PATENT SPECIFICATION

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The inventors of this invention in the sense of being the actual devisers thereof within the meaning of Section 16 of the Patents Act, 1949, are Jorgen Schlichtkrull of 34B, Bellahojvej, Copenhagen, Denmark and Inger Merete Noring of 26 Ornekuldsvej, Charlottenlund, Denmark both subjects of the King of Denmark.

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COMPLETE SPECIFICATION

Improvements in or relating to the Production of Insulin Crystals

We, NOVO TERAPEUTISK LABORATORIUM A/S, of 115, Fuglebakkevej, Copenhagen, Denmark, a limited liability company, organised under the laws of 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:—

Injectable insulin preparations are known the protracted effect of which is exclusively or mainly based on the presence of insulin crystals in aqueous suspension. Like other injectable insulin preparations these preparations are marketed in ampoules having a closure entirely or partly consisting of rubber so that the cannula of an injection syringe can be pierced through the rubber in order to take up the desired dose in the syringe. Ordinarily, an ampoule contains the aqueous insulin crystal suspension in an amount sufficient for several doses.

Any time a dose is to be removed from the ampoule it is necessary beforehand to shake the ampoule so that the settled insulin crystals are dispersed in order to form a suspension in which the insulin crystals are uniformly distributed. If it has not been ascertained that the insulin crystals are uniformly distributed in the aqueous suspension medium before the suspension is taken up into the injection syringe, each dose will not contain the same amount of insulin.

As the administration of insulin is usually carried out by the patients themselves it is important that a uniform suspension is obtainable without difficulties by a single shaking of an ampoule so that one is absolutely sure that each dose contains the same amount of insulin.

If the aqueous crystal suspension contains insulin crystals which due to their size are difficult to keep suspended, this security will not be present. Therefore it will be of importance that the crystal suspension does not contain particularly big crystals, or in other words that the largest insulin crystals have a size which does not exceed a predetermined size making it impossible with certainty to ensure that, after the patient has shaken the ampoule and until the desired dose has been taken up by the injection syringe, the same insulin concentration will be present throughout the suspension, and thus no crystals will be settled out on the bottom of the ampoule.

According to the hitherto known processes of making crystalline insulin nothing has been done in order to adjust the size of the produced crystals. An adjustment would, besides, have had no pharmaceutical purpose as formerly the insulin crystals have not been made constituents of insulin preparations for clinical use.

The crystallization of insulin, as is well known, is in principle carried out by causing the insulin to crystallize from an aqueous medium by changing the *p*H-value of the medium to in the neighbourhood of the isoelectric point of the insulin. If the insulin itself does not contain beforehand sufficient amounts of a crystallization-promoting metal, ordinarily zinc, for instance in the form of zinc chloride, the crystallization medium is given the necessary content thereof, and, moreover, use is generally made of buffer substances to fix the *p*H-value during the crystallization.

One of the purposes of the present invention is to provide a process of producing insulin crystals having a size which does not exceed a predetermined size, so that directly in the crystallization process a crystal charge will be obtained which may be used as such for clinical purposes and which need not be subjected to a fractionating sedimentation to separate too big crystals.

The process according to the invention is of the type in which the insulin is caused to

[*Price 3s. 0d.*]

crystallize from an aqueous medium having a *p*H-value between 5 and 7 in the presence of a crystallization-promoting metal, and the characteristic feature of the invention consists in that the crystallization takes place while seeding with insulin crystals in an amount corresponding to the following equation:—

$$P = I \times \frac{d_p^3}{d_i^3 - d_p^3}$$

where P indicates the quantity in grams of seed crystals used, I the weight in grams of insulin to be crystallized, d_p the largest size in μ of the seed crystals and d_i the desired upper limit of the size in μ of the insulin crystals to be produced.

Here and in the following description the size of the insulin crystals is to be understood as the length in μ (micron) of the longest diagonal of the crystal.

If, for instance, it is desired that the insulin crystals do not exceed about 30 μ, and it is desired to crystallize 100 grams of insulin, the seed crystals must be employed in an amount of about 30 mgs. if the seed crystals have a size of about 2 μ and less, and in an amount of about 240 mgs. if the seed crystals have a size of about 4 μ and less.

With injectable insulin preparations, the protracted effect of which is based on the presence of insulin crystals in suspension, it is, moreover, important that the suspended insulin crystals have mainly the same size, that is to say that the main part of the insulin present has the form of crystals which do not or only slightly differ from each other in size. This will secure a constant and completely reproducible clinical effect.

A further purpose of the present invention consists in providing such conditions during the crystallization that the main part of the insulin will crystallize as crystals the size of which is in the neighbourhood of the desired upper limit.

This may be obtained by preventing, as far as possible, the formation of insulin crystals due to spontaneous crystallization and by using seed crystals of mainly the same size. In this way it is possible to obtain the result that the main part of the insulin of a crystal charge will be present in mainly the same crystal size. Under these circumstances d_p will indicate approximately the size of the seed crystals and d_i the crystal size in which the main part of the insulin will crystallize. For example, it may be mentioned that by suppression of the spontaneous crystallization in the process according to the invention 90% by weight of the insulin of a crystal charge will crystallize in the form of crystals having a size between 28 and 36μ.

In order to prevent as far as possible spontaneous crystallization there may, according to the invention, be used an addition of a buffer substance, the anion of which does not bind the crystallization-promoting metal at neutral reaction. Examples of such buffer substances are the borate, diethylbarbiturate, maleate and acetate buffers, among which the last mentioned buffer is preferred, when the produced crystal suspensions are to be used directly in the production of injectable insulin preparations.

It is also possible to make use of the process referred to in British Patent Application No. 16635/53 (Serial No. 733,740) and according to which the crystallization takes place in the presence of halogen in ionic form in a concentration above 0.2 mole per litre. It is preferable to provide this halogen ion concentration by adding a sufficient quantity of a halogen salt of an alkali metal, ammonia or one or the alkaline earth metals or a mixture of such salts. In order to obtain maximal crystal crops it is not expedient to use higher halogen ion concentrations than about 1 mole per litre.

If crystallization is carried out as indicated above it is not necessary to use buffer substances the anion of which does not bind the crystallization-promoting metal at neutral reaction. Phosphate or citrate buffers or mixtures thereof may also be used.

In carrying out the process according to the invention the insulin-containing aqueous crystallization medium may be produced in a manner known *per se*. It is most common to produce an acid aqueous insulin solution with the necessary content of one or more crystallization-promoting metals (zinc, cobalt, nickel, cadmium, copper, manganese and iron, among which use is almost always made of zinc), and, if desired, buffer substances, and to adjust the solution to the *p*H of the crystallization, but it is also possible to precipitate the insulin amorphously in an aqueous medium without the necessary metal content and then to cause the insulin to change into crystalline form by subsequent addition of the necessary amount of meal, for instance in the form of an aqueous solution of the metal salt. Finally, it is also possible to approach the *p*H of the crystallization from the basic side by using basic insulin solutions.

The seed crystals are added either in the form of an aqueous suspension, for instance suspended in the aqueous medium in which the seed crystals are produced, or in dry, for instance freeze-dried, form. If the insulin is present in precipitated amorphous form without the necessary metal content in the crystallization medium, the seed crystals may be added together with the missing metal content, or the aqueous suspension of the seed crystals may in itself exhibit a sufficient metal content.

As mentioned, the crystallization takes place at a *p*H-value between 5 and 7, and within this range it is expedient to use 5.3 to 6.5 dependent on the composition of the crystallization medium.

The seed crystals used may for instance be produced according to the method referred to in British Patent Application No. 29685/54 (Serial No. 766,994).

To further illustrate the process according to the invention reference is made to the following examples, of which Examples 1 and 3 illustrate the production of insulin crystals having a size not exceeding a predetermined size, while the remaining examples illustrate the production of insulin crystals having mainly the same size.

EXAMPLE 1.

500 mgs. of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 0.6 millilitre of 1 N HCl. Then 50 millilitres of a buffer solution are added containing 7 grams of NaCl and 10 millilitres of an aqueous solution containing 10% by weight of citric acid, 0.4% by weight of zinc (as zinc chloride) and sufficient NaOH to give the solution a *p*H-value of 6.3. If necessary, the *p*H is adjusted to 6.0. Before spontaneous crystallization begins, 2 millilitres of a seed crystal suspension are added, said suspension having the following composition: 0.03% by weight of insulin crystals of a size of 3μ and less, 2.5 mgs. per 100 millilitres of zinc (as zinc chloride), 2.5 mgs. per 100 millilitres of citric acid, 0.08% by weight of methyl-*p*-hydroxy-benzoate and NaOH so as to obtain a *p*H of 7.2 to 7.4. After stirring for some hours the crystallization is completed, and the crystals have obtained a size of about 4 to 25μ. Without seeding crystals having a size varying from about 5 to 50μ will be obtained.

EXAMPLE 2.

500 mgs. of crystalline insulin from pig pancreas are dissolved in 50 millilitres of water containing 6 millilitres of 0.1 N HCl 15 millilitres of acetone are added and then 10 millilitres of a buffer solution containing 10% by weight of citric acid, 0.4% by weight of Zn (as zinc chloride) and sufficient NaOH to give the buffer solution a *p*H-value of about 6.3. Then water is added to form a total volume of 100 millilitres. The *p*H is adjusted to about 6.2. Before spontanoeous crystallization begins 2 millilitres of a seed crystal suspension are added said suspension having the same composition as in Example 1 crystal size 3μ. After cautious stirring for some hours the crystallization is completed, and the main part of the insulin has crystallized in the form of crystals having a size of 20 to 25μ.

EXAMPLE 3.

1.12 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of a solution are added which contains 7 grams of NaCl and 1.7 millilitres of 1 N NaOH. Immediately thereafter are added 2.5 millilitres of a seed crystal suspension having the same composition as in Example 1. The *p*H is adjusted to 5.4 to 5.6. After stirring for 15 to 20 hours the crystallization is completed. The size of crystals is less than 30μ.

EXAMPLE 4.

1.6 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of a buffer solution are added which contains 1.36 grams of CH_3COONa, $3H_2O$, 7 grams of NaCl and 1 millilitre of 1 N NaOH. Immediately thereafter 3.5 millilitres of a seed crystal suspension as in Example 2 are added. The *p*H is adjusted to 5.4 to 5.6. After stirring for 15 to 20 hours the crystallization is completed, and the main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

EXAMPLE 5.

1.12 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of an aqueous solution are added which contains 1.36 grams of CH_3COONa, $3H_2O$, 11.9 grams of KBr and 1 millilitre of 1 N NaOH. immediately thereafter are added 2.5 millilitres of a seed crystal suspension as described in Example 2. The *p*H is adjusted to form 5.4 to 5.6. After stirring for 15 to 20 hours the crystallization is completed. The main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

EXAMPLE 6.

1.6 grams of crystalline insulin are dissolved in 500 millilitres of water containing 100 mgs. of Zn (as zinc chloride) and 20 millilitres of 1 N HCl. Then 500 millilitres of a buffer solution are added which contains 13.6 grams of CH_3COONa, $3H_2O$, 70 grams of NaCl and 10 millilitres of IN NaOH. Then 1.8 millilitres of a seed crystal suspension as described in Example 2 are added. The *p*H is adjusted to from 5.4 to 5.6. After stirring for 15 to 20 hours the crystallization is completed. The main part of the insulin has crystallized in the form of crystals of a size of about 40μ.

EXAMPLE 7.

3.2 grams of crystalline insulin are dissolved in 50 millilitres of an aqueous solution containing 20 mgs. of Zn (as zinc chloride) and 3 millilitres of 1 N HCl. Then 50 millilitres of an aqueous solution are added which contains 1.36 grams of CH_3COONa, $3H_2O$, 7 grams of NaCl and 2 millilitres of 1 N NaOH. Then 5 millilitres of a seed crystal suspension are added being, as regards all constituents, 10 times as concentrated as that referred to in

Example 2. After the course of about 20 hours crystallization is completed. The main part of the insulin has crystallized in the form of crystals of a size of about 15μ.

EXAMPLE 8.

The crystallization is carried out as indicated in Example 4, but instead of adding 3.5 millilitres of a suspension of seed crystals an equivalent amount in the form of a freeze-dried crystalline powder is added. By this procedure the same crystal size will be obtained as indicated in Example 4.

The process according to the invention may be employed in conjunction with the process for producing injectable insulin preparations with protracted effect referred to in the British Patent Specification No. 709,927. If so, the process according to the invention comprises effecting the crystallization under aseptic conditions, using an injectable crystallization medium, and adding after the crystallization a sterile solution of a crystallization-promoting metal, preferably zinc, in such amounts that the insulin crystal suspension thus produced contains in total at least $13 \times A \times 10^{-3}$ milliequivalents of the crystallization-promoting metal per litre of the suspension. A indicating the number of international units of insulin per millilitre of the suspension.

Furthemore, the sterile insulin crystal suspension should be given a predetermined insulin content per unit volume and a sufficient amount of isotonics and a preserving agent.

Finally, as crystallization buffer, use should not be made of substances the anions of which bind the crystallization-promoting metals at neutral reaction.

The example below illustrates how this combined process may be carried out:—

EXAMPLE 9.

1.6 grams of crystalline insulin are dissolved in 50 millilitres of water containing 10 mgs. of zinc (as zinc chloride) and 2 millilitres of 1 N HCl. The solution is sterile-filtered, and the filter is washed with 25 millilitres of water. To the 75 millilitres of filtrate are added 25 millilitres of a buffer solution prepared under aseptic conditions and containing 1.36 grams of CH_3COONa, $3H_2O$, 7 grams of NaCl and 1 millilitre of 1 N NaOH. Then 3.5 millilitres of a seed crystal suspension prepared under aseptic conditions and having the same composition as in Example 2 are added. The pH is adjusted to 5.5 After stirring for 15 to 20 hours the crystallization is completed. The main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

The crystal suspension is transferred into an injectable preparation, ready for use, by diluting it with 900 millilires of a solution, prepared under aseptic conditions and containing 70 mgs. of zinc (as zinc chloride), 0.11% methyl-*p*-hydroxy-benzoate and 2.7 millilitres of 1 N NaOH. The pH should be about 7.3 and the suspension adjusted to this value by means of 0.1 N NaOH or HCl.

The final preparation contains 40 international units of insulin per millilitre.

Other ways in which the present invention may be employed directly in the production of injectable preparations ready for use, appear from the specification to the said patent.

What we claim is:—

1. A process in producing insulin crystals, in which the insulin is caused to crystallize from an aqueous medium having a pH-value of between 5 and 7 in the presence of a crystallization-promoting metal, characterized in that crystallization takes place while seeding with insulin crystals in an amount corresponding to the equation:—

$$P = I \times \frac{d_p^3}{d_i^3 - d_p^3}$$

in which P indicates the quantity in grams of seed crystals used, I the amount in grams of insulin to be crystallized, d_p the greatest size in μ of the seed crystals and d_i the desired upper limit for the size in μ of the insulin crystals to be produced.

2. A process according to Claim 1, characterized in that the formation of insulin crystals due to a spontaneous crystallization is prevented as much as possible, and use is made of seed crystals having mainly the same size.

3. A process according to Claim 2, in which the crystallization takes place in the presence of a buffer substance characterized in that use is made of a buffer substance, the anion of which does not bind the crystallization-promoting metal at neutral reaction, preferably acetate buffer.

4. A process according to any one of claims 1 to 3, characterized in that crystallization takes place in the presence of halogen in ionic form in a concentration above 0.2 mole per litre.

5. A process according to any one of the preceding claims, characterized in effecting the crystallization under aseptic conditions, using an injectable crystallization medium, and adding after the crystallization a sterile solution of a crystallization-promoting metal, preferably zinc, in such amounts that the insulin crystal suspension thus produced contains in total at least $13 \times A \times 10^{-3}$ milliequivalents of the crystallization-promoting metal per litre of the suspension, A indicating the number of international units of insulin per millilitre of the suspension.

6. A process according to Claim 5, characerized in giving the sterile insulin crystal suspension a predetermined insulin content per unit volume and a sufficient amount of isotonics and a preserving agent.

7. A process of producing insulin crystals substantially as hereinbefore described with

Example 2. After the course of about 20 hours crystallization is completed. The main part of the insulin has crystallized in the form of crystals of a size of about 15μ.

EXAMPLE 8.

The crystallization is carried out as indicated in Example 4, but instead of adding 3.5 millilitres of a suspension of seed crystals an equivalent amount in the form of a freeze-dried crystalline powder is added. By this procedure the same crystal size will be obtained as indicated in Example 4.

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Furthemore, the sterile insulin crystal suspension should be given a predetermined insulin content per unit volume and a sufficient amount of isotonics and a preserving agent.

Finally, as crystallization buffer, use should not be made of substances the anions of which bind the crystallization-promoting metals at neutral reaction.

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EXAMPLE 9.

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The final preparation contains 40 international units of insulin per millilitre.

Other ways in which the present invention may be employed directly in the production of injectable preparations ready for use, appear from the specification to the said patent.

What we claim is:—

1. A process in producing insulin crystals, in which the insulin is caused to crystallize from an aqueous medium having a *p*H-value of between 5 and 7 in the presence of a crystallization-promoting metal, characterized in that crystallization takes place while seeding with insulin crystals in an amount corresponding to the equation:—

$$P = I \times \frac{d_p^3}{d_i^3 - d_p^3}$$

in which P indicates the quantity in grams of seed crystals used, I the amount in grams of insulin to be crystallized, d_p the greatest size in μ of the seed crystals and d_i the desired upper limit for the size in μ of the insulin crystals to be produced.

2. A process according to Claim 1, characterized in that the formation of insulin crystals due to a spontaneous crystallization is prevented as much as possible, and use is made of seed crystals having mainly the same size.

3. A process according to Claim 2, in which the crystallization takes place in the presence of a buffer substance characterized in that use is made of a buffer substance, the anion of which does not bind the crystallization-promoting metal at neutral reaction, preferably acetate buffer.

4. A process according to any one of claims 1 to 3, characterized in that crystallization takes place in the presence of halogen in ionic form in a concentration above 0.2 mole per litre.

5. A process according to any one of the preceding claims, characterized in effecting the crystallization under aseptic conditions, using an injectable crystallization medium, and adding after the crystallization a sterile solution of a crystallization-promoting metal, preferably zinc, in such amounts that the insulin crystal suspension thus produced contains in total at least $13 \times A \times 10^{-3}$ milliequivalents of the crystallization-promoting metal per litre of the suspension, A indicating the number of international units of insulin per millilitre of the suspension.

6. A process according to Claim 5, characterized in giving the sterile insulin crystal suspension a predetermined insulin content per unit volume and a sufficient amount of isotonics and a preserving agent.

7. A process of producing insulin crystals substantially as hereinbefore described with

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particular reference to the foregoing examples.

8. Crystalline insulin preparations when produced by the process claimed in any one of the preceding claims.

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