

Extraction, Purification, Drying and Analysis of Camel Insulin

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Abstract: The increasing number of diabetic patients in Sudan in addition to the large quantities of wasted camel pancreas in the slaughter houses stimulated the research in this project aimed at extracting, purifying and analyze camel insulin from the local sources. In this study, collected fresh camel pancreatic glands were trimmed small pieces then homogenized, centrifuged after adjustment of pH and then filtered. Crude insulin is precipitated from the filtrate and dissolved in acetic acid. Insulin was again precipitated iso-electrically and crystallized using zinc acetate. After the processes of extraction the processes purification, amino acid analysis and study of the presence and function in the camel insulin. The products of insulin scale were subjected to a wide range of quality control testes including yield percent, zinc content (mg/100 μ), nitrogen percent and purity test by electrophoresis amino acid analysis is a test to determine the quantities of each individual amino acid in camel insulin and study of the function groups of each amino acid.

Key words: Insulin, camel pancreatic gland, extraction, purification, amino acid analysis, function groups amino acid tests, Sudan

INTRODUCTION

Diabetes mellitus is a major public health problem associated with enormous personal social and economic burdens (Weinstock, 2003). World Health Organizations (WHO) defines diabetes as a metabolic disorder of multiple a etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion insulin action or both (Wild *et al.*, 2004). According to the study conducted by ministry of health in Khartoum, the prevalence of diabetes type 1 (IDDM) in Sudan is about 6% the population.

Taking into account, the population of Sudan according the lateral census of 26 million people which means that there one about one and 7086 and 91 cases of insulin-dependent diabetes mellitus (Ashford *et al.*, 1969; Zimmet *et al.*, 2001). These patients would cost about 74.880.000 vials of insulin per annum which would cost about 748.800000 US dollars (According to the prices obtained from the ministry of health). However, the actual quantity of all types of insulin imported one for less than this calculated quantity. For examples, the total quantity of all types of insulin imported by both the private and public sectors in 2005 was 310.000 vials. On the other

hand in 2005, the estimated camels population in Sudan was 3.98.000 head camels in about 37.000 were slaughtered and consumed locally in addition to 242.000 h slaughtered and exported. These together can produce a total amount of pancreatic glands of about 121.000 kg which can be used manufacture about vials per year (each vials contains 400 units of insulin and each kilogram of pancreatic glands produces 2000 units) (Romans *et al.*, 1940). According to a preliminary feasibility study camel out during the early phase of the present project, a locally manufactured vial of insulin would cost half the price of imported vial. Therefore, local manufacturing of insulin would meet the growing local demand, save the hard currency and may provide surplus of the product for export.

MATERIALS AND METHODS

Animal: Healthy local bred camels with variable ages and weights when slaughtered in the slaughter house were used as a source of pancreatic glands.

Chemical and reagents: All chemicals and reagents used in different stage of insulin extraction, purification and assay were of pharmaceutical or analytical grades.

Preparation of crystalline zinc insulin

Insulin extraction: The collected camel pancreatic glands from Omdurman and Tambol slaughter-house were frozen and transferred to the laboratory as soon as possible. The excessive fatty tissue was immediately removed and the frozen glands were stored until several 1000 pounds of gland have been collected. The glands were minced and placed into glass-lined extraction solution consisted of 100 L of 93% denatured alcohol (ethanol containing 10% of methanol) and 1000 mL of concentrated hydrochloric acid for each 45 kg of glands. The mixture was stirred slowly for 3 h, centrifuged in a rubber-lined basket centrifuge which separated the alcoholic extract (Supernatant) from most of the insoluble material (residue). The residue was re-extracted for about 2 h with fresh 100 L of 65-7% alcohol for each 45 kg of the original material.

The mixture was also centrifuged and the 1st and 2nd alcoholic extracts were pooled and made basic (pH = 8) with ammonium hydroxide. The heavy inactive precipitate formed was removed by filtration through a wooden filter press. The filtrate was acidified with sulfuric acid (pH, 2.5-2.5). The filtrate was concentrated in vacuum at 20°C to approximately one 7th of the original volume. Immediately before being removed from the still, the concentrate was heated to 50°C and then filtered through soft study to remove fat and other insoluble materials.

The active principle insulin was precipitated from the cool filtrate by the addition of sodium chloride to a 25% concentration. After standing for at least 2 h, the active protein precipitate was easily removed from the top of the brine. When the precipitate from 2177 kg of pancreas has been collected, it was added to 220 L of water and the mixture was acidified with hydrochloric acid pH 1.8-2.2. By stirring for about 2 h, the active principle was dissolved. The insoluble material was removed by filtration through asbestos clarifying films.

To the clear filtrate sodium hydroxide was added to a concentration 0.15%. After standing for at least 3 h, the precipitate was removed and allowed to drain on filter study. The brine was discarded. The precipitate was then dissolved in 40 L of water acidified with hydrochloric acid to pH 2.0-2.5. The solution was divided into two equal parts and the process of purification continued (Romans *et al.*, 1940).

Insulin purification and drying: To 20 L of the above solution, 25 mL of a 90% solution of phenol were added. Sodium hydroxide solution (5 N) was then added slowly to adjust the acidity to pH 5.0-5.4, the isoelectric point of

insulin. After standing for 48 h at refrigerator temperature, the precipitate was removed by filtration. The filtrate contained a small amount of insulin, the active material which was precipitated by storage at room temperature for 3-4 days. The precipitate formed was removed by filtration. Several such precipitates were then purified or the precipitates were added individually to the precipitate obtained by addition of sodium chloride to the still concentrate. The iso-electric precipitate was dissolved in 18 L of water containing 200 mL of glacial acetic acid. After addition of 12 L of acetone, the volume was adjusted to 40 L and the acidity to pH 5.9-6.0 by addition of dilute ammonium hydroxide (approximately 200 mL of concentrated ammonium hydroxide in 10 L of water). After standing for about 2 h at room temperature, a flocculent precipitate was removed by filtration through soft filter study. This precipitate contained only a small amount of activity but was extracted with 60% alcohol acidified with sulfuric acid.

The extract was added to that which was ready to be concentrated in the still. The acetone filtrate was diluted to a total volume of 60 L by addition of an ammonium acetate buffer, pH 5.9-6.0, prepared by diluting approximately 200 mL of glacial acetic acid and approximately 200 mL of concentrated ammonium hydroxide to 20 L with water. After ensuring that the acidity of this dilute filtrate corresponded to pH 5.9-6.0, 75 mL of zinc acetate solution (25 mg of zinc mL⁻¹) were added with stirring. The mixture was stored at room temperature for 24 h and at refrigerator temperature for 24 h.

During this time, the precipitate containing the active principle settled and was separated from the mother liquor by means of a siphon and filtration. The precipitate was dissolved in 30 L of distilled water containing 525 mL of glacial acetic acid. After filtration through hardened filter study, 6 mL of a zinc acetate solution (25 mg of zinc mL⁻¹) were added. The acidity was then adjusted to pH 6.1-6.6 and the volume to 605 mL of concentrated ammonium hydroxide with distilled water.

The acidity was then carefully adjusted to pH 5.9 by drop wise addition of 1-1 mL of glacial acetic acid as may be required. Within an hour, crystals of zinc-insulin started to form and in 48 h at room temperature, the crystals were separated from the supernatant liquid by filtration. The precipitate was washed with distilled water and centrifuged slowly (about 1400 rpm) for approximately an hour. During this time, the crystals were moved to the bottom of the centrifuge tubes and amorphous material present in the upper layer. After draining, the tubes were

placed in vacuum at 40°C until the precipitate was dry. The amorphous material present would form a dark layer which was readily removed prior to complete drying of the precipitate.

Determination of insulin zinc content using atomic absorption: It may be determined by the following method (method of direct calibration) by preparing the solution of the substance being examined (test solution). Three reference (standard) solutions of eluent were prepared to determine the concentration which spanned the expected value in the test solution. Any reagents used in the preparation of the test solution were added to the reference (standard) and blank solutions at the same concentration.

The test solution and each reference (standard) solution were injected into the instrument at least 3 times and the steady reading was recorded. The substance being examined was insulin standard in 0.01 N_{HCl} measuring at 213.9 nm, using zinc hollow cathode lamp.

Determination of nitrogen content: Nitrogen content was determined by a semi-micro Kjeldahl method. A 0.2 g sample was transferred to the digestion flask of a semi-micro Kjeldahl apparatus. About 1 g of powder mixture of potassium sulfate and cupric sulfate (10+1) was added; 3.5 mL of a concentrated nitrogen-free sulfuric acid was added. The flask with its contents was heated over an electric heater until the solution attained a clear blue color and the contents of the flask were then transferred to a steam distillation unit and 20 mL of 40% sodium hydroxide solution were added and distillation was carried out with steam. The distillate was collected in 10 mL of boric acid solution (2%). Three drops of methyl red-methyl blue indicator were added and titrated against 0.01 N_{HCl}. The same procedure was carried out for the blank. Nitrogen content was calculated according to the following equation:

$$N\% = \frac{V_t \times 14 \times N_{HCl} \times 100}{Wt_s \times 100}$$

Where:

V_t = Titration volume

N_{HCl} = Normality of HCl neutralizing the distillate

Wt_s = Sample weight

Insulin purity testing using electrophoresis: This test was carried by adopting the technique reported in instructions by Sebia Spain (2003).

For sample analysis

Migration: The gel was unpacked and conveniently placed on top of the closed gel box for further processing. Excess of liquid was blotted rapidly off the surface by

Table 1: Yield of insulin

Samples	Weight of pancreas (g)	Insulin (g)	Yield (%)
A	410	0.454	0.110
B ₁	800	6.175	1.330
B ₂		4.483	
C ₁	100	22.835	4.698
C ₂		24.149	
D ₁	1000	18.196	3.575
D ₂		17.557	

placing a filter study strip on the gel surface so that its centre was lined up with the arrows printed at the edge of the gel plastic packing. The sample application template was placed onto the gel surface aligning the two outside slits of the template with the arrows on the gel packing. Total 5 µL of diluted sample were applied in each template slit. The sample was left to diffuse into the gel for 5 min. Excess of the sample was blotted with a filter study strip. The sample application template was taken off. The gel was put into an appropriate electrophoresis chamber. Samples were placed on the cathodic side.

When using Sebia K20 chamber, the Hydragel was placed on the bridge with the gel side facing down, the gel dipped about 1 cm into the buffer on each side. The chamber was plugged on the power supply.

Migration conditions

Volume of buffer per compartment: Volume of buffer per compartment: 150 mL; Total buffer volume: 300 mL; Migration time: 30 min.; Constant voltage: 70 V and Constant current (per gel): 12 Am. After migration, the chamber was unplugged off and the gel plate removed.

Fixation: The gel was placed into a gel holder for further processing. One tank was filled with 300 mL of fixative solution. The gel was immersed vertically in the fixative solution for 15 min. The gel was removed and dried with hot (80°C) airflow incubator dryer.

Staining and de-staining: The dried and cold gel was immersed in the stain for 5 min. The gel was de-stained in three successive baths of the de-staining solution until the background was completely color less and clear. Excess liquid was soaked up of the gel surface with a tissue study and the gel was dried with hot (80°C) airflow if needed. The backside (the plastic support side) of the dry film was cleaned with a wet tissue study.

Scanning: The sample was scanned using a densito-meter with a yellow filter at 570 nm (Table 1).

RESULTS AND DISCUSSION

Study of large volume solution (Large scale) and formulation study of samples E and D. Initially, 1340 g of beef pancreas were extracted. It produced 0.3755 g of insulin (Sample designated D, yield was 0.028%). Then,

280 g of beef pancreas were extracted. This produced 0.6895 g of insulin (Sample designated E, yield was 0.02573%) (Table 2).

Determinations of zinc in insulin using atomic absorption: About 5 samples of standard insulin weighing 0.1 mg were dissolved in 1 mL of 0.01 N_{HCl} and read at 213 nm using hollow cathode lamp. Zinc content of these samples is shown in Table 3. From the stocks of samples, A-C (1.6 mg mL⁻¹) of crystalline zinc insulin were prepared. The following concentrations were prepared in 0.1 mg. Results complied with BP and Lilly specifications, the range (0.004-0.016 mg/100 units). About 7 samples of substances being prepared from samples A-D of insulin weighing 0.1 mg were dissolved in 1 mL 0.01 N_{HCl} and read at 213 nm hollow cathode lamp. Zinc contents are shown in Table 4.

Determination of nitrogen content: About 3 samples of insulin weighing 0.016 and 0.02 g were taken from C, B

Table 2: Insulin yield obtained from 2 samples of camel pancreas

Camel pancreas (g)	Weight of insulin (g)	Samples	Yield (%)
1340	0.3755	D	0.02800
2680	0.6895	E	0.02573

Table 3: Zinc content of 6 samples of standard insulin

Sample	Weight of insulin (g)	Zinc (mg/100 units)
1	0.1	0.0139
2	0.1	0.0207
3	0.1	0.0188
4	0.1	0.0177
5	0.1	0.0090

Table 4: Zinc content of different samples of different insulin

Name of sample	Weight (mg)	Zinc (mg/100 units)
A	0.1	0.0210
B1	0.1	0.0150
B2	0.1	0.0040
C1	0.1	0.0160
C2	0.1	0.0120
D1	0.1	0.0104
D2	0.1	0.0101
Mixed	0.1	0.0150

Results complied with BP and Lilly specification, range (0.004-0.016 mg/100 units)

Table 5: Nitrogen content of different samples of insulin

Sample code, (C), (B) and (D)	Weight (mg) (C), (B) and (A)	Titration volume (C)	Titration volume (B)	Nitrogen (%) (C)	Nitrogen (%) (B)	Nitrogen (%) (D)
C ₁	0.016	0.70	0.54	98	95	105
C ₂	0.020	0.58	0.68	102	95	95
C ₃	0.016	0.60	0.70	105	98	98
C ₄	0.020	0.72	0.71	101	99	98
B ₁	0.016	0.56	0.56	98	98	99
D ₁	0.020	0.68	0.60	95	105	102
Mixed (D)	0.61	0.54	0.58	95	102	-
Mixed (E)	0.200	0.71	0.70	99	95	-

and digestion and then distilled with 15-10 mL of sodium D of crystalline insulin dissolved in 10 mL of sulphuric acid with 11 g of catalyst and submitted to the process of hydroxide (40%), collected with boric acid solution and titrated against 0.01 N_{HCl}. Nitrogen content of each sample is shown in Table 5. Results complied with BP and Lilly specification, range (95-105%).

Study of insulin purity of insulin samples by electrophoresis: From the electrophoresis run, all samples showed migration times similar to that of the standard insulin and complied with the BP and each sample showed a single band indicating a pure sample as a quality control, human normal serum was used.

CONCLUSION

In this study, it was shown that insulin was extracted, purified and dried. Extraction was carried out from fresh glands obtained from beef slaughtered for local consumption and for export. The purity of insulin obtained was compared with that of leading manufacturing companies using some analytical techniques such as electrophoresis and atomic absorption and amino acid analyzer. The quality of prepared insulin products met the official pharmacopoeia and manufacturers specifications. The project is of paramount importance since it provides the scientific basis for utilization of locally available beef pancreas for production of different insulin products needed by the increasing numbers of diabetic patients.

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