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(54) Title: METHOD FOR IMPROVING THE STABILITY OF PURIFIED FACTOR VIII AFTER RECONSTITUTION

(57) Abstract: The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule. The invention further pertains to a method for improving the bioavailability of Factor VIII after intravenous and non- intravenous injection.

Method for improving the stability of purified Factor VIII after reconstitution

The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule. The invention further pertains to a method for improving the bioavailability of Factor VIII after intravenous and non-intravenous injection.

BACKGROUND OF THE INVENTION

Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation Factor VIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency. Before treatment with Factor VIII concentrates was introduced the mean life span for a person with severe hemophilia was less than 20 years. The use of concentrates of Factor VIII from plasma has considerably improved the situation for the hemophilia patients increasing the mean life span extensively, giving most of them the possibility to live a more or less normal life. However, there have been certain problems with the plasma derived concentrates and their use, the most serious of which have been the transmission of viruses. So far, viruses causing AIDS, hepatitis B, and non-A non-B hepatitis have hit the population seriously. Since then different virus inactivation methods and new highly

purified Factor VIII concentrates have recently been developed which established a very high safety standard also for plasma derived Factor VIII.

Several recombinant and plasma-derived, therapeutic polypeptides, e.g. blood coagulation factors, are commercially available for therapeutic and prophylactic use in humans. FVIII is a blood plasma glycoprotein of up to about 280 kDa molecular mass, produced in the liver of mammals. It is a critical component of the cascade of coagulation reactions that lead to blood clotting. Within this cascade is a step in which factor IXa (FIXa), in conjunction with activated factor VIII (FVIIIa), converts factor X (FX) to an activated form, FXa. FVIIIa acts as a cofactor at this step, being required together with calcium ions and phospholipids for maximizing the activity of FIXa.

An important advance in the treatment of hemophilia A has been the isolation of cDNA clones encoding the complete 2,351 amino acid sequence of human FVIII (United States Patent No. 4,757,006) and the provision of the human FVIII gene DNA sequence and recombinant methods for its production).

Factor VIII is synthesized as a single polypeptide chain with a molecular weight of about 280 kDa. The amino-terminal signal peptide is removed upon translocation of factor VIII into the endoplasmatic reticulum, and the mature (i.e. after the cleavage of the signal peptide) native Factor VIII molecule is then proteolytically cleaved after amino acid residues 1313 and 1648 in the course of its secretion. This results in the release of a heterodimer which consists of a C-terminal light chain of about 80 kDa in a metal ion-dependent association with an about 160-200 kDa N-terminal heavy chain fragment. (See review by Kaufman, Transfusion Med. Revs. 6:235 (1992)).

Physiological activation of the heterodimer occurs through proteolytic cleavage of the protein chains by thrombin. Thrombin cleaves the heavy chain to a 90 kDa protein, and then to 54 kDa and 44 kDa fragments. Thrombin also cleaves the 80 kDa light chain to a 72 kDa protein. It is the latter protein, and the two heavy chain

fragments (54 kDa and 44 kDa above), held together by calcium ions, that constitute active FVIII. Inactivation occurs when the 44 kDa A2 heavy chain fragment dissociates from the molecule or when the 72 kDa and 54 kDa proteins are further cleaved by thrombin, activated protein C or FXa. In plasma, FVIII is stabilized by association with a 50-fold molar excess of VWF protein ("VWF"), which appears to inhibit proteolytic destruction of FVIII as described above.

The amino acid sequence of FVIII is organized into three structural domains: a triplicated A domain of 330 amino acids, a single B domain of 980 amino acids, and a duplicated C domain of 150 amino acids. The B domain has no homology to other proteins and provides 18 of the 25 potential asparagine(N)-linked glycosylation sites of this protein. The B domain has apparently no function in coagulation and can be deleted with the B-domain deleted FVIII molecule still having procoagulatory activity.

The Factor VIII products on the market are currently presented as a lyophilized formulation of Factor VIII either produced by recombinant technology or purified from pooled plasma. The lyophilized product is reconstituted prior to administration. Once reconstituted, shelf-life of the Factor VIII is relatively short. Factor VIII is a relatively unstable protein, particularly in aqueous solutions. Stabilization during manufacturing and storage by complexing with other plasma proteins, particularly von Willebrand factor (vWF) and albumin, has been described. See, for example, US Patent No. 6,228,613. US Patent No. 5,565,427 discloses a stabilized formulation of Factor VIII comprising an amino acid or one of its salts or homologues and a detergent or an organic polymer such as polyethyleneglycol. US Patent No. 5,605,884 discloses stabilized formulations of Factor VIII in high ionic strength media based on histidine buffer in the presence of calcium chloride and a high concentration of sodium chloride or potassium chloride. Such compositions were shown to improve significantly the stability of Factor VIII in aqueous form following reconstitution. The importance of calcium ions in the formulations of Factor VIII is generally recognized. According to US Patent No. 6,599,724, the

presence of other divalent cations, namely Cu^{2+} and Zn^{2+}, optionally in the presence of Ca^{2+} ions or Mn^{2+} ions improves the stability of Factor VIII. Also WO 2011/027152 A1 describes stable aqueous Factor VIII compositions comprising various additives.

In view of the short shelf life of Factor VIII after reconstitution of a lyophilisate, there is a need for methods to increase the stability of reconstituted Factor VIII in aqueous solution. To provide a purified FVIII preparation with increased stability in the liquid phase is desirable for different reasons. First of all, it is of advantage to have a sufficient time span at ambient temperature to support manufacturing of the purified FVIII product at ambient temperature. In particular, the filling step necessitates some storage of a liquid bulk to increase flexibility in manufacturing. Secondly, an increased stability of the liquid purified FVIII would be of advantage for physician and patient if the product could not be applied directly after reconstitution. And finally, the use of FVIII under continuous infusion conditions e.g. upon surgery in hospitalized patients is depending on a preferably high product stability after reconstitution (Takedani H., Haemophilia 2010, 16: 740-746). A FVIII molecule with increased stability would also be an advantage for development of a FVIII preparation suitable for long term storage under liquid conditions.

The inventors of this application surprisingly found that the stability of purified Factor VIII after reconstitution of a lyophilisate is substantially enhanced in single-chain Factor VIII constructs. Such constructs can be obtained by preventing the proteolytic cleavage which typically occurs in the Golgi compartment prior to secretion of Factor VIII. The single-chain constructs exhibit a better stability in solution after purificationand/or a better bioavailability upon subcutaneous administration.

SUMMARY OF THE INVENTION

In a first aspect the present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain.

The first aspect encompasses a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule.

The first aspect further encompasses a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain prior to the purification of the Factor VIII molecule.

With regard to these methods according to the invention the terms “throughout manufacturing of the Factor VIII molecule” and “prior to the purification of the Factor VIII molecule” are intended to mean that the methods of the invention prevent the cleavage of Factor VIII into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain but the methods according to the invention do not prevent the activation cleavage of Factor VIII which may occur after administration of the reconstituted Factor VIII molecule. The Factor VIII molecules

generated by the methods of the invention can still be activated by thrombin which cleaves the Factor VIII molecule after Arg 372, Arg 740 and Arg 1689.

In a second aspect, the present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising inactivating the proteolytic cleavage sites which are cleaved during secretion of said Factor VIII molecule by the host cell expressing the Factor VIII molecule except the cleavage site between the signal sequence and the mature Factor VIII. Typically, at least 50% of the Factor VIII molecules expressed and secreted by the host cells are single-chain Factor VIII molecules. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% of the Factor VIII molecules expressed and secreted by the host cells are single-chain Factor VIII molecules.

Preferably, the method comprises inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314. The inactivation of the proteolytic cleavage site may be effected by deleting one or more residues of the protease recognition sequence. For example, inactivation step may comprise deleting at least Arg1648 from the Factor VIII sequence. In one embodiment, the inactivation step comprises deleting at least the amino acid sequence from Arg1313 to Arg1648 from the Factor VIII sequence.

In another embodiment of the first aspect of the invention the inactivation of the proteolytic cleavage site is effected by substituting one or more amino acid residues forming the protease recognition sequence.

In yet another embodiment (concerning those FVIII variants which retain the part of the B-domain comprising Arg1313) the method further comprises inactivating the proteolytic cleavage site between Arg1313 and Ala1314 by deletion or substitution of one or more residues forming the protease recognition sequence. In a

particularly preferred embodiment, the method comprises deleting at least a portion from the Factor VIII amino acid sequence which comprises both protease cleavage sites after residues Arg1313 and Arg1648.

It is further preferred that a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the cleavage site between Arg1313 and Ala1314 is inactivated.

In another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased stability in aqueous solution. The loss of activity of the modified Factor VIII molecule, in aqueous solution, after storage for 7 days at 25°C is preferably less than 15%.

In another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased stability in aqueous solution after reconstitution.

In yet another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased bioavailability after non-intravenous injection, as compared to the bioavailability of human wild type Factor VIII or as compared to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner. In yet another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased bioavailability after non-intravenous injection, as compared to the bioavailability of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner. The bioavailability of the modified FVIII is preferably increased by at least 25%, as

compared to the bioavailability of human wild type Factor VIII or of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, each administered at the same dose and in the same manner. In another preferred embodiment the non-intravenous injection is subcutaneous, transdermal or intramuscular injection.

Another preferred embodiment is a method wherein (i) the Factor VIII exhibits improved plasma half-life after intravenous administration relative to human wild type Factor VIII; preferably wherein the plasma half-life is improved by at least 40% relative to human wild type Factor VIII, or (ii) wherein the Factor VIII exhibits a longer time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII; preferably wherein this time period is prolonged by at least 10 hours relative to human wild type Factor VIII, or (iii) wherein the Factor VIII retains a higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C; preferably wherein the retained activity of the Factor VIII is at least 10% higher relative to that of a human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

The methods may further comprise the steps of

(i) providing a nucleic acid encoding a modified Factor VIII molecule in which the proteolytic cleavage sites between Arg1648 and Glu1649, and between Arg1313 and Ala1314, are inactivated,

(ii) transforming a host cell with said nucleic acid,

(iii) culturing the transformed host cell under conditions such that the modified Factor VIII molecule is expressed, and

(iv) recovering the modified Factor VIII molecule from the host cells or from the culture medium.

In another aspect, the present invention relates to a method for improving the bioavailability of a Factor VIII molecule after non-intravenous administration, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314. Preferably, the non-intravenous injection is subcutaneous injection. The bioavailability after subcutaneous injection is preferably increased by at least 25% as compared to that of human wild type Factor VIII or of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, each administered at the same dose and in the same manner.

In another aspect, the present invention relates to a method for improving the plasma half-life of a Factor VIII molecule after intravenous administration relative to human wild-type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

In another aspect, the present invention relates to a method for prolonging the time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration of a Factor VIII molecule relative to human wild type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

In another aspect, the present invention relates to a method for retaining a higher activity for a Factor VIII molecule as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

A preferred embodiment of the methods described above are methods wherein a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314 is inactivated.

The preferred embodiments of the different aspects are applicable *mutatis mutandis*.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by (i) on the one hand non-intravenous administration, wherein the bioavailability of said single chain Factor VIII molecule is increased by at least 25% as compared to human wild type Factor VIII or as compared to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner, or (ii) on the other hand by intravenous administration, wherein (a) the plasma half-life of said single chain Factor VIII molecule after intravenous administration is increased by at least 40%, relative to human wild type Factor VIII, administered at the same dose and in the same manner, or (b) the single chain Factor VIII molecule exhibits a time period prolonged by at least 10 hours for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII, administered at the same dose and in the same manner.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, wherein the single

chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by non-intravenous administration, wherein the dose of said FVIII molecule can be decreased by at least 25% as compared to that of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner to achieve the same hemostatic activity in blood.

In another aspect, the present invention relates to the use of a single chain Factor VIII molecule for achieving an increased stability after reconstitution or a longer shelf life of a pharmaceutical preparation for treating a bleeding disorder, wherein (i) the Factor VIII activity of the pharmaceutical preparation comprising the single chain Factor VIII molecule, after reconstitution and storage at room temperature for 7 days after reconstitution is at least 10% higher than that of a pharmaceutical preparation comprising the same amount of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, or (ii) wherein the single chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay when incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C at the same concentration.

DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the results of Example 1. Various Factor VIII molecules have been provided as aqueous solutions, and their stability has been monitored over a time period of seven days. The loss in activity after seven days of storage is much less for the single chain Factor VIII molecule as compared to heterodimeric (two-chain) full length Factor VIII molecules (Beriate® and Helixate®) and to heterodimeric (two-chain) B-domain deleted constructs (ReFacto®).

Figure 2 depicts the results of Example 2. Various lyophilized Factor VIII preparations were reconstituted to aqueous solutions, and their stability has been monitored over a time period of seven days. The loss in activity after seven days of storage is much less for the single chain Factor VIII molecule as compared to a heterodimeric (two-chain) full length Factor VIII molecule (Advate®) and to a heterodimeric (two-chain) and B-domain deleted construct (ReFacto®).

Figure 3 depicts the results of Example 3. Three different Factor VIII molecules have been injected subcutaneously in mice and their bioavailability has been determined as described in Example 2. The bioavailability of the single chain Factor VIII molecule is substantially higher than that of a two chain and full length Factor VIII (Advate®) or a heterodimeric (two chain) B-domain deleted construct (ReFacto®).

Figure 4 depicts the results of Example 4. The Factor VIII molecule of the invention and 2 commercially obtainable FVIII preparations were incubated at 37°C after purification, lyophilization and reconstitution. The FVIII- samples were incubated at 37°C for varying time periods (0, 0.25, 1, 2, 4 and 8 days) and the FVIII:C activity was determined by an one-stage-coagulation assay. The values shown represent the average and standard deviation of two samples (except 0.25 days only one sample).

Figure 5 depicts part of the results of Example 5. The pharmacokinetic (PK) profiles of scFVIII and full-length rFVIII (Advate®, Baxter Healthcare) were determined following a single I.V. injection to cynomolgus monkeys at a dose of 250 IU/kg.

Figure 6 depicts part of the results of Example 5. The pharmacokinetic (PK) profiles of full-length rFVIII (Advate®, Baxter Healthcare) were determined following a single I.V. injection to hemophilia A mice at a dose of 100 IU/kg.

Figure 7 depicts part of the results of Example 6. The average peak thrombin levels from days1-8 were determined after scFVIII or full-length rFVIII (Advate®, Baxter Healthcare) were administered to hemophilia A mice at a dose of 250 IU/kg.

Figure 8 depicts the results of Example 7. The pharmacokinetic (PK) profiles of full-length rFVIII (Advate®, Baxter Healthcare) and of a B-domain deleted Factor VIII (ReFacto®, Pfizer) was determined following a single I.V. injection to VWF deficient mice at a dose of 100 IU/kg.

DETAILED DESCRIPTION

The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain.

This invention further pertains to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, optionally inactivating the proteolytic cleavage site between Arg1313 and Ala1314, if present in the Factor VIII molecule.

Factor VIII

The terms "blood coagulation Factor VIII", "Factor VIII" and FVIII" are used interchangeably herein. Mature human Factor VIII consists of 2332 amino acids which are arranged in the following domain structure:

A1:	residues 1-336,
A2:	residues 373-710,
B:	residues 741-1648,
A3.	residues 1690-2019,
C1:	residues 2020-2172,
and	
C2:	residues 2173-2332.

In addition, there are three acidic regions *a1* (337-372), *a2* (711-740), and *a3* (1649-1689). It is known that the acidic region *a3* is involved in the binding of the Factor VIII molecule to von Willebrand Factor (vWF) which plays an important role in blood coagulation. During secretion, the FVIII is cleaved between the B-domain and the a3 acidic region, resulting in a heterodimeric polypeptide The factor VIII heterodimer consists of a light chain (comprising A3, C1 and C2) and a variably sized heavy chain (comprising A1, A2 and B). The latter is heterogeneous due to limited proteolysis within the B-domain. In case of heterodimeric B-domain deleted constructs the "heavy chain" comprises A1 and A2 but lacks part or all of the B-domain.

The amino acid sequence of the mature wild type form of human blood coagulation Factor VIII is shown in SEQ ID NO:2. The reference to an amino acid position of a specific sequence means the position of said amino acid in the FVIII wild-type

protein and does not exclude the presence of mutations, e.g. deletions, insertions and/or substitutions at other positions in the sequence referred to. For example, a mutation in "Glu2004" referring to SEQ ID NO:2 does not exclude that in the modified homologue one or more amino acids at positions 1 through 2332 of SEQ ID NO:2 are missing. A DNA sequence encoding SEQ ID NO:2 is shown in SEQ ID NO:1.

"Blood coagulation Factor VIII" includes wild type blood coagulation Factor VIII as well as derivatives of wild type blood coagulation Factor VIII having the procoagulant activity of wild type blood coagulation Factor VIII. Derivatives may have deletions, insertions and/or additions compared with the amino acid sequence of wild type Factor VIII. Preferred derivatives are FVIII molecules in which all or part of the B-domain has been deleted. Amino acid positions indicated throughout this application always refer to the position of the respective amino acid in the full length mature (i.e. after signal peptide cleavage) wild-type FVIII.

The term "factor VIII" includes any factor VIII variants or mutants having at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild type factor VIII. A suitable test to determine the biological activity of Factor VIII is the one stage or the two stage coagulation assay (Rizza et al. 1982. Coagulation assay of FVIII:C and FIXa in Bloom ed. The Hemophilias. NY Churchchill Livingston 1992) or the chromogenic substrate FVIII:C assay (S. Rosen, 1984. Scand J Haematol 33: 139-145, suppl.). The content of these references is incorporated herein by reference.

As non-limiting examples, Factor VIII molecules include Factor VIII mutants preventing or reducing APC cleavage (Amano 1998. Thromb. Haemost. 79:557-563), albumin-fused FVIII molecules (WO 2011/020866 A2), FVIII-Fc fusion molecules (WO 04/101740 A), Factor VIII mutants further stabilizing the A2 domain (WO 97/40145), FVIII mutants resulting in increased expression (Swaroop et al. 1997. JBC 272:24121-24124), Factor VIII mutants with reduced immunogenicity

(Lollar 1999. Thromb. Haemost. 82:505-508), FVIII reconstituted from differently expressed heavy and light chains (Oh et al. 1999. Exp. Mol. Med. 31:95-100), FVIII mutants reducing binding to receptors leading to catabolism of FVIII like HSPG (heparan sulfate proteoglycans) and/or LRP (low density lipoprotein receptor related protein) (Ananyeva et al. 2001. TCM, 11:251-257), disulfide bond-stabilized FVIII variants (Gale et al., 2006. J. Thromb. Hemost. 4:1315-1322), FVIII mutants with improved secretion properties (Miao et al., 2004. Blood 103:3412-3419), FVIII mutants with increased cofactor specific activity (Wakabayashi et al., 2005. Biochemistry 44:10298-304), FVIII mutants with improved biosynthesis and secretion, reduced ER chaperone interaction, improved ER-Golgi transport, increased activation or resistance to inactivation and improved half-life (summarized by Pipe 2004. Sem. Thromb. Hemost. 30:227-237), and FVIII mutants having a deletion of all or part of the B-domain (see, e.g., WO 2004/067566 A1, WO 02/102850 A2, WO 00/24759 A1 and US patent No. 4,868,112). All of these factor VIII mutants and variants are incorporated herein by reference in their entirety.

The term "single-chain Factor VIII" refers to a Factor VIII molecule which has not been proteolytically cleaved into two chains (e.g. a heavy chain and a light chain) during secretion from the cells expressing said FVIII molecule and, accordingly, is present as a single polypeptide chain.

Preventing cleavage

The method of the invention comprises preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain. The term "preventing proteolytic cleavage" includes partially preventing proteolytic cleavage and completely preventing proteolytic cleavage. It further includes the embodiment "reducing proteolytic cleavage". In other words, "preventing proteolytic cleavage of the Factor VIII molecule" does not

require completely abolishing any proteolytic cleavage such that substantially 100% of the Factor VIII molecules expressed and secreted by the host cells are single chain molecules (though this embodiment is encompassed by the method of the invention). Usually, the proteolytic cleavage of the Factor VIII molecule is prevented in a manner such that at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, most preferably at least 95% of the Factor VIII molecules expressed and secreted by the host cells are single chain molecules. The incomplete prevention of cleavage may, at least in part, be due to the fact that there can be some minor cleavage sites within the B domain which can lead to proteolytic cleavage of a small portion of the Factor VIII molecules even if the major cleavage sites (at R1313 and R1648) are absent. This minor cleavage may or may not be prevented in accordance with this invention.

The first fragment comprises essentially the A1 domain and the A2 domain of Factor VIII. The first fragment may comprise the A1 domain and the A2 domain, each domain having exactly the amino acid sequence indicated above. For example, the first fragment may comprise at least amino acids 1 to 740 of the amino acid sequence of SEQ ID NO:2. Alternatively, the first fragment may comprise a variant of this sequence, having amino acid deletions, substitutions and/or insertions which do not substantially affect the Factor VIII activity. The first fragment may additionally comprise an N-terminal part of the B domain of Factor VIII.

The second fragment comprises essentially the A3 domain, the C1 domain and the C2 domain. The second fragment may comprise the A3 domain, the C1 domain and the C2 domain, each domain having exactly the amino acid sequence indicated above. For example, the second fragment may comprise at least amino acids 1690 to 2332 of the amino acid sequence shown in SEQ ID NO:2. Alternatively, the second fragment may comprise a variant of this sequence, having amino acid deletions, substitutions and/or insertions which do not substantially affect the Factor

VIII activity. The second fragment may additionally comprise a C-terminal part of the acidic *a3* region.

The method of the invention comprises preventing the proteolytic cleavage during secretion of the recombinantly expressed FVIII molecule, which would result in a heterodimeric (two-chain) polypeptide. That is, the method includes obtaining a single-chain Factor VIIII molecule. This can be achieved in various ways, e.g. by inactivating the proteolytic cleavage sites involved in the intracellular processing of the mature, one-chain FVIII into the heterodimeric FVIII eventually secreted by the host cells.

In one embodiment the step of inactivating the proteolytic cleavage site between Arg1648 and Glu1649 comprises deleting one or more amino acids forming the protease recognition sequence. The cleavage site after residue 1648 is a furin-type cleavage site. The recognition sequence for the protease in the Factor VIII sequence is LKRHQR. Preferably, the inactivation step comprises deleting one, two, three, four, five or more of these amino acid residues forming the recognition sequence. Preferably, the inactivation step comprises deleting at least one basic amino acid within the recognition sequence, more preferably, the inactivation step comprises deleting at least the arginine at position 1648. Still more preferably, the inactivation step comprises deleting at least amino acids 1643 to 1648 of the Factor VIII sequence. If the respective FVIII derivative comprises Arg1313, the inactivation step comprises also deleting at least the arginine at position Arg 1313. Still preferably is deleting at least amino acids 1313 to 1648 of the Factor VIII sequence to inactivate both cleavage sites after 1313 and 1648, respectively.

Most preferably, the inactivation step comprises deleting at least the amino acid sequence from residues 800 to 1648 from the Factor VIII sequence, e.g. the amino acid sequence from residues 741 to 1648 from the Factor VIII sequence. In another preferred embodiment, a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected

from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage during secretion is prevented. Preferred deletions are as follows:

- amino acid 740 is fused to amino acid 1650, whereby amino acids 741 to 1649 are deleted;
- amino acid 740 is fused to amino acid 1690, whereby amino acids 741 to 1689 are deleted;
- amino acid 740 is fused to amino acid 1669, whereby amino acids 741 to 1668 are deleted;
- amino acid 743 is fused to amino acid 1650, whereby amino acids 744 to 1649 are deleted;
- amino acid 764 is fused to amino acid 1650, whereby amino acids 765 to 1649 are deleted;
- amino acid 764 is fused to amino acid 1653, whereby amino acids 765 to 1652 are deleted;
- amino acid 764 is fused to amino acid 1656, whereby amino acids 765 to 1655 are deleted;
- amino acid 745 is fused to amino acid 1650, whereby amino acids 746 to 1649 are deleted;
- amino acid 745 is fused to amino acid 1653, whereby amino acids 746 to 1652 are deleted;
- amino acid 745 is fused to amino acid 1656, whereby amino acids 746 to 1655 are deleted;
- amino acid 757 is fused to amino acid 1650, whereby amino acids 758 to 1649 are deleted;
- amino acid 757 is fused to amino acid 1653, whereby amino acids 758 to 1652 are deleted;
- amino acid 757 is fused to amino acid 1656, whereby amino acids 758 to 1655 are deleted;

- amino acid 793 is fused to amino acid 1649, whereby amino acids 794 to 1648 are deleted;
- amino acid 793 is fused to amino acid 1690, whereby amino acids 794 to 1689 are deleted;
- amino acid 747 is fused to amino acid 1649, whereby amino acids 748 to 1648 are deleted;
- amino acid 751 is fused to amino acid 1649, whereby amino acids 752 to 1648 are deleted;
- amino acid 776 is fused to amino acid 1649, whereby amino acids 777 to 1648 are deleted;
- amino acid 770 is fused to amino acid 1667, whereby amino acids 771 to 1666 are deleted.

The molecules resulting from the deletion are usually obtained in the form of single chain Factor VIII molecules.

Preferred single chain FVIII molecules have a deletion of all or part of the B-domain and a deletion of all or a part of the acidic a3 region, so that the cleavage site at Arg1648 (which is usually cleaved during secretion) is deleted. Single chain FVIII molecules are disclosed in, e.g., WO 2004/067566 A1; US 2002/132306 A1; Krishnan et al. (1991) European Journal of Biochemistry vol. 195, no. 3, pages 637-644; Herlitschka et al. (1998) Journal of Biotechnology, vol. 61, no. 3, pages 165-173; Donath et al. (1995) Biochem. J., vol. 312, pages 49-55. These single-chain Factor VIII molecules described in these references are incorporated herein by reference.

The fusions referred to above may be direct fusions or indirect fusions. In the latter case, the deleted amino acids are replaced by a heterologous spacer. This embodiment is described in more detail hereinafter. It is possible that the deleted amino acids are replaced with a peptidic linker consisting of about 1 to about 500

amino acids, or about 2 to 250 amino acids, or about 3 to about 100 amino acids, or about 4 to about 50 amino acids, or about 5 to about 10 amino acids. The peptidic linker should be flexible and not immunogenic (Robinson et al.; PNAS (1998), Vol 95, p5929). The peptidic linkers may consist of Gly preceded N-terminally to said Gly by multimers of the amino acid sequence GlyGlySer or GlyGlySerSer or any combination thereof, in a specific embodiment the peptidic linker consists of 80 to 120 amino acids.

In an alternative embodiment, one or more amino acids which form the protease recognition site at residue 1313 and 1648 may be substituted with another amino acid such that the cleavage does not occur. For example, the basic amino acids may be replaced with hydrophobic amino acids.

Preparation of single-chain Factor VIII

The step of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" is carried out prior to the purification, lyophilisation and reconstitution of the Factor VIII. The step of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" is typically carried out during the preparation of the Factor VIII molecule. The method of the invention may include preventing the proteolytic cleavage during expression of the Factor VIII molecule (in host cells), or inactivating the proteolytic cleavage site at Arg1313 and/or Arg1648 during the preparation of the nucleic acid encoding the Factor VIII molecule.

These steps of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" may include removing, from a nucleic acid encoding Factor VIII, a portion encoding the proteolytic cleavage site at Arg1313 and/or Arg1648, in accordance with the embodiments described above. This typically results in a nucleic acid encoding single chain Factor VIII. Generally, the method of the

invention may further include providing a nucleic acid encoding the single-chain Factor VIII, e.g. in an expression plasmid or vector.

The nucleic acid, the expression vector or the expression plasmid may then be introduced into host cells, preferably mammalian host cells, for expression. The method of the invention may further comprise culturing the host cells under suitable conditions such that the modified Factor VIII molecule, e.g. the single chain Factor VIII molecule, is expressed; and optionally recovering (e.g. purifying) the modified Factor VIII molecule from the host cells or from the culture medium. Generally, techniques of manipulating the nucleic acid encoding Factor VIII, of culturing mammalian cells to allow expression of the Factor VIII, and of purifying Factor VIII from the cell culture medium are known in the art.

It is preferred to purify the single chain Factor VIII molecule to ≥ 80% purity, more preferably ≥ 95% purity and particularly preferred is a pharmaceutically pure state that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins or/and nucleic acids, and free of infectious and pyrogenic agents. Preferably, an isolated or purified modified Factor VIII molecule is substantially free of other polypeptides.

The methods of the invention may further comprise the steps of purifying, lyophilizing, and reconstituting the single chain Factor VIII. The reconstitution is preferably carried out by using water, e.g. "water for injection".

Stability

The Factor VIII molecules prepared in accordance with the present invention exhibit enhanced stability relative to full length Factor VIII and/or relative to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

As used herein, the term "stability" refers to stability in aqueous solution, preferably to stability in aqueous solution after reconstitution of a lyophilized Factor VIII preparation, e.g. by adding water to the lyophilized Factor VIII preparation. Typically, the lyophilized Factor VIII preparation is reconstituted with "water for injection".

The stability in aqueous solution can be determined by providing the Factor VIII molecule in aqueous solution and incubating it for a certain period of time. In a preferred embodiment, the conditions for determining the storage stability of the Factor VIII molecule are as follows:

The Factor VIII molecule is provided in aqueous solution having the following composition:

L-histidine	25 mM
NaCl	225 mM
calcium chloride	4 mM
Tween® 80	0.03% (w/w)
sucrose	2% (w/w)
D-mannitol	8% (w/w)
pH 7.0.	

This solution is referred to hereinafter as "Buffer A". The initial Factor VIII activity in the aqueous solution is preferably between 100 IU/ml and 1,500 IU/ml, preferably it is 100 IU/ml.

The so prepared Factor VIII solution can then be incubated at 25°C for at least 24 hours, preferably for at least two days, more preferably for at least five days, most preferably for seven or eight days. After the incubation period the stability is determined by measuring the Factor VIII activity in the solution, preferably by using a chromogenic substrate assay (e.g. Coamatic® Factor VIII, Chromogenix). The

lower the loss in activity relative to the initial activity, the higher is the stability of the Factor VIII molecule. Most preferably, the stability is determined as in Example 1 or 2 below.

According to the present invention the loss in Factor VIII activity of the single-chain Factor VIII after seven days of storage under the above-identified conditions is less than 15%, preferably less than 12%, most preferably less than 10%.

Typically, the initial Factor VIII activity at the start of the incubation period (t=0) is normalized to 100%. The remaining Factor VIII activity after 24 hours of storage in Buffer A at 25°C is preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 48 hours of storage in Buffer A at 25°C is preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 4 days of storage in Buffer A at 25°C is preferably at least 90%, more preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 7 days of storage in Buffer A at 25°C is preferably at least 85%, more preferably at least 90%, most preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 8 days of storage in Buffer A at 25°C is preferably at least 85%, more preferably at least 90%, most preferably at least 95% of the initial Factor VIII activity.

The remaining Factor VIII activity of the single chain Factor VIII is usually higher than that of two-chain Factor VIII molecules (assuming that both molecules have been incubated under identical conditions for the same period of time).

The term “human full length two-chain Factor VIII” is used herein interchangeably with the term “human wild-type Factor VIII”.

In one embodiment, the remaining Factor VIII activity of the single chain Factor VIII is higher than that of human full length two-chain Factor VIII. In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII is

higher than that of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

Preferably, the remaining Factor VIII activity of the single chain Factor VIII after 48 hours of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 4 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 48 hours of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 4 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 4 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 4 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 7 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5, preferably by at least 10 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 7 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5, preferably by at least 10 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 8 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5, preferably by at least 10 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 8 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5, preferably by at least 10 percentage points.

Alternatively to Buffer A other buffers may also be used like, for example the buffer used in Example 2 of the present invention.

The preferred pH range for the buffers of the present invention is a pH range from 5.5 to 9.0, preferably a pH range from 6.0 to 8.5 and especially preferred a pH range from 6.5 to 8.0.

The activity of Factor VIII can be determined by a chromogenic or clotting assay, or any other bioassay. Preferably, the Factor VIII activity is determined as shown in Example 1 below.

Bioavailability

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibits improved bioavailability after non-intravenous injection, as compared to two chain human wild type Factor VIII or compared to two chain human B-domain deleted Factor VIII. The non-intravenous injection is preferably subcutaneous, transdermal or intramuscular injection. Most preferably, the non-intravenous injection is subcutaneous injection.

The term „bioavailability", as used herein, refers to the proportion of an administered dose of a Factor VIII or a FVIII-related preparation that can be detected in plasma at predetermined times until a final time point after subcutaneous, intravenous or intradermal administration. Typically, bioavailability is measured in test animals by administering a dose of between 10 IU/kg and 1000 IU/kg of the preparation (e.g. 400 IU/kg body weight); obtaining plasma samples at pre-determined time points after administration; and determining the content of the Factor VIII or Factor VIII-related polypeptides in the samples using one or more of a chromogenic or clotting assay (or any bioassay), an immunoassay, or an equivalent thereof. The bioavailability is expressed as the area under the curve (AUC) of the concentration or activity of the coagulation factor in plasma on the y-axis and the time after administration on the x-axis until a predefined final time point after administration. Preferably, this predefined time point is 72 or 48 hours after administration. Most preferably, the bioavailability is determined as shown in Example 3 herein below. Relative bioavailability of a test preparation refers to the ratio between the AUC of the test preparation (here: single chain Factor VIII) and that of the reference preparation (e.g. full length recombinant two-chain Factor VIII or two-chain B-domain deleted Factor VIII) which is administered in the same dose and way (e.g. intravenous, subcutaneous or intradermal) as the test preparation.

According to the present invention, the bioavailability of the single chain Factor VIII after subcutaneous injection is higher than that of the two-chain human wild type Factor VIII or of two-chain human B-domain deleted Factor VIII. Preferably, the bioavailability (AUC over 72 hours after subcutaneous injection) is increased by at least 10%, more preferably by at least 25%, more preferably by at least 50%, most preferably by at least 75%, relative to wild type FVIII. In another embodiment, the bioavailability (AUC over 72 hours after subcutaneous injection) is increased by at least 10%, more preferably by at least 20%, more preferably by at least 30%, most preferably by at least 40%, relative to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

Improvement of plasma half-life (in –vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibit increased pharmacokinetic (PK) parameters.

Factor VIII molecules of the invention can be tested by i.v. injection into different species like hemophilia A mice or cynomolgus monkeys e.g. at a dose of 100 IU/kg or 250 IU/kg respectively e.g. as determined in a chromogenic assay. Blood samples are drawn at various time points after administration e.g. until 72 hours (hrs) in hemophilia A mice and e.g. until 24 hrs in cynomolgus monkeys. Citrate plasma is prepared immediately and used for quantification of FVIII:C e.g. by a chromogenic assay system (FVIII:C) (Chromogenix - Instrumentation Laboratory SpA, Milan, Italy).

The AUC of the FVIII levels in plasma is calculated using the linear trapezoidal rule to calculate AUC_{last} : from t=0 to last observation. Terminal half-life ($t_{1/2\beta}$) is determined by a log-linear regression using the points of the terminal phase selected by the adjusted R2 criterion. AUC: from t=0 to infinity (extrapolated by using the regression model of the terminal phase).

The single chain FVIII molecules according to the invention show at least a 40%, preferably at least a 50%, even more preferably at least a 60% increased terminal half life as compared to the terminal half-life a human wild-type Factor VIII administered at the same dose and in the same manner.

Preferably the plasma half-life is determined as shown in Example 5.

Prolongation of efficacy as determined in a thrombin generation assay (in-vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibit a longer time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII. This test show that also the functionality of FVIII is stabilized in the molecules according to the invention.

FVIII molecules according to the invention can be tested by first administering the FVIII molecule of the invention at an equimolar dose (e.g. at 250 IU/kg) intravenously into hemophilia A mice. At different time points (e.g. daily from day 1 to 8) citrated blood is collected and a thrombin generation assay (TGA) is performed e.g. by calibrated thrombinography (CAT) (Thrombinoscope, Netherlands) after intrinsic activation in presence of Phospholipid (e.g. Rossix, Mölndal, Sweden) / Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (1:30). Thrombin peak levels are recorded. The average AUC of peak thrombin levels from days 1-8 is calculated by the linear trapezoidal rule. The AUC of the two Factor VIII products are compared using an approximate F-test for the difference in AUC in a linear model with variable variances per time-point and treatment group resulting in a estimated time until peak levels of thrombin drop below a defined limit ranging of 50 nM.

Preferably the efficacy in a thrombin generation assay is determined as shown in Example 6.

In hemophilia A mice scFVIII shows a favorable hemostatic activity compared to human wild-type Factor VIII. This translates into an averaged at least 10 hrs longer, preferably at least 15 hours longer and even more preferred at least 20 hours longer thrombin generation activity value for scFVIII versus full-length rFVIII before the thrombin peak level falls below a level of 50 nM.

Retaining higher FVIII:C activity in plasma (ex vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention retain a higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C; preferably wherein the retained activity of the Factor VIII is at least 10% higher relative to that of a human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

Samples with Factor VIII molecules according to the invention can be tested by diluting them into with FVIII deficient plasma (e.g. from Siemens Healthcare Diagnostics) to 1 IU/mL FVIII:C (based on values determined by the chromogenic substrate assay). The FVIII- samples are then incubated at 37°C for varying time periods (e.g. for 0, 0.25, 1, 2, 4 and 8 days) in presence of 0.05% Na-azide. After each incubation period, FVIII:C is then determined by a one-stage-coagulation assay e.g. by using Pathromtin-SL (Siemens Healthcare Diagnostics) as activator, normalized to the value at t= 0 (% FVIII:C) and plotted versus the incubation time.

After a 4 day incubation at 37°C the Factor VIII molecule of the invention has retained at least a 10% higher FVIII:C activity, preferably at least 15% higher FVIII:C activity, preferably at least a 20% higher FVIII:C activity, preferably at least a 25% higher FVIII:C activity, preferably at least a 30% higher FVIII:C activity.

Preferably the activity in plasma is determined as shown in Example 4.

Treatment and prophylaxis

The single-chain Factor VIII constructs in accordance with the present invention having increased stability after reconstitution can be administered in the treatment or prophylaxis of bleeding disorders.

As used herein, the term “bleeding disorders” includes familial and acquired hemophilia A and B, familial or acquired von Willebrand disease, familial or acquired deficiency of any coagulation factor, all types of trauma, blunt or penetrating, leading to severe hemorrhage either from a single organ, a bone fraction or from polytrauma, bleeding during surgical procedures including peri- or postoperative haemorrhage, bleeding due to cardiac surgery including patients undergoing extracorporal circulation and hemodilution in pediatric cardiac surgery, intracerebral hemorrhage, subarachnoid hemorrhage, sub-or epidural bleeding, bleedings due to blood loss and hemodilution, by non-plasmatic volume substitution leading to reduced levels of coagulation factors in affected patients, bleedings due to disseminated intravascular coagulation (DIC) and a consumption coagulopathy, thrombocyte dysfunctions, depletion and coagulopathies, bleeding due to liver cirrhosis, liver dysfunction and fulminant liver failure, liver biopsy in patients with liver disease, bleeding after liver and other organ transplantations, bleeding from gastric varices and peptic ulcer bleeding, gynaecological bleedings as dysfunctional uterine bleeding (DUB), premature detachment of the placenta, periventricular haemorrhage in low birth weight children, post partum haemorrhage, fatal distress of newborns, bleeding associated with burns, bleeding associated with amyloidosis, hematopoietic stem cell transplantation associated with platelet disorder, bleedings associated with malignancies, infections with haemorrhaging viruses, bleeding associated with pancreatitis.

The components of the pharmaceutical preparation may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical excipients to provide the pharmaceutical preparation. The components of the pharmaceutical preparation may already contain all necessary pharmaceutical, physiologically compatible excipients and may be dissolved in water for injection to provide the pharmaceutical preparation.

Such pharmaceutical carriers and excipients as well as the preparation of suitable pharmaceutical formulations are well known in the art (see for example

"Pharmaceutical Formulation Development of Peptides and Proteins", Frokjaer et al., Taylor & Francis (2000) or "Handbook of Pharmaceutical Excipients", 3rd edition, Kibbe et al., Pharmaceutical Press (2000)). In certain embodiments, a pharmaceutical composition can comprise at least one additive such as a bulking agent, buffer, or stabilizer. Standard pharmaceutical formulation techniques are well known to persons skilled in the art (see, e.g., 2005 Physicians' Desk Reference®, Thomson Healthcare: Montvale, NJ, 2004; Remington: The Science and Practice of Pharmacy, 20th ed., Gennaro et al., Eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2000). Suitable pharmaceutical additives include, e.g., sugars like mannitol, sorbitol, lactose, sucrose, trehalose, or others, amino acids like histidine, arginine, lysine, glycine, alanine, leucine, serine, threonine, glutamic acid, aspartic acid, glutamine, asparagine, phenylalanine, or others, additives to achieve isotonic conditions like sodium chloride or other salts, stabilizers like Polysorbate 80, Polysorbate 20, Polyethylene glycol, propylene glycol, calcium chloride, or others, physiological pH buffering agents like Tris(hydroxymethyl)aminomethan, and the like. In certain embodiments, the pharmaceutical compositions may contain pH buffering reagents and wetting or emulsifying agents. In further embodiments, the compositions may contain preservatives or stabilizers. In particular, the pharmaceutical preparation comprising the blood coagulation factor may be formulated in lyophilized or stable soluble form. The blood coagulation factor may be lyophilized by a variety of procedures known in the art. Lyophilized formulations are reconstituted prior to use by the addition of one or more pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution or a suitable buffer solution.

The composition(s) contained in the pharmaceutical preparation of the invention may be delivered to the individual by any pharmaceutically suitable means. Various delivery systems are known and can be used to administer the composition by any convenient route. Preferably, the composition(s) contained in the pharmaceutical preparation of the invention are delivered to the individual by non-intravenous injection. More preferably, the composition(s) of the invention are formulated for

subcutaneous, intramuscular, intraperitoneal, intracerebral, intrapulmonar, intranasal, intradermal or transdermal adminstration, most preferably for subcutaneous, intramuscular or transdermal administration according to conventional methods. The formulations can be administered continuously by infusion or by bolus injection. Some formulations may encompass slow release systems.

The composition(s) of the pharmaceutical preparation of the present invention is/are administered to patients in a therapeutically effective dose, meaning a dose that is sufficient to produce the desired effects, preventing or lessening the severity or spread of the condition or indication being treated without reaching a dose which produces intolerable adverse side effects. The exact dose depends on many factors as e.g. the indication, formulation, mode of administration and has to be determined in preclinical and clinical trials for each respective indication.

In one embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 5 hours after injection to 8 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 5%, more preferably higher than 8%, most preferably higher than 10%, of the normal plasma level of the coagulation factor in healthy subjects. The plasma level is to be determined as shown hereinafter in Example 3.

In one embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 4 hours after injection to 16 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 5%, more preferably higher than 8%, most preferably higher than 10%, of the normal plasma level of the coagulation factor in healthy subjects.

In another embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 3 hours after injection to 24 hours after non-intravenous injection, continuously higher than 2%, preferably higher than

4%, more preferably higher than 6%, most preferably higher than 8%, of the normal plasma level of the coagulation factor in healthy subjects.

In another embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 2 hours after injection to 32 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 3%, more preferably higher than 4%, most preferably higher than 5%, of the normal plasma level of the coagulation factor in healthy subjects.

Preferably, the dose of single-chain Factor VIII for one non-intravenous injection is less than 1,000 IU/kg body weight, or less than 800 IU/kg body weight, or less than 600 IU/kg body weight, or less than 400 IU/kg body weight, e.g. at a dose of from about 10 IU/kg body weight to about 1,000 IU/kg body weight, or from about 20 IU/kg body weight to about 800 IU/kg body weight, or from about 30 IU/kg body weight to about 700 IU/kg body weight, or from about 40 IU/kg body weight to about 600 IU/kg body weight, or from about 50 IU/kg body weight to about 500 IU/kg body weight, or from about 75 IU/kg body weight to about 400 IU/kg body weight, or from about 100 IU/kg body weight to about 300 IU/kg body weight, or from about 50 IU/kg body weight to about 1,000 IU/kg body weight, or from about 50 IU/kg body weight to about 800 IU/kg body weight, or from about 50 IU/kg body weight to about 700 IU/kg body weight, or from about 50 IU/kg body weight to about 600 IU/kg body weight, or from about 50 IU/kg body weight to about 500 IU/kg body weight, or from about 50 IU/kg body weight to about 400 IU/kg body weight, or from about 50 IU/kg body weight to about 300 IU/kg body weight, or about 50 IU/kg body weight to about 200 IU/kg body weight. The FVIII can be administered on its own, or as a complex with VWF.

The pharmaceutical composition(s) of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical.

Examples

Example 1: Stability of purified Factor VIII molecules after reconstitution

The following Factor VIII preparations were used in this Example:

Beriate®, a lyophilized human coagulation Factor VIII concentrate, was obtained from CSL Behring GmbH. Beriate® comprises plasma-derived Factor VIII in heterodimeric form.

Helixate®, a lyophilized, recombinant coagulation Factor VIII was obtained from CSL Behring GmbH. Helixate® contains recombinantly produced heterodimeric Factor VIII.

ReFacto® is a lyophilized Factor VIII preparation containing heterodimeric, B-domain-deleted Factor VIII produced by recombinant technology. It can be obtained from, e.g., Pfizer Pharma GmbH, Germany.

Beriate®, Helixate®, and ReFacto® are predominantly heterodimeric two-chain polypeptides.

The construct termed "scFVIII" is a single-chain Factor VIII produced by recombinant expression in mammalian cell culture cells. The single-chain Factor VIII used in this Example was obtained by directly fusing Asn764 with Thr1653, and provided in lyophilized form after purification. That is, "scFVIII" is a single chain polypeptide consisting substantially of amino acids 1-764 and 1653-2332 of SEQ ID NO:2.

Beriate®, Helixate®, and ReFacto® were reconstituted according the manufacturer's instructions as given in the package insert. "scFVIII" was reconstituted by dissolving the purified and lyophilized FVIII preparation in water for injection resulting in a composition containing 25 mM L-histidine, 225 mM NaCl, 4 mM $CaCl_2$, 0.03 % Tween 80, 2% sucrose, 8% D-mannitol, pH 7.0.

The reconstituted FVIII products were incubated at 25°C. The FVIII activity of the products was determined in duplicates by a chromogenic substrate assay (Coamatic® Factor VIII, Chromogenix) at the following time points: 0 h, 6h, 1day, 2 days, 4 days, 7 days. Activity values were normalized to time point 0.

The results are shown in the following Table and in Figure 1.

Table 1: Factor VIII activity over time

Time (days)	0 d	0.25 d	1 d	2 d	4 d	7 d
Beriate®	100.0	103.8	92.7	97.9	90.0	82.7
Helixate®	100.0	101.4	103.3	95.5	85.0	78.6
ReFacto®	100.0	106.7	83.7	94.0	93.6	77.3
scFVIII	100.0	107.8	99.4	102.0	99.0	93.3

As can be seen, "scFVIII" shows the lowest loss in activity and, consequently, is the most stable Factor VIII molecule.

Example 2: Stability of purified Factor VIII molecules after reconstitution

The following Factor VIII preparations were used in this Example:

ReFacto® and "scFVIII" were the same as used in Example 1, with the difference that “scFVIII”, provided by CSL Behring GmbH, was applied in a formulation containing different excipients. Advate® is a full-length, heterodimeric, recombinant Factor VIII preparation which was purchased in lyophilized form from Baxter.

Advate® and ReFacto® were reconstituted according the manufacturer's instructions as given in the package insert. "scFVIII" was reconstituted in water for injection resulting in a composition containing 20 mM L-histidine, 280 mM NaCl, 3.4 mM $CaCl_2$, 0.02 % Tween 80, 0.6% sucrose, pH 7.0. The sample "scFVIII 001" had an initial FVIII activity of 100 IU/ml, the sample "scFVIII 0006" had an initial FVIII activity of 400 IU/ml. The reconstituted FVIII products were incubated at 25°C. The FVIII activity of the products was determined in duplicates by a chromogenic substrate assay (Coamatic® Factor VIII, Chromogenix) at the following time points: 0 h, 6h, 1day, 2 days, 4 days, 8 days. Activity values were normalized to time point 0.

The results are shown in the following Table and in Figure 2.

Table 2: Factor VIII activity over time

Time (days)	0 d	0.25 d	1 d	2 d	4 d	8 d
Advate	100.0	94.9	100.6	89.8	74.1	73.7
ReFacto	100.0	92.0	88.8	92.1	74.3	80.6
scFVIII 001	100.0	99.2	95.6	99.2	89.0	95.1
scFVIII 006	100.0	96.8	97.6	100.0	88.9	92.3

As can be seen, "scFVIII" shows the lowest loss in activity and, consequently, is the most stable Factor VIII molecule.

Example 3: Bioavailability of Factor VIII molecules

Advate®, ReFacto® and "scFVIII" were the same as used in Example 2 and reconstituted as described in Example 2.

Factor VIII knockout mice were used as animal model for hemophilia A. These mice lack exons 16 and 17 and thus do not express FVIII (Bi L. et al, Nature genetics,

1995, Vol 10(1), 119-121; Bi L. et al, Blood, 1996, Vol 88(9), 3446-3450). This allows the analysis of FVIII levels following treatment by quantification of FVIII activity in the plasma of the ko mice.

To assess whether extravascular injections might be an option for an improved therapy with human FVIII, subcutaneous injection was chosen. The design of the non-clinical pharmacokinetic study performed is detailed in table 3 below. Plasma levels of Factor VIII activity were determined following a single subcutaneous injection of the respective FVIII preparation (detailed treatment groups in table 3) to a hemophilia A model.

Corresponding groups were treated with the same dose of FVIII:chromogen activity. For a single application the Factor VIII was provided in a volume of 200 µL (identical volumes for all groups) prior to subcutaneous application to FVIII knockout (ko) mice weighing about 25 g. The treatment groups are summarized in table 3.

Under short term anesthesia, blood samples were drawn, anticoagulated using sodium citrate to 10 % citrate blood, processed to plasma and stored at –70°C for the determination of FVIII activity. The sampling time points are detailed in table 4. Quantification of FVIII activity in plasma was performed by a standard, aPTT based approach (Behring Coagulation Timer). The animals were kept at standard housing conditions.

Table 3: Treatment groups

No.	Treatment	FVIII:chromogen / Additive Dose	volume [mL/kg]	schedule	route	N
1	Advate®	400 IU/kg	8	single injection (t=0)	s.c.	25
2	ReFacto®	400 IU/kg	8	single injection (t=0)	s.c.	25
3	"scFVIII"	400 IU/kg	8	single injection (t=0)	s.c.	20

Results

The results are summarized in Table 4 and Figure 3.

Table 4:

timepoint [hours]	scFVIII			ReFacto®			Advate®		
	mean	SD	n	mean	SD	n	mean	SD	n
1	5.83	1.84	5	6.35	7.62	5	3.82	3.30	5
4	11.53	5.35	5	4.77	3.47	5	3.16	2.72	5
8	20.03	9.91	5	9.81	6.06	5	5.52	3.92	5
16	7.75	4.78	5	4.91	1.98	5	2.77	2.10	5
24	3.00	3.25	5	0.66	0.69	5	0.31	0.68	5
32	3.30	2.36	5	2.11	1.53	5	3.13	1.78	5
48	3.71	1.52	5	6.01	5.28	5	4.15	1.95	5
72	1.23	2.63	5	0.00	0.00	5	0.00	0.00	5
AUC_{0-72h} (h × % of the norm)	**383.9**			**275.1**			**195.1**		

Subcutaneous injection of 400 IU/kg single chain FVIII ("scFVIII") to FVIII ko mice resulted in a significant increase of FVIII activity in plasma level as compared to administration of heterodimeric full length FVIII (Advate®) or heterodimeric B-

domain-deleted FVIII (ReFacto®). That is, the single chain Factor VIII molecule shows the highest in vivo bioavailability after subcutaneous injection into mice. The two chain full length construct Advate®, as well as the two chain B-domain deleted preparation ReFacto® showed substantially lower bioavailability.

Example 4: Stability of Factor VIII molecules in plasma (in-vitro)

Different FVIII products (Advate®, ReFacto AF® and two lots of scFVIII as used in Example 2) were diluted with FVIII deficient plasma (Siemens Healthcare Diagnostics) to 1 IU/mL FVIII:C (based on values determined by the chromogenic substrate assay). The FVIII- samples were incubated at 37°C for varying time periods (0, 0.25, 1, 2, 4 and 8 days) in presence of 0.05% Na-azide. After each incubation period, FVIII:C was determined by one-stage-coagulation assay using Pathromtin-SL (Siemens Healthcare Diagnostics) as activator, normalized to the value at t= 0 (% FVIII:C) and plotted versus the incubation time. The values shown represent the average and standard deviation of two samples (except 0.25 days only one sample).

Table 5: Average % Activity Compared to Time 0

	0 d	0,25 d	1 d	2 d	4 d	8 d
Advate	100,00	90,63	81,25	79,69	70,31	59,38
ReFacto	100,00	92,31	86,15	83,08	70,77	58,46
scFVIII 001	100,00	93,33	93,33	96,67	93,33	93,33
scFVIII 006	100,00	96,88	96,88	93,75	93,75	90,63

Example 5: Stability of Factor VIII molecules in plasma (in-vivo)

The pharmacokinetic (PK) profiles of scFVIII and full-length rFVIII (Advate®, Baxter Healthcare) was determined following a single I.V. injection to cynomolgus monkeys (Figure 5 and Table 6) and hemophilia A mice (Figure 6 and Table 7) at doses of 250 IU/kg and 100 IU/kg, respectively. Test items were dosed according to labeled activity for Advate® and chromogenic activity (FVIII:C) for scFVIII. Blood samples were drawn predose (monkeys only) and at various time points after administration until 72 hours (hrs) in hemophilia A mice and until 24 hrs in cynomolgus monkeys. Citrate plasma was prepared immediately and used for quantification of FVIII:C by a chromogenic assay system (FVIII:C) (Chromogenix - Instrumentation Laboratory SpA, Milan, Italy).

The AUC of the FVIII levels in plasma was calculated using the linear trapezoidal rule to calculate AUC_{last} : from t=0 to last observation. Terminal half-life ($t_{1/2\beta}$) was determined by a log-linear regression using the points of the terminal phase selected by the adjusted R2 criterion. AUC: from t=0 to infinity (extrapolated by using the regression model of the terminal phase).

In cynomolgous monkeys scFVIII showed a ~1.6 fold enhanced $AUC_{0\text{-}tlast}$ or $t_{1/2\beta}$ with a correspondingly ~2 fold lower clearance (CL), while FVIII activity peak levels (C_{max}), representative of in vivo recovery (IVR), and volume of distribution at steady state (V_{ss}) appeared more similar versus full-length rFVIII. These PK parameter results were obtained from n=10 animals after toxicokinetic data from 8 additional monkeys, when dosed during the GLP- toxicity studies with 250 IU/kg of scFVIII, were included (Table 6 and Figure 5).

In hemophilia A mice enhancement of $AUC_{0\text{-}tlast}$, of mean residence time (MRT), time until 5% FVIII activity trough levels, terminal half-life and a correspondingly lower CL ranged between 1.6-2 fold for scFVIII, while C_{max}, representative of IVR, and V_{ss} appeared similar versus full-length rFVIII. $AUC_{0\text{-}tlast}$ and $t_{1/2\beta}$ results

obtained after rVIII-SingleChain treatment were significantly better than for full-length rFVIII with an $AUC_{0\text{-}tlast}$ ratio of 1.97 (90% confidence interval (CI): 1.7-2.3; p-value(ratio=1):< 0.0001), and a $t_{1/2\beta}$ ratio of 1.65 (90% CI: 1.11-2.70; p-value(ratio=1): 0.036 (Table 7 and Figure 6).

Table 6: PK parameters of scFVIII and full-length rFVIII in cynomolgus monkeys

Parameters	scFVIII (n=10)	Full-length rFVIII (n=2)
$AUC_{0\text{-}tlast}$ (hrs·IU/mL)	78.4	49.1
C_{max} (IU/mL)	7.8	8.7
CL ((mL/hrs)/kg)	2.1	4.7
$t_{1/2\beta}$ (hrs)	11.0	6.8

Table 7: PK parameters of scFVIII and full-length rFVIII in hemophilia A mice

Parameters	scFVIII	Full-length rFVIII
$AUC_{0\text{-last}}$ (hrs·IU/mL)	35	18
C_{max} (IU/mL)	2.3	2.2
CL ((mL/hrs)/kg)	2.7	5.5
MRT (hrs)	18	10
V_{SS} (mL/kg)	50	57
$t_{1/2\beta}$ (hrs)	15.9	9.7
Time until 0.05 IU/mL (hrs)	73	39

Both sets of PK parameters reflect the increased stability of scFVIII after purification, lyophilization, reconstitution *in vivo* in plasma after administration to the two animal species tested.

Example 6: Thrombin generation assay in hemophilia A mice (ex vivo)

Citrate- (10% v/v) hemophilia A mouse blood was terminally collected under deep anesthesia at different time-points (days 1-8) when scFVIII or full-length rFVIII (Advate®), were dosed @ a level of 250 IU/kg. TGA was performed by calibrated thrombinography (CAT, Thrombinoscope, Netherlands) after intrinsic activation in presence of Phospholipid (Rossix, Mölndal, Sweden) / Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (1:30). Thrombin

peak levels were recorded. The average AUC of peak thrombin levels from days1-8 was calculated by the linear trapezoidal rule. The AUC of the two Factor VIII products were compared using an approximate F-test for the difference in AUC in a linear model with variable variances per time-point and treatment group resulting in estimated time until peak levels of thrombin drop below a defined limit ranging between 50-250 nM.

In hemophilia A mice scFVIII showed a favorable hemostatic activity compared to full-length rFVIII as indicated by the estimated time until peak levels of thrombin drop below a defined limit ranging from 50-250 nm peak level (Figure 8 and Table 8). This translated into an averaged 20 hrs longer thrombin generation activity value for scFVIII versus full-length rFVIII for the thrombin peak level interval between 50 and 250 nM. When assessing the area under the peak curve between days 1-8 the thrombin generation activity of scFVIII was significantly better with $p(AUC_{TGA\ Peak}$-ratio=1) = 0.0002 (estimated ratio 1.26, 90% CI: 1.14-1.39) compared to full-length FVIII, or in other words it took significantly longer for scFVIII to fall below a thrombin peak level of 50nm after administration than for the human wild-type Factor VIII Advate®.

These results again confirmed the increased functional stability of scFVIII after purification, lyophilization and reconstitution.

Table 8: Peak nm Thrombin

	CSL627 (N=8-14)		Advate (N=7-14)	
time [hrs]	mean	SD	mean	SD
0	0	0	0	0
24	325,5	40,2	343,4	40,41
32	294	102,8	305,2	72,13
48	277,1	22,44	279,6	30,92
72	283,1	42,88	163,6	79,87
96	115,4	29,63	75,44	35,28
120	91,85	57,63	55,17	30,89
144	45,6	36,15	24,61	13,52
168	8,453	12,66	14,13	19,44
192	3,901	9,384	4,71	8,1

Example 6: Stability of Factor VIII molecules in vWF deficient plasma (in-vivo)

scFVIII was reconstituted in 2.5 mL water for injection. ReFacto AF® and Advate® were reconstituted according to the description in the package insert. All test articles were aliquoted and stored immediately frozen at approximately -70°C. Prior to administration test articles were diluted with formulation buffer for CSL 627 to get a minimum practical volume ensuring a reliable administration.

12 VWF ko mice (6 female/6 male) per group received a single i.v. injection of 100 IU/kg of either scFVIII based on chromogenic FVIII activity and ReFacto AF® or Advate® based on the labeled FVIII activity into the lateral tail vein. Following administration of the different test items blood samples were drawn for determination of FVIII plasma levels at 0.083, 0.5, 1, 2, 4, 7, 16 and 24 hours from n= 2-3 mice per time point. Blood samples were processed to 10% citrate (3.13% w/v) plasma and subsequently subjected to FVIII plasma level analysis using the

chromogenic assays system The chromogenic FVIII activity was determined using the COAMATIC® FVIII test kit from Chromogenix, Italy.

The AUC of the FVIII levels in plasma was calculated using the linear trapezoidal rule to calculate AUClast : from t=0 to last observation.

Likewise to results obtained after i.v. administration to FVIII ko mice and normal monkeys as well as s.c. administration to FVIII ko mice analysis the exposure to CSL627 was higher compared to ReFacto AF® and Advate®. Since analysis of the AUC, the most relevant and representative PK parameter for systemic exposure yielded a 30 % higher AUC value after administration of CSL627 compared to both ReFacto AF® and Advate®. Again. these observations reflect the increased intrinsic stability of scFVIII after purification, lyophilization, reconstitution in vivo in plasma after administration to mice lacking systemic, circulating VWF, hence in absence of its shielding and protective effect for systemic, circulating FVIII.

Table 9: Plasma levels of scFVIII compared to Advate® and ReFacto AF® after administration to VWF deficient mice at a dose level of 100 IU/kg

	CSL627	Advate®	ReFacto AF®
Baseline	0,0	0,0	0,0
Total Area	2427	1654	1714
Total Peak Area	2427	1654	1714
Number of Peaks	1,000	1,000	1,000
Peak 1			
First X=	0,0830	0,0830	0,0830
Last X=	24,00	24,00	24,00
Peak X=	0,0830	0,0830	0,0830
Peak Y=	1770	1131	1248
Area under curve=	2427	1654	1714
%Area=	100,0	100,0	100,0

Claims

1. A method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule.

2. The method of claim 1, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

3. The method of claim 2, wherein the inactivation step comprises deleting at least Arg1648 from the Factor VIII sequence.

4. The method of claim 3, wherein the inactivation step comprises deleting at least the amino acid sequence from Arg1313 to Arg1648 from the Factor VIII sequence.

5. The method of any one of claims 1 to 4, wherein a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314 is inactivated.

6. The method of any one of claims 3 to 5, wherein the deleted amino acids are replaced with a peptidic spacer having a length of 1 to 50 amino acids.

7. The method of claim 2, wherein the inactivation step comprises substituting Arg1648 and, if present in the Factor VIII molecule, Arg1313 with a different amino acid.

8. The method of any one of claims 1 to 7, wherein the loss of activity of the Factor VIII molecule, after reconstitution in aqueous solution and storage for 7 days at 25°C is less than 15%.

9. The method of any one of claims 2 to 8, wherein the in vitro stability of the Factor VIII after reconstitution in aqueous solution is increased by inactivation of said cleavage site(s).

10. The method of any one of claims 1 to 9, wherein the Factor VIII exhibits improved bioavailability after non-intravenous injection relative to human wild type Factor VIII or relative to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner; preferably wherein the bioavailability after non-intravenous injection is increased by at least 25% relative to human wild type Factor VIII or relative to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640 administered in the same dose and in the same manner.

11. The method of claim 10, wherein said non-intravenous injection is subcutaneous, transdermal or intramuscular injection.

12. The method of any one of claims 1 to 9, wherein

(i) the Factor VIII exhibits improved plasma half-life after intravenous administration relative to human wild type Factor VIII; preferably wherein the

plasma half-life is improved by at least 40% relative to human wild type Factor VIII,

or

(ii) wherein the Factor VIII exhibits a longer time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII; preferably wherein this time period is prolonged by at least 10 hours relative to human wild type Factor VIII,

or

(iii) wherein the Factor VIII retains a higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C; preferably wherein the retained activity of the Factor VIII is at least 10% higher relative to that of a human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

13. The method of any one of the preceding claims, comprising the steps of

(i) providing a nucleic acid encoding a modified Factor VIII molecule in which the proteolytic cleavage sites between Arg1648 and Glu1649, and between Arg1313 and Ala1314, are inactivated,

(ii) transforming a host cell with said nucleic acid,

(iii) culturing the transformed host cell under conditions such that the modified Factor VIII molecule is expressed, and

(iv) recovering the modified Factor VIII molecule from the host cells or from the cell culture medium.

14. A method for improving the bioavailability of a Factor VIII molecule after non-intravenous administration relative to human wild type Factor VIII or relative to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640, comprising inactivating the proteolytic cleavage site between Arg1648 and

Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

15. The method of claim 14, wherein said non-intravenous administration is subcutaneous, transdermal or intramuscular injection.

16. A method for improving the plasma half-life of a Factor VIII molecule after intravenous administration relative to human wild-type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

17. A method for prolonging the time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration of a Factor VIII molecule relative to human wild type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

18. A method for retaining a higher activity for a Factor VIII molecule as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

19. The method of claims 14 to 18, wherein a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and

Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314 is inactivated.

20. A pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by

(i) non-intravenous administration, wherein

the bioavailability of said single chain Factor VIII molecule is increased by at least 25% as compared to human wild type Factor VIII or as compared to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner,

or

(ii) by intravenous administration, wherein

(a) the plasma half-life of said single chain Factor VIII molecule after intravenous administration is increased by at least 40%, relative to human wild type Factor VIII, administered at the same dose and in the same manner,

or

(b) the single chain Factor VIII molecule exhibits a time period prolonged by at least 10 hours for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII, administered at the same dose and in the same manner.

21. A pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, wherein the single chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay after having been incubated for

4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

22. A pharmaceutical solution comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by non-intravenous administration, wherein the dose of said FVIII molecule can be decreased by at least 25% as compared to that of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner to achieve the same hemostatic activity in blood.

23. The use of a single chain Factor VIII molecule for achieving an increased stability after reconstitution or a longer shelf life of a pharmaceutical preparation for treating a bleeding disorder, wherein

(i) the Factor VIII activity of the pharmaceutical preparation comprising the single chain Factor VIII molecule, after reconstitution and storage at room temperature for 7 days after reconstitution is at least 10% higher than that of a pharmaceutical preparation comprising the same amount of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, or

(ii) wherein the single chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay when incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C at the same concentration.

Figure 1

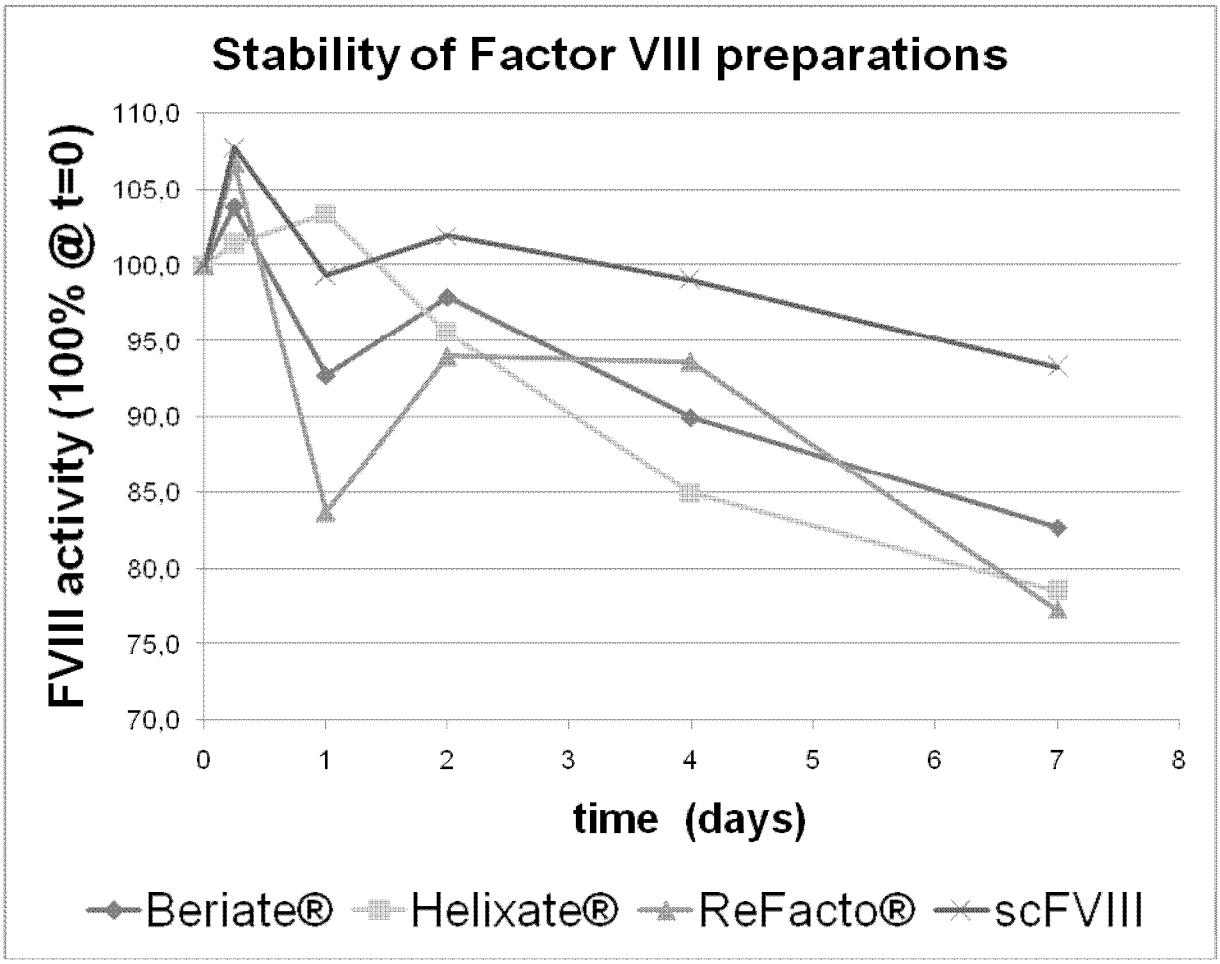

Stability of Factor VIII preparations
FVIII activity (100% @ t=0)
110,0
105,0
100,0
95,0
90,0
85,0
80,0
75,0
70,0
0
1
2
3
4
5
6
7
8
time (days)
Beriate®
Helixate®
ReFacto®
scFVIII

Figure 2

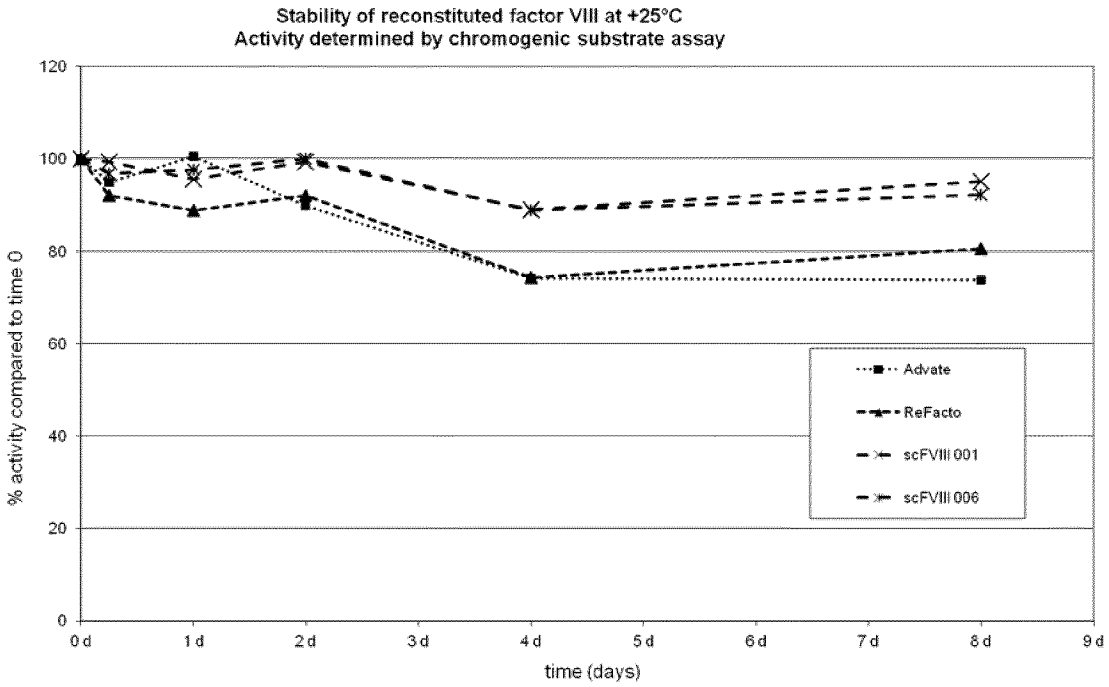

Stability of reconstituted factor VIII at +25°C
Activity determined by chromogenic substrate assay
% activity compared to time 0
time (days)
0
20
40
60
80
100
120
0 d
1 d
2 d
3 d
4 d
5 d
6 d
7 d
8 d
9 d
Advate
ReFacto
scFVIII 001
scFVIII 006

Figure 3

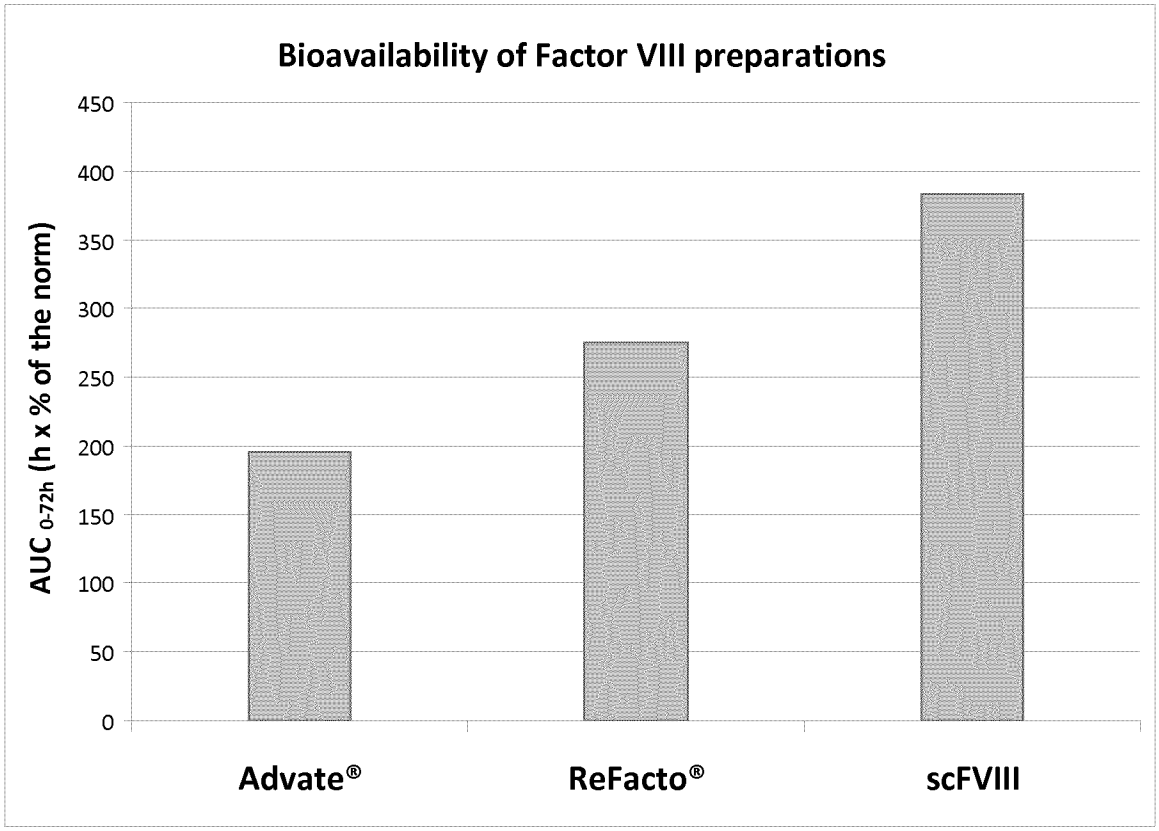

Bioavailability of Factor VIII preparations
AUC 0-72h (h x % of the norm)
450
400
350
300
250
200
150
100
50
0
Advate®
ReFacto®
scFVIII

Figure 4

Stability of FVIII molecules in plasma (ex-vivo)

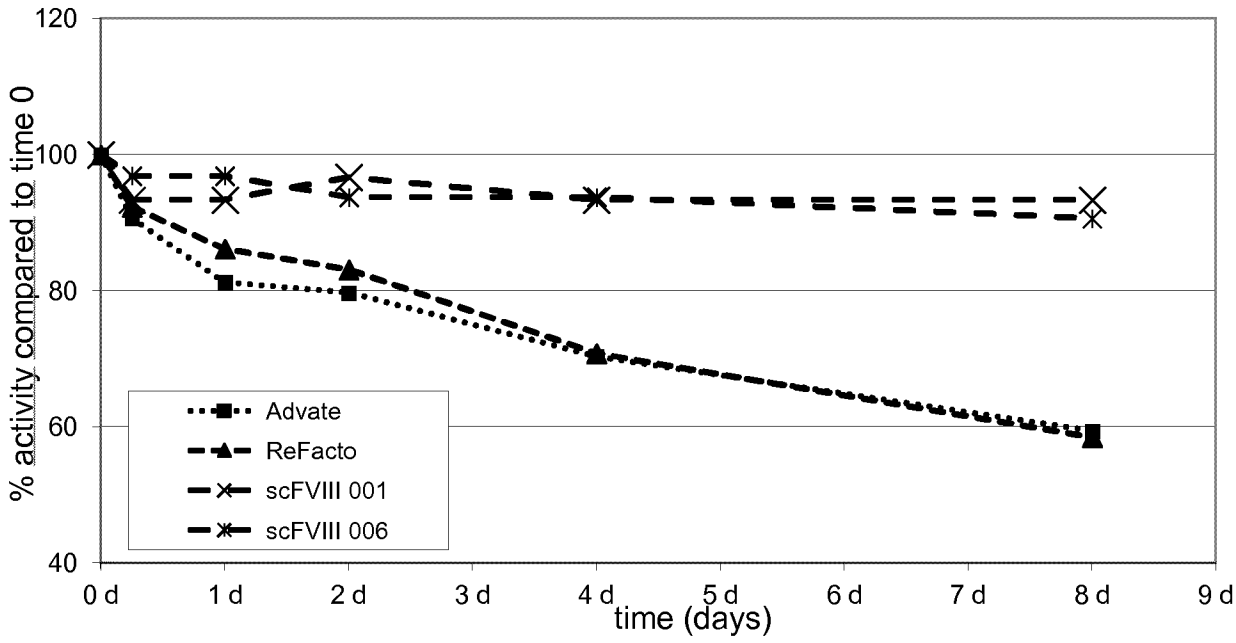


Figure 5

Stability of FVIII molecules in plasma (in-vivo) of cynomolgus monkeys (dosed at 250 IU/kg)

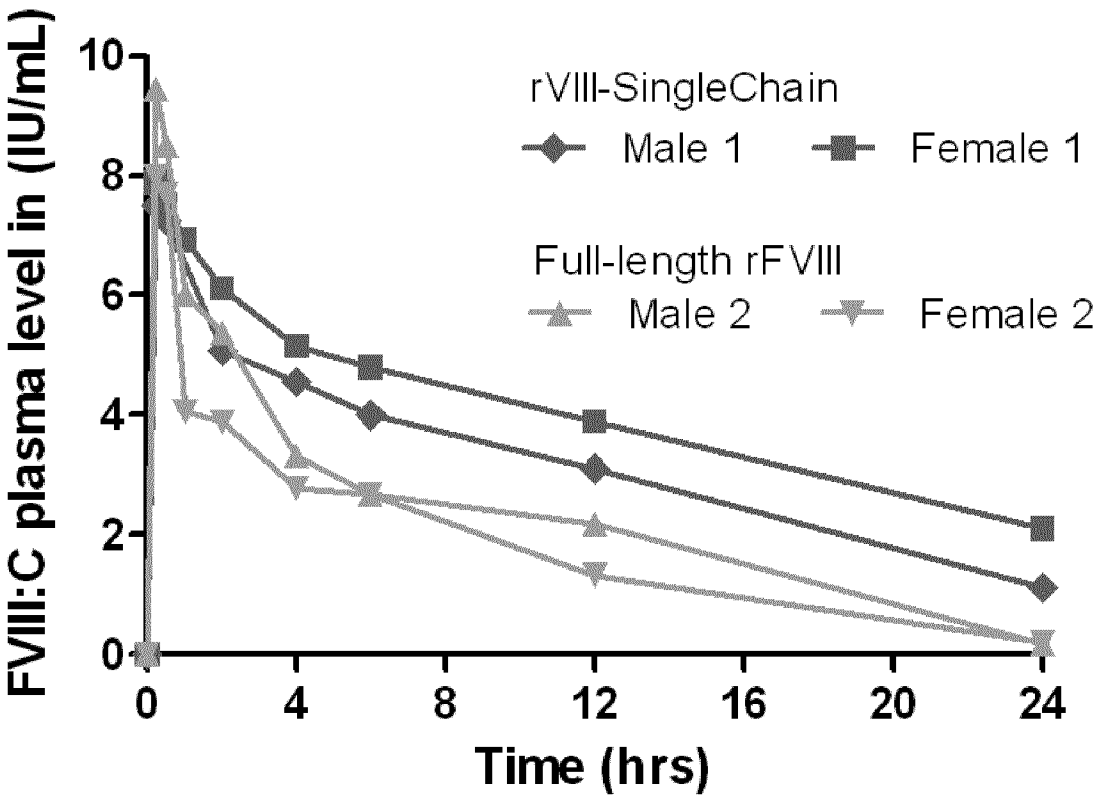


Figure 6

Stability of FVIII molecules in plasma (in-vivo) of in hemophilia A mice (dosed at 100 IU/kg)

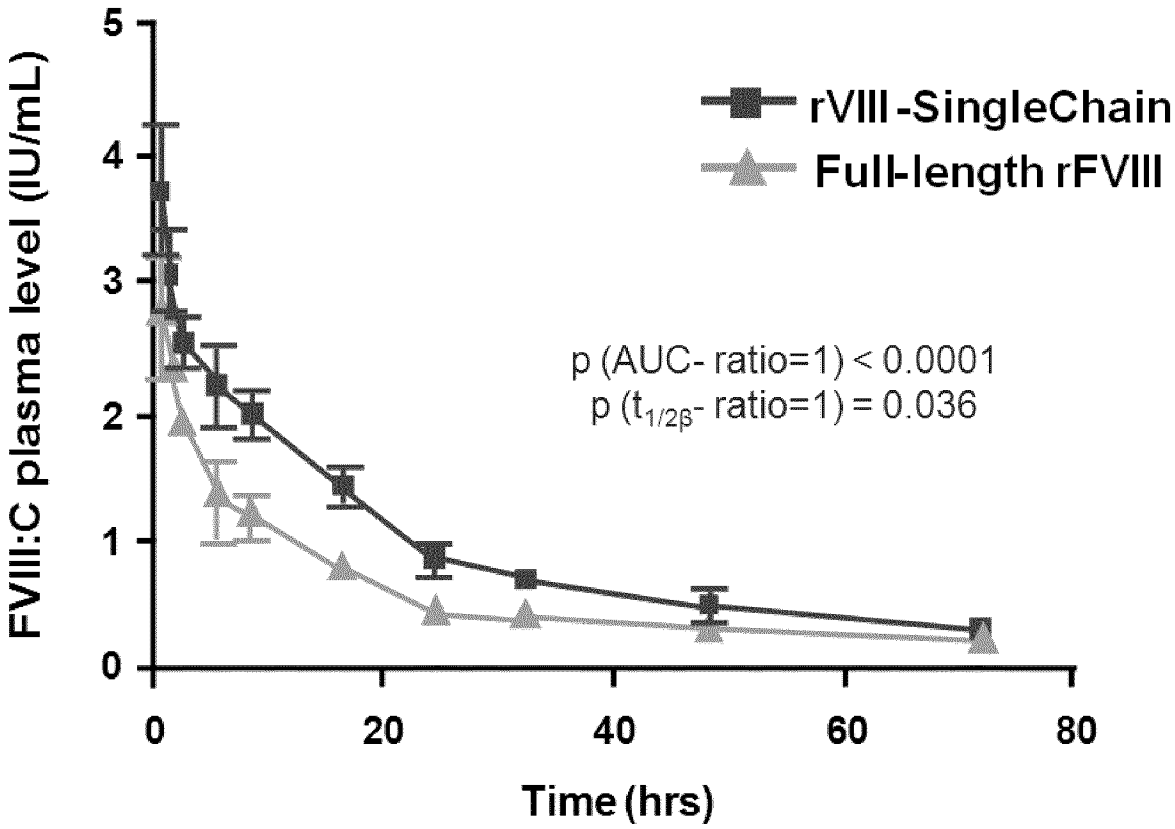


Figure 7

Thrombin generation in hemophilia A mice (ex vivo)

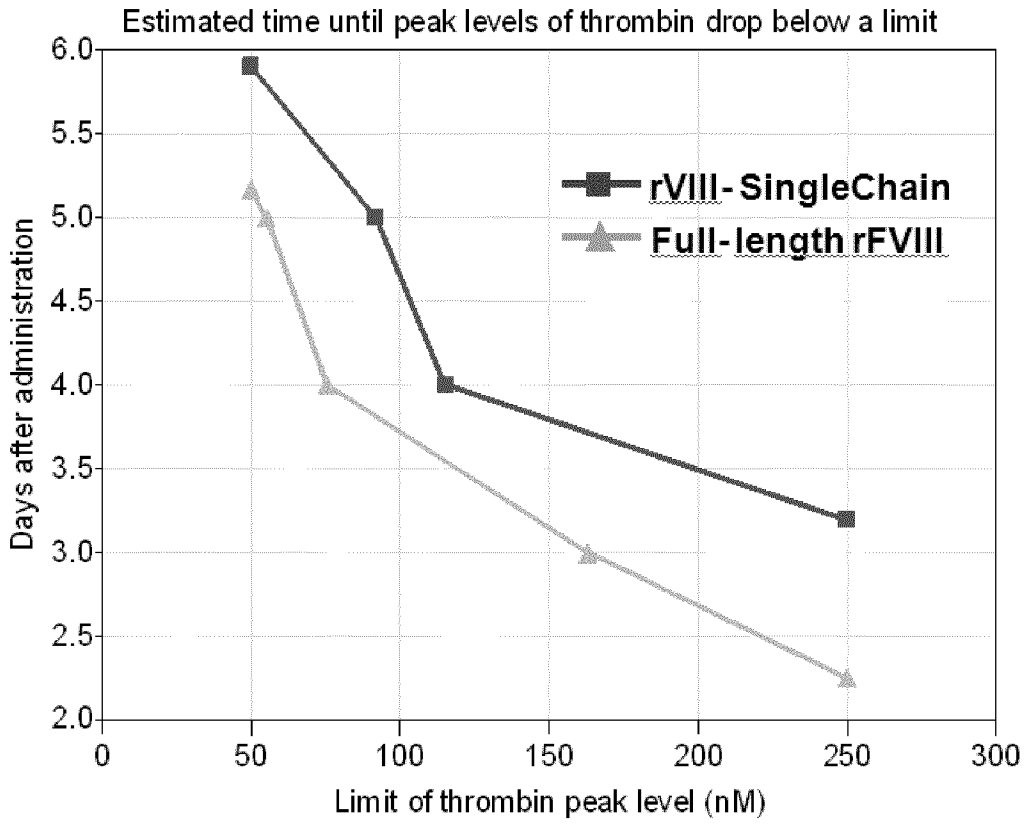
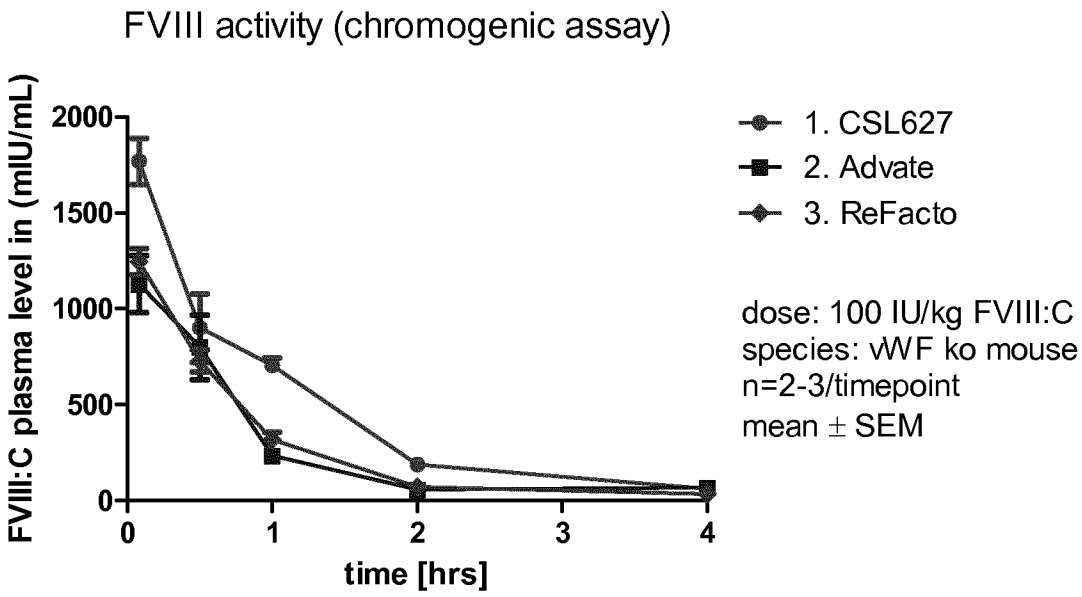


Figure 8

Stability of FVIII molecules in plasma (in-vivo) of vWF deficient mice



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/070701

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/755 A61K38/37
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/067566 A1 (IN2GEN CO LTD [KR]) 12 August 2004 (2004-08-12) pages 2-7 claims 1-20 -----	1-23
X	WO 2008/077616 A1 (CSL BEHRING GMBH [DE]; SCHULTE STEFAN [DE]; WEIMER THOMAS [DE]; METZNE) 3 July 2008 (2008-07-03) page 5 - page 9 pages 15, 21 figure 1 compounds 1-32 -----	1-23
X	WO 2010/111414 A1 (BAYER HEALTHCARE LLC [US]; ZHAO XIAO-YAN [US]; KRETSCHMER PETER JOHN [) 30 September 2010 (2010-09-30) figure 3 claims 1-52 ----- -/--	1-23

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

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
11 December 2012	02/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Behrens, Joyce

3

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SANDBERG H ET AL: "STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF B-DOMAIN DELETED RECOMBINANT FACTOR VIII", SEMINARS IN HEMATOLOGY, PHILADELPHIA, PA, US, vol. 38, no. 2, SUPPL. 04, 1 April 2001 (2001-04-01), pages 4-12, XP001223580, ISSN: 0037-1963, DOI: 10.1053/SHEM.2001.25888 abstract	1-23
X	EATON D L ET AL: "CONSTRUCTION AND CHARACTERIZATION OF AN ACTIVE FACTOR VIII VARIANT LACKING THE CENTRAL ONE-THIRD OF THE MOLECULE", BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, US, vol. 25, no. 26, 1 January 1986 (1986-01-01), pages 8343-8347, XP002226817, ISSN: 0006-2960, DOI: 10.1021/BI00374A001 abstract	1-23
X	DONATH MARIE-JOSE S H ET AL: "Characterization of des-(741-1668)-factor VIII, a single-chain factor VIII variant with a fusion site susceptible to proteolysis by thrombin and factor Xa", BIOCHEMICAL JOURNAL, THE BIOCHEMICAL SOCIETY, LONDON, GB, vol. 312, no. 1, 15 November 1995 (1995-11-15), pages 49-55, XP009155492, ISSN: 0264-6021 abstract	1-23
A	TOOLE J J ET AL: "A LARGE REGION (=95 KDA) OF HUMAN FACTOR VIII IS DISPENSABLE FOR IN VITRO PROCOAGULANT ACTIVITY", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC; US, vol. 83, 1 August 1986 (1986-08-01), pages 5939-5942, XP000651924, ISSN: 0027-8424, DOI: 10.1073/PNAS.83.16.5939 abstract	1-23

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DI PAOLA J ET AL: "ReFacto and Advate: a single-dose, randomized, two-period crossover pharmacokinetics study in subjects with haemophilia A", HAEMOPHILIA, BLACKWELL SCIENCE, OXFORD, GB, vol. 13, no. 2, 1 March 2007 (2007-03-01), pages 124-130, XP009155617, ISSN: 1351-8216 [retrieved on 2007-01-25] abstract -----	1-23
A	LENTING PETER J ET AL: "The life cycle of coagulation factor VIII in view of its structure and function", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 92, no. 11, 1 December 1998 (1998-12-01), pages 3983-3996, XP002333863, ISSN: 0006-4971 abstract -----	1-23
A	ESMON P C ET AL: "CHARACTERIZATION OF RECOMBINANT FACTOR VIII AND A RECOMBINANT FACTOR VIII DELETION MUTANT USING A RABBIT IMMUNOGENICITY MODEL SYSTEM", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 76, no. 8, 15 October 1990 (1990-10-15), pages 1593-1600, XP009155616, ISSN: 0006-4971 abstract -----	1-23

3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/070701

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004067566 A1	12-08-2004	AU 2003274766 A1	23-08-2004
		CA 2514646 A1	12-08-2004
		CN 1745100 A	08-03-2006
		EP 1587832 A1	26-10-2005
		EP 2253645 A2	24-11-2010
		KR 20050044776 A	12-05-2005
		US 2004147436 A1	29-07-2004
		WO 2004067566 A1	12-08-2004
WO 2008077616 A1	03-07-2008	AU 2007338298 A1	03-07-2008
		CA 2673459 A1	03-07-2008
		EP 2097096 A1	09-09-2009
		JP 2010512768 A	30-04-2010
		KR 20090102795 A	30-09-2009
		US 2010120664 A1	13-05-2010
		WO 2008077616 A1	03-07-2008
WO 2010111414 A1	30-09-2010	CA 2756197 A1	30-09-2010
		CN 102427823 A	25-04-2012
		EP 2411024 A1	01-02-2012
		JP 2012522490 A	27-09-2012
		US 2012142593 A1	07-06-2012
		WO 2010111414 A1	30-09-2010