 2050 2055

Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val
2060 2065 2070

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly
2075 2080 2085

Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile
2090 2095 2100

Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn
2105 2110 2115

Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser
2120 2125 2130

Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr
2135 2140 2145

Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
2150 2155 2160

Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu
2165 2170 2175

Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser
2180 2185 2190

Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
2195 2200 2205

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val
2210 2215 2220

Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met

2225 2230 2235

Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr
2240 2245 2250

Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly
2255 2260 2265

His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe
2270 2275 2280

Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp
2285 2290 2295

Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
2300 2305 2310

Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala
2315 2320 2325

Gln Asp Leu Tyr
2330

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<220>
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Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe
1 5 10 15

Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser
20 25 30

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
35 40 45

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
50 55 60

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
65 70 75 80

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
85 90 95

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
100 105 110

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His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
        115                 120                 125

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
    130                 135                 140

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
145                 150                 155                 160

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
                165                 170                 175

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
            180                 185                 190

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
        195                 200                 205

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
    210                 215                 220

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
225                 230                 235                 240

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
                245                 250                 255

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
            260                 265                 270

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
        275                 280                 285

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
    290                 295                 300

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
305                 310                 315                 320

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
                325                 330                 335

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
            340                 345                 350

Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
        355                 360                 365

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
    370                 375                 380
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Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
385                 390                 395                 400

Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro
                405                 410                 415

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
            420                 425                 430

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
        435                 440                 445

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
    450                 455                 460

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
465                 470                 475                 480

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
                485                 490                 495

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
            500                 505                 510

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
        515                 520                 525

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
    530                 535                 540

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
545                 550                 555                 560

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
                565                 570                 575

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
            580                 585                 590

Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
        595                 600                 605

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
    610                 615                 620

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
625                 630                 635                 640

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
                645                 650                 655
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Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
            660                 665                 670

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
        675                 680                 685

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
    690                 695                 700

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
705                 710                 715                 720

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
                725                 730                 735

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
            740                 745                 750

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Pro Pro Val Leu
        755                 760                 765

Lys Arg His Gln Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln
    770                 775                 780

Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu
785                 790                 795                 800

Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe
                805                 810                 815

Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp
            820                 825                 830

Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln
        835                 840                 845

Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr
    850                 855                 860

Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His
865                 870                 875                 880

Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile
                885                 890                 895

Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser
            900                 905                 910

Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
        915                 920                 925
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Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val
930 935 940

Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp
945 950 955 960

Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu
965 970 975

Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro Ala His
980 985 990

Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe
995 1000 1005

Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn
1010 1015 1020

Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys
1025 1030 1035

Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr
1040 1045 1050

Leu Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr
1055 1060 1065

Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe
1070 1075 1080

Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met
1085 1090 1095

Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met
1100 1105 1110

Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly
1115 1120 1125

Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser
1130 1135 1140

Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile Arg
1145 1150 1155

Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
1160 1165 1170

Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser
1175 1180 1185

```
Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val Asp Leu  Leu Ala Pro
    1190                 1195                 1200


Met Ile  Ile His Gly Ile Lys  Thr Gln Gly Ala Arg  Gln Lys Phe
    1205                 1210                 1215


Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile Met Tyr  Ser Leu Asp
    1220                 1225                 1230


Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn Ser Thr  Gly Thr Leu
    1235                 1240                 1245


Met Val  Phe Phe Gly Asn Val  Asp Ser Ser Gly Ile  Lys His Asn
    1250                 1255                 1260


Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr Ile Arg  Leu His Pro
    1265                 1270                 1275


Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg Met Glu  Leu Met Gly
    1280                 1285                 1290


Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu Gly Met  Glu Ser Lys
    1295                 1300                 1305


Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser Ser Tyr  Phe Thr Asn
    1310                 1315                 1320


Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala Arg Leu  His Leu Gln
    1325                 1330                 1335


Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val Asn Asn  Pro Lys Glu
    1340                 1345                 1350


Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met Lys Val  Thr Gly Val
    1355                 1360                 1365


Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr Ser Met  Tyr Val Lys
    1370                 1375                 1380


Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly His Gln  Trp Thr Leu
    1385                 1390                 1395


Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe Gln Gly  Asn Gln Asp
    1400                 1405                 1410


Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp Pro Pro  Leu Leu Thr
    1415                 1420                 1425


Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp Val His  Gln Ile Ala
    1430                 1435                 1440
```

```
Leu Arg  Met Glu Val Leu Gly  Cys Glu Ala Gln Asp  Leu Tyr
    1445                1450                1455


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<213>  Homo sapiens

<400>  4

Ser Phe Ser Gln
1


<210>  5
<211>  10
<212>  PRT
<213>  Homo sapiens

<400>  5

Asn Pro Pro Val Leu Lys Arg His Gln Arg
1               5                   10
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