(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 December 2013 (19.12.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/186138 A1

(51) International Patent Classification:
A61K 31/198 (2006.01) *A61K 38/28* (2006.01)
A61K 31/465 (2006.01) *A61K 47/18* (2006.01)
A61K 33/30 (2006.01) *A61P 3/10* (2006.01)

(21) International Application Number:
PCT/EP2013/061795

(22) International Filing Date:
7 June 2013 (07.06.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12172071.8 14 June 2012 (14.06.2012) EP
61/661,398 19 June 2012 (19.06.2012) US

(71) Applicant: **NOVO NORDISK A/S** [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(71) Applicants : **HEMMINGSEN, Pernille Kristine Høyrup** [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). **JENSEN, Steen** [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2013/186138 A1

(54) **Title**: PREPARATION COMPRISING INSULIN, NICOTINAMIDE AND ARGININE

(57) **Abstract**: Insulin preparations comprising an insulin compound or a mixture of two or more insulin compounds, a nicotinic compound and arginine.

PREPARATION COMPRISING INSULIN, NICOTINAMIDE AND ARGININE

FIELD OF THE INVENTION

The present invention relates to pharmaceutical preparations comprising an insulin compound, nicotinamide and arginine.

BACKGROUND OF THE INVENTION

Diabetes mellitus is a metabolic disorder in which the ability to utilize glucose is partly or completely lost. About 5% of all people suffer from diabetes and the disorder approaches epidemic proportions.

Since the introduction of insulin in the 1920's, continuous improvements have been made in the treatment of diabetes. To help avoid high glycaemia levels, diabetic patients often practice multiple injection therapy, whereby insulin is administered with each meal. As diabetic patients have been treated with insulin for several decades, there is a major need for safe and life-quality improving insulin preparations. Among the commercially available insulin preparations, rapid-acting, intermediate-acting and prolonged-acting preparations can be mentioned.

In the treatment of diabetes mellitus, many varieties of pharmaceutical preparations of insulin have been suggested and used, such as regular insulin (such as Actrapid®), isophane insulin (designated NPH), insulin zinc suspensions (such as Semilente®, Lente®, and Ultralente®), and biphasic isophane insulin (such as NovoMix®). Human insulin analogues and derivatives have also been developed, designed for particular profiles of action, i.e. fast action or prolonged action. Some of the commercially available insulin preparations comprising such rapid acting insulin analogues include NovoRapid® (preparation of B28Asp human insulin), Humalog® (preparation of B28LysB29Pro human insulin) and Apidra® (preparation of B3LysB29Glu human insulin).

Most often pharmaceutical preparations of insulins are administered by subcutaneous injection. Important for the patient is the action profile of the insulin, meaning the action of insulin on glucose metabolism as a function of time from injection. In this profile, *inter alia*, the time of the onset, the maximum value and the total duration of action are important. In the case of bolus insulins, a variety of insulin preparations with different action profiles are desired and requested by the patients. One patient may, on the same day, use insulin preparations with very different action profiles. The action profile desired for example, depends on the time of the day and the amount and composition of the meal eaten by the patient.

Equally important for the patient is the chemical stability of the insulin preparations, for example, due to the abundant use of pen-like injection devices such as devices which contain Penfill® cartridges, in which an insulin preparation is stored until the entire cartridge is empty which may be at least 1 to 2 weeks for devices containing 1.5-3.0ml cartridges. During storage, covalent chemical changes in the insulin structure occur. This may lead to formation of molecules which may be less active and/or potentially immunogenic such as deamidation products and higher molecular weight transformation products (dimers, polymers). Furthermore, also important is the physical stability of the insulin preparations, since long term storage may eventually lead to formation of insoluble fibrils, which are biologically inactive and potentially immunogenic.

SUMMARY OF THE INVENTION

The invention relates to insulin preparations with the most favourable balance between an ultra-fast absorption rate and favourable chemical and physical stability. The present invention relates to insulin preparations comprising human insulin and/or analogues thereof, nicotinamide or nicotinic acid and/or salts thereof, arginine and phosphate buffer.

In one embodiment, the present invention relates to an insulin preparation comprising:

- B28Asp human insulin present in the amount from 0.5mM to 0.7mM,
- nicotinamide or salts thereof, present in the amount from 160mM to 180mM,
- arginine present in the amount from 10mM to 30mM,
- zinc, and
- a phosphate buffer.

In another embodiment, the present invention also contemplates a method for the treatment of diabetes mellitus in a subject or for reducing the blood glucose level in a subject comprising administering to a subject or mammal an insulin preparation according to the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the glucose concentration in plasma (mean +/- SEM, N=8, each pig dosed twice) after subcutaneous injection in pigs of a 1 nmol/kg dose at 0 minutes of preparations according to this invention. The letter **A** refers to a NovoRapid® reference and number **11** corresponds to a insulin aspart preparation as described in Table 3 of Example 1. Compared

to the NovoRapid® preparation (preparation **A)**, the initial rate of plasma glucose lowering is faster for a preparation with a combination of nicotinamide and arginine (preparation **11)**.

Figure 2 shows the insulin aspart concentration in plasma (mean +/- SEM, N=8, each pig dosed twice) after subcutaneous injection in pigs of a 1 nmol/kg dose at 0 minutes of preparations according to this invention. The letter **A** refers to a NovoRapid® reference and number **11** corresponds to a insulin aspart preparation as described in Table 3 of Example 1. Compared to the NovoRapid® preparation (preparation **A)**, the initial absorption rate of the insulin component of the preparations with nicotinamide and arginine (preparation **11)** is markedly faster.

Figure 3 shows ThT fluorescence over time. The constant t_0 is the time needed to reach 50% of maximum fluorescence. Two parameters describing fibril formation are the lag-time calculated by $t_0 - 2\tau$ and the apparent rate constant $k_{app} = 1/\tau$.

DESCRIPTION OF THE INVENTION

The absorption after subcutaneous injection of the insulin compound in the insulin preparations of the present invention was surprisingly found to be faster than that of the reference insulin preparations. This property is useful for rapid-acting insulins, in particular in connection with a multiple injection regimen where insulin is given before each meal. With faster onset of action, the insulin can conveniently be taken closer to the meal than with conventional rapid acting insulin solutions. Furthermore, a faster disappearance of insulin probably diminishes the risk of post-meal hypoglycaemia.

The insulin preparations of the present invention are ultra-fast acting insulin preparations comprising an insulin compound such as insulin aspart, a nicotinic compound, such as nicotinamide and the amino acid arginine. Optionally, the insulin preparations of the present invention may comprise further amino acids. These insulin preparations have a rapid absorption profile that mimics normal physiology more closely than existing therapies. Furthermore, the insulin preparations of the present invention have chemical and physical stability suitable for commercial pharmaceutical preparations.

The insulin preparations of the present invention provide ultra-fast-acting insulin preparations which are not only physically stable, but surprisingly also chemically stable. The insulin preparations of the present invention provide the most favourable balance between an ultra-fast absorption rate and favourable chemical and physical stability. The insulin prepara-

tions of the present invention provide an even faster onset of action compared with existing insulin therapies. Such ultra-fast insulin preparations have the advantage of restoring first phase insulin release, injection convenience and shutting down hepatic glucose production. The insulin preparations of the present invention have a favourable absorption rate from subcutis into plasma with an increase in initial absorption rate ranging from 1.5 to 5 times, when compared to conventional preparations such as NovoRapid®, as suggested by several PK/PD experiments in pigs. This faster absorption rate may improve glycaemic control and convenience and may allow for a shift from pre-meal to post-meal dosing. The present invention is based in part, on the surprising discovery that although, the addition of nicotinamide allows the increase in absorption rate, it also has a negative effect on chemical stability by significantly increasing the amount of HMWP. The insulin preparations of the present invention have an improved chemical stability by addition of arginine, which is reflected in e.g. a reduction in the formation of dimers and polymers and desamido insulins after storage. The insulin preparations of the present invention may furthermore also have improved physical stability. Surprisingly, the insulin preparations of the present invention are also ready to use preparations, advantageous for use in pumps.

The present invention provides an insulin preparation comprising an insulin compound according to the present invention which is present in a concentration from about 0.1 mM to about 10.0mM, and wherein said preparation has a pH from 3 to 8.5. The preparation also comprises a nicotinic compound and arginine. The preparation may further comprise protease inhibitor(s), metal ions, a buffer system, preservative(s), tonicity agent(s), chelating agent(s), stabilizers and surfactants.

In one embodiment the metal ion is zinc, wherein zinc is added as zinc acetate or zinc chloride.

In one embodiment the insulin preparations comprise a human insulin, an analogue or combinations thereof, nicotinamide and/or nicotinic acid and/or salts thereof and arginine and/or salts thereof.

In one embodiment, the insulin preparations according to the present invention comprise an aqueous solution of B28Asp human insulin, nicotinamide and arginine.

The content of B28Asp human insulin in the solutions of this invention may be in the range of 15 to 500 international units (IU)/ml, preferably in the range of 50 to 333 IU/ml, in preparations for injection. However, for other purposes of parenteral administration, the content of insulin compound may be higher.

In the present context the unit "U" corresponds to 6 nmol.

The term “insulin aspart” refers to the human insulin analogue B28Asp human insulin.

The term “onset” refers to the time from injection until the PK curve shifts to an increase.

The term “absorption rate” refers to the slope of the PK curve.

An “insulin compound” according to the invention is herein to be understood as human insulin, an insulin analogue and/or any combination thereof.

The term “human insulin” as used herein means the human hormone whose structure and properties are well-known. Human insulin has two polypeptide chains that are connected by disulphide bridges between cysteine residues, namely the A-chain and the B-chain. The A-chain is a 21 amino acid peptide and the B-chain is a 30 amino acid peptide, the two chains being connected by three disulphide bridges: one between the cysteines in position 6 and 11 of the A-chain, the second between the cysteine in position 7 of the A-chain and the cysteine in position 7 of the B-chain, and the third between the cysteine in position 20 of the A-chain and the cysteine in position 19 of the B-chain.

By "insulin analogue" as used herein is meant a polypeptide derived from the primary structure of a naturally occurring insulin, for example that of human insulin, by mutation. One or more mutations are made by deleting and/or substituting at least one amino acid residue occurring in the naturally occurring insulin and/or by adding at least one amino acid residue. The added and/or substituted amino acid residues can either be codable amino acid residues or other naturally occurring amino acid residues.

In one embodiment an insulin analogue comprises less than 8 modifications (substitutions, deletions, additions and any combination thereof) relative to the parent insulin, alternatively less than 7 modifications relative to the parent insulin, alternatively less than 6 modifications relative to the parent insulin, alternatively less than 5 modifications relative to the parent insulin, alternatively less than 4 modifications relative to the parent insulin, alternatively less than 3 modifications relative to the parent insulin, alternatively less than 2 modifications relative to the parent insulin.

Mutations in the insulin molecule are denoted stating the chain (A or B), the position, and the three letter code for the amino acid substituting the native amino acid. By “desB30” or “B(1-29)” is meant a natural insulin B chain or analogue thereof lacking the B30 amino acid residue, and by B28Asp human insulin is meant human insulin wherein the amino acid residue in position 28 of the B chain has been substituted with Asp.

Examples of insulin analogues are such wherein Pro in position 28 of the B chain is mutated with Asp, Lys, Leu, Val, Ala and/or Lys at position B29 is mutated with Pro, Glu or

Asp. Furthermore, Asn at position B3 may be mutated with Thr, Lys, Gln, Glu or Asp. The amino acid residue in position A21 may be mutated with Gly. The amino acid in position B1 may be mutated with Glu. The amino acid in position B16 may be mutated with Glu or His. Further examples of insulin analogues are the deletion analogues e.g. analogues where the B30 amino acid in human insulin has been deleted (des(B30) human insulin), insulin analogues wherein the B1 amino acid in human insulin has been deleted (des(B1) human insulin), des(B28-B30) human insulin and des(B27) human insulin. Insulin analogues wherein the A-chain and/or the B-chain have an N-terminal extension and insulin analogues wherein the A-chain and/or the B-chain have a C-terminal extension such as with two arginine residues added to the C-terminal of the B-chain are also examples of insulin analogues. Further examples are insulin analogues comprising combinations of the mentioned mutations. Insulin analogues wherein the amino acid in position A14 is Asn, Gln, Glu, Arg, Asp, Gly or His, the amino acid in position B25 is His and which optionally further comprises one or more additional mutations are further examples of insulin analogues. Insulin analogues of human insulin wherein the amino acid residue in position A21 is Gly and wherein the insulin analogue is further extended in the C-terminal with two arginine residues are also examples of insulin analogues.

Further examples of insulin analogues include, but are not limited to: DesB30 human insulin; AspB28 human insulin; AspB28,desB30 human insulin; LysB3,GluB29 human insulin; LysB28,ProB29 human insulin; GlyA21,ArgB31,ArgB32 human insulin; GluA14,HisB25 human insulin; HisA14,HisB25 human insulin; GluA14,HisB25,desB30 human insulin; HisA14, HisB25,desB30 human insulin; GluA14,HisB25,desB27,desB28,desB29,desB30 human insulin; GluA14,HisB25,GluB27,desB30 human insulin; GluA14,HisB16,HisB25,desB30 human insulin; HisA14,HisB16,HisB25,desB30 human insulin;
HisA8,GluA14,HisB25,GluB27,desB30 human insulin;
HisA8,GluA14,GluB1,GluB16,HisB25,GluB27,desB30 human insulin; and
HisA8,GluA14,GluB16,HisB25,desB30 human insulin.

The term “nicotinic compound” includes nicotinamide, nicotinic acid, niacin, niacin amide and vitamin B3 and/or salts thereof and/or any combination thereof.

The term “arginine” or “Arg” includes the amino acid arginine and/or a salt thereof, e.g. arginine hydrochloride or arginine glutamate.

The term “pharmaceutical preparation” or “insulin preparation” or “formulation” or “insulin formulation” as used herein, means a product comprising an insulin compound, i.e., a human insulin, an analogue thereof and/or combinations thereof, a nicotinic compound and an amino acid, optionally together with other excipients such as preservatives, chelating

agents, tonicity modifiers, bulking agents, stabilizers, antioxidants, polymers, surfactants, metal ions, oleaginous vehicles and proteins (e.g., human serum albumin or gelatine) said insulin preparation being useful for treating, preventing or reducing the severity of a disease or disorder by administration of said insulin preparation to a person. Thus, an insulin preparation is also known in the art as a pharmaceutical preparation or pharmaceutical composition.

The buffer may be selected from the group consisting of, but not limited to, sodium acetate, sodium carbonate, citrate, sodium dihydrogen phosphate, disodium hydrogen phosphate, disodium-phosphate dihydrate, sodium phosphate, and tris(hydroxymethyl)-aminomethan (tris), bicine, tricine, malic acid, succinate, maleic acid, fumaric acid, tartaric acid, aspartic acid, ethylendiamine or mixtures thereof. Each one of these specific buffers constitutes an alternative embodiment of the invention. The buffer used in the present invention is not arginine.

The insulin preparation of the present invention may further comprise other ingredients common to insulin preparations, for example zinc complexing agents such as citrate, and phosphate buffers.

Glycerol and/or mannitol and/or sodium chloride may be present in an amount corresponding to a concentration of 0 to 250mM, 0 to 200mM or 0 to 100mM.

Stabilizers, surfactants and preservatives may also be present in the insulin preparations of this invention.

The insulin preparations of the present invention may further comprise a pharmaceutically acceptable preservative. The preservative may be present in an amount sufficient to obtain a preserving effect. The amount of preservative in an insulin preparation may be determined from e.g. literature in the field and/or the known amount(s) of preservative in e.g. commercial products. Each one of these specific preservatives constitutes an alternative embodiment of the invention. The use of a preservative in pharmaceutical preparations is described, for example in Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

The preservative present in the insulin preparation of this invention may be as in the heretofore conventional insulin preparations, for example phenol, m-cresol, methylparaben and various combinations thereof.

The insulin preparation of the present invention may further comprise a chelating agent, selected from for example, but not limited to, EDTA. The use of a chelating agent in pharmaceutical preparations is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

The insulin preparation of the present invention may further comprise a stabilizer. The term "stabilizer" as used herein refers to chemicals added to polypeptide containing pharmaceutical preparations in order to stabilize the peptide, i.e. to increase the shelf life and/or in-use time of such preparations. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

The insulin preparation of the present invention may further comprise a surfactant. The term "surfactant" as used herein refers to any molecules or ions that are comprised of a water-soluble (hydrophilic) part and a fat-soluble (lipophilic) part. Surfactants accumulate preferably at interfaces, which the hydrophilic part is orientated towards the water (hydrophilic phase) and the lipophilic part towards the oil- or hydrophobic phase (*i.e.* glass, air, oil etc.). The concentration at which surfactants begin to form micelles is known as the critical micelle concentration ("CMC"). Furthermore, surfactants lower the surface tension of a liquid. Surfactants are also known as amphipathic compounds. The term "detergent" is a synonym used for surfactants in general. The use of a surfactant in pharmaceutical preparations is well-known to the skilled person. For convenience reference is made to Remington: *The Science and Practice of Pharmacy*, 19th edition, 1995.

In a further embodiment the invention relates to an insulin preparation comprising an aqueous solution of an insulin compound of the present invention, and a buffer, wherein said insulin compound is present in a concentration from 0.1mM or above, and wherein said preparation has a pH from about 3.0 to about 8.5 at room temperature (~25°C).

In one embodiment, the method for making insulin preparations of the invention comprises:

a) preparing a solution of preservative agents, tonicity agent, Arginine or Arginine salt, nicotinic compound, sodium hydroxide and buffer into water

b) preparing a solution by dissolving the insulin compound in water; adding metal ion salt and HCl to acidify this solution;

c) adding solution of b) to solution of a) and stirring;

d) adjusting pH of c) with NaOH/HCl to desired pH.

In one embodiment, the method for making insulin preparations of the invention comprises:

a) preparing a solution of preservative agents, tonicity agent, Arginine or Arginine salt, nicotinic compound, and buffer into water

b) preparing a solution by dissolving the insulin compound in water; adding metal ion salt and HCl to acidify this solution;

c) adding solution of b) to solution of a) and stirring;

d) adjusting pH of c) with NaOH/HCl to desired pH.

The insulin preparations of the present invention can be used in the treatment of diabetes by parenteral administration. It is recommended that the dosage of the insulin preparations of this invention which is to be administered to the patient be selected by a physician.

Parenteral administration may be performed by subcutaneous, intramuscular, intraperitoneal or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. As a further option, the insulin preparations containing the insulin compound of the invention can also be adapted to transdermal administration, *e.g.* by needle-free injection or from a patch, optionally an iontophoretic patch, or transmucosal, *e.g.* buccal administration.

Insulin preparations according to the present invention may be administered to a patient in need of such treatment at several sites, for example, at topical sites, for example, skin and mucosal sites, at sites which bypass absorption, for example, administration in an artery, in a vein, in the heart, and at sites which involve absorption, for example, administration in the skin, under the skin, in a muscle or in the abdomen.

In one embodiment of the invention the insulin preparation is an aqueous preparation, i.e. preparation comprising water. Such preparation is typically a solution or a suspension. In a further embodiment of the invention the insulin preparation is an aqueous solution.

The term “aqueous preparation” is defined as a preparation comprising at least 50 %w/w water. Likewise, the term “aqueous solution” is defined as a solution comprising at least 50 %w/w water, and the term “aqueous suspension” is defined as a suspension comprising at least 50 %w/w water.

Aqueous suspensions may contain the active compounds in admixture with excipients suitable for the manufacture of aqueous suspensions.

In one embodiment, the insulin preparations of this invention are well-suited for application in pen-like devices used for insulin therapy by injection.

In one embodiment the insulin preparations of the present invention can be used in pumps for continuous subcutaneous insulin administration.

The term “physical stability” of the insulin preparation as used herein refers to the tendency of the protein to form biologically inactive and/or insoluble aggregates of the protein as a result of exposure of the protein to thermo-mechanical stresses and/or interaction with

interfaces and surfaces that are destabilizing, such as hydrophobic surfaces and interfaces. Physical stability of the aqueous protein preparations is evaluated by means of visual inspection and/or turbidity measurements after exposing the preparation filled in suitable containers (e.g. cartridges or vials) to mechanical/physical stress (e.g. agitation) at different temperatures for various time periods. Visual inspection of the preparations is performed in a sharp focused light with a dark background. The turbidity of the preparation is characterized by a visual score ranking the degree of turbidity for instance on a scale from 0 to 3 (a preparation showing no turbidity corresponds to a visual score 0, and a preparation showing visual turbidity in daylight corresponds to visual score 3). A preparation is classified physically unstable with respect to protein aggregation, when it shows visual turbidity in daylight. Alternatively, the turbidity of the preparation can be evaluated by simple turbidity measurements well-known to the skilled person. Physical stability of the aqueous protein preparations can also be evaluated by using a spectroscopic agent or probe of the conformational status of the protein. The probe is preferably a small molecule that preferentially binds to a non-native conformer of the protein. One example of a small molecular spectroscopic probe of protein structure is Thioflavin T. Thioflavin T is a fluorescent dye that has been widely used for the detection of amyloid fibrils. In the presence of fibrils, and perhaps other protein configurations as well, Thioflavin T gives rise to a new excitation maximum at about 450 nm and enhanced emission at about 482 nm when bound to a fibril protein form. Unbound Thioflavin T is essentially non-fluorescent at the wavelengths.

The term “chemical stability” of the protein preparation as used herein refers to changes in the covalent protein structure leading to formation of chemical degradation products with potential less biological potency and/or potential increased immunogenic properties compared to the native protein structure. Various chemical degradation products can be formed depending on the type and nature of the native protein and the environment to which the protein is exposed. Increasing amounts of chemical degradation products are often seen during storage and use of the protein preparation. Most proteins are prone to deamidation, a process in which the side chain amide group in glutaminyl or asparaginyl residues is hydrolysed to form a free carboxylic acid or asparaginyl residues to form an IsoAsp derivative. Other degradations pathways involves formation of high molecular weight products where two or more protein molecules are covalently bound to each other through transamidation and/or disulfide interactions leading to formation of covalently bound dimer, oligomer and polymer degradation products (*Stability of Protein Pharmaceuticals, Ahern. T.J. & Manning M.C., Plenum Press, New York 1992*). Oxidation (of for instance methionine residues) can be mentioned as another variant of chemical degradation. The chemical stability of the protein

preparation can be evaluated by measuring the amount of the chemical degradation products at various time-points after exposure to different environmental conditions (the formation of degradation products can often be accelerated by for instance increasing temperature). The amount of each individual degradation product is often determined by separation of the degradation products depending on molecule size and/or charge using various chromatography techniques (e.g. SEC-HPLC and/or RP-HPLC). Since high molecular weight protein (HMWP) products are potentially immunogenic and not biologically active, low levels of HMWP are advantageous.

The term “stabilized preparation” or “favorable preparation stability” refers to a preparation with favorable physical stability, favorable chemical stability or balance between favorable physical and chemical stability, suitable for pharmaceutical formulations. In general, a preparation must be stable during use and storage (in compliance with recommended use and storage conditions) until the expiration date is reached.

The term “diabetes” or “diabetes mellitus” includes type 1 diabetes, type 2 diabetes, gestational diabetes (during pregnancy) and other states that cause hyperglycaemia. The term is used for a metabolic disorder in which the pancreas produces insufficient amounts of insulin, or in which the cells of the body fail to respond appropriately to insulin thus preventing cells from absorbing glucose. As a result, glucose builds up in the blood.

Type 1 diabetes, also called insulin-dependent diabetes mellitus (IDDM) and juvenile-onset diabetes, is caused by B-cell destruction, usually leading to absolute insulin deficiency.

Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM) and adult-onset diabetes, is associated with predominant insulin resistance and thus relative insulin deficiency and/or a predominantly insulin secretory defect with insulin resistance.

The term “pharmaceutically acceptable” as used herein means suited for normal pharmaceutical applications, i.e., not giving rise to any serious adverse events in patients.

The term “treatment of a disease” as used herein means the management and care of a patient having developed the disease, condition or disorder and includes treatment, prevention or alleviation of the disease. The purpose of treatment is to combat the disease, condition or disorder. Treatment includes the administration of the active compounds to eliminate or control the disease, condition or disorder as well as to alleviate the symptoms or complications associated with the disease, condition or disorder, and prevention of the disease, condition or disorder.

In its broadest sense, the term a “critically ill patient”, as used herein refers to a patient who has sustained or are at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury, a patient who is being operated on and where

complications supervene, and a patient who has been operated in a vital organ within the last week or has been subject to major surgery within the last week. In a more restricted sense, the term a “critically ill patient”, as used herein refers to a patient who has sustained or is at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury, or a patient who is being operated and where complications supervene. In an even more restricted sense, the term a “critically ill patient”, as used herein refers to a patient who has sustained or are at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury. Similarly, these definitions apply to similar expressions such as “critical illness in a patient” and a “patient is critically ill”. Examples of a critically ill patient is a patient in need of cardiac surgery, cerebral surgery, thoracic surgery, abdominal surgery, vascular surgery or transplantation, or a patient suffering from neurological diseases, cerebral trauma, respiratory insufficiency, abdominal peritonitis, multiple trauma or severe burns, or critical illness polyneuropathy.

The term “anabolism” as used herein, means the set of metabolic pathways that construct molecules from smaller units. These reactions require energy. One way of categorizing metabolic processes, whether at the cellular, organ or organism level is as 'anabolic' or as 'catabolic', which is the opposite of anabolic. Anabolism is powered by catabolism, where large molecules are broken down into smaller parts and then used up in respiration. Many anabolic processes are powered by adenosine triphosphate (ATP). Anabolic processes tend toward "building up" organs and tissues. These processes produce growth and differentiation of cells and increase in body size, a process that involves synthesis of complex molecules. Examples of anabolic processes include the growth and mineralization of bone and increases in muscle mass. Endocrinologists have traditionally classified hormones as anabolic or catabolic, depending on which part of metabolism they stimulate. The balance between anabolism and catabolism is also regulated by circadian rhythms with processes such as glucose metabolism fluctuating to match an animal's normal periods of activity throughout the day. Some examples of the “anabolic effects” of these hormones are increased protein synthesis from amino acids, increased appetite, increased bone remodeling and growth, and stimulation of bone marrow, which increases the production of red blood cells. Through a number of mechanisms anabolic hormones stimulate the formation of muscle cells and hence cause an increase in the size of skeletal muscles, leading to increased strength.

In another embodiment, an insulin analogue according to the invention is used as a medicament for delaying or preventing disease progression in type 2 diabetes.

In one embodiment of the present invention, the insulin preparation according to the invention is for use as a medicament for the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders is provided.

In a further embodiment of the present invention, a method for the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, coronary heart disease and other cardiovascular disorders, stroke, the method comprising administering to a patient in need of such treatment an effective amount for such treatment of an insulin preparation according to the invention, is provided.

The treatment with an insulin preparation according to the present invention may also be combined with a second or more pharmacologically active substances, e.g. selected from antidiabetic agents, antiobesity agents, appetite regulating agents, antihypertensive agents, agents for the treatment and/or prevention of complications resulting from or associated with diabetes and agents for the treatment and/or prevention of complications and disorders resulting from or associated with obesity.

The treatment with an insulin preparation according to the present invention may also be combined with bariatric surgery - a surgery that influences the glucose levels and/or lipid homeostasis, such as gastric banding or gastric bypass.

The production of polypeptides, e.g., insulins, is well known in the art. An insulin analogue according to the invention may for instance be produced by classical peptide synthesis, e.g. solid phase peptide synthesis using t-Boc or Fmoc chemistry or other well established techniques, see e.g. Greene and Wuts, “Protective Groups in Organic Synthesis”, John Wiley & Sons, 1999. The insulin analogue may also be produced by a method which comprises culturing a host cell containing a DNA sequence encoding the analogue and capable of expressing the insulin analogue in a suitable nutrient medium under conditions permitting the expression of the insulin analogue. For insulin analogues comprising non-natural amino acid residues, the recombinant cell should be modified such that the non-natural amino acids are incorporated into the analogue, for instance by use of tRNA mutants. Hence, briefly, the insulin analogues according to the invention are prepared analogously to the preparation of known insulin analogues.

Several methods may be used for the production of human insulin and human insulin analogues. For example, three major methods which are used in the production of insulin in microorganisms are disclosed in WO2008034881. Two of these involve *Escherichia coli*, with either the expression of a large fusion protein in the cytoplasm (Frank et al. (1981) in Peptides: Proceedings of the 7th American Peptide Chemistry Symposium (Rich & Gross, eds.), Pierce Chemical Co., Rockford, Ill. pp 729-739), or use of a signal peptide to enable secretion into the periplasmic space (Chan et al. (1981) PNAS 78:5401-5404). A third method utilizes *Saccharomyces cerevisiae* to secrete an insulin precursor into the medium (Thim et al. (1986) PNAS 83:6766-6770). The prior art discloses a number of insulin precursors which are expressed in either *E. coli* or *S. cerevisiae*, vide U.S. 5,962,267, WO 95/16708, EP 0055945, EP 0163529, EP 0347845 and EP 0741188.

The insulin analogues are produced by expressing a DNA sequence encoding the insulin analogue in question in a suitable host cell by well-known technique as disclosed in e.g. US 6,500,645. The insulin analogue is either expressed directly or as a precursor molecule which has an N-terminal extension on the B-chain or a C-terminal extension on the B-chain. The N-terminal extension may have the function of increasing the yield of the directly expressed product and may be of up to 15 amino acid residues long. The N-terminal extension is to be cleaved off in vitro after isolation from the culture broth and will therefore have a cleavage site next to B1. N-terminal extensions of the type suitable in the present invention are disclosed in US 5,395,922, and EP 765,395. The C-terminal extension may have the function of protecting the mature insulin or insulin analogue molecule against intracellular proteolytic processing by host cell exoproteases. The C-terminal extension is to be cleaved of either extra-cellularly in the culture broth by secreted, active carboxypeptidase or in vitro after isolation from the culture broth. A method for producing mature insulin and insulin analogs with C-terminal extensions on the B-chain that are removed by carboxypetidase are disclosed in WO 08/037735. The target insulin product of the process may either be a two-chain human insulin or a two-chain human insulin analogue which may or may not have a short C-terminal extension of the B-chain. If the target insulin product will have no C-terminal extension of the B-chain, then said C-terminal extension should be capable of subsequently being cleaved off from the B-chain before further purification steps.

The present invention also contemplates the following non-limiting list of embodiments, which are further described elsewhere herein:

1. An insulin preparation comprising:
   - B28Asp human insulin present in an amount from 0.5mM to 0.7mM,
   - nicotinamide or a salt thereof, present in an amount from 160mM to 180mM,
   - arginine present in an amount from 10mM to 30mM,
   - zinc, wherein the zinc:insulin molar ratio is from about 2.0:6 to about 3.5:6, and
   - a phosphate buffer present in an amount from 1mM to 10mM.
2. The insulin preparation according to any of the preceding embodiments, wherein the insulin compound is present in the amount of about 0.5mM.
3. The insulin preparation according to any of the preceding embodiments, wherein the insulin compound is present in the amount of about 0.6mM.
4. The insulin preparation according to any of the preceding embodiments, wherein the insulin compound is present in the amount of about 0.7mM.
5. The insulin preparation according to any of the preceding embodiments, comprising from about 165-175mM of nicotinamide.
6. The insulin preparation according to any of the preceding embodiments, comprising about 160mM of the nicotinamide.
7. The insulin preparation according to any of the preceding embodiments, comprising about 165mM of the nicotinamide.
8. The insulin preparation according to any of the preceding embodiments, comprising about 170mM of the nicotinamide.
9. The insulin preparation according to any of the preceding embodiments, comprising about 175mM of the nicotinamide.
10. The insulin preparation according to any of the preceding embodiments, comprising about 180mM of the nicotinamide.
11. The insulin preparation according to any of the preceding embodiments, wherein arginine is present in an amount selected from the following: 10mM, 15mM, 20mM, 25mM or 30mM.
12. The insulin preparation according to any of the preceding embodiments, comprising 15-25mM of arginine.
13. The insulin preparation according to any of the preceding embodiments, comprising 10mM of arginine.
14. The insulin preparation according to any of the preceding embodiments, comprising 15mM of arginine.
15. The insulin preparation according to any of the preceding embodiments, comprising 16mM of arginine.

16. The insulin preparation according to any of the preceding embodiments, comprising 17mM of arginine.
17. The insulin preparation according to any of the preceding embodiments, comprising 18mM of arginine.
18. The insulin preparation according to any of the preceding embodiments, comprising 19mM of arginine.
19. The insulin preparation according to any of the preceding embodiments, comprising 20mM of arginine.
20. The insulin preparation according to any of the preceding embodiments, comprising 21mM of arginine.
21. The insulin preparation according to any of the preceding embodiments, comprising 22mM of arginine.
22. The insulin preparation according to any of the preceding embodiments, comprising 23mM of arginine.
23. The insulin preparation according to any of the preceding embodiments, comprising 24mM of arginine.
24. The insulin preparation according to any of the preceding embodiments, comprising 25mM of arginine.
25. The insulin preparation according to any of the preceding embodiments, comprising 30mM of arginine.
26. The insulin preparation according to any one of the preceding embodiments, comprising from 1mM to 4mM of phosphate buffer.
27. The insulin preparation according to embodiment 26, comprising from about 2.5mM to 3.5mM of phosphate buffer.
28. The insulin preparation according to embodiment 26, comprising about 1mM of phosphate buffer.
29. The insulin preparation according to embodiment 26, comprising about 2mM of phosphate buffer.
30. The insulin preparation according to embodiment 26, comprising about 3mM of phosphate buffer.
31. The insulin preparation according to embodiment 26, comprising about 4mM of phosphate buffer.
32. The insulin preparation according to embodiment 26, comprising about 5mM of phosphate buffer.
33. The insulin preparation according to any of embodiments 1-32, wherein the phosphate

buffer is disodium-phosphate dihydrate.

34. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is from about 2:6 to about 3:6.
35. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is from about 2.5:6 to about 3:6.
36. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is from about 2.5:6 to about 3.5:6.
37. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is 2.5:6 or 3:6.
38. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2:6.
39. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.1:6.
40. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.2:6.
41. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.3:6.
42. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.4:6.
43. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.5:6.
44. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.6:6.
45. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.7:6.
46. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.8:6.
47. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 2.9:6.
48. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3:6.
49. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3.1:6.
50. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3.2:6.

51. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3.3:6.
52. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3.4:6.
53. The insulin preparation according to any one of the preceding embodiments, wherein the zinc:insulin molar ratio is about 3.5:6.
54. The insulin preparation according to any one of the preceding embodiments, which further comprises one or more preservative agent(s).
55. The insulin preparation according to embodiment 54, wherein the preservative is a phenolic compound, such as phenol.
56. The insulin preparation according to embodiment 54, wherein said phenolic compound is present in an amount of from about 5 to about 100mM.
57. The insulin preparation according to embodiment 54, wherein said phenolic compound is present in an amount of from about 5 to about 50mM.
58. The insulin preparation according to embodiment 54, wherein said phenolic compound is present in an amount of from about 5 to about 30mM.
59. The insulin preparation according to embodiment 54, wherein said phenolic compound is present in the amount of about 16mM.
60. The insulin preparation according to embodiment 54, wherein said preservative is m-cresol.
61. The insulin preparation according to embodiment 60, wherein m-cresol is present in an amount of from about 5 to about 100mM.
62. The insulin preparation according to embodiment 60, wherein m-cresol is present in an amount of from about 5 to about 50mM.
63. The insulin preparation according to embodiment 60, wherein m-cresol is present in an amount of from about 5 to about 30mM.
64. The insulin preparation according to embodiment 60, wherein m-cresol is present in an amount of about 16mM.
65. The insulin preparation according to any one of the preceding embodiments, which further comprises a stabilizer(s).
66. The insulin preparation according to embodiment 65, wherein the stabilizer is a non-ionic detergent.
67. The insulin preparation according to embodiment 66, wherein the non-ionic detergent is polysorbate 20 (Tween 20) or polysorbate 80 (Tween 80).
68. The insulin preparation according to any one of the preceding embodiments, further

comprising glycerol.

69. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 0.5 to about 2.5%.
70. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 0.7 to about 2.0%.
71. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 1.0 to about 1.5%.
72. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount of about 1.25%.
73. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 5 to about 100mM.
74. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 5 to about 85mM.
75. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount from about 25 to about 45mM.
76. The insulin preparation according to any one of the preceding embodiments, wherein the glycerol is in an amount of about 35.8mM.
77. The insulin preparation according to any one of the preceding embodiments, wherein the pH is neutral to weakly basic.
78. The insulin preparation according to any one of the preceding embodiments, wherein the pH is from about 6.8 to about 7.4.
79. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 6.8.
80. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 6.9.
81. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 7.0.
82. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 7.1.
83. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 7.2.
84. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 7.3.
85. The insulin preparation according to any one of the preceding embodiments, wherein the pH is about 7.4.

86. A method of reducing the blood glucose level in mammals by administering to a patient in need of such treatment a therapeutically active dose of an insulin preparation according to any one of the preceding embodiments.
87. A method for the treatment of diabetes mellitus in a subject comprising administering to a subject an insulin preparation according to any one of the preceding embodiments.
88. A method according to any of embodiments 86-87, for parenteral administration.
89. An insulin preparation according to any one of the preceding embodiments, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders and treatment of critically ill diabetic and non-diabetic patients.
90. The insulin preparation according to embodiment 89, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns and operation wounds, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders.
91. The insulin preparation according to embodiments 89-90, for use in the treatment of hyperglycemia type 2 diabetes and type 1 diabetes.

Further embodiments of the invention relate to the following:

92. An insulin preparation consisting essentially of:
    - B28Asp human insulin present in the amount from 0.5mM to 0.7mM,
    - nicotinamide or a salt thereof, present in the amount from 160mM to 180mM,
    - arginine present in the amount from 10mM to 30mM,
    - disodium dihydrate phosphate present in the amount from 2mM to 4mM,

    wherein the preparation has a pH of about 7.1.
93. A method of reducing the blood glucose level in mammals by administering to a mammal in need of such treatment a therapeutically active dose of an insulin preparation according to embodiment 92.
94. A method for the treatment of diabetes mellitus in a subject comprising administering to a subject an insulin preparation according to embodiment 92.
95. An insulin preparation according to any one of the preceding embodiments, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and

other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders and treatment of critically ill diabetic and non-diabetic patients.

96. An insulin preparation according to embodiment 95, for use in the treatment of hyperglycemia type 2 diabetes and type 1 diabetes.

97. An insulin preparation consisting of:

a. B28Asp human insulin present in a concentration of 0.6 mM,
b. nicotinamide present in a concentration of about 170mM,
c. zinc, wherein about 2.5 or 3 zinc ions are present per six B28Asp human insulin molecules,
d. arginine present in a concentration of about 20mM,
e. a phosphate buffer present in a concentration of about 3mM,

wherein the preparation has a pH of about 7.1.

98. A method of reducing the blood glucose level in mammals by administering to a mammal in need of such treatment a therapeutically active dose of an insulin preparation according to embodiment 97.

99. A method for the treatment of diabetes mellitus in a subject comprising administering to a subject an insulin preparation according to any one of the preceding embodiments.

100. An insulin preparation according to any one of the preceding embodiments, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders and treatment of critically ill diabetic and non-diabetic patients.

101. An insulin preparation according to embodiment 100, for use in the treatment of hyperglycemia type 2 diabetes and type 1 diabetes.

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference in their entirety and to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein (to the maximum extent permitted by law).

All headings and sub-headings are used herein for convenience only and should not be construed as limiting the invention in any way.

The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

EXAMPLES

Example 1

Preparation of pharmaceutical preparations

The pharmaceutical preparations of the present invention may be formulated as an aqueous solution. The aqueous medium is made isotonic, for example, with sodium chloride or glycerol. Furthermore, the aqueous medium may contain zinc ions, for example added as zinc acetate or zinc chloride, buffers and preservatives. Arginine may be added as Arg, HCl. The pH value of the preparation is adjusted to the desired value and may be between about 3 to about 8.5, between about 3 and about 5 or about 6.5 and about 7.5 depending on the isoelectric point, pI, of the insulin in question.

Table 1. Composition of insulin preparations according to this invention

	Insulin aspart (mM)	Zn (mM)	Phenol (mM)	m-cresol (mM)	NaCl (mM)	Phosphate (mM)	Tris (mM)	Glycerol (%w/v)	Arginine, HCl (mM)	Nicotinamide (mM)	pH
A*	0.6	0.3	16	16	10	7		1.6			7.4
10	0.6	0.35	16	16			7	1.3		80	7.4
11	0.6	0.35	16	16			7	1.3	10	80	7.4
12	0.6	0.35	16	16			7	1.3	30	80	7.4
13	0.6	0.35	16	16			7	1.3	50	80	7.4

* Commercially available NovoRapid®

Table 2. Composition of further insulin preparations according to this invention

Prepara-tion nr.	[Insulin aspart] mM	[Zn2+] mM	[phenol] mM	[m-cresol] mM	[Arg] mM	[Glycerol] %w/vol	[Phos-phate] mg/mL	[Nicotin amide] mM	pH
14	0.6	0.30	16	16	20	1.08	3	80	7.1
15	0.6	0.30	16	16	20	-	3	230	7.1
16	0.6	0.20	16	16	20	-	3	230	7.1
17	0.6	0.24	16	16	20	0.24	3	180	7.1
18	0.6	0.28	16	16	20	0.45	3	155	7.1
19	0.6	0.25	16	16	20	0.75	3	120	7.1
20	0.6	0.20	16	16	20	1.08	3	80	7.1

5

Table 3. Batches of insulin aspart formulations Q and R were prepared according to the following compositions:

Preparation	**Q**	**R**
Insulin aspart	600 nmol	600 nmol
Zinc (as zinc acetate)	19,6 µg (3 Zn/hex)	16,35 µg (2,5 Zn/hex)
Phenol	1,50 mg (16 mM)	1,50 mg (16 mM)
m-Cresol	1,72 mg (16 mM)	1,72 mg (16 mM)
Disodium-Phosphate di-hydrate	0,53 mg (3 mM)	0,53 mg (3 mM)
Glycerol	3,3 mg/ml (35,8 mM)	3,3 mg/ml (35,8 mM)
Arginine (as L-Arginine HCl)	3,48 mg/ml (20 mM)	3,48 mg/ml (20 mM)
Nicotinamide	20,76 mg (170 mM)	20,76 mg (170 mM)
Sodium hydroxide	q.s	q.s
Hydrochloric acid	q.s	q.s
pH	7.1	7.1

Example 2

Analysis of insulin chemical stability

Size Exclusion Chromatography

Quantitative determination of high molecular weight protein (HMWP) and monomer insulin aspart was performed on Waters insulin (300 x 7.8mm, part nr wat 201549) with an eluent containing 2.5M acetic acid, 4mM L-arginine and 20 %(V/V) acetonitrile at a flow rate of 1ml/min. and 40°C. Detection was performed with a tuneable absorbance detector (Waters 486) at 276nm. Injection volume was 40µl and a 600µM human insulin standard. HMWP and concentration of the preparations were measured at each sampling point.

Reverse phase chromatography (UPLC)

Determination of the insulin aspart related impurities were performed on a UPLC system using a BEH RP C8 2.1 x 100mm column, particle size of 1,7µm (Waters part no 186002878) with a flow rate of 0,5ml/min., at 40° C detection at 220nm. Elution was performed with a mobile phase consisting of the following:

A. 10 % (w/V) acetonititrile, 2.8% (w/w) sodium sulphate, 0.3 % (w/w) o-phosphoric acid, pH 3.5.

B. 70 % (w/V) acetonitrile. Gradient: 0-11 min isocratic with 73%/27% of A/B, 11-12 linear change to 52%/48% A/B, 13-15 min. linear change to 73%/27% of A/B, 15-20 min. isocratic gradient at 73%/27% of A/B.

The amount of B28iso-aspartate, desamido and other related impurities were determined as absorbance area measured in percent of total absorbance area determined after elution of the preservatives. The RP-UPLC method is equivalent to the analytical method used for quality control of Novo Nordisk marketed insulin aspart pharmaceuticals.

Addition of arginine reduces the amount of degradation products formed, especially HMWP and desamido forms, increasing the concentration of arginine in the range 10 to 50mM leads to further reduction of degradation. The physical stability measured as lag time in the ThT assay is reduced upon addition of arginine and is increasingly reduced when the arginine concentration is increased. The overall performance of 50mM arginine is superior to 50 mM glycine, 50mM glutamic acid, or 50mM histidine regarding reduction of the formation of degradation products, as is shown in Table 4 below.

The insulin preparations of the present invention provide ultra-fast-acting insulin preparations which are not only physically stable, but surprisingly also chemically stable.

Table 4. Chemical stability data for insulin preparations 10-13 of Table 1

Preparation nr.	Chemical stability Content of degradation product (%) measured as difference between content after incubation for 2 weeks at 37˚C and at 4˚C			
	B28 IsoAsp	desamido forms	Other related impurities	HMWP
10	1.35	1.90	1.71	0.91
11	1.45	1.44	0.98	0.39
12	1.43	1.07	0.75	0.21
13	1.46	0.99	0.84	0.16

Table 5. Physical and chemical stability data for insulin preparations 14-20 of Table 2

Preparation nr.	Physical stability, lag time (min) in ThT assay	Chemical stability Content of degradation product (%) measured after incubation for 4 weeks at 37˚C			
		B28 IsoAsp	desamido forms	Other related impurities	HMWP
14	133	2.7	2.9	2.1	0.4
15	87	2.7	3.6	2.3	0.4
16	50	2.8	4.4	2.7	0.4
17	65	2.7	3.5	2.3	0.4
18	107	2.7	3.3	2.2	0.5
19	85	2.6	3.1	2.3	0.5
20	59	2.6	3.3	2.3	0.5

Table 6. Physical and chemical stability data for insulin preparations Q and R

Preparation	Chemical stability Content of degradation product (%) measured after incubation for 4 weeks at 37°C			
	B28 IsoAsp	desamido forms	Other related impurities	HMWP
Q	3.8	3.8	2.6	0.7
R	3.2	4.2	2.6	0.7

Example 3

Pharmacokinetic (PK)/ Pharmacodynamic (PD) studies in LYD pig model and plasma analysis assay

PK/PD studies in LYD pigs

The PK/PD studies were performed on domestic female pigs, LYD cross-breed, weighing between 55 and 110kg. The pigs were catheterised into the jugular vein through an ear vein at least 2 days before start of the study. The last meal before the start of the study was served to the animals approx. 18 hours prior to the injection of the test preparation, and the animals had free access to water at all time during the fasting period and the test period.

At time 0 hours the test preparation was given subcutaneous on the lateral side of the neck. A blood sample was drawn prior dosing and at regular time intervals after dosing samples were drawn from the catheter and sampled into 1.5ml glass tubes pre-coated with heparin. The blood samples were kept in ice water until separation of plasma by centrifugation for 10min. 3000rpm at 4°C, which was done within the first 30 minutes. Plasma samples were stored at 4°C for short time (2-3 hours) or at -18°C for long term storage and were analysed for glucose on YSI or Konelab 30i and for insulin aspart concentration by LOCI.

Luminescent Oxygen Channeling Immunoassay (LOCI) for Insulin Aspart quantification

The insulin aspart LOCI is a monoclonal antibody-based sandwich immunoassay and applies the proximity of two beads, the europium-coated acceptor beads and the streptavidin coated donor-beads. The acceptor beads were coated with a specific antibody against human insulin and recognize insulin aspart in plasma samples. A second biotinylated antibody bind specific to insulin aspart and together with the streptavidin coated beads, they make up the

sandwich. Illumination of the beads-aggregate-immunocomplex releases singlet oxygen from the donor beads which channels into the acceptor beads and triggers chemiluminescence. The chemiluminescence was measured and the amount of light generated is proportional to the concentration of insulin aspart.

Compared to the marketed product NovoRapid®, the initial rate of plasma glucose lowering is faster for the preparations of the present invention (Figures 1 and 2). Likewise, when compared to NovoRapid®, the initial absorption rate of the insulin component of the preparations of the present invention, is markedly faster (Figure 2).

Example 4

General introduction to ThT fibrillation assays for the assessment of physical stability of protein formulations

Low physical stability of a peptide may lead to amyloid fibril formation, which is observed as well-ordered, thread-like macromolecular structures in the sample eventually resulting in gel formation. This has traditionally been measured by visual inspection of the sample. However, that kind of measurement is very subjective and depending on the observer. Therefore, the application of a small molecule indicator probe is much more advantageous. Thioflavin T (ThT) is such a probe and has a distinct fluorescence signature when binding to fibrils [Naiki et al. (1989) Anal. Biochem. **177**, 244-249; LeVine (1999) Methods. Enzymol. **309**, 274-284]. The time course for fibril formation can be described by a sigmoidal curve with the following expression [Nielsen et al. (2001) Biochemistry **40**, 6036-6046]:

$$F = f_i + m_i t + \frac{f_f + m_f t}{1 + e^{-[(t-t_0)/\tau]}} \quad \text{Eq.(1)}$$

Here, F is the ThT fluorescence at the time t. The constant t_0 is the time needed to reach 50% of maximum fluorescence. The two important parameters describing fibril formation are the lag-time calculated by $t_0 - 2\tau$ and the apparent rate constant $k_{app} = 1/\tau$ (see Figure 3).

Formation of a partially folded intermediate of the peptide is suggested as a general initiating mechanism for fibrillation. Few of those intermediates nucleate to form a template onto which further intermediates may assembly and the fibrillation proceeds. The lag-time

corresponds to the interval in which the critical mass of nucleus is built up and the apparent rate constant is the rate with which the fibril itself is formed.

Sample preparation

Samples were prepared freshly before each assay. Each sample composition is described in each example. The pH of the sample was adjusted to the desired value using appropriate amounts of concentrated NaOH and $HClO_4$ or HCl. Thioflavin T was added to the samples from a stock solution in H_2O to a final concentration of 1 μM.

Sample aliquots of 200μl were placed in a 96 well microtiter plate (Packard Opti-Plate™-96, white polystyrene). Usually, four or eight replicas of each sample (corresponding to one test condition) were placed in one column of wells. The plate was sealed with Scotch Pad (Qiagen).

Incubation and fluorescence measurement

Incubation at given temperature, shaking and measurement of the ThT fluorescence emission were done in a Fluoroskan Ascent FL fluorescence platereader or Varioskan platereader (Thermo Labsystems). The temperature was adjusted to 37 °C. The orbital shaking was adjusted to 960rpm with an amplitude of 1mm in all the presented data. Fluorescence measurement was done using excitation through a 444nm filter and measurement of emission through a 485nm filter.

Each run was initiated by incubating the plate at the assay temperature for 10 min. The plate was measured every 20 minutes for a desired period of time. Between each measurement, the plate was shaken and heated as described.

Data handling

The measurement points were saved in Microsoft Excel format for further processing, and curve drawing and fitting were performed using GraphPad Prism. The background emission from ThT in the absence of fibrils was negligible. The data points are typically a mean of four or eight samples and shown with standard deviation error bars. Only data obtained in the same experiment (i.e. samples on the same plate) are presented in the same graph ensuring a relative measure of fibrillation between experiments.

The data set may be fitted to Eq. (1). However, since full sigmodial curves are not always achieved during the measurement time, lag times were here visually determined from the ThT fluorescence curve as the time point at which the ThT fluorescence is different than the background level.

Measurement of initial and final concentrations

The peptide concentration in each of the tested formulations was measured both before application in the ThT fibrillation assay ("Initial") and after completion of the ThT fibrillation ("After ThT assay"). Concentrations were determined by reverse HPLC methods using a pramlintide standard as a reference. Before measurement after completion 150µl was collected from each of the replica and transferred to an Eppendorf tube. These were centrifuged at 30000 G for 40mins. The supernatants were filtered through a 0.22µm filter before application on the HPLC system.

Example 5

In-use stability

Mechanical stress such as shaking or continuous movement may cause peptides and proteins to fibrillate or in other way change the physical and chemical attributes of the drug product. In-use stability studies are performed to evaluate the physical and chemical stability for the intended use of the drug product.

One study to investigate in-use stability is penetration studies.
The container containing the product is placed at controlled temperature: e.g. 25°C, 30°C or 37°C and turned a specified number of times, e.g. 15 times or 20 times, at specified intervals, e.g. 1 time every day or 3 times every week. The container is penetrated a specified number of times, e.g. 7 times or 15 times, with a syringe needle at specified intervals, e.g. 1 time every week or 1 time every day or 3 times every day.

Another study to investigate is an accelerated method using rotation of the sample.
The container containing the product is placed at controlled temperature: e.g. 25°C, 30°C or 37°C and rotated for 4 hours at 30 RPM every day.

The drug product is tested for physical and chemical stability at defined time points, e.g. 1, 2, 3 and 4 weeks. Particularly the drug product can be tested for purity, content, and impurities. Furthermore the product can be tested by means of ThT and macroscopy analysis as well as other physical chemical or microscopic methods such as for example turbidimetry, DLS, MFI or TEM.

The batches of formulations Q and R were filled in 3 ml cartridges and 10 ml vials. Each formulation was tested for in-use stability by penetration studies and rotation studies according to the schedules below.

Table 7. Stability scheme for rotation studies of formulations Q and R of Table 3

Rotation study		
Container	Cartridge	
Temperature	25°C ± 2°C	30°C ± 2°C
Rpm	30	
Hours/day	4	
Analysis	Macroscopy + turbidimetry chemical analysis at time zero and end of study	

Table 8. In-use stability scheme for penetration studies of formulations Q and R of Table 3

Penetration study				
Container	Cartridge		Vial	
Temperature	25°C ± 2°C	30°C ± 2°C	25°C ± 2°C	30°C ± 2°C
Movement	Turned 20 times 3 times per week and 40 times 2 times per week			
Penetration	3 penetrations, 5 times per week			
Macroscopi/turbidimetry	1 to 3 times a week for the duration of the study.			
ThT, DLS	-	1 time per week	-	
Chemical analysis	Chemical analysis at time zero and end of experiment			

The results show that both that the product does not change appearance as tested by macroscopy (see Table 9). “Complies” means that the penfill was essentially free from particulate matter and thereby no fibrillation at the naked eye.

Table 9. Results of in-use stability of formulations of Table 3

Design	Timepoint (weeks)	Macroscopy result

Rotation, 30°C	0	Complies
Rotation, 25°C	0	Complies
Penetration, 30°C	0	Complies
	1	Complies
Penetration, 25°C	0	Complies
	1	Complies

The preparations with the most favourable balance between an ultra-fast absorption rate and favourable chemical and physical stability are shown in the results of the tables above.

Example 6

Free insulin aspart assay

Human serum samples are precipitated with PEG to obtain a supernatant containing free Insulin aspart.

A monoclonal specific antibody for insulin aspart (HUI-018) is coated onto a microplate used as capture antibody. Subsequent to removing excess capture antibody, the microplate wells are blocked by using blocking buffer. Standards, QC samples and unknown samples in human serum are added to the appropriate wells of the coated microplate. This is followed by the addition of a biotinylated antibody specific for insulin aspart (X14-6 F34 – Biotin). During an overnight incubation period, insulin aspart in the samples is captured by the immobilized capture antibody and in parallel binds the biotinylated antibody. Unbound materials are removed subsequently by a wash step followed by the addition of a horseradish peroxidase avidin D (HRP) conjugate. The avidin D HRP conjugate binds to the biotin on the bound antibody.

Following an incubation period and a wash step, tetramethylbenzidine (TMB) solution is added to the wells, creating a colorimetric signal that is proportional to the amount of insulin aspart bound in the plate. Colour development is stopped using 2N sulfuric acid and the intensity of the colour (optical density (OD)) is measured at 450 nm - 620 nm using a plate reader.

CLAIMS

1. An insulin preparation comprising:
   - B28Asp human insulin present in an amount from 0.5mM to 0.7mM,
   - nicotinamide or a salt thereof, present in an amount from 160mM to 180mM,
   - arginine present in an amount from 10mM to 30mM,
   - zinc, wherein a zinc:insulin molar ratio is from about 2.0:6 to about 3.5:6, and
   - a phosphate buffer present in the amount from 1mM to 10mM.
2. The insulin preparation according to claim 1, wherein the insulin compound is present in the amount of about 0.6mM.
3. The insulin preparation according to any of claims 1 or 2, comprising about 170mM nicotinamide.
4. The insulin preparation according to any of claims 1-3, comprising 20mM of arginine.
5. The insulin preparation according to any of claims 1-4, comprising about 3mM of phosphate buffer.
6. The insulin preparation according to any one of the preceding claims, wherein the zinc:insulin molar ratio is 2.5:6 or 3:6.
7. The insulin preparation according to any one of the preceding claims, which further comprises one or more preservative agents.
8. The insulin preparation according to claim 7, wherein one preservative is a phenolic compound, such as phenol.
9. The insulin preparation according to claim 8, wherein said phenolic compound is present in the amount of 16mM.
10. The insulin preparation according to claim 7, wherein one preservative is m-cresol.
11. The insulin preparation according to claim 10, wherein m-cresol is present in an amount of about 16mM.
12. The insulin preparation according to any one of the preceding claims, which further comprises one or more stabilizers.
13. The insulin preparation according to any one of the preceding claims, further comprising glycerol, wherein the glycerol is in an amount from about 25mM to about 45mM.
14. The insulin preparation according to any one of the preceding claims, wherein the preparation has a pH of about 7.1.
15. An insulin preparation comprising:
    a. B28Asp human insulin, wherein the B28Asp human insulin is present in a

concentration of about 0.6 mM,

b. Nicotinamide, wherein the nicotinamide is present at a concentration of about 170mM,

c. Arginine, wherein the arginine is present in a concentration of about 20 mM,

d. Zinc, wherein about 2.5 or about 3 zinc ions are present per six B28Asp human insulin molecules; and

e. a phosphate buffer present in a concentration of about 3mM

wherein the preparation has a pH of about 7.1.

16. An insulin preparation consisting essentially of:

a. B28Asp human insulin present in a concentration of 0.6 mM,

b. nicotinamide present in a concentration of 170mM;

c. arginine present in a concentration of 20mM;

d. zinc, wherein 2.5 or 3 zinc ions are present per six B28Asp human insulin molecules, and

e. a phosphate buffer present in a concentration of 3mM,

wherein the preparation has a pH of about 7.1.

17. An aqueous insulin preparation consisting of:

a. B28Asp human insulin present in a concentration of 0.6 mM,

b. Nicotinamide present in a concentration of 170mM;

c. Arginine present in a concentration of 20mM;

d. Zinc, wherein 2.5 or 3 zinc ions are present per six B28Asp human insulin molecules;

e. a phosphate buffer present in a concentration of 3mM;

f. phenol present in a concentration of 16mM;

g. m-cresol present in a concentration of 16mM;

h. glycerol present in a concentration of 35,8mM, and

i. NaOH and/or HCl so that the preparation has a pH of about 7.1.

18. An insulin preparation according to any one of claims 1-17, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarc-

tion, stroke, coronary heart disease and other cardiovascular disorders and treatment of critically ill diabetic and non-diabetic patients.

19. The insulin preparation according to claim 18, for use in the treatment of hyperglycemia type 2 diabetes and type 1 diabetes.
20. A method of reducing the blood glucose level in mammals by administering to a patient in need of such treatment a therapeutically active dose of an insulin preparation according to any one of the preceding claims.
21. A method for the treatment of diabetes mellitus in a subject comprising administering to a subject an insulin preparation according to any one of the preceding claims.
22. A method according to any of claims 20-21, for parenteral administration.
23. An insulin preparation according to any one of the preceding claims, for use in the treatment or prevention of hyperglycemia including stress induced hyperglycemia, type 2 diabetes, impaired glucose tolerance, type 1 diabetes, and burns, operation wounds and other diseases or injuries where an anabolic effect is needed in the treatment, myocardial infarction, stroke, coronary heart disease and other cardiovascular disorders and treatment of critically ill diabetic and non-diabetic patients.
24. The insulin preparation according to claim 23, for use in the treatment of hyperglycemia type 2 diabetes and type 1 diabetes.

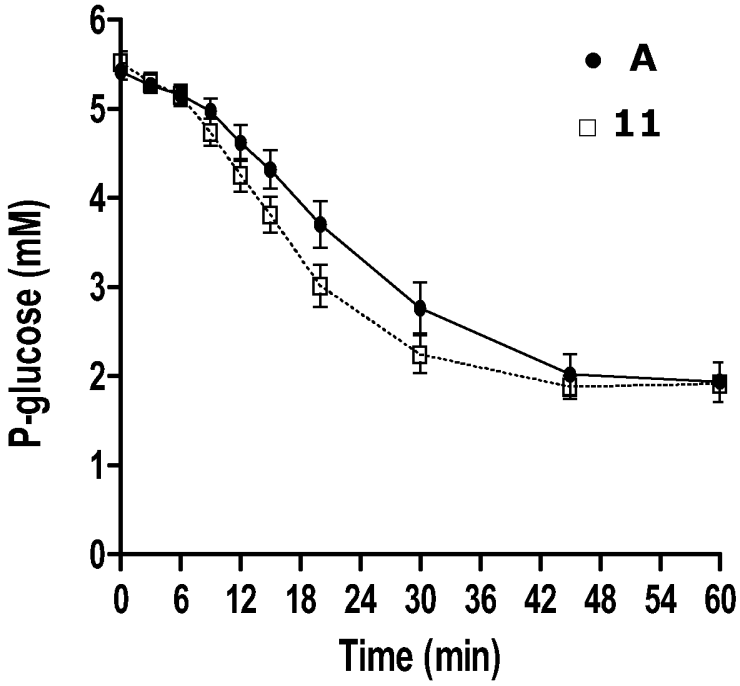
P-glucose (mM)
Time (min)
A
11
0
1
2
3
4
5
6
0 6 12 18 24 30 36 42 48 54 60

Fig. 1/3

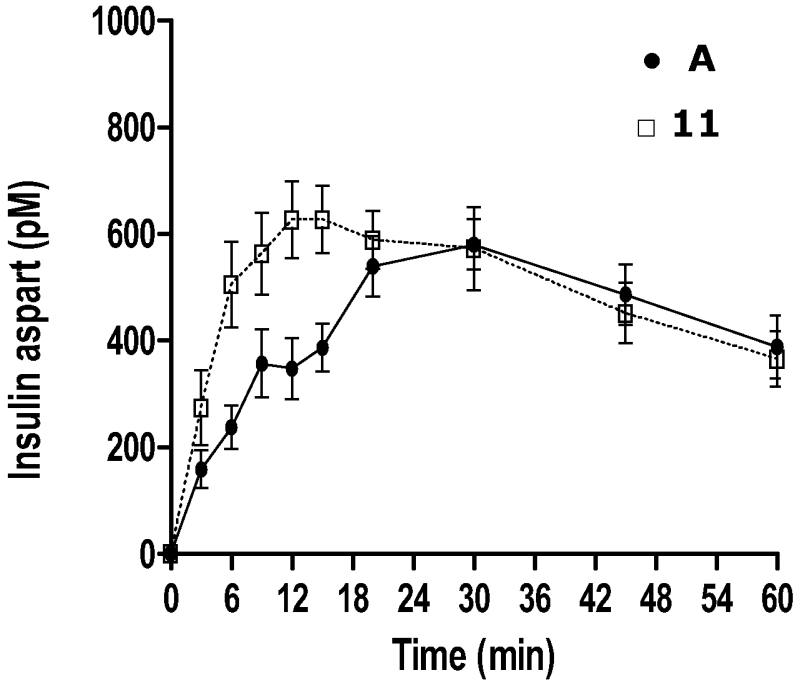
Insulin aspart (pM)
Time (min)
A
11
0
200
400
600
800
1000
0 6 12 18 24 30 36 42 48 54 60

Fig. 2/3

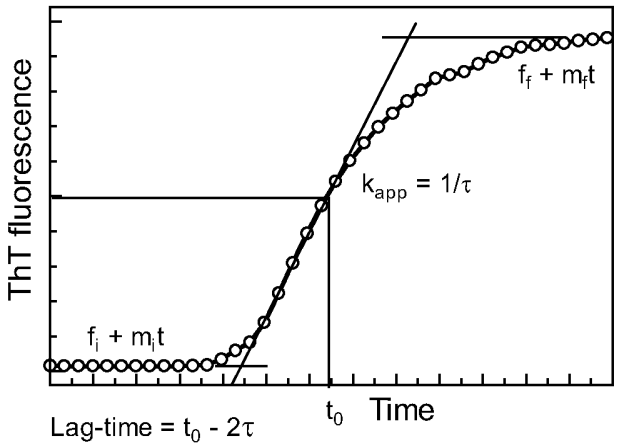
ThT fluorescence
$f_f + m_f t$
$k_{app} = 1/\tau$
$f_i + m_i t$
t_0
Time
Lag-time = $t_0 - 2\tau$

Fig. 3/3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/061795

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/198 A61K31/465 A61K33/30 A61K38/28 A61K47/18
A61P3/10
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/149772 A1 (NOVO NORDISK AS [DK]; OLSEN HELLE BIRK [DK]; HAVELUND SVEND [DK]; RIBE) 29 December 2010 (2010-12-29) page 2, lines 15-28 page 16, line 7 - page 17, line 16 page 24; example 1	1-24
A	EP 1 283 051 A1 (LILLY CO ELI [US]) 12 February 2003 (2003-02-12) page 1, paragraph 1 page 3, paragraph 24 page 5; table 2 page 5, paragraph 34 - page 6, paragraph 44	1-24
X,P	WO 2012/080362 A1 (NOVO NORDISK AS [DK]; OLSEN HELLE BIRK [DK]; HAVELUND SVEND [DK]; RIBE) 21 June 2012 (2012-06-21) page 35; example 17	1-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family 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 application or patent 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 referring to an oral disclosure, use, exhibition or other means

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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 2013	16/07/2013

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

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/061795

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010149772 A1	29-12-2010	AU 2010264636 A1	12-01-2012
		CA 2764423 A1	29-12-2010
		CN 102470165 A	23-05-2012
		EP 2340033 A1	06-07-2011
		EP 2612677 A1	10-07-2013
		JP 2012530768 A	06-12-2012
		KR 20120047226 A	11-05-2012
		TW 201100093 A	01-01-2011
		US 2012135920 A1	31-05-2012
		WO 2010149772 A1	29-12-2010
EP 1283051 A1	12-02-2003	DK 1283051 T3	16-10-2006
		EP 1283051 A1	12-02-2003
		SI 884053 T1	28-02-2003
WO 2012080362 A1	21-06-2012	NONE	