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# PATENT SPECIFICATION

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## (54) PRODUCTION OF LONG ACTING INSULIN PREPARATIONS

(71) We, NORDISK INSULIN-LABORATORIUM, a Danish body corporate, of Ved Stadion 2, DK-2820 Gentofte, Denmark, do hereby declare the invention, for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a therapeutic insulin preparation and a process for producing a stable insulin preparation with protracted effect and low antigenicity by reacting insulin with an organic compound comprising basic groups.

In the treatment of diabetes mellitus insulin preparations derived from swine or ox pancreas are generally used. Thus approximately 30% of the world consumption of insulin is based on swine insulin and approximately 70% on ox insulin. Insulin from other animals has been suggested, for instance sheep insulin, but so far it has not attained any major commercial significance.

Insulin therapy has previously involved many disadvantages, including the manifestation of allergy reactions and lipodystrophy.

It has long been known that conventional insulin treatment, in most patients, results in the formation of insulin antibodies, which may lead to increased insulin requirements, in that unknown amounts of insulin may be bonded to the antibodies and will, while bonded, be ineffective in regulating blood sugar levels.

The primary cause of the antibody formation and the consequent high insulin consumption has been attributed to the presence of various impurities in normal commercial insulin. Such impurities include, for example, insulin dimer, proinsulin, intermediary insulin (the stage between proinsulin and insulin), arginine insulin, ethylester insulin, mono-desamido insulin and didesamido insulin. The first three of these compounds are

known to be highly antigenic. It is also known that the fourth or the fifth, or both, are highly antigenic, while the sixth and seventh, and the insulin molecule itself, are non-antigenic or only slightly antigenic. Cf. Schlichtcrull, Monocomponent Insulin and its Clinical Implications, 16th March, 1973.

It is known to remove the impurities by gel filtration and/or ion exchange, so that it is possible to obtain highly purified insulin containing substantially only a single component. In recent years insulin preparations which have been freed of at least some of the impurities have been marketed, and in many cases they have resulted in reduced antibody formation in diabetics.

It has been found that quick-acting preparations of both swine and ox insulin can be produced so pure that they will not, or at any rate only to a very small degree, cause insulin antibody formation, cf. Schlichtscrull, Monocomponent Insulin and its Clinical Implications, 16th March 1973. Further, it has proved to be possible to produce preparations with protracted action and having substantially reduce antigenicity if the production is based on highly purified swine insulin, cf. T. Deckert *et al.* Diabetologia, vol. 10, pp. 703-8, 1974. However, it has been found that even if ox insulin is produced in a purity that with the present analytical methods must be regarded as just as high as the highly purified swine insulin that can be made today and so pure that, as stated above, it will not, or at any rate only to a very low degree, cause antibody formation when used in quick-acting preparations, protracted action low-antigenic preparations based on highly purified ox insulin have not yet been marketed. This is a serious drawback because protracted-action preparations based on ox insulin are preferred internationally.

Antibody formation in live organisms against chemical compounds such as insulin

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may be due to a number of factors e.g. that there are chemical differences in the primary structure, or that the chemical compound has total dimensions (aggregation) such that it is incompatible with and repelled by the organism (Arquilla E.R. *et al.*, Immuno-chemistry of Insulin, Handbook of Physiology, Ch. 9, p. 160, 1972), or that the chemical compound has a steric structure which is incompatible with and repelled by the organism (Arquilla, E.R. *et al.*, Immunology Conformation and Biological Activity in Insulin, Diabetes, vol. 18, p 194, 1969).

It is also possible to cause antibody formation by administering together with the chemical compound a component capable of activating the immunity mechanism.

Tests tend to indicate that the physical state of insulin plays an essential part in antibody formation. (Kumar *et al.* Horn. Metab. Res. 6, (1974) pp. 185-177; and Piers *et al.* Neth J. Med. 17 (1974) pp. 234-238).

The reason why quick-acting highly purified insulin preparations do not, or only to a limited degree, cause antibody formation is probably that in those cases the insulin, besides being highly purified, is present in monomer or only loosely aggregated form, whereas in previously produced and marketed protracted-action highly purified insulin preparations the insulin appeared in an aggregated form and with properties that resulted in antibody formation.

This problem is very pronounced with ox insulin, which is probably due to the fact that ox insulin is more inclined to form aggregates than swine insulin.

Ox insulin without additives is known to have a wider isoelectric precipitation zone or interval than swine insulin. It is also known that this wide isoelectric precipitation zone may be restricted to the same width as known for swine insulin by the addition of certain substances such as phenol or *m*-cresol, as in British Patent Specification No. 1,222,00. The wider isoelectric precipitation zone of ox insulin is assumed to be due to the fact that ox insulin aggregates at pH values close to neutral.

We have now found a process by which it is possible to stabilize purified insulin, i.e. insulin which is substantially free of antigenic impurities, so that it will not form aggregates that might cause antibody formation during production or in protracted-action preparations during storage or in use.

According to the present invention, the process comprises reacting purified insulin (as hereinbefore defined), in the presence

of a stabiliser which maintains the insulin in stabilised monomer or loosely aggregated (as hereinafter defined) form, with an organic compound comprising amino and/or substituted amino groups, so that the amino groups react with the carboxyl groups of the insulin. The insulin which is used in the process of the invention must be maintained in stabilised monomer form, by which we mean that the insulin is monomeric and is stabilised against the natural tendency of insulin to aggregate, or in loosely aggregated form, by which we mean that any insulin aggregate is such that it can break down into insulin monomers. Accordingly, the purified insulin which is used in the invention must not be in irreversibly aggregated form, i.e. a form which is known to be antigenic and which cannot split up into monomeric insulin in the reaction.

Preferred organic compounds comprising amino and/or substituted amino groups for use in the invention are polypeptides such as polarginine, somatostatin, protamine or globin. Another suitable compound is 1,3-bis(4amino-2-methyl-6-quinolyl) urea.

The reaction is advantageously carried out in the presence of glucose or a phenol such as *m*-cresol.

The invention may be used with insulin derived from many different animal species, such as swine, oxen and sheep, but it is particularly significant in connection with ox insulin.

In a preferred embodiment of the invention, a solution of insulin and a stabiliser as defined above is subjected to ion exchange by elution with an eluent containing a stabiliser as defined above. A solution containing a polypeptide is mixed with an eluted fraction containing insulin thus freed of impurities; and the insulin complex which is precipitated is isolated. This procedure provides effective security against aggregation, in that the purified insulin is not first isolated. Further, the process is very simple in that the protracted-action insulin preparation is directly precipitated in a stable form.

The preferred stabiliser for use in the invention is urea.

Therapeutic compositions according to the invention comprise an insulin preparation produced according to the invention in association with a pharmaceutically acceptable carrier.

The following Examples illustrate the invention.

#### EXAMPLE 1

250 mg of recrystallized ox insulin was dissolved in 5.2 ml of stabilized buffer solution consisting of 7 M deionized urea

and 0.02 M of tris buffer having a pH of 8.1. The solution was mixed with 5.2 ml of 7 M urea. The pH of the mixture was adjusted to 8.1. A column, 5 cm in diameter, was packed with a layer 2.1 cm high DEAE cellulose (Whatman DE 52—"Whatman" in a registered Trade Mark) and equilibrated with a buffer solution of the above composition. The insulin solution was introduced in the column and elution performed at a rate of 75 ml per hour according to the following schedule:

- 2.5 hours with a buffer of the composition defined above;
- 3 hours with a buffer of the composition defined above to which had been added 0.0045 moles of sodium chloride per liter; and
- 12 hours with a buffer of the composition defined above to which had been added 0.011 moles of sodium chloride per liter.

The eluate was divided into fractions. The highly purified fractions were collected and the content of insulin determined. In a 7 M urea solution of the same volume as the mixture of the highly purified fractions, protamine was dissolved in the amount necessary for obtaining the isophanic ratio of highly purified insulin to protamine, i.e. the ratio at which the maximum haze of the suspension formed by the reaction is achieved.

The insulin solution was added slowly and dropwise to the protamine solution while stirring and diluted to a urea concentration of 1 M. A protamine-insulin complex in amorphous state is precipitated. The precipitate was isolated by centrifugation when 300  $\mu$ g of the precipitate was subjected to isoelectric focusing in polyacrylamide gel and using 2% w/v of ampholine (pH 3-10) in 6 M of deionized urea, essentially only one band was visible.

#### EXAMPLE 2

The procedure of Example 1 was repeated, except that 0.5% w/w zn ions, based on the amount of insulin (as  $\text{ZnCl}_2$ ) and 0.3% w/v *m*-cresol, based on the volume of the mixture, was added to the protamine solution. The protamine insulin complex was then precipitated in crystalline form.

The precipitate was isolated by centrifugation. When 300  $\mu$ g of the precipitate was subjected to isoelectric focusing in polyacrylamide gel using 2% w/v of ampholine (pH 3-10) in 6 M deionized urea, essentially only one band was visible.

#### EXAMPLE 3

The procedure of Example 1 was repeated, but recrystallised ox insulin purified by gel filtration on Sephadex G-50 was used as starting material. ("Sephadex" is a registered Trade Mark).

The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 4

The procedures of Examples 1 and 2 were repeated, but as starting material swine insulin was used, prepared by salting-out an aqueous crude extract formed in the production of insulin by addition of salt to obtain 3.5 M at pH 8.5. The obtained salt cake was desalted in conventional manner prior to the ion exchange.

The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 5

The procedure of Example 4 was repeated and the obtained salt cake was subjected to gel filtration on Sephadex G-50.

The product was of the same purity as the product described in Example 1.

#### EXAMPLE 6

The procedures of Examples 1-3 were repeated, but swine insulin was used instead of ox insulin.

The formed protamine-swine insulin complex was as pure as the complex prepared in Example 1.

#### EXAMPLE 7

The procedure of Example 1 was repeated but, instead of protamine poly-L-arginine with a degree of polymerization 295 was used. The precipitate was isolated in the manner described in Example 1.

#### EXAMPLE 8

The procedure of Example 1 was repeated, but, to the pooled highly purified insulin fractions, 0.2% w/v aqueous solution of bis-(4-amino-2-methyl-6-quinoly)-urea hydrochloride was added in an amount equivalent to 10% by weight of the insulin amount present in the fractions. By diluting the product to a urea concentration of 1M with 0.025 M sodium phosphate buffer (pH 7.3), an insulin complex was precipitated, which was maintained some time and then isolated by centrifuging.

The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 9

The procedure of Example 8 was

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repeated, but as starting material was used recrystallized ox insulin purified by gel filtration on Sephadex G-50. The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 10

The procedures of Examples 8 and 9 were repeated, but swine insulin was used instead of ox insulin.

The formed swine insulin complex was as pure as the complex prepared in Example 1.

#### EXAMPLE 11

The procedure of Example 8 was repeated, but as starting material was used swine insulin, produced by salting-out an aqueous crude extract formed in the production of insulin by addition of salt to obtain 3.5 M at pH 8.5. The obtained salt cake was desalted in conventional manner prior to the ion exchange.

The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 12

The procedure of Example 11 was repeated, and the obtained salt cake was subject to gel filtration on Sephadex G-50.

The product was of the same purity as the product described in Example 1.

#### WHAT WE CLAIM IS:—

1. A process for the production of a stable insulin preparation with protracted action and low antigenicity, which comprises reacting purified insulin (as hereinbefore define), in the presence of a stabiliser which maintains the insulin in stabilised monomer or loosely aggregated (as hereinbefore defined) form, with an organic compound comprising amino and/or substituted amino groups, such that

the amino groups react with the carboxyl groups of the insulin.

2. A process according to Claim 1 in which the purified insulin is ox insulin.

3. A process according to Claim 1 or Claim 2 in which the organic compound is a basic polypeptide.

4. A process according to Claim 3 in which the basic polypeptide is a protamine.

5. A process according to any preceding claim in which the stabiliser is urea.

6. A process for the production of a stable insulin preparation with protracted action and low antigenicity, which comprises subjecting a solution of insulin and a stabiliser which maintains the insulin as solubilised monomer or loosely aggregated (as hereinbefore defined) form to ion exchange by elution with an eluent containing a basic polypeptide with an eluted fraction containing insulin thus freed of impurities; and isolating the insulin complex which is precipitated.

7. A process according to Claim 6 in which the stabiliser is urea.

8. A process according to Claim 6 or Claim 7 in which the basic polypeptide is polyarginine, somatostatin, protamine or globin.

9. A process according to Claim 1 substantially as herein described with reference to any of the Examples.

10. A therapeutic insulin preparation when produced by a process according to any preceding claim.

11. A therapeutic composition comprising a preparation as claimed in Claim 10 in association with a pharmaceutically acceptable carrier.

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repeated, but as starting material was used recrystallized ox insulin purified by gel filtration on Sephadex G-50. The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 10

The procedures of Examples 8 and 9 were repeated, but swine insulin was used instead of ox insulin.

The formed swine insulin complex was as pure as the complex prepared in Example 1.

#### EXAMPLE 11

The procedure of Example 8 was repeated, but as starting material was used swine insulin, produced by salting-out an aqueous crude extract formed in the production of insulin by addition of salt to obtain 3.5 M at pH 8.5. The obtained salt cake was desalted in conventional manner prior to the ion exchange.

The prepared product was of the same purity as the product described in Example 1.

#### EXAMPLE 12

The procedure of Example 11 was repeated and the obtained salt cake was subject to gel filtration on Sephadex G-50.

The product was of the same purity as the product described in Example 1.

#### WHAT WE CLAIM IS:—

1. A process for the production of a stable insulin preparation with protracted action and low antigenicity, which comprises reacting purified insulin (as hereinbefore define), in the presence of a stabiliser which maintains the insulin in stabilised monomer or loosely aggregated (as hereinbefore defined) form, with an organic compound comprising amino and/or substituted amino groups, such that

the amino groups react with the carboxyl groups of the insulin.

2. A process according to Claim 1 in which the purified insulin is ox insulin.

3. A process according to Claim 1 or Claim 2 in which the organic compound is a basic polypeptide.

4. A process according to Claim 3 in which the basic polypeptide is a protamine.

5. A process according to any preceding claim in which the stabiliser is urea.

6. A process for the production of a stable insulin preparation with protracted action and low antigenicity, which comprises subjecting a solution of insulin and a stabiliser which maintains the insulin as solubilised monomer or loosely aggregated (as hereinbefore defined) form to ion exchange by elution with an eluent containing a basic polypeptide with an eluted fraction containing insulin thus freed of impurities; and isolating the insulin complex which is precipitated.

7. A process according to Claim 6 in which the stabiliser is urea.

8. A process according to Claim 6 or Claim 7 in which the basic polypeptide is polyarginine, somatostatin, protamine or globin.

9. A process according to Claim 1 substantially as herein described with reference to any of the Examples.

10. A therapeutic insulin preparation when produced by a process according to any preceding claim.

11. A therapeutic composition comprising a preparation as claimed in Claim 10 in association with a pharmaceutically acceptable carrier.

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