PATENT SPECIFICATION

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COMPLETE SPECIFICATION

Improved Process for the Preparation of Prolonged Effect Insulin Products

We, NORDISK INSULINLABORATORIUM, of Brogaardsvej 40A, Gentofte, Denmark, a Body Corporate organized and existing under the Laws of Denmark, do hereby declare the nature of this invention and in what manner the same is to be performed, to be particularly described and ascertained in and by the following statement:—

Various "insulin" products of prolonged effect are known; especially, protamine-insulin suspensions with or without zinc have been in use. The characteristic feature of these products is their low solubility in water at the pH of the blood.

The present invention concerns the production of similar preparations, but, in contrast to those known at present, the new insulin product is obtained in a crystalline state. The crystalline state is a direct criterion for the purity, as complete crystallization demands well-purified starting materials. Moreover, the crystals do not tend to clot or to adhere to the glass, and by shaking a uniform suspension is easily obtained. The crystals are fairly resistant to various actions; for example, they can be separated from the mother liquor without being damaged and can be suspended in a medium of different composition. In pure water at 2—4° C., for instance, they can be preserved unchanged for months. Applied to the treatment of diabetes these products of our invention secure a uniform and prolonged effect with a minimum addition of foreign substances such as protamine and zinc. While the commercial zinc-protamine-insulin suspensions usually contain 4—5% zinc in relation to the quantity of insulin, the crystalline products described in the following can be prepared with, for example, 0.2% zinc. Since the crystalline products can advantageously be prepared without excess of protamine, they can be mixed with solutions of ordinary insulin without the latter being precipitated to any great extent, further the free insulin is not adsorbed on the crystals to the same extent as on an amorphous protamine insulin product; thus, the effect of one mixed insulin injection is similar to the result which would be obtained by injecting the two solutions separately at different sites of the body.

It is well-known how to prepare insulin products with prolonged effect by bringing together in an aqueous medium insulin and a zinc salt with an alkaline protein or protein split-product and phenol. Hereby, however, no crystalline product is obtained and phenol is added only to inhibit bacterial growth in the preparation. According to our invention, the mentioned substances are mixed at approximately isophanous conditions at a pH between 6 and 8, whereby a precipitate is formed which—immediately or after standing——becomes completely or predominantly crystalline.

According to our invention, isophanous conditions prevail in a preparation when the following conditions are fulfilled:

After the precipitate is centrifuged off and the clear supernatant liquid is separated into two parts, isophanous conditions exist if the addition of insulin to one of the portions and of an alkaline protein or protein split-product to the other portion produces the same degree of opalescence in both portions. If e.g. the first portion becomes more opalescent than the second one, the preparation contains too much alkaline protein or protein split-product and thus isophanous conditions

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do not prevail. The additions of insulin and protein must be made in such a way that no change of pH takes place and so that complete precipitation and subsequently maximal opalescence is obtained. Cf. Schweiz. Med. Wschr. *68* (1938) pp. 37—41, especially p. 38, column 2 and p. 39, column 1.

For the preparation according to our invention, preponderantly crystalline insulin which contains zinc is used. The concentration of insulin in the crystallization medium can be chosen rather arbitrarily. Amorphous, zinc-free insulin can also be used if zinc is added, for example as $ZnCl_2$. Preferably, less than 0.5% zinc relative to the amount of insulin is applied, but one may very well work with, for example, 5% zinc.

Among the alkaline proteins and protein split-products suited to this procedure there may be mentioned, for example, histones and protamines as well as some of their split-products. Crystallization has, for instance been carried through with salmiridine, clupeine, scombrine, sturine, thynine, cyprinine, cyclopterine, with a split-product of salmiridine and with thymus histone. The quantity of these substances necessary for crystallization is determined relative to the quantity of insulin in units of weight. This ratio is designated as P/I. The most favourable crystallization conditions are obtained when chosing such quantities that isophanous conditions prevail. These conditions are present for most protamines if P/I = 1/10; the P/I corresponding to isophanous conditions varies, however, with the respective substances, the pH-value, the salt concentration, the temperature and phenol derivative concentration. It is not absolutely necessary to crystallize at exactly isophanous conditions. If, for example, for a given alkaline protein, at a given pH, temperature, salt concentration, and phenol derivative concentration the P/I corresponding to isophanous conditions is equal to 1/10 one can still work at, for example, P/I = 1/8 or 1/15, provided that the conditions otherwise are the same. In the first mentioned case the crystals will be formed rapidly and they will therefore become relatively small, showing a certain tendency to deformations so that it may be difficult to distinguish the crystal shape. In the latter case, crystallization will occur at a slower rate, but, on the other hand, the crystals will become more distinct, thick and short. If the deviation from the P/I value which corresponds to isophanous conditions is too great, the product does not—or at least not predominantly—become crystalline. Great quantities of zinc demand relatively more alkaline protein or protein split-product.

The presence of phenol or certain phenol derivatives is a condition for the formation of crystals. The concentration of these substances in the crystallization medium can be chosen rather arbitrarily; preferably 0.1—1% are added, (calculated in per cent of the final volume), or possibly more, dependent on the substance chosen. The meta-derivatives are generally best suited. Moreover, among others ortho-, meta-, and paracresol, meta- and para-chloro-phenol, meta- and para-nitrophenol and resorcin have led to satisfactory results while, for example, ortho-chloro-phenol, ortho-nitro-phenol, and sodium salicylate are unsuited. The choice of the substance and the concentration are of some influence on the isophanous condition.

Crystallization must take place at a pH-value between 6 and 8. With regard to the durability of the product it would not be practical to work at a higher pH-value. The pH can be adjusted in an arbitrary way, however, preferably by means of a buffer mixture, for example both sodium phosphate buffer and sodium acetate buffer have been used. The P/I corresponding to isophanous conditions increases with increasing pH-value and at the same time the rate of crystallization is increased. At a low pH-value, crystallization can occur so slowly that it becomes incomplete, and deformed crystals may be obtained.

The pH of the solution from which the crystallization occurs is dependent on the phenol or the phenols used and of the concentration thereof.

It may be opportune—yet not necessary—to crystallize at an ionic concentration greater than that which is obtained after mixing the original substances, together with any substitute required for pH-adjustment, as a greater ionic concentration promotes crystallization. According to our invention, additional quantities of salts can therefore be added, such as sodium chloride which is usually applied. For experimental purposes, crystallization has also been attempted with an arbitrary selection of other salts. Likewise a high buffer concentration can produce the desired effect. Even a small increase in salt concentration favours the crystallization which then proceeds more rapidly. One may add salt in such an amount that the final suspension of crystals becomes just isotonic, but crystallization can also be carried through at a still higher salt concentration. In a 1 *n* NaCl-solution, for example, crystals of a length

up to 0.3 mm. were obtained. Isotony can then be reached by addition of, for example, glucose.

Generally it will be convenient to work at room temperature, however, the course of crystallization—e.g. the rate of crystallization—may very well be somewhat affected by a suitable temperature, by cooling or heating. An increase in temperature, however, demands addition of more alkaline proteins or protein split-products in order to maintain isophanous conditions.

The mentioned substances can be mixed in arbitrary sequence. It may, however, be convenient to dissolve the different substances separately, or some of them together at a suitable pH-value before the final mixture takes place. The final reaction can, for example, be adjusted after the individual substances are mixed at, for instance, a relatively acid reaction or by a suitable choice of the pH of the respective solutions. The crystallization can be almost complete in the course of a few minutes, especially in the case of preparations with small crystals; in certain other cases, however, it may last several days.

Once formed the crystals can be separated from the mother liquor but, since the preparations most frequently are used in the treatment of diabetes, it may be advantageous to perform the operations in a sterile manner and to use the sterile suspension for the injections. In practice, these considerations will also influence the choice of the quantities and concentrations of the various components, since the preparations to be used without further treatment after shaking must contain a given number of insulin units per unit of volume. The crystallization proper, however, may just as well be performed at other concentrations.

The shape of the crystals is distinctly different from that of insulin crystals which most frequently are rhombohedrons or prisms, but which are also described in the literature as being double spindle-shaped. In the products of our invention, the crystals most frequently show a marked development in length, perpendicular to the longest axis they show a square or rectangular cross-section and a characteristic pyramidal shape at both ends. Apart from this shape, there may occur all possible intermediates between long, thin, bi-pyramidal crystals and short, thick, sometimes octahedral-shaped crystals, dependent on the conditions chosen for crystallization.

The crystal formation can be regarded as a function of the components chosen in the procedure, their concentrations and, moreover, the physico-chemical conditions prevailing during their mixture. However, these variables are not independent of one another. Therefore, only a few of them can be chosen freely within the limits outlined above for the respective variables, while the rest of them must be determined with due regard to those chosen first, preferably by determining the isophanous conditions.

EXAMPLE 1.

1.6 g. of crystalline insulin containing 0.4% zinc are dissolved in water with the aid of 25 ml. 0.1 *n* hydrochloric acid. There are added aqueous solutions of 3 ml. tricresol (consisting of 90% meta-cresol, 5% orthocresol and 5% para-cresol), 7.6 g. of sodium chloride and so much sodium phosphate buffer that the final concentration is 1/75 molar and pH 6.9. Finally, 0.14 g. of salmiridine sulphate dissolved in water are added while shaking, whereby protamine-insulin containing zinc is precipitated. The solution is made up to 1000 ml., shaken again, and after 1 hour of standing the precipitated protamine-insulin becomes crystalline.

EXAMPLE 2.

3.2 g. of crystalline insulin containing 0.4% zinc, 50 ml. 0.1 *n* hydrochloric acid, 3 ml. meta-cresol, so much sodium phosphate buffer that the final concentration is 1/75 molar and the pH-value 6.67, 0.25 g. of salmiridine sulphate, eventually 50 g. of glucose and water are mixed as in example 1, so that a final volume of 1000 ml. is obtained. The suspension is stored at 20° C. until a microscopical test indicates that crystallization is complete.

EXAMPLE 3.

1.6 g. of crystalline insulin containing 0.4% zinc, 25 ml. 0.1 *n* hydrochloric acid, 2 ml. meta-cresol, 7.6 g. sodium chloride, so much sodium phosphate buffer and, if necessary, sodium hydroxide that the final concentration becomes 1/75 molar and the pH-value 7.35, 0.16 g. of salmiridine sulphate and water are mixed as in example 1, so that a final volume of 1000 ml. is obtained. After standing, the suspension becomes crystalline.

EXAMPLE 4.

1.6 g. of crystalline insulin containing 0.4% zinc, 25 ml. 0.1 *n* hydrochloric acid, 0.15 g. of zinc chloride, 3 ml. meta-cresol, 7.6 g. of sodium chloride, so much sodium phosphate buffer that the final concentration becomes 1/75 molar and the pH-value 6.8, 0.15 g. of salmiridine sulphate and water are mixed as in example 1, so that the final volume of 1000 ml. is obtained.

The suspension which contains approximately 5% zinc in relation to the quantity of insulin becomes crystalline by standing to the following day.

EXAMPLE 5.

A 50 ml. suspension is prepared from 80 mg. of crystalline insulin containing 0.4% zinc, 7.25 mg. salmiridine sulphate, 0.3% meta-cresol, 0.76% sodium chloride, and so much sodium phosphate buffer that the suspension becomes 1/75 molar and the pH-value becomes 6.69. The product which contains 7.5% more protamine than corresponds to isophanous conditions P/I becomes crystalline in the course of 15—20 minutes.

EXAMPLE 6.

A 50 ml. suspension is prepared from 80 mg. of crystalline insulin containing 0.4% zinc, 0.2% para-chloro phenol, 0.76% sodium chloride, so much sodium phosphate buffer that the suspension becomes 1/75 molar and the pH-value becomes 6.91, and 6 mg. of clupeine sulphate. After standing, the preparation becomes crystalline. The quantity of protamine used is 25% smaller than corresponds to isophanous conditions P/I.

Having now particularly described and ascertained the nature of our said invention and in what manner the same is to be performed, we declare that what we claim is:—

1. A process for the preparation of insulin products of prolonged effect in which zinc-containing insulin or insulin and a zinc salt and an alkaline protein or protein split-product together with phenol or a phenol derivative are brought together in an aqueous medium, which process is characterised by the fact that the mentioned substances for the preparation of a crystalline product are mixed together in the proximity of isophanous conditions at a pH-value between 6 and 8.

2. A process according to claim 1, wherein a crystalline product is prepared with an ionic concentration greater than that obtained by mixing the reactants by the addition to the reaction mixture of an inorganic salt, e.g. sodium chloride.

3. A process for the preparation of prolonged effect insulin products substantially as described in the foregoing Examples.

4. Prolonged effect insulin products whenever prepared or produced by the process claimed in any of the preceding claims.

Dated this 20th day of December, 1945.

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The suspension which contains approximately 5% zinc in relation to the quantity of insulin becomes crystalline by standing to the following day.

EXAMPLE 5.

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