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The inventor of this invention in the sense of being the actual deviser thereof within the meaning of Section 16 of the Patents Act 1949 is Jørgen Schlichtkrull, a Danish Subject of 28B, Ved Bellahøj, Bronshøj, Denmark.

COMPLETE SPECIFICATION

Improvements in or relating to Insulin Preparations

We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish Body Corporate, of 115 Fuglebakkevej, Copenhagen, 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:

The present invention relates to a process for the treatment of insulin crystals and for the production of insulin preparations therefrom.

It is known that crystalline insulin is produced by adding a buffering substance to an aqueous insulin solution containing one or more of the metal ions prerequisite to the crystallization of the insulin, such as zinc, cadmium, cobalt, nickel and copper, preferably zinc, and adjusting the pH value to between 5·5 and 6·5. In order to avoid a temporary precipitation of insulin in the amorphous state during the crystallization organic solvents, such as acetone, may be added, as they will prevent precipitation of amorphous insulin but not of the crystals.

As buffering substances, use has been made of alkali phosphates, but these have the disadvantage of precipitating a part of the zinc ions in the form of very slightly soluble zinc phosphate, which pollutes the insulin crystals. In subsequent recrystallization of the insulin precipitate from a solution that has been buffered with ammonium acetate, it is possible to remove the zinc phosphate and the insulin crystals thus produced are found to contain 0·52 per cent of zinc, which, incidentally, corresponds to three atoms of zinc in the rhombohedral unit cell of the crystals. As buffering substance, use may also be made of borate, diethylbarbiturate or maleate buffers, which are not precipitated or in other way bind the zinc ions of the solution. When this kind of buffer substance is used, care must be taken that there is not so much zinc or other crystallization-promoting metal ions present that the insulin when being bound to the metal is rendered slightly soluble so that crystallization is hindered. A buffer that is especially suitable is sodium citrate, which binds the zinc ions in a strongly complex compound having just such strength that the amount of zinc prerequisite to the crystallization of the insulin (0·35 per cent or two atoms of zinc per unit cell) is bound to the insulin. The insulin crystals produced with citrate buffer thus contain 0·35 per cent of zinc or an equimolar amount of the other crystallization-promoting metals (cadmium, cobalt, nickel and so on).

The crystallization of insulin may be a step in the aseptic production of pharmaceutical insulin preparations, viz., the so called insulin-zinc suspensions, which are sterile zinc-containing suspensions of insulin crystals and possess a prolonged insulin action. In such case no isolation of the crystals from the mother-liquor will usually be performed, but the crystal suspension will be diluted in an appropriate way so as to get the desired composition of the final preparation. In all other cases the insulin crystals appear as an intermediate product in the manufacture, for which reason they are isolated and, if desired, washed and dried so that they only contain 2 to 8 per cent of water. The contents of zinc and other crystallization-promoting metals in the insulin crystals, as mentioned hereinbefore and hereinafter, have thus been calculated on the weight of the dried crystals. The undried crystals are estimated to contain about 60 per cent of solid matter.

If the insulin crystals produced according to the above-mentioned crystallization methods are suspended in distilled water, and the suspension is injected subcutaneously, the insulin action obtained will not be substantially different from the brief action of dissolved insulin.

For many years the opinion prevailed that in order to obtain a prolonged insulin action of practical importance it was necessary to

inject the insulin together with auxiliary substances foreign to the human body, such as protamine, globin and surfen, bis (2-methyl-4-amino-chinolyl-6) carbamide, which have the effect of delaying the resorption of the insulin.

Later it was realized that the employment of such foreign substances is not necessary. Thus, it is possible to obtain a prolonged insulin action by means of aqueous insulin crystal suspensions of such composition that, if the suspension medium is adjusted to pH 7, the suspended insulin crystals will show a particularly high content of the above-mentioned metals, that is, more than 0·25 milli-equivalent per gram, preferably at least 0·35 milli-equivalent per gram, whereby the crystals will remain practically undissolved at this pH.

The present invention relates to a process for treatment of insulin crystals by which it is possible to impart to the crystals such properties that on parenteral injection after suspension in an aqueous medium they will give a prolonged insulin action without the necessity of giving the aqueous medium beforehand a certain minimum content of the crystallization-promoting metals and such composition that the crystals will take up metal from the medium at neutral reaction or without the necessity of giving the insulin crystals beforehand an increased metal content.

According to the invention there is provided a process wherein insulin crystals that have entirely or partly been produced from bovine pancreas glands are subjected to a thermal treatment, while maintaining the water content of the crystals above 8%, at a temperature above 20°C. and during such a period of time that, after suspension in an aqueous injectable medium and subsequent subcutaneous injection, the crystals will show an insulin action that is protracted compared with the action of the untreated crystals under the same conditions.

The prolonged insulin action will be the more prolonged the higher the thermal treatment temperature is and the longer the treatment period has been. If, as starting material, use is made of ox insulin crystals, a very prolonged insulin action may be obtained by storing the crystals at 45°C. for 3 weeks, or at 70°C. for 24 hours. According to the invention, it is preferred to subject the crystals when present in suspension to a temperature between 40 and 70°C. Temperatures above 70°C. will normally be unsuitable, as the crystals will tend to stick together, whereas the employment of temperatures below 40°C. requires a period of time that is too long for practical manufacture. Any desired degree of prolongation of the insulin action can easily be obtained by variation of temperature and time of the thermal action. By mixing suspensions having different actions, it is possible to obtain an infinite number of different combination actions.

It is appropriate that the thermal treatment of the crystals according to the invention should take place while the crystals are in suspension, for example as they are obtained by a crystallization. In this state the crystals contain, as mentioned above, about 60 per cent of solid matter. The effect of the thermal treatment is weakened when the water content of the crystals is decreased. If during the thermal treatment the water content of the crystals is about 20 per cent it is estimated that it will be necessary to use twice the time in treating the crystals in order to obtain the same protracted effect as by treating non-dried crystals, the other conditions being identical. Ordinary crystalline dry insulin containing between 2 and 8 per cent of water will hardly be influenced by the thermal treatment. Therefore, the water content of the crystals should be maintained above 8% during the thermal treatment.

According to the invention it has been found that the pH value of the medium with which the crystals are or have been in balance has a decisive influence on the therapeutic effect of the thermal action. Thus tests have shown that the prolonged action of the treated crystals will be the longer, the lower this pH value within the interval of 5-8 is. If the pH value is in the neighbourhood of the pH of the blood (7·4), the thermal action will hardly have any effect. Consequently, in the production of crystals having a slow action, according to the invention the pH value used is less than 7, for example, 5, 5·5 or 6.

As suspension medium for the crystals when these are subjected to the thermal treatment, the mother-liquor from the crystallization may be used. Other media may also be employed provided that, when making use of the current knowledge about insulin, care is taken that the crystals do not go into solution in the medium, which, moreover, may not contain substances that are detrimental to the biological activity of the insulin. In order to fix the pH value of the medium it is appropriate to make use of a buffering substance, examples of which are mentioned in what follows. It may be mentioned that buffering substances, such as citrate, that when bound to the zinc ions may abolish the prolonged action of the crystalline insulin-zinc suspensions do not hinder the effect of the thermal treatment. The medium may also contain solvents that are miscible with water, such as acetone, which will happen when it is, for example, the mother-liquor from a crystallization according to the citrate method. In practice it is most appropriate that the medium in which the thermal action takes place has such a composition that it is suitable for injection purposes.

In such case it is possible by a suitable dilution procedure and by adjusting the pH value to 7 to convert the suspension into an insulin preparation ready for use, when working under sterile conditions.

The insulin crystals produced according to the invention do not differ from the crystals as they are before the thermal treatment in appearance or in the amino-acid composition, but they have a somewhat lower solubility in a solution of sodium chloride in hydrochloric acid and in a neutral phosphate buffer. The crystallization ability of the insulin is decreased during the thermal treatment and this may easily be ascertained by recrystallizing the treated crystals.

In the process according to the invention use may be made of insulin crystals produced by means of any crystallization method. Particularly applicable is a special crystallization method mentioned in United Kingdom Patent Specification No. 733,740.

In view of the therapeutical application it is appropriate that the crystals should not be too big, and in order to avoid sedimentation in ampoules their size should not exceed 40μ. The biological activity of the crystal suspensions is somewhat dependent on the crystal size, as the prolongation of the activity increases with increasing crystal size. Consequently, it is appropriate that the crystals are produced with the same size distribution from one crystal charge to the other.

The process according to the invention may also be applied to insulin crystals having a particularly high metal content (more than 0·25 milli-equivalent per gram), as these thereby may be given a more protracted effect in aqueous suspension than without the thermal treatment according to the invention.

The invention also relates to a process for the production of insulin preparations with protracted effect, the said process being of the kind in which sterile insulin crystals are suspended under sterile conditions in a sterile aqueous medium appropriate for injection purposes, and the characteristic feature of this process is that use is made of the insulin crystals produced according to the invention, and the aqueous medium has or is given a pH value of about 7 (6·5 to 7·5). As appears from the above, such neutral preparations are completely stable even when stored for a long period of time, owing to the fact that the aqueous medium has a pH value of about 7.

If in the preparations no use is made of the auxiliary substances such as protamine and zinc, which are now superfluous, they are miscible with ordinary quick-acting insulin solutions while maintaining the quick action of the ordinary insulin and the prolonged action of the crystals. It is even possible in the production of the preparations to give them a content of dissolved quick-acting insulin in order to obtain a strong initial effect. In such case use may in the dissolved fraction be made of swine insulin, which has a relatively high solubility at neutral reaction. Finally, composite preparations may be produced containing crystals having several different breadths of activity, so that there is practically an unlimited number of possibilities of obtaining appropriate polyphasic actions. Thus, the new discovery and the production methods developed according to the invention not only represent a novel manner in which to obtain a prolonged insulin action but form the basis of an insulin therapy that is more flexible and can be individualized to a much larger extent than has previously been possible and, in particular, without the use of auxiliary substances.

There is no reason why zinc ions, for example, should not be added to the said suspensions, whereby a still more prolonged insulin action may be obtained, but this is not necessary, as within the scope of the invention it is possible to produce insulin crystal suspensions having an even more prolonged insulin action than has previously been obtainable with the hitherto known insulin crystals in suspensions containing zinc at concentrations suitable for application for therapeutic purposes.

The invention will now be further described in and illustrated by the following non-limiting Examples.

In the following Examples use has been made of a stock suspension (S_1) having the following composition:

40 units per ml. of insulin crystals from ox pancreas glands,
1·4 mg. of Zn^{++} per 100 ml. (as chloride),
0·01 M sodium acetate,
0·7 per cent of sodium chloride,
0·1 per cent of methyl *p*-hydroxybenzoate and hydrochloric acid to give a pH value of 5·5.

The suspension is produced by diluting 1 volume of concentrated crystal suspension with 9 volumes of methyl *p*-hydroxybenzoate solution containing 0·11 per cent of methyl *p*-hydroxybenzoate. The concentrated crystal suspension is produced by mixing solutions *A* and *B*, which have the following composition, and subsequently agitating while the insulin crystallizes:

A. 3·48 g. of ox insulin crystals containing 0·78 per cent of zinc are suspended in 100 ml. of water and dissolved by adding 4 ml. of N-hydrochloric acid. The solution is filtered under sterile conditions and diluted to 150 ml.

B. 0·02 mole of sodium acetate and 14 g. of sodium chloride are dissolved in water and diluted to 50 ml. This solu-

tion should be of such alkalinity that the mixture of *A* plus *B* has a pH of 5·5.

EXAMPLE 1.

Suspension S_1 is stored at 45°C. for 2 weeks. On injection into rabbits it shows a protracted effect resembling that of the zinc-protamine-insulin. The crystals are collected by filtration under suction and dried.

EXAMPLE 2.

The dried crystals produced as described in Example 1 are suspended in a sterile injectable medium having the same composition as that in which the crystals received the thermal action. After suspension of the crystals, the pH value is adjusted to 7·0 with N-sodium hydroxide. The preparation has almost the same effect as zinc-protamine-insulin. The prolonged action of the preparation does not change under the usual storing conditions for insulin preparations.

EXAMPLE 3.

Suspension S_1 is stored at 45°C. for 4 weeks. Hereby the activity of the crystals becomes so protracted that after subsequent injection of the suspension into rabbits hardly any action will be observed during the first 8 hours after the injection. The biological activity of the insulin is on the contrary intact, and this may be ascertained by dissolving the crystals in hydrochloric acid and performing a biological potency determination.

EXAMPLE 4.

To suspension S_1 are added sodium phosphate and hydrochloric acid without the volume being substantially increased so that the suspension becomes 0·01 M with respect to phosphate and has a pH value of 5. After standing for 2 weeks at 45°C. the crystals have obtained a highly protracted activity and may be isolated and dried, if desired.

EXAMPLE 5.

To suspension S_1 N hydrochloric acid is added until the pH is 5·0. After standing for 7 hours at 70°C. the crystals have changed so much that the suspension has obtained a highly protracted activity.

EXAMPLE 6.

A crystallization mixture having the following composition is produced:

0·8 per cent of ox insulin,
0·040 per cent of Zn^{++} (as chloride),
0·05 M sodium citrate,
15 per cent of acetone, and
hydrochloric acid to give a pH value of 6·0.

The crystallization takes place while stirring for some hours, whereafter the acetone is evaporated, with continued stirring, for 24 hours at 45°C. Then the suspension is diluted with a solution of sodium chloride and methyl *p*-hydroxybenzoate, whereby it obtains the following composition:

40 units of insulin crystals per ml.,
97 μ g. of Zn^{++} per ml.,
0·01 M sodium citrate,
0·7 per cent of sodium chloride, and
0·1 per cent of methyl *p*-hydroxybenzoate,

whereafter the pH is adjusted to 5·5 with N hydrochloric acid. After being stored for 2 weeks at 45°C., the suspension will show a highly protracted insulin action, and the crystals are separated.

EXAMPLE 7.

A crystallization is carried out as described in the preceding Example, but the crystals are filtered off, washed with 60 per cent aqueous acetone and dried before the thermal treatment. The dried crystals are suspended in a suspension medium so that a suspension of the same chemical composition as S_1 is obtained. After standing for 2 weeks at 45°C., the suspension shows a highly protracted action and the crystals are separated, or the suspension is adjusted to pH 7, whereby an insulin preparation having a protracted effect is directly obtained.

EXAMPLE 8.

A suspension S_2 is produced, the procedure being that described for S_1, with the exception that the addition of sodium chloride is omitted. After standing for 2 weeks at 45°C., S_2 appears to possess a highly protracted insulin action.

EXAMPLE 9.

A crystallization as described for S_1 is performed, but instead of diluting the crystal suspension the crystals are separated and dried. Then the dried crystals are suspended in an aqueous medium so that a suspension having the same chemical composition as S_1 is obtained. After standing for 2 weeks at 45°C., the suspension will show a highly protracted effect, and the crystals may then be separated.

In the above Examples use has been made of zinc insulin crystals. The process according to the invention may, however, also be applied to insulin crystals containing other metals, such as cobalt, cadmium, nickel or copper. In order to illustrate this, reference may be made to the following Example, in which use has been made of copper insulin crystals.

EXAMPLE 10.

A crystallization mixture having the following composition is produced:

0·8 per cent of zinc-free ox insulin,
0·040 per cent of Cu^{++} (as chloride),
0·05 M sodium citrate,
15 per cent of acetone, and
hydrochloric acid to give a pH of 6·0.

The crystallization takes place while stirring until the following day, whereafter the acetone is evaporated and the suspension is diluted as described in Example 7. Hereby the suspension gets the following composition:

40 units of insulin crystals per ml.,
87 μ g. Cu^{++} per ml.,
0·01 M sodium citrate,
0·7 per cent of sodium chloride, and

0·1 per cent of methyl *p*-hydroxybenzoate, whereafter the pH is adjusted to 5·5 with N hydrochloric acid. After standing for 2 weeks at 45°C., the suspension will show a highly protracted insulin action.

Instead of subjecting the insulin crystals to the thermal treatment in suspension the crystals may as such be subjected to the treatment, care being taken that the crystals are not allowed to become dry during the treatment. This may be ensured by storing the separated crystals in a container such as a desiccator containing, for example, an aqueous saline solution, so that a vapour pressure is maintained such that the water content of the crystals is kept constant at between 20 and 40 per cent.

When carrying out the process according to the invention, the following procedure may be adopted.

EXAMPLE 11

Crystals from suspension S_1 are filtered off and weighed on a tared glass filter, and then they are placed *in vacuo* (1 to 2 mm. of Hg) in a desiccator containing a 5 per cent sodium chloride solution. After 3 days at room temperature equilibrium is reached, and the desiccator is kept at 45°C. for 2 weeks. Thereby the equilibrium is only slightly changed, and the crystals are found to contain about 20 per cent of water. The crystals are suspended in a suspension medium so that the suspension will have the same composition as S_1. The suspension shows a somewhat prolonged action.

WHAT WE CLAIM IS:

1. A process for the treatment of insulin crystals, wherein insulin crystals produced entirely or partly from bovine pancreas glands are subjected to a thermal treatment, while maintaining the water content of the crystals above 8%, at a temperature above 20°C. and during such a period of time that, after suspension in an aqueous injectable medium and subsequent subcutaneous injection, the crystals will show an insulin action that is protracted compared with the action of the untreated crystals under equal conditions.

2. A process as claimed in claim 1, wherein the crystals are subjected to the thermal treatment while suspended in an aqueous medium having a pH value below 7.

3. A process as claimed in claim 2, wherein the crystals are subjected to a temperature between 40 and 70°C.

4. A process as claimed in claim 2, wherein the aqueous medium has such a composition that it is suitable for injection purposes.

5. A process for the production of insulin preparations having a protracted action, in which process sterile insulin crystals are suspended under sterile conditions in a sterile aqueous medium suitable for injection purposes, wherein use is made of insulin crystals that have been treated as claimed in any one of the preceding claims, and wherein the aqueous medium has or is adjusted to have a pH value of 6·5 to 7·5.

6. A process for the treatment of insulin crystals substantially as hereinbefore set forth, with particular reference to the Examples.

7. A process for producing insulin crystals having a protracted action substantially as hereinbefore set forth, with particular reference to the Examples.

8. Insulin crystals whenever treated by a process as claimed in any one of claims 1 to 4.

9. An insulin preparation having a protracted action, comprising a suspension of insulin crystals, treated as claimed in claims 1 to 4, in an aqueous medium having a pH value of 6·5 to 7·5.

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0·1 per cent of methyl *p*-hydroxybenzoate, whereafter the pH is adjusted to 5·5 with N hydrochloric acid. After standing for 2 weeks at 45°C., the suspension will show a highly protracted insulin action.

Instead of subjecting the insulin crystals to the thermal treatment in suspension the crystals may as such be subjected to the treatment, care being taken that the crystals are not allowed to become dry during the treatment. This may be ensured by storing the separated crystals in a container such as a desiccator containing, for example, an aqueous saline solution, so that a vapour pressure is maintained such that the water content of the crystals is kept constant at between 20 and 40 per cent.

When carrying out the process according to the invention, the following procedure may be adopted.

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Crystals from suspension S_1 are filtered off and weighed on a tared glass filter, and then they are placed *in vacuo* (1 to 2 mm. of Hg) in a desiccator containing a 5 per cent sodium chloride solution. After 3 days at room temperature equilibrium is reached, and the desiccator is kept at 45°C. for 2 weeks. Thereby the equilibrium is only slightly changed, and the crystals are found to contain about 20 per cent of water. The crystals are suspended in a suspension medium so that the suspension will have the same composition as S_1. The suspension shows a somewhat prolonged action.

WHAT WE CLAIM IS:

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2. A process as claimed in claim 1, wherein the crystals are subjected to the thermal treatment while suspended in an aqueous medium having a pH value below 7.

3. A process as claimed in claim 2, wherein the crystals are subjected to a temperature between 40 and 70°C.

4. A process as claimed in claim 2, wherein the aqueous medium has such a composition that it is suitable for injection purposes.

5. A process for the production of insulin preparations having a protracted action, in which process sterile insulin crystals are suspended under sterile conditions in a sterile aqueous medium suitable for injection purposes, wherein use is made of insulin crystals that have been treated as claimed in any one of the preceding claims, and wherein the aqueous medium has or is adjusted to have a pH value of 6·5 to 7·5.

6. A process for the treatment of insulin crystals substantially as hereinbefore set forth, with particular reference to the Examples.

7. A process for producing insulin crystals having a protracted action substantially as hereinbefore set forth, with particular reference to the Examples.

8. Insulin crystals whenever treated by a process as claimed in any one of claims 1 to 4.

9. An insulin preparation having a protracted action, comprising a suspension of insulin crystals, treated as claimed in claims 1 to 4, in an aqueous medium having a pH value of 6·5 to 7·5.

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