FORMULATIONS OF VITAMIN K-DEPENDENT POLYPEPTIDES AND SULFOALKYL ETHER CYCLOEX-TRINS

C01

![Graph showing the effect of Captisol® on U/mg at different temperatures]

**Abstract:** Stabilized formulations, in particular liquid formulations such as an aqueous solution, comprising a vitamin K-dependent polypeptide such as protein C or factor VII/VIIa and a sulfoalkyl ether cycloextrim derivative, e.g., a salt of a beta cycloextrim sulfobutyl ether.

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FORMULATIONS OF VITAMIN K-DEPENDENT POLYPEPTIDES AND SULFOALKYL ETHER CYCLODEXTRINS

FIELD OF THE INVENTION

The present invention relates to formulations of vitamin K-dependent polypeptides, in particular stabilized formulations comprising a vitamin K-dependent polypeptide such as protein C or factor VII and a sulfoalkyl ether cyclodextrin derivative.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually give rise to a fibrin clot. Generally, blood components participating in the coagulation “cascade” are proenzymes or zymogens, i.e. enzymatically inactive proteins that are converted into an active form by action of an activator. Regulation of blood coagulation is accomplished enzymatically by proteolytic inactivation of the procoagulation factors Va and VIIIa achieved by activated protein C (Esmon, J Biol Chem 1989; 264; 4743-4746).

Vitamin K-dependent proteins contain 9 to 13 gamma-carboxyglutamic acid (Gla) residues in their amino terminal 45 residues. The Gla residues are produced by enzymes in the liver that utilize vitamin K to carboxylate the side chains of glutamic acid residues in protein precursors.

Vitamin K-dependent proteins are involved in a number of biological processes, of which the most well-described is blood coagulation (reviewed in Furie et al. Cell (1998) 53:505-518). Vitamin K-dependent proteins include protein Z, protein S, prothrombin, factor X (FX), factor IX (FIX), protein C, factor VII (FVII) and Gas6. The latter protein functions in cell growth regulation (Matsubara et al. Dev. Biol. (1996) 180:499-510) The Gla residues are needed for proper calcium binding and membrane interaction by these proteins. Although the Gla-containing regions of these proteins show a high degree of sequence homology, they have at least a 1000-fold range in membrane affinity (McDonald et al. Biochemistry (1997) 36:5120-5137. Vitamin K-dependent proteins are reviewed e.g. by Nelsestuen et al., Vitam. Horm. (2000) 58:355-89.

Protein C is a serine protease that circulates in the plasma as a zymogen with a half-life of approximately 7 hours, and plasma levels are typically in the range of 3-5 μg/l. It is produced in vivo in the liver as a single chain precursor polypeptide of 461 amino acids. The protein C precursor comprises a 42 amino acid residue signal and propeptide sequence that includes a conserved 18 amino acid propeptide sequence found in all vitamin K-dependent
proteins (Stanley et al., *Biochemistry* (1999) 38:15681-7). This precursor polypeptide undergoes multiple post-translational modifications, including a) cleavage of the signal sequence and the propeptide sequence; b) cleavage of lysine and arginine residues (positions 156 and 157) to make a two-chain inactive zymogen (a 155 amino acid light chain attached via a disulfide bridge to a 262 amino acid heavy chain); c) vitamin K-dependent carboxylation of nine glutamic acid residues of the light chain resulting in nine gamma-carboxyglutamic acid residues in the N-terminal region of the light chain; and d) carbohydrate attachment at four sites (one in the light chain and three in the heavy chain). Finally, the two-chain zymogen may be activated by removal of a dodecapeptide (the activation peptide) at the N-terminus of the heavy chain (positions 158-169), producing the activated protein C (APC).

Protein C is activated by limited proteolysis by thrombin in complex with thrombomodulin on the lumenal surface of the endothelial cell. As explained above, activation liberates a 12 amino acid activation peptide from the N-terminal of the heavy chain. The APC has a half-life of approximately 15 minutes in plasma.

In the presence of its cofactor, protein S, APC proteolytically inactivates factors Va and VIIIa, thereby reducing thrombin generation (Esmen, *Thromb Haemost* 1993; 70; 29-35). Protein S circulates reversibly bound to another plasma protein, C4b-binding protein. Only free protein S serves as a cofactor for APC. Since C4b-binding protein is an acute phase reactant, the plasma levels of this protein vary greatly in many diseases and thus influence the anticoagulant activity of the protein C system.

The gene encoding human protein C maps to chromosome 2q13-q14 (Patracchini et al., *Hum Genet* 1989; 81; 191-192), spans over 11 kb, and comprises a coding region (exons II to IX) and a 5’ untranslatable region encompassing exon I. The protein domains encoded by exons II to IX show considerable homology with other vitamin K-dependent coagulation proteins such as factor IX and X. Exon II codes for a signal peptide, while exon III codes for a propeptide and a 38 amino acid sequence containing 9 Glu residues. The propeptide contains a binding site for the carboxylase that transforms the Glu residues into dicarboxylic acid (Gla) able to bind calcium ions, a step required for phospholipid binding (Cheung et al., *Arch Biochem Biophys* 1989; 274; 574-581). Exons IV, V and VI encode a short connection sequence and two EGF-like domains, respectively. Exon VII encodes both a domain encompassing the 12 amino acid activation peptide and the dipeptide 156-157 which, when cleaved off, yields the mature two-chain form of the protein. Exons VIII and IX encode the serine protease domain.
The complete amino acid sequence of human protein C has been reported by Foster et al., *PNAS USA* 1986; 82; 4673-4677 and includes a signal peptide, a propeptide, a light chain, a heavy chain and an activation peptide. The sequence is available from the Swiss-Prot protein sequence database under entry name PRTC_HUMAN and primary accession number P04070.

APC is inhibited in plasma by the protein C inhibitor as well as by alpha-1-antitrypsin and alpha-2-macroglobulin.

The experimental three-dimensional structure of human APC (in a Gla-domainless form) has been determined to 2.8 Å resolution and reported by Mather et al., *EMBO J* 1996; 15; 6822-6831. The structure included a covalently bound inhibitor (D-Phe-Pro-Arg chloromethylketone, PPACK).

APC is used for the treatment of genetic and acquired protein C deficiency and has been suggested for use as an anticoagulant in patients with some forms of Lupus, following stroke or myocardial infarction, after venous thrombosis, disseminated intravascular coagulation (DIC), septic shock, emboli such as pulmonary emboli, transplantation, such as bone marrow transplantation, burns, major surgery/trauma and adult respiratory stress syndrome (ARDS).

Recombinant APC is produced by Eli Lilly and Co. and marketed under the name Xigris®. Xigris® is sold as a lyophilized powder to be reconstituted with sterile water immediately prior to administration.

Since APC is used for treatment of acute conditions such as septic shock, it would be advantageous to be able to store APC as a stable, ready-to-use formulation, e.g. an aqueous solution, that could be rapidly administered without the need to first reconstitute a dry powder in water. However, the lack of stability of APC in solution has thus far prevented the development of such stable liquid formulations.

APC is known to degrade at increasing temperature as well as elevated pH. A major degradation pathway is the cleavage of the K308-E309 peptide bond in the heavy chain, leading to two fragments of approximately equal size, with N-terminals starting at L170 and E309, respectively. The latter 111 amino acid fragment, constituting the C-terminal end of the heavy chain from E309 to P419, is referred to as the "EAK fragment" in EP 0662513 A1, which discloses methods for minimizing degradation of protein C by maintaining the molecule at a lowered pH, in a denaturing agent or at an extreme salt concentration, in particular by maintaining APC at a pH between 6.3 and 7.0. Although APC was found to have maxi-
mum amido-lytic activity at pH 7.4, EP 0662513 A1 teaches reducing autodegradation by avoiding pH conditions at which APC has maximal activity, i.e. by lowering pH to below 7.0.

US 6,162,629 describes another APC degradation pathway involving autodegradation on either side of H10 near the N-terminal of the light chain, resulting in removal of the first nine or ten residues of the light chain, including Gla residues at positions 6 and 7 that are involved in anticoagulation. It is described that at low pH of less than 6.3 this autodegradation pathway at the N-terminal of the light chain predominates over autodegradation between residues 308-309 of the heavy chain, but that the N-terminal autodegradation can be reduced by use of a pH of 5.5-6.3, preferably about 6.0, in combination with an ionic strength of greater than 150 mM. Although the use of low pH and a high ionic strength reportedly reduced the autodegradation of APC, it is worth noting that processing of APC under these conditions is described as in the context of ultimately isolating the protein in lyophilized form.

Thus, in spite of the clinical advantages that would be provided by a stable, liquid formulation of activated protein C, such formulations have yet to be developed.

FVII is synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem. 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (TF) is able to convert both FIX and FX into their activated forms, followed by reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, PNAS USA 1977; 74:5260-5264).

FVII undergoes post-translational modifications, including vitamin K-dependent carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the molecule. Thus, residues number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:2 are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-translational modifications include sugar moiety attachment at two naturally occurring N-glycosylation sites at positions 145 and 322, respectively, and at two naturally occurring O-glycosylation sites at positions 52 and 60, respectively.

The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-pter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., PNAS USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla do-
main; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like domains; and exons 6 through 8 the serine protease catalytic domain (Yoshitake et al., *Biochemistry* 1985; 24: 3736-3750).


Reports exist on expression of FVII in BHK or other mammalian cells (WO 92/15686, WO 91/11514 and WO 88/10295) and co-expression of FVII and kex2 endoprotease in eukaryotic cells (WO 00/28065).

An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are modified has been reported in WO 91/11514. These amino acids are located at the activation site. WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor. Inactivation by carbamylation of FVIIa at the α-amino acid group 1153 has been described by Petersen et al., *Eur. J. Biochem.*, 1999;261:124-129. The inactivated form is capable of competing with hFVII or hFVIIa for binding to TF and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients in a hypercoagulable state, such as patients with sepsis, or at risk of myocardial infarction or thrombotic stroke.

WO 98/32466 suggests that FVII, among many other proteins, may be PEGylated (i.e. bound to polyethylene glycol) but does not contain any further information in this respect. WO 01/58935 discloses a new strategy for developing FVII or FVIIa molecules having *inter alia* an increased half-life by means of directed conjugation.

Commercial preparations of recombinant human FVIIa (rhFVIIa) are sold under the trade name NovoSeven®. NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients and is currently the only rhFVIIa product available for effective and reliable treatment of bleeding episodes. NovoSeven® is a freeze-dried FVIIa product which is reconstituted before use; it contains a relatively low FVIIa concentration. A vial (1.2 mg) of NovoSeven® contains 1.2 mg rhFVIIa, 5.84 mg NaCl, 2.94 mg CaCl₂, 2H₂O, 2.64 mg
glycy1glycine, 0.14 mg polysorbate 80 and 60.0 mg mannitol; it is reconstituted to pH 5.5 by
2.0 ml water for injection. When reconstituted, the protein is stable for use for 24 hours. Ac-
ccordingly, no liquid ready-for-use or concentrated FVII products are currently commercially
available.

A stable soluble preparation of FVIIa would provide the advantage of easier hand-
ing for patients, medical care personnel, etc. Further, in case of emergencies such as severe
bleeding caused e.g. by acute trauma or surgery, a ready-to-use soluble formulation of FVIIa
would be able to be administered quicker than a dry formulation that must first be reconsti-
tuted with water, which could potentially be life saving and/or prevent complications caused
by loss of blood.

Attempts to prevent proteolytic degradation by site-directed mutagenesis at major
proteolytic sites are disclosed in WO 88/10295, and attempts to prepare stabilized liquid
compositions of FVIIa are disclosed in WO 03/055511 and WO 03/055512. Nevertheless, in
spite of the clinical advantages that would be provided by a stable, liquid formulation of
FVIIa, such formulations have yet to be developed.

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a stabilized formulation for vitamin
K-dependent polypeptides, preferably a liquid formulation having a sufficient stability to
allow storage in liquid form for a desired length of time, without the need for e.g. freezing or
freeze-drying, while maintaining a sufficient level of protein activity. This would e.g. make it
possible to rapidly administer the vitamin K-dependent polypeptide to a patient without first
having to reconstitute a freeze-dried powder in water, thus saving valuable time in acute
clinical situations.

Surprisingly, it has been found that adding a sulfoalkyl ether cyclodextrin derivative
to a liquid formulation of a vitamin K-dependent polypeptide in its activated form results in
improved stability of the polypeptide. In a first aspect, the invention thus relates to a com-
position comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin de-

In another aspect, the invention relates to a method for increasing the stability of a
vitamin K-dependent polypeptide in a pharmaceutical composition, the method comprising
incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally
at least one buffering agent.
In an additional aspect, the invention relates to a method for preparing the above composition, comprising mixing a vitamin K-dependent polypeptide with a sulfoalkyl ether cyclodextrin derivative.

In further aspects, the invention relates to a method for treating a condition treatable by administration of a vitamin K-dependent polypeptide comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of a vitamin K-dependent polypeptide.

In a particular aspect, the invention relates to a composition comprising an activated protein C polypeptide and a sulfoalkyl ether cyclodextrin derivative; a method for increasing the stability of an activated protein C polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally at least one buffering agent; a method of treating a condition treatable by administration of activated protein C, e.g. sepsis or septic shock, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention comprising APC; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of activated protein C.

In another particular aspect, the invention relates to a composition comprising a factor VIIa polypeptide and a sulfoalkyl ether cyclodextrin derivative; a method for increasing the stability of a factor VIIa polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative and optionally at least one buffering agent; a method of treating a condition treatable by administration of FVIIa, e.g. patients with clotting factor deficiencies (such as hemophilia A or B or deficiency of clotting factors XI or VII) or trauma patients, comprising administering to a patient in need thereof a therapeutically effective amount of a composition of the invention comprising FVIIa; and use of a composition of the invention for the manufacture of a medicament for treatment of a condition treatable by administration of FVIIa.

**DRAWING DESCRIPTION**

Figures 1-4 show the amidolytic activity of APC samples treated with 0, 50 or 100 mM Captisol®. Samples stored at –20°C (white) or 37°C (shaded) were analyzed for their ability to cleave a synthetic substrate. Figure 1 shows the activity of Xigris®, Figure 2 shows the activity of recombinant APC having the wild-type sequence (“WT”), Figure 3 shows the activity of the APC variant “C01”, and Figure 4 shows the activity of APC variant “C02”.
DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application the following definitions apply:

The term “stabilized” is intended to mean that the composition has increased storage stability as compared to a composition which does not comprise the sulfoalkyl ether cyclo-dextrin derivative. For instance, the increased storage stability may be observed in a liquid formulation stored as such or stored in a frozen state and thawed prior to use, in a dried form, e.g. lyophilized, spray-dried or air-dried form, for later reconstitution into a liquid form prior to use, in a solid form, e.g. intended for pulmonary or nasal delivery, and/or in any other form, e.g. made for a special drug delivery system (such as microspheres or the like). The increased storage stability may e.g. be measured in terms of increased activity as compared to a reference composition when subjected to the same storage conditions and/or in terms of reduced aggregate formation. The increased storage stability is thus intended to comprise physical stability, e.g. reduced aggregate formation, and/or chemical stability, e.g. reduced degradation.

The term “activity” is intended to mean an in vitro and/or in vivo activity as determined by any suitable assay known to be relevant for the vitamin K-dependent polypeptide in question. For APC, an example of a suitable activity assay is the analysis for enzyme activity (amidolytic activity) described below. Suitable activity assays for FVIIa are also described below.

The term “aggregate formation” is intended to mean a physical interaction between vitamin K-dependent polypeptides that results in formation of oligomers. Aggregate formation is undesirable since in most cases this leads to reduced or even lost activity and/or increased immunogenicity.

The term “protein C precursor” refers to the DNA-encoded form of protein C that includes an 18-residue propeptide, the light chain (residues 1-155), the Lys-Arg dipeptide (residues 156-157) and the heavy chain (residues 158-419), including the activation peptide (residues 158-169). The protein C precursor may also include a signal peptide, e.g. the native signal peptide of human protein C, or alternatively an altered version of the human protein C signal peptide or a heterologous signal peptide selected according the particular expression system used.

The term “one-chain zymogen protein C” refers to the one-chain inactive form of protein C, which includes the light chain (residues 1-155), the Lys-Arg dipeptide (residues
156-157), and the heavy chain (residues 158-419), including the activation peptide (residues 158-169), as shown in SEQ ID NO:1.

The term “two-chain zymogen protein C” refers to the two-chain inactive form of protein C, which includes the light chain (residues 1-155) and the heavy chain (residues 158-419), including the activation peptide (residues 158-169) (but without the Lys-Arg dipeptide between the light chain and the heavy chain), shown in SEQ ID NO:1. The term “zymogen protein C” is intended to refer to both the one-chain form and the two-chain form of the zymogen protein C.

The terms “activated protein C”, “activated human protein C”, “APC” or “human APC” are used about the activated zymogen and include the light chain (residues 1-155) and the heavy chain without the activation peptide (i.e. residues 170-419) of SEQ ID NO:1. The amino acid sequence of activated protein C may be referred to as “the APC part” of the amino acid sequence of SEQ ID NO:1.

The term “protein C” encompasses all of the above-mentioned forms of protein C, i.e. the “protein C precursor” form, the “zymogen protein C” form (the one-chain form as well as the two-chain form) and “activated protein C”, as well as variants thereof.

The “Gla domain” of protein C comprises amino acid residues 1-45 of SEQ ID NO:1.

The “EGF domains” of protein C comprise amino acid residues 55-134 of SEQ ID NO:1.


The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form and includes human wild-type FVII as well as variants thereof. The term "FVIIa" or “FVIIa polypeptide” refers to a FVIIa molecule provided in its activated two-chain form.
and includes human wild-type FVIIa as well as variants thereof. When the amino acid sequence of SEQ ID NO:2 is used to describe the amino acid sequence of FVIIa it will be understood that the peptide bond between R152 and I153 of the single-chain form has been cleaved, and that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa polypeptides produced by recombinant techniques.

The terms “hFVII” and “hFVIIa” refer to human wild-type FVII and FVIIa, respectively, where hFVII has the amino acid sequence shown in SEQ ID NO:2.

The terms “rhFVII” and “rhFVIIa” refer to human wild-type FVII and FVIIa, respectively, produced by recombinant means. An example of rhFVIIa is NovoSeven®.

The term “vitamin K-dependent polypeptide” is intended to cover any polypeptide which requires vitamin K as a cofactor for carboxylation of glutamic acid residues, including precursor, activated or variant forms thereof. The formation of Gla residues in vitamin K-dependent polypeptides is necessary to allow the protein to chelate calcium ions and hence exert its biological activity. Specific examples of vitamin K-dependent polypeptides include protein C, FVII, FIX, protein S, protein Z, prothrombin, FX and Gas6 (including activated and/or variant forms thereof).

The term “amino acid residue” is intended to include any natural or synthetic amino acid residue, and is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e. selected from the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W) and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions/substitutions is illustrated as follows with reference to the sequence of human protein C (SEQ ID NO:1) as an example: A39 in a given amino acid sequence indicates that position number 39 is occupied by an alanine residue. A39S indicates that the alanine residue of position 39 is substituted with a serine residue. Alternative substitutions are indicated with a “+”, e.g., A39S/T means that the alanine residue of position 39 is substituted with either a serine residue or a threonine residue. Multiple substitutions are indicated with a “++”, e.g., A39S+K251N means that the alanine residue of position 39 is substituted with a serine residue and that the lysine residue in posi-
tion 251 is substituted with an asparagine residue. The insertion of an additional amino acid residue is indicated in the following way: Insertion of a serine residue after A39 is indicated by A39AS. An insertion may alternatively be indicated by an asterisk, e.g. addition of an arginine residue after position 156 may be indicated by *156R. Deletion of an amino acid residue is indicated by an asterisk. For example, deletion of the alanine residue in position 39 is indicated by A39*.

The term “non-polypeptide moiety” refers to a non-polypeptide molecule that is capable of conjugating to an attachment group of the vitamin K-dependent polypeptide. Examples of such non-polypeptide moieties include polymer molecules, sugar moieties, lipophilic compounds and organic derivatizing agents. The non-polypeptide moiety can be directly covalently joined to the attachment group or it can be indirectly covalently joined to the attachment group through an intervening moiety, such as a bridge, spacer or linker moiety or moieties. Preferred examples of non-polypeptide moieties are a polymer molecule, in particular a linear or branched polyethylene glycol (PEG) or other polyalkylene glycol, and a sugar moiety, in particular an N- or O-linked oligosaccharide generally attached by in vivo glycosylation, typically an N-linked oligosaccharide.

The term “differs” or “differs from” refers to the fact that the vitamin K-dependent polypeptides in the compositions of the invention may comprise various substitutions, insertions, additions or deletions in relation to the native (typically human wild-type) polypeptide. Such alterations, e.g. with the aim of introducing at least one site for conjugation to a non-polypeptide moiety, may e.g. be performed with the aim of improving or altering the effect of the polypeptide or increasing the half-life. For APC, desired alterations/improvements may include increasing the anti-inflammatory effect and/or lowering the anticoagulant activity of the variant. For FVII, desired alterations/improvements may include increasing the phospholipid membrane-binding properties and/or the clotting activity. Additional alterations may further include, for example, truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus, e.g. addition of a methionine residue at the N-terminus as well as “conservative amino acid substitutions”, i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

Examples of conservative substitutions include amino acids within the respective groups listed in the table below.
<table>
<thead>
<tr>
<th>1</th>
<th>Alanine (A)</th>
<th>Glycine (G)</th>
<th>Serine (S)</th>
<th>Threonine (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Aspartic acid (D)</td>
<td>Glutamic acid (E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Asparagine (N)</td>
<td>Glutamine (Q)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Arginine (R)</td>
<td>Histidine (H)</td>
<td>Lysine (K)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Isoleucine (I)</td>
<td>Leucine (L)</td>
<td>Methionine (M)</td>
<td>Valine (V)</td>
</tr>
<tr>
<td>6</td>
<td>Phenylalanine (F)</td>
<td>Tyrosine (Y)</td>
<td>Tryptophan (W)</td>
<td></td>
</tr>
</tbody>
</table>

Vitamin K-dependent polypeptides that may be used in the composition of the invention thus include not only the human form of such vitamin K-dependent polypeptides, but also variants thereof.

The term “variant” is intended to cover a vitamin K-dependent polypeptide that differs in one or more amino acid residues from its parent polypeptide, normally in 1-15 amino acid residues (such as in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues), e.g. in 1-10, 1-8, 1-6, 1-5, 1-4 or 1-3 amino acid residues, e.g. one or two amino acid residues. The parent polypeptide in the present context is in particular the wild-type human form of the vitamin K-dependent polypeptide, such as human protein C (SEQ ID NO:1) or human FVII (SEQ ID NO:2), or the activated form of a wild-type human vitamin K-dependent polypeptide, e.g. the APC part of SEQ ID NO:1.

The term “modified” or “modification” includes a substitution, an insertion or a deletion.

The term “introduce” is primarily intended to mean substitution of an existing amino acid residue, but may also mean insertion of an additional amino acid residue.

The term “remove” is primarily intended to mean substitution of the amino acid residue to be removed with another amino acid residue, but may also mean deletion (without substitution) of the amino acid residue to be removed.

The term “nucleotide sequence” is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semi-synthetic or synthetic origin, or any combination thereof.

In the present description and claims, any reference to “a” component, e.g. in the context of a non-polypeptide moiety, an amino acid residue, a substitution, a buffer, a cation, etc., is intended to refer to one or more of such components, unless stated otherwise or unless it is clear from the particular context that this is not the case. For example, the expression “a component selected from the group consisting of A, B and C” is intended to include all combinations of A, B and C, i.e. A, B, C, A+B, A+C, B+C or A+B+C.
The sulfoalkyl ether cyclodextrin derivative

Sulfoalkyl ether cyclodextrin derivatives suitable for use in the compositions of the invention include any of the derivatives described in WO 91/11172, US 5,134,127, US 5,376,645 and US 5,874,418, the contents of which are incorporated herein by reference.

In a preferred embodiment, the sulfoalkyl ether cyclodextrin derivative is a compound of the following formula (I):

\[
\begin{align*}
&\text{S}_4\text{R}_4 \\
&\text{R}_5\text{S}_5 \\
&\text{O} \\
&\text{R}_6\text{S}_6 \\
&\text{R}_7\text{S}_7 \\
&\text{O} \\
&\text{R}_8\text{S}_8 \\
&\text{S}_9 \\
&\text{R}_9\text{S}_9 \\
&\text{n}
\end{align*}
\]

wherein:

- \( n \) is 4, 5 or 6,
- \( \text{R}_1, \text{R}_2, \text{R}_3, \text{R}_4, \text{R}_5, \text{R}_6, \text{R}_7, \text{R}_8, \) and \( \text{R}_9 \) are each independently \(-\text{O}-\) or a \(-\text{O}-\text{(C}_2\text{-C}_6\text{ alkyl)}\)-\text{SO}_3\text{-} group, wherein at least one of \( \text{R}_1 \) and \( \text{R}_2 \) is an \(-\text{O}-\text{(C}_2\text{-C}_6\text{ alkyl)}\)-\text{SO}_3\text{-} group, and
- \( \text{S}_1, \text{S}_2, \text{S}_3, \text{S}_4, \text{S}_5, \text{S}_6, \text{S}_7, \text{S}_8, \) and \( \text{S}_9 \) are each independently a pharmaceutically acceptable cation.

In one particular embodiment of the compound of formula (I), \( n \) is 4 ("alpha cyclodextrin"). In another embodiment of the compound of formula (I), \( n \) is 5 ("beta cyclodextrin"). In a further embodiment of the compound of formula (I), \( n \) is 6 ("gamma cyclodextrin"). Preferably, the compound is a derivative of beta cyclodextrin.

In a further embodiment of the compound of formula (I) at least one of \( \text{R}_1 \) and \( \text{R}_2 \) is

\[-\text{O}(\text{CH}_2)_m\text{SO}_3^-, \text{ and } m = 2, 3, 4, 5 \text{ or } 6.\]

In a further embodiment of the compound of formula (I) each of \( \text{R}_1 \) and \( \text{R}_2 \) are independently selected from \(-\text{OCH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-\) and \(-\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{SO}_3^-\).

In a further embodiment of the compound of formula (I) at least one of \( \text{R}_4, \text{R}_6, \) and \( \text{R}_8 \) is independently \(-\text{O}\text{(C}_2\text{-C}_6\text{ alkyl)}\)-\text{SO}_3^-; and \( \text{R}_5, \text{R}_7, \) and \( \text{R}_9 \) are all \(-\text{O}\).

In a further embodiment of the compound of formula (I) \( \text{S}_1, \text{S}_2, \text{S}_3, \text{S}_4, \text{S}_5, \text{S}_6, \text{S}_7, \text{S}_8 \) and \( \text{S}_9 \) are each independently a pharmaceutically acceptable cation selected from \( \text{H}^+ \), an alkali metal (e.g. \( \text{Li}^+, \text{Na}^+, \text{K}^+ \)), an alkaline earth metal (e.g., \( \text{Ca}^{2+}, \text{Mg}^{2+} \)), an ammonium ion
and an amine cation such as the cations of (C₁-C₆) alkylamines, piperidine, pyrazine, (C₁-C₆) alkanolamine and (C₄-C₅)cycloalkanolamine.

In a further embodiment of the compound of formula (I) S₁, S₂, S₃, S₄, S₅, S₆, S₇, S₈ and S₉ are independently selected from H⁺, an alkali metal cation, an alkaline earth metal cation, a quaternary ammonium cation, a tertiary ammonium cation, and a secondary ammonium cation.

In a further embodiment at least one of R₄, R₆, and R₈ is independently -O-(C₂-C₆ alkyl)-SO₃⁻; and R₅, R₇, and R₉ are all -O⁻.

The “alkyl” group as used herein may be linear, cyclic or branched, and saturated or unsaturated (i.e., containing at least one double bond), including divalent alkylene groups and monovalent alkyl groups, respectively. The term “alkanol” likewise includes linear, cyclic and branched, as well as saturated and unsaturated alkyl components of the alkanol groups, in which the hydroxy groups may be situated at any position on the alkyl moiety. The term “cycloalkanol” includes unsubstituted or substituted (e.g., by methyl or ethyl) cyclic alcohols.

The presently preferred sulfoalkyl ether cyclodextrin derivative is a salt of beta cyclodextrin sulfoethyl ether, e.g. based on any one or more of the pharmaceutically acceptable cations listed above. In particular, it may be in the form of an alkali metal or an alkaline earth metal salt, such as a lithium salt, a sodium salt, a potassium salt, a calcium salt or a magnesium salt, or an ammonium salt. An example of a preferred salt form is a sodium salt.

A suitable beta cyclodextrin sulfoethyl ether is commercially available as Captisol® from CyDex Inc., Overland Park, Kansas, USA). Captisol®, also termed SBE7-β-CD, is in the form of a sodium salt having approximately seven sulfoethyl ether substitutions per molecule.

The sulfoalkyl ether cyclodextrin derivative will generally be present in compositions of the invention in a concentration of from about 1 mg/ml to about 200 mg/ml. Typically, the concentration of the sulfoalkyl ether cyclodextrin derivative will be at least about 10 mg/ml, such as at least about 20 mg/ml, at least about 30 mg/ml or at least about 40 mg/ml, more typically at least about 50 mg/ml, and up to about 190 mg/ml, such as up to about 180 mg/ml, up to about 170 mg/ml, up to about 160 mg/ml or up to about 150 mg/ml. The sulfoalkyl ether cyclodextrin derivative may thus be present in a concentration range of, for example, about 10-190 mg/ml, 20-180 mg/ml, 30-170 mg/ml or 40-160 mg/ml. A more typical concentration will be in the range of about 50-150 mg/ml.

Compositions of the invention comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin derivative will generally have a pH in the range of from about 5
to about 8, such as a range of from about 5 to about 7, about 6 to about 7, about 6 to about 8, or about 7 to about 8. Surprisingly, it has been found that use of a sulfobutyl ether cyclodextrin derivative (Captisol®) results in improved stability of liquid formulations of protein C at a pH of above 7. In one particular embodiment, the composition of the invention thus has a pH of above about 7.0, for example from about 7.1 to about 8.5, preferably from about 7.2 to about 8.0, such as from about 7.2 to about 7.8 or about 7.2 to about 7.6.

It will be apparent that any reference to “a” or “the” sulfoalkyl ether cyclodextrin derivative is intended to refer to at least one sulfoalkyl ether cyclodextrin derivative, i.e. the invention is intended to encompass compositions comprising more than one such derivative, even though the compositions of the invention will typically contain only a single sulfoalkyl ether cyclodextrin derivative.

**Vitamin K-dependent polypeptides**

As explained above, the vitamin K-dependent polypeptide is in particular selected from the group consisting of protein C, FVII, FIX, protein S, protein Z, prothrombin, FX and Gas6 (including activated and/or variant forms thereof).

In one aspect, the invention thus relates to a composition that comprises a sulfoalkyl ether cyclodextrin derivative and a vitamin K-dependent polypeptide selected from the group consisting of:

- human wild-type protein C, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein C,
- human wild-type FVII, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FVII,
- human wild-type Factor IX (FIX), preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FIX,
- human wild-type protein S, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein S,
- human wild-type protein Z, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type protein Z,
human wild-type prothrombin, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type prothrombin,

- human wild-type Factor X (FX), preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type FX, and
- human wild-type Gas6, preferably in its activated form, or a variant thereof comprising 1-15 amino acid modifications relative to the amino acid sequence of human wild-type Gas6.

Examples of variants of the above-mentioned vitamin K-dependent polypeptides are given in US 6,017,882. Thus, the vitamin K-dependent polypeptides mentioned above may comprise at least one amino acid substitution in the Gla domain, such as at least substitution in a position selected from the group consisting of position 10, 11, 28, 32 and 33. Preferably, the amino acid substitution is at amino acid 12, 32 or 33. It will be understood that since FIX contains one additional amino acid residue in the Gla domain, the above-mentioned positions must be corrected accordingly, i.e. in case of FIX the at least one substitution is preferably selected from the group consisting of position 11, 12, 29, 33 and 34, in particular position 11, 33 or 34. These substitutions may be either conservative or non-conservative. Non-conservative amino acid substitutions replace an amino acid with an amino acid of a different class and may result in a substantial change in the hydrophobicity of the vitamin K-dependent polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions may make a substantial change in the charge of the vitamin K-dependent polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions include substitution of a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid.

Protein C polypeptides

Activated protein C polypeptides suitable for use in compositions of the invention include human APC with the wild-type sequence as well as variants of hAPC having one or more mutations, e.g. having at least one substitution, insertion or deletion, typically at least one substitution. These additional mutations may e.g. be aimed at increasing the anti-inflammatory effect, increasing the half-life and/or lowering the anticoagulant activity of the resulting polypeptide. Typically, such mutations are aimed at introducing and/or at removing
at least one amino acid residue comprising an attachment group for a non-polypeptide moiety.


G383N+G385T and L386N+H388T; in particular from the group consisting of D189N+K191T, D214N, D251N, and L386N+H388T. Particularly preferred substitutions include D214N and K251N. See WO 02/32461 for further information on variants of protein C with introduced glycosylation sites.

As is also described in WO 02/32461, at least some of the charged residues of protein C interact with each other. For example, K251 is believed to form a salt bridge to D214. Moreover, a cluster of negatively charged amino acid residues (D214, E215 and E357) is present in the protein. Therefore, another group of amino acid substitutions of interest include substitutions where a charged amino acid residue in the active site region and having at least 25% of its side chain exposed to the surface (as defined in WO 02/32461) is substituted with an amino acid residue having no charge, in particular an amino acid residue having no charge but a polar side chain (Gly, Ser, Thr, Cys, Tyr, Asn or Gln) or with an amino acid residue having an opposite charge.


It will be understood that when the APC polypeptide variant comprises one or more in vivo glycosylation sites, the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Preferred host cells thus include, in particular, mammalian cells. Further information on suitable host cells is provided below.
In a further embodiment, at least one attachment group for a non-polypeptide moiety may be introduced in the EGF domains of protein C with the aim of producing variants that have a decreased anticoagulant activity as compared to human APC and with anti-inflammatory properties that have not been substantially changed as compared to human APC. Such variants are disclosed in US 2003/0018175 A1, which is hereby incorporated by reference.

More particularly, such variants comprise at least one sugar moiety covalently attached to a protein C polypeptide having an amino acid sequence which differs from that of a parent protein C polypeptide, in particular human protein C (SEQ ID NO:1), in that at least one in vivo glycosylation site has been introduced by a substitution selected from the group consisting of H66N, H66N+T68S, I73N, I73N+S75T, S75N, S75N+S77T, D79N+R81S, D79N+R81T, E92N, E92N+S94T, G104N, G104N+T106S, R117N, R117N+S119T, D128N+L130S and D128N+L130T, preferably from the group consisting of H66N, I73N+S75T, S75N+S77T, D79N+R81T, E92N, G104N, R117N+S119T and D128N+L130T. A particularly preferred substitution in the EGF domains is H66N.

In another embodiment, the protein C polypeptide may include at least one introduced amino acid residue comprising an attachment group for a polymer molecule, in particular an introduced cysteine residue. Preferably, such a cysteine residue is introduced in a position selected from the group consisting of D172, D189, S190, K191, K192, K193, D214, E215, S216, K217, K218, L220, V243, V245, S250, K251, S252, T253, T254, L296, Y302, H303, S304, S305, T315, F316, V334, S336, N337, M338, I348, L349, D351, R352, E357, G383, L386, L387 and H388; more preferably from the group consisting of D189, S190, K191, D214, K217, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; such as from the group consisting of D189, K191, D214, K251, S252, T253, Y302, S336, N337, M338, G383 and L386; in particular from the group consisting of D189, D214, K251 and L386. The polymer molecule to be covalently attached to said introduced cysteine residue is in particular a linear or branched polyethylene glycol or other polyalkylene oxide.

Specific examples of activated PEG polymers particularly preferred for coupling to cysteine residues include the following linear PEGs: vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG) and orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG). Such PEG or mPEG polymers will generally have a size of from about 1 kDa to about 40 kDa, such as from about 1 kDa to about 20 kDa, e.g. from
about 2 kDa to about 15 kDa, such as from about 3 kDa to about 10 kDa; for example about 5 kDa, about 6 kDa, about 10 kD, about 12 kDa or about 20 kDa.

For PEGylation to cysteine residues the protein C variant is usually treated with a reducing agent, such as dithiothreitol (DDT) prior to PEGylation. The reducing agent is subsequently removed by any conventional method, such as by desalting. Conjugation of PEG to a cysteine residue typically takes place in a suitable buffer at about pH 6-9 at temperatures of about 4°C to 25°C for periods up to about 16 hours.

It will be understood that the APC variant according to this aspect of the invention may contain combinations of more than one of the alterations described above. For example, in addition to having at least one introduced glycosylation site, the APC variant may comprise at least one introduced amino acid residue comprising an attachment site for a polymer molecule such as PEG, and/or at least one introduced amino acid residue with an altered charge. The APC polypeptide variant may in this case e.g. contain a total of 1-6 attachment groups that are available for conjugation, such as 1, 2, 3, 4, 5, or 6 such attachment groups.

In a further embodiment, the protein C polypeptide may comprise at least one amino acid modification in the autolysis loop constituted by the amino acid residues in position 306-314 relative to SEQ ID NO:1 in order to achieve a reduced anticoagulant activity. This modification may e.g. include substitution of at least one of R306, E307, K308, E309, K311, R312 and R314 with an uncharged amino acid residue, e.g. A, V, L, I, F, W, P, G, S, T, Y, N or Q. By way of example, in one preferred embodiment, one, two, three, four or five of the residues in these positions may be substituted with an alanine residue.

Further information on protein C variants comprising advantageous substitutions of the type described above as well as conjugation of such variants to one or more non-polypeptide moieties is found in WO 02/32461, US 2003/0018175 A1 and PCT/DK03/00392, which are hereby incorporated herein by reference.

**FVII polypeptides**

FVIIa polypeptides suitable for use in compositions of the invention include human FVIIa (hFVIIa) with the wild-type sequence as well as variants of hFVIIa having one or more mutations, e.g. having at least one substitution, insertion or deletion, typically at least one substitution. These additional mutations may e.g. be aimed at increasing the half-life and/or increasing the clotting activity of the resulting polypeptide.
The term “clotting activity” is used to mean the activity measured in the “Whole Blood Assay” described herein. It will be understood that the activity measured in the “Whole Blood Assay” is the time needed to obtain clot formation. Thus, a lower clotting time corresponds to a higher clotting activity.

The term “increased clotting activity” is used to indicate that the clotting time of the polypeptide variant is statistically significantly decreased relative to that generated by rhFVIIa as determined under comparable conditions and when measured in the “Whole Blood Assay” described herein.

In one embodiment, the FVIIa polypeptide is a variant of hFVIIa which comprises at least one modification in the Gla domain. A number of modifications in the FVII Gla domain leading to an increased membrane binding affinity have been described in the art (see, for example, WO 99/20767 and WO 00/66753). Mutations of particular interest in the Gla domain are substitutions in one or more of P10, K32, D33, A34 and R36, as well as insertion of an amino acid residue between A3 and F4. Particularly preferred positions are P10 and K32. Preferably, the substitution in position 32 is K32E, the substitution in position 10 is P10Q, the substitution in position 33 is D33F, the substitution in position 34 is A34E, the substitution in position 36 is R36E, and the insertion between A3 and F4 is A3AY.

In another embodiment, the FVIIa polypeptide has been modified to result in a variant with an increased functional in vivo half-life, an increased plasma half-life and/or an increased Area Under the Curve when administered intravenously (AUC<sub>v</sub>), in particular as determined by intravenous administration to rats, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation. Such variants may allow lower doses and/or a longer duration between injections.

Interesting FVIIa variants include those wherein at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced, and which have at least one non-polypeptide moiety covalently attached to at least one of said attachment groups. Numerous examples of relevant amino acid substitutions of this type are given in WO 01/58935, which is hereby incorporated by reference. See also WO 03/093465, incorporated by reference, which discloses FVII or FVIIa variants comprising an amino acid substitution in positions 10 and 32 and having one or more introduced N-glycosylation sites located outside the Gla domain.

Also, the polypeptide variant may be attached to a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide or, alternatively, one of the active-site amino acid
residues may be mutated (such as S344A) to render the resulting molecule catalytically inactive.

The amino acid residue comprising an attachment group for a non-polypeptide moiety is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the FVII polypeptide and the non-polypeptide moiety is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule, amino acid residues comprising an attachment group may be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably lysine, cysteine, aspartic acid and glutamic acid, more preferably lysine and cysteine, in particular cysteine.

When an attachment group for a non-polypeptide moiety is to be introduced into the FVII polypeptide, the position of the amino acid residue to be modified is preferably located at the surface of the FVII polypeptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the surface preferably more than 50% of its side chain exposed to the surface. Such positions have been identified on the basis of an analysis of a 3D structure of the hFVII molecule as described in WO 01/58935.

Furthermore, the position is preferably selected from a part of the FVII polypeptide that is located outside the tissue factor binding site, the Gla domain, the active site region and/or the ridge of the active site binding cleft. These sites/regions are identified in Example 1 of WO 01/58935. In certain situations, however, e.g. in case an inactivated FVII polypeptide is desired, it may be advantageous to perform modifications in or close to the active site region and/or the ridge of the active site binding cleft. For example, it is contemplated that one or more attachment groups for the non-polypeptide moieties, such as attachment groups for in vivo N-glycosylation sites, may advantageously be introduced in the active site region or at the ridge of the active site binding cleft of the FVII polypeptide.

Functional in vivo half-life is inter alia dependent on the molecular weight of the protein, and the number of attachment groups needed for providing increased half-life thus depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment, the FVIIa polypeptide has a molecular weight of at least 67 kDa, in particular at least 70 kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), p680-85. FVIIa itself has a molecular weight of about 53 kDa, and therefore an additional 10-20 kDa is required to obtain such an effect. This may, e.g., be provided by conjugating 2-4 10 kDa PEG molecules or otherwise as described in WO 01/58935.
The FVII polypeptide may contain 1-10 non-polypeptide moieties, typically 1-8 or 2-8 non-polypeptide moieties, preferably 1-5 or 2-5 non-polypeptide moieties, such as 1-4 or 1-3 non-polypeptide moieties, e.g. 1, 2 or 3 non-polypeptide moieties. Preferred non-polypeptide moieties include PEG, such as mPEG, or sugar moieties. It will also be apparent that a FVII polypeptide may contain, for example, at least one PEG moiety and at least one sugar moiety.

In one embodiment, the non-polypeptide moiety attached to a FVII variant may thus be a sugar moiety, i.e. the FVII polypeptide variant comprises at least one sugar moiety covalently attached to an introduced glycosylation site. Preferably said glycosylation site is an in vivo glycosylation site, in particular an in vivo N-glycosylation site, which has been introduced by substitution.

As used herein in the context of FVII, the term "naturally occurring glycosylation site" covers the glycosylation sites at positions N145, N322, S52 and S60. In a similar way, the term "naturally occurring in vivo O-glycosylation site" includes the positions S52 and S60, and the term "naturally occurring in vivo N-glycosylation site" includes positions N145 and N322. When the FVIIa polypeptide variant comprises one or more in vivo glycosylation sites, it will be apparent that the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s). Preferred host cells thus include, in particular, mammalian cells. Further information on suitable host cells is provided below.

Positions where glycosylation sites may be introduced in FVII are preferably selected from a part of the molecule that is located outside the TF binding site, the Gla domain, the active site region and the ridge of the active site cleft; see WO 01/58935. Specific examples of substitutions creating an in vivo N-glycosylation site include those selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205S, I205T, V253N, T267N, T267N+S269T, S314N+K316S, S314N+K316T, R315N+V317S, R315N+V317T, K316N+G318S, K316N+G318T, G318N and D334N. Preferred substitutions include those selected from the group consisting of A51N, G58N, T106N, K109N, G124N, K143N+N145T, A175T, I205T, V253N, T267N+S269T, S314N+K316T, R315N+V317T, K316N+G318T, G318N and D334N, more preferably those selected from the group consisting of T106N, A175T, I205T, V253N and T267N+S269T, in particular I205T or V253N.

In one embodiment, one in vivo N-glycosylation site has been introduced into FVII by substitution. In another embodiment, at least two in vivo N-glycosylation site sites have
In a still further embodiment of this aspect of the invention at least three \textit{in vivo} N-glycosylation site sites have been introduced by substitution. Specific examples of substitutions creating three \textit{in vivo} N-glycosylation sites include I205T+V253N+T267N+S269T and T106N+I205T+V253N.

As indicated above, the FVIIa variant according to this aspect of the invention may contain, in addition to at least one sugar moiety, at least one additional non-polypeptide moiety, in particular a polymer molecule such as PEG, i.e. one or more substitutions aimed at introducing at least one \textit{in vivo} glycosylation site may be combined with one or more substitutions aimed at introducing at least one site for attachment of e.g. a PEG moiety. The FVII or FVIIa polypeptide variant may e.g. contain a total of 1-6 attachment groups that are available for conjugation, such as 1, 2, 3, 4, 5, or 6 such attachment groups.

As mentioned above, when the non-polypeptide moiety is a polymer molecule such as PEG, the attachment group in a preferred embodiment is cysteine. FVII/FVIIa contains 22 cysteine residues located outside the Gla domain, and disulfide bridges are established between the following cysteine residues: C50 and C61, C55 and C70, C72 and C81, C91 and C102, C98 and C112, C114 and C127, C135 and C262, C159 and C164, C178 and C194, C310 and C329, and between C340 and C368.

Thus, in an interesting embodiment at least one cysteine residue has been introduced, preferably by substitution, in the FVIIa polypeptide. Typically 1-10 cysteine residues have been introduced, preferably 1-8, 1-6, 1-4 or 1-3. In particular 1, 2 or 3 cysteine residues have been introduced by way of substitution.

In one embodiment, a cysteine residue may be introduced near or at the C-terminus. For example, a cysteine residue may be introduced, either by substitution or insertion, in position 400-406. Specific examples of substitutions include: L400C, L401C, R402C, A403C, P404C, F405C and P406C, in particular P406C. Specific examples of insertions include L400LC, L401LC, R402RC, A403AC, P404PC, F405FC and P406PC, in particular P406PC.

The polymer molecule to be attached to a cysteine residue is preferably a linear or branched polyethylene glycol or another polyalkylene oxide, in particular vinylsulfone-PEG (VS-PEG), preferably vinylsulfone-mPEG (VS-mPEG); maleimide-PEG (MAL-PEG), preferably maleimide-mPEG (MAL-mPEG); or orthopyridyl-disulfide-PEG (OPSS-PEG), preferably orthopyridyl-disulfide-mPEG (OPSS-mPEG).

When the FVIIa polypeptide variant comprises only one conjugatable cysteine residue, this residue is preferably conjugated to a non-polypeptide moiety with a molecular weight of from about 5 kDa to about 20 kDa, e.g. from about 10 kDa to about 20 kDa, such
as a molecular weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO 99/55377). When the FVIIa polypeptide variant comprises two or more conjugatable cysteine residues, normally each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa, such as about 5 kDa or about 10 kDa.

In a further embodiment of the present invention, the FVIIa variant may, optionally in addition to the modifications described in the sections above, also contain mutations which are known to increase the intrinsic activity of the polypeptide, such as those described in WO 02/22776. Examples of such substitutions are those selected from the group consisting of V158D, E296D, M298Q, L305V and K337A. More preferably, said substitutions are selected from the group consisting of V158D+E296D+M298Q+L305V+K337A, V158D+E296D+M298Q+K337A, V158D+E296D+M298Q+L305V, V158D+E296D+M298Q, M298Q, L305V+K337A, L305V and K337A.

Moreover, the FVII variant may, optionally in addition to the modifications mentioned above, contain modifications which increase the tissue factor (TF) binding affinity. Examples of such modifications include at least one substitution selected from the group consisting of L39E, L39Q, L39H, I42R, S43H, S43Q, K62E, K62R, L65Q, L65S, F71D, F71Y, F71E, F71Q, F71N, E82Q, E82N, E82K, F275H and combinations thereof, in particular L65Q, F71Y, K62E, S43Q and combinations thereof.

In a further embodiment, the FVIIa variant may, optionally in addition to the modifications described in the sections above, also contain mutations which cause a decreased inhibition by TFPI. One such example is the substitution K341Q (disclosed by Neuen-schwanter et al., Biochemistry 1995; 34:8701-8707). Other examples include D196K, D196N, G237L, G237GAA and combinations thereof.

Methods for preparing polypeptides

Vitamin K-dependent polypeptides, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is a gamma-carboxylating host cell, in particular a mammalian cell.

A nucleotide sequence encoding a polypeptide precursor may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent vitamin K-dependent polypeptide precursor, and then changing the nucleotide sequence so as to effect introduction (i.e.
insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid
residue(s).

The nucleotide sequence may conveniently be modified by site-directed mutagenesis
in accordance with conventional methods. Alternatively, the nucleotide sequence may be
prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein
oligonucleotides are designed based on the amino acid sequence of the desired polypeptide,
and preferably selecting those codons that are favored in the host cell in which the
recombinant polypeptide will be produced.

Persons skilled in the art will be capable of selecting suitable vectors, expression
control sequences and hosts for expressing the polypeptide. The nucleotide sequence encoding
a vitamin K-dependent polypeptide precursor, whether prepared by site-directed
mutagenesis, synthesis, PCR or other methods, will generally include a nucleotide sequence
that encodes a suitable signal peptide to allow secretion from the host cells.

Suitable host cells that may be used to produce the polypeptide precursor include, in
particular, mammalian cells. Examples of suitable mammalian host cells include Chinese
hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines
(COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g.
NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10),
and human cells (e.g. HEK 293 (ATCC CRL-1573)).

The resulting polypeptide may be recovered from the nutrient medium by methods
known in the art, e.g. using conventional procedures such as centrifugation, filtration, ultra-
filtration, extraction and/or precipitation, and may 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., prepara-
trative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation) or
extraction (see, e.g., Protein Purification (2nd Edition), Janson and Ryden, editors, Wiley,

For further information on preparing vitamin K-dependent polypeptides, see e.g.
WO 00/66753.

Information on methods for preparing protein C and variants thereof may be found
e.g. in WO 02/32461. Activation of the zymogen form of protein C to result in activated pro-
tein C may be performed in a manner known in the art, e.g. by using thrombin or thrombin
and thrombomodulin to cleave the activation peptide, or alternatively by use of a snake
venom activator. Another approach for producing activated protein C is to alter or replace the
native activation peptide. For example, EP 0319312 A2 describes replacement of the activation peptide of protein C with a non-native proteolytic cleavage site that leads to direct expression of activated protein C in recombinant host cells. A similar approach is disclosed in WO 03/035861, which describes chimeric protein C in which the native activation peptide has been replaced with a thrombin-cleavable sequence of fibrinopeptide A or a portion thereof in order to obtain enhanced activation by thrombin in the absence of thrombomodulin.

For further information on preparing FVII and FVIIa polypeptides, see e.g. WO 01/58935 and WO 03/093465.

Pharmaceutical compositions and use

Vitamin K-dependent polypeptides produced according to the present invention may be formulated as known in the art in a pharmaceutical composition comprising a polypeptide and at least one pharmaceutically acceptable carrier or excipient. In the present context, the term "pharmaceutically acceptable" means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the patients to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 19th edition, A. R. Gennaro, Ed., Mack Publishing Company, 1995; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis, 2000; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press, 2000).

Compositions of the invention comprising APC may in particular be used for the manufacture of a medicament for treating or preventing a disease selected from the group consisting of stroke; myocardial infarction; after venous thrombosis; disseminated intravascular coagulation (DIC); sepsis; septic shock; emboli, such as pulmonary emboli; transplantation, such as bone marrow transplantation; burns; major surgery/trauma or adult respiratory stress syndrome (ARDS), in particular for the treatment of sepsis, including septic shock. The invention thus includes a method for treating or preventing such diseases or conditions by administering to a patient in need thereof an effective amount of a composition of the invention comprising activated protein C or a variant thereof and an sulfoalkyl ether cyclodextrin derivative.

Compositions of the invention comprising FVIIa may in particular be used for the manufacture of a medicament for treatment of a condition treatable by administration of
FVIIa, in particular for treating or preventing clotting factor deficiencies (such as hemophilia A or B or deficiency of clotting factors XI or VII) or trauma. The invention thus includes a method for treating or preventing such diseases or conditions by administering to a patient in need thereof an effective amount of a composition of the invention comprising FVIIa or a variant thereof and an sulfoalkyl ether cyclodextrin derivative.

A "patient" for the purposes of the present invention includes both humans and other mammals, i.e. the methods are applicable to both human therapy and veterinary applications.

The vitamin K-dependent polypeptides will be administered to patients in an effective dose. By "effective dose" herein is meant a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the disorder to be treated, and will be ascertainable by one skilled in the art using known techniques.

The polypeptides can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may by present as a crystalline and/or amorphous structure.

The pharmaceutical composition 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 composition or may be administered separately, either concurrently or in accordance with another treatment schedule. In addition, the pharmaceutical composition of the invention may be used as an adjuvant to other therapies.

The composition of the invention is preferably in the form of an aqueous solution or suspension, typically an aqueous solution.

The administration of the formulations of the present invention can be performed in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, intraocularly, or in any other acceptable manner. The liquid formulations of the invention will typically be administered continuously by infusion, although bolus injection may also be used in certain cases.

In the general discussion below regarding pharmaceutical compositions, it will be understood that compositions according to the invention will in all cases contain a sulfoalkyl ether cyclodextrin derivative as described above.
**Parenteral compositions**

An example of a pharmaceutical composition is a solution designed for parenteral administration. Although in many cases pharmaceutical solution formulations are provided in liquid form, appropriate for immediate use, such parenteral formulations may also be provided in frozen or in lyophilized form.

In case of parenterals, they are prepared for storage as lyophilized formulations or aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically employed in the art (all of which are termed "excipients"), for example buffering agents, stabilizing agents, preservatives, isotonicifiers, non-ionic detergents, antioxidants and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates physiological conditions; see also the discussion of pH above. They are typically present at a concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents for use with the present invention include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium glyconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

Preservatives are added to retard microbial growth, and are typically added in amounts of e.g. about 0.1%-2% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g. benzalkonium chloride,
bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, omithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinositol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e. <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Non-ionic surfactants or detergents (also known as "wetting agents") may be present to help solubilize the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols, polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g. starch), chelating agents (e.g. EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and cosolvents.

The active ingredient may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example hydroxymethylcellulose, gelatin or poly-(methylmethacrylate) microcapsules, in colloidal drug delivery systems
(for example liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This is readily accomplished, for example, by filtration through sterile filtration membranes.

In addition to parenteral formulations, it is also possible to administer the vitamin K-dependent polypeptide in the form of a sustained release formulation, e.g. as described in WO 02/32461.

The invention is further illustrated by the following non-limiting examples.

**Example 1**

**Stabilization of activated protein C**

Experiments were carried out to demonstrate the stabilizing effect of Captisol® (a beta-cyclodextrin pharmaceutical excipient) on the degradation as well as aggregation of activated protein C (APC) at a pH above 7. Each of the samples were formulated to final concentrations of 50 mg/ml Captisol® and 100 mg/ml Captisol®, respectively, and as a control without Captisol®. Storage conditions were either at −20°C or 37°C, and samples were stored for 96 hours.

Four different APC samples were studied:

- Xigris®: recombinant APC commercially available from Eli Lilly, produced in HEK293 cells, and formulated in 16 mM citrate, 150 mM NaCl, 12.5 mg/ml sucrose, pH 6.0;
- recombinant APC having the wild-type sequence (“WT”);
- APC variant C01: *156R, 157R, D214N*, i.e. with an N-glycosylation site introduced in position 214 and an R-R insertion in position 156-157; and
- APC variant C02: K251N, i.e. with an N-glycosylation site introduced in position 251.

The recombinant WT APC and the APC variants C01 and C02 were produced in CHO cells and were formulated in 20 mM Tris, 150 mM NaCl, pH 7.4.
Samples were analyzed for aggregation, degradation, and enzymatic activity. In all analyses Captisol® was shown to improve the stability of at least one of the samples that were formulated at pH 7.4.

Materials and Methods

Sample preparation

All samples, except Xigris®, were diafiltered to 20 mM Tris, 150 mM NaCl, pH 7.4 using a 6 ml Vivaspin cartridge with a molecular weight cut-off of 10 kDa. Xigris® was maintained in its commercial formulation conditions: 16 mM citrate, 150 mM NaCl, 12.5 mg/ml sucrose, pH 6.0.

A 200 mg/ml stock solution of Captisol® (CyDex, Overland Park, KS, USA) was added to each of the Captisol® samples, to final concentrations of 100 and 50 mg/ml. The 50 mg/ml samples were adjusted to the same volume as the 100 mg/ml samples with buffer of the corresponding pH. Non-Captisol® samples were diluted with buffer to the same volumes as the Captisol® samples.

The protein concentrations were as follows:
- Xigris: 1.08 mg/ml
- WT: 0.84 mg/ml
- C01: 0.65 mg/ml
- C02: 0.64 mg/ml

Samples were split in two, and one aliquot was stored at -20°C and the other at 37°C. The latter was frozen at -20°C after 96 hours.

Analyses

Thawed samples were analyzed using SDS-PAGE (for degradation), size-exclusion chromatography (for aggregation), as well as for enzyme activity.

SDS-PAGE:

20 µl of sample was mixed with 10µl Reducing Cocktail (250 µl NuPAGE® LDS Sample Buffer (4x) and 100 µl 0.5M DTT) and heated for 5 minutes at 95°C. Samples were applied on NuPAGE® Novex Bis-Tris 4-12% gels and electrophoresed in an MES buffer system for 35 minutes at a constant 200 V. Gels were incubated in fixing solution (50% (v/v)
methanol, 10% (v/v) acetic acid, 40% (v/v) deionized water, and protein was visualized by staining with Novex Colloidal Blue using the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA).

Size-exclusion chromatography:

A Dionex Summit HPLC system consisting of a pump, degasser, autosampler, and diode-array detector, and controlled by Chromeleon version 6.30 software (Dionex, Sunnyvale, CA, USA), was connected to a Superdex 200 HR 30/10 SEC HPLC column (Amersham Biosciences, Uppsala, Sweden). The buffer used was 50 mM Na$_2$HPO$_4$, 500 mM NaCl, pH 7.0, and the flow rate was 1 ml/min. Eluting protein was detected by measurement of OD$_{214\text{nm}}$. 5 μg of sample (between 5 and 8 μl) was injected for each analysis.

Enzyme activity (Amidolytic Assay):

Amidolytic activity was determined using the synthetic peptide substrate SPEC-TROZYME PCs with the formula H-D-Lys(γ-Cbo)-Pro-Arg-pNA.2AcOH (American Diagnostica Inc, product # 336) at a final concentration of 0.5 mM. Assays were performed in 96 well microtiter plates at 23°C in 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, 5 mM CaCl$_2$ and 0.1% BSA. The rate of hydrolysis was recorded for 3 min at 405 nm as the change in absorbance units/min using a SpectraMax Plus 384 kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Xigris® was used as a standard.

Results and Discussion

SDS-PAGE

When APC is analyzed under the conditions described above, the heavy chain (HC) and the light chain (LC) are separated due to reduction of the disulphide bridge that holds them together. The heavy chain gives rise to several protein bands due to glycosylation heterogeneity; this was especially noticeable in C01, which contains a fully occupied extra glycosylation site at position 214. The light chain normally only gives rise to one band at approximately 22 kDa.

As explained above, protein C is known to degrade at increasing temperature as well as elevated pH, a major degradation pathway being cleavage of the K308-E309 peptide bond in the heavy chain. In these experiments, it was found that degradation in all of the APC variants stored at 37°C was substantially decreased with increasing concentrations of Captisol®
(50 and 100 mg/ml). No formulation effect was seen in the analogous samples that were immediately frozen at –20°C.

The diminished formation of low molecular weight degradation products was primarily seen in WT and C02, and to a lesser extent in Xigris®. Xigris® is formulated at pH 6.0, whereas the other samples had a pH of 7.4, and this could be a cause of the different degradation profiles. The effect of Captisol® on C01 degradation was most apparent in its effect on a heavy chain fragment band migrating just above the light chain band.

Size-exclusion chromatography

Size-exclusion chromatography separates molecules according to their hydrodynamic radius. Under these experimental conditions, APC is in its native conformation, and all interactions, including van der Waal’s forces, hydrogen bonds and hydrophobic interactions, are preserved. It is thus possible to monitor aggregation of APC molecules caused not only by covalent interaction but also by these weaker forces.

The results of the size-exclusion chromatography analyses showed that the aggregated forms of APC decrease with increasing amounts of Captisol®, indicating that this excipient prevents the formation of impurities in the form of aggregates. This was seen most clearly for WT APC stored at 37°C.

Enzyme activity

The results of the determinations of enzyme activity are shown in Figures 1-4, which show that the specific amidolytic activity of APC (U/mg) in the absence of Captisol® was lower for samples stored at 37°C (shaded bars) than at –20°C (white bars) for all samples except Xigris®, where the difference was negligible. Addition of Captisol® led to a stabilization (increase) of enzyme activity in WT and C01, whereas the effect was negligible in Xigris® and C02. The effect of Captisol® on samples stored immediately at –20°C was also negligible, except for C02, in which addition of Captisol® resulted in a decrease in specific activity.

No effect of Captisol® was seen on the specific activity of samples that were analyzed immediately after formulation (data not shown).
Example 2

Stabilization of FVII variants

Materials and Methods

Determination of Phospholipid Membrane Binding Affinity

Phospholipid membrane binding affinity may be determined as described in Nelsестuen et al., *Biochemistry*, 1977; 30;10819-10824 or as described in Example 1 in US 6,017,882.

TF-independent Factor X Activation Assay

This assay has been described in detail on page 39826 in Nelsестuen et al., *J Biol Chem*, 2001; 276:39825-39831.

Briefly, the molecule to be assayed is mixed with a source of phospholipid (preferably phosphatidylcholine and phosphatidylserine in a ratio of 8:2) and relipidated Factor X in Tris buffer containing BSA. After a specified incubation time the reaction is stopped by addition of excess EDTA. The concentration of factor Xa is then measured from the absorbance change at 405 nm after addition of a chromogenic substrate (S-2222™, Chromogenix). After correction for background the tissue factor independent activity of rhFVIIa (ait) is determined as the absorbance change after 10 minutes and the tissue factor independent activity of the FVIIa variant (avariant) is also determined as the absorbance change after 10 minutes. The ratio between the activity of the FVIIa variant, in its activated form, and the activity of rhFVIIa is defined as avariant/avit.

Clotting Assay

The clotting activity of the FVIIa or variants thereof may be measured in one-stage assays and the clotting times recorded on a Thrombotrack IV coagulometer (Medinor). Factor VII-depleted human plasma (American Diagnostica) is reconstituted and equilibrated at room temperature for 15-20 minutes. 50 microliters of plasma is then transferred to the coagulometer cups.
FVIIa or variants thereof are diluted in glyoxaline buffer (5.7 mM barbiturate, 4.3 mM sodium citrate, 117 mM NaCl, 1 mg/ml BSA, pH 7.35). The samples are added to the cup in 50 µl portions and incubated at 37°C for 2 minutes.

Thromboplastin (Medinor) is reconstituted with water and CaCl₂ is added to a final concentration of 4.5 mM. The reaction is initiated by adding 100 µl of thromboplastin. To measure the clotting activity in the absence of TF the same assay is used without addition of thromboplastin. Data may be analysed using PRISM software.

**Whole Blood Assay**

The clotting activity of FVIIa or variants thereof may be measured in one-stage assays and the clotting times recorded on a Thrombotrack IV coagulometer (Medinor). 100 µl of FVIIa or variants thereof are diluted in a buffer containing 10 mM glycylglycine, 50 mM NaCl, 37.5 mM CaCl₂, pH 7.35 and transferred to the reaction cup. The clotting reaction is initiated by addition of 50 µl blood containing 10% 0.13 M tri-sodium citrate as an anticoagulant. Data may be analysed using Excel or PRISM software.

**Amidolytic Assay**

The ability of FVII or variants thereof to cleave small peptide substrates can be measured using the chromogenic substrate S-2288™ (D-Ile-Pro-Arg-p-nitroanilide, Chromogenix). FVIIa is diluted to about 10-90 nM in assay buffer (50 mM Na-Hepes pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.1% BSA, 1U/ml heparin). Furthermore, soluble TF (sTF) is diluted to 50-450 nM in assay buffer. 120 µl of assay buffer is mixed with 20 µl of the FVIIa sample and 20 µl sTF. After 5 min. of incubation at room temperature with gentle shaking, followed by 10 min. of incubation at 37°C, the reaction is started by addition of the S-2288™ substrate to 1 mM and the absorption at 405 nm is determined at several time points.

**ELISA Assay**

FVII/FVIIa (or variant) concentrations may be determined by ELISA. Wells of a microtiter plate are coated with an antibody directed against the protease domain using a solution of 2 µg/ml in PBS (100 µl per well). After overnight coating at room temperature, the wells are washed 4 times with THT buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2, 0.05% Tween®-20). Subsequently, 200 µl of 1% casein (diluted from 2.5% stock using 100 mM NaCl, 50 mM Tris-HCl pH 7.2) is added per well for blocking. After 1 hr of incubation at
room temperature, the wells are emptied, and 100 µl of sample (optionally diluted in dilution buffer (THT + 0.1% casein)) is added. After another incubation of 1 hr at room temperature, the wells are washed 4 times with THT buffer, and 100 µl of a biotin-labelled antibody directed against the EGF-like domain (1 µg/ml) is added. After another 1 hr of incubation at room temperature, followed by 4 more washes with THT buffer, 100 µl of streptavidin-horse radish peroxidase (DAKO A/S, Glostrup, Denmark, diluted 1/10000) is added. After another 1 hr of incubation at room temperature, followed by 4 more washes with THT buffer, 100 µl of TMB (3,3',5,5'-tetramethylbenzidine, Kem-en-Tech A/S, Denmark) is added. After 30 min of incubation at room temperature in the dark, 100 µl of 1 M H₂SO₄ is added and OD₄₅₀ is determined. A standard curve is prepared using rhFVIIa (NovoSeven®).

Alternatively, FVII/FVIIa or variants may be quantified via the Gla domain rather than via the protease domain. In this ELISA set-up, wells are coated overnight with an antibody directed against the EGF-like domain and for detection a calcium-dependent biotin-labelled monoclonal anti-Gla domain antibody is used (2 µg/ml, 100 µl per well). 5 mM CaCl₂ is added to the THT and dilution buffers.

**DSC of formulations containing variant [L280Q]rhFVIIa**

Samples of the [L280Q]rhFVIIa variant were analyzed by Differential Scanning Calorimetry (DSC) to study the unfolding (i.e. denaturation) of the protein and, in particular, to determine the melting temperature (Tₘ) for the protein in each run.

The starting material for DSC analysis was a solution of the protein in 10 mM Gly-Gly buffer containing 50 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 (adjusted to pH 5.5). A series of solutions were prepared with varying excipients added to give a final protein concentration of 0.25 mg/ml and a final sodium chloride concentration of 86 mM.

The addition of excipient was done by carefully adding a pre-made solution of the excipient in a buffer consisting of 10 mM Gly-Gly buffer containing 140 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 (adjusted to pH 5.5) to a portion of the thawed bulk solution of the protein. Water for injection was used to serve as blanks for DSC, since the object was only to determine shifts in Tₘ values. Prior to being subjected to DSC analysis, all solutions were degassed under vacuum for a sufficient time period as described by MicroCal Inc.
The behavior of the protein was evaluated by using a DSC apparatus from MicroCal Inc. (model VP-DSC). The temperature of the solution in question was gradually increased from 15°C to 120°C at a rate of 1.5°C per minute.

Adding 0 mg/ml, 2 mg/ml, 10 mg/ml or 80 mg/ml Captisol® to the original solution resulted in different DSC profiles.

In the first three samples (containing 0, 2, or 10 mg/ml Captisol®), two events occurred as the temperature was increased. The first event was an unfolding reaction (endothermic), and was observed as an upward peak in the scans. The second event was a precipitation (exothermic reaction), and was observed as a downward peak in the scans. The shifts in the observed $\Delta T_m$-values were 0 and +20°C for samples with 2 and 10 mg/ml Captisol®, respectively. $\Delta T_m$ is defined as:

$$\Delta T_m = (T_{m2} - T_{m1})$$

where $T_{m1}$ is the temperature related to maximum of the denaturation peak in the DSC scan of the original solution without additional excipients added (i.e. the solution with 0 mg/ml Captisol®). $T_{m2}$ is the temperature related to the maximum of the denaturation peak in each of the DSC scans of the solutions containing either 2 mg/ml or 10 mg/ml Captisol®.

The DSC profile for the 80 mg/ml Captisol® sample was flat and without a downward peak, indicating an exothermic precipitation. Thus, it was not possible to calculate $\Delta T_m$ for this sample.

The observed changes in $T_m$ values are summarized in the table below:

<table>
<thead>
<tr>
<th>Captisol® conc. (mg/ml)</th>
<th>$\Delta T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>80</td>
<td>*</td>
</tr>
</tbody>
</table>

*) Not possible to determine

These results clearly demonstrate that addition of Captisol® stabilizes an aqueous solution of the Factor VIIa variant.
Activity of formulations containing the [L280Q]rhFVIIa variant upon freezing and thawing.

An excess of the solutions originally made for DSC analysis as described above was transferred into Eppendorf tubes and frozen at −20°C. The samples were later thawed and analyzed for activity in the “Clotting Assay” and the “Amidolytic Assay” described herein. Adding either 0 mg/ml, 2 mg/ml or 10 mg/ml Captisol® to the original solution resulted in rather different activities (see below). The activity of the formulation sample containing 0 mg/ml Captisol® was defined as 100% in both assays.

<table>
<thead>
<tr>
<th>Captisol® conc. (mg/ml)</th>
<th>Activity (Clotting Assay)</th>
<th>Activity (Amidolytic Assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U/mg)</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>18481</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>58797</td>
<td>318</td>
</tr>
<tr>
<td>10</td>
<td>37956</td>
<td>205</td>
</tr>
</tbody>
</table>

Captisol® was shown not to influence the clotting assay result in itself. This was shown by addition of either 2 or 10 mg/ml Captisol® to the formulation buffer (10 mM Gly-Gly buffer containing 50 mM sodium chloride, 10 mM calcium chloride and 0.01% Tween® 80 - adjusted to pH 5.5). The clotting time for these samples did not differ from the clotting time for the assay buffer (110, 119 and 114 seconds, respectively).

These results clearly demonstrate that addition of Captisol® stabilizes the Factor VIIa variant during freezing and thawing.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above may be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.
SEQ ID NO: 1

Human protein C (inactive one-chain zymogen)

5 Ala Asn Ser Phe Leu Glu Glu Leu Arg His Ser Ser Leu Glu Arg Glu
1  5 10 15

Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln
20  25 30

Asn Val Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp
35 40 45

Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys
50 55 60

Gly His Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys
65 70 75 80

Arg Ser Gly Trp Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu
85 90 95

Asn Cys Ser Leu Asp Asn Gly Gly Cys Thr His Tyr Cys Leu Glu Glu
100 105 110

Val Gly Trp Arg Arg Cys Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp
115 120 125

Asp Leu Leu Gln Cys His Pro Ala Val Lys Phe Pro Cys Gly Arg Pro
130 135 140

Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys Arg Asp Thr Glu
145 150 155 160

Asp Gln Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys Met Thr
165 170 175

Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys
180 185 190

Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr
195 200 205

Ala Ala His Cys Met Asp Glu Ser Lys Leu Leu Val Arg Leu Gly
210 215 220

Glu Tyr Asp Leu Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile
225 230 235 240

Lys Glu Val Phe Val His Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn
245 250 255

Asp Ile Ala Leu Leu His Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr
260 265 270

41
Ile Val Pro Ile Cys Leu Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu 275 280 285
Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp Gly Tyr His Ser 290 295 300
Ser Arg Glu Lys Ala Lys Arg Asn Arg Thr Phe Val Leu Asn Phe 305 310 315 320
Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser 325 330 335
Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg 340 345 350
Gln Asp Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe 355 360 365
His Gly Thr Trp Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys 370 375 380
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Trp Ala Pro

SEQ ID NO:2

Human factor VII

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Leu Gin Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80
Cys Glu Thr His Lys Asp Gin Leu Ile Cys Val Asn Glu Gin Gly 85 90 95
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42
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  Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160
  Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175
  Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190
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  His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270
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  Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335
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  Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380
  Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400
  Leu Arg Ala Pro Phe Pro 405
CLAIMS

1. A composition comprising a vitamin K-dependent polypeptide and a sulfoalkyl ether cyclodextrin derivative.

2. The composition of claim 1, wherein the vitamin K-dependent polypeptide is selected from the group consisting of human protein C, human factor VII, human protein Z, human protein S, human prothrombin, human factor X, human factor IX and human Gas6; and variants thereof comprising 1-15 amino acid modifications relative to the human amino acid sequence.

3. The composition of claim 2, wherein the vitamin K-dependent polypeptide is human factor VIIa (hFVIIa) or a variant thereof that differs from hFVIIa in 1-15 amino acid residues.

4. The composition of claim 2, wherein the vitamin K-dependent polypeptide is human activated protein C (hAPC) or a variant thereof that differs from hAPC in 1-15 amino acid residues.

5. The composition of any of the preceding claims which has a pH of from about 5 to about 8.

6. The composition of claim 4 which has a pH of above about 7.0.

7. The composition of claim 6 which has a pH of from about 7.1 to about 8.5.

8. The composition of claim 6 which has a pH of from about 7.2 to about 8.0.

9. The composition of claim 6, which has a pH of from about 7.2 to about 7.8.

10. The composition of any of the preceding claims, wherein the polypeptide comprises at least one covalently attached non-polypeptide moiety.

11. The composition of claim 10, wherein the polypeptide comprises at least one introduced glycosylation site.

12. The composition of any of the preceding claims, wherein the sulfoalkyl ether cyclodextrin derivative is a compound of formula (I):
wherein:

n is 4, 5 or 6,

R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈, and R₉ are each independently -O- or a -O-(C₂-C₆ alkyl)-
SO₃- group, wherein at least one of R₁ and R₂ is an -O-(C₂-C₆ alkyl)-SO₃- group, and

S₁, S₂, S₃, S₄, S₅, S₆, S₇, S₈, and S₉ are each independently a pharmaceutically acceptable cation.

13. The composition of claim 12, wherein the sulfoalkyl ether cyclodextrin is a beta cyclodextrin sulfobutyl ether.

14. The composition of claim 13, wherein the beta cyclodextrin sulfobutyl ether is in the form of an alkali metal or alkaline earth metal salt.

15. The composition of claim 13, wherein the beta cyclodextrin sulfobutyl ether is Captisol®.

16. The composition of any of the preceding claims, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from 1 mg/ml to 200 mg/ml.

17. The composition of claim 16, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from 50 mg/ml to 150 mg/ml.

18. The composition of any of the preceding claims, further comprising at least one buffering agent.

19. The composition of any of the preceding claims, further comprising at least one tonicity modifying agent.
20. The composition of any of the preceding claims, further comprising at least one surfactant.

21. The composition of any of the preceding claims, in the form of a liquid formulation.

22. The composition of claim 21, in the form of an aqueous solution or suspension.

23. A method for preparing a composition according to any of the preceding claims, comprising mixing a vitamin K-dependent polypeptide with a sulfoalkyl ether cyclodextrin derivative.

24. A method for increasing the stability of a vitamin K-dependent polypeptide in a pharmaceutical composition, the method comprising incorporating into said composition a sulfoalkyl ether cyclodextrin derivative.

25. The method of claim 24, wherein the vitamin K-dependent polypeptide is selected from the group consisting of human protein C, human factor VII, human protein Z, human protein S, human prothrombin, human factor X, human factor IX and human Gas6; and variants thereof comprising 1-15 amino acid modifications relative to the human amino acid sequence.

26. The method of claim 25, wherein the vitamin K-dependent polypeptide is human factor VIIa (hFVIIa) or a variant thereof that differs from hFVIIa in 1-15 amino acid residues.

27. The method of claim 25, wherein the vitamin K-dependent polypeptide is human activated protein C (hAPC) or a variant thereof that differs from hAPC in 1-15 amino acid residues.

28. The method of claim 27 wherein the composition comprises at least one buffering agent and the pH of the composition is adjusted to above about 7.0.

29. The method of claim 28, wherein the pH of the composition is from about 7.1 to about 8.5.

30. The method of claim 28, wherein the pH of the composition is from about 7.2 to about 8.0.
31. The method of claim 28, wherein the pH of the composition is from about 7.2 to about 7.8.

32. The method of claim 24, wherein the sulfoalkyl ether cyclodextrin derivative is a compound of formula (I):

\[
\begin{align*}
&\text{wherein:} \\
&n \text{ is } 4, 5 \text{ or } 6, \\
&R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, \text{ and } R_9 \text{ are each independently } -O- \text{ or a } -O-(C_2-C_6 \text{ alkyl})-SO_3- \text{ group, wherein at least one of } R_1 \text{ and } R_2 \text{ is an } -O-(C_2-C_6 \text{ alkyl})-SO_3- \text{ group, and} \\
&S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, \text{ and } S_9 \text{ are each independently a pharmaceutically acceptable cation.}
\end{align*}
\]

33. The method of claim 32, wherein the sulfoalkyl ether cyclodextrin is a beta cyclodextrin sulfobutyl ether.

34. The method of claim 33, wherein the beta cyclodextrin sulfobutyl ether is in the form of an alkali metal or alkaline earth metal salt.

35. The method of claim 33, wherein the beta cyclodextrin sulfobutyl ether is Captisol®.

36. The method of any of claims 23-35, wherein the sulfoalkyl ether cyclodextrin derivative is present in a concentration from about 1 mg/ml to about 200 mg/ml.

37. A method of treating a condition treatable by administration of factor VIIa, comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 3.
38. The method of claim 37, wherein the condition is a clotting factor deficiency or trauma.

39. A method of treating a condition treatable by administration of activated protein C, comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 4.

40. The method of claim 39, wherein the condition is sepsis or septic shock.

41. A composition according to any of claims 1-22 for use as a medicine.

42. Use of a composition according to claim 3 for the manufacture of a medicament for treatment of a condition treatable by administration of factor VIIa.

43. Use according to claim 42, wherein the condition is a clotting factor deficiency or trauma.

44. Use of a composition according to claim 4 for the manufacture of a medicament for treatment of a condition treatable by administration of activated protein C.

45. Use according to claim 44, wherein the condition is sepsis or septic shock.
Fig. 1

Fig. 2
Fig. 3

Fig. 4
SEQUENCE LISTING

Maxygen Holdings, Ltd.
Maxygen ApS

FORMULATIONS OF VITAMIN K-DEPENDENT POLYPEPTIDES

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2003-09-05
60/526,326
2003-12-01
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PatentIn version 3.2

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Homo sapiens

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

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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)

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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)

BIOSIS, EPO-Internal, WPI Data, PAJ, EMBASE, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of box C.  
X Patent family members are listed in annex.

* 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 priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

*O* document relating 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

*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

*S* document member of the same patent family

Date of the actual completion of the international search

29 November 2004

Date of mailing of the international search report

21/12/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaak 2 NL – 2208 HV Rijswijk

Tel: (+31-70) 340-2040, Tx: 31 651 epo nl, Fax: (+31-70) 340-3016

Authorized officer

Durrenberger, A
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<td>UEKAMA KANETO ET AL: &quot;Stabilizing and solubilizing effects of sulfobutyl ether beta-cyclodextrin on prostaglandin E1 analogue&quot; PHARMACEUTICAL RESEARCH (NEW YORK), vol. 18, no. 11, November 2001 (2001-11), pages 1578-1585, XP002308050 ISSN: 0724-8741 the whole document</td>
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1. [X] Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 37-40 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).

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<td>This International Searching Authority found multiple inventions in this international application, as follows:</td>
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1. [ ] 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 fee, this Authority did not invite payment of any additional fee.

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.:

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