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(54) PROCESS OF PURIFYING INSULIN

(71) We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish Company of 215, Nordre Fasanvej, DK-2200 Copenhagen N, 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 of purifying insulin.

This application is a divisional application from our copending Application No. 38081/68 (Serial No. 1,285,023) which describes and claims an injectable insulin preparation containing insulin recovered from pancreas glands and being dissolved or suspended in an injectable aqueous medium, said insulin being free of proteins of pancreatic origin with a molecular weight above 6000 and said insulin showing essentially a single component when a solution thereof is analysed by polyacrylamide gel electrophoresis (DISC PAGE).

As insulin starting material in the purification process use is ordinarily made of commercial insulin, i.e. amorphous insulin or crystalline insulin which may have been crystallized several times. However, use may also be made of crude insulin, such as the insulin-containing salt cake which is formed during the recovery of insulin from pancreas glands, and usually contains 10 to 30 per cent by weight of insulin, but the high contents of impurities make this starting material less suited for the purification according to the invention. A better starting material may be obtained by subjecting a solution of the salt cake to an isoelectric precipitation by adjusting the pH-valve of the solution to 5.5 and isolating the precipitate by centrifugation.

Originally, the general opinion was that properly recrystallized insulin in the form of sharp-edged rhombohedrons bounded by plane crystal faces constituted the pure protein, insulin, the configuration of which was determined by Sanger and co-workers, vide e.g. Biochemical Journal 60 (1955), 556. Later it was found, however, that it was not so. Scientific analytical investigations showed that the crystalline insulin referred to contains proteins of pancreatic origin with a molecular weight above that of the pure insulin and in addition thereto insulin-like substances of about the same molecular weight as that of pure insulin (about 6000).

Thus, it has been demonstrated that crystalline insulin dissolved in 1 M acetic acid may be fractionated into components (a), (b) and (c), respectively, by gel filtration on a column of Sephadex G-50 ("Sephadex" is a Registered Trade Mark) which is dextran cross-linked with epichlorohydrin to give an exclusive limit of penetration of about 10,000 (minimum mole weight of excluded substances), being in bead form (diameter 20 to 80 μ) and produced by PHARMACIA, Uppsala, Sweden. These three components may be isolated from their solutions by conventional methods, e.g. salting out, precipitation with a zinc salt at neutral reaction, freeze-drying etc. Both component (a) and component (b) contain proteins of pancreatic origin having a molecular weight above 6000, while component (c) contains pure insulin contaminated by insulin- like proteins of about the same molecular weight as that of the pure insulin.

The amount of component (a) may be 2—5 per cent by weight of crystallized insulin and less than 1 per cent by weight of recrystallized insulin. The amount of component (b) may in both cases be 4—8 per cent, and the amount of the insulin-like proteins in compound (c) may be 5—13 per cent by weight of component (c).

Immunological experiments carried out in animals and forming the background for the present invention have shown that the components (a) and (b) referred to above and to some extent the insulin-like proteins present in component (c) are in principle responsible for the antigenic behaviour of the insulin preparations hitherto known.

The purpose of the invention is to remove in a simple and effective manner the impurities contained in not only crude insulin, but

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also and in particular in commercial insulin so that the purified insulin shows no or materially reduced antigenicity.

According to the invention, there is provided a process of purifying commercial or crude insulin by column chromatography, which process comprises applying a water-containing solution of the insulin to be purified to a column of an anion exchanger, the solution having a pH in the range from 6 to 10, eluting the column in fractions with a water-miscible organic solvent which contains water in an amount of from 20% to 70% by volume and which has a pH in the range from 6 to 10 collecting the eluate fraction(s) showing essentially a single component when analysed by polyacrylamide gel electrophoresis (DISC PAGE), and recovering the insulin thus purified from the collected fraction(s).

For analytical purposes use has previously been made of discontinuous polyacrylamide gel electrophoresis (DISC PAGE), also in connection with crystalline insulin. This specific gel electrophoresis was developed by B. J. Davis and L. Ornstein and is described in Ann. N Y Acad Sci., 121, p. 321—349 and 404—427 (1964).

The anion exchangers, which should preferably be of the strongly basic type, used in the process according to the invention are known in the art. Examples of weakly basic anion exchangers are Bio-Gel DM produced on the basis of polyacrylamides, DEAE-Sephadex produced from cross linked dextrans and DEAE-Cellulose produced from cellulose. Examples of strongly basic anion exchangers are Dowex 1 ("Dowex" is a Register Trade Mark) produced from polystyrenes and QAE-Sephadex produced from cross-linked dextrans. The sephadex anion exchangers are produced by PHARMACIA, Uppsala, Sweden.

Examples of water-containing, water-miscible organic solvent eluents are tetramethyl urea, dimethyl formamide, dioxane, water-miscible ketones, such as acetone, and alkanols such as methanol, ethanol and propanols. The alkanols are preferred organic solvent eluents for use in the process of the invention and when used are preferably employed in a form which contains from 30% to 50% by volume of water.

The eluent always contains a buffer substance to control the pH-value of the eluent. It is preferred to operate the process of the invention at a constant pH-value. The pH of the eluent should be kept preferably in the range from 6.5 to 8.5, measured with a glass electrode adjusted against a standard buffer in the usual manner.

Suitable buffer substances can be found in the literature available.

It is essential that the temperature is constant during the ion exchange operation. The temperature can be chosen within the range of −10°C. to 40°C, preferably 0° to 30°C. The purified insulin from the collected fractions may be recovered in the usual way, e.g. by evaporation, salting out or precipitation in amorphous or crystalline form in the presence of zinc ions.

For the purpose of recovering insulin it is known to subject acid or neutralized alcoholic extracts from pancreas to column chromatography on a cation exchanger, such as carboxymethyl cellulose (British Patent Specification No. 1,106,076), sulphonated resins synthesized from the telomer of styrene and divinyl benzene (British Patent Specification No. 1,054,523) or on an anion exchanger, such as diethylaminoethyl cellulose (British Patent Specification No. 931,954). However, the insulin obtained by these processes contains impurities as does the crystalline insulin obtained by the conventional recovery processes including precipitation several times by salting out of the crude insulin solutions with sodium chloride.

It is also known from British Patent Specification No. 881,855 to subject unspecified zinc insulin to column chromatography on the cation exchanger "Dowex 50-X1" (a sulphonated styrene-divinylbenzene copolymer in bead form produced by The Dow Chemical company, Midland, U.S.A.), using aqueous ethanol as eluent, but even when recrystallized insulin is used as starting material, the recovered insulin will show mainly the same impurities as does the starting material.

Furthermore it is known from British Patent Specification No. 913,042 to purify crystalline insulin mixed with amorphous or microcrystalline foreign materials by washing the impure crystallizate with an aqueous liquid of which the pH is held at from 3.8 to 4.3 with the aid of a weak acid or a buffer mixture. However, this purification step does not remove such impurities as proinsulin, the dimer or the intermediate.

Finally, it is known from British Patent Specification No. 871,541 to subject 6 times crystallized insulin and insulin purified as described by Lens (Biochem. et Biophys. Acta (1948), 2, 76) to column chromatography on a styrene-divinyl benzene copolymer cation exchanger deposited on an inert extender of large superficial area, but the insulin will after this chromatography still contain impurities such as proinsulin, the dimer and the intermediate together with arginine insulin and monodesamido insulin since the presence of these impurities will not prevent the chromatogram from looking as if the material eluted from the column were essentially homogeneous.

The following examples illustrate the process of the invention.

EXAMPLE 1

A buffer of the following composition is prepared:

0.1 M NH_4Cl
0.02 M NH_3
60 per cent (v/v) ethanol
The pH is 8.3 at 25°C.

15 grams of the anion exchanger Dowex 1×2 (mesh: 50/100) are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 1.6 cm and a height of 25 cm. The column is equilibrated with the buffer. Dowex 1×2 is a copolymer of styrene and 2 per cent divinylbenzene produced by The Dow Chemical Co. of Midland, U.S.A., said copolymer carrying as functional groups benzyl trimethylammonium groups and being in bead form.

500 mg of insulin crystallized once from a citrate buffer are dissolved in a mixture of 20 mg of ethylene diamine tetra acetic acid disodium salt, 5 ml of the buffer, 5 ml of 60 per cent (v/v) ethanol and 0.04 ml of 13.3 M NH_3 (final pH 8.5). The insoluble material is removed by centrifugation and the clear solution is applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and a rate of 7.5 ml/h. Fractions of 5 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding 100 ml of water, 1.3 ml of 1 N HCl and 2 ml of 1 M zinc acetate per 100 ml of the pool. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield of purified insulin is 200 mg.

EXAMPLE 2

A buffer of the following composition is prepared:

25 grams of tris(hydroxymethyl)aminomethane
29.0 ml of 6 N HCl
1.2 liters of methanol
Water to make a total volume of 2 liters
The pH is 7.3 at 25°C.

40 grams of the anion exchanger QAE-Sephadex A-25 are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 2.5 cm and a height of 25 cm. The column is equilibrated with the buffer. QAE-Sephadex A-25 is dextran cross-linked with epichlorohydrin to give an exclusive limit of penetration of about 5000 (minimum mole weight of excluded substances) carrying as functional groups diethyl - (2 - hydroxypropyl) - aminoethyl groups, being in bead form (diameter 40 to 120 μ) and produced by PHARMACIA, Uppsala, Sweden.

500 mg of insulin crystallized once from a citrate buffer are dissolved in a mixture of 20 mg of ethylene diamine tetra acetic acid disodium salt, 94 mg of tris(hydroxymethyl) - aminomethane, 10 ml of 60 per cent (v/v) methanol and 0.073 ml of 6 N HCl (final pH: 7.3). The insoluble material is removed by centrifugation and the clear solution is applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 30 ml/h. Fractions of 5 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding 100 ml of water and 2 ml of 1 M zinc acetate per 100 ml of the pool. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield of purified insulin is 250 mg.

EXAMPLE 3

A buffer of the following composition is prepared:

0.06 M tris(hydroxymethyl)aminomethane
0.02 N HCl
0.075 M NaCl
60 per cent (v/v) ethanol
The pH is 8.3 at 25°C.

80 grams of the anion exchanger mentioned in Example 2 are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 2.5 cm and a height of 50 cm. The column is equilibrated with the buffer.

2.5 grams of insulin crystallized once from a citrate buffer are dissolved at 0°C in a mixture of 450 mg of tris(hydroxymethyl) - aminomethane, 100 mg of ethylene diamine-tetra acetic acid tetrasodium salt, 25 ml of buffer, 25 ml of 60 per cent (v/v) ethanol and 0.08 ml of 4 N HCl (final pH at 25°C: 8.4). The insoluble material is removed by centrifugation at 2 to 4°C and the clear supernatant is applicated on the column. The elution is carried out with the buffer at a temperature of 4°C and at a rate of 26 ml/h. Fractions of 20 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding

one pool volume of 0.02 M zinc acetate, 0.05 N HCl. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield is 1.3 grams of purified insulin.

EXAMPLE 4

A buffer of the following composition is prepared:

14.9 grams of histidinium monochloride
38.7 ml of 1 N NaOH
16.1 grams of NaCl
3.12 liters of 96 per cent (v/v) ethanol
Water to make a total volume of 5 liters
The pH is 6.5 at 25°C.

35 grams of the anion exchanger mentioned in Example 2 are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 2.5 cm and a height of 25 cm. The column is equilibrated with the buffer.

500 mg of insulin crystallized once from a citrate buffer are dissolved in a mixture of 20 mg of ethylene diamine tetra acetic acid disodium salt, 6.5 ml of the buffer and 6 ml of 60 per cent (v/v) ethanol, the pH being adjusted to 6.8 with 1 N NaOH. The insoluble material is removed by centrifugation and the clear solution is applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 30 ml/h. Fractions of 5 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding one pool volume of 0.02 M zinc acetate. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield of purified insulin is 210 mg.

EXAMPLE 5

A buffer of the following composition is prepared:

1.25 kg of tris(hydroxymethyl)aminomethane
725 ml of 12.3 N HCl
62.4 liters of 96 per cent (v/v) ethanol
Water to make a total volume of 100 liters.
The pH is 7.3 at 25°C.

1.3 kg of the anion exchanger mentioned in Example 2 are swollen in the buffer and the finest particles removed by decantation. The material is used for the packing of a column with a diameter of 15 cm and a height of 2.5 cm. The column is equilibrated with the buffer.

18 grams of recrystallized pork or beef insulin containing 0.4 per cent Zn are dissolved in a mixture of 0.72 gram of ethylene diamine tetra acetic acid disodium salt, 10.2 grams of tris(hydroxymethyl)aminomethane and 720 ml of 60 per cent (v/v) ethanol. The insoluble material is removed by centrifugation. 10.7 ml of 6 N HCl are added to the clear solution which is then applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 1.2 liters/h. Fractions of 0.5 liter are collected.

The extinctions at 276 nm (E_{276}) are measured and plotted against the volumes of eluate. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled. The insulin is precipitated from the pool by adding one pool volume of 0.02 zinc acetate. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner.

The yield is 8.2 grams of purified insulin.

The insulin is recrystallized from a buffer of the following composition:

2.0 per cent of insulin
0.8 per cent of Zn^{++} (as chloride) calculated on the weight of insulin
0.1 M sodium acetate
7.0 per cent of sodium chloride
HCl to pH 5.45 to 5.55.

The insulin is dissolved in water containing HCl and the $ZnCl_2$, whereupon a solution of the proper quantities of sodium acetate and sodium chloride is added. The crystallization is carried out at room temperature with mechanical stirring. The crystallization is completed within one or two days. The insulin is isolated by filtration, washed with water and dried in vacuo. The yield is 8.0 grams.

The purified insulin can be used for preparation of all kinds of pharmaceutical insulin preparations for clinical use.

WHAT WE CLAIM IS:—

1. A process of purifying commercial or crude insulin by column chromatography, which process comprises applying a water-containing solution of the insulin to be purified to a column of an anion exchanger, the solution having a pH in the range from 6 to 10, eluting the column in fractions with a water-miscible organic solvent which contains water in an amount of from 20% to 70% by volume and which has a pH in the range from 6 to 10, collecting the eluate fraction(s) showing essentially a single component when analysed by polyacrylamide gel electrophoresis (DISC PAGE), and recovering the insulin thus purified from the collected fraction(s).

one pool volume of 0.02 M zinc acetate, 0.03 N HCl. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield is 1.3 grams of purified insulin.

EXAMPLE 4

A buffer of the following composition is prepared:

14.9 grams of histidinium monochloride
38.7 ml of 1 N NaOH
16.1 grams of NaCl
3.12 liters of 96 per cent (v/v) ethanol
Water to make a total volume of 5 liters
The pH is 6.5 at 25°C.

35 grams of the anion exchanger mentioned in Example 2 are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 2.5 cm and a height of 25 cm. The column is equilibrated with the buffer.

500 mg of insulin crystallized once from a citrate buffer are dissolved in a mixture of 20 mg of ethylene diamine tetra acetic acid disodium salt, 6.5 ml of the buffer and 6 ml of 60 per cent (v/v) ethanol, the pH being adjusted to 6.8 with 1 N NaOH. The insoluble material is removed by centrifugation and the clear solution is applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 30 ml/h. Fractions of 5 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding one pool volume of 0.02 M zinc acetate. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield of purified insulin is 210 mg.

EXAMPLE 5

A buffer of the following composition is prepared:

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62.4 liters of 96 per cent (v/v) ethanol
Water to make a total volume of 100 liters.
The pH is 7.3 at 25°C.

1.3 kg of the anion exchanger mentioned in Example 2 are swollen in the buffer and the finest particles removed by decantation. The material is used for the packing of a column with a diameter of 15 cm and a height of 2.5 cm. The column is equilibrated with the buffer.

18 grams of recrystallized pork or beef insulin containing 0.4 per cent Zn are dissolved in a mixture of 0.72 gram of ethylene diamine tetra acetic acid disodium salt, 10.2 grams of tris(hydroxymethyl)aminomethane and 720 ml of 60 per cent (v/v) ethanol. The insoluble material is removed by centrifugation. 10.7 ml of 6 N HCl are added to the clear solution which is then applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 1.2 liters/h. Fractions of 0.5 liter are collected.

The extinctions at 276 nm (E_{276}) are measured and plotted against the volumes of eluate. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled. The insulin is precipitated from the pool by adding one pool volume of 0.02 zinc acetate. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner.

The yield is 8.2 grams of purified insulin.

The insulin is recrystallized from a buffer of the following composition:

2.0 per cent of insulin
0.8 per cent of Zn^{++} (as chloride) calculated on the weight of insulin
0.1 M sodium acetate
7.0 per cent of sodium chloride
HCl to pH 5.45 to 5.55.

The insulin is dissolved in water containing HCl and the $ZnCl_2$, whereupon a solution of the proper quantities of sodium acetate and sodium chloride is added. The crystallization is carried out at room temperature with mechanical stirring. The crystallization is completed within one or two days. The insulin is isolated by filtration, washed with water and dried in vacuo. The yield is 8.0 grams.

The purified insulin can be used for preparation of all kinds of pharmaceutical insulin preparations for clinical use.

WHAT WE CLAIM IS:—

1. A process of purifying commercial or crude insulin by column chromatography, which process comprises applying a water-containing solution of the insulin to be purified to a column of an anion exchanger, the solution having a pH in the range from 6 to 10, eluting the column in fractions with a water-miscible organic solvent which contains water in an amount of from 20% to 70% by volume and which has a pH in the range from 6 to 10, collecting the eluate fraction(s) showing essentially a single component when analysed by polyacrylamide gel electrophoresis (DISC PAGE), and recovering the insulin thus purified from the collected fraction(s).

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2. A process as claimed in claim 1 wherein the eluent is a water-miscible alkanol containing from 30% to 50% by volume of water.

3. A process as claimed in claim 1 or claim 2, wherein the pH of the eluent is within the range from 6.5 to 8.5.

4. A process as claimed in claim 1 and substantially as hereinbefore described in any one of the foregoing specific Examples.

5. Insulin purified by a process as claimed in any one of claims 1 to 4.

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