LYOPHILIZED RECOMBINANT VWF FORMULATIONS

FIELD OF THE INVENTION

[0001] Generally, the invention relates to formulations of lyophilized recombinant VWF and methods for making a lyophilized composition comprising recombinant VWF.

BACKGROUND OF THE INVENTION

[0002] Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of VWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. VWF mediates initial platelet adhesion to the sub-endothelium of the damaged vessel wall. Only the larger multimers exhibit hemostatic activity. It is assumed that endothelial cells secrete large polymeric forms of VWF and those forms of VWF which have a low molecular weight (low molecular weight VWF) arise from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Pallade bodies of endothelial cells and liberated upon stimulation.

[0003] VWF is synthesized by endothelial cells and megakaryocytes as prepro-VWF that consists to a large extent of repeated domains. Upon cleavage of the signal peptide, pro-VWF dimerizes through disulfide linkages at its C-terminal region. The dimers serve as protomers for multimerization, which is governed by disulfide linkages between the free end termini. The assembly to multimers is followed by the proteolytic removal of the propeptide sequence (Leyte et al., Biochem. J. 274 (1991), 257-261).

[0004] The primary translation product predicted from the cloned cDNA of VWF is a 2813-residue precursor polypeptide (prepro-VWF). The prepro-VWF consists of a 22 amino acid signal peptide and a 741 amino acid propeptide, with the mature VWF comprising 2050 amino acids (Ruggeri Z.A., and Ware, J., FASEB J., 308-316 (1993)).

[0005] Defects in VWF are causal to Von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, and VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects

of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms, some being associated with the loss or the decrease of high molecular weight multimers. Von Willebrand syndrome type 2a (VWS-2A) is characterized by a loss of both intermediate and large multimers. VWS-2B is characterized by a loss of highest-molecular-weight multimers. Other diseases and disorders related to VWF are known in the art.

[0006] US. Patent Nos. 6,531,577, 7,166,709, and European Patent Application No. 04380188.5, describe plasma-derived VWF formulations. However, in addition to quantity and purity issues with plasma-derived VWF, there is also a risk of blood-born pathogens (e.g., viruses and Variant Creutzfeldt-Jakob disease (vCJD). Further, VWF is known to form aggregates during stress conditions.

[0007] Thus there exists a need in the art to develop a stable pharmaceutical formulation comprising recombinant VWF.

SUMMARY OF THE INVENTION

[0008] The present invention provides formulations useful for lyophilization of recombinant VWF, resulting in a highly stable pharmaceutical composition. The stable pharmaceutical composition is useful as a therapeutic agent in the treatment of individuals suffering from disorders or conditions that can benefit from the administration of recombinant VWF.

[0009] In one embodiment, a stable lyophilized pharmaceutical formulation of a recombinant von Willebrand Factor (rVWF) is provided comprising: (a) a rVWF; (b) one or more buffering agents; (c) one or more amino acids; (d) one or more stabilizing agents; and (e) one or more surfactants; the rVWF comprising a polypeptide selected from the group consisting of: a) the amino acid sequence set out in SEQ ID NO: 3; b) a biologically active analog, fragment or variant of a); c) a polypeptide encoded by the polynucleotide set out in SEQ ID NO: 1; d) a biologically active analog, fragment or variant of c); and e) a polypeptide encoded by a polynucleotide that hybridizes to the polynucleotide set out in SEQ ID NO: 1 under moderately stringent hybridization conditions; the buffer is comprising of a pH buffering agent in a range of about 0.1 mM to about 500 mM and the pH is in a range of about 2.0 to about 12.0; the amino acid is at a concentration of about 1 to about 500 mM; the stabilizing agent is at a concentration of about 0.1 to about 1000 mM; and the surfactant is at a concentration of about 0.01 g/L to about 0.5 g/L.

[0010] In another embodiment, the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3. In still another embodiment, the buffering agent is selected from the group consisting of citrate, glycine, histidine, HEPES, Tris and combinations of these agents. In yet another embodiment, the buffering agent is citrate. In various embodiments, the pH is in the range of about 6.0 to about 8.0, about 6.5 to about 7.5, or about 7.3. In another embodiment, the pH is about 7.3.

[0011] In another embodiment, the aforementioned amino acid is selected from the group consisting of glycine, histidine, proline, serine, alanine and arginine. In another embodiment, the amino acid is at a concentration range of about 0.5 mM to about 300 mM. In still another embodiment, the amino acid is glycine at a concentration of about 15 mM.

[0012] In one embodiment of the invention, the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate and the pH is about 7.3; and wherein the amino acid is glycine at a concentration of about 15 mM.

[0013] In still another embodiment of the invention, the aforementioned one or more stabilizing agents is selected from the group consisting of mannitol, lactose, sorbitol, xylitol, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose, gentiobiose, isomaltose, arabinose, glucosamine, fructose and combinations of these stabilizing agents. In one embodiment, the stabilizing agents are trehalose at a concentration of about 10 g/L mM and mannitol at a concentration of about 20 g/L.

[0014] In yet another embodiment of the invention, the aforementioned surfactant is selected from the group consisting of digitonin, Triton X-100, Triton X-114, TWEEN-20, TWEEN-80 and combinations of these surfactants. In still another embodiment, the surfactant is TWEEN-80 at about 0.01 g/L.

[0015] In another embodiment of the invention, the rVWF comprises amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate at a concentration of about 15 mM at about pH 7.3; wherein the amino acid is glycine at a concentration of about 15 mM; wherein the stabilizing agents are trehalose at a concentration of about 10 g/L and mannitol at a concentration of about 20 g/L.; and wherein the surfactant is TWEEN-80 at about 0.1 g/L.

BRIEF DESCRIPTION OF THE FIGURES

[0016] **Figure 1** shows ANCOVA analysis of pooled VWF:RCo activity in lots evaluated for stability (stored at 5°C ± 3°C).

[0017] **Figure 2** shows the increase in residual moisture in rVWF FDP stored at 5°C ± 3°C.

[0018] **Figure 3** shows the increase in residual moisture in rVWF FDP stored at 40°C ± 2°C.

DETAILED DESCRIPTION OF THE INVENTION

Definition of terms

[0019] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger, et al. (eds.), Springer Verlag (1991); and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991).

[0020] Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

[0021] It is noted here that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0022] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0023] The term "comprising," with respect to a peptide compound, means that a compound may include additional amino acids at either or both amino and carboxy termini of the given sequence. Of course, these additional amino acids should not significantly interfere with the activity of the compound. With respect to a composition of the instant invention, the term "comprising" means that a composition may include additional components. These additional components should not significantly interfere with the activity of the composition.

[0024] The term "pharmacologically active" means that a substance so described is determined to have activity that affects a medical parameter (e.g., but not limited to blood pressure, blood cell count, cholesterol level) or disease state (e.g., but not limited to cancer, autoimmune disorders).

[0025] As used herein the terms "express," "expressing" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g. the resulting protein, may also be said to be "expressed." An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means inside a cell. The term "extracellular" means outside a cell, such as a transmembrane protein. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

[0026] As used herein a "polypeptide" refers to a polymer composed of amino acid residues, structural variants, related naturally-occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds. Synthetic polypeptides are prepared, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides.

[0027] As used herein a "fragment" of a polypeptide is meant to refer to any portion of a polypeptide or protein smaller than the full-length polypeptide or protein expression product.

[0028] As used herein an “analog” refers to any of two or more polypeptides substantially similar in structure and having the same biological activity, but can have varying degrees of activity, to either the entire molecule, or to a fragment thereof. Analogs differ in the composition of their amino acid sequences based on one or more mutations involving substitution, deletion, insertionand/or addition of one or more amino acids for other amino acids. Substitutions can be conservative or non-conservative based on the physico-chemical or functional relatedness of the amino acid that is being replaced and the amino acid replacing it.

[0029] As used herein a "variant" refers to a polypeptide, protein or analog thereof that is modified to comprise additional chemical moieties not normally a part of the molecule. Such

moieties may modulate the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule and eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedure for coupling such moieties to a molecule are well known in the art. For example and without limitation, in one aspect the variant is a blood clotting factor having a chemical modification which confers a longer half-life in vivo to the protein. In various aspects, polypeptides are modified by glycosylation, pegylation, and/or polysialylation.

Recombinant VWF

[0030] The polynucleotide and amino acid sequences of prepro-VWF are set out in SEQ ID NO:1 and SEQ ID NO:2, respectively, and are available at GenBank Accession Nos. NM_000552 and NP_000543, respectively. The amino acid sequence corresponding to the mature VWF protein is set out in SEQ ID NO: 3 (corresponding to amino acids 764-2813 of the full length prepro-VWF amino acid sequence).

[0031] One form of useful rVWF has at least the property of in vivo-stabilizing, e.g. binding, of at least one Factor VIII (FVIII) molecule and having optionally a glycosylation pattern which is pharmacologically acceptable. Specific examples thereof include VWF without the A2 domain thus resistant to proteolysis (Lankhof et al., Thromb. Haemost. 77: 1008-1013, 1997), and a VWF fragment from Val 449 to Asn 730 including the glycoprotein lb-binding domain and binding sites for collagen and heparin (Pietu et al., Biochem. Biophys. Res. Commun. 164: 1339-1347, 1989). The determination of the ability of a VWF to stabilize at least one FVIII molecule is, in one aspect, carried out in VWF-deficient mammals according to methods known in the state in the art.

[0032] The rVWF of the present invention is produced by any method known in the art. One specific example is disclosed in WO86/06096 published on Oct. 23, 1986 and U.S. Patent Application No. 07/559,509, filed on Jul. 23, 1990, which is incorporated herein by reference with respect to the methods of producing recombinant VWF. Thus, methods are known in the art for (i) the production of recombinant DNA by genetic engineering, e.g. via reverse transcription of RNA and/or amplification of DNA, (ii) introducing recombinant DNA into procaryotic or eucaryotic cells by transfection, e.g. via electroporation or microinjection, (iii) cultivating the transformed cells, e.g. in a continuous or batchwise manner, (iv) expressing VWF, e.g. constitutively or upon induction, and (v) isolating the

VWF, e.g. from the culture medium or by harvesting the transformed cells, in order to (vi) obtain purified rVWF, e.g. via anion exchange chromatography or affinity chromatography. A recombinant VWF is, in one aspect, made in transformed host cells using recombinant DNA techniques well known in the art. For instance, sequences coding for the polypeptide could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule is, in another aspect, synthesized using chemical synthesis techniques, such as the phosphoramidate method. Also, in still another aspect, a combination of these techniques is used.

[0033] The invention also provides vectors encoding polypeptides of the invention in an appropriate host. The vector comprises the polynucleotide that encodes the polypeptide operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the polynucleotide is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation. The resulting vector having the polynucleotide therein is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

[0034] Any of a large number of available and well-known host cells are used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art, including, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all host cells are equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells include, without limitation, bacteria, yeast and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

[0035] Transformed host cells are cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art. Finally, the polypeptides are purified from culture media or the host cells themselves by methods well known in the art.

[0036] Depending on the host cell utilized to express a compound of the invention, carbohydrate (oligosaccharide) groups are optionally attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, in one aspect, confers acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). In other aspects, such sites are glycosylated by synthetic or semi-synthetic procedures known in the art.

[0037] Alternatively, the compounds are made by synthetic methods using, for example, solid phase synthesis techniques. Suitable techniques are well known in the art, and include those described in Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

Fragments, variants and analogs of VWF

[0038] Methods for preparing polypeptide fragments, variants or analogs are well-known in the art.

[0039] Fragments of a polypeptide are prepared using, without limitation, enzymatic cleavage (e.g., trypsin, chymotrypsin) and also using recombinant means to generate a polypeptide fragments having a specific amino acid sequence. Polypeptide fragments may be

generated comprising a region of the protein having a particular activity, such as a multimerization domain or any other identifiable VWF domain known in the art.

[0040] Methods of making polypeptide analogs are also well-known. Amino acid sequence analogs of a polypeptide can be substitutional, insertional, addition or deletion analogs. Deletion analogs, including fragments of a polypeptide, lack one or more residues of the native protein which are not essential for function or immunogenic activity. Insertional analogs involve the addition of, e.g., amino acid(s) at a non-terminal point in the polypeptide. This analog may include, for example and without limitation, insertion of an immunoreactive epitope or simply a single residue. Addition analogs, including fragments of a polypeptide, include the addition of one or more amino acids at either or both termini of a protein and include, for example, fusion proteins. Combinations of the aforementioned analogs are also contemplated.

[0041] Substitutional analogs typically exchange one amino acid of the wild-type for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide without the complete loss of other functions or properties. In one aspect, substitutions are conservative substitutions. "Conservative amino acid substitution" is substitution of an amino acid with an amino acid having a side chain or a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine).

[0042] In one aspect, analogs are substantially homologous or substantially identical to the recombinant VWF from which they are derived. Analogs include those which retain at least some of the biological activity of the wild-type polypeptide, e.g. blood clotting activity.

[0043] Polypeptide variants contemplated include, without limitation, polypeptides chemically modified by such techniques as ubiquitination, glycosylation, including polysialation, conjugation to therapeutic or diagnostic agents, labeling, covalent polymer attachment such as pegylation (derivatization with polyethylene glycol), introduction of non-hydrolyzable bonds, and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins. Variants retain the same or

essentially the same binding properties of non-modified molecules of the invention. Such chemical modification may include direct or indirect (e.g., via a linker) attachment of an agent to the VWF polypeptide. In the case of indirect attachment, it is contemplated that the linker may be hydrolyzable or non-hydrolyzable.

[0044] Preparing pegylated polypeptide analogs will in one aspect comprise the steps of (a) reacting the polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the binding construct polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions are determined based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product. In some embodiments, the binding construct has a single PEG moiety at the N-terminus. Polyethylene glycol (PEG) may be attached to the blood clotting factor to, for example, provide a longer half-life *in vivo*. The PEG group may be of any convenient molecular weight and is linear or branched. The average molecular weight of the PEG ranges from about 2 kiloDalton ("kD") to about 100 kDa, from about 5 kDa to about 50 kDa, or from about 5 kDa to about 10 kDa. In certain aspects, the PEG groups are attached to the blood clotting factor via acylation or reductive alkylation through a natural or engineered reactive group on the PEG moiety (e.g., an aldehyde, amino, thiol, or ester group) to a reactive group on the blood clotting factor (e.g., an aldehyde, amino, or ester group) or by any other technique known in the art.

[0045] Methods for preparing polysialylated polypeptide are described in United States Patent Publication 20060160948, Fernandes et Gregoriadis; Biochim. Biophys. Acta 1341: 26-34, 1997, and Saenko et al., Haemophilia 12:42-51, 2006. Briefly, a solution of colominic acid (CA) containing 0.1 M NaIO4 is stirred in the dark at room temperature to oxidize the CA. The activated CA solution is dialyzed against, e.g., 0.05 M sodium phosphate buffer, pH 7.2 in the dark and this solution was added to a rVWF solution and incubated for 18 h at room temperature in the dark under gentle shaking. Free reagents are optionally be separated from the rVWF-polysialic acid conjugate by, for example, ultrafiltration/diafiltration. Conjugation of rVWF with polysialic acid is achieved using glutaraldehyde as cross-linking reagent (Migneault et al., Biotechniques 37: 790-796, 2004).

[0046] It is further contemplated in another aspect that a polypeptide of the invention is a fusion protein with a second agent which is a polypeptide. In one embodiment, the second agent which is a polypeptide, without limitation, is an enzyme, a growth factor, an antibody, a

cytokine, a chemokine, a cell-surface receptor, the extracellular domain of a cell surface receptor, a cell adhesion molecule, or fragment or active domain of a protein described above. In a related embodiment, the second agent is a blood clotting factor such as Factor VIII, Factor VII, Factor IX. The fusion protein contemplated is made by chemical or recombinant techniques well-known in the art.

[0047] It is also contemplated in another aspect that prepro-VWF and pro-VWF polypeptides will provide a therapeutic benefit in the formulations of the present invention. For example, US Patent No. 7,005,502 describes a pharmaceutical preparation comprising substantial amounts of pro-VWF that induces thrombin gerneation in vitro. In addition to recombinant, biologically active fragments, variants, or other analogs of the naturally-occuring mature VWF, the present invention contemplates the use of recombinant biologically active fragments, variants, or analogs of the prepro-VWF (set out in SEQ ID NO:2) or pro-VWF polypeptides (amino acid residues 23 to 764 of SEQ ID NO: 2) in the formulations described herein.

[0048] Polynucleotides encoding fragments, variants and analogs may be readily generated by a worker of skill to encode biologically active fragments, variants, or analogs of the naturally-occurring molecule that possess the same or similar biological activity to the naturally-occurring molecule. In various aspects, these polynucleotides are prepared using PCR techniques, digestion/ligation of DNA encoding molecule, and the like. Thus, one of skill in the art will be able to generate single base changes in the DNA strand to result in an altered codon and a missense mutation, using any method known in the art, including, but not limited to site-specific mutagenesis. As used herein, the phrase "moderately stringent hybridization conditions" means, for example, hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook et al., 9.47-9.51 in Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Lyophilization

[0049] In one aspect, the formulations comprising a VWF polypeptide of the invention are lyophilized prior to administration. Lyophilization is carried out using techniques common in

the art and should be optimized for the composition being developed [Tang et al., Pharm Res. 21:191-200, (2004) and Chang et al., Pharm Res. 13:243-9 (1996)].

[0050] A lyophilization cycle is, in one aspect, composed of three steps: freezing, primary drying, and secondary drying [A.P. Mackenzie, Phil Trans R Soc London, Ser B, Biol 278:167 (1977)]. In the freezing step, the solution is cooled to initiate ice formation. Furthermore, this step induces the crystallization of the bulking agent. The ice sublimes in the primary drying stage, which is conducted by reducing chamber pressure below the vapor pressure of the ice, using a vacuum and introducing heat to promote sublimation. Finally, adsorbed or bound water is removed at the secondary drying stage under reduced chamber pressure and at an elevated shelf temperature. The process produces a material known as a lyophilized cake. Thereafter the cake can be reconstituted with either sterile water or suitable diluent for injection.

[0051] The lyophilization cycle not only determines the final physical state of excipients but also affects other parameters such as reconstitution time, appearance, stability and final moisture content. The composition structure in the frozen state proceeds through several transitions (e.g., glass transitions, wettings, and crystallizations) that occur at specific temperatures and the structure may be used to understand and optimize the lyophilization process. The glass transition temperature (Tg and/or Tg') can provide information about the physical state of a solute and can be determined by differential scanning calorimetry (DSC). Tg and Tg' are an important parameter that must be taken into account when designing the lyophilization cycle. For example, Tg' is important for primary drying. Furthermore, in the dried state, the glass transition temperature provides information on the storage temperature of the final product.

Formulations and excipients in general

[0052] Excipients are additives that either impart or enhance the stability and delivery of a drug product (e.g., protein). Regardless of the reason for their inclusion, excipients are an integral component of a formulation and therefore need to be safe and well tolerated by patients. For protein drugs, the choice of excipients is particularly important because they can affect both efficacy and immunogenicity of the drug. Hence, protein formulations need to be developed with appropriate selection of excipients that afford suitable stability, safety, and marketability.

[0053] A lyophilized formulation is, in one aspect, at least comprised of one or more of a buffer, a bulking agent, and a stabilizer. In this aspect, the utility of a surfactant is evaluated and selected in cases where aggregation during the lyophilization step or during reconstitution becomes an issue. An appropriate buffering agent is included to maintain the formulation within stable zones of pH during lyophilization. A comparison of the excipient components contemplated for liquid and lyophilized protein formulations is provided in Table A.

[0054]

Table A: Excipient components of lyophilized protein formulations

Excipient component	Function in lyophilized formulation
Buffer	○ Maintain pH of formulation during lyophilization and upon reconstitution
Tonicity agent/ stabilizer	○ Stabilizers include cryo and lyoprotectants ○ Examples include Polyols, sugars and polymers ○ Cryoprotectants protect proteins from freezing stresses ○ Lyoprotectants stabilize proteins in the freeze-dried state
Bulking agent	○ Used to enhance product elegance and to prevent blowout ○ Provides structural strength to the lyo cake ○ Examples include mannitol and glycine
Surfactant	○ Employed if aggregation during the lyophilization process is an issue ○ May serve to reduce reconstitution times ○ Examples include polysorbate 20 and 80
Anti-oxidant	○ Usually not employed, molecular reactions in the lyo cake are greatly retarded
Metal ions/chelating agent	○ May be included if a specific metal ion is included only as a co-factor or where the metal is required for protease activity ○ Chelating agents are generally not needed in lyo formulations
Preservative	○ For multi-dose formulations only ○ Provides protection against microbial growth in formulation ○ Is usually included in the reconstitution diluent (e.g. bWFI)

[0055] The principal challenge in developing formulations for proteins is stabilizing the product against the stresses of manufacturing, shipping and storage. The role of formulation

excipients is to provide stabilization against these stresses. Excipients are also be employed to reduce viscosity of high concentration protein formulations in order to enable their delivery and enhance patient convenience. In general, excipients can be classified on the basis of the mechanisms by which they stabilize proteins against various chemical and physical stresses. Some excipients are used to alleviate the effects of a specific stress or to regulate a particular susceptibility of a specific protein. Other excipients have more general effects on the physical and covalent stabilities of proteins. The excipients described herein are organized either by their chemical type or their functional role in formulations. Brief descriptions of the modes of stabilization are provided when discussing each excipient type.

[0056] Given the teachings and guidance provided herein, those skilled in the art will know what amount or range of excipient can be included in any particular formulation to achieve a biopharmaceutical formulation of the invention that promotes retention in stability of the biopharmaceutical (e.g., a protein). For example, the amount and type of a salt to be included in a biopharmaceutical formulation of the invention is selected based on the desired osmolality (i.e., isotonic, hypotonic or hypertonic) of the final solution as well as the amounts and osmolality of other components to be included in the formulation.

[0057] By way of example, inclusion of about 5% sorbitol can achieve isotonicity while about 9% of a sucrose excipient is needed to achieve isotonicity. Selection of the amount or range of concentrations of one or more excipients that can be included within a biopharmaceutical formulation of the invention has been exemplified above by reference to salts, polyols and sugars. However, those skilled in the art will understand that the considerations described herein and further exemplified by reference to specific excipients are equally applicable to all types and combinations of excipients including, for example, salts, amino acids, other tonicity agents, surfactants, stabilizers, bulking agents, cryoprotectants, lyoprotectants, anti-oxidants, metal ions, chelating agents and/or preservatives.

[0058] Further, where a particular excipient is reported in molar concentration, those skilled in the art will recognize that the equivalent percent (%) w/v (e.g., (grams of substance in a solution sample/mL of solution) X 100%) of solution is also contemplated.

[0059] Of course, a person having ordinary skill in the art would recognize that the concentrations of the excipients described herein share an interdependency within a particular formulation. By way of example, the concentration of a bulking agent may be lowered

where, e.g., there is a high protein concentration or where, e.g., there is a high stabilizing agent concentration. In addition, a person having ordinary skill in the art would recognize that, in order to maintain the isotonicity of a particular formulation in which there is no bulking agent, the concentration of a stabilizing agent would be adjusted accordingly (i.e., a "tonicifying" amount of stabilizer would be used). Common excipients are known in the art and can be found in Powell et al., Compendium of Excipients fir Parenteral Formulations (1998), PDA J. Pharm. Sci. Technology, 52:238-311.

Buffers and buffering agents

[0060] The stability of a pharmacologically active protein formulation is usually observed to be maximal in a narrow pH range. This pH range of optimal stability needs to be identified early during pre-formulation studies. Several approaches, such as accelerated stability studies and calorimetric screening studies, are useful in this endeavor (Remmele R.L. Jr., et al., *Biochemistry*, 38(16): 5241-7 (1999)). Once a formulation is finalized, the protein must be manufactured and maintained throughout its shelf-life. Hence, buffering agents are almost always employed to control pH in the formulation.

[0061] The buffer capacity of the buffering species is maximal at a pH equal to the pKa and decreases as pH increases or decreases away from this value. Ninety percent of the buffering capacity exists within one pH unit of its pKa. Buffer capacity also increases proportionally with increasing buffer concentration.

[0062] Several factors need to be considered when choosing a buffer. First and foremost, the buffer species and its concentration need to be defined based on its pKa and the desired formulation pH. Equally important is to ensure that the buffer is compatible with the protein and other formulation excipients, and does not catalyze any degradation reactions. A third important aspect to be considered is the sensation of stinging and irritation the buffer may induce upon administration. For example, citrate is known to cause stinging upon injection (Laursen T, et al., *Basic Clin Pharmacol Toxicol.*, 98(2): 218-21 (2006)). The potential for stinging and irritation is greater for drugs that are administered via the subcutaneous (SC) or intramuscular (IM) routes, where the drug solution remains at the site for a relatively longer period of time than when administered by the IV route where the formulation gets diluted rapidly into the blood upon administration. For formulations that are administered by direct IV infusion, the total amount of buffer (and any other formulation component) needs to be monitored. One has to be particularly careful about potassium ions administered in the form

of the potassium phosphate buffer, which can induce cardiovascular effects in a patient (Hollander-Rodriguez JC, et al., *Am. Fam. Physician.*, 73(2): 283-90 (2006)).

[0063] Buffers for lyophilized formulations need additional consideration. Some buffers like sodium phosphate can crystallize out of the protein amorphous phase during freezing resulting in shifts in pH. Other common buffers such as acetate and imidazole may sublime or evaporate during the lyophilization process, thereby shifting the pH of formulation during lyophilization or after reconstitution.

[0064] The buffer system present in the compositions is selected to be physiologically compatible and to maintain a desired pH of the pharmaceutical formulation. In one embodiment, the pH of the solution is between pH 2.0 and pH 12.0. For example, the pH of the solution may be 2.0, 2.3, 2.5, 2.7, 3.0, 3.3, 3.5, 3.7, 4.0, 4.3, 4.5, 4.7, 5.0, 5.3, 5.5, 5.7, 6.0, 6.3, 6.5, 6.7, 7.0, 7.3, 7.5, 7.7, 8.0, 8.3, 8.5, 8.7, 9.0, 9.3, 9.5, 9.7, 10.0, 10.3, 10.5, 10.7, 11.0, 11.3, 11.5, 11.7, or 12.0.

[0065] The pH buffering compound may be present in any amount suitable to maintain the pH of the formulation at a predetermined level. In one embodiment, the pH buffering concentration is between 0.1 mM and 500 mM (1 M). For example, it is contemplated that the pH buffering agent is at least 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 200, or 500 mM.

[0066] Exemplary pH buffering agents used to buffer the formulation as set out herein include, but are not limited to organic acids, glycine, histidine, glutamate, succinate, phosphate, acetate, citrate, Tris, HEPES, and amino acids or mixtures of amino acids, including, but not limited to aspartate, histidine, and glycine. In one embodiment of the present invention, the buffering agent is citrate.

Stabilizers and bulking agents

[0067] In one aspect of the present pharmaceutical formulations, a stabilizer (or a combination of stabilizers) is added to prevent or reduce storage-induced aggregation and chemical degradation. A hazy or turbid solution upon reconstitution indicates that the protein has precipitated or at least aggregated. The term "stabilizer" means an excipient capable of preventing aggregation or physical degradation, including chemical degradation (for example, autolysis, deamidation, oxidation, etc.) in an aqueous state. Stabilizers contemplated include, but are not limited to, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose, gentiobiose, isomaltose, arabinose, glucosamine, fructose,

mannitol, sorbitol, glycine, arginine HCL, poly-hydroxy compounds, including polysaccharides such as dextran, starch, hydroxyethyl starch, cyclodextrins, N-methyl pyrollidene, cellulose and hyaluronic acid, sodium chloride, [Carpenter et al., Develop. Biol. Standard 74:225, (1991)]. In the present formulations, the stabilizer is incorporated in a concentration of about 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 500, 700, 900, or 1000 mM. In one embodiment f the present invention, mannitol and trehalose are used as stabilizing agents.

[0068] If desired, the formulations also include appropriate amounts of bulking and osmolarity regulating agents. Bulking agents include, for example and without limitation, mannitol, glycine, sucrose, polymers such as dextran, polyvinylpyrolidone, carboxymethylcellulose, lactose, sorbitol, trehalose, or xylitol. In one embodiment, the bulking agent is mannitol. The bulking agent is incorporated in a concentration of about 0.1, 0.5, 0.7, 0.8 0.9, 1.0, 1.2, 1.5, 1.7, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 500, 700, 900, or 1000 mM.

Surfactants

[0069] Proteins have a high propensity to interact with surfaces making them susceptible to adsorption and denaturation at air-liquid, vial-liquid, and liquid-liquid (silicone oil) interfaces. This degradation pathway has been observed to be inversely dependent on protein concentration and results in either the formation of soluble and insoluble protein aggregates or the loss of protein from solution via adsorption to surfaces. In addition to container surface adsorption, surface-induced degradation is exacerbated with physical agitation, as would be experienced during shipping and handling of the product.

[0070] Surfactants are commonly used in protein formulations to prevent surface-induced degradation. Surfactants are amphipathic molecules with the capability of out-competing proteins for interfacial positions. Hydrophobic portions of the surfactant molecules occupy interfacial positions (e.g., air/liquid), while hydrophilic portions of the molecules remain oriented towards the bulk solvent. At sufficient concentrations (typically around the detergent's critical micellar concentration), a surface layer of surfactant molecules serve to prevent protein molecules from adsorbing at the interface. Thereby, surface-induced degradation is minimized. Surfactants contemplated herein include, without limitation, fatty acid esters of sorbitan polyethoxylates, i.e. polysorbate 20 and polysorbate 80. The two differ

only in the length of the aliphatic chain that imparts hydrophobic character to the molecules, C-12 and C-18, respectively. Accordingly, polysorbate-80 is more surface-active and has a lower critical micellar concentration than polysorbate-20.

[0071] Detergents can also affect the thermodynamic conformational stability of proteins. Here again, the effects of a given detergent excipient will be protein specific. For example, polysorbates have been shown to reduce the stability of some proteins and increase the stability of others. Detergent destabilization of proteins can be rationalized in terms of the hydrophobic tails of the detergent molecules that can engage in specific binding with partially or wholly unfolded protein states. These types of interactions could cause a shift in the conformational equilibrium towards the more expanded protein states (i.e. increasing the exposure of hydrophobic portions of the protein molecule in complement to binding polysorbate). Alternatively, if the protein native state exhibits some hydrophobic surfaces, detergent binding to the native state may stabilize that conformation.

[0072] Another aspect of polysorbates is that they are inherently susceptible to oxidative degradation. Often, as raw materials, they contain sufficient quantities of peroxides to cause oxidation of protein residue side-chains, especially methionine. The potential for oxidative damage arising from the addition of stabilizer emphasizes the point that the lowest effective concentrations of excipients should be used in formulations. For surfactants, the effective concentration for a given protein will depend on the mechanism of stabilization.

[0073] Surfactants are also added in appropriate amounts to prevent surface related aggregation phenomenon during freezing and drying [Chang, B, J. Pharm. Sci. 85:1325, (1996)]. Thus, exemplary surfactants include, without limitation, anionic, cationic, nonionic, zwitterionic, and amphoteric surfactants including surfactants derived from naturally-occurring amino acids. Anionic surfactants include, but are not limited to, sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate, chenodeoxycholic acid, N-lauroylsarcosine sodium salt, lithium dodecyl sulfate, 1-octanesulfonic acid sodium salt, sodium cholate hydrate, sodium deoxycholate, and glycodeoxycholic acid sodium salt. Cationic surfactants include, but are not limited to, benzalkonium chloride or benzethonium chloride, cetylpyridinium chloride monohydrate, and hexadecyltrimethylammonium bromide. Zwitterionic surfactants include, but are not limited to, CHAPS, CHAPSO, SB3-10, and SB3-12. Non-ionic surfactants include, but are not limited to, digitonin, Triton X-100, Triton X-114, TWEEN-20, and TWEEN-80. Surfactants also include, but are not limited to lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 40, 50

and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, soy lecithin and other phospholipids such as dioleyl phosphatidyl choline (DOPC), dimyristoylphosphatidyl glycerol (DMPG), dimyristoylphosphatidyl choline (DMPC), and (dioleyl phosphatidyl glycerol) DOPG; sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. Compositions comprising these surfactants, either individually or as a mixture in different ratios, are therefore further provided. In one embodiment of the present invention, the surfactant is TWEEN-80. In the present formulations, the surfactant is incorporated in a concentration of about 0.01 to about 0.5 g/L. In formulations provided, the surfactant concentration is 0.005, 0.01, 0.02, 0.03, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 g/L.

Salts

[0074] Salts are often added to increase the ionic strength of the formulation, which can be important for protein solubility, physical stability, and isotonicity. Salts can affect the physical stability of proteins in a variety of ways. Ions can stabilize the native state of proteins by binding to charged residues on the protein's surface. Alternatively, salts can stabilize the denatured state by binding to peptide groups along the protein backbone (-CONH-). Salts can also stabilize the protein native conformation by shielding repulsive electrostatic interactions between residues within a protein molecule. Salts in protein formulations can also shield attractive electrostatic interactions between protein molecules that can lead to protein aggregation and insolubility. In formulations provided, the salt concentration is between 0.1, 1, 10, 20, 30, 40, 50, 80, 100, 120, 150, 200, 300, and 500 mM.

Other common excipient components

Amino acids

[0075] Amino acids have found versatile use in protein formulations as buffers, bulking agents, stabilizers and antioxidants. Thus, in one aspect histidine and glutamic acid are employed to buffer protein formulations in the pH range of 5.5 – 6.5 and 4.0 – 5.5 respectively. The imidazole group of histidine has a pKa = 6.0 and the carboxyl group of glutamic acid side chain has a pKa of 4.3 which makes these amino acids suitable for buffering in their respective pH ranges. Glutamic acid is particularly useful in such cases. Histidine is commonly found in marketed protein formulations, and this amino acid provides an alternative to citrate, a buffer known to sting upon injection. Interestingly, histidine has also been reported to have a stabilizing effect, with respect to aggregation when used at high

concentrations in both liquid and lyophilized presentations (Chen B, et al., *Pharm Res.*, 20(12): 1952-60 (2003)). Histidine was also observed by others to reduce the viscosity of a high protein concentration formulation. However, in the same study, the authors observed increased aggregation and discoloration in histidine containing formulations during freeze-thaw studies of the antibody in stainless steel containers. Another note of caution with histidine is that it undergoes photo-oxidation in the presence of metal ions (Tomita M, et al., *Biochemistry*, 8(12): 5149-60 (1969)). The use of methionine as an antioxidant in formulations appears promising; it has been observed to be effective against a number of oxidative stresses (Lam XM, et al., *J Pharm Sci.*, 86(11): 1250-5 (1997)).

[0076] In various aspects, formulations are provided which include one or more of the amino acids glycine, proline, serine, arginine and alanine have been shown to stabilize proteins by the mechanism of preferential exclusion. Glycine is also a commonly used bulking agent in lyophilized formulations. Arginine has been shown to be an effective agent in inhibiting aggregation and has been used in both liquid and lyophilized formulations.

[0077] In formulations provided, the amino acid concentration is between 0.1, 1, 10, 20, 30, 40, 50, 80, 100, 120, 150, 200, 300, and 500 mM. In one embodiment of the present invention, the amino acid is glycine.

Antioxidants

[0078] Oxidation of protein residues arises from a number of different sources. Beyond the addition of specific antioxidants, the prevention of oxidative protein damage involves the careful control of a number of factors throughout the manufacturing process and storage of the product such as atmospheric oxygen, temperature, light exposure, and chemical contamination. The invention therefore contemplates the use of the pharmaceutical antioxidants including, without limitation, reducing agents, oxygen/free-radical scavengers, or chelating agents. Antioxidants in therapeutic protein formulations are, in one aspect, water-soluble and remain active throughout the product shelf-life. Reducing agents and oxygen/free-radical scavengers work by ablating active oxygen species in solution. Chelating agents such as EDTA are effective by binding trace metal contaminants that promote free-radical formation. For example, EDTA was utilized in the liquid formulation of acidic fibroblast growth factor to inhibit the metal ion catalyzed oxidation of cysteine residues.

[0079] In addition to the effectiveness of various excipients to prevent protein oxidation, the potential for the antioxidants themselves to induce other covalent or physical changes to

the protein is of concern. For example, reducing agents can cause disruption of intramolecular disulfide linkages, which can lead to disulfide shuffling. In the presence of transition metal ions, ascorbic acid and EDTA have been shown to promote methionine oxidation in a number of proteins and peptides (Akers MJ, and Defelippis MR. Peptides and Proteins as Parenteral Solutions. In: Pharmaceutical Formulation Development of Peptides and Proteins. Sven Frokjaer, Lars Hovgaard, editors. Pharmaceutical Science. Taylor and Francis, UK (1999)); Fransson J.R., *J. Pharm. Sci.* 86(9): 4046-1050 (1997); Yin J, et al., *Pharm Res.*, 21(12): 2377-83 (2004)). Sodium thiosulfate has been reported to reduce the levels of light and temperature induced methionine-oxidation in rhuMab HER2; however, the formation of a thiosulfate-protein adduct was also reported in this study (Lam XM, Yang JY, et al., *J Pharm Sci.* 86(11): 1250-5 (1997)). Selection of an appropriate antioxidant is made according to the specific stresses and sensitivities of the protein. Antioxidants contemplated in certain aspects include, without limitation, reducing agents and oxygen/free-radical scavengers, EDTA, and sodium thiosulfate.

Metal ions

[0080] In general, transition metal ions are undesired in protein formulations because they can catalyze physical and chemical degradation reactions in proteins. However, specific metal ions are included in formulations when they are co-factors to proteins and in suspension formulations of proteins where they form coordination complexes (e.g., zinc suspension of insulin). Recently, the use of magnesium ions (10 –120 mM) has been proposed to inhibit the isomerization of aspartic acid to isoaspartic acid (WO 2004039337).

[0081] Two examples where metal ions confer stability or increased activity in proteins are human deoxyribonuclease (rhDNase, Pulmozyme®), and Factor VIII. In the case of rhDNase, Ca^{+2} ions (up to 100 mM) increased the stability of the enzyme through a specific binding site (Chen B, et al., *J Pharm Sci.*, 88(4): 477-82 (1999)). In fact, removal of calcium ions from the solution with EGTA caused an increase in deamidation and aggregation. However, this effect was observed only with Ca^{+2} ions; other divalent cations Mg^{+2}, Mn^{+2} and Zn^{+2} were observed to destabilize rhDNase. Similar effects were observed in Factor VIII. Ca^{+2} and Sr^{+2} ions stabilized the protein while others like Mg^{+2}, Mn^{+2} and Zn^{+2}, Cu^{+2} and Fe^{+2} destabilized the enzyme (Fatouros, A., et al., *Int. J. Pharm.*, 155, 121–131 (1997). In a separate study with Factor VIII, a significant increase in aggregation rate was observed in the presence of Al^{+3} ions (Derrick TS, et al., *J. Pharm. Sci.*, 93(10): 2549-57 (2004)). The authors note that

other excipients like buffer salts are often contaminated with Al^{+3} ions and illustrate the need to use excipients of appropriate quality in formulated products.

Preservatives

[0082] Preservatives are necessary when developing multi-use parenteral formulations that involve more than one extraction from the same container. Their primary function is to inhibit microbial growth and ensure product sterility throughout the shelf-life or term of use of the drug product. Commonly used preservatives include, without limitation, benzyl alcohol, phenol and m-cresol. Although preservatives have a long history of use, the development of protein formulations that includes preservatives can be challenging. Preservatives almost always have a destabilizing effect (aggregation) on proteins, and this has become a major factor in limiting their use in multi-dose protein formulations (Roy S, et al., J Pharm Sci., 94(2): 382-96 (2005)).

[0083] To date, most protein drugs have been formulated for single-use only. However, when multi-dose formulations are possible, they have the added advantage of enabling patient convenience, and increased marketability. A good example is that of human growth hormone (hGH) where the development of preserved formulations has led to commercialization of more convenient, multi-use injection pen presentations. At least four such pen devices containing preserved formulations of hGH are currently available on the market. Norditropin® (liquid, Novo Nordisk), Nutropin AQ® (liquid, Genentech) & Genotropin (lyophilized – dual chamber cartridge, Pharmacia & Upjohn) contain phenol while Somatrope® (Eli Lilly) is formulated with m-cresol.

[0084] Several aspects need to be considered during the formulation development of preserved dosage forms. The effective preservative concentration in the drug product must be optimized. This requires testing a given preservative in the dosage form with concentration ranges that confer anti-microbial effectiveness without compromising protein stability. For example, three preservatives were successfully screened in the development of a liquid formulation for interleukin-1 receptor (Type I), using differential scanning calorimetry (DSC). The preservatives were rank ordered based on their impact on stability at concentrations commonly used in marketed products (Remmele RL Jr., et al., *Pharm Res.*, 15(2): 200-8 (1998)).

[0085] Development of liquid formulations containing preservatives are more challenging than lyophilized formulations. Freeze-dried products can be lyophilized without the

preservative and reconstituted with a preservative containing diluent at the time of use. This shortens the time for which a preservative is in contact with the protein significantly minimizing the associated stability risks. With liquid formulations, preservative effectiveness and stability have to be maintained over the entire product shelf-life (~ 18 –24 months). An important point to note is that preservative effectiveness has to be demonstrated in the final formulation containing the active drug and all excipient components.

[0086] Some preservatives can cause injection site reactions, which is another factor that needs consideration when choosing a preservative. In clinical trials that focused on the evaluation of preservatives and buffers in Norditropin, pain perception was observed to be lower in formulations containing phenol and benzyl alcohol as compared to a formulation containing m-cresol (Kappelgaard A.M., Horm Res. 62 Suppl 3:98-103 (2004)). Interestingly, among the commonly used preservative, benzyl alcohol possesses anesthetic properties (Minogue SC, and Sun DA., *Anesth Analg.*, 100(3): 683-6 (2005)). In various aspects the use of preservatives provide a benefit that outweighs any side effects.

Methods of Preparation

[0087] The present invention further contemplates methods for the preparation of pharmaceutical formulations.

[0088] The present methods further comprise one or more of the following steps: adding a stabilizing agent as described herein to said mixture prior to lyophilizing, adding at least one agent selected from a bulking agent, an osmolarity regulating agent, and a surfactant, each of which as described herein, to said mixture prior to lyophilization.

[0089] The standard reconstitution practice for lyophilized material is to add back a volume of pure water or sterile water for injection (WFI) (typically equivalent to the volume removed during lyophilization), although dilute solutions of antibacterial agents are sometimes used in the production of pharmaceuticals for parenteral administration [Chen, Drug Development and Industrial Pharmacy, 18:1311-1354 (1992)]. Accordingly, methods are provided for preparation of reconstituted rVWF compositions comprising the step of adding a diluent to a lyophilized rVWFcomposition of the invention.

[0090] The lyophilized material may be reconstituted as an aqueous solution. A variety of aqueous carriers, e.g., sterile water for injection, water with preservatives for multi dose use, or water with appropriate amounts of surfactants (for example, an aqueous suspension that contains the active compound in admixture with excipients suitable for the manufacture of

aqueous suspensions). In various aspects, such excipients are suspending agents, for example and without limitation, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents are a naturally-occurring phosphatide, for example and without limitation, lecithin, or condensation products of an alkylene oxide with fatty acids, for example and without limitation, polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example and without limitation, heptadecaethyl-eneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example and without limitation, polyethylene sorbitan monooleate. In various aspects, the aqueous suspensions also contain one or more preservatives, for example and without limitation, ethyl, or n-propyl, p-hydroxybenzoate.

Administration

[0091] To administer compositions to human or test animals, in one aspect, the compositions comprises one or more pharmaceutically acceptable carriers. The phrases "pharmaceutically" or "pharmacologically" acceptable refer to molecular entities and compositions that are stable, inhibit protein degradation such as aggregation and cleavage products, and in addition do not produce allergic, or other adverse reactions when administered using routes well-known in the art, as described below. "Pharmaceutically acceptable carriers" include any and all clinically useful solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like, including those agents disclosed above.

[0092] The pharmaceutical formulations are administered orally, topically, transdermally, parenterally, by inhalation spray, vaginally, rectally, or by intracranial injection. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intracisternal injection, or infusion techniques. Administration by intravenous, intradermal, intramusclar, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary injection and or surgical implantation at a particular site is contemplated as well. Generally, compositions are essentially free of pyrogens, as well as other impurities that could be harmful to the recipient.

[0093] Single or multiple administrations of the compositions are carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage depends on the type of disease to be treated, as defined above, the severity and course of the disease, whether drug is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the drug, and the discretion of the attending physician.

Kits

[0094] As an additional aspect, the invention includes kits which comprise one or more lyophilized compositions packaged in a manner which facilitates their use for administration to subjects. In one embodiment, such a kit includes pharmaceutical formulation described herein (e.g., a composition comprising a therapeutic protein or peptide), packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. In one embodiment, the pharmaceutical formulation is packaged in the container such that the amount of headspace in the container (e.g., the amount of air between the liquid formulation and the top of the container) is very small. Preferably, the amount of headspace is negligible (i.e., almost none). In one embodiment, the kit contains a first container having a therapeutic protein or peptide composition and a second container having a physiologically acceptable reconstitution solution for the composition. In one aspect, the pharmaceutical formulation is packaged in a unit dosage form. The kit may further include a device suitable for administering the pharmaceutical formulation according to a specific route of administration. Preferably, the kit contains a label that describes use of the pharmaceutical formulations.

Dosages

[0095] The dosage regimen involved in a method for treating a condition described herein will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. By way of example, a typical dose of a recombinant VWF of the present invention is approximately 50 U/kg, equal to 500 μg/kg.

[0096] In one aspect, formulations of the invention are administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. As another example, the inventive compound is administered as a one-time dose. Those of

ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing depends on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation is determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose is calculated according to body weight, body surface area or organ size. Appropriate dosages may be ascertained through use of established assays for determining blood level dosages in conjunction with appropriate dose-response data. The final dosage regimen is determined by the attending physician, considering various factors which modify the action of drugs, e.g. the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

[0097] The following examples are not intended to be limiting but only exemplary of specific embodiments of the invention.

Example 1

Shaking experiments

[0098] In order to determine the amount of precipitation of rVWF in various formulations, the extent of aggregation of rVWF following turbulent shaking was tested under a variety of conditions.

[0099] As shown in Table 1 below, various rVWF formulations were assessed in a 20 mM citrate buffer, pH 7.3. Shaking experiments were designed to simulate mechanical stress conditions. 1-2 ml of each formulation was shaken with a laboratory shaker for 10 minutes at 1200 rpm.

TABLE 1

Lyo 25	Lysine	Histidine	Glycine	Serine	Mannitol	PEG 1500	Tween 80	Sucrose	Trehalose	Raffinose
18	30mM				5g/L					
19		30mM			5g/L					
20			30mM		5g/L					
21				30mM	5g/L					
22	30mM					5g/L				
23		30mM				5g/L				
24			30mM			5g/L				
25				30mM		5g/L				
26	30mM						0.1g/L			
27		30mM					0.1g/L			
28			30mM				0.1g/L			
29				30mM			0.1g/L			
30	30mM				5g/L	5g/L				
31		30mM			5g/L	5g/L				
32			30mM		5g/L	5g/L				
33				30mM	5g/L	5g/L				
34	30mM				5g/L		0.1g/L			
35		30mM			5g/L		0.1g/L			
36			30mM		5g/L		0.1g/L			
37				30mM	5g/L		0.1g/L			
38	30mM				5g/L	5g/L	0.1g/L			
39		30mM			5g/L	5g/L	0.1g/L			
40			30mM		5g/L	5g/L	0.1g/L			
41				30mM	5g/L	5g/L	0.1g/L			
42					5g/L					
43								5g/L		
44									5g/L	
45										5g/L
46					5g/L	5g/L				
47						5g/L		5g/L		
48						5g/L			5g/L	
49						5g/L				5g/L
50					5g/L		0.1g/L			
51							0.1g/L	5g/L		
52							0.1g/L		5g/L	
53							0.1g/L			5g/L

[00100] The assessment of the visible VWF aggregates was done according to the scheme shown below. "Visible aggregates," in most cases, are gelatinous fibers ranging in size from about 100 nm to 1-2 cm.

SCHEME

Particles	
A	no particles
B	several particles, rarely visible (dots)
B1	many particles, rarely visible (dots)
C	several particles, easily visible (fibers)
D	many particles, easily visible (fibers)
E	visible particles (>1mm fibers)
E1	fluffy white precipitate (swims on the surface)
E2	jellyfish

[00101] The results of the shaking experiments are shown in Table 2, below.

TABLE 2

Lyo 25 Samples	Lysine	Histidine	Glycine	Serine	Mannitol	PEG 1500	Tween 80	Shaking 1-2mL 1200rpm 30min
18	30mM				5g/L			E1
19		30mM			5g/L			E1
20			30mM		5g/L			E1
21				30mM	5g/L			E1
22	30mM					5g/L		E1
23		30mM				5g/L		E1
24			30mM			5g/L		E1
25				30mM		5g/L		E1
26	30mM						0.1g/L	E2 big
27		30mM					0.1g/L	E2 big
28			30mM				0.1g/L	E2 ~6mm
29				30mM			0.1g/L	E2 ~3mm
30	30mM				5g/L	5g/L		E1
31		30mM			5g/L	5g/L		E1
32			30mM		5g/L	5g/L		E1
33				30mM	5g/L	5g/L		E1
34	30mM				5g/L		0.1g/L	B1
35		30mM			5g/L		0.1g/L	B
36			30mM		5g/L		0.1g/L	E2 big
37				30mM	5g/L		0.1g/L	E2 big
38	30mM				5g/L	5g/L	0.1g/L	D
39		30mM			5g/L	5g/L	0.1g/L	D
40			30mM		5g/L	5g/L	0.1g/L	B
41				30mM	5g/L	5g/L	0.1g/L	B

[00102] In summary, the shaking experiments described above indicate that formulations containing Tween-80 and Mannitol provide the best results (i.e., the least amount of aggregation).

Example 2

Freeze-thaw experiments

[00103] Freeze-thaw experiments were designed to assess the impact of stress caused by repeated freezing and thawing. In addition to the formulations described above for the shaking experiments (Table 1), the following formulations were assessed (Table 3 and Table 4):

TABLE 3

Lyo 25 Samples	Mannitol	PEG 1500	Tween 80	Sucrose	Trehalose	Raffinose
42	5g/L					
43				5g/L		
44					5g/L	
45						5g/L
46	5g/L	5g/L				
47		5g/L				
48		5g/L				
49		5g/L				
50	5g/L		0.1g/L			
51			0.1g/L	5g/L		
52			0.1g/L		5g/L	
53			0.1g/L			5g/L

TABLE 4

Lyo 25 Samples	Lysine	Histidine	Glycine	Serine	Mannitol	PEG 1500	Tween-80	Sucrose	Trehalose	Raffinose
76					20g/L		0.2g/L	20g/L		
77					20g/L		0.2g/L		10g/L	
78					20g/L		0.2g/L			10g/L
79		15mM			20g/L		0.2g/L	20g/L		
80		15mM			20g/L		0.2g/L		10g/L	
81		15mM			20g/L		0.2g/L			10g/L
82		15mM	15mM		20g/L		0.2g/L	20g/L		
83		15mM	15mM		20g/L		0.2g/L		10g/L	
84		15mM	15mM		20g/L		0.2g/L			10g/L
85		15mM	15mM		20g/L	5g/L	0.2g/L			
86		15mM	15mM		20g/L	5g/L	0.2g/L		10g/L	
87		15mM	15mM		20g/L	15g/L	0.2g/L		10g/L	
88		15mM	15mM		20g/L		0.2g/L			5g/L
89		15mM	15mM		20g/L		0.2g/L			15g/L
90		15mM			20g/L		0.2g/L			15g/L
92		15mM	15mM		20g/L		0.2g/L		10g/L	
93	30mM				20g/L		0.2g/L		10g/L	
94		30mM			20g/L		0.2g/L		10g/L	
95			30mM		20g/L		0.2g/L		10g/L	
96				30mM	20g/L		0.2g/L		10g/L	
97		15mM			20g/L		0.2g/L		10g/L	
98		15mM	15mM		20g/L		0.2g/L		10g/L	
99		15mM	15mM		20g/L		0.2g/L		5g/L	
100		15mM			20g/L		0.2g/L		10g/L	
		15mM			20g/L		0.2g/L		10g/L	

[00104] All formulations were frozen at -20° C in a freezer for approximately 1 hour and then thawed at room temperature. The results are shown in Table 5 below.

TABLE 5

Lyo 25 Samples	Lysine	Histidine	Glycine	Serine	Mannitol	PEG 1500	Tween-80	Sucrose	Trehalose	Raffinose	Freeze/ Thaw (4 times)	Freeze/ Thaw (~10 times)
18	30mM				5g/L						E1	15
19		30mM			5g/L						E1	19
20			30mM		5g/L						C	20
21				30m	5g/L						C	21
				M								
22	30mM					5g/L					C	22
23		30mM				5g/L					C/B1	23
24			30mM			5g/L					C	24
25				30m		5g/L					C	25
				M								
26	30mM						0.1g/L				B	26
27		30mM					0.1g/L				B-B1	27
28			30mM				0.1g/L				B	28
29				30m			0.1g/L				E	29
				M								
30	30mM				5g/L	5g/L					E	30
31		30mM			5g/L	5g/L					D	31
32			30mM		5g/L	5g/L					B1-C	32
33				30m	5g/L	5g/L					C/D	33
				M								
34	30mM				5g/L		0.1g/L				E2 (rest B)	34
35		30mM			5g/L		0.1g/L				E	35
36			30mM		5g/L		0.1g/L				E	36
37				30m	5g/L		0.1g/L				B	37
				M								
38	30mM				5g/L	5g/L	0.1g/L				B	38
39		30mM			5g/L	5g/L	0.1g/L				B	39
40			30mM		5g/L	5g/L	0.1g/L				B	40
41				30m	5g/L	5g/L	0.1g/L				A	41
				M								
42					5g/L						D	42
43								5g/L			D	43
44									5g/L		E1	44
45										5g/L	E1	45
46					5g/L	5g/L					D	46
47						5g/L		5g/L			D	47
48						5g/L			5g/L		D-E	48
49						5g/L				5g/L	E	49
50					5g/L		0.1g/L				B1	50
51							0.1g/L	5g/L			C-D	51
52							0.1g/L		5g/L		B1	52
53							0.1g/L			5g/L	B1	53

[00105] As shown above, Trehalose provided the best results (i.e., the least amount of aggregation).

Example 3

Lyophilization experiments

[00106] Lyophilization experiments were designed to assess the ability o various formulations to allow the formation of a lyo-cake which dissolves in less than 10 minutes and results in a clear solution. An accelerated stability study was also performed to demonstrate that no significant loss of biological activity.

[00107] The formulations shown in Table 6 below were lyophilized with a nitrogen lyophilizer TS20002 according to the manufacturer's instructions. The total time for lyophilization was approximately 72 hours. Each of the formulations below also contained 20 g/L Mannitol and 0.1 g/L Tween-80.

TABLE 6

Lyo 26	Citrate	HEPES	Glycine	Histidine	Acetate	Tris	Phosphate	Lysine	Histidine	Glycine	Trehalose	Raffinose
1	15mM										10g/L	
2	15mM							15mM			10g/L	
3	15mM								15mM		10g/L	
4	15mM									15mM	10g/L	
5	15mM							15mM		15mM	10g/L	
6	15mM							30mM			10g/L	
7	15mM											10g/L
8	15mM								15mM			10g/L
9		15mM									10g/L	
10		15mM						15mM			10g/L	
11		15mM							15mM		10g/L	
12		15mM								15mM	10g/L	
13		15mM						15mM		15mM	10g/L	
14		15mM						15mM		15mM		10g/L
15			15mM								10g/L	
16			15mM						15mM		10g/L	
17				15mM							10g/L	
18				15mM				15mM			10g/L	
19					15mM			15mM		15mM	10g/L	
20						15mM		15mM		15mM	10g/L	
21							15mM	15mM		15mM	10g/L	

[00108] The results of the lyophilzation experiments are shown in Table 7 below.

TABLE 7

			Buffer					--	Excipents	--	--	--
Lyo 26	Citrate	HEPES	Glycine	Histidine	Acetate	Tris	Phosphate	Lysine	Histidine	Glycine	Trehalose	Raffinose
1	15mM										10g/L	
2	15mM							15mM			10g/L	
3	15mM								15mM		10g/L	
4	15mM									15mM	10g/L	
5	15mM							15mM			10g/L	
6	15mM							30mM			10g/L	
7	15mM											10g/L
8	15mM								15mM			10g/L
9		15mM									10g/L	
10		15mM						15mM			10g/L	
11		15mM							15mM		10g/L	
12		15mM								15mM	10g/L	
13		15mM						15mM		15mM	10g/L	
14		15mM						15mM		15mM		10g/L
15			15mM								10g/L	
16			15mM						15mM		10g/L	
17				15mM							10g/L	
18				15mM				15mM			10g/L	
19					15mM			15mM		15mM	10g/L	
20						15mM		15mM		15mM	10g/L	
21							15mM	15mM		15mM	10g/L	

[00109] As shown above, either a Citrate or HEPES buffer in combination with an amino acid provided the clearest solution.

[00110] In order to assess stability of the reconstituted lyophilized rVWF, VWF:Ag and VWF:RCo tests were performed. VWF:Ag corresponds to the amount of VWF which can be detected in an VWF-specific ELISA using polyclonal anti-VWF antibody, while VWF:RCo corresponds to the amount of VWF which causes agglutination of stabilized platelets in the presence of ristocetin. Samples were stored at 40o C. Assuming applicability of the Arrhenius equation, one month stability at 40° C is equivalent to approximately one year at 4° C. The results of the stability experiments are shown in Table 8 and Table 9 below.

TABLE 8

		Weeks at 40°			
rVWF:Ag formulation		0	4	5	8
1		121.1	89.8	113.0	106.6
2		121.8	102.0	114.0	112.8
3		119.9	102.0	105.,0	112.7
4		117.3	100.0	108.0	114.4
5		121.2	98.2	117.0	114.9
6		123.8	96.6	107.0	--
7		135.2	96.6	112.0	112.4
8		130.6	82.2	108.0	115.7
9		112.0	89.5	109.0	107.0
10		122.4	87.1	106.0	107.7
11		119.3	97.5	115.0	114.2
12		124.2	109.0	109.0	103.4
13		110.2	92.3	106.0	112.4
14		108.9	107.0	103.0	109.0

TABLE 9

		Weeks at 40°			
rVWF:RCo formulation		0	4	5	8
1		86	102	97.0	93.0
2		84	97	88.0	89.0
3		85	100	87.0	93.0
4			102	81.0	98.0
5		85	89	88.0	98.0
6		83	102	88.0	
7			92	97.0	95.0
8		88	94	90.0	104.0
9		93	91	97.0	100.0
10		95	87	87.0	87.0
11		86	93	89.0	99.0
12		84	91	89.0	95.0
13		88	87	96.0	89.0
14		90	91	86.0	92.0

[00111] The standard deviation for the ELISA is in the range of 10-20%. The results above indicate that all of the formulations tested provide good stability over 8 weeks at 40o C.

[00112] Additional stability experiments were performed where different amino acids were used in the formulations (e.g., glycine, lysine or histidine at 15 mMor 20 m), and where the citrate buffer was varied (e.g., 15 , 20 or 25 mM). As described above, stability of rVWF was monitored using the VWF:RCo activity assay. Even after 13 months no significant differences were observed for VWF:RCo activity values of rVWF stability samples stored at 40o C. the significane of the measurements were tested with a t-Test. The intermediate precision of the assay was determined by calculating the Coefficient of Variance. In all series of the stability data the CV was below 20% and met the validation criteria of a CV<20%. Based on the above, it can be concluded that rVWF is stable in all citrate buffer systems tested, independent of buffer molarity and amino acids added. rVWF remains stable for at least 13 months even when stored at 40o C. The potency determination using the VWF:RCo activity assay shows good intermediate precision with CV values below 20%.

[00113] Thus, in view of the data presented herein, a formulation was proposed for rVWF including 15 mM citrate ($Na_3Citrate \times 2\ H_2O$), 15 mM glycine, 10 g/L Trehalose, 20 g/L Mannitol, 0.1 g/L Tween-80, pH 7.3.

Example 4

Long term stability

[00114] **Accelerated and long-term stability testing**

[00115] Studies were conducted to evaluate the stability of the rVWF final drug product (FDP) stored at both the recommended and elevated storage conditions. Data from the elevated storage conditions provides assurance that deviations in the temperature will not impact the quality of the rVWF FDP and will be used to extrapolate the acceptable expiry condition of the material in the absence of real-time, real-condition stability data.

[00116] The current specification is $\leq 3.0\%$ residual moisture (as determined using the Karl Fischer Method). Lots rVWFF#4FC, rVWFF#5FC, rVWFF#6FC and rVWFF#7FC were released with moisture levels of 1.2%, 1.3%, 1.2%, and 1.5% respectively. Based on the past experience with other products with similar vial and stopper configurations, it is expected that any rVWF lots released with approximately 1.3% residual moisture will meet the specification limit of $\leq 3.0\%$ at the end of the proposed shelf life (i.e. 24 months at the intended storage temperature of $5°C \pm 3°C$).

[00117] Long-term stability studies at the recommended storage condition (i.e. 5°C ± 3°C) and elevated temperatures (i.e. 40°C ± 2°C) were conducted with four rVWF FDP lots that have been manufactured. These studies have provided sufficient data to compare the stability behavior of the individual clinical lots.

[00118] The stability protocol, including a description of the stability-indicating assays and stability-acceptance criteria, can be found in Table 10 which also contains information related to the rVWF FDP lots evaluated in the stability studies.

Table 10

Storage Conditions (°C)	Batch Number	Completed (and Proposed) Test Intervals
5°C ± 3°C	rVWF#1FC	0, 1, 2, 3, 6, 9, 12, 18, 24 months
30°C	rVWF#1FC	0, 1, 3, 6 months
5°C ± 3°C	rVWF#2FC	0, 1, 2, 3, 6, months
5°C ± 3°C	rVWF#3FC	0, 1, 2, 3, 6, 9, 12, 18, 24 months
30°C	rVWF#3FC	0, 0.5, 1, 2, 3, 6 months
40°C	rVWF#3FC	0, 0.5, 1, 2, 3 months
5°C ± 3°C	rVWF#4FC	0, 1, 2, 3, 6, 9, 12, 18, 24, (30) months
40°C	rVWF#4FC	0, 1, 2, 3, 6, 9 months
5°C ± 3°C	rVWF#5FC	0, 1, 2, 3, 6, 9, 12, 18, (24, 30) months
40°C	rVWF#5FC	0, 1, 2, 3 6, 9 months
5°C ± 3°C	rVWF#6FC	0, 1, 2, 3, 6, 9, 12, (18, 24, 30) months
40°C	rVWF#6FC	0, 1, 2, 3, 6, 9 months
5°C ± 3°C	rVWF#7FC	0, 1, 2, 3, 6, 9, 12, (18, 24, 30) months
40°C	rVWF#7FC	0, 1, 2, 3, 6, 9 months

[00119] Summary and discussion of overall stability (24 Months)

[00120] The rVWF FDP stability data presented is comprised of the following:

[00121] 1. 24 months data of long-term studies at 5°C ± 3°C (complete testing) and 6 months intermediate data at 30°C ± 2°C (complete testing) for lot rVWF#1FC;

[00122] 2. 6 months data at 5°C ± 3°C (complete testing) for lot rVWF#2FC;

[00123] 3. 24 months data of long-term studies at 2-8° (complete testing), 6 months data at 30°C ± 2°C and 3 months data at 40°C ± 2°C (complete testing) for lot rVWF#3FC;

[00124] 4. 24 months stability data at 5°C ± 3°C and 9 months data at 40°C ± 2°C for lot rVWFF#4FC;

[00125] 5. 24 months stability data at 5°C ± 3°C and 9 months data at 40°C ± 2°C for lot rVWFF#5FC;

[00126] 6. 12 months stability data at 5°C ± 3°C and 9 months data at 40°C ± 2°C for lot rVWFF#6FC; and

[00127] 7. 12 months stability data at 5°C ± 3°C and 9 months data at 40°C ± 2°C for lot rVWFF#7FC

[00128] The variation observed in residual moisture for lots rVWFF#4FC, rVWFF#5FC, rVWFF#6FC and rVWFF#7FC has remained well below the acceptance criterion ≤ 3%, and has not impacted the functional activity (VWF:RCo). There was no observable change in the stability results for qualitative analytical techniques (i.e. appearance, SDS-PAGE analysis, etc.) for the lots manufactured to be suitable for use in the non-clinical and clinical studies. Similarly, there was no trend in decreasing stability for the total protein analysis, the VWF:Ag analysis or the observed number of VWF multimers during storage.

[00129] Variation in both the ratio of VWF:RCo activity to VWF:Ag activity and the VWF:RCo data presented for lots rVWF#1FC, rVWF#2FC and rVWF#3FC was likely the result of variation of the test method, the fact that the individual VWF:RCo stability test results consisted of data from a single determination of one stability sample, and/or data from the non-Ph. Eur.-conforming method assay methodology. All testing time points for the non-clinical lots subsequent to the modification of the assay methodology to the Ph.Eur.-conforming assay were tested using both the original and new assay methodology.

[00130] The rVWF FDP manufactured at a large-scale exhibited similar stability characteristics to the rVWF FDP lots manufactured at an experimental scale. These rVWF FDP lots maintained VWF:RCo activity for up to 24 months of storage at 5°C ± 3°C. There

was no change in the VWF multimer pattern in samples of the large-scale lots currently on stability, even after 6 months of storage at 30°C ± 2°C or 9 months storage at 40°C ± 2°C. Table 11 shows results for VWF:RCo, VWF:Ag and VWF multimer pattern of the batches rVWF#4FC, rVWF#5FC, rVWF#6FC and rVWF#7FC stored under stress condition at 40°C ± 2°C. The results indicate stability at elevated temperature storage conditions for 9 months which can be extrapolated into a shelf life of more than 3 years at ambient temperatures or even more under refrigerated conditions.

Table 11

Stability Data for rVWF#4FC at 40°C ± 2°C							
Attribute	**Specification**	**Results at Time (Months)**					
		0	**1**	**2**	**3**	**6**	**9**
VWF:RCo Activity [U/ml][1)]	70-150	130	117	118	127	132	142
VWF:Ag ELISA (U/ml)	Report result	86	87	79	81	79	86
VWF multimer analysis	Report result	21	20	20	20	21	18
Stability Data for rVWF#5FC at 40°C ± 2°C							
Attribute	**Specification**	**Results at Time (Months)**					
		0	**1**	**2**	**3**	**6**	**9**
VWF:RCo Activity [U/ml][1)]	70-150	107	119	120	116	132	134
VWF:Ag ELISA (U/ml)	Report result	94	86	84	91	90	79
VWF multimer analysis	Report result	20	20	18	19	20	19
Stability Data for rVWF#6FC at 40°C ± 2°C							
Attribute	**Specification**	**Results at Time (Months)**					
		0	**1**	**2**	**3**	**6**	**9**
VWF:RCo Activity [U/ml][1)]	70-150	118	111	126	129	130	119
VWF:Ag ELISA (U/ml)	Report result	85	95	86.3	73.5	80.8	70.3
VWF multimer analysis	Report result	20	19	20	20	20	n.t.
Stability Data for rVWF#7FC at 40°C ± 2°C							
Attribute	**Specification**	**Results at Time (Months)**					
		0	**1**	**2**	**3**	**6**	**9**
VWF:RCo Activity [U/ml][1)]	70-150	111	115	122	105	99	112
VWF:Ag ELISA (U/ml)	Report result	87.3	85.3	77.5	68.8	75	73.8
VWF multimer analysis	Report result	21	20	20	19	19	19

[00131] An analysis of covariance (ANCOVA analysis) demonstrated that the difference in slopes of the regression lines (lots rVWFF#4FC, rVWFF#5FC, rVWFF#6FC and rVWFF#7FC stored at 5°C ± 3°C) is not significant (p=0.906), allowing the VWF:RCo activity data to be pooled as described in ICH Q1A (R2). The difference in elevation of the trend lines of the individual lots is also not significant. Extrapolation of the pooled worse case slope, as shown in Figure 1, shows that the confidence intervals are well within the acceptance criteria for a minimum of 24 months. The lower confidence interval for the mean curve decreases to 80% of initial activity at 51 months (80% is also the maximum difference between estimated potency and stated potency for Human von Willebrand Factor in Ph.Eur). The pooled worse case slope shows a decrease of 0.0344 U VWF:RCo per month. This comparison shows that stability characteristics of the rVWF FDP, specifically the VWF:RCo activity, did not change as a result of the changes in the production process. The above extrapolation supports the extension of the provisional shelf life of rVWF FDP to 24 months when stored at the recommended storage temperature.

[00132] The transfer of moisture from the stopper to the lyophilized product is dependent on the stopper material and is influenced by the residual moisture of the stopper after sterilization, the humidity at which the sample is stored and the intrinsic moisture transfer rate of the stopper. The residual moisture in the lots rVWFF#4FC, rVWFF#5FC, rVWFF#6FC and rVWFF#7FC stored at 5°C ± 3°C was comparable (the difference in comparison of slopes being not significant, with p=0.734), as shown in Figure 2. Lots stored at the elevated temperature condition 40°C ± 2°C also showed a comparable increase in residual moisture over 9 months (Figure 3). ANCOVA analysis demonstrates here that the difference in slope of the regression lines is comparable (p = 0.546). Figure 3 shows the extrapolation of the worse case pooled slope up to 24 months.

[00133] These are sufficient data to support the use of lots rVWFF#6FC and rVWFF#7FC for the duration of the described expiry period of 24 months when stored at 5°C ± 3°C.

[00134] Proposed Storage Conditions and Shelf Life

[00135] The recommended storage condition for the rVWF FDP is 5°C ± 3°C. A provisional shelf life of 24 months for the rVWF FDP is therefore proposed when stored at the recommended storage condition. The shelf life for the rVWF FDP lots likely can be further extended based on additional data to be generated for longer storage periods.

What is claimed is:

1. A stable lyophilized pharmaceutical formulation of a recombinant von Willebrand Factor (rVWF) comprising: (a) a rVWF; (b) one or more buffering agents; (c) one or more amino acids; (d) one or more stabilizing agents; and (e) one or more surfactants;

said rVWF comprising a polypeptide selected from the group consisting of:

a) the amino acid sequence set out in SEQ ID NO: 3;

b) a biologically active analog, fragment or variant of a);

c) a polypeptide encoded by the polynucleotide set out in SEQ ID NO: 1;

d) a biologically active analog, fragment or variant of c); and

e) a polypeptide encoded by a polynucleotide that hybridizes to the polynucleotide set out in SEQ ID NO: 1 under moderately stringent hybridization conditions;

said buffer is comprising of a pH buffering agent in a range of about 0.1 mM to about 500 mM and said pH is in a range of about 2.0 to about 12.0;

said amino acid is at a concentration of about 1 to about 500 mM;

said stabilizing agent is at a concentration of about 0.1 to about 1000 mM; and

said surfactant is at a concentration of about 0.01 g/L to about 0.5 g/L.

2. The formulation of claim 1 wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3.

3. The formulation of claim 1 wherein the buffering agent is selected from the group consisting of citrate, glycine, histidine, HEPES, Tris and combinations of these agents.

4. The formulation of claim 3 wherein the buffering agent is citrate.

5. The formulation of claim 1 wherein pH is in the range of about 6.0 to about 8.0.

6. The formulation of claim 5 wherein pH is in the range of about 6.5 to about 7.5.

7. The formulation of claim 4 wherein the pH is about 7.3.

8. The formulation of claim 1 wherein the buffering agent is citrate and the pH is about 7.3.

9. The formulation of claim 1 wherein the amino acid is selected from the group consisting of glycine, histidine, proline, serine, alanine and arginine.

10. The formulation of claim 9 wherein the amino acid is at a concentration range of about 0.5 mM to about 300 mM.

11. The formulation of claim 10 wherein the amino acid is glycine at a concentration of about 15 mM.

12. The formulation of claim 1 wherein the rVWF comprises the amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate and the pH is about 7.3; and wherein the amino acid is glycine at a concentration of about 15 mM.

13. The formulation of claim 1 wherein the one or more stabilizing agents is selected from the group consisting of mannitol, lactose, sorbitol, xylitol, sucrose, trehalose, mannose, maltose, lactose, glucose, raffinose, cellobiose, gentiobiose, isomaltose, arabinose, glucosamine, fructose and combinations of these stabilizing agents.

14. The formulation of claim 13 wherein the stabilizing agents are trehalose at a concentration of about 10 g/L mM and mannitol at a concentration of about 20 g/L.

15. The formulation of claim 1 wherein the surfactant is selected from the group consisting of digitonin, Triton X-100, Triton X-114, TWEEN-20, TWEEN-80 and combinations of these surfactants.

16. The formulation of claim 15 wherein the surfactant is TWEEN-80 at about 0.01 g/L.

17. The formulation of claim 1 wherein the rVWF comprises amino acid sequence set out in SEQ ID NO: 3; wherein the buffering agent is citrate at a concentration of about 15 mM at about pH 7.3; wherein the amino acid is glycine at a concentration of about 15 mM; wherein the stabilizing agents are trehalose at a concentration of about 10 g/L and mannitol at a concentration of about 20 g/L.; and wherein the surfactant is TWEEN-80 at about 0.1 g/L.