The invention relates to the use of PEGylated Factor IX polypeptide for the manufacture of a medicament for treatment of a condition affectable by Factor IX, said medicament being for subcutaneous administration.
SUBCUTANEOUS ADMINISTRATION OF COAGULATION FACTOR IX

FIELD OF THE INVENTION

The invention relates to the use of PEGylated Factor IX polypeptides for the manufacture of a medicament for prevention or treatment of conditions affectable by Factor IX or the Factor IX polypeptide, wherein the medicament is for subcutaneous or intramuscular administration.

BACKGROUND OF INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually gives rise to a fibrin clot. Generally, the blood components that participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins that are converted to proteolytic enzymes by the action of an activator, which is itself an activated clotting Factor. Coagulation factors that have undergone such a conversion are generally referred to as "active factors", and are designated by the addition of a lower case "a" suffix (e.g., Factor Vila).

Activated Factor X ("Xa") is required to convert prothrombin to thrombin, which then converts fibrinogen to fibrin as a final stage in forming a fibrin clot. There are two systems, or pathways, that promote the activation of Factor X. The "intrinsic pathway" refers to those reactions that lead to thrombin formation through utilisation of factors present only in plasma. A series of protease-mediated activations ultimately generates Factor IXa, which, in conjunction with Factor Villa, cleaves Factor X into Xa. An identical proteolysis is effected by Factor Vila and its co-Factor, tissue factor, in the "extrinsic pathway" of blood coagulation. Tissue factor is a membrane bound protein and does not normally circulate in plasma. Upon vessel disruption, however, it can complex with Factor Vila to catalyse Factor X activation or Factor IX activation in the presence of Ca++ and phospholipid. The relative importance of the two coagulation pathways in haemostasis is still unclear.

Factor IXa (FIXa) is a trypsin-like serine protease that serves a key role in haemostasis by generating, as part of the Xase complex, most of the Factor Xa required to support proper thrombin formation during coagulation (reviewed in Hoffman M. and Monroe D. M., Ill (2001) A cell-based model of hemostasis. Thromb Haemost 85, 958-965).

Congenital deficiency of Factor IXa activity is the cause of the X-linked bleeding disorder
haemophilia B affecting approximately 1:100,000 males. These haemophilia patients are currently treated by replacement therapy with either recombinant or plasma-derived coagulation Factor IX. Administration of Factor IX is either as on-demand or prophylactic basis which requires venipuncture. The venipuncture procedure can be painful, difficult and time consuming, resulting in delayed treatment and considerable stress for the patient.

Factor IX is a vitamin K-dependent coagulation factor with structural similarities to Factor VII, Factor X, and protein C. The circulating zymogen form, which has a plasma half-life of about 18-30 hours, consists of 415 amino acids divided into four distinct domains comprising an N-terminal γ-carboxyglutamic acid rich (Gla) domain, two EGF domains, and a C-terminal trypsin-like serine protease domain. Activation of Factor IX occurs by limited proteolysis at Arg145-Ala146 and Arg180-Val181 releasing a 35-aa fragment, the so-called activation peptide (Schmidt A. E. and Bajaj S. P. (2003) Structure-function relationships in Factor IX and Factor IXa. Trends Cardiovasc Med 13, 39-45). The activation peptide is heavily glycosylated containing two N-linked and up to four O-linked glycans.


As mentioned above, current administration of Factor IX is either as on-demand or prophylactic basis which requires venipuncture. Subcutaneous administration would be a significant benefit, especially in patients where venous access represents a barrier to optimal treatment compliance.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the primary structure of plasma derived FIX.

Figure 2 illustrates blood concentration of FIX (ng/ml) as a function of time (h) for the studied compounds after iv and sc administration in FIX KO mice (mean ± SD, n=3).

SUMMARY OF THE INVENTION

The present invention provides a pharmaceutical formulation for prevention or treatment of Factor IX-responsive syndromes such as, e.g., hemophilia comprising a therapeutically effective amount of a PEGylated Factor IX polypeptide together with a pharmaceutically acceptable carrier for subcutaneous or intramuscular administration.

The present invention provides methods for preventing or treating Factor IX-responsive syndromes such as, e.g., hemophilia which are carried out by administering to a patient in need of such treatment an effective amount for such treatment of a PEGylated Factor IX polypeptide via a subcutaneous or intramuscular route of administration.

The invention provides in another aspect administration of PEGylated Factor IX polypeptides via a subcutaneous or intramuscular route, providing therapeutically beneficial methods for preventing and/or treating Factor IX-responsive syndromes, such as, e.g., hemophilia.

DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses the subcutaneous or intramuscular administration of PEGylated Factor IX polypeptides, i.e., PEGylated polypeptides that have been modified relative to the same non-pegylated Factor IX polypeptide to enhance their therapeutic applications. It has now been found that such PEGylated Factor IX polypeptides can be effectively administered via a subcutaneous route, providing therapeutically beneficial methods for preventing and/or treating Factor IX-responsive syndromes, such as, e.g., hemophilia. Subcutaneous administration is a significant benefit, especially in patients where venous access represents a barrier to optimal treatment compliance.
The present invention relates in one aspect to a pharmaceutical formulation comprising a PEGylated Factor IX polypeptide together with a pharmaceutically acceptable carrier, wherein the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 0.1 mg/ml to about 200 mg/ml. It is to be understood that the calculation of the concentration of the PEGylated Factor IX polypeptide, is based on the weight of the corresponding Factor IX polypeptide without the weight of the PEG-moiety included.

In a further aspect, the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 1 mg/ml to about 50 mg/ml.

The present invention relates in one aspect to a pharmaceutical formulation for subcutaneous treatment of a haemophilia patient comprising a therapeutically effective amount of a PEGylated Factor IX polypeptide together with a pharmaceutically acceptable carrier, in which pharmaceutical formulation the concentration of the PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is from about 0.1 mg/ml to about 200 mg/ml.

The term "PEGylated Factor IX polypeptide" refers to any Factor IX polypeptide to which one or more PEG moieties has been conjugated by chemical bonding. The PEG molecule may be attached to any part of the Factor IX polypeptide, including any amino acid residue or carbohydrate moiety of the Factor IX polypeptide.

The term "cysteine-PEGylated human Factor IX" refers to a Factor IX polypeptide having a PEG molecule conjugated to a sulfhydryl group of a cysteine introduced in human Factor IX to form a Factor IX sequence variant as e.g. described in WO 200614492.9 and US provisional 60/809456.

The term "Factor IX polypeptide" refers to both wild-type plasma derived Factor IX molecules and recombinantly produced Factor IX molecules, including recombinant wild-type human Factor IX. It is envisaged that in one embodiment Factor IX polypeptide may be a derivative, an amino acid variant or analogue of Factor IX.

The term "non-PEGylated Factor IX polypeptide" refers to the corresponding Factor IX polypeptide without the PEG-moiety(s).
Examples of Factor IX variants are described in WO99/03496 such as variants of Factor IX having an amino acid substitution at amino acid position 338. Substitutions in amino acid position 338 are, for example, a substitution of an arginine residue for an amino acid selected from the group consisting of alanine, valine, leucine, isoleucine, phenylalanine, tryptophan, methionine, glycine, serine, and threonine. Preferred substitutions include the substitution of alanine, leucine or valine for the arginine at amino acid position 338. Further non-limiting examples of Factor IX variants include: K5A and VIOK as described by Wing-Fai Cheung et-al, Proc. Natl. Acad. ScL, Vol. 93, pp 11068-11073, October 1996, Medical Sciences and by Tong Gui et al, Blood, 1 July 2002, Vol. 100, number 1, page 153-158.

"Factor IX" or "FIX" as used herein refers to a human plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. Unless otherwise specified or indicated, as used herein Factor IX polypeptide means any functional human Factor IX protein molecule in its normal role in coagulation, including any fragment, analogue or variant and derivative thereof.

"Native Factor IX" is the full length human Factor IX molecule as shown in SEQ ID NO: 1. The numbering of the amino acid residue position is according to SEQ ID NO: 1 where the first N-terminal amino acid residue is number 1 and so on.

The terms "analogue" and "variant" are used interchangeably herein and is intended to designate a Factor IX polypeptide having the sequence of SEQ ID NO: 1, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in protein and/or wherein one or more amino acids have been added to the parent protein. Such addition can take place either at the N-terminal end or at the C-terminal end of the parent protein or both. The "analogue" or "variant" within this definition still have "Factor IX biological activity".

Unless otherwise specified, Factor IX domains include the following amino acid residues: Gla domain being the region from reside Tyr1 to residue Lys43; EGFl being the region from residue Gln44 to residue Leu84; EGF2 being the region from residue Asp85 to residue Arg145; the Activation Peptide being the region from residue Ala46 to residue Arg180; and the Protease Domain being the region from residue Val181 to Thr415. The light chain refers to the region encompassing the Gla domain, EGFl and EGF2, while the heavy chain refers to the Protease Domain.
In a preferred embodiment, the PEGylated Factor IX polypeptide has a terminal half-life of at least 1.2 times, such as at least 1.3 times, such as at least 1.4 times, such as at least 1.5 times, such as at least 2 times, such as at least 3 times or such as at least 4 times that of the same non-PEGylated Factor IX polypeptide i.e. the same Factor IX polypeptide without the PEGylation, in the same assay or model.

The term "half-life" or "V/2", as used herein in the context of administering a peptide drug to a patient, is defined as the time required for plasma concentration of a drug in a patient to be reduced by one half. There may be more than one half-life associated with the peptide drug depending on multiple clearance mechanisms, redistribution, and other mechanisms well known in the art. Usually, alpha and beta half-lives are defined such that the alpha phase is associated with redistribution, and the beta phase is associated with clearance. However, with protein drugs that are, for the most part, confined to the bloodstream, there can be at least two clearance half-lives. For some glycosylated peptides, rapid beta phase clearance may be mediated via receptors on macrophages, or endothelial cells that recognize terminal galactose, N-acetylgalactosamine, N-acetylgalactosamine, mannose, or fucose. Slower beta phase clearance may occur via renal glomerular filtration for molecules with an effective radius < 2 nm (approximately 68 kD) and/or specific or non-specific uptake and metabolism in tissues. GlycoPEGylation may cap terminal sugars (e.g., galactose or N-acetylgalactosamine) and thereby block rapid alpha phase clearance via receptors that recognize these sugars. It may also confer a larger effective radius and thereby decrease the volume of distribution and tissue uptake, thereby prolonging the late beta phase. Thus, the precise impact of glycoPEGylation on alpha phase and beta phase half-lives will vary depending upon the size, state of glycosylation, and other parameters, as is well known in the art. Further explanation of "half-life" is found in Pharmaceutical Biotechnology (1997, D.F. Crommelin and R.D. Sindelar, eds., Harwood Publishers, Amsterdam, pp 101 - 120). The term "terminal half life" or "terminal V/2" refers to the half-life of the elimination phase. For example, for the two compartment model, the beta phase constant (beta = elimination constant) can be used to calculate the half-life of the elimination phase, e.g., \( t_{1/2} = 0.693/\beta \).

In a preferred embodiment, the PEGylated Factor IX polypeptide, when measured in a clotting assay, has a biological activity of at least 25%, 50%, 75%, 100%, 125%, 150%, 200%, or 500% of the same non-pegylated Factor IX polypeptide i.e. the same Factor IX polypeptide without the PEGylation, in the same assay or model. "Factor IX activity" or "Factor IX biological activity" is defined as the ability to function in the coagulation cascade, induce the formation of FXa via interaction with FVIIIa on an activated platelet, and support the formation of a blood clot. The activity can be assessed in vitro by techniques such as clot
analysis, as described in e.g. McCarthy et al. Thromb Haemost. 2002 May;87(5):824-30, and other techniques known to people skilled in the art. For example a modified one stage activated partial thromboplastin time assay, which uses equal amounts of test-sample, FIX deficient plasma (immunodepleted, ILs), ellagic acid activator (Synthefax, ILs), and CaCl₂ (0.02M). The clotting times can be determined on the ACL9000 instrument (ILs). In this assay, activity can be measured against normal pooled human plasma (ILs) which has been calibrated against WHO human FIX standard (NIBSC).

The mature human Factor IX has the sequence shown in SEQ ID NO 1, where glutamic acid residues at position 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36, and 40 are posttranslationally gamma-carboxylated to gamma-carboxyglutamic acid (or Gla) residues. YNSGKLEEFVGGNLEREMEKKSFEEAREFENTETFTFQYVGDQ CESNPCGGGSKCDINSEWCPFGEGKNCELVTNCNKNRCEQFKC NSSDNKVCSCTEGYYRLAEQKSCPAVPHCGRVSQTSKLTRAEAVF PDDYVNSTAEATLDNQTGSFNDFTWGGEDAKPGQFPWQWNLNG KTVDAFCGGSIVNEKWITAAHVCKETGAKTVVEGHNIEEETEHRQKRNV IRIPHHINNAIKHYNDFLLEPVLSYVTPIADKEYTNFL KFGGSGYVSGWGRVFHKGMRALQVLRVPLVDRATCLRTKFIYNNMFC AGFHEGGRDSCQGDSGGPHVTEVEGTSLGTIIWGGECAMKGYGIYTK VSRYVNWIKEKTTLT. (SEQ ID NO 1)

In one embodiment of the invention, the Factor IX polypeptide comprises or consists of the amino acid sequence of SEQ ID NO 1. However, it is envisaged that a Factor IX variant may comprise one or more inserted amino acid residues, such as between 1 and 10 inserted amino acids, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 inserted amino acids.

In another embodiment of the invention, the Factor IX polypeptide carries a threonine at position 148, which is a known dimorphism in the natural population (McGraw et al. (1985) Evidence for a prevalent dimorphism in the activation peptide of human coagulation factor IX. Proc.Natl.Acad.Sci, 82, 2847-2851)

Suitable the Factor IX variant may, in one embodiment be at least 90% identical at the amino acid level with SEQ ID NO 1, such as at least 91, 92, 93, 94, 95, 96, 97, 98 or 99% identical at the amino acid level with SEQ ID NO 1.

Factor IX variants are well known in the art, as described in, for example, U.S. Pat. Nos. 4,770,999, 5,521,070 in which a tyrosine is replaced by an alanine in the first position, U.S. Pat. No. 6,037,452, in which Factor IX is linked to an alkylene oxide group, and U.S. Pat.
No. 6,046,380, in which the DNA encoding Factor IX is modified in at least one splice site. As demonstrated herein, variants of Factor IX are well known in the art, and the present disclosure encompasses those variants known or to be developed or discovered in the future.

Preferred PEGylated Factor IX polypeptides with prolonged circulatory half life, wherein at least one of the natural amino residues in position 44, 46, 47, 50, 53, 57, 66, 67, 68, 70, 72, 74, 80, 84, 87, 89, 90, 91, 94, 100, 101, 102, 103, 104, 105, 106, 108, 113, 116, 119, 120, 121, 123, 125, 129, 138, 140, 141, 142, 146-180, 185, 186, 188, 189, 201, 202, 203, 224, 225, 228, 239, 240, 241, 243, 247, 249, 252, 257, 260, 261, 262, 263, 265, 277, 280, 314, 316, 318, 321, 341, 372, 374, 391, 392, 406, 410, 413 or 415 is substituted for a cysteine amino acid residue conjugated with a chemical group increasing the molecular weight of the Factor IX polypeptide, are disclosed in EP 06114492.9, US provisional 60/809456.

Methods for determining the activity of a mutant or modified Factor IX can be carried out using the methods described in the art, such as a one stage activated partial thromboplastin time assay as described in, for example, Biggs (1972, Human Blood Coagulation Haemostasis and Thrombosis (Ed. 1), Oxford, Blackwell, Scientific, pg. 614). Briefly, to assay the biological activity of a Factor IX molecule as described herein, the assay can be performed with equal volumes of activated partial thromboplastin reagent, Factor IX deficient plasma isolated from a patient with hemophilia B using sterile phlebotomy techniques well known in the art or Factor IX immunodepleted plasma (commercial available from e.g. Helena Laboratories or ILS), normal pooled plasma as standard, or the sample, and 25 mM calcium. In this assay, one unit of activity is defined as that amount present in one milliliter of normal pooled plasma. Alternatively, the activity can be measured against a normal human plasma pool which has been calibrated against a WHO human FIX standard (NIBSC). Further, an assay for biological activity based on the ability of Factor IX to reduce the clotting time of plasma from Factor IX-deficient patients to normal can be performed as described in, for example, Proctor and Rapaport (Amer. J. Clin. Path. 36: 212 (1961)).

The activity of a mutant or modified Factor IX can also be determined in thrombin generation assays comprising human platelets or other phospholipid sources. The assay can either be a reconstituted model system as described (Allen GA, et al. J Thromb Haemost 2004; 2: 402-413), or a plasma-based assay (Hemker HC et al Pathophysiol Haemost Thromb 2003; 33: 4-15). In case the plasma-based assay is used platelet-rich plasma deficient of Factor IX should be applied. In case congenital Factor IX platelet-rich plasma is not available the assay can be performed as follows: Platelet-rich plasma are prepared from a normal donor
by 15 min centrifugation at 330xg, and platelets isolated by gel filtration on a Sepharose CL2B column (Amersham) equilibrated with hepes/Tyrodes buffer (15 mM HEPES, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 5.5 mM dextrose and 1 mg/ml BSA, pH 7.4). The platelets are counted e.g. in a FACSCanto flow cytometer (BD) using TruCount tubes (BD) as described by the manufactures. The platelet density is adjusted to 150x 10⁹/L with hepes/Tyrodes buffer (final density 150x 10⁹/L). Lyophilized Factor IX-deficient plasma (Helena Technologies or ILS) is reconstituted with the platelet suspension thereby making Factor IX-deficient platelet-rich plasma. Eighty µl (80 µl) of this PRP is mixed with 10 µl relipidated tissue factor (Innovin, Dade, final dilution 1:50000), the Factor IX sample (modified factor IX, mutant or reference factor IX protein) diluted in 10 µl 20 mM hepes, 150 mM NaCl, pH 7.4, 1 mg/ml BSA, and 20 µl fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, final concentration 417 nM) mixed with CaCl₂ (final concentration 16.7 mM). A GPVI agonist e.g. convulxin (0.1 µg/ml final concentration, Pentapharm) may be included to ensure strong platelet activation. Emission at 460 nm after excitation at 390 nm is measured continuously for 30 min in a Fluoroskan Ascent plate reader (Thermo Electron Corporation). The fluorescence signal are corrected for α₂ macroglobulin-bound thrombin activity and converted to thrombin concentration by use of a calibrator and Thrombinoscope software (Synapse BV) as described (Hemker HC et al Pathophysiol Haemost Thromb 2003; 33: 4-15).

Modification of plasma derived Factor IX or Factor IX sequence variants for use in the invention includes, without limitation, chemical and/or enzymatic modification, such as, e.g. by one or more of alkylation, glycosylation, acylation, phosphorylation, or other ester formation or amide formation or the like.


The term "polyethylene glycol" or "PEG" means a polyethylene glycol compound or a derivative thereof, with or without coupling agents, coupling or activating moieties (e.g., with thiol, triflate, tresylate, azidine, oxirane, pyridyldithio, vinyl sulfone, N-hydroxysuccinimid ester, haloacetamide, or preferably with a maleimide moiety). Compounds such as maleimido monomethoxy PEG are exemplary of activated PEG compounds of the invention.
PEG is a suitable polymer molecule, since it has only few reactive groups capable of cross-linking compared to polysaccharides such as dextran. In particular, monofunctional PEG, e.g. methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.


The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R. F. Taylor, (1991),
"Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g. being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinylsulfone or haloacetate). The PEGylation may be directed towards conjugation to all available attachment groups on the polypeptide (i.e. such attachment groups that are exposed at the surface of the polypeptide) or may be directed towards one or more specific attachment groups, e.g. the N-terminal amino group (U.S. Pat. No. 5,985,265). Furthermore, the conjugation may be achieved in one step or in a stepwise manner (e.g. as described in WO 99/55377).

Chemical and/or enzymatic modification of Factor IX and Factor IX variants may be achieved by any means known in the art. Suitable modifications include, without limitation, chemical glycan modifications as described in EP application, EP06120000 (Novo Nordisk A/S), C-terminal modifications as described in WO2006013202, glycosyltransferase mediated modification as described in WO2006035057, dendrimer modification as described in WO2005014049, carboxypeptidase mediated terminal modification (as described, e.g., in WO2005035553-A2, WO9520039-A, and WO9838285-A), transglutaminase-mediated modification (as described, e.g., WO2005070468), autocatalytic/endopeptidase-mediated transpeptidation (as described, e.g., in WO2006013202-A2); thiol-mediated modification (as described, e.g., in WO2002077218 and PCT/EP/2006063310) and amine-mediated modification (as described, e.g., in WO02/02764).

It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g. whether they are linear or branched), and where in the polypeptide such molecules are attached. The molecular weight of the polymer to be used will be chosen taking into consideration the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight and larger size (e.g. to reduce renal clearance), one may choose to conjugate either one or a few high molecular weight polymer molecules or a number of polymer molecules with a smaller molecular weight to obtain the desired effect.

Furthermore, it has been reported that excessive polymer conjugation can lead to a loss of activity of the protein (e.g. Factor IX polypeptide) to which the chemical group (e.g. a non-
polypeptide moiety) is conjugated. This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by reversible blocking the functional site prior to conjugation so that the functional site of the protein is blocked during conjugation. Specifically, the conjugation between the protein and the chemical group (e.g. non-polypeptide moiety) may be conducted under conditions where the functional site of the protein is blocked by a helper molecule e.g. a serine protease inhibitor. Preferably, the helper molecule is one, which specifically recognizes a functional site of the protein, such as a receptor or active-site inhibitor binding to and thus protecting the area around the catalytic triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the protein (e.g. Factor IX polypeptide). In particular, the helper molecule may be a neutralizing monoclonal antibody.

The protein is preferably to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the protein (e.g. Factor IX polypeptide) is shielded or protected and consequently unavailable for derivatization by the chemical group (e.g. non-polypeptide moiety) such, as a polymer.

Following its elution from the helper molecule, the conjugate of the chemical group and the protein can be recovered with at least a partially preserved functional site

In one interesting embodiment, the Factor IX polypeptide has been PEGylated with polyethyleneglycol (PEG), in particular one having an average molecular weight in the range of 500-100,000, such as 1000-75,000, or 5000-60,000. The PEG has in a preferred embodiment an average molecular weight in the range of 20,000-60,000 or 35,000-55,000 such as e.g. 40,000.

 Preferentially, the PEG can be added selectively to the glycans on the Factor IX polypeptide such as e.g. described in US 20060040856, introduced Cys residues, or other selective chemistries, but may also be coupled to the Factor IX polypeptide in a non-selective manner. In a preferred aspect, the PEG is conjugated selectively to glycans on the Factor IX polypeptide.

An important aspect is that the solubility of the derivative must be such that the required amount of protein can be injected conveniently. Solubility of a PEGylated peptide may be determined by any standard method known in the art.
Conjugation can be conducted as disclosed in WO 02/077218 A1 and WO 01/58935 A2.

As disclosed in US 20060040856 PEGylated or glycopegylated Factor IX molecules of the invention may be produced by the enzyme mediated formation of a conjugate between a glycosylated or non-glycosylated Factor IX peptide and an enzymatically transferable saccharyl moiety that includes a poly(ethylene glycol) moiety within its structure. The PEG moiety, which is typically one having an average molecular weight of in the range of 500-100,000, such as 1000-75,000, or 2,000-60,000, is attached to the saccharyl moiety directly (i.e., through a single group formed by the reaction of two reactive groups) or through a linker moiety, e.g., substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, etc.

PEGylated or glycopegylated Factor IX molecules suitably include at least one N-linked or O-linked glycosylation site, at least one of which is conjugated to a glycosyl residue that includes a PEG moiety. The PEG is covalently attached to the peptide via an intact glycosyl linking group. The glycosyl linking group is covalently attached to either an amino acid residue or a glycosyl residue of the peptide. Alternatively, the glycosyl linking group is attached to one or more glycosyl units of a glycopeptide. The invention also provides conjugates in which the glycosyl linking group is attached to both an amino acid residue and a glycosyl residue.

Alternatively, PEG molecules may be attached to glycan moieties present in the FIX molecule. In one embodiment, FIX is initially treated with sialidase (neuraminidase) to expose galactose residues present in the FIX N-glycans. The galactose residues are then oxidized using galactose oxidase and the resulting aldehydes (6-aldogalactoside residues) are reacted with PEG molecules containing suitable reactive handles, such as semicarbazides, semithiocarbazides, hydroxylamines, hydrazines ect. Non-reacted aldehydes may subsequently be capped using e.g. methoxylamine. A general procedure can be found in WO2005014035.

PEG molecules may also be attached to glycosyl residues that have been oxidized under mild chemical conditions. In one embodiment, the FIX molecule is treated with sodium periodate to generate reactive aldehyde functionalities. The aldehyde functionalities are reacted with PEG molecules containing suitable reactive handles, such as semicarbazides, semithiocarbazides, hydroxylamines, hydrazines ect. Non-reacted aldehydes may subsequently be capped using e.g. methoxylamine. A general procedure can be found in WO 2008025856 A2.
The PEG moiety is typically attached to an intact glycosyl linker directly, or via a non-glycosyl linker, e.g., substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl.

In a preferred embodiment, the PEG is a linear or branched PEG. In one aspect the PEGylated Factor IX polypeptides include from 1 to about 9 PEG moieties per peptide. In another aspect, the PEG is a linear PEG and the PEGylated Factor IX polypeptide includes approximately 1 to 5 PEG moieties per peptide molecule. In another aspect, the PEG is a branched PEG and the PEGylated Factor IX polypeptide includes approximately 1 to 5 PEG moieties per peptide molecule. In exemplary embodiments, in which the PEG is a linear species, the PEG moiety has a molecular weight which is from about 1 kD to about 60 kD. In another aspect, in which the PEG moiety is a linear PEG moiety, the molecular weight of the linear PEG is at least about 1 kD, more preferably, at least about 5 kD, even more preferably, at least about 10 kD, more preferably, at least about 20 kD, such as e.g. 30 kD.

In another aspect in which the PEG species is branched, the branched PEG includes from 2 to 6 linear PEG arms. Exemplary PEG arms have a molecular weight from about 200 D to about 30 kD. It is generally preferred that each arm has an individually selected molecular weight that is at least about 2 kD, preferably, at least about 5 kD, more preferably, at least about 10 kD, still more preferably, at least about 15 kD, such as e.g. 20 kD. In one aspect the PEG species has two PEG arms. In one aspect the the two-arm branched structure is based on an amino acid. Preferred amino acids include serine, cysteine and lysine. In one aspect of the invention the two-arm branched structure is based on glycerol.

In one aspect the PEGylated Factor IX polypeptide includes from 1-4 branched PEG moieties, in which the branched PEG is based upon an amino acid (i.e., the PEG arms are covalently linked to an amino acid core). In this embodiment, the branched PEG species have a molecular weight of at least about 15 kD, preferably, at least about 20, kD, and more preferably, at least about 30 kD, such as e.g. 40 kD. In another aspect, the PEG species has a molecular weight of about 30 kD. In yet another aspect, the branched PEG species includes two linear PEG moieties covalently attached to an amino acid which is a member selected from lysine, serine and cysteine. In another aspect, the PEG species includes two linear PEG moieties covalently attached to a glycerol moiety. Each branched PEG moiety is covalently attached to an amino acid or a glycosyl residue of the Factor IX peptide.

Pharmacokinetic Variants
In one series of embodiments, the PEGylated Factor IX polypeptides for use in the present invention encompass those for which one or more pharmacokinetic properties has been altered relative to the non-PEGylated Factor IX polypeptide in the same assay or model.

In practicing the present invention, pharmacokinetic properties may be calculated using, e.g., WinNonlin Professional Version 3.1 (Pharsight Inc., Mountain View, CA, USA). Calculations are performed using mean concentration values at each time point, if more than one value was present.

The following pharmacokinetic parameters may be calculated: AUC, AUC\(_{0-\infty}\)\(_{\text{Extrap}}\), \(C_{\text{max}}\), \(t_{\text{max}}\), \(\lambda_z\), \(t_{1/2}\), CL, and \(V_z\) using the following formulas:

\[
\text{AUC} \quad \text{Area under the plasma concentration-time curve from time 0 to infinity. Calculated using the linear/log trapezoidal rule with extrapolation to infinity.}
\]

The linear trapezoidal rule is used from time 0 to \(t_{\text{max}}\):

\[
AUC(0-t_{\text{max}}) = \frac{\sum_{i=1}^{n-1} C(i) + C(i+1)}{2} \cdot \frac{1}{(t(i+1)-t(i))}
\]

The log trapezoidal rule is used from time \(t_{\text{max}}\) to the last time point \(t\):

\[
AUC(t_{\text{max}}-t) = \sum_{i=1}^{n} \left( \ln \left( \frac{C(i)}{C(i+1)} \right) \cdot (t(i+1)-t(i)) \right)
\]

Extrapolation to infinity is performed using:

\[
AUC(t-\infty) = \frac{C(t)}{\lambda_z}
\]

\(AUC_{0-\infty}\)\(_{\text{Extrap}}\) Percentage of AUC that is due to extrapolation from the last concentration to infinity:

\[
AUC_{0-\infty}\text{Extrap} = \frac{AUC(t-\infty)}{AUC} \cdot 100\%
\]

\(C_{\text{max}}\) Maximum plasma concentration

CL Total body clearance:

\[
CL = \frac{Doss}{AUC} \quad \text{(bolus dose)}
\]

CL/f Total body clearance for extravascular administration:
Bioavailability of drug after extravascular administration

\[
f = \frac{\text{AUC}_{\text{ev}}}{\text{Dose}_{\text{ev}}} \cdot \frac{\text{Dose}_{\text{iv}}}{\text{AUC}_{\text{iv}}}
\]

\( t_{\text{max}} \) Time at which maximum plasma concentration is observed.

\( t_{1/2} \) Half-life:

\[
t_{1/2} = \frac{\ln 2}{k}
\]

\( \lambda_z \) Terminal rate constant. Calculated by log-linear regression of (mean) concentrations versus time.

\( V_z \) Volume of distribution based on the terminal phase:

\[
V_z = \frac{\text{Dose}}{\text{AUC} - \lambda_z} \quad \text{(bolus dose)}
\]

\( V_{z/f} \) Extravascular volume of distribution based on the terminal phase:

\[
\frac{V_z}{f} = \frac{\text{Dose}}{\text{AUC} - \lambda_z} \quad \text{(bolus dose)}
\]

Non-limiting examples of useful PEGylated Factor IX polypeptides include those in which the ratio between absorption and clearance has been altered to provide increased AUC and/or \( t_{1/2} \).

In some embodiments, PEGylated Factor IX polypeptides are those in which the modification leads to an improvement in bioavailability (\( f \)) of at least about 25%, 50%, 75%, 100%, 125%, 150%, 200%, or 500% of the bioavailability of the non-PEGylated Factor IX polypeptide.

**Preparation of compound**

**Mutating, expressing & purifying**

The Factor IX analogues may be produced by means of recombinant nucleic acid techniques. In general, a cloned human nucleic acid sequence is modified to encode the desired Factor IX analogue and is then inserted into an expression vector, which is in turn transformed or transfected into host cells. Higher eukaryotic cells, in particular cultured mammalian cells, are preferred as host cells.

The amino acid sequence alterations may be accomplished by a variety of techniques. Modification of the nucleic acid sequence may be by site-specific mutagenesis. Techniques
for site-specific mutagenesis are well known in the art and are described in, for example, Zoller and Smith (DNA 3:479-488, 1984) or "Splicing by extension overlap", Horton et al., Gene 77, 1989, pp. 61-68. Thus, using the nucleotide and amino acid sequences of Factor IX, one may introduce the alteration(s) of choice. Likewise, procedures for preparing a DNA construct using polymerase chain reaction using specific primers are well known to persons skilled in the art (cf. PCR Protocols, 1990, Academic Press, San Diego, California, USA). The nucleic acid construct encoding the Factor IX analogue may be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of Factor IX by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed. Cold Spring Harbor Labora-tory, Cold Spring Harbor, New York, 1989).

The nucleic acid construct encoding the Factor IX polypeptide analogue may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in suitable vectors. The DNA sequences encoding the human Factor IX polypeptides may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202, Saiki et al., Science 239 (1988), 487 - 491, or Sambrook et al., supra.

Furthermore, the nucleic acid construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire nucleic acid construct, in accordance with standard techniques.

The DNA sequences encoding the Factor IX polypeptides are usually inserted into a recombinant vector which may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.
The vector is preferably an expression vector in which the DNA sequence encoding the Factor IX analogue is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

Expression vectors for use in expressing Factor IX analogues will comprise a promoter capable of directing the transcription of a cloned gene or cDNA. The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the Factor IX analogues in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854-864), the MT-I (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809-814), the CMV promoter (Boshart et al., Cell 41:521-530, 1985) or the adenovirus 2 major late promoter (Kaufman and Sharp, Mol. Cell. Biol, 2:1304-1319, 1982).

The DNA sequences encoding the Factor IX analogue may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., Science 222, 1983, pp. 809-814) or the TP II (Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or ADH3 (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) terminators. Expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the insertion site for the Factor IX sequence itself. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signal from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 E1b region, the human growth hormone gene terminator (DeNoto et al. Nucl. Acids Res. 9:3719-3730, 1981) or the polyadenylation signal from the human Factor IX gene. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites; and enhancer sequences, such as the SV40 enhancer.

To direct the Factor IX analogue into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequences encoding the Factor IX analogues in the correct reading frame. Secretory signal
sequences are commonly positioned 5' to the DNA sequence encoding the peptide. The secretory signal sequence may be that, normally associated with the protein or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the Factor IX analogues, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).


Cloned DNA sequences are introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725-732, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and Van der Eb, Virology 52d:456-467, 1973) or electroporation (Neumann et al., EMBO J. 1:841-845, 1982). To identify and select cells that express the exogenous DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into cells along with the gene or cDNA of interest. Preferred selectable markers include genes that confer resistance to drugs such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is a dihydrofolate reductase (DHFR) sequence. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, incorporated herein by reference). The person skilled in the art will easily be able to choose suitable selectable markers.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

After the cells have taken up the DNA, they are grown in an appropriate growth medium, typically 1-2 days, to begin expressing the gene of interest. As used herein the term "appropriate growth medium" means a medium containing nutrients and other components
required for the growth of cells and the expression of the Factor IX analogues. Media generally include a carbon source, a nitrogen source, essential amino acids, essential sugars, vitamins, salts, phospholipids, protein and growth factors. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion.

For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of the Factor IX analogue.

Examples of mammalian cell lines for use in the present invention are the COS-I (ATCC CRL 1650), baby hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk- tsl3 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79: 1106-1110, 1982, incorporated herein by reference), hereinafter referred to as BHK 570 cells. The BHK 570 cell line has been deposited with the American Type Culture Collection, 12301 Parklawn Dr., Rockville, Md. 20852, under ATCC accession number CRL 10314. A tk- tsl3 BHK cell line is also available from the ATCC under accession number CRL 1632. In addition, a number of other cell lines may be used within the present invention, including Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61) and CHO-DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980).

Factor IX analogues are recovered from cell culture medium and can then be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). Preferably, they may be purified by affinity chromatography on an anti-Factor IX antibody column. Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification are known in the art, and may be applied to the purification of the novel Factor IX polypeptides described herein (see, for example, Scopes, R., Protein Purification, Springer-Verlag, N.Y., 1982).

For therapeutic purposes it is preferred that the Factor IX analogue is purified to at least about 90 to 95% homogeneity, preferably to at least about 98% homogeneity. Purity may be assessed by e.g. gel electrophoresis and amino-terminal amino acid sequencing.

**Pharmaceutical administration**
The regimen for any patient to be treated with PEGylated Factor IX polypeptides as mentioned herein should be determined by those skilled in the art. The dose to be administered in therapy may depend on numerous factors, such as, e.g., the weight and the condition of the patient, and can be determined by examining different points in a matrix of treatment and correlating to clinical outcomes.

In one series of embodiments, a PEGylated Factor IX polypeptide is administered subcutaneously and in an amount of about 5-1000 units per kg body weight, such as, e.g., in an amount of about 20-400 units per kg body weight.

In one embodiment, the method according to the invention comprises at least once weekly treatment, such as e.g. once every day or once every second day. In another embodiment, the treatment comprises only once weekly treatment.

**Combination treatments:**

The present invention encompasses combined administration of an additional agent in concert with a PEGylated Factor IX. In some embodiments, the additional agent comprises a coagulant, including, without limitation, a coagulation factor such as, e.g., Factor VII, Factor VIII, Factor V, Factor XI, or Factor XIII; or an inhibitor of the fibrinolytic system, such as, e.g., PAI-I, aprotinin, ε-aminocaproic acid or tranexamic acid.

It will be understood that, in embodiments comprising administration of combinations of PEGylated Factor IX polypeptide with other agents, the dosage of the PEGylated Factor IX polypeptide may on its own comprise an effective amount and additional agent(s) may further augment the therapeutic benefit to the patient. Alternatively, the combination of PEGylated Factor IX polypeptide and the second agent may together comprise an effective amount. It will also be understood that effective amounts may be defined in the context of particular treatment regimens, including, e.g., timing and number of administrations, modes of administrations, formulations, etc.

**Formulation of medicament**

An intravenous injection is normally 5-20 ml. It is normally preferred that an injection given subcutaneously is between 0.05 to 2 ml. The concentration of the PEGylated Factor IX polypeptide must therefore be relatively high in such a medicament. The volume administered can in one aspect be less than 2 ml. In a further aspect, less than 1.5 ml,
such as less than 1 ml, such as less than 0.5, such as less than 0.1, such as less than 0.05 ml. In another aspect, the volume administered can be between 0.05 to 2 ml such as, e.g., 0.1-2 ml, 0.05-1 ml, 0.05-0.1, 0.25-1.5 ml, and 0.5-1 ml.


Additives increasing the bioavailability of the PEGylated Factor IX polypeptide are suitably organic compounds per se, salts thereof, emulsions or dispersions containing organic compounds per se or salts thereof, e.g. dispersions of polar lipids, or any combination or sequence of addition thereof. Organic compounds useful in the invention are e.g. amino acids, peptides, proteins, and polysaccharides. Peptides include dipeptides, tripeptides, oligopeptides, such as collagen and gelatine. The collagen and gelatine is preferably hydrolysed. Polysaccharides include e.g. chitosans, cyclodextrins, starch, hyaluronic acids, dextrans, cellulose, and any derivatives, combinations and/or sequence of addition thereof. The starch is preferably hydrolysed. The emulsions include oil-in-water emulsions with oil as the dispersed phase and water-in-oil dispersions with oil as the continuous phase. The oil can be of vegetable or of animal origin or synthetically produced. Suitably, the vegetable oil of the emulsions is soybean oil or safflower oil, or any combination thereof. Suitably the polar liquids are one or more phospholipids or glycolipids or any combination thereof. The additives increasing the bioavailability of Factor IX polypeptide could be added to the formulation before drying or upon reconstitution, or can be added to a stable solution or dispersion containing Factor IX polypeptide.

Before administration, one or more aqueous solutions or dispersions can be added, in any mixture or sequence, to the medicament of the present invention, which is a stable aqueous solution, a dispersion or in dried form.

The medicament can be in a dried form, preferably freeze-dried. Before administration, the dried product or composition can be reconstituted with an aqueous solution or a dispersion e.g. a suspension, a liposomal formulation or an emulsion.

The medicament can also be a stable aqueous solution ready for administration. It can also be a dispersion, e.g. a suspension, a liposomal formulation or an emulsion.
The medicament is preferably given subcutaneously. The concentration of PEGylated Factor IX polypeptide in the formulation calculated based on the corresponding non-PEGylated Factor IX is preferably from about from 0.1 mg/ml to 200 mg/ml, e.g. from 0.1 mg/ml to about 100 mg/ml, such as, e.g., from about 0.3 mg/ml to about 80 mg/ml, from about 0.6 mg/ml to about 60 mg/ml or from about 1 mg/ml to about 50 mg/ml.

In one aspect of the invention, the concentration of PEGylated Factor IX polypeptide in the formulation calculated based on the corresponding non-PEGylated Factor IX is from 0.1 mg/ml to 200 mg/ml. In a further aspect of the invention, the concentration of PEGylated Factor IX polypeptide in the formulation calculated based on the corresponding non-PEGylated Factor IX is from 1 mg/ml to 50 mg/ml.

The medicament may also comprise one or more salts in order to give an isotonic solution, e.g. NaCl, KCl, and/or it may comprise one or more other isotonicity establishing compounds, preferably in an amount of more than 1.0 mg/ml.

Calcium, or other divalent metal ions, e.g. zinc, may be used as necessary for the maintenance of the Factor IX activity. It may be added as, for example, calcium chloride, but other salts such as calcium gluconate, calcium gluconate or calcium gluceptate may also be used. In one aspect, the medicament comprises calcium chloride in an amount of more than 0.15 mg/ml. In another embodiment, the pharmaceutical formulation according to the invention does not comprise calcium.

An amino acid is preferably used to buffer the system and it also protects the protein if the formulation is freeze-dried. A suitable buffer can be glycine, lysine, arginine, histidine or glycyclglycine, preferred is histidine.

A non-ionic surfactant may also be present in the medicament. The surfactant is preferable chosen from block-copolymers, such as a poloxamer, e.g. poloxamer 188, or a polyoxethyene sorbitan fatty acid ester, such as polyoxyethylene-(20)-sorbitan monolaurate or polyoxyethylene-(80)-sorbitan monooleate. Preferred are polyoxyethylene-(80)-sorbitan monooleate (Tween 80). Tween 80 is preferably used in a concentration of at least 0.01 mg/ml. The non-ionic surfactant, if used, should preferably be present in an amount above the critical micelle concentration (CMC). See Wan and Lee, *Journal of Pharm Sci*, 63, p. 136, 1974.

Mono- or disaccharides (e.g. sucrose), polysaccharides such as low molecular weight dextrins, or sugar alcohols (e.g. sorbitol, glycerol or mannitol) may be added. The
medicament may also comprise antioxidants such as bisulfite, ascorbate glutathione, acetylcystein, tocopherol, methionin, EDTA, citric acid, butyl hydroxy toluene and/or butyl hydroxy anisole. Complexing agents, such as EDTA and citric acid can also be present in small concentrations for stabilising the Factor IX molecules, if they exhibit a stronger affinity for destabilising metal ions than for calcium or other divalent metal ions, e.g. zn2+. The medicament may also contain cyclodextrins, in particular sulfoalkyl ether cyclodextrins (WO2005023308). Furthermore, preservatives such as benzyl alcohol, phenol, sorbic acid, parabens, m-cresol and chlorocresol may be added.

The adjuvants are generally present in a concentration of from 0.001 to 4% w/v. The pharmaceutical preparation may also contain protease inhibitors, e.g. aprotinin or tranexamic acid.

In one aspect of the invention, the pH of the formulation is adjusted to a value in the interval of 2 - 9. Formulations having a pH from about 5.0 to about 7.5 are preferred, such as from about 5.0 to about 7.0. In one aspect of the invention, the formulation has a pH from about 6.0 to about 7.0. In a further aspect of the invention, the formulation has a pH from about 5.0 to about 6.0. In yet a further aspect of the invention, the formulation has a pH from about 5.5 to about 6.5.

Conventional techniques for preparing pharmaceutical compositions, which can be used according to the present invention, are, for example, described in Remington: The Science and Practice of Pharmacy, 19th ed., 1995.

The medicaments may be sterilised by, for example, filtration through a bacteria-retaining filter, by incorporating sterilising agents into the medicaments, by irradiating the medicaments, or by heating the medicaments. They can also be manufactured in the form of sterile solid medicaments which can be dissolved in sterile water, or some other sterile injectable medium prior to or immediately before use.

The present invention is further illustrated by the following examples. The presented examples are meant as an illustration of the invention, not as a limitation.

**EXAMPLES**

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference in their entirety and to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein (to the maximum extent permitted by law), regard-
less of any separately provided incorporation of particular documents made elsewhere herein.

Unless otherwise stated, all exact values provided herein are representative of corresponding approximate values (e.g., all exact exemplary values provided with respect to a particular factor or measurement can be considered to also provide a corresponding approximate measurement, modified by "about," where appropriate).

Example 1: Pharmacokinetics of factor IX and PEG40k-FIX after intravenous and subcutaneous administration in factor IX knock out mice

The pharmacokinetics of FIX and PEG40k-FIX was studied in FIX knock out (FIX KO) mice after intravenous and subcutaneous administration. The study included 107 female (64) and male (43) mice from Taconic M&B Tornbjergvej 40, DK4623 Lille Skensved, Denmark. The animals weighted 21 ± 3 g (minimum 14 g and maximum 27g) and had free access to food and water (Altromin 1320) throughout the study period. The study was performed in a thermostated room.

As only a small amount of blood can be drawn, full profiles from individual mice cannot be obtained. Accordingly, the present study used a sparse sampling regimen that allows mean profiles to be obtained based on 3 samples pr. time point and 3 samples pr. mouse. The dose was 1.5 mg/kg body weight intravenous in the tail vein and 4.5 mg/kg subcutaneous in the right flank.

The concentration of FIX in blood samples was determined by ELISA and clot activity analysis.

The ELISA assay is a two-sited enzyme-linked immunosorbent assay using two antibodies against FIX. A monoclonal antibody specific for human FIX, was immobilized to the surface of micro plate wells. Plasma samples or calibrators were then added to the appropriate wells followed by peroxidase-conjugated secondary antibody. The concentration of peroxidase was determined enzymatically with the generation of a colorimetric signal. By relating the generated signal to a standard curve made with known concentrations of FIX or PEG40k-FIX the concentration of FIX in the plasma could be determined. All plasma samples were tested diluted at least 1/50 to avoid matrix effects from the plasma.
Results from ELISA were subjected to non-compartmental pharmacokinetic analysis (NCA) using the PC based software WinNonlin (Pharsight Corporation). Bioavailability (F) was calculated as:

\[ F = \frac{AUC_{ss} \cdot \text{Dose}_{iv}}{AUC_{iv} \cdot \text{Dose}_{sc}} \]

In Figure 2 is illustrated the ELISA versus time profiles obtained for the two compounds and Table 1 summarizes the calculated pharmacokinetic parameters, including terminal half-life (HL) and bioavailability (F).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route</th>
<th>Dose (mg/kg)</th>
<th>Cmax (ng/ml)</th>
<th>HL (h)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG40k-FIX</td>
<td>iv</td>
<td>1.23</td>
<td>13160</td>
<td>41</td>
<td>n/a</td>
</tr>
<tr>
<td>FIX</td>
<td>iv</td>
<td>1.4</td>
<td>6720</td>
<td>19</td>
<td>n/a</td>
</tr>
<tr>
<td>PEG40k-FIX</td>
<td>sc</td>
<td>3.7</td>
<td>5444</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>FIX</td>
<td>sc</td>
<td>4.2</td>
<td>635</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>

In FIX KO mice, the terminal half-life of PEG40k-FIX was found to be significantly prolonged compared to unmodified FIX. After intravenous and subcutaneous administration the half-lives of PEG40k-FIX were increased by approximately 200% relative to FIX. Similarly, PEGylation had a significant positive effect on the bioavailability of FIX after sc administration. The bioavailability of PEG40k-FIX was 47% compared to 22% for FIX.

**Example 2 : Specific activity of FIX and PEG40k-FIX in clotting assay**

The potencies of FIX and PEG40k-FIX were assessed using a FIX clotting assay, which measures FIX activity-dependent time to fibrin clot formation. It is a modified one stage activated partial thromboplastin time assay (APTT) that measures the intrinsic coagulation cascade. The clotting times were determined on an ACL9000 coagulation analyzer (ILS). Equal amounts (4Oul) of test-sample, human FIX deficient plasma (immunodepleted, ILS), APTT reagent (Synthefax, ILS), and CaCl2 (0.02M) were used. Test compounds were diluted in HBS/BSA (0.5%) FIX dilution buffer. The activity was measured against a normal pooled human plasma calibrator (ILS) that has been calibrated against WHO human FIX standard (NIBSC). Results are given in Table 2 and demonstrate that FIX retains 73% of its specific clot activity after modification with a 40k-PEG.
Results are given as mean of 3 determinations ± standard deviation.

EMBODIMENTS OF THE INVENTION

1. A pharmaceutical formulation comprising a PEGylated Factor IX polypeptide together with a pharmaceutically acceptable carrier, wherein the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 0.1 mg/ml to about 200 mg/ml.

2. The pharmaceutical formulation according to embodiment 1, wherein the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 1 mg/ml to about 50 mg/ml.

3. The pharmaceutical formulation according to any one of the embodiments 1-2, wherein the pH of the formulation is about 5.0 to about 7.0.

4. The pharmaceutical formulation according to any one of the embodiments 1-3, wherein the PEGylated Factor IX polypeptide has been PEGylated with polyethyleneglycol (PEG), in particular one having an average molecular weight in the range of 500-100,000, such as 1000-75,000, or 5000-60,000.

5. The pharmaceutical formulation according to any one of the embodiments 1-4, wherein the PEG is conjugated selectively to glycans on the Factor IX polypeptide.

6. The pharmaceutical formulation according to any one of the embodiments 1-5, wherein the PEGylated Factor IX polypeptide exhibits a bioavailability of at least about 125% relative to the bioavailability of the non-PEGylated Factor IX polypeptide in the same assay or model.

7. The pharmaceutical formulation according to any one of the embodiments 1-6, wherein the PEGylated Factor IX polypeptide has a terminal half-life of at least 1.2 times that of the non-PEGylated Factor IX polypeptide as measured in the same assay or model.
8. A method for preventing and/or treating Factor IX-responsive syndromes such as haemophilia, the method comprising administering to a patient in need of such treatment an effective amount for such treatment of a PEGylated Factor IX polypeptide, wherein the administering is via a subcutaneous or intramuscular route.

9. The method according to embodiment 8, wherein the treatment comprises at least once weekly treatment.

10. The method according to embodiment 9, wherein the treatment comprises only once weekly treatment.

11. The method according to any one of the embodiments 8-10, wherein the PEGylated Factor IX polypeptide is administered in a concentration calculated based on the corresponding non-PEGylated Factor IX polypeptide of about 0.1 mg/ml to about 200 mg/ml.

12. The method according to any one of the embodiments 8-11, wherein the PEGylated Factor IX polypeptide is administered in a concentration calculated based on the corresponding non-PEGylated Factor IX polypeptide of about 1 mg/ml to about 50 mg/ml.

13. The method according to any one of the embodiments 8-12, wherein the administration is with a pharmaceutical formulation having a pH of about 5.0 to about 7.0.

14. The method according according to any one of the embodiments 8-13, wherein the Factor IX polypeptide has been PEGylated with polyethylene glycol (PEG), in particular one having an average molecular weight in the range of 500-100,000, such as 1000-75,000, or 5000-60,000.

15. The method according to any one of the embodiments 8-14, wherein the PEG is conjugated selectively to glycans on the Factor IX polypeptide.

16. The method according to any one of the embodiments 8-15, wherein the PEGylated Factor IX polypeptide exhibits a bioavailability of at least about 125% relative to the bioavailability of the non-PEGylated Factor IX polypeptide as measured in the same assay or model.
17. The method according to any one of the embodiments 8-16, wherein the PEGylated Factor IX polypeptide has a terminal half-life of at least 1.2 times that of the same non-PEGylated Factor IX polypeptide as measured in the same assay or model.

18. The method according to any one of the embodiments 8-17, wherein the PEGylated Factor IX polypeptide is administered via a subcutaneous route.
CLAIMS

1. A pharmaceutical formulation comprising a PEGylated Factor IX polypeptide together with a pharmaceutically acceptable carrier, wherein the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 0.1 mg/ml to about 200 mg/ml.

2. The pharmaceutical formulation according to claim 1, wherein the concentration of PEGylated Factor IX polypeptide calculated based on the corresponding non-PEGylated Factor IX polypeptide is about 1 mg/ml to about 50 mg/ml.

3. The pharmaceutical formulation according to any one of the claims 1-2, wherein the pH of the formulation is about 5.0 to about 7.0.

4. The pharmaceutical formulation according to any one of the claims 1-3, wherein the PEGylated Factor IX polypeptide has been PEGylated with polyethyleneglycol (PEG) having an average molecular weight in the range of 500-100,000.

5. The pharmaceutical formulation according to any one of the claims 1-4, wherein the PEG is conjugated selectively to glycans on the Factor IX polypeptide.

6. The pharmaceutical formulation according to any one of the claims 1-5, wherein the PEGylated Factor IX polypeptide exhibits a bioavailability of at least about 125% relative to the bioavailability of the non-PEGylated Factor IX polypeptide in the same assay or model.

7. The pharmaceutical formulation according to any one of the claims 1-6, wherein the PEGylated Factor IX polypeptide has a terminal half-life of at least 1.2 times that of the non-PEGylated Factor IX polypeptide as measured in the same assay or model.

8. A method of treatment of haemophilia, the method comprising administering to a patient in need of such treatment an effective amount for such treatment of a PEGylated Factor IX polypeptide, wherein the administering is via a subcutaneous or intramuscular route.
9. The method according to claim 8, wherein the treatment comprises at least once weekly treatment.

10. The method according to claim 9, wherein the treatment comprises only once weekly treatment.

11. The method according to any one of the claims 8-10, wherein the PEGylated Factor IX polypeptide is administered in a concentration calculated based on the corresponding non-PEGylated Factor IX polypeptide of about 0.1 mg/ml to about 200 mg/ml.

12. The method according to any one of the claims 8-11, wherein the PEGylated Factor IX polypeptide is administered in a concentration calculated based on the corresponding non-PEGylated Factor IX polypeptide of about 1 mg/ml to about 50 mg/ml.

13. The method according to any one of the claims 8-12, wherein the administration is with a pharmaceutical formulation having a pH of about 5.0 to about 7.0.

14. The method according to any one of the claims 8-13, wherein the Factor IX polypeptide has been PEGylated with polyethylene glycol (PEG) having an average molecular weight in the range of 500-100,000.

15. The method according to any one of the claims 8-14, wherein the PEG is conjugated selectively to glycans on the Factor IX polypeptide.

16. The method according to any one of the claims 8-15, wherein the PEGylated Factor IX polypeptide exhibits a bioavailability of at least about 125% relative to the bioavailability of the non-PEGylated Factor IX polypeptide as measured in the same assay or model.

17. The method according to any one of the claims 8-16, wherein the PEGylated Factor IX polypeptide has a terminal half-life of at least 1.2 times that of the same non-PEGylated Factor IX polypeptide as measured in the same assay or model.

18. The method according to any one of the claims 8-17, wherein the PEGylated Factor IX polypeptide is administered via a subcutaneous route.
Figure 1. Blood concentration of FIX (ng/ml) as a function of time (h)
INTERNATIONAL SEARCH REPORT

A CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48

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

B RELD5 SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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 practical search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

C DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>X</td>
<td>WO 2006/005058 A (NEKTAR THERAPEUTICS AL CORP [US]; BOSSARD MARY J [US]; STEPHENSON GAYL) 12 January 2006 (2006-01-12) claims 1,6,12,46,60</td>
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<td>A</td>
<td>WO 2006/103298 A (NOVO NORDISK AS [DK]; OESTERGAARD HENRIK [DK]; BOLT GERT [DK]; DOCK ST) 5 October 2006 (2006-10-05) abstract</td>
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Further documents are listed in the continuation of Box C

Special categories of cited documents

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on patentability claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure use exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search

9 July 2008

Date of mailing of the international search report

16/07/2008

Name and mailing address of the ISA/

European Patent Office
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Tel (+31-70) 340-2040
Fax (+31-70) 340-3016

Authorized officer

Kukolka, Florian

From PCT/ISA/210 (second sheet) (April 2005)
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</table>
**INTERNATIONAL SEARCH REPORT**

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
   
   Although claims 8-18 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. **D** As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- [ ] No protest accompanied the payment of additional search fees.
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