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(54) Title: NOVEL INSULIN DERIVATIVES AND PHARMACEUTICAL PREPARATIONS CONTAINING THESE DERIVATIVES (57) Abstract Insulin derivatives wherein the B30 carboxyl group is blocked with an uncharged group and optionally one to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and B21 are blocked with or converted into an uncharged group provided that when only the B30 carboxyl group is blocked with an uncharged group, said group comprises an alkyl group having at least 8 carbon atoms, and pharmaceutical preparations containing these insulin derivatives.		

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Novel insulin derivatives and pharmaceutical preparations containing these derivatives.

TECHNICAL FIELD

The present invention relates to novel insulin derivatives
5 and to insulin preparations showing protracted action and comprising at least one of the novel insulin derivatives and, if desired, a fast acting insulin.

BACKGROUND ART

In severe or chronic cases the disease of Diabetes is usually
10 treated with injection preparations containing insulin, e.g. porcine insulin, bovine insulin or human insulin.

Soluble insulin preparations are usually fast acting, but in return the action ceases after few hours. Therefore, it is necessary to give frequent injections, normally several
15 times a day.

In order to overcome this disadvantage insulin preparations with protracted action have been formulated so that the action is maintained for several hours or even up to 24 hours or longer. Using such protracted preparations the diabetic
20 patient only has to receive a smaller number of injections, e.g. a single or two injections during 24 hours.

Such a protracted action can be achieved by converting the insulin to a slightly soluble salt, such as zinc insulin or protamin insulin. The slightly soluble insulin salts are
25 used in the form of suspensions from which the insulin is gradually released after subcutaneous injection.

Recently other methods have been invoked to achieve a pro-

tracted action. An example hereof is the encapsulation of insulin crystals in polymerized serum albumin. Another example is continuously acting infusion devices, so-called insulin pumps, which however may be uncomfortable and entail
5 a risk to the patient.

The specifications of Danish patent applications No. 3582/84 and No. 3583/84 disclose the preparation and use of insulin derivatives wherein the C-terminus of the B-chain is extended with an organic group carrying at least one positive
10 charge, preferably Arg-OH or Arg-Arg-OH. Preparations containing suspensions of such insulin derivatives exhibit a protracted action.

DISCLOSURE OF THE INVENTION

It has now surprisingly been found that by the use of insulin derivatives, wherein the B30 carboxyl group is blocked
15 with an uncharged group and optionally up to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and B21 are blocked with or converted into an uncharged group, in insulin preparations, in
20 particular injection preparations, a hitherto unknown and desirably long-lasting protracted action is achieved. This effect is particularly pronounced, if the B30 carboxyl group is blocked with a group containing an alkyl chain of
at least 8 carbon atoms, or if the B30 carboxyl group is
25 blocked with an uncharged group and at least one and not more than four additional carboxyl groups at the same time are blocked with or converted into an uncharged group.

Accordingly, the invention relates to insulin derivatives wherein the B30 carboxyl group is blocked with an uncharged
30 group and optionally one to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and B21 are blocked with or converted into an uncharged group provided that when only the B30 carboxyl group is blocked with an uncharged group, said group comprises an

alkyl group having at least 8 carbon atoms.

The invention relates specifically to insulin derivatives wherein only the B30 carboxyl group is blocked with an uncharged group which comprises an alkyl group of 8-12 carbon atoms.

The invention relates in particular to insulin derivatives in which the B30 carboxyl group is blocked as an alkyl amide having at least 8 carbon atoms.

The invention relates particularly to insulin derivatives wherein one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is blocked with or converted into an uncharged group and the B30 carboxyl group is blocked as an amide or with an uncharged group comprising an alkyl group of 1-12 carbon atoms.

The invention relates specifically to insulin derivatives wherein one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is blocked with or converted into an uncharged group and the B30 carboxyl group is blocked with an uncharged group comprising an alkyl group of 8-12 carbon atoms.

The invention also relates to insulin preparations containing at least one insulin derivative wherein the B30 carboxyl group is blocked with an uncharged group and optionally one to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and B21 are blocked with or converted into an uncharged group, provided that when only the B30 carboxyl group is blocked with an uncharged group, said group comprises an alkyl group having at least 8 carbon atoms and, if desired, also containing a fast acting insulin.

A particularly advantageous embodiment of the insulin pre-

paration of the invention is an injection preparation exhibiting protracted insulin action and comprising a suspension of at least one insulin derivative of the invention in an isotonic aqueous medium having a neutral pH and optionally containing a buffer and/or a preserving agent and, if desired, a fast acting insulin.

Insulins which are normally used for the treatment of Diabetes, such as porcine, bovine, ovine, and human insulin, do all contain six free carboxylic groups, viz. in the A4-, A17-, A21-, B13-, B21- and B30-amino acid residues. The numbering is referring to the positions in the insulin A and B chain, respectively, starting from the N termini. It is known that an insulin in which the B30 carboxyl group is converted into a methyl ester or amide possesses unchanged biological activity, vide H. Zahn, et al.: *Naturwissenschaften*, 68, (1981), p. 56-62, and M. Kobayashi, et al.: *Diabetes*, 30 (1981), p. 519-22. A protracted action was not mentioned. The biological activity of insulin will usually decrease by an increasing degree of derivatization. However, the biological activity is influenced surprisingly little even at high degrees of derivatization of the free carboxylic acid groups; however, when all six free carboxylic acid groups are derivatized the biological activity is completely abolished, vide D. Levy: *Biochem. Biophys. Acta*, 310 (1973), pages 406-415.

By means of the insulin preparations of the invention the insulin activity can be maintained, and at the same time a surprisingly protracted action can be achieved. This protracting effect is dependent on the properties of the specific derivative, i.e. not only on the number of carboxylic acid groups being blocked, but also on the size and the chemical composition of the blocking group. Thus, it is possible to vary the protracted action at will.

In the case of Insulin-Dependent Diabetes a frequently used

therapy consists in two daily injections of a protracted insulin preparation, one in the morning and one in the evening just before bedtime, in order to create a basic insulin level. Additionally, three injections of a fast acting insulin preparation are given prior to the principal meals. The disadvantage of this therapy is that the late injection of the protracted preparation may result in a dangerously low blood glucose level in the course of the night. This situation may be avoided by injecting a mixed preparation of a protracted and a fast acting insulin before supper, whereby hypoglycemia will, if at all, occur during the evening, where it can be averted by means of a light snack. However, this type of the therapy often results in hyperglycemia during the morning, as the most used protracted insulin preparations "Insulatard" [®] and "Monotard" [®] do not act long enough. Therefore, it is desirable to provide the diabetic patient with insulin preparations that act longer than the preparations commonly in use, in particular if one injection of such preparations will suffice for one or even several days. Insulin preparations prepared according to the invention exhibit a protracted insulin action of the same or longer duration than that of the commonly used protracted insulin preparation "Insulatard" [®].

With particular insulin derivatives of the invention in which only the B30 carboxyl group is blocked with an uncharged group, derivatives are preferred in which the blocking takes place in the form of alkyl ester or alkyl amide, wherein the alkyl group contains from 8 to 18 carbon atoms, in particular in the form of N-alkyl. If the alkyl group is smaller, the prolongation will not become substantially different from that of "Insulatard" [®], and if the alkyl group is larger, solubility problems will arise which make the preparation of the derivatives very difficult.

Among the insulin derivatives of the invention wherein the B30 carboxyl group and additionally from 1 to 4 carboxyl groups are blocked with an uncharged group, derivatives are

preferred in which the blocking takes place in the form of amide, alkyl ester and, as far as the B30 carboxyl group is concerned, also in the form of alkyl amide, whereby an alkyl group in the B30 carboxyl group may contain up to
5 18 carbon atoms, but in the remaining carboxyl groups not more than four carbon atoms only.

The invention relates in particular to the following specific compounds:

Insulin-B30-octyl ester, insulin-B30-dodecyl amide,
10 insulin-B30-hexadecyl amide, insulin-(B21,B30)-dimethyl ester, insulin-(A17,B30)-dimethyl ester, insulin-(A4,B30)-diamide, insulin-A17-amide-B30-octyl ester, insulin-(A4,B13)-diamide-B30-hexyl amide, insulin-(A4,A17,B21,B30)-tetramide, insulin-(A17,B30)-di-
15 amide, A4-ala-insulin-B30-amide, B30-leu-insulin-(A4,B30)-diamide.

When the insulin preparations of the invention contain a fast acting insulin, this insulin may e.g. be human or porcine insulin.

20 The insulin derivatives in question may be derived from porcine, bovine, ovine, rabbit, or in particular human insulin, including other such insulins wherein the B30 amino acid residue has been exchanged with another naturally occurring amino acid residue, e.g. by semisynthesis.

25 The insulin derivatives can be prepared in a manner known per se for the conversion of a carboxylic acid group into an uncharged group, e.g. by esterification or amidation. Thus, an insulin derivative wherein two or more of the total of six carboxylic acid groups are methylated, is e.g.
30 prepared by reacting human insulin-B30-methyl ester in borontrifluoride/methanol for a suitable time, followed by a fractionation of the modified insulin derivatives on an anion exchange column at a slightly basic pH value. Finally,

the possible products can be fractionated and isolated by preparative HPLC.

Derivatization of the B30 carboxyl group can take place by enzymatic catalysis, e.g. by following the process disclosed in Danish Patent Specification No. 146,482. According to this process the B30-amino acid is removed by digesting e.g. porcine insulin with carboxypeptidase A, and in the second step the des-B30-insulin formed is reacted in the presence of trypsin or a trypsin-like enzyme with a neutral amino acid ester, especially the threonine methyl ester.

A similar process is known from Danish Patent Application No. 574/80, according to which process the B30-amino acid in an insulin by a one pot method is exchanged by tryptic catalysis with the desired amino acid ester or amide. According to the above application the reaction may even be carried out using as starting material a proinsulin.

Some of the carboxyl group blockings preferred according to the invention can be established by biotechnological methods. This applies to the blocking of the carboxyl groups in the positions of A4, A17, B13 and B21 in the form of amides, or by changing some of or all the glutamic acids located at these positions with naturally occurring amino acids carrying an uncharged side chain.

Usually the amino acids in these positions are glutamic acid, but in some of the insulin derivatives of the invention they are exchanged with glutamine.

It is obvious to prepare these modified insulins by biosynthesis, as by changing only a single base in the codon in the DNA strand coding for glutamic acid this codon can be made code for e.g. glutamine. By changing bases in the DNA strand coding for insulin (or proinsulin) it is thus

possible to make modifications which would be difficult or impossible to make chemically due to limitations as to isolation.

There exists a number of different methods to prepare human insulin by biosynthesis. It is common to all of them that the DNA strand coding for either the entire proinsulin, a modified version thereof or the A- and B-chain separately has been inserted into a replicable plasmide containing a suitable promotor. By transforming this system into a given host organism a product can be produced, which can be converted into authentic human insulin in a manner known per se.

Some known methods for biosynthesis of proinsulin or A- and B-chain and their conversion to insulin are described below.

15 In the well known Genentech process an intracellular preparation in *E. coli* of polypeptides is made, wherein these polypeptides are a fusion between β -galactosidase and the A- and B-chain respectively, of insulin. After fermentation the two products are isolated, the A- and B-chains are split off from β -galactosidase by means of cyanogen bromide, whereafter the sulphitated chains are combined under reducing conditions in the presence of dithiothreitol at a pH value of 10.5. Finally human insulin is isolated from this reaction mixture.

25 In the process disclosed in EPO 55945 the product is synthesized as a fusion protein with another protein separated by a cleaving site. This may be a protease cleaving site or a site which can be cleaved chemically (e.g. methionine). The gene for proinsulin is e.g. cloned as a genefusion with another protein by means of recombinant DNA techniques. After isolation, the chimeric protein is cleaved by using a suitable enzyme or CNBr, whereupon the denatured proinsulin is isolated in a sulphitolyzed form. This product

is renatured under reducing conditions using β -mercapto ethanol at a pH of 10.5, as described by Frank, followed by trypsin/CpB cleavage as described by Kemmler (1971). The resulting insulin is isolated in a manner known per se.

5 Finally, proinsulin can be prepared biosynthetically by using the method disclosed in EPO 116201. In this method proteins are secreted into the culture medium by using the α -factor system from *Saccharomyces cerevisiae*. By inserting the gene coding for proinsulin into this system, pro-
10 insulin can be isolated from the culture medium by using the system described in DK patent application No. 3091/84. Hereafter, proinsulin can be converted into insulin by the known methods described above.

In the above, methods are described in which the product is
15 either proinsulin or A- and B-chain separately. Furthermore, these products are either synthesized together with a signal sequence, the purpose of which is to bring the product through to the cell surface where it is split, or as a fusion with a protein, the purpose of which is to stabilize
20 the product.

Moreover it is possible to prepare modified proinsulins biosynthetically. European Patent Application No. 82303071.3 discloses proinsulins wherein the C-chain is modified by means of recombinant DNA techniques.

25 The above-mentioned modifications of insulin can be prepared by expressing the modified DNA sequence in a suitable expression system. The changes of the DNA sequence can be made by in vitro mutagenesis of the insulin gene by a method described by Thomas A. Kunkel, Proc. Natl. Acad.
30 Sci., USA, Vol 82, pp. 448-492 (1985). According to this method the insulin gene is cloned into the single-stranded DNA bacteriophage M13. DNA from this phage is purified, and hereto a primer, typically a 15-25-mer, which contains the

given mutation (one mismatch) as well as a homology on each side of this mutation is annealed. The primer is then extended by use of a DNA polymerase in the total length of the phagegenome and thus provides the complete double-
5 -strand molecule, wherein one strand contains the mutation, and the other contains the wild type gene. By introducing this molecule into an E. coli cell, the phage progeny will partly be phages containing the wild type gene, partly phages containing the mutation. Among these phages it is
10 then possible to screen for the desired type by hybridization with the mutagenization primer to single-stranded DNA of the phage progeny. The primer will have complete homology to the mutated strand, but will be different from the wild type at a single nucleotide. After screening, double
15 strand DNA can be isolated from the E. coli cells, wherein the phage is replicated, and the modified insulin gene can be isolated herefrom. This gene is thereafter reinserted into the original expression system.

The injection preparations can comprise the insulin derivatives of the invention as amorphous and/or crystalline
20 suspensions, either separately or in combination with other such insulin derivatives and/or naturally occurring insulins. It has been found that a majority of these derivatives can be crystallized with zinc, either at a pH value
25 being higher than their isoelectric points, or at a pH value about the isoelectric pH value, when the crystallization medium contains a phenol. It is also possible to crystallize the insulin derivatives of the invention in the presence of protamin or an excess of zinc, which causes a
30 further protracted activity.

MODES FOR CARRYING OUT THE INVENTION

The invention is further illustrated by means of the following examples. However, Example 5 illustrates the preparation of an insulin derivative falling outside the scope
35 of the invention and used as comparative compound in Ex-

ample 6. Example 9 illustrates the preparation of starting materials used in Examples 1 and 5.

Example 1

Preparation of human insulin-B30-n-dodecyl amide

5 970 mg of L-threonine-n-dodecyl amide were dissolved in a mixture of 3.60 ml of t.butanol, 1.20 ml of dimethyl formamide and 1.40 ml of water. 450 mg of des-B30-alanine porcine insulin were dissolved in the mixture with stirring and the pH value adjusted to 6.4 - 6.5 with acetic acid. A
10 solution of 8 mg of trypsin in 0.20 ml of 0.001 M calcium acetate was added and the reaction mixture left at 20° C. for 2 hours. A sample was analyzed by reverse phase high pressure liquid chromatography which showed that 71 % of the des-B30-insulin was converted into insulin-B30-n-do-
15 decyl amide.

The reaction mixture was poured into 5 ml of 2-propanol and left for 15 minutes. The precipitated protein was isolated by centrifugation and the precipitate washed with 25 ml of 2-propanol and centrifuged again.

20 The precipitate was redissolved in 15 ml of 1 M acetic acid in 7 M urea, and after filtration the solution was applied to a 2.6 x 90 cm column packed with Sephadex® G50 Superfine and equilibrated with 1 M acetic acid. The protein was eluted with the same buffer at a rate of 25 ml per hour,
25 and fractions of 5 ml were collected. The optical density at 277 nm of the fractions were examined, whereupon the proteinaceous main fraction was collected and freeze-dried.

The freeze-dried protein was redissolved in 25 ml of 60 % by volume ethanol and the pH adjusted to 9.0 with 1 M TRIS.
30 After filtration, the solution was applied to a 1.6 x 20 cm column of Q-Sepharose® CL-6B FF, equilibrated with 0.02 M TRIS/hydrochloric acid in 60 % by volume ethanol, pH 8.25.

The column was eluted at 20° C. at a rate of 50 ml per hour, first for 1.5 hour with equilibration buffer and then with a linear gradient of from 0 to 0.1 M sodium chloride in the same buffer for 16 hours. The proteinaceous main fraction
5 eluted at a salt strength of about 0.05 M sodium chloride was collected, whereupon the ethanol was removed by evacuation. The pH value of the aqueous residue was adjusted to 6.3 with hydrochloric acid and centrifuged after standing at 4° C. for 1 hour, whereafter the isolated human insulin-
10 -B30-n-dodecyl amide was freeze-dried. Yield: 96 mg.

Example 2

Formulation and biological effect of an insulin preparation containing human insulin-B30-n-dodecyl amide.

30 mg of human insulin-B30-n-dodecyl amide were dissolved
15 in 10 ml of 0.05 M m-cresol by the addition of a small amount of hydrochloric acid, whereafter the solution was sterilized by filtration. 5 ml of a sterile solution being 0.05 M in respect to sodium dihydrogen phosphate and 0.7 M in respect to glycerol were then added to the solution,
20 whereupon the pH value was adjusted to 7.3 with sodium hydroxide solution. Finally, the volume was increased to 20 ml by addition of sterile water.

When the resulting suspension preparation was injected subcutaneously into guinea pigs (20 µlitres per kg) hypoglycemia persisted substantially longer than the hypoglycemic
25 effect produced by the standard NPH insulin preparation "Insulatard" ®.

Example 3

Preparation of human insulin-(B21,B30)-dimethyl ester and
30 human insulin-(A17,B30)-dimethyl ester, respectively.

500 mg of human insulin-B30-methyl ester were suspended in 50 ml of dry methanol. 2.0 ml of 20 % by weight borontri-

fluoride in methanol were added with stirring. After standing at ambient temperature for 2 hours the sample was poured into 400 ml of ether and centrifuged for 20 minutes at 2500 x g, 4° C. The precipitate was washed twice with
5 ether, resuspended in 150 ml of ether, evaporated in vacuo and dissolved in 50 ml of 20 mM TRIS/hydrochloride, 60 % by volume ethanol, pH value 8.25. The sample was then applied to a 1.6 x 21 cm Q-Sepharose (R) CL-6B Fast Flow column equilibrated with the same buffer. After 1 hour of isocratic
10 tic elution at a flow rate of 24 cm/hour the sodium chloride concentration was increased linearly from 0 to 0.1 M during the following 16 hours. The peak containing insulin dimethyl ester was collected in 3 main pools.

The first eluted pool was further fractionated on a
15 4 x 250 mm LiChrosorb (R) RP18 column (Merck 50333) equilibrated with a buffer containing 0.125 M ammonium sulphate, pH value 4.0 and acetonitrile. 1 - 2 mg of protein was applied to the column and eluted with the above buffer containing 38 % acetonitrile. The main proteinaceous fraction
20 eluted after 20 - 25 minutes. The acetonitrile was removed from the sample in vacuo and the protein applied to a SepPak (R) C18 column (Waters Ass. No. 51910), washed with H₂O and eluted with 70 % methanol. The latter was removed in vacuo, and the protein was freeze-dried. Hereby 20 ml of
25 human insulin-B21,B30-dimethyl ester were obtained.

The next eluted pool was treated in a corresponding manner, whereby 15 mg of human insulin-A17,B30-dimethyl ester were obtained.

Both human insulin dimethyl esters were characterized by
30 Plasma Desorption Mass Spectrometry, whereby the correct molecular weight was found, and by decomposition with S. Aureus protease, whereby the esterified glutamic acids were identified.

Example 4

Biological effect of preparations containing human insulin dimethyl esters

- 25 ml of a medium containing 1.6 % (w/v) of glycerol,
 5 0.33 % (w/v) of m-cresol, 1/75 M of sodium phosphate, pH value 7.3, were prepared. The solution was sterilized by filtration. 2.1 mg of human insulin-(B21,B30)-dimethyl ester, prepared as described in Example 3, were suspended in 1.0 ml of medium.
- 10 Analogously, 3.2 mg of human insulin-(A17,B30)-dimethyl ester, prepared as described in Example 3, were suspended in 1.0 ml of medium.

- Strongly protracted hypoglycemia was observed by administering these zinc-free preparations subcutaneously to guinea pigs. As it is apparent from the Table below, the protraction is comparable to the commonly used crystalline protamin-insulin preparation "Insulatard" [®].

Time (hours)		0	1	2	4	6
Insulatard ^R 11 µg/kg		100	74	45	82	98
20	Human insulin-(B21,B30)- -dimethyl ester 16 µg/kg	100	64	51	79	94
	Human insulin-(A17,B30)-di- methyl ester 24 µg/kg	100	50	38	55	94

The Table shows the blood glucose value in % of the starting value (n = 6 animals).

Example 5

Preparation of human insulin-B30-n-hexyl amide

290 mg of L-threonine-n-hexyl amide, prepared as described in Example 9, were dissolved in a mixture of 2.60 ml of 5 96 % by volume ethanol, 0.80 ml of water and 0.16 ml of acetic acid. 240 mg of des-B30-alanine porcine insulin were added with stirring and the pH value adjusted to 6.3 - 6.4 with triethylamine. A solution of 5 mg of trypsin in 0.10 ml of 0.001 M calcium acetate was then added and the reac- 10 tion mixture left at 12° C. After two hours the reaction mixture was poured into 20 ml of 2-propanol, and after standing for 15 minutes the precipitated protein was isolated by centrifugation. The precipitate was washed with 20 ml of 2-propanol and centrifuged again.

15 Analysis of the precipitate by reverse phase high pressure liquid chromatography showed a conversion of 65 %.

The precipitate was redissolved in 10 ml of 1 M acetic acid in 7 M urea, and, after filtration, the solution was applied to a 2.6 x 90 cm Sephadex® G50 Superfine column 20 equilibrated with 1 M acetic acid. This column was eluted with the same buffer at a rate of 25 ml per hour. The proteinaceous main fraction was collected and freeze-dried.

The freeze-dried protein was redissolved in 20 ml of 60 % by volume ethanol by adjusting the pH value to 8.25 with 25 1 M tris-(hydroxymethyl)-amino methane (TRIS). After filtration, the solution was applied to a 1.5 x 20 cm Q-Sepharose® CL-6B FF column, equilibrated with 0.02 M TRIS/-hydrochloric acid in 60 % by volume ethanol, pH 8.25. The column was eluted at 20° C. at a rate of 50 ml per hour, 30 first with equilibration buffer for 1.5 hour and for the following 16 hours with a linear gradient of from 0 to 0.1 M sodium chloride. The protein material eluting at a salt strength of ~ 0.05 M sodium chloride was collected,

whereupon the ethanol was removed in vacuo. The pH value of the aqueous evaporation residue was adjusted to 6.3 with hydrochloric acid and centrifuged after standing at 4° C. for 4 hours. The isolated human insulin-B30-n-hexyl amide
5 was freeze-dried. Yield: 50 mg.

Example 6

Formulation and biological effect of an insulin preparation containing human insulin-B30-n-hexyl amide

30 mg of human insulin-B30-n-hexyl amide were dissolved in
10 10 ml of 0.05 M m-cresol by adding a small amount of hydrochloric acid, whereupon the solution was sterilized by filtration. 5 ml of a sterile solution being 0.05 M in respect to sodium dihydrogen phosphate and 0.7 M in respect to glycerol were added to the solution and the pH value adjusted
15 to 7.3 with a sterile sodium hydroxide solution. Finally, sterile water was added to give a final volume of 20 ml.

Subcutaneous injection (20 plitres/kg) of this preparation into guinea pigs resulted in a protracted hypoglycemia corresponding to the hypoglycemia induced by a standard NPH
20 insulin preparation ("Insulatard" [®]).

Example 7

Preparation of human insulin-A4-amide-B30-ethyl ester

50 mg of human proinsulin, wherein the glutamic acid of position A4 has been replaced by glutamine, introduced by one
25 of the biotechnological methods described in the introductory part, were dissolved in a mixture containing 400 plitres of N,N-dimethyl formamide, 100 plitres of water, and 125 mg of L-threonine ethyl ester. The pH value of the solution was adjusted to 6.5 with acetic acid. 2.5 mg of porcine trypsin,
30 dissolved in 100 plitres of 0.001 M calcium acetate, was added and the reaction mixture left at 12° C. The reaction

was stopped after 48 hours by precipitating the proteins with 10 ml of 2-propanol. The precipitate was isolated by centrifugation and subsequently fractionated by gelfiltration and ion-exchange chromatography as described in Example 5.

- 5 Hereby 15 mg of human insulin-A4-amide-B30-ethyl ester were obtained.

Example 8

Formulation and biological effect of an insulin preparation containing human insulin-A4-amide-B30-ethyl ester

- 10 15 mg of human insulin-A4-amide-B30-ethyl ester were suspended in 5 ml of an aqueous solution containing 15 mg of sodium acetate trihydrate, 400 mg of mannitol, and 30 mg of phenol, whereupon the pH value was adjusted to 7.3, and water was added to give a final volume of 10 ml.
- 15 Subcutaneous injection (20 μ litres/kg) of this preparation into guinea pigs resulted in a hypoglycemia of longer duration than the hypoglycemia induced by a standard NPH-insulin preparation ("Insulatard" [®]).

Example 9

- 20 Preparation of L-threonine alkyl amides

- 10 mmoles of t-butyloxy-carbonyl-L-threonine and 10 mmoles of alkylamine were dissolved in 25 ml of chloroform, whereupon 11 mmoles of N-ethyloxy-carbonyl-2-ethyloxy-1,2-dihydroquinoline (EEDQ) were added. The reaction mixture was
- 25 stirred at 20° C. for 16 hours, whereupon the solvent was removed by evaporation in vacuo. The resulting viscous oil was redissolved in 100 ml of ethyl acetate, washed with 2 x 50 ml of 10 % citric acid, 2 x 50 ml of 1 M sodium bicarbonate and 2 x 50 ml of water. The organic phase was
- 30 dried over anhydrous sodium sulphate and removed by evaporation in vacuo.

To splitt off the protecting group the residue was dissolved in 25 ml of trifluoroacetic acid and left at 20° C. for 30 minutes. After removal of trifluoroacetic acid by evaporation the resulting viscous oil was mixed with 50 ml
5 of 1 M sodium carbonate and the mixture extracted with 3 x 50 ml of chloroform. The combined extracts were dried over anhydrous sodium sulphate and evaporated in vacuo. The residue solidified to waxy crystals upon standing at 4° C. Yield: 50 - 85 %.

CLAIMS

1. Insulin derivative, c h a r a c t e r i z e d in that the B30 carboxyl group is blocked with an uncharged group and that optionally one to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and
5 B21 are blocked with or converted into an uncharged group provided that when only the B30 carboxyl group is blocked with an uncharged group, said group comprises an alkyl group having at least 8 carbon atoms.
2. Insulin derivative according to claim 1, c h a r a c -
10 t e r i z e d in that only the B30 carboxyl group is blocked with an uncharged group which comprises an alkyl group of 8-12 carbon atoms.
3. Insulin derivative according to claim 1, c h a r a c -
t e r i z e d in that the B30 carboxyl group is blocked as
15 an alkyl amide having at least 8 carbon atoms.
4. Insulin derivative according to claim 1, c h a r a c -
t e r i z e d in that one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is blocked with or converted into an uncharged group and that
20 the B30 carboxyl group is blocked as an amide or with an uncharged group comprising an alkyl group of 1-12 carbon atoms.
5. Insulin derivative according to claim 1, c h a r a c -
t e r i z e d in that one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is
25 blocked with or converted into an uncharged group and that the B30 carboxyl group is blocked with an uncharged group comprising an alkyl group of 8-12 carbon atoms.
6. Insulin derivative according to claim 1, c h a r a c -
t e r i z e d in being an insulin-(A4-B30)-diamide.

7. Insulin preparation, characterized by containing at least one insulin derivative wherein the B30 carboxyl group is blocked with an uncharged group and optionally one to four of the carboxylic acid groups on the amino acid residues of positions A4, A17, A21, B13 and B21 are blocked with or converted into an uncharged group, provided that when only the B30 carboxyl group is blocked with an uncharged group, said group comprises an alkyl group having at least 8 carbon atoms and, if desired, also containing a fast acting insulin.

8. Insulin preparation according to claim 7, characterized by comprising at least one insulin derivative, wherein only the B30 carboxyl group is blocked with an uncharged group comprising an alkyl group having 8-12 carbon atoms.

9. Insulin preparation according to claim 7, characterized by comprising at least one insulin derivative, wherein the B30 carboxyl group is blocked as an alkyl amide having at least 8 carbon atoms.

10. Insulin preparation according to claim 7, characterized by comprising at least one insulin derivative, wherein one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is blocked with or converted into an uncharged group and that the B30 carboxyl group is blocked with an uncharged group comprising an alkyl group of 1-12 carbon atoms.

11. Insulin preparation according to claim 7, characterized by comprising at least one insulin derivative, wherein one of the carboxylic acid groups on the amino acid residues of positions A4, A17, B13 and B21 is blocked with or converted into an uncharged group and that the B30 carboxyl group is blocked with an uncharged group comprising an alkyl group of 8-12 carbon atoms.

12. Insulin preparation according to claim 7, c h a r a c -
t e r i z e d by comprising an insulin-(A4-B30)-diamide.

13. Preparation according to claim 7, c h a r a c t e r -
i z e d by being an injection preparation exhibiting pro-
5 tracted insulin action and comprising a suspension of at
least one insulin derivative in an isotonic aqueous medium
having a neutral pH and optionally containing a buffer and/-
or a preserving agent and, if desired, a fast acting insulin.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK86/00022

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 C 07 K 7/40, A 61 K 37/26 // C 12 P 21/02								
II. FIELDS SEARCHED Minimum Documentation Searched ⁷ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">IPC</td> <td style="padding: 5px;">A 61 K 37/26; C 07 C 103/52; C 07 K 7/40, /42; C 12 P 21/02</td> </tr> <tr> <td style="padding: 5px;">US Cl</td> <td style="padding: 5px;">260:112.7; 424:178; 514:3, 4</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸			Classification System	Classification Symbols	IPC	A 61 K 37/26; C 07 C 103/52; C 07 K 7/40, /42; C 12 P 21/02	US Cl	260:112.7; 424:178; 514:3, 4
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US Cl	260:112.7; 424:178; 514:3, 4							
SE, NO, DK, FI classes as above								
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹								
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³						
Y	DK, B, 146 482 (SHIONOGI & CO LTD) 17 October 1983	1-13						
Y	DK, B, 147 437 (NOVO INDUSTRI A/S) 6 August 1984	1-6						
Y A	US, A, 3 903 068 (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY OF HEALTH, EDUCATION, AND WELFARE) 2 September 1975	1-6 7-13						
Y	US, A, 4 421 685 (ELI LILLY AND COMPANY) 20 December 1983 & EP, 0037256 GB, 2072680 JP, 56154443 AU, 68844/81 AT, E, 4638 CA, 1155109 AU, 540645 .../...	1-6						
¹⁰ * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document 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. "Z" document member of the same patent family								
IV. CERTIFICATION								
Date of the Actual Completion of the International Search 1986-06-10	Date of Mailing of this International Search Report 1986 -06- 13							
International Searching Authority Swedish Patent Office	Signature of Authorized Officer Elisabeth Carlberg							

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A1, 45 187 (DE FORENEDE BRYGGERIER A/S) 3 February 1982 & WO, 82/00301 AU, 74508/81 CA, 1175372 AT, E, 9820 AU, 543687	1-6
Y	Biochimica et Biophysica Acta, 33 (1973), p 406-415 D Levy, The Esterification of Insulin with Triethyloxonium Tetrafluoroborat.	1-6
Y	Chemical Abstracts, Vol 87 (1977), abstract No 161917r Hoppe-Seyler's Z. Physiol. Chem. 1977, 358 (1), p 105-113 (Eng.)	1-6