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(54) Title: NOVEL INSULIN ANALOGS

(57) Abstract

Insulin analogs have been developed which are well suited for transdermal administration by iontophoresis. The insulin analogs have a negative charge at neutral pH and a reduced tendency to association. They are prepared by altering the human insulin molecule by substituting at least two of the amino acid residues of human insulin by Glu and/or Asp at selected positions in the molecule.

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NOVEL INSULIN ANALOGS

FIELD OF THE INVENTION

The present invention relates to novel insulin analogs for transdermal administration by iontophoresis, to compositions containing the novel insulin analogs and to the use of the insulin analogs for the treatment of diabetes by transdermal administration by iontophoresis.

BACKGROUND OF THE INVENTION

The transdermal administration of drugs in order to obtain systemic effects has gained increasing recognition in recent years and this administration method is increasingly being used for delivery of low molecular weight drugs. The conventional transdermal delivery by passive diffusional transport of the drug across the skin barrier is useful only for delivery of drugs which are relatively small in molecular size and lipophilic (hydrophobic) in nature. The anti-sea sickness patch and the nitroglycerin patch to which this mode of administration has been applied are both low molecular weight drugs which are lipophilic (hydrophobic) in character.

However, passive transdermal delivery is not well suited to, and may not even be possible for drugs which are low in skin permeability. Unfortunately, such drugs include pharmacologically active hydrophilic peptides or proteins.

In order to obviate the limitations on passive forms of transdermal delivery, the art has considered iontophoresis for systemic delivery of ionizied hydrophilic drugs. Iontophoresis may be described as being the transfer of solutes through a biologic membrane under the influence of an electrical current.

Transport through the skin by iontophoresis is believed to occur primarily through aqueous pores in the skin,

e.g. sweat ducts. Since the net polarity of the pores in human skin is negative at neutral pH, delivery of positively charged molecules therethrough can be facilitated by application of an electrical current.

The efforts made by the art, heretofore, to administer insulin through iontophoresis have been without great success. See Stephen, R. L. et al. "Potential novel methods for insulin administration: I. Iontophoresis", Biomed. Biochim. Acta **43** (1984) 553 - 558. Stephen et al. attributed their failure to force insulin across the skin barrier in pharmacologically adequate amounts to the relatively large size of the hexamer form of the insulin molecules (in solution). Stephen et al. postulated that a more strongly ionized predominantly monomeric form of insulin might sucessfully be administered transdermally by iontophoresis.

Subsequent to the work of Stephen et al. other investigators have reported achieving a more sucessful transdermal transport of human insulin by iontophoresis, see Meyer et al., "Transdermal Delivery of Human Insulin to Albino Rabbits Using Electrical Current", The American Journal of Medical Sciences, **297** (1989) 321 - 325 and Ovais Siddiqui et al., "Facilitated Transdermal of Insulin", Journal of Pharmaceutical sciences, **76** (1987) 341 - 345.

In the treatment of diabetes, parenteral administration of a predetermined quantity of insulin is presently required in order to maintain a therapeutically effective level of insulin in the blood. Due to the discomfort of the several daily (sub-cutaneous) injections of insulin that typically are administered, much effort has been expended by the art over the years to find an administration route for insulin, other than through sub-cutanous injection. Transdermal iontophoretic delivery offers the potential for being a superior route for administration of insulin. However, molecules of the usual insulin compound administered, e.g., the porcine insulin or human insulin, that heretofore has been conventional in the pharmaceutical compositions, is present in solution in such compositions in an associated

form, notably as dimers and hexamers, the latter being the predominant association species.

The insulin hexamer has the shape of a slightly flattened sphere. The diameter of the insulin hexamer is approximately 50Å, which diameter is believed to be too large for transdermal delivery (through the pores in the skin) in sufficient quantities to generate the therapeutically needed blood levels of insulin.

It is the purpose of the present invention to provide novel human insulin analogs with a smaller diameter which can be driven across the skin barrier through iontophoresis in therapeutically effective amounts.

SUMMARY OF THE INVENTION

Briefly stated, the present invention relates to a method for transdermally administering an insulin analog with a negative charge at neutral pH and with a reduced tendency to association. The method comprises placing an aqueous medium of the human insulin analog in contact with human skin then applying direct current to said insulin analog medium and therethrough to the skin in contact with the medium. Pharmacologically effective quantities of the insulin analog then cross the skin barrier through iontophoresis. The human insulin analog employed in practice of this invention is characterized by exhibiting a greater negative charge of at least one unit at neutral pH than that exhibited by human insulin.

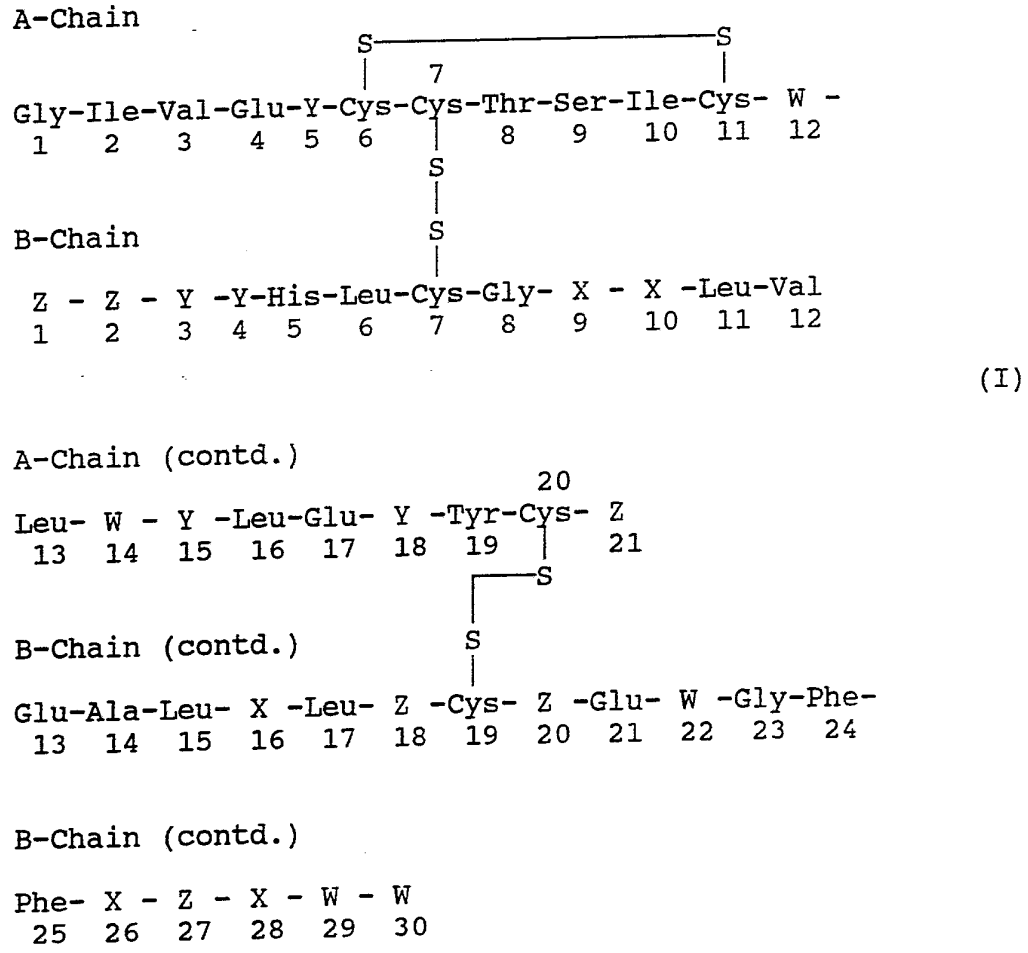
By "insulin analogs" as used herein is meant a compound having a molecular structure similar to that of human insulin including the disulphide bridges between Cys(A7) and Cys(B7) and between Cys(A20) and Cys(B19) and an internal disulphide bridge between Cys(A6) and Cys(A11) and with insulin activity.

Desirably, the insulin analog medium is made the cathode in the electrical circuit through the skin and the pH

of the insulin analog medium is in the range of pH 4 - 8.

The present invention also relates to novel human insulin analogs characterized by having improved properties for delivery by iontophoresis. These analogs have a reduced tendency to association and although they carry an increased net negative charge at neutral pH, analogs of this type have in iontophoretic <u>in vitro</u> experiments surprisingly been shown to have substantially enhanced transport through skin as compared to human insulin. The negative net charge is provided by substituting at least two of the amino acid residues in human insulin by a Glu or Asp residue.

The present insulin analogs can be characterized by having the following formula I:



wherein the amino acid residue in at least one of the positions designated X or Z and furthermore the amino acid residue in at least one of the positions designated Y or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

One group of the above insulin analogs are such wherein in formula I one amino acid residue in a position designated X and furthermore the amino acid residue in at least one of the positions designated Y, Z or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

Another preferred group are such wherein in formula I two amino acid residues in positions designated X or Z and furthermore the amino acid residue in at least one of the positions designated Y or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

In the above insulin analogs one, two or three amino acid residues in a position designated W may independently be substituted with Asp or Glu. Also one, two or three of the amino acid residues in a position designated Y may independently be substituted by Asp or Glu.

If no W is substituted in formula I, or if the terminal ends of the B-chain have not been extended, then the total number of substitutions is preferably at least five. Preferred X positions for substitution are B9, B16, B26 and B28.

Examples of insulin analogs according to the present invention are:

Glu(B9),Glu(A12) human insulin,
Asp(B9),Glu(A12) human insulin,
Glu(B9),Asp(A12) human insulin,
Glu(B9),Glu(A14) human insulin,
Glu(B9),Glu(B22),Glu(B26) human insulin,
Asp(B9),Glu(B22),Glu(B26),Asp(A21) human insulin,
Glu(B3),Glu(B16),Asp(A12),Asp(A21) human insulin,
Glu(B16),Glu(B22) human insulin,
Asp(B16),Glu(B22) human insulin,
Glu(B16),Asp(A14) human insulin,
Glu(B16),Glu(B21),Asp(A14) human insulin,
Glu(B26),Glu(B29) human insulin,
Asp(B26),Glu(B30) human insulin,
Asp(B28),Glu(A12) human insulin,
Asp(B2),Glu(B16),Asp(A12),Asp(A21) human insulin,
Glu(B3),Glu(B9),Glu(B29) human insulin,
Glu(B9),Asp(B22),Glu(B31) human insulin,
Asp(B9),Asp(B16),Glu(B26),Glu(B28),Asp(A21) human insulin,
Asp(B16),Asp(B26),Glu(B31),Asp(B32) human insulin,
Asp(B22),Glu(B26) human insulin,
Glu(B9),Asp(B22),Glu(B31) human insulin and
Asp(B1),Asp(B4),Asp(B10),Asp(B16),Glu(B27) human insulin.

DETAILED DESCRIPTION OF THE INVENTION

The novel insulin analogs according to the present invention can be prepared by altering the proinsulin gene through replacement of codon(s) at the appropriate site in the native human proinsulin gene by codon(s) encoding the desired amino acid residue substitute(s) or by synthesizing the whole DNA-sequence encoding the desired insulin analog. The gene encoding the desired insulin analog is then inserted into a suitable expression vector which when transferred to a

suitable host organism, e.g. E. coli, Bacillus or yeast, generates the desired product. The expressed product is then isolated from the cells or the culture broth depending on whether the expressed product is secreted from the cells or not.

The novel insulin analogs may also be prepared by chemical synthesis by methods analogue to the method described by Märki et al. (Hoppe-Seyler's Z. Physiol.Chem., 360 (1979), 1619 - 1632). They may also be formed from separately in vitro prepared A- and B-chains containing the appropriate amino acid residue substitutions, whereupon the modified A- and B-chains are linked together by establishing disulphide bridges according to known methods (e.g. Chance et al., In: Rick, D.H., Gross, E. (Editors) Peptides: Synthesis - Structure - Function. Proceedings of the Seventh American Peptide Symposium, Illinois, pp. 721 - 728).

The insulin analogs may furthermore be prepared by a method analogue to the method described in EP patent application No. 0163529A, the disclosure of which is incorporated by reference hereinto. By such a method an insulin precursor of human insulin wherein Lys(B29) is connected to Gly(A21) by means of either a peptide bond or a peptide chain of varying length with correctly positioned disulphide bridges is expressed and secreted by yeast and then converted into human insulin by the so-called transpeptidation reaction.

Accordingly, the present insulin analogs may be prepared by inserting a DNA-sequence encoding a precursor of the insulin analog in question into a suitable yeast expression vehicle which when transferred to yeast is capable of expressing and secreting the precursor of the insulin analog in which Lys(B29) is connected to Gly(A21) by a peptide bond or a peptide chain with the formula II

$$-R_n-R^1- \qquad (II)$$

wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R^1 is Lys or Arg when culturing

the transformed yeast strain in a suitable nutrient medium. The precursor is then recovered from the culture broth and reacted with an amino compound with the formula III

$$R^2\text{-}R^3\text{-}R^4\text{-}OR^5 \qquad \text{(III)}$$

wherein R^2 is Thr, Asp or Glu, R^3 and R^4 are each either Glu or Asp or a peptide bond, and R^5 is a carboxy protecting group (e.g. methyl or tert-butyl), using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents analogously as described in US patent specification No. 4,343,898 (the disclosure of which is incorporated by reference hereinto) whereupon the carboxy protecting group is removed and the insulin analog is isolated from the reaction mixture.

The insulin analogs may also be prepared by a method analogous to the method described in EP patent application No. 86302133.3 the disclosure of which is incorporated by reference hereinto. By this method insulin precursors of the type having a bridge between the A- and B-chain consisting of a single pair of basic amino acid (Lys, Arg) are made in yeast and then converted into insulin by an enzymatic conversion.

Genes encoding the precursors of the insulin analog can be prepared by modification of genes encoding the corresponding human insulin precursors by site specific mutagenesis to insert or substitute with codons encoding the desired mutation. A DNA-sequence encoding the precursor of the insulin analog may also be made by enzymatic synthesis form oligonucleotides corresponding in whole or part to the insulin analog precursor gene.

DNA-sequences containing a gene with the desired mutation are then combined with a suitable promoter sequence, e.g. fragments coding for the TPI promoter (TPIp) (Alber, T. and Kawasaki, G., Nucleotide Sequence of the triose Phosphate Isomerase Gene of Saccharomyces cerevisiae. J.Mol. Applied Genet. **1** (1982), 419 - 434), a suitable leader sequence and

possible transcription termination sequence, e.g. from TPI of S. cerevisiae (TPI_T). These fragments provide sequences to ensure a high rate of transcription for the precursor encoding gene and also provide a presequence which can effect the localization of precursor into the secretory pathway and its eventual excretion into the growth medium. The expression units are furthermore provided with a yeast origin of replication, for instance the 2μ origin, and a selectable marker, for instance LEU 2.

For the purpose of this invention we prepared the genes encoding the insulin analog precursor in question by ligation of oligonucleotides followed by insertion of the gene into a yeast expression plasmid as described by L. Thim et al., Proc.Natl.Acad.Sci. USA **83** (1986), 6766 - 6770, and J. Markussen et al., Protein Engineering **1** (1987), 215 - 223.

Example 1

Production of Glu(B9),Glu(A12) human insulin

A synthetic gene for the precursor Glu(B9),B(1-29)-Ala-Ala-Lys-Glu(A21),A(1-21) was constructed from oligonucleotides by ligation. The oligonucleotides were synthesized on an automatic DNA synthesizer using phosphoramidite chemistry on a controlled pore glass support (Beaucage, S.L. and Caruthers, M.H. Tetrahydron Letters **22** (1981), 1859 - 1869). The synthetic precursor gene was composed as follows:

		B1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
K	R	F	V	N	Q	H	L	C	G	E	H	L	V	E	A	L	Y	L

AAAGATTCGTTAACCAACACTTGTGCGGTGAGCACTTGGTTGAAGCTTTGTACTTGG-
AAGCAATTGGTTGTGAACACGCCACTGGTGAACCAACTTCGAAACATGAACC-

```
18 19 20 21 22 23 24 25 26 27 28 29          A1 2  3  4
 V  C  G  E  R  G  F  F  Y  T  P  K  A  A  K  G  I  V  E

TTTGCGGTGAAAGAGGTTTCTTCTACACTCCTAAGGCTGCTAAGGGTATTGTCGAAC-
AAACGCCACTTTCTCCAAAGAAGATGTGAGGATTCCGACGATTCCCATAACAGCTTG-

 5  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21
 Q  C  C  T  S  I  C  E  L  Y  Q  L  E  N  Y  C  N

AATGCTGTACCTCCATCTGCGAATTATACCAATTGGAAAACTACTGCAACT-
TTACGACATGGAGGTAGACGCTTAATATGGTTAACCTTTTGATGACGTTGA-

AGACGCAGCCCGCAGGCT
TCTGCGTCGGGCGTCCGAGATC
```

Letters and numbers above the DNA-sequence indicate the corresponding amino acid residues (using the one letter abbreviation) and their position, respectively, in the B- and in the A-chain. The sequence AAK (AlaAlaLys) is the bridge which connects the position B29 of the B-chain and position A1 of the A-chain.

By a method analogous to that described in WO 89/10937 a plasmid encoding the following sequence:

TPI_P-MFα1-signal-leader(1-85)-precursor gene-TPI_T

where MFα1 is the S. cerevisiae MFα1 coding sequence (Kurjan, J. and Herskowitz, I., Cell **30** (1982) 933 - 943) and signal-leader(1-85) means that the sequence contains the first 85 amino acid residues of the MFα1 signal-leader sequence was obtained.

An S. cerevisiae (E2-7B XE11-36 a/α, Δtpi Δtpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an optical density at 600 nm of 0.6.

100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of a solution containing 1.2 M sorbitol, 25 mM Na_2EDTA pH = 8.0, and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate, pH = 5.8, and 2 mg Novozym® 234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl (Tris = Tris(hydroxymethyl)aminomethan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 μg of the above described plasmid and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 μg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, resisolated and used to start liquid cultures.

Transformant strains were grown on YPD medium (1% yeast extract, 2% peptone (from Difco laboratories), and 2% glucose) in a 1500 liter tank at 30°C. The expressed product was isolated from the culture broth.

Transpeptidation

L-threonine methyl ester acetate (38.6 g, 0.2 mole) was dissolved in dimethylformamide to give 100 ml of solution, 50 ml 76.5% v/v dimethylformamide in water was added and 10 g of crude Glu(B9),B(1-29)-Ala-Ala-Lys-Glu(A12),A(1-21) human insulin was dissolved in the mixture, which was thermostated at 12°C. Then 1 g of trypsin in 25 ml 0.05 M calcium acetate was added and after 24 hours at 12°C the mixture was added to 2 liters of acetone and the precipitated peptides were isolated by centrifugation and dried in vacuo. The Glu(B9),Glu(A21),B30Thr-OMe human insulin was purified on a preparative HPLC column with silica-C18 as column material.

The Glu(B9),Glu(A12),B30Thr-OMe human insulin was dispersed in water to 1% (w/v) and was dissolved by addition of 1 N sodium hydroxide to a pH value of 10.0. The pH value was kept constant at 10.0 for 24 hours at 25°C. The Glu(B9),Glu(A12) human insulin formed was precipitated by addition of sodium chloride to about 8% (w/v), sodium acetate trihydrate to about 1.4% (w/v), and zinc acetate dihydrate to about 0.01% (w/v) followed by addition of 1 N hydrochloric acid to pH 5.5. The precipitate of Glu(B9),Glu(A12) human insulin was isolated by centrifugation and purified by anion exchange chromotography and desalted by gel filtration.

Example 2

Production of Asp(B1),Asp(B4),Asp(B10),Asp(B16),Glu(B27) human insulin.

The title product was prepared by a method analogous to the method described in Example 1, using a synthetic precursor gene having the following composition:

```
      B1 2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17
   K  R  D  V  N  D  H  L  C  G  S  D  L  V  E  A  L  D  L

AAAGAGACGTTAACGATCACTTGTGCGGTTCCGACTTGGTTGAAGCTTTGGACTTGG-
    CTGCAATTGCTAGTGAACACGCCAAGGCTGAACCAACTTCGAAACCTGAACC-

 5 18 19 20 21 22 23 24 25 26 27 28 29          A1 2  3  4
   V  C  G  E  R  G  F  F  Y  E  P  K  A  A  K  G  I  V  E

TTTGCGGTGAAAGAGGTTTCTTCTACGAACCTAAGGCTGCTAAGGGTATTGTCGAAC-
AAACGCCACTTTCTCCAAAGAAGATGCTTGGATTCCGACGATTCCCATAACAGCTTG-

   5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21
10 Q  C  C  T  S  I  C  S  L  Y  Q  L  E  N  Y  C  N

AATGCTGTACCTCCATCTGCTCCTTGTACCAATTGGAAAACTACTGCAACT-
TTACGACATGGAGGTAGACGAGGAACATGGTTAACCTTTTGATGACGTTGA-

AGACGCAGCCCGCAGGCT
TCTGCGTCGGGCGTCCGAGATC
```

15 Testing of Insulins by in vitro Iontophoresis.

Three different insulins, 1) human insulin, (1), 2) a monomeric insulin analog (2), with two extra negative charges in the molecule, Asp(B9),Glu(B27) human insulin, obtained as described in EP application No. 86306721.1, and 3) 20 a monomeric insulin analog (3), with five extra negative charges in the molecule, Asp(B1),Asp(B4),Asp(B10),Asp(B16),-Glu(B27) human insulin, obtained as described in the present Example 2, were compared in vitro with respect to transdermal flux by iontophoresis.

The measurements were made in a cell as described by Glikfeld, P. et al., Pharmaceutical Research 5 (1988) 443 - 446. The anode and the cathode were positioned on the same side of a piece of hairless mouse skin which was stripped twenty times with adhesive tape. Ag/AgCl electrodes, used in order to minimize pH changes in the electrode chambers, were mounted 3 mm from the surface of the skin. A phosphate buffered (0.03 M, pH 7.4) insulin solution was placed in the cathode chamber and a similar buffer containing saline but without insulin was placed in the anode chamber. The cell was assembled with the skin separating the electrode compartments and the receptor compartment. The latter compartment was filled with phosphate buffer containing 0.3% bovine serum albumin and perfused with the same solution at a rate of 2 ml per hour. Iontophoresis was carried out at a constant current of 0.5 mA/cm^2 supplied to the electrodes from a commercial power supply for 6 hours. In the experiment the exposed area of the mouse skin was 0.55 cm^2 and the current applied was 0.28 mA. The 2 ml fractions of perfusion liquid collected every hour were analysed by insulin radio immunoassay.

Table 1 reports the results of the experiments. The concentration of insulin in the fractions collected during the experiment with human insulin (1) was below the detection limit. The transport per <u>in vitro</u> cell of the insulin analogs (2) and (3) which did pass the mouse skin is expressed relative to the concentration of the analog in question in the donor chamber, i.e. transport normalized by concentration.

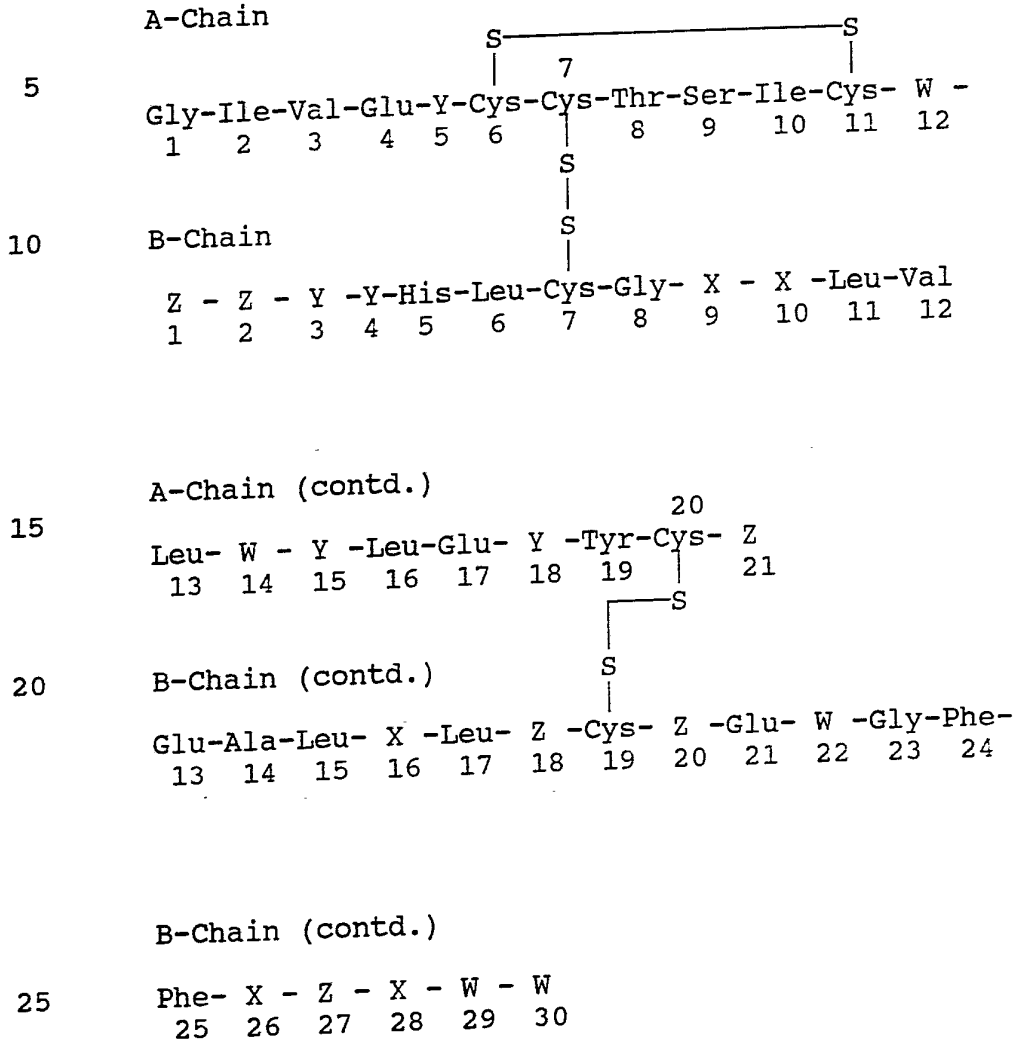
Table 1

Hours	Accumulated transport of test Compound X 10^3 (cm^3)		
	(1)	(2)	(3)
0	0	0	0
1	0	0.1	0.6
2	0	0.6	2.3
3	0	1.4	4.6
4	0	2.1	5.9
5	0	2.6	6.9
6	0	3.3	8.4

In summary, whereas no measurable flux of human insulin through the mouse skin could be detected during the 6 hours iontophoresis, a nearly constant flux was observed with the two monomeric analogs with the most negatively charged analog (3) exhibiting a flux 2-3 times higher than the flux of (2).

CLAIMS

1. Insulin analogs having the general formula I:



(I)

wherein the amino acid residue in at least one of the positions designated X or Z and furthermore the amino acid residue in at least one of the positions designated Y or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the

B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

2. Insulin analogs according to Claim 1 wherein one amino acid residue in a position designated X and furthermore the amino acid residue in at least one of the positions designated Y, Z or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

3. Insulin analogs according to Claim 2 wherein two amino acid residues in positions designated X or Z and furthermore the amino acid residue in at least one of the positions Y or W independently is substituted by Asp or Glu while any remaining X, Y, Z or W designates the amino acid residue occuring in human insulin in the pertinent position, the terminal ends of the B-chain optionally being extended by one or two amino acid residues which can be chosen independently among Asp and Glu.

4. Insulin analogs according to any of the predecing claims wherein the total number of substitutions is at least five.

5. Insulin analogs according to any of the preceding claims wherein one amino acid residue in a position designated W is substituted by Asp or Glu.

6. Insulin analogs according to any of the claims 1 -4 wherein two amino acid residues in positions designated W independently are substituted by Asp or Glu.

7. Insulin analogs according to any of the claims 1 -4 wherein three amino acid residues in positions designated W independently are substituted by Asp or Glu.

8. Insulin analogs according to any of the claims 1 -4 wherein one amino acid residue in a position designated Y is substitued by Asp or Glu.

9. Insulin analogs according to any of the claims 1 -4 wherein two amino acid residues in positions designated Y independently are substituted by Asp or Glu.

10. Insulin analogs according to any of the claims 1 -4 wherein three amino acid residues in positions designated Y independently are substituted by Asp or Glu.

11. A pharmaceutical composition comprising an insulin analog according to any of the preceding claims.

12. The use of an insulin analog according to any of the claims 1 - 10 for the manufacture of a pharmaceutical composition for the treatment of diabetes.

13. Any novel feature or combination of features as herein described.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00019

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/40, A 61 K 37/26 // C 07 K 99:26

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO, A1, 8910937 (NOVO-NORDISK A/S) 16 November 1989, see example 2 and the claims --	1-13
A	NATURE, Vol 333, Nr 6174, 16 June 1988, J. Brange et al: "Monomeric insulins obtained by protein engineering and their medical implications", p 679-682 --	1-13
A	EP, A2, 0214826 (NOVO INDUSTRI A/S) 18 March 1987, see the whole document --	1-13
A	WO, A1, 9007522 (NOVO-NORDISK A/S) 12 July 1990, see the whole document --	1-13

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* Special categories of cited documents:[10]

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

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

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

"O" document 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

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th April 1992	1992 -05- 05
International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Elisabeth Carlborg

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00019

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 28/03/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8910937	89-11-16	AU-B-	615760	91-10-10
		AU-D-	3692889	89-11-29
		EP-A-	0419504	91-04-03
		JP-T-	3504240	91-09-19
EP-A2- 0214826	87-03-18	AU-B-	593274	90-02-08
		AU-D-	6206686	87-03-05
		JP-A-	62053999	87-03-09
WO-A1- 9007522	90-07-12	AU-D-	4834490	90-08-01
		CA-A-	2006578	90-06-23
		EP-A-	0375437	90-06-27