1.72 mg/ml m-cresol;
200mM glycerol;
3mM phosphate;
40 mM of nicotinamide; and
pH of 7.4.

90. The pharmaceutical composition according to preceding embodiments, comprising 0.6 mM, 1.2mM, 1.8mM, 2.4mM or 3.0mM of insulin analogue A9E, B3E, B27E, B28E, desB30;
1.5mg/ml phenol;
1.72 mg/ml m-cresol;
185mM glycerol;
3mM phosphate;
20 mM of nicotinamide;
10 mM of citrate; and
pH of 7.4.

91. The insulin analogue according to any one of embodiments 1-24 or a pharmaceutically acceptable salt thereof or the pharmaceutical composition according to any one of embodiments 25-90, for use as a medicament.

92. The insulin analogue according to any one of embodiments 1-24 or a pharmaceutically acceptable salt thereof or the pharmaceutical composition according to any one of embodiments 25-90, for use in the prevention or alleviation or treatment of a disease or disorder or condition of a human body, wherein the disease, disorder or condition relates to diabetes, in particular Type 1 diabetes, or Type 2 diabetes.

93. A method of treatment, prevention or alleviation of a disease or disorder or condition of a human body, wherein the disease, disorder or condition relates to diabetes, in particular Type 1 diabetes, or Type 2 diabetes, wherein the method comprises the step of administering to such a living animal body in need thereof, a therapeutically effective amount of the insulin analogue according to any one of embodiments 1-24 or pharmaceutical composition according to any one embodiments 25-90.

The invention is even further described by the following non-limiting embodiments:

1. An insulin analogue, wherein the analogue comprises A9E or A9D and further comprises B3E and/or desB30 relative to human insulin.

2. The insulin analogue according to embodiment 1, wherein the analogue further comprises at least one of B26E, B27E and/ or B28E.

3. The insulin analogue according to embodiment 2, wherein the analogue additionally comprises the A21A substitution.

4. The insulin analogue according to any of the preceding embodiments, wherein the analogue is

A9D, B3E;
A9E, B3E;
A9D, desB30;
A9E, desB30;
A9D, B3E, desB30;
A9E, B3E, desB30;
A9D, B3E, B26E;
A9E, B3E, B26E;
A9D, B26E, desB30;
A9E, B26E, desB30;
A9D, B3E, B26E, desB30;
A9E, B3E, B26E, desB30;
A9D, A21A, B3E, B26E;
A9E, A21A, B3E, B26E;
A9D, A21A, B26E, desB30;
A9E, A21A, B26E, desB30;
A9D, A21A, B3E, B26E, desB30;
A9E, A21A, B3E, B26E, desB30;
A9D, B3E, B27E;
A9E, B3E, B27E;
A9D, B27E, desB30;
A9E, B27E, desB30;
A9D, B3E, B27E, desB30;
A9E, B3E, B27E, desB30;
A9D, A21A, B3E, B27E;
A9E, A21A, B3E, B27E;
A9D, A21A, B27E, desB30;
A9E, A21A, B27E, desB30;
A9D, A21A, B3E, B27E, desB30;
A9E, A21A, B3E, B27E, desB30;

A9D, B3E, B28E;
A9E, B3E, B28E;
A9D, B28E, desB30;
A9E, B28E, desB30;
A9D, B3E, B28E, desB30;
A9E, B3E, B28E, desB30;
A9D, A21A, B3E, B28E;
A9E, A21A, B3E, B28E;
A9D, A21A, B28E, desB30;
A9E, A21A, B28E, desB30;
A9D, A21A, B3E, B28E, desB30;
A9E, A21A, B3E, B28E, desB30;
A9D, B3E, B26E, B27E;
A9E, B3E, B26E, B27E;
A9D, B26E, B27E, desB30;
A9E, B26E, B27E, desB30;
A9D, B3E, B26E, B27E, desB30;
A9E, B3E, B26E, B27E, desB30;
A9D, A21A, B3E, B26E, B27E;
A9E, A21A, B3E, B26E, B27E;
A9D, A21A, B26E, B27E, desB30;
A9E, A21A, B26E, B27E, desB30;
A9D, A21A, B3E, B26E, B27E, desB30;
A9E, A21A, B3E, B26E, B27E, desB30;
A9D, B3E, B27E, B28E;
A9E, B3E, B27E, B28E;
A9D, B27E, B28E, desB30;
A9E, B27E, B28E, desB30;
A9D, B3E, B27E, B28E, desB30;
A9E, B3E, B27E, B28E, desB30;
A9D, A21A, B3E, B27E, B28E;
A9E, A21A, B3E, B27E, B28E;
A9D, A21A, B27E, B28E, desB30;
A9E, A21A, B27E, B28E, desB30;
A9D, A21A, B3E, B27E, B28E, desB30;

A9E, A21A, B3E, B27E, B28E, desB30;
A9D, B3E, B26E, B28E;
A9E, B3E, B26E, B28E;
A9D, B26E, B28E, desB30;
A9E, B26E, B28E, desB30;
A9D, B3E, B26E, B28E, desB30;
A9E, B3E, B26E, B28E, desB30;
A9D, A21A, B3E, B26E, B28E;
A9E, A21A, B3E, B26E, B28E;
A9D, A21A, B26E, B28E, desB30;
A9E, A21A, B26E, B28E, desB30;
A9D, A21A, B3E, B26E, B28E, desB30;
A9E, A21A, B3E, B26E, B28E, desB30;
A9D, B3E, B26E, B27E, B28E;
A9E, B3E, B26E, B27E, B28E;
A9D, B26E, B27E, B28E, desB30;
A9E, B26E, B27E, B28E, desB30;
A9D, B3E, B26E, B27E, B28E, desB30;
A9E, B3E, B26E, B27E, B28E, desB30;
A9D, A21A, B3E, B26E, B27E, B28E;
A9E, A21A, B3E, B26E, B27E, B28E;
A9D, A21A, B26E, B27E, B28E, desB30;
A9E, A21A, B26E, B27E, B28E, desB30;
A9D, A21A, B3E, B26E, B27E, B28E, desB30; or
A9E, A21A, B3E, B26E, B27E, B28E, desB30.

5. The insulin analogue according to embodiment 4, wherein the analogue is
A9E, B3E, B26E;
A9E, B3E, B27E, B28E;
A9E, B3E, B26E, desB30;
A9E, B3E, B27E, B28E, desB30;
A9D, B3E, B26E, desB30; or
A9E, A21A, B3E, B26E, desB30.

6. The insulin analogue according to embodiment 5, wherein the analogue is A9E, B3E, B26E, desB30.

7. A pharmaceutical composition comprising an insulin analogue according to any one of embodiments 1-6, and one or more pharmaceutically acceptable excipients.

8. The pharmaceutical composition according to embodiment 7, wherein the insulin analogue is in a concentration of from 0.6mM to 3mM.

9. The pharmaceutical composition according to embodiments 7-8, wherein the composition is free of zinc.

10. The pharmaceutical composition according to embodiments 7-9, comprising a nicotinic compound, and in particular nicotinamide.

11. The pharmaceutical composition according to embodiments 7-9, wherein the composition is free of a nicotinic compound, and in particular nicotinamide.

12. The insulin analogue according to any of the embodiments 1-6 is monomeric in a zinc free pharmaceutical composition.

13. The insulin analogue according to embodiment 7 is chemically and/or physically stable.

14. The insulin analogue according to any one of embodiments 1-6 or a pharmaceutically acceptable salt thereof or the pharmaceutical composition according to any one of embodiments 7-11, for use as a medicament.

15. Use of the insulin analogue according to any one of embodiments 1-7 in the manufacture of a medicament for the treatment, prevention or alleviation of a disease or disorder or condition of a human body, wherein the disease, disorder or condition relates to diabetes, in particular Type 1 diabetes, or Type 2 diabetes.

**Examples**

Materials and Methods

List of Abbreviations

ACN - Acetonitrile

ALP – Achromobactor lyticus protease

AUC – Area under the curve

C-peptide – connecting peptide

DCM – dichloromethane

DIC – diisopropylcarbodiimide;

DIPEA = DIEA – N, N-disopropylethylamine

DMF – N, N-dmethylformamide

DMSO – dimethyl sulphoxide

Fmoc – fluorenylmethoxycarbonyl

Glu (gGlu) – gamma L-glutamyl

HFIP - Hexafluoro-2-propanol

HPLC - high-performance liquid chromatography

IR – insulin receptor

IGF-1R insulin-like growth factor 1 receptor

LC – liquid chromatography

MALDI-TOF - matrix-assisted laser desorption ionisation time-of-flight

MS – mass spectrometry

NMP – N-methylpyrrolidone

PAL - Peptide Amide Linker

PCR – polymerase chain reaction

PD – pharmacodynamics (blood/plasma glucose lowering effect)

PK – pharmacodynamics (blood/plasma insulin concentrations versus time profiles)

RT – room temperature

tBu – tert-butyl

TFA – trifluoroacetic acid; and

TRIS – tris (hydroxy methyl) aminomethane.

**General Methods of Preparation**

General procedure (A)

Solid phase synthesis and purification of analogues of the invention

A general procedure for the solid phase synthesis and purification of the insulin analogues of the invention is described in detail below and has been applied to the synthesis of additional compounds as indicated below.

Insulin A and B chains were prepared on a Prelude peptide synthesiser using a general Fmoc based solid phase peptide coupling method.

Resins used:

Fmoc-Thr(OtBu)-Wang; and Fmoc-Asp-OtBu coupled to PAL resin.

Amino acids (listed below) and oxyma were dissolved in DMF to a concentration of 0.3 M:

Fmoc-Ala-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp(OtBu)-OH; Fmoc-Cys(Trt)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Glu(OtBu)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Met-OH; Fmoc-Phe-OH; Fmoc-Pro-

OH; Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(tBu)-OH; and Fmoc-Val-OH.

Special/unnatural amino acids: Boc-Phe-OH; Boc-Gly-OH; and Fmoc-Cys(Acm)-OH.

Procedure

Scale: 0.25 mmol.

Standard coupling conditions used on resins were: 8 eq amino acid, DIC, collidine and oxyma (ethyl (hydroxyimino)cyanoacetate) in NMP for 1 hour, in the case of Fmoc-Arg(Pbf)-OH, a double coupling protocol (2x1h) was used.

Standard deprotection conditions used were: 20% piperdine in NMP (2x 5.5 ml for 2x 7.5 min or 2x 10 min), followed by washing with NMP and DCM.

A6C-A11C disulfide formation

The resin was treated for 15 min with a 0.5% solution of iodine in DCM/HFIP (30 mL of 1:1 mixture). After removal of solvent by filtration the resin was washed with DCM (3x20ml) and dried over a nitrogen stream.

A-Chain cleavage from the resin and activation of A20-Cys as S-S-Pyridyl

The resin was treated with a solution of TFA (30mL), triisopropylsilane (1 ml), water (0.75ml) and dithiodipyridine (0.75 g) for 3 h, and then the filtrate was collected and added to 150ml ether (split into 6 plastic NUNC tubes) to precipitate the peptide. The tubes were centrifuged at 3500rpm for 3 min, the ether layer was decanted, and this ether step was repeated a further 3 times. The crude material was combined and allowed to dry overnight at RT to give the desired peptide A-chain.

B-Chain cleavage from the resin

The resin was treated with a solution of TFA (30mL), triisopropylsilane (1 ml), water (0.75 ml) and dithiothreitol (0.5 g) for 3 h, and then the filtrate was collected and added to ether (150 ml, split into 6 plastic NUNC tubes) to precipitate the peptide. The tubes were centrifuged at 3500rpm for 3min, the ether layer was decanted, and this ether step was repeated a further 3 times. The crude material was allowed to dry overnight at RT to give the desired peptide B-chain.

A20C-B19C disulfide formation

To a mixture of A-chain (0.33 g) and B-chain (0.33 g) was added DMSO (8 mL) and DIPEA (1 mL) and the mixture stirred for 20 min, then added drop-wise with stirring to 140 ml of neutral buffer solution (water, TRIS (10 mM), ammonium sulphate (15 mM), 20% acetonitrile) to a total volume of approx. 150 ml.

The mixture was then purified by reverse phase chromatography using following set up

- Phenomenex Gemini 5 µM 5u C18 110Å 30x250mm column, running at 20 mL/min 10% B to 60% B over 40 min
- Eluant A=10mM TRIS, 15 mM ammonium sulfate, pH =7.3, 20% ACN in milliQ water
- Eluant B=20% miliQ water in acetonitrile

Pure fractions were pooled, flash frozen and freeze dried.

A7C-B7C disulfide formation

Freeze dried intermediate from the previous step was re-dissolved in 5 mL DMSO. Acetic acid (20 mL) and water (4 mL) was added, followed by iodine in AcOH (3 mL of 40 mM)

After total reaction time of 20min, the reaction quenched with 1M sodium ascorbate, and then added to a stirred solution of water (90 mL).

The mixture was then purified by reverse phase chromatography using following set up

- Phenomenex Gemini 5µM 5u C18 110Å 30x250mm column, running at 20 mL/min 10% B to 45% B over 40 min
- Eluant A=0.1% TFA in milliQ water
- Eluant B=0.1% TFA in acetonitrile

Pure fractions were pooled, flash frozen and freeze dried to give the desired product.

General procedure (B)

Insulin analogue expression and purification

Insulin analogue expression in S. cerevisiae

The insulin analogue, i.e. the two-chain non-acylated insulin analogues, for use according to the invention are produced recombinantly by expressing a DNA sequence encoding the insulin analogue in question in a suitable host cell by well-known techniques, e.g. as disclosed in US 6500645. The insulin analogue is either expressed directly or as a precursor molecule which may have an N-terminal extension on the B-chain and/or a connecting peptide

(C-peptide) between the B-chain and the A-chain. This N-terminal extension and C-peptide are cleaved off in vitro by a suitable protease, e.g. Achromobactor lyticus protease (ALP) or trypsin, and will therefore have a cleavage site next to position B1 and A1, respectively. N-terminal extensions and C-peptides of the type suitable for use according to this invention are disclosed in e.g. US 5395922, EP 765395 and WO 9828429.

The polynucleotide sequence encoding the insulin analogue precursor for use according to the invention may be prepared synthetically by established methods, e.g. the phosphoamidite method described by Beaucage et al. (1981) Tetrahedron Letters 22 1859-1869, or the method described by Matthes et al. (1984) EMBO Journal 3 801-805. According to the phosphoamidite method, oligonucleotides are synthesised in e.g. an automatic DNA synthesiser, purified, duplexed, and ligated to form the synthetic DNA construct. A currently preferred way of preparing the DNA construct is by polymerase chain reaction (PCR).

The recombinant method will typically make use of a vector which is capable of replicating in the selected microorganism or host cell and which carries a polynucleotide sequence encoding the insulin analogue precursor for use according to the present invention. The recombinant vector may be an autonomously replicating vector, i.e., a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used. The vector may be linear or closed circular plasmids and will preferably contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

The recombinant expression vector may be one capable of replicating in yeast. Examples of sequences which enable the vector to replicate in yeast are the yeast plasmid 2 µm replication genes REP 1-3 and origin of replication.

The vector may contain one or more selectable markers, which permit easy selection of trans-formed cells. A selectable marker is a gene product, which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Selectable markers for use in a filamentous fungal host cell include

amdS (acetamidase), argB (ornithine carbamoyltransferase), pyrG (orotidine-5'-phosphate decarboxylase) and trpC (anthranilate synthase). Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3 . A well suited selectable marker for yeast is the Schizosaccharomyces pompe TPI gene (Russell (1985) Gene 40 125-130).

In the vector, the polynucleotide sequence is operably connected to a suitable promoter sequence. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extra-cellular or intra-cellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription in a bacterial host cell, are the promoters obtained from the E. coli lac operon, Streptomyces coelicolor agarase gene (dagA), Bacillus subtilis levansucrase gene (sacB), Bacillus licheniformis alpha-amylase gene (amyL), Bacillus stearothermophilus maltogenic amylase gene (amyM), Bacillus amyloliquefaciens alpha-amylase gene (amyQ), and Bacillus licheniformis penicillinase gene (penP). Examples of suitable promoters for di-recting the transcription in a filamentous fungal host cell are promoters obtained from the genes for Aspergillus oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, Aspergillus niger neutral alpha-amylase, and Aspergillus niger acid stable alpha-amylase. In a yeast host, useful promoters are the Saccharomyces cerevisiae Ma1, TPI, ADH, TDH3 or PGK promoters.

The polynucleotide sequence encoding the insulin peptide backbone for use according to the invention also will typically be operably connected to a suitable terminator. In yeast, a suitable terminator is the TPI terminator (Alber et al. (1982) J. Mol. Appl. Genet. 1 419-434).

The procedures used to combine the polynucleotide sequence encoding the insulin analogue for use according to the invention, the promoter and the terminator, respectively, and to insert them into a suitable vector containing the information necessary for replication in the selected host, are well known to persons skilled in the art. It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire DNA sequence encoding the insulin backbones for use according to the invention, and subsequently inserting this fragment into a suitable expression vector, or by sequentially inserting DNA fragments containing genetic information for the individual elements such as the signal and pro-peptide (N-terminal extension of the B-chain), C- peptide, A- and B-chains, followed by ligation.

The vector comprising the polynucleotide sequence encoding the insulin analogue for use according to the invention is introduced into a host cell, so that the vector is maintained as

a chromosomal integrant, or as a self-replicating extra-chromosomal vector. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The host cell may be a unicellular microorganism, e.g. a prokaryote, or a non-unicellular microorganism, e.g. a eukaryote. Useful unicellular cells are bacterial cells such as gram-positive bacteria including, but not limited to, a Bacillus cell, a Streptomyces cell, or a gram-negative bacteria such as E. coli and Pseudomonas sp. Eukaryote cells may be mammalian, insect, plant, or fungal cells.

The host cell may in particular be a yeast cell. The yeast organism may be any suitable yeast organism which, on cultivation, secretes the insulin peptide backbone or the precursor hereof into the culture medium. Examples of suitable yeast organisms include strains selected from *Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Sacchoromyces uvarum, Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Pichia methanolica, Pichia kluyveri, Yarrowia lipolytica, Candida sp., Candida utilis, Candida cacaoi, Geotrichum sp., and Geotrichum fermentans*.

The transformation of the yeast cells may for instance be affected by protoplast formation followed by transformation by known methods. The medium used to cultivate the cells may be any conventional medium suitable for growing yeast organisms.

Insulin analogue purification

The secreted insulin analogue or precursor hereof may be recovered from the medium by conventional procedures including separating the yeast cells from the medium by centrifugation, by filtration or by catching or adsorbing the insulin analogue or precursor hereof on an ion exchange matrix or on a reverse phase absorption matrix, precipitating the proteinaceous components of the supernatant, or by filtration by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, etc.

The purification and digestion of the insulin peptide backbones of this invention is carried out as follows:

The single-chain insulin analogue precursor, which may contain an N-terminal extension of the B-chain and a modified C-peptide between the B-chain and the A-chain, is purified and concentrated from the yeast culture supernatant by cation exchange (Kjeldsen et al. (1998) Prot. Expr. Pur. 14 309-316).

The single-chain insulin analogue precursor is matured into two-chain insulin peptide backbone by digestion with lysine-specific immobilised ALP (Kristensen et al. (1997) J. Biol.

Chem. 20 12978-12983) or by use of trypsin to cleave off the N-terminal extension of the B-chain, if present, and the C-peptide.

ALP digestion

The eluate from the cation exchange chromatography step containing the insulin peptide backbone precursor is diluted with water to an ethanol concentration of 15-20%. Sodium glutamate is added to a concentration of 15 mM and pH is adjusted to 9.7 by NaOH. Immobilised ALP (4 gram/L) is added in a proportion of 1:100 (volume:volume) and digestion is allowed to proceed with mild stirring at room temperature overnight.

The digestion reaction is analysed by analytical LC on a Waters Acquity Ultra-Performance Liquid Chromatography system using a C18 column and the molecular weight is confirmed by Matrix-Assisted Laser Desorption Ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS) (Bruker Daltonics Autoflex II TOF/TOF).

The immobilised ALP is removed by filtration using a 0.2 µm filter. The two-chain insulin peptide backbone is purified by reversed phase HPLC (Waters 600 system) on a C18 column using an acetonitrile gradient. The desired insulin is recovered by lyophilisation.

Purity is determined by analytical LC on a Waters Acquity Ultra-Performance Liquid Chromatography system using a C18 column, and the molecular weight is confirmed by MALDI-TOF MS.

**Example 1**

Prepared by General procedure (B)

A9E, B3E, B26E, desB30 human insulin (SEQ ID NOS: 3 and 4)

**Example 2**

Prepared by General procedure (B)

A9E, B3E, B27E, B28E, desB30 human insulin (SEQ ID NOS: 3 and 5)

**Example 3**

Prepared by General procedure (A)

A9E, B3E, B26E human insulin; (SEQ ID NOS:3 and 6)

**Example 4**

Prepared by General procedure (A)

A9E, B3E, B27E, B28E human insulin; (SEQ ID NOS: 3 and 7)

**Example 5**

Prepared by General procedure (B)

A9D, B3E, B26E, desB30 (SEQ ID NOS: 8 and 4)

**Example 6**

Prepared by General procedure (B)

A9E, A21A, B3E, B26E, desB30 (SEQ ID NOS: 9 and 4)

**Comparator compound 1**

B3E, B26E, desB30 human insulin;

**Comparator compound 2**

B3E, B27E, B28E, desB30 human insulin;

**Comparator compound 3**

B28D human insulin (Insulin Aspart)

**Comparator compound 4**

A21A, B3E, B26E, desB30 human insulin

**Example 7**

**Insulin receptor affinity of selected insulin derivatives of the invention, measured on solubilised receptors**

The relative binding affinity of the insulin analogues of the invention for the human insulin receptor (IR) is determined by competition binding in a scintillation proximity assay (SPA) (according to Glendorf T et al. (2008) Biochemistry 47 4743-4751).

In brief, dilution series of a human insulin standard and the insulin analogue to be tested are performed in 96-well Optiplates (Perkin-Elmer Life Sciences) followed by the addition of [125I-A14Y]-human insulin, anti-IR mouse antibody 83–7, solubilised human IR-A (semipurified by wheat germ agglutinin chromatography from baby hamster kidney (BHK) cells overexpressing the IR-A holoreceptor), and SPA beads (Anti-Mouse polyvinyltoluene SPA Beads, GE Healthcare) in binding buffer consisting of 100 mM HEPES (pH 7.8), 100 mM NaCl, 10 mM MgSO4, and 0.025% (v/v) Tween 20. Plates are incubated with gentle shaking for 22-

24 h at 22°C, centrifuged at 2000 rpm for 2 minutes and counted on a TopCount NXT (Perkin-Elmer Life Sciences).

Data from the SPA are analysed according to the four-parameter logistic model (Vølund A (1978) Biometrics 34 357-365), and the binding affinities of the analogues calculated relative to that of the human insulin standard measured within the same plate.

Insulin receptor affinities and other *in vitro* data of selected insulin analogues of the invention are presented in Table 1, below.

Table 1: Insulin receptor affinities, IGF-1 receptor affinities and functional lipogenesis potencies of insulins of the invention

| **Example No.** | **hIRA (% rel to HI) Ex 7** | **hIRA mem (% rel to HI) Ex 8** | **hIGF1R mem (% rel to HI) Ex 8** | **Lipogenesis (% rel to HI) Ex 9** |
|---|---|---|---|---|
| 1 | 83.2 | 55.8 | 10.2 | 68.2 |
| 2 | 59.5 | 35.4 | 16.5 | 46.5 |
| 5 | 75.5 | ND | ND | ND |
| 6 | 35.3 | ND | ND | ND |

In conclusion, insulin analogues of the invention have high affinity for the insulin receptor, and they are able to activate the receptor and elicit functional response. Furthermore, relative affinity of the insulin analogues of the invention for IGF-1 receptor is lower compared to affinity for insulin receptor. This represents a potential benefit with regards to the safety of the analogues of the invention in clinical practice as mitogenic response would be lower compared to the metabolic response.

**Example 8**

**Insulin and Insulin-Like Growth factor-1 receptor affinities of selected insulin derivatives of the invention, measured on membrane associated receptors**

Membrane-associated human IR and IGF-1R are purified from BHK cells stably transfected with the pZem219B vector containing either the human IR-A, IR-B or IGF-IR insert. BHK cells are harvested and homogenized in ice-cold buffer (25 mM HEPES pH 7.4, 25 mM CaCl2 and 1 mM MgCl2, 250 mg/L bacitracin, 0.1 mM Pefablock). The homogenates are

layered on a 41% (w/v) sucrose cushion and centrifuged for 75 minutes at 95000g at 4°C. The plasma membranes are collected, diluted 1:5 with buffer (as above) and centrifuged again for 45 minutes at 40000g at 4°C. The pellets are re-suspended in a minimal volume of buffer and drawn through a needle (size 23) three times before storage at -80°C until usage.

The relative binding affinity for either of the membrane-associated human IR-A, IR-B or IGF-1R is determined by competition binding in a SPA setup. IR assays are performed in duplicate in 96-well OptiPlates (Perkin-Elmer Life Sciences). Membrane protein is incubated with gentle agitation for 150 minutes at 25°C with 50 pM [125I-A14Y]-human insulin in a total volume of 200 µL assay buffer (50 mM HEPES, 150 mM NaCl, 5 mM MgSO4, 0.01% Triton X−100, 0.1% (w/v) HSA (Sigma A1887), Complete EDTA-free protease inhibitors), 50 µg of wheat germ agglutinate (WGA)-coated PVT microspheres (GE Healthcare) and increasing concentrations of ligand. Assays are terminated by centrifugation of the plate at 2000 rpm for 2 minutes and bound radioactivity quantified by counting on a TopCount NXT (Perkin-Elmer Life Sciences).

IGF-1R assays are conducted essentially as for the IR binding assays except that membrane-associated IGF-1R and 50 pM [125I-Tyr31]-human IGF-1 were employed. Data from the SPA are analysed according to the four-parameter logistic model (Vølund A (1978) Biometrics 34 357-365), and the binding affinities of the analogues to be tested are calculated relative to that of the human insulin standard measured within the same plate.

IR (A isoform), and IGF-1R binding data of selected insulin analogues of the invention are given in the Table 1 above.

**Example 9**

**Lipogenesis in rat adipocytes**

As a measure of in vitro potency of the insulin analogues of the invention, lipogenesis can be used.

Primary rat adipocytes are isolated from the epididymale fat pads and incubated with 3H-glucose in buffer containing e.g. 0.1% fat free HSA and either standard (human insulin, HI) or insulin of the invention. The labelled glucose is converted into extractable lipids in a dose dependent way, resulting in full dose response curves. The result is expressed as relative potency (%) with 95% confidence limits of insulin of the invention compared to standard (HI).

Data are given in the Table 1, above.

**Example 10**

**Preparation of insulin compositions**

Insulin compositions of the present invention may be prepared as aqueous solutions. The aqueous solution is made isotonic, for example, with sodium chloride and/or glycerol. Furthermore, the aqueous medium may contain buffers and preservatives. 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, or about 7.4, or about 7.5, depending on the isoelectric point, pI, of the insulin analogue in question.

Preparation of zinc-free insulin compositions

Zinc-free insulin analogues were dissolved in aqueous solution, which in the final composition contained between 0.1 mM and 10 mM insulin analogue, 16 mM m-cresol, 16 mM phenol, and appropriate amounts of nicotinamide and glycerol, and the pH was adjusted to the desired value (between 7.0-8.0, measured at room temperature) using 1 N hydrochloric acid/1 N NaOH. Water was added to the final volume and the solution was sterile-filtered through a 0.2 µm filter. The composition was filled into vials (2 ml vials sealed using crimp caps, penfills sealed using crimp caps, or HPLC vials with screw tops).

Table 2: Exemplary compositions of insulin preparations

| **Composition** | **Insulin (mM)** | **Phenol (mM)** | **m-cresol (mM)** | **Nicotinamide (mM)** | **Glycerol** | | **Propylene glycol %** | **Phosphate (mM)** | **Tris (mM)** | **pH** | **Citrate (mM)** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | **%** | **mM** | | | | | |
| A | 0.6 | 16 | 16 | 0 | 2.15 | 233 | 0 | 3 | | 7.4 | |
| B | 0.6 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.4 | |
| B1 | 0.6 | 16 | 16 | 40 | 1.5 | 163 | 0 | 3 | | 7.4 | 5 |
| B2 | 0.6 | 16 | 16 | 40 | 1.5 | 163 | 0 | 3 | | 7.4 | 10 |
| B3 | 0.6 | 16 | 16 | 40 | 1.2 | 130 | 0 | 3 | | 7.4 | 15 |
| B4 | 0.6 | 16 | 16 | 40 | 1.2 | 130 | 0 | 3 | | 7.4 | 20 |
| C | 0.6 | 16 | 16 | 170 | 0.95 | 103 | 0 | 3 | | 7.4 | |
| I | 0.6 | 16 | 16 | 40 | 1.7 | 185 | 0 | 3 | | 7.2 | |
| I1 | 0.6 | 16 | 16 | 20 | 1.7 | 185 | 0 | 3 | | 7.4 | 10 |
| L | 0.6 | 16 | 16 | 40 | 0 | 0 | 1.5 | | 7 | 7.4 | |
| M | 0.6 | 16 | 16 | 40 | 1.8 | 195 | 0 | | 7 | 7.4 | |
| M1 | 0.6 | 16 | 16 | 10 | 1.8 | 195 | 0 | 3 | | 7.4 | 10 |
| D | 1.2 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.4 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D1 | 1.2 | 16 | 16 | 40 | 1.5 | 163 | 0 | 3 | | 7.4 | 10 |
| E | 1.8 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.4 | |
| F | 2.4 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.4 | |
| G | 3.0 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.4 | |
| H | 3.0 | 16 | 16 | 40 | 1.85 | 200 | 0 | 3 | | 7.0 | |
| J | 3.0 | 16 | 16 | 0 | 1.8 | 195 | 0 | 3 | | 7.0 | |
| K | 3.0 | 16 | 16 | 0 | 2.1 | 230 | 0 | 3 | | 7.4 | |
| K1 | 3.0 | 16 | 16 | 0 | 2.1 | 230 | 0 | 3 | | 7.4 | 20 |
| O | 3.0 | 19 | 19 | 40 | 1.7 | 185 | 0 | 3 | | 7.2 | |
| P | 3.0 | 19 | 19 | 40 | 1.7 | 185 | 0 | 3 | | 7.6 | |
| Q | 3.0 | 19 | 19 | 40 | 1.7 | 185 | 0 | 3 | | 8.0 | |

**Example 11**

**Self-association of insulin compositions measured by Small Angle X-ray Scattering (SAXS)**

SAXS data was used to estimate self-association state of the insulin analogues of the invention under different conditions.

Scattering curve of each experiment was described by the average radius of gyration (Rg) and maximal dimension (Dmax).

Furthermore, the relative amounts of monomer, dimer and larger species in the scattering curve was estimated using the fact that a SAXS scattering profile has an intensity contribution from all individual components in a multicomponent mixture. By using intensities (form factors) from each component it is possible to estimate the volume fraction contribution of each component in the mixture. A system of linear equations using the algorithm of nonnegative or unconstrained least-squares is used to minimize the discrepancy between the experimental and calculated scattering curves. Form factors are calculated from crystal structures of a monomer, dimer, hexamer etc. The volume fractions are expressed in percentages (%).

Results obtained from derivatives of the invention and of derivatives of the prior art are shown in Table 3, below.

Table 3: SAXS data of derivatives of the invention, and the comparator insulin analogues

| **Example No.** | **Composition** | **SAXS* formulation as described in example 10** | |
|---|---|---|---|
| | | M | D+>D |
| 1 | A | 100 | 0 |
| 1 | B | 100 | 0 |
| 1 | C | 100 | 0 |
| 1 | I | 99 | 1 |
| 1 | I1 | 100 | 0 |
| 1 | L | 98 | 2 |
| 1 | M | 100 | 0 |
| 1 | M1 | 100 | 0 |
| 1 | D | 100 | 0 |
| 1 | D1 | 97 | 3 |
| 1 | E | 100 | 0 |
| 1 | F | 100 | 0 |
| 1 | G | 100 | 0 |
| 1 | H | 79 | 21 |
| 1 | O | 90 | 10 |
| 1 | P | 100 | 0 |
| 1 | Q | 100 | 0 |
| 1 | B1 | 100 | 0 |
| 1 | B2 | 100 | 0 |
| 1 | B3 | 100 | 0 |
| 1 | B4 | 100 | 0 |
| 2 | A | 100 | 0 |
| 2 | I | 100 | 0 |
| 2 | B | 100 | 0 |
| 2 | L | 100 | 0 |
| 2 | M | 100 | 0 |
| 2 | O | 100 | 0 |
| 2 | P | 100 | 0 |
| 2 | Q | 100 | 0 |
| 5 | B | 98 | 2 |
| 5 | G | 95 | 5 |
| 6 | B | 91 | 9 |
| Comparator 1 | B | 97 | 3 |
| Comparator 1 | B4 | 95 | 5 |
| Comparator 1 | G | 77 | 23 |
| Comparator 1 | H | 4 | 96 |
| Comparator 2 | A | 100 | 0 |

| | | | |
|---|---|---|---|
| Comparator 2 | J | 0 | 100 |
| Comparator 2 | K | 87 | 13 |
| Comparator 2 | K1 | 0 | 100 |
| Comparator 3 | A# | 35 | 65 |
| Comparator 3 | B# | 0 | 100 |
| Comparator 3 | A+Zn | 0 | 100 |
| Comparator 4 | A | 86 | 14 |
| Comparator 4 | K | 42 | 58 |
| Comparator 4 | B4 | 87 | 13 |
| Comparator 4 | K1 | 0 | 100 |

*) M: Percentage of monomeric species in composition; D: Percentage of dimeric species in composition; >D: Percentage of species larger than dimeric in composition;

#) phosphate concentration 7 mM instead of 3 mM

In conclusion, the insulin analogues of the invention are less self-associated than the comparator compounds according to the SAXS results. This difference is more pronounced at higher concentrations of the insulin analogues of the invention. It will thus be expected that the insulin analogues of the invention will be absorbed more quickly after subcutaneous injection of especially high concentration compositions.

**Example 12**

**Physical stability analysis for insulin compositions**

Compositions (1 ml) of the insulin analogues of the invention as described in example 10 were transferred into 2 ml vials and sealed using crimp caps. The vials were incubated at 30 °C lying down. Every 2 days, the vial was picked by a robotic arm, and a picture of a standing vial was taken with the background light of 10000 lux. The resulting images were analyzed for the presence of particles using a pixel analysis algorithm. The first day when particles were detected is given in the table below.

Table 4. physical and chemical stability of insulin analogues of the invention in clinically relevant compositions

| **Example No.** | **Composition** | **Physical Stability Lag time (days)** | **HMWP formation (4 week at 37°C)** |
|---|---|---|---|
| 1 | A | >35 | 0.1 |

| | | | |
|---|---|---|---|
| 1 | B | >35 | 0.2 |
| 1 | C | >35 | 0.2 |
| 1 | I | >35 | 0.1 |
| 1 | L | ND | 0.2 |
| 1 | M | ND | 0.4 |
| 1 | D | >35 | 0.3 |
| 1 | E | >35 | 0.8 |
| 1 | F | >35 | 1.3 |
| 1 | G | >35 | 1.9 |
| 1 | H | 30 | 1.6 |
| 1 | O | >35 | 1.6 |
| 1 | P | >35 | 3.8 |
| 1 | Q | >35 | 8.6 |
| 1 | B1 | >35 | 0.1 |
| 1 | B2 | >35 | 0.8 |
| 1 | B3 | >35 | 0.6 |
| 1 | B4 | >35 | 0.1 |
| 2 | A | >35 | 0.1 |
| 2 | I | >36 | 0.1 |
| 2 | B | >40 | 0.1 |
| 2 | L | >40 | 0.1 |
| 2 | M | >40 | 0.1 |
| 2 | O | >36 | 0.7 |
| 2 | P | >36 | 0.6 |
| 2 | Q | >36 | 1.1 |
| 5 | B | >35 | 0.4 |
| 6 | B | >35 | -0.1 |
| 6 | G | 15 | 2.1* |
| Comparator 1 | B | 12 | 1.9 |
| Comparator 1 | B4 | 2 | 1.7* |
| Comparator 1 | G | 2 | 3.5* |
| Comparator 1 | H | 4 | 3.2* |
| Comparator 2 | A | >36 | 0.8 |
| Comparator 2 | J | 24 | 2.4* |
| Comparator 2 | K | 13 | 3.9* |
| Comparator 2 | K1 | 5 | 1.1* |
| Comparator 3 | A# | 5.3 | 1.6* |
| Comparator 3 | B# | 3.3 | 1.8* |
| Comparator 3 | A+Zn | >43 | 0.5 |
| Comparator 4 | A | 14 | 1.6* |
| Comparator 4 | B4 | 4 | 2.9* |

#) phosphate concentration 7 mM instead of 3 mM

ND: Not Determined

*) HMWP was determined in supernatants for samples containing particles; particles were not solubilized.

In conclusion, the insulin analogues of the invention have higher physical stability in clinically relevant Zn-free compositions, especially at high insulin concentration.

**Example 13**

**Chemical stability analysis for insulin compositions**

HMWP content

High molecular weight proteins (HMWP) were separated from the monomeric form of insulin by size-exclusion chromatography using Waters Insulin HMWP column (125Å, 10 µm, WAT 201549, 7.8 x 300 mm) with 0.5 ml/min flow of an eluent containing 50% (v/v) isopropanol, 600 mM NaCl, 20 mM NaH2PO4 and UV detection at 215 nm.

Typical injection volume was 5 µl of 600 µM insulin composition.

The results are shown in Table 4, above.

**Example 14**

**Subcutaneous PK/PD profiles in LYD pigs**

The insulin analogues of the invention may be tested by subcutaneous administration to pigs, e.g. comparing with insulin aspart in the clinical composition (FIASP®) or comparing with similar insulin analogues of the prior art according to this protocol. The analogues may be tested for pharmacokinetic and/or pharmacodynamic parameters.

General methods used

During anaesthesia for placement of permanent intravenous catheters, the pigs are examined by ultrasound with and Esaote ultrasound scanner model "MyLabFive" and a linear probe type "LA435 6-18 MHz".

Mid neck between ear and scapula, on the right and left side, an area of 2 x 2 cm with no underlying muscle and suitable for subcutaneous injection is identified and marked by tattoo.

Feeding schedule

The pigs are fasted (no breakfast) prior to the experiment.

The pigs are in their normal pens during the entire experiment and they are not anaesthetized. The pigs are fasted until the 5-hour blood sample has been collected, but with free access to water. After the 5-hour blood sample the pigs are fed food and apples.

Subcutaneous dosing

The Penfill is mounted in a NovoPen Echo® with Novofine 30G 8mm needle, using a needle reducer with 5mm depth, is used for each pig. Each pig is dosed 10U=60 microL of compositions containing 600 nmol/ml insulin analog.

The pig is dosed in the subcutis laterally on the right or left side (opposite the catheter) of the neck and the needle is kept in the subcutis for a minimum of 10 seconds after injection to secure deposition of compound.

Treatment of hypoglycaemia

After subcutaneous dosing, glucose solution should be ready for i.v. injection to prevent hypoglycaemia, i.e. 4-5 syringes (20 mL) are filled with sterile 20% glucose, ready for use. Diagnosis of hypoglycemia is based on clinical symptoms and blood glucose measurements on a glucometer (Glucocard X-meter).

Treatment consists of slow i.v. injection of 50-100 ml 20% glucose (10-20 g glucose). The glucose is given in fractions over 5-10 minutes until effect.

Blood sampling

The patency of the jugular catheters is checked prior to the experiment with sterile 0.9% NaCl without addition of 10 IU/mL heparin.

Before and after the dosing, blood samples will be taken in the stable from a central venous catheter at the following time points:

Predose (-15, -5), 3, 6, 9, 12, 15, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 240, 300 min.

Samples are taken with a flowswitch. 4-5 ml of waste blood is withdrawn and discarded before taking the sample.

Blood samples of 0.8 ml are collected into tubes coated with EDTA for glucose and insulin analysis.

After each blood sample the catheter is flushed with 5 ml of sterile 0.9% NaCl without addition of 10 IU/mL heparin.

The tube is tilted gently a minimum of 10 times to ensure sufficient mixing of blood and anticoagulant (EDTA) and after one minute it is placed on wet ice. The tubes are spun for

10 min at 3000 rpm and 4ºC within 1 hour after sampling. The samples are stored on wet ice until pipetting.

Aseptic technique is demanded to avoid bacterial growth in the catheter with increased risk of clotting.

Closure of the catheters after the experiment

After the last sample has been collected at the days of dosing, a single intravenous treatment with 1 ml per 10 kg Pentrexyl® (1 g of ampicillin dissolved in 10 ml 0.9 % NaCl) is administered slowly i.v. via the catheter used for blood sampling. Following this treatment, the catheter is flushed with 10 ml 0.9% NaCl.

The catheters are closed with 0.5 ml of TauroLockHep500 is injected through the membrane as a lock for the catheter.

Analysis of blood samples

Plasma glucose: 10 ul of plasma is pipetted into 500 ul of buffer solution for measurements of glucose concentration in plasma in the BIOSEN autoanalyser.

Plasma insulin: 1 x 50 µl of plasma are pipetted into 0.65 ml Micronic® tubes (ELISA/LOCI/SPA setup) for analysis.

Plasma is stored frozen at -20°C.

PK calculations: Area under the curve (AUC) to infinity and partial AUCs, AUC0-5, AUC0-10, AUC0-15, AUC0-20 and AUC0-30, were calculated by non-compartmental analysis applying the linear up log up calculation method in Phoenix 64 (Certara, US). The ratio between a partial AUC/AUCinf was used as a measure for absorption rate based on the similar iv properties for the insulin analogues in LYD pig.

Fig 1 and Fig 2 show that the analogues of the invention are better suited for use in insulin pumps than FIASP® as their onset of action is faster compared to FIASP®; the minimum glucose levels for the insulin analogues of the invention is lower compared to FIASP®. Furthermore, the offset of action is faster for the insulin analogues of the invention when compared to FIASP®, Faster offset of action is beneficial for insulin pumps.

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.

**CLAIMS**

1. An insulin analogue, wherein the analogue comprises A9E or A9D and further comprises B3E and/or desB30 relative to human insulin.

2. The insulin analogue according to claim 1, wherein the analogue further comprises at least one of B26E, B27E and/ or B28E.

3. The insulin analogue according to claim 2, wherein the analogue additionally comprises the A21A substitution.

4. The insulin analogue according to any of the preceding claims, wherein the analogue is

A9D, B3E;
A9E, B3E;
A9D, desB30;
A9E, desB30;
A9D, B3E, desB30;
A9E, B3E, desB30;
A9D, B3E, B26E;
A9E, B3E, B26E;
A9D, B26E, desB30;
A9E, B26E, desB30;
A9D, B3E, B26E, desB30;
A9E, B3E, B26E, desB30;
A9D, A21A, B3E, B26E;
A9E, A21A, B3E, B26E;
A9D, A21A, B26E, desB30;
A9E, A21A, B26E, desB30;
A9D, A21A, B3E, B26E, desB30;
A9E, A21A, B3E, B26E, desB30;
A9D, B3E, B27E;
A9E, B3E, B27E;
A9D, B27E, desB30;
A9E, B27E, desB30;

A9D, B3E, B27E, desB30;
A9E, B3E, B27E, desB30;
A9D, A21A, B3E, B27E;
A9E, A21A, B3E, B27E;
A9D, A21A, B27E, desB30;
A9E, A21A, B27E, desB30;
A9D, A21A, B3E, B27E, desB30;
A9E, A21A, B3E, B27E, desB30;
A9D, B3E, B28E;
A9E, B3E, B28E;
A9D, B28E, desB30;
A9E, B28E, desB30;
A9D, B3E, B28E, desB30;
A9E, B3E, B28E, desB30;
A9D, A21A, B3E, B28E;
A9E, A21A, B3E, B28E;
A9D, A21A, B28E, desB30;
A9E, A21A, B28E, desB30;
A9D, A21A, B3E, B28E, desB30;
A9E, A21A, B3E, B28E, desB30;
A9D, B3E, B26E, B27E;
A9E, B3E, B26E, B27E;
A9D, B26E, B27E, desB30;
A9E, B26E, B27E, desB30;
A9D, B3E, B26E, B27E, desB30;
A9E, B3E, B26E, B27E, desB30;
A9D, A21A, B3E, B26E, B27E;
A9E, A21A, B3E, B26E, B27E;
A9D, A21A, B26E, B27E, desB30;
A9E, A21A, B26E, B27E, desB30;
A9D, A21A, B3E, B26E, B27E, desB30;
A9E, A21A, B3E, B26E, B27E, desB30;
A9D, B3E, B27E, B28E;
A9E, B3E, B27E, B28E;
A9D, B27E, B28E, desB30;

A9E, B27E, B28E, desB30;
A9D, B3E, B27E, B28E, desB30;
A9E, B3E, B27E, B28E, desB30;
A9D, A21A, B3E, B27E, B28E;
A9E, A21A, B3E, B27E, B28E;
A9D, A21A, B27E, B28E, desB30;
A9E, A21A, B27E, B28E, desB30;
A9D, A21A, B3E, B27E, B28E, desB30;
A9E, A21A, B3E, B27E, B28E, desB30;
A9D, B3E, B26E, B28E;
A9E, B3E, B26E, B28E;
A9D, B26E, B28E, desB30;
A9E, B26E, B28E, desB30;
A9D, B3E, B26E, B28E, desB30;
A9E, B3E, B26E, B28E, desB30;
A9D, A21A, B3E, B26E, B28E;
A9E, A21A, B3E, B26E, B28E;
A9D, A21A, B26E, B28E, desB30;
A9E, A21A, B26E, B28E, desB30;
A9D, A21A, B3E, B26E, B28E, desB30;
A9E, A21A, B3E, B26E, B28E, desB30;
A9D, B3E, B26E, B27E, B28E;
A9E, B3E, B26E, B27E, B28E;
A9D, B26E, B27E, B28E, desB30;
A9E, B26E, B27E, B28E, desB30;
A9D, B3E, B26E, B27E, B28E, desB30;
A9E, B3E, B26E, B27E, B28E, desB30;
A9D, A21A, B3E, B26E, B27E, B28E;
A9E, A21A, B3E, B26E, B27E, B28E;
A9D, A21A, B26E, B27E, B28E, desB30;
A9E, A21A, B26E, B27E, B28E, desB30;
A9D, A21A, B3E, B26E, B27E, B28E, desB30; or
A9E, A21A, B3E, B26E, B27E, B28E, desB30.

5. The insulin analogue according to claim 4, wherein the analogue is

A9E, B3E, B26E;
A9E, B3E, B27E, B28E;
A9E, B3E, B26E, desB30;
A9E, B3E, B27E, B28E, desB30;
A9D, B3E, B26E, desB30; or
A9E, A21A, B3E, B26E, desB30.

6. The insulin analogue according to claim 5, wherein the analogue is A9E, B3E, B26E, desB30.

7. The insulin analogue according to claim 5, wherein the analogue is A9E, B3E, B27E, B28E, desB30.

8. A pharmaceutical composition comprising an insulin analogue according to any one of claims 1-6, and one or more pharmaceutically acceptable excipients.

9. The pharmaceutical composition according to claim 8, wherein the insulin analogue is in a concentration of from 0.6mM to 3mM.

10. The pharmaceutical composition according to any one of claims 8-9, wherein the composition is free of zinc.

11. The pharmaceutical composition according to any one of claims 8-10, comprising a nicotinic compound, and in particular nicotinamide.

12. The pharmaceutical composition according to any one of claims 8-10, wherein the composition is free of a nicotinic compound, and in particular nicotinamide.

13. The insulin analogue according to any one of claims 1-7, wherein the insulin analogue is monomeric in a zinc free pharmaceutical composition.

14. The insulin analogue according to any one of claims 1-7, wherein the insulin analogue is chemically and/or physically stable in a zinc free pharmaceutical composition.

15. The insulin analogue according to any one of claims 1-7 or a pharmaceutically acceptable salt thereof or the pharmaceutical composition according to any one of claims 8-12, for use as a medicament.

16. Use of the insulin analogue according to any one of claims 1-7 in the manufacture of a medicament for the treatment, prevention or alleviation of a disease or disorder or condition of a human body, wherein the disease, disorder or condition relates to diabetes, in particular Type 1 diabetes, or Type 2 diabetes.