

Determination of the Potency of Extracted, Purified and Formulated Insulin from the Pancreatic Organs of the Sudanese Beef Cattle

Abdella Emam Abdella Baragob¹, Waleed Hassan AlMalki¹, Imran Shahid^{1*},
Ragia Mahmood Hegazy¹, Khojali Salwa Muhammed², Samia Abdella²

¹Department of Pharmacology and Toxicology, College of Pharmacy, Umm Al Qura University, Makkah, KSA; ²Veterinary Research Institute, Khartoum, Sudan.

Email: aeabaragob@yahoo.com, whmalki@uqu.edu.sa, iyshahid@uqu.edu.sa, salwamuhammed@hotmail.com,
r.m.hegazy@hotmail.com, samiah11@gmail.com

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ABSTRACT

The treatment of type 1 diabetes is mainly dependent on insulin therapy and current formulated insulin formulations are used for its control all over the world. The presented study was designed to evaluate the potency of extracted, purified and formulated insulin from the pancreatic organs of the Sudanese beef cattle. Twenty healthy rabbits were used to conduct the study following subcutaneous administration of the sample insulin, to determine the hypoglycemic effect and to analyze the potency of the testing insulin by the hypoglycemic seizure method, blood sugar method and glucose enzymatic colorimetric test (GOD-PAP) respectively. The potency of the injected insulin samples was estimated by comparing the variation in blood glucose levels produced in the treated animals with that produced by a standard insulin preparation under the suitable conditions of the blood sugar method. The results revealed that the potency of the testing beef insulin samples was slightly higher (*i.e.*, 2.2 USP units/ml, 9%) compared to the standard and assumed potency of the prepared insulin preparations (*i.e.*, 1 - 2 USP units/ml) which indicated that the solvents and diluents used to prepare the assay dilution might be of higher potency and must be diluted to such an extent that the testing insulin potency must be compatible with the standard dilutions. Furthermore, to determine the choice of an assay to analyze the potency of insulin preparations, not only the accuracy of the result but also the purpose for which the test is to be used and the time limit must be taken into consideration.

Keywords: Blood Sugar Method; Glucose Oxidase Method; Hypoglycemic Effect; Insulin Potency

1. Introduction

Blood sugar level refers to the amount of glucose in the blood and is a prime source of energy to the body cells, and it was transported via the bloodstream [1]. Insulin produced by the pancreatic beta cells plays a pivotal role in regulating the blood sugar level as a part of metabolic homeostasis [2]. The normal blood glucose level maintains between about 4 - 8 mmole/L (*i.e.*, 70 - 150 mg/dl), but it fluctuates around the clock throughout the day. The blood glucose levels are the lowest in the morning (for non-diabetics 70 - 100 mg/dl) and rise after meals for an hour or two [3]. According to the American Diabetes Association, the blood glucose range for a diabetic patient should be 70 - 130 mg/dl before meals and less than

180 mg/dl after meals as measured by a glucose monitor [4]. The blood sugar levels outside the normal range indicates a medical condition where a persistently higher level is termed as hyperglycemia and the lower level as hypoglycemia [5]. Diabetes mellitus is a disease characterized by persistent hyperglycemia and the most prominent disease related to failure of the blood glucose regulation. Different Insulin formulations from some esteemed manufacturing companies like Novo Nordisk, Sanofi-Aventis, Biocon and Eli Lilly are used to lower the blood glucose levels (as hypoglycemic agent) and these are very much effective to regulate the blood glucose levels in diabetic patients [6]. The presented study was aimed to determine the potency of some unknown extracted, purified, and formulated insulin preparations

*Corresponding author.

from the pancreas of the Sudanese beef cattle. The potency of the testing insulin preparations was determined in rabbits by administering subcutaneous injections and by comparing the decreased glucose level to the standard insulin preparations by the blood sugar method [7] modified with the glucose enzymatic colorimetric test [8]. The hypoglycemic seizures method which produced convulsions in treating animals after administering hypoglycemic agents was used to confirm that the testing samples possess hypoglycemic activity. The results revealed that the testing insulin dilutions possess potent hypoglycemic activity compared to standard insulin dilutions and these preparations must be diluted to such an extent that the final dilutions are compatible with standard insulin dilutions in term of potency.

2. Materials and Methods

2.1. Materials

2.1.1. The Animal

Twenty suitable healthy rabbits weighing 1.8 - 2.2 kg of either sex were used in this study. The animals were housed not in cages less than one week before the start of the assay under constant environmental and nutritional conditions throughout the period of investigation. The animals were allowed a free access to water and diet consisting of standard chow at all times except during the assay [9].

2.1.2. Chemicals and Reagents

All the chemicals and reagents used in different stages of the study were of pharmaceutical and analytical grades. The preparations of stock, dilutions of standard and sample preparations were according to the manufacturer's protocols and standard reference book procedures [10].

2.1.3. Standard Insulin Solution

A quantity of USP beef insulin reference standard weighed accurately was dissolved in double distilled water containing 0.1% - 0.25% (w/v) of either phenol or cresol, 1.4% - 1.8% of glycerin and sufficient hydrochloric acid (HCl) to adjust pH between 2.5 - 3.5 to make a standard beef insulin solution containing 40 USP insulin units per ml. The solution was kept at 4°C in a cool place protecting from freezing [11].

2.1.4. Standard Insulin Dilutions

The portions of the standard insulin solution were diluted in double distilled water containing 0.1% - 0.25% (w/v) of either phenol or cresol, 1.4% - 1.8% of glycerin and sufficient hydrochloric acid (HCl) to adjust pH between 2.5 - 3.5 to make two standard insulin dilutions, one to contain 1.0 USP units per ml (standard dilution 1), and

the other to contain 2.0 USP insulin units per ml (standard dilution 2) and stored in a cool dry place at 4°C protecting from freezing.

2.1.5. Assay Dilutions or Sample Beef Insulin Dilutions

Applying the same diluent and other ingredients as used in preparing the standard dilutions, we prepared two dilutions of the beef insulin to be assayed, one of which may be expected on the basis of the assumed potency to contain 1.0 USP insulin unit per ml (assay dilution 1), and the other to contain 2.0 USP insulin units per ml (assay dilution 2) and stored in a cool dry place at 4°C protecting from freezing.

2.1.6. Volume of the Standard and Assay Dilution to Be Injected

The volume of standard and assay dilution to be injected was in between 0.3 - 0.5 ml for each animal in the groups. A total volume of 0.4 ml was injected for each standard and assay dilution.

2.1.7. Identification of Insulin

Six rabbits of either sex weighing 1.8 - 2.2 kg from which food had been withheld for the previous 18 - 24 hrs were used to identify the extracted, purified and formulated samples contain insulin. Each animal injected subcutaneously with sample insulin (0.4 ml) till the symptoms of hypoglycemia appeared (*i.e.*, convulsions), then each animal received a subcutaneous dose of dextrose (5 ml of 50% dextrose solution) to remove the hypoglycemic symptoms. The animals were closely observed for the following three days. The same procedure was followed for the standard insulin preparations [12].

2.2. Rabbit Blood Sugar Method

This method is used to determine the potency of insulin reference standards, validation and stability of new insulin preparations and to determine the specific activity of insulin analogs. Although the procedure is relatively cumbersome, it has an accurate influence on the diabetic patients. The rabbit blood sugar method used in this study is according to the protocol described in US pharmacopeia [7]. The blood glucose levels were determined by the glucose oxidase (GOD-PAP) method [8].

2.3. Experimental Design and Treatment Protocol

The animals were divided into four groups (five rabbits in each group). On the preceding day, approximately 20 hours before the assay, each animal was provided with water and food which must consume within 6 hours. The

same feeding schedule was followed before each test day. During the assay, all food and water were withheld until after the final blood specimen was taken. The rabbits were handled with care in order to avoid undue excitement, the doses were injected subcutaneously as shown in **Table 1**. The second injection was made on the day after the first injection and not more than one week later. The time between the first and second injection was the same for all rabbits throughout the experiment. The blood sample was collected after one hour or maximum 2.5 hrs after the treatment from a marginal ear vein of each rabbit.

2.4. Determination of Glucose Level in Blood Specimens

In a falcon tube, 0.8 ml blood was collected and added 0.5 ml of sodium fluoride as an anticoagulant. Centrifuged at 3000 rpm for 5 minutes and serum was separated in another eppendorf tube. 10 µl of the serum was shifted in another eppendorf tube and 1000 µl of working reagent (buffer + enzyme) were added. The same procedure was repeated for the standard in another eppendorf tube while adding 10 µl of standard reagent instead of serum and incubated the eppendorf tubes (sample + standard) at room temperature (25°C) for 10 minutes. Then determined the absorbance at 420 nm in a reading colorimeter or spectrophotometer at 490 - 520 nm [8]. The absorbance of sample (A) was determined as:

$$\begin{aligned} & \text{Absorbance of sample (A)} \\ & = \text{absorbance of sample} - \text{absorbance of blank} \\ & \text{Absorbance of standard known} \\ & \text{concentration (100 mg/dl)} \\ & = \text{The concentration of glucose in sample (A)} \\ & = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of standard} \end{aligned}$$

3. Data Analysis

The variation in blood glucose level of each rabbit injected with standard and testing insulin dilution was calculated from the sum of the two blood-sugar values and

Table 1. Treatment protocol for standard and sample insulins solution to be injected.

Group	First Injection	Second Injection
1	Standard Dilution 2	Assay Dilution 1
2	Standard Dilution 1	Assay Dilution 2
3	Assay Dilution 2	Standard Dilution 1
4	Assay Dilution 1	Standard Dilution 2

subtracted its response to standard dilution 1 from that to standard dilution 2 disregarding the chronological order in which the responses were observed to obtain the individual differences “y” as shown in the **Table 2**.

As the number of rabbits carried through the experiment was the same in each group, so total the individual response (Y's) in each group and computed;

$$T_a = -T_1 + T_2 + T_3 - T_4$$

and

$$T_b = T_1 + T_2 + T_3 + T_4.$$

The logarithm of the relative potency of the test dilutions is

$$M' = 0.301 T_a / T_b.$$

The potency of the insulin injection in USP units per ml equals to the antilog (log R + M')

where

$$R = V_s / V_u$$

V_s = the volume of the standard
insulin dilution (in ml),

and

V_u = the volume of the sample
insulin dilution (in ml).

T_a = t-test for testing insulin samples

T_b = t-test for standard insulin samples

The confidence interval between different groups < 0.0816, which corresponds to $P < 0.95$, was considered statistically significant.

4. Results

Five rabbits in group 1 injected subcutaneously with 0.4 ml insulin dilutions (standard and testing) and their blood samples were taken to determine the blood glucose level by the glucose oxidase method. From each rabbit, two blood samples were taken and mean of the two blood glucose level was taken as the final value. The same

Table 2. Flow chart to determine the variation in blood glucose level in treating rabbits in each group when injected with standard and testing insulin dilutions.

Group	Differences	Individual Response (Y)	Total Response (T)
1	Standard Dilution 2 – Assay Dilution 1	Y1	T1
2	Assay Dilution 2 – Standard Dilution 1	Y2	T2
3	Assay Dilution 2 – Standard Dilution 1	Y3	T3
4	Standard Dilution 2 – Assay Dilution 1	Y4	T4

protocol was followed for the other animal groups. Group 1 rabbits were injected subcutaneously with standard dilution 2 and assay dilution 1 respectively. The response to assay dilution 1 was subtracted from standard dilution 2 to get the individual difference in blood glucose level (**Table 3**). The confidence interval between different groups <0.0816 , which corresponds to $P < 0.95$, was considered statistically significant.

In group 2, the rabbits were treated with standard dilution 1 and assay dilution 2 respectively. The response to standard dilution 1 was subtracted from assay dilution 2 to obtain the individual difference in blood glucose concentration (**Table 4**).

In group 3, the rabbits were injected first with 0.4 ml

assay dilution 2 and then standard dilution 1 respectively. The response to standard dilution 1 was subtracted from assay dilution 2 to obtain the individual differences in blood glucose level (**Table 5**).

In group 4, the rabbits were injected first with 0.4 ml assay dilution 1 and then standard dilution 2 respectively. The response to assay dilution 1 was subtracted from standard dilution 2 to get the individual differences in blood glucose level (**Table 6**).

The T (student t-test) value of each group was calculated by determining the total response of each group by adding the individual response difference between the first injected dose and the second dose respectively (as directed in **Table 7**).

Table 3. Individual differences in blood glucose concentrations in group 1 treating animals.

Group 1	Standard Dilution 2 (ml)	Glucose Conc. (mg/100ml)	Assay Dilution 1 (ml)	Glucose Conc. (mg/100ml)	Difference (mg/100ml)
1	0.4	105	0.4	102	3
2	0.4	104	0.4	109.8	-5.8
3	0.4	90	0.4	94.5	-4.5
4	0.4	100.5	0.4	95.2	5.3
5	0.4	78	0.4	86.5	-8.5

Table 4. Blood glucose level and Individual differences in blood glucose concentrations in group 2 treating animals.

Group 2	Standard Dilution 1 (ml)	Glucose Conc. (mg/100ml)	Assay Dilution 2 (ml)	Glucose Conc. (mg/100ml)	Difference (mg/100ml)
1	0.4	103	0.4	107	4
2	0.4	105.6	0.4	105.6	0
3	0.4	108.6	0.4	112	3.4
4	0.4	88	0.4	88	0
5	0.4	104.6	0.4	108	3.4

Table 5. Blood glucose level and Individual differences in blood glucose concentrations in group 3 treating animals.

Group 3	Assay Dilution 2 (ml)	Glucose conc. (mg/100ml)	Standard Dilution 1 (ml)	Glucose Conc. (mg/100ml)	Difference (mg/100ml)
1	0.4	111.5	0.4	108.3	3.2
2	0.4	92.8	0.4	88	4.8
3	0.4	105.6	0.4	102	3.6
4	0.4	111	0.4	108	3
5	0.4	103	0.4	100	3

Table 6. Individual differences in blood glucose concentrations in group 4 treating animals with assay dilution 1 and standard dilution 2.

Group 4	Assay Dilution 1 (ml)	Glucose Conc. (mg/100ml)	Standard Dilution 2 (ml)	Glucose Conc. (mg/100ml)	Difference (mg/100ml)
1	0.4	116	0.4	113.5	-2.5
2	0.4	113.9	0.4	110	-3.9
3	0.4	104.6	0.4	100	-4.6
4	0.4	118	0.4	115	-3
5	0.4	109	0.4	112	-3

Table 7. T-value of each group injected with standard and sample insulin preparations.

Group	T Value	Differences in Individual Response between Different Insulin Dilutions (mg/100ml)					Total Response (mg/100ml)
1	-T1	3	-5.8	-4.5	5.3	-8.5	-10.5
2	T2	4	0	3.4	0	3.4	10.8
3	T3	3.2	4.8	3.6	3	3	17.6
4	-T4	-2.5	-3.9	-4.6	-3	3	-11

Calculated the “Ta” and “Tb” by putting the values in the following equation

$$\begin{aligned}
 T_a &= -T_1 + T_2 + T_3 - T_4 \\
 T_a &= -(-10.5 \text{ mg/100ml}) + (10.8 \text{ mg/100ml}) \\
 &\quad + (17.6 \text{ mg/100ml}) - (-11 \text{ mg/100ml}) \\
 &= 49.9 \text{ mg/100ml.} \\
 T_b &= T_1 + T_2 + T_3 + T_4. \\
 T_b &= (-10.5 \text{ mg/100ml}) + (10.8 \text{ mg/100ml}) \\
 &\quad + (17.6 \text{ mg/100ml}) + (-11 \text{ mg/100ml}) \\
 &= 6.9 \text{ mg/100ml}
 \end{aligned}$$

The logarithm of the relative potency of the test dilution was M:

$$\begin{aligned}
 \text{Potency} &= P \\
 &= \text{Antilogarithm } M \\
 &= (\text{Antilogarithm } M')R \\
 M' &= 0.301 \log T_a/T_b [8]
 \end{aligned}$$

where

$$\begin{aligned}
 &“T_a = 49.9 \text{ mg/100ml, } T_b = 6.9 \text{ mg/100ml}” \\
 M' &= 0.301 \frac{49.9}{6.9} = 2.176 = 0.338
 \end{aligned}$$

Potency of the testing insulin injection in USP units per ml = The Antilog (logs R + M), where:

$$R = \frac{V_s}{V_u}$$

and V_s = the volume of the standard insulin dilution (in ml) and V_u = the volume of the assay insulin dilution (in ml).

$$\begin{aligned}
 &\text{Potency of the injection} \\
 &\text{in USP units per ml} \\
 &= \text{Antilog} \left(\log \frac{0.4}{0.4} + 0.338 \right) \\
 &= \text{Antilog} (0 + 0.338) \\
 &= 2.177 \\
 &= 2.2 \text{ units} \\
 &= 2.2 \text{ USP units/ml.}
 \end{aligned}$$

5. Discussion

The presented study described an effort to evaluate the potency of unknown insulin preparations extracted from Sudanese beef cattle using long-standing rabbit blood sugar and glucose oxidase methods. Despite the advancement in practical and sophisticated methods (e.g., liquid and high pressure liquid chromatography) to measure insulin potency has resulted in more accurate and precise procedures for insulin and insulin products, the bioidentity of insulin and insulin products cannot be assessed by these methods [13]. Similarly, in several pharmacopoeias the biological assays have been replaced by chemical methods, but the rabbit blood sugar method is widely used and still applicable to determine the bioidentity and potency of unknown insulin samples [8]. The results obtained were in accordance with as described by J. W. Young [14], and individual monographs in standard official books [8]. The method of hypoglycemic seizures in the rabbit was used to confirm the identifying of testing samples as insulin samples. We observed that after administering subcutaneous insulin all rabbits developed rapidly symptoms of hypoglycemia (convulsions) which confirmed that the injected samples contain insulin. Administration of 5 ml dextrose (50% dextrose solution) reversed the hypoglycemic effect of testing insulin and four of the injected animals remained alive for three days. These results are in line with the designed experiments as mentioned in the official books for identification of insulin methods [15].

The blood glucose level in treating rabbits was determined by the glucose oxidase method. We used the enzymatic method which is highly specific for glucose and exploit the non-specific reducing property of glucose in a reaction with an indicator substance that acquires or changes color on its reduction. The results obtained in each group treating with insulin assay dilution 1 and 2 were remarkably similar in term of variation of blood glucose level when compared to the standard dilutions. We calculated 2.2 USP units/ml, the potency of the testing insulin sample after performing all the experimental procedure and analyzing the whole data which indicated moderately high potency (9%) compared to the assumed potency of the assay and standard dilution (*i.e.*, 1 - 2 USP units/ml). The reason behind that might be the solution

used in the study were of higher concentrations and tested insulin samples must be diluted to such an extent that their assumed potency compatible to the standard insulin dilutions. Furthermore, we noted that the glucose oxidase method was reliable to determine blood glucose level as the precision, accuracy and similarity of the variations of blood glucose in treating animals was remarkably uniform. It may conclude here that for the choice of an assay to determine the bioidentity and potency of insulin samples, not only the accuracy of the result but also the purpose for which the test is to be used and the time limit must be taken into consideration. Moreover, the selection of the method to determine the insulin potency of unknown preparations may be of great values if the assay is conducted with insulin preparations not only of unknown concentration but also of purity.

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