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Protein engineering design of thermostable insulin for use in various medication

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ABSTRACT

Background: Insulin is utilized to sustenance type 1 diabetes mellitus, a chronic and severe condition. Numerous drawbacks exist with subcutaneous insulin injection, such as the fact that it is a thermolability medicine that cannot be kept at room temperature and must instead be kept in a refrigerator (2-8 0C). **Methodology**: In the present study, thermostable insulin was developed that could be kept at ambient temperature away from the refrigerator. In order to manage diabetes mellitus types 1 and 2, insulin is a crucial bio-molecule. Insulin subcutaneous injections have many drawbacks, but the new thermostable insulin has helped to overcome them. Via the inclusion of cysteine and cysteine next to one another in the alpha helices of the core of the two sub-units of insulin, insulin was created utilizing recombinant DNA technology and bio-informatics technology in this study. Trans-dermal drug delivery techniques, such as insulin patches and all forms of insulin injections, as well as other drug delivery systems might be used to create modified thermostable insulin (subcutaneous and intravenous injections). **Results**: The effectiveness of the test insulin was evaluated using animal models and compared to that of regular subcutaneous insulin. Although the test insulin was thermostable and could be kept at ambient temperature outside of the refrigerator, it was only about 90% as effective as the normal one. **Conclusion**: The current investigation was promising since thermostable insulin has evolved with the application of fungal recombinant DNA technology.

Introduction

The burden of diabetes mellitus illness is widespread and persistent. Diabetes mellitus, a metabolic condition brought on by insulin insufficiency and/or insulin resistance, is characterized by hyperglycemia and sometimes ketoacidosis as a result of the beta-oxidation of fatty acids for energy. Type 1 diabetes mellitus is responsible for 10% of all diabetics globally. It is characterized by a complete lack of insulin brought on by an autoimmune assault on the pancreatic beta

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cells of Langerhans. Diabetes mellitus type 1 must be treated with insulin. Strong hereditary influences have a role in type 2 diabetes. It happens as a result of both insulin resistance and damaged beta cells. The most typical cause of insulin resistance is obesity.[1]

There are two primary categories for it (I and II).[2] Insulin produced by the beta islets of the pancreas is the sole treatment for type I diabetes mellitus.[3] Oral hypoglycemic medications are the mainstay of treatment for type II diabetes;[4] although insulin may be needed in life-threatening situations if there is severe hyperglycemia.[5] In an adult person, the endocrine pancreas is made up of around 1 million islets of Langerhans that are distributed throughout the pancreatic gland.[6] Four or more hormone-producing cells can be found inside the islets.[7] These hormones include Insulin, the body's storage and anabolism hormone;[8] Islet amyloid polypeptide (IAPP, or Amylin), which regulates hunger, stomach emptying, and Glucagon and insulin production;[9] Glucagon, the hyperglycemia factor that mobilizes glycogen stores; and others.[10]

Somatostatin, a general secretory cell inhibitor,[11] pancreatic poly-peptide, a tiny protein that speeds up digestion by a mechanism that is yet unclear.[12] Pancreatic hormones: Insulin is secreted by beta cells, glucagon by alpha cells, somatostatin by sigma cells, which locally controls insulin and glucagon release, and pancreatic poly-peptide by Somatostatin-producing cells make up the endocrine component of the pancreatic islets of Langerhans.[13] A protein with a molecular weight of roughly 6000 Dalton, Insulin is a tiny protein.[14] Di-sulfide bonds hold the two chains that make up the substance together.[15] Pharmacological actions of insulin:

Insulin has anabolism effects; it promotes glucose absorption and storage by various tissues as well as protein synthesis by boosting cellular uptake of amino acids and by raising ribosome activity. Both type 1 and type 2 diabetics need insulin to reduce their blood sugar levels; moreover, diabetes mellitus in pregnancy. A young surgeon by the name of Frederick Bunting and his helper Charles Best discovered how to extract insulin from a dog's pancreas in 1921. Recombinant human insulin and insulin analogues were created from animal pancreas crude extracts as therapeutic insulin. In order to more closely resemble the natural insulin response, the time-response profiles of insulin and formulations have been changed.[16] Insulin Delivery Systems: Subcutaneous injection using normal disposable needles and syringes is the typical method of administering insulin treatment.[17] Insulin injection's primary drawback: Drug with thermolability.[18] In this work, the objective was to overcome the limitation by employing innovative drug delivery systems incorporating thermostable insulin that had been changed by genetic engineering and peptidomimetics to increase its physicochemical properties.

Methodology

Ethical statement:

The use of humans and animals was conducted in accordance with all applicable national, international, and/or institutional rules in the current study. The ethical committee for the handling of humans and animals at Cairo University (ECAHCU), located at Cairo University, Egypt's college of pharmacy, approved all procedures used in the study, including those involving people and animals, with permission number P-2-2-2021. Every possible measures were taken to reduce the number of people and animals used in the study as well as their suffering.

Equipment:

The type of the study:

experimental study of screening.

Place and the date of the study:

The current investigation was conducted at Cairo University's pharmacy school in Egypt.

Material:

The chemical and biological components were bought from the pharmaceutical companies Algomhoria in Cairo, Egypt, and Alnasr in Abo zabal Alkhanka, Qalyubia, Egypt.

Source of animal models:

They were legally acquired from the faculty of pharmacy at Cairo University at Egypt's pharmacology and toxicology department.

Inclusion criteria for animal models are:

Fat adult male animals (rabbits or mice) (ii) Hyperglycemia may be generated in animal models. (iii) It is simple to estimate the blood glucose levels of animals. Obesity animal models are (iv). In the current investigation, obese male albino mice weighing between 40 and 50 gm and adult obese male rabbits weighing around 2 kg each were used. Prior to the trial, mice underwent a week of

acclimatization. At 50% humidity, a 12-hour light/dark cycle, and a set temperature (25 0C). Mice were fed a naturally occurring commercially available chow diet (Elnasr pharmaceutical and chemical company).

Exclusion criteria are:

Infant animals. (ii) Animals in gestation.

Animal blood glucose levels are difficult to assess (ii). (iv) Animals without obesity.

Methods

Primer construction for expression of recombinant human insulin:

This was accomplished using the NCBI website and the Microsoft NEBcutter version 3 application.

Forward expression primer:

ACATTGGTGCTACCAGCCTC

Tm= 60.04 0C, Ta=55.04 0C

Reverse expression primer:

GCGGGTATCGCTGGTATGAA

Tm= 59.97 0C, Ta=54.97 0C

Fungal production of recombinant thermostable human insulin:

Using the NCBI website, it was possible to identify the most flexible regions in the core of the human insulin protein; following this, bioinformatics and genetic engineering were used to change two residues in these flexible regions. The mutations involved single nucleotide polymorphisms designed using Pymol software, which placed two cysteines next to one another not more than 0.2 nm apart, forming a disulfide bond that increased the protein's stability. Pymol and pdb 2 max in gomacs software packages were used to achieve this. Also, the design of a physically stable insulin was accomplished by introducing two cysteine residues next to one another in the alpha helices in the insulin protein's core region. A stationary Saccharomyces cerevisiae culture in LB medium was exposed to 60 0C gamma radiation while being continuously flushed with oxygen to produce mutants.

Saccharomyces cerevisiae BJ1824 was used as the expression host to successfully synthesise customised insulin using fungal recombinant DNA technology. The C-terminal was 6x histidine, the inducer was methanol, the promotor was AUG1, and the expression system vector was PYES2-DEST52.

Upstream process:

Biosynthesis of recombinant thermostable insulin:

By using Saccharomyces cerevisiae BJ1824 as an expression host, recombinant DNA technology was used to synthesise human insulin. The expression vector was PYES2-DEST52, the promoter was AUG1, the inducer was methanol, and the C-terminus was 6x histidine. The target gene from human insulin was cloned using primers for polymerase chain reaction (PCR) expression of the human insulin; for plasmid digestion, it was sub-cloned into PYES2-DEST52 recombinant plasmids using HindIII and EcoRI restriction endonuclease II; this was followed by ligase enzyme ligation. After being created and grown in E. coli Top 10 (Invitrogen, USA), recombinant plasmids were converted into the expression host Saccharomyces cerevisiae BJ1824. Yeast transformants were grown at 30 °C using YNBG selective medium (0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% galactose), and were further maintained in YPG-rich medium (2% bacteriopeptone, 1% yeast extract, and 2% galactose). 2% galactose was used as an inducer for the production of insulin.

Downstream process:

Later on centrifuge for three minutes at 4000 rpm, the purification of the soluble insulin protein precursor from 100 ml of the culture's supernatant was partially completed by precipitation with 52 ml of 60% ethanol. After this, recombinant soluble insulin was completely purified using nickel affinity chromatography.

Clarification and purification of recombinant human insulin:

Three minutes were spent centrifuging at 4000 rpm. After precipitating (salting out) 100 ml of culture supernatant with 53 ml of 4.1 M ammonium sulphate with ammonium sulphate, the soluble insulin protein precursors from the culture supernatants were clarified. Nickel affinity chromatography was then used to separate the clarified proteins. Immobilized metal affinity chromatography could be used to quickly purify recombinant fusion insulin proteins with polyhistidine-tagged proteins from supernatants on nickel columns (The metal ligand was a nickel metal ion, but the target bio-molecule was a polyhistidine-tagged fusion protein.). Before being put into its final form, the preparation was sterilized by

filtration via sterile Whatman 1541-042 filter paper, which was purchased in the USA.

Formulation of recombinant human soluble insulin:

Clear, colourless solution at 30 mg/ml in 0.01 M HCL or diluted (1%) acetic acid. Moreover, insulin might dissolve in 125 mM NaHCO3. At a neutral PH, insulin demonstrated limited solubility. At 6–10 mg/ml in diluted acetic[1%] or hydrochloric acid, PH 2-3, it might be solubilized. Due to the limited solubility of insulin at neutral PH, soluble insulin was created by dissolving white recombinant human insulin at a concentration of 6–10 mg/ml in 1% acetic acid or 0.01 M HCL. The phamacokinetic and pharmacodynamic properties of test insulin were similar to those of quick acting insulin. Recombinant human soluble insulin was available in 5 formulations [F1-F5] that ranged in strength from 6 to 10 mg/ml, respectively.

Determination of pharmacologic effects of recombinant insulin in preclinical trials animal testing:

Screening and bio-assay of test recombinant insulin was achieved using:

Rabbit blood glucose sugar method:

Principle: In rabbits, insulin lowers blood sugar levels, and the amount of blood sugar reduction is exactly proportional to the dosage. 10 rabbits, each weighing around 2 kg, were employed in the current investigation.

Procedure: There were One hundred rabbits [each rabbit weighted close to 2 kilo-gramm] utilized. In a preliminary experiment, the positive control group of rabbits received subcutaneous injections of standard insulin in graduated doses (0.1–0.5 IU/kg), while the test group received subcutaneous injections of test insulin in graduated doses (0.1–0.5 IU/kg) after fasting for 18 hours for both groups of animal models (positive control and standard). Every bunny that had convulsions within five hours was eliminated. The rabbits were then divided into four groups at random, fasted for at least 18 hours, and a blood sample was collected from an ear vein to gauge the starting blood sugar level (BGL). Following the administration of an insulin dosage in accordance with the 2 and 2-dose assay to each group, blood samples were obtained every hour for a total of 5 hours. The BGL of the combined sample was calculated after pooling the samples from each rabbit. There was a drop in blood glucose levels. The next day, a cross over test was conducted. The relative potency was computed based on the average BGL reduction for each selected dosage.

Mouse convulsion method using 2 and 2 dose assay technique:

Principle: In mice, insulin lowers blood glucose levels. The hypoglycemia convulsion happens when it reaches a crucial point. The number of convulsing mice increases in direct proportion to dosage.

Procedure: Techniques for 2 and 2 dosage assays were used. 100 mice weighing 40–50 g were maintained at a constant temperature of 29–35 0C while fasting for 12–24 hours. The test insulin had graduated doses of insulin (0.1-0.5 IU/kg) and was administered subcutaneously, whereas the standard insulin was administered intraperitoneally (IP) with graded doses of insulin (0.1-0.5 IU/kg). The animals were then monitored for 1.5 hours. The proportion of animals in each group that passed away, displayed convulsions, or stayed on their backs for two to three seconds after being turned over was computed to establish the relative potency. Because the animals would perish, cross-over testing could not be performed.

Evaluation tests of test soluble human insulin given par-eternally:

These tests were performed in accordance with British Pharmacopoeia 2021 guidelines.

Compatibility study:

By using FT-IR (Perkin-Elmer 1600 FTIR spectrophotometer), spectroscopy, and DSC (Shimadzu-DSC 50), the compatibility of recombinant test human insulin and various excipients used in the creation of par-enteral formulations were examined. The improved powder formulation was mixed with 200 mg of KBr, compacted into discs, and scanned at a speed of 5 mm/sec with a resolution of 1 cm-1 across a range of 4000-200 cm-1. Using several scanning calorimeters, experiments with thermal analysis were conducted (DSC). In hermetically sealed aluminium pans, we heated samples of the improved formulation at a continuous pace of 110 °C per minute while purging the air with nitrogen at a rate of 35 ml per minute. The temperature range we used was 0–4000 °C.

Determination of uniformity of drug content:

Test recombinant human insulin powder weighing 10 mg was combined with 100 ml of distilled water to form a solution. We processed the

mixture through Whatman filter paper No. 40 after sonicating it for 170 seconds. After that, the filtrate was thinned down with distilled water, and the absorbance at 280 nm was calculated as a result of covalent insulin dityrosine dimerization and disulfide photolysis brought on by UV light exposure.

In vitro drug release profile:

The dissolve media (300 ml) was distilled water, which was employed at 37 0C, PH 3, and 50 rpm (paddle). According to USP pharmacopoeia requirements 2021, we took samples (25ml) at intervals of 3, 6, 8, 11, 16, 19, 60, 120, and 240 minutes. The withdrawn quantities were then substituted with corresponding amounts of the simple dissolving medium. Due to covalent insulin dityrosine dimerization and disulfide photolysis brought on by UV light exposure, the quantity of insulin released was determined at 280 nm using a UV spectrophotometer. Dissolution testers of the DISi brand were created in Copley, England.

Stability study:

According to USP Pharmacopoeia 2021, a stability test of an insulin subcutaneous injection formulation was performed on three groups:

In this test, a negative control group of insulin series vials kept at 2-8 0C for two months was used. During the stability test technique, a typical group of a number of positive control insulin vials was used.

The current stability test experiment also included a number of test vials of insulin. Insulin vials used for testing as well as the standard (positive control) were both kept at room temperature for two months, with temperature swings between 25 and 37 0C. For the three groups of the insulin stability experiment, HPLC chromatography and UV spectroscopy analysis were used to determine the potency of the insulin. The parameters of the stability test included observing the colour of the insulin solution visually and measuring its potency using UV spectroscopy at a wavelength of 280 NM, as well as conducting in vivo tests on mouse convulsions and rabbit blood glucose using the 2 and 2 dose assay technique in both experiments. At a wavelength of 280 nm, the samples were evaluated for UV content. During animal model fasting, the mouse convulsion technique and the rabbit blood sugar test were conducted.

Human evaluation of human soluble insulin drug subcutaneous drug delivery system via human clinical trials phases 1/2:

The current study comprised three groups of adult volunteers with type 1 diabetes who had fasting hyperglycemia of 200 mg/dl or above. These patients were treated at Al-Qasr Ainy and Zagazig general hospitals. 100 subjects made up each group.

Graded doses of the placebo (0.2–0.5U/kg) were given to Group 1 (the negative control group) through subcutaneous injection. Gradually varying doses of the conventional insulin (0.2-0.5U/kg) were subcutaneously and intravenously administered to Group 2 (the positive control group).

Graded doses of the test recombinant human insulin (0.2–0.3U/kg of insulin via subcutaneous injection) were given to Group 3 (the test group). The drop in blood glucose level while fasting served as a proxy for measuring insulin action.

In vivo bio-availability and phamacokinetic studies:

0.7–0.9 ml of samples were taken both before and after the subcutaneous insulin injection, at intervals of 30, 60, 120, and 240 minutes. Within an hour after collection, blood samples were further chilled and centrifuged at 4 0C. Using HPLC, insulin concentrations were identified. Phosphate buffer (PH 4.4) and acetonitrile (660/340, v/v) were used as the mobile phase in an HPLC study using a reversed phase column with a flow rate of 0.9 ml/min. The 280 NM wavelength was the upper limit for UV assessment of blood insulin levels. The proportion of relative bioavailability and the area under the curve (AUC) were evaluated. The following formula was used to calculate the relative bio-availability as a percentage: % Relative bio-availability=(AUC Subcutaneous/AUC Intravenous)(Dose Intravenous/Dose Subcutaneous)100%.

The standard and control groups underwent the identical treatments.

Statistical analysis

Triplets were used for all civilizations. They used means and standard deviation to present their findings. The means for doing statistical analysis, including statistical analysis based on Excel spreadsheet software, were one way analysis of variance (p value.05). In this investigation, the F test was used.

Results

The smallest dose of insulin that was still able to reduce the normal blood glucose of rabbits from 0.1% to.039 mg/dL was 2.7 mg (as shown in table 2). Prior to using the mouse convulsion technique using the 2 and 2 dose test, mice were fasted for 24 hours. It demonstrated that 75% of the mice experienced convulsions due to the hypoglycemic effect of insulin starting at 2.9 mg of dose (as displayed in table 3). The FT-IR and DSC investigation demonstrated that there was no potential for interaction between recombinant human insulin and excipients. All formulations' drug content percentages ranged from 97.24% to 98.77% of recombinant insulin, which was in an average range.

The subcutaneous insulin injection had a release time that varied from 97.81% to 99.26% in 15 to 18 minutes at 37 °C and 50 rpm. The medication was released more quickly from Batch F4 than from any previous batch. Batch F4 showed 98.26% total drug release in 15 minutes. The instant release insulin subcutaneous injection's biological half life (t50%) for batch F4 was found to be 4 minutes.

It was found that the temperature range between 2 and 8 0C was ideal for storing insulin for subcutaneous injection (batch F1 to F5). We conducted an in vivo investigation using formulation F4 and compared the results to an intravenous insulin injection. The blood samples were taken at various intervals, and their drug concentration was then determined by HPLC. Test subcutaneous recombinant human insulin had a T max and C max of 1 hour and 435 micrograms/ml at an average dosage of 0.2–0.3 U/kg. Equation 1 provided an estimate of the relative bioavailability percentage, which was 91%. With an average dosage of 0.2–0.3 U/kg, SC fast acting insulin had a T max of one hour and a C max of around 480 micrograms per millilitre.

In addition to having an estimated 5-hour duration of action and a 90% relative bioavailability, SC insulin's beginning of action was the same for both the test and regular formulations. Both the test and regular insulin had a 100% bioavailability when administered intravenously.

The bio-availability of the test recombinant insulin subcutaneous injection approached 90% during phases 1 and 2 of human clinical trials, while the effectiveness was close to 90%. During a human clinical investigation, the pharmacokinetic profile of insulin subcutaneous injection showed a quick start of action (10–15 minutes), a biological half-life of 3 hours, and an action duration of roughly 5 hours. The liver and the kidneys were shown to be responsible for the bulk of insulin catabolism. When insulin was no longer being supplied directly to the portal vein when the test insulin was administered exogenously, the destructive metabolism profile was altered. Around 30% of the insulin was rejected by the liver, with the remaining 60% by the kidney. Insulin's impact was prolonged and its clearance was reduced as a result of renal impairment.

The delivery of endogenous and exogenous insulin allowed for the detection of this ablated elimination. Health institution, a gradual decrease in the need for exogenous and endogenous insulin as well as an increased risk of hypoglycemia resulted from a reduction in renal utility. After more than 60 days of storage at room temperature, which fluctuated between 25 and 37 0C, test insulin had a discernible yellow colour and insoluble insulin clumps. The range of USP stability test criteria [100 10%] was discovered to apply to test insulin. Visual investigation revealed insoluble clumps of conventional insulin with a yellow tint after one month. Also, after one month of storage at ambient temperature, which varied between 25 and 37 0C, regular insulin's efficacy lost 15% of its original strength; but, after more than 35 days of storage at the same temperature range, it entirely diminished. Insulin vials in the negative control group that were kept in the refrigerator (2-8 0C) maintained 99% of their potency and effectiveness. When compared to the negative and positive control insulin series vial groups at almost two months, test insulin maintained 91% of its potency and efficacy. Test insulin demonstrated therm-stability under ambient temperature varied between 25 and 37 0C for 60 days.

Primer for expression of physically stable insulin[5--3-]:

Forward primer:

ACATTGGTGCTACCAGCCTC

Tm=60.04 0C, Ta=55.040C

Reverse primer :

GCGGGTATCGCTGGTATGAA

Tm=59.97 0C, Ta=54.97 0C.

The insulin screening and bioassay technique for rabbit blood glucose was shown in Table 1. Table 2 displayed a bioassay approach using mouse convulsions (log dose of insulin). The

batch formulations F1 through F5 had consistent drug contents, as shown in Table 7. Figure 4 shows the mouse convulsion technique used for an insulin bioassay. In clinical trial stages 1 and 2, the hypoglycemic effect of the typical rapid-acting subcutaneous par-enteral insulin formulation is depicted in Figure 5. The release profile of sublingual insulin tablets is shown in Table 6. Table 3 provides an estimate of the hypoglycemic impact of test insulin subcutaneous injection throughout phases 1 and 2 of clinical trials. The estimated area under the curve (AUC) of test soluble human insulin administered by subcutaneous injection when fasting is shown in Table 5.

In clinical trial stages 1 and 2, the hypoglycemic effect of normal insulin subcutaneous injection is estimated in Table 4. Figure 1 shows the Saccharomyces cerevisiae produced recombinant human insulin protein's three-dimensional structure. In Figure 2, immobilized metal affinity chromatography using nickel affinity resins is used to purify recombinant human insulin. Recombinant insulin had a purity level of roughly 85%. Figure 3 depicts a method for screening and bio-evaluating thermostable insulin using rabbit blood glucose. Figure 4 shows the mouse convulsion technique used for an insulin bioassay. In clinical trial stages 1 and 2, the hypoglycemic effect of the typical rapid-acting subcutaneous par-enteral insulin formulation is depicted in Figure 5. The estimated hypoglycemic effect of the test quick acting subcutaneous par-enteral insulin formulation throughout clinical trial phases 1 and 2 is shown in Figure 6. Injection of test soluble insulin beneath the skin during a fast is depicted in Figure 7 as an assessment of the area under the curve (AUC). Figure 8 depicts FTIR spectroscopy results that showed there was no interaction between excipients and recombinant human insulin. Figure 9 illustrates DSC thermal analysis, which indicated that there was little chance that recombinant human insulin and excipients would interact. A comparison of the subcutaneous insulin release profile for a stability study between the first release and one month later is shown in Figure 10.

Table 1. List of instruments:.

Instrument	Model and manufacturer
Autoclaves	Tomy, japan
Aerobic incubator	Sanyo, Japan
Digital balance	Mettler Toledo, Switzerland
Oven	Binder, Germany
Deep freezer -80 ^{0}C	Artikel
Refrigerator 5	Whirpool
PH meter electrode	Mettler-toledo, UK
Deep freezer -20 ^{0}C	whirlpool
Gyratory shaker	Corning gyratory shaker, Japan
190-1100nm Ultraviolet-visible spectrophotometer	UV1600PC, China
Light(optical) microscope	Amscope 120X-1200X, China

Table 2. Rabbit Blood Glucose Method for Screening & Bio-assay of Insulin:

Dose of Insulin (mg)	***Decrease in Blood Glucose Level %***
1	0.10%
2	0.04%
3	0.04%
4	0.03%
5	0.03%
6	0.03%
7	0.03%
8	0.025%
9	0.02%
10	0.02%

Table 3. Mouse Convulsion Method for Bioassay (Log Dose of Insulin):

% of Mice Showing Convulsion	*Log Dose of Insulin*
50%	0.0000
60%	0.3010
75%	0.4771
76%	0.4773
78%	0.6020
79%	0.6980
82%	0.8450
86%	0.9030
89%	0.9540
90%	1.0000

Table 4. Estimation of hypoglycemic effect of test insulin subcutaneous injection during clinical trials phases 1/2:

S.C insulin dose(mg/l)	**Blood glucose level(mg/dl)**
10	183
20	166
30	151
40	129
50	121
60	111
70	107
80	89
90	85
100	67

Table 5. Estimation of hypoglycemic impact of standard insulin subcutaneous injection in clinical trials phases 1/2:

S.C insulin dose(mg/l)	Blood glucose level(mg/dl)
10	179
20	165
30	150
40	130
50	122
60	113
70	111
80	89
90	79
100	66

Table 6. It shows the estimation of area under the curve(AUC) of test soluble human insulin given by subcutaneous injection during fasting:

Time(h)	C(µg/ml)
0	0
0.5	15
1	30
2	49.35
3	42
4	37

Table 7. It represents sublingual insulin tablets release profile:

Time(min)	% release
2	40
5	60
10	85
15	99
20	99

Table 8. I t shows drug content uniformity of batch formulation F1-F5:

Batch	**Drug content uniformity**
F1	99.15 ±2.24
F2	97.78 ±1.36
F3	97.24 ±0.99
F4	98.77 ±1.78
F5	98.61 ±2.07

Table 9. It shows stability assay of subcutaneous test insulin compared to subcutaneous standard insulin according to USP pharmacopoeia:

Parameter	**Standard insulin(+ve control) stored at ambient temperature[25-37 ^{0}C].**	**Test insulin stored at ambient temperature[25-37 ^{0}C].**	**-ve control insulin stored at refrigerator temperature[2-8 ^{0}C].**
Potency	Lost 15% after 28 days; while, lost 100 after 35 days.	Lost 9% at approximately 60 days.	Retained its potency completely after more than 2 months.
Therm-stability	Therm-stable for approximately one month	Therm-stable for approximately two months.	Thermolability when stored at ambient temperature[25-37 ^{0}C].
Solution color at 28 days	Clear solution	Clear solution	Clear solution
Solution color at 60 days	yellow solution	Yellow solution after storage more than 60 days at ambient temperature	Colorless transparent solution
Turbidity of solution	Turbid after more than 28 days of storage at ambient temperature with the formation of insoluble aggregates	Turbid after more than 60 days of storage at ambient temperature with the formation of insoluble aggregates.	No turbidity and/ or the formation of insoluble aggregates were noticed after more than 2 months of storage at 2-8 ^{0}C.
Efficacy	Lost completely after more than 35 days of storage at ambient temperature	Retained 90% of efficacy within 2 months.	Retained 99% of efficacy after one month. As well It retained 97% of efficacy after 2 months

Figure 1. It displays the recombinant human insulin protein's three-dimensional structure, which was created by Saccharomyces cerevisiae.

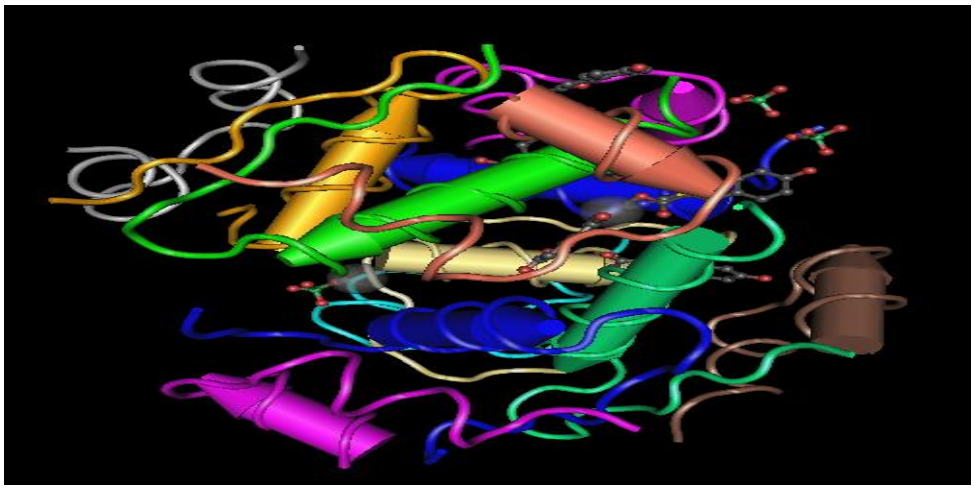


Figure 2. It demonstrates the purification of recombinant human insulin utilizing nickel columns and nickel affinity resins in immobilized metal affinity chromatography. Recombinant insulin had a purity level of roughly 85%.

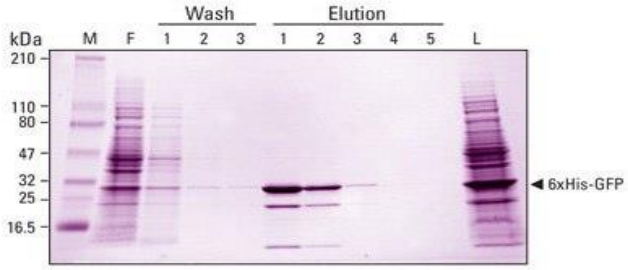


Figure 3. It demonstrates a method for screening and bioassaying thermostable insulin using rabbit blood glucose.

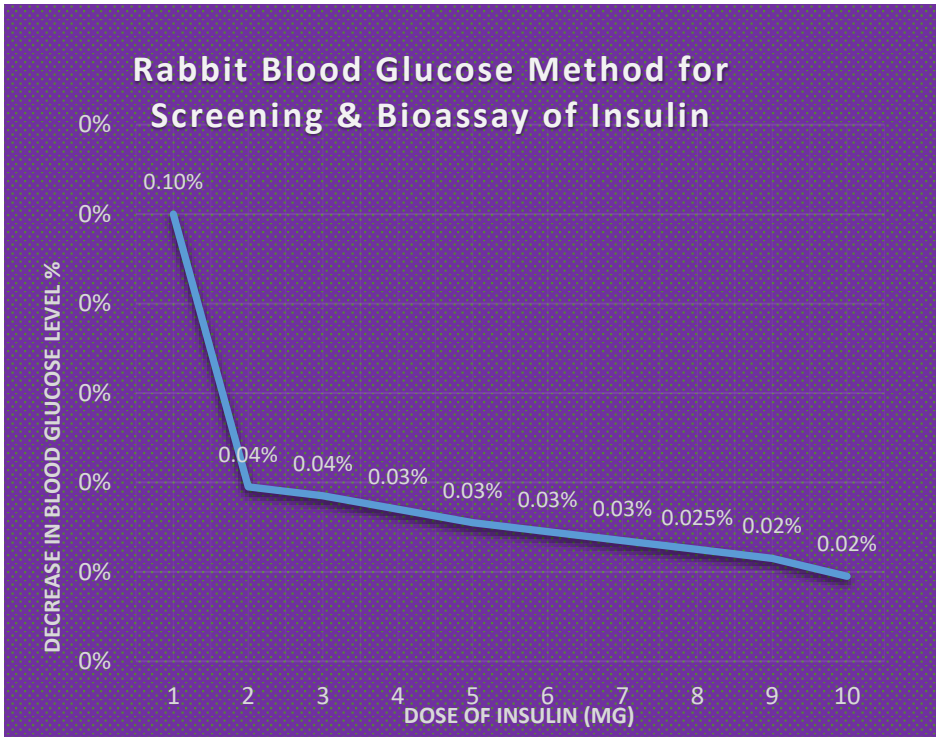


Figure 4. It demonstrates the use of a mouse convulsion test for the bioassay of insulin.

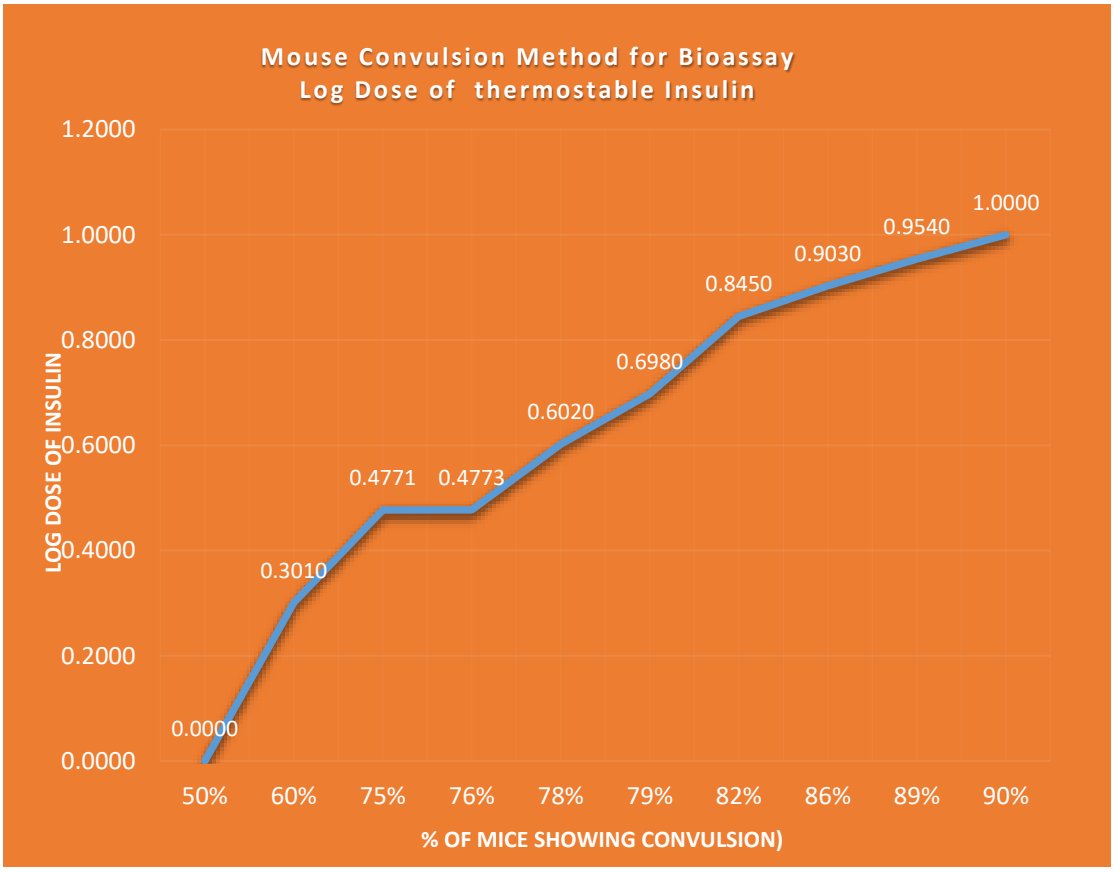


Figure 5. It displays the estimation of hypoglycemic impact of standard rapid acting subcutaneous par-enteral insulin formulation during clinical trials phases 1/2.

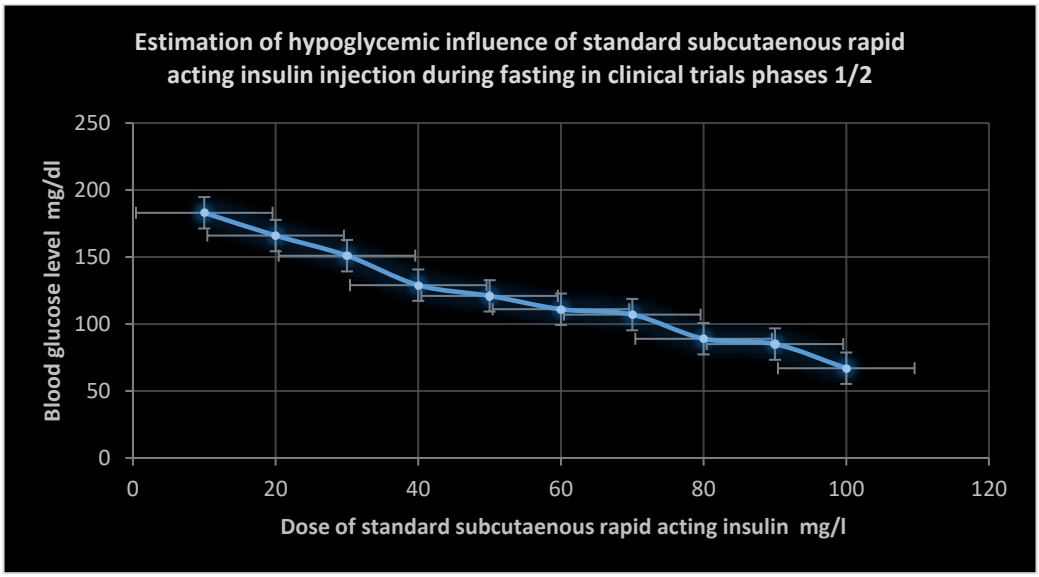


Figure 6. It shows the estimation of hypoglycemic impact of test rapid acting subcutaneous par-enteral insulin formulation during clinical trials phases 1/2.

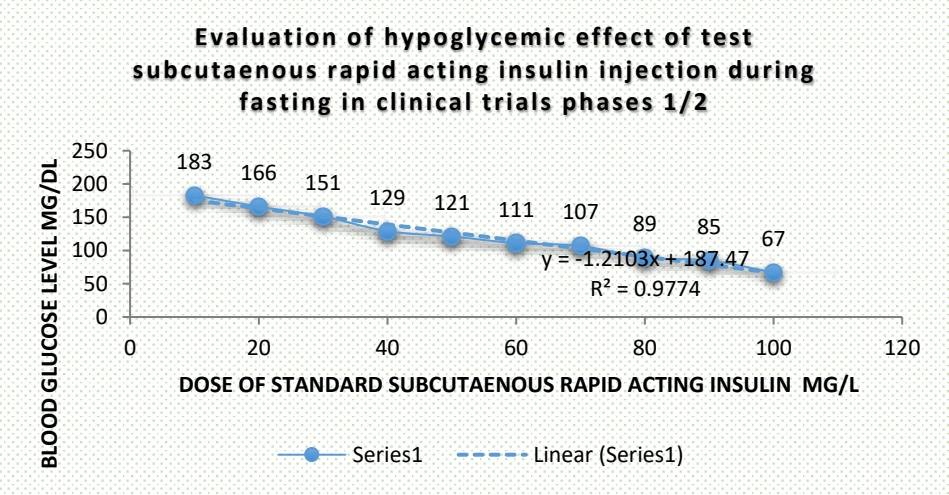


Figure 7. It displays the estimation of area under the curve(AUC) of test soluble insulin given via subcutaneous injection during fasting.

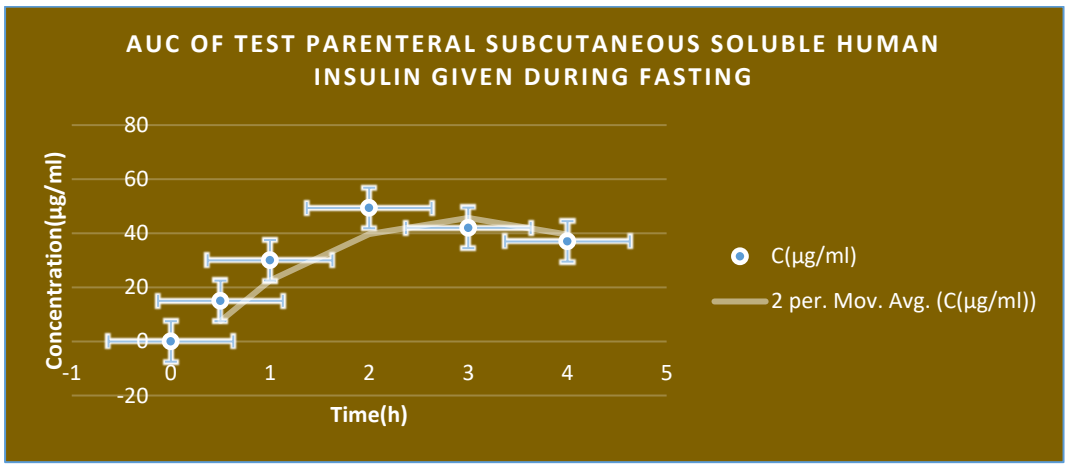


Figure 8. FTIR spectroscopy displays no fundamental interaction between recombinant human insulin and excipients.

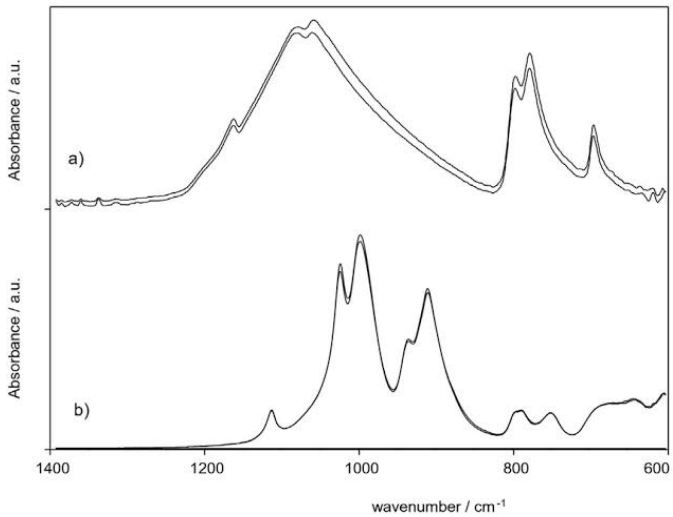


Figure 9. DSC thermal analysis displays impossibility of interaction between recombinant human insulin and excipients.

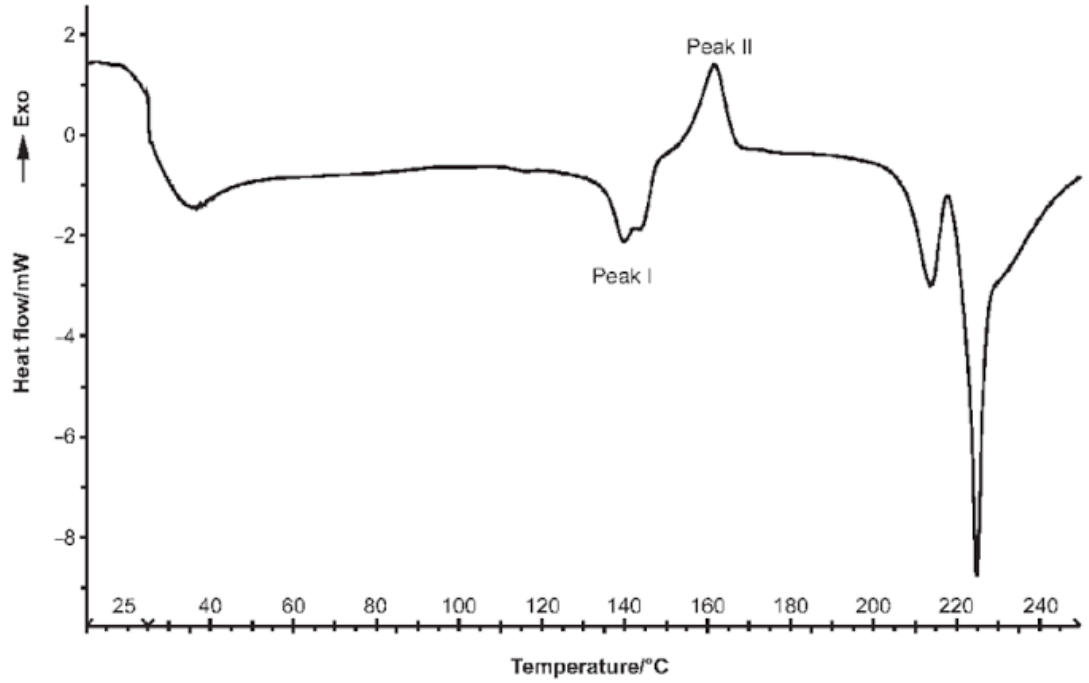
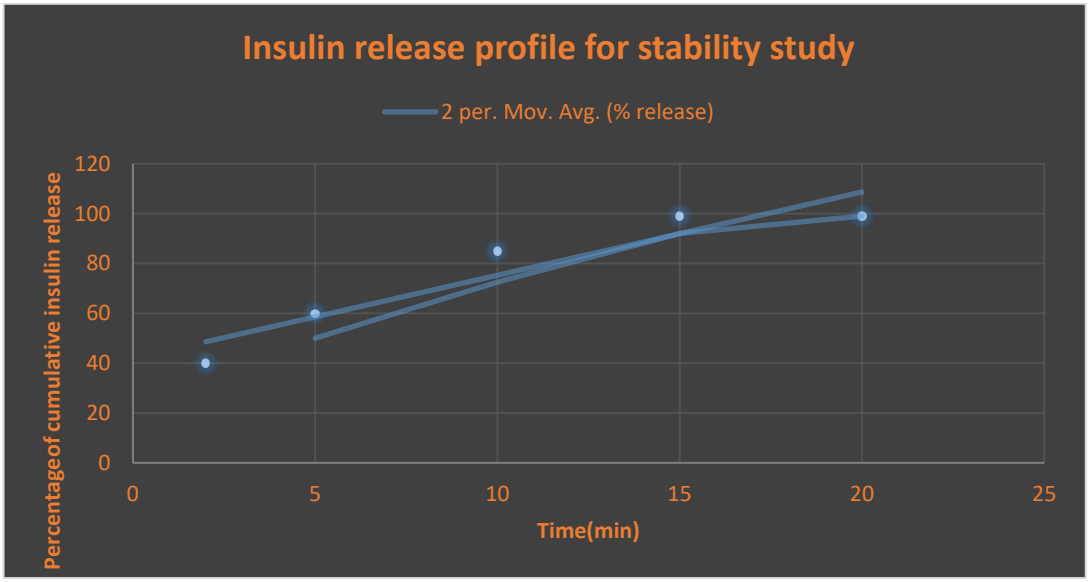


Figure 10. It represents a comparison of subcutaneous insulin release profile for stability study between initial release and after one month.



Discussion

The lowest effective dosage of modified thermostable insulin that lowered normal rabbit

blood glucose from 0.1% to.039 was 2.7 mg of insulin, according to screening and bioassay results of various drug delivery systems including graded doses of the substance ranging from 1 to 10 mg. Using the mouse convulsion technique and a two-dose experiment on fasting mice for 24 hours, several drug delivery systems containing graduated dosages of modified thermostable insulin were bioassayed. As opposed to 3 mg of conventional insulin as a standard hypoglycemic medication, it was shown that 75% of mice had convulsions as a result of the hypoglycemic impact of insulin starting at doses of 2.9 mg of insulin.[19]

In all tests, hypoglycemia became apparent after 3 to 4 hours, and the insulin's effects lasted for 7-8 hours. This showed that insulin that had been genetically altered and had two neighbouring cysteines added to the alpha helices of the hormone's core had enhanced physicochemical qualities.

In contrast to a previous study (Ahmed G et al., 2018) carried out in Australia, the current study showed that the efficacy of subcutaneous insulin delivery system was just about 90% and bio-availability was 91% in clinical trials phases1/2; the prior study, however, showed that the efficacy of rapid acting insulin subcutaneous injection formulation did not exceed 90%, while bio-availability was, on the other hand, about 90%.[20]

Recombinant DNA technology was effectively used to create subcutaneous par-enteral formulations of insulin with increased bioavailability. According to the results of the DSC and FTIR spectroscopy studies, there were no potential interactions between the medication and polymers. Using batch F4 as the optimised formulation was a wise decision. Studies on batch F4's stability and in vivo performance were completed. According to the stability study, there was not much change after one month. A good homogeneity of the drug content was shown by Batch F4. Thermo-stability and storage at room temperature without a refrigerator: In the current study, test insulin could be kept at room temperature without a refrigerator for almost two months; however, standard insulin was destroyed and damaged when kept at room temperature without a refrigerator for longer than 30 days.[21]

Subcutaneous par-enteral formulations have been shown to increase the bioavailability of test insulin, according to in vivo investigations. When both types of insulin were administered via the subcutaneous method, test insulin also showed a greater percentage of relative bio-availability than regular insulin.

Recombinant DNA technology used in bacteria is used to produce human insulin. The forms that are available offer 4 rates of onset and 4 different lengths of action, ranging from rapid-acting to long-acting. Insulin treatment aims to reduce the risk of hypoglycemia while controlling basal and postprandial (after a meal) glucose levels. To accomplish these objectives, insulin formulations with various rates of onset and action are frequently mixed.

Rapid-acting: Insulin lispro, insulin aspart, and insulin glulisine all have quick onsets and early peaks of action, allowing for the regulation of postprandial glucose levels. Little changes to the main amino acid sequences of the three rapid-acting insulins hasten their circulation without changing how they interact with insulin receptors. The preferred insulin for continuous subcutaneous infusion devices is rapid-acting insulin, which is administered right before a meal. They can also be used to treat diabetic ketoacidosis that is not difficult in an emergency.[22]

Regular insulin is injected subcutaneously during routine maintenance procedures or intravenously during emergencies. It can be used alone or in combination with intermediate- or long-acting formulations. It was the main kind of insulin used to manage postprandial glucose concentrations before to the introduction of rapid-acting insulins, but it had to be administered at least an hour before a meal. Neutral protamine, intermediate-acting Regular insulin and protamine, a highly basic protein also used to counteract the effects of unfractionated heparin, are combined to create Hagedorn insulin (also known as NPH insulin), which has a delayed start and peak of activity. Regular and rapid-acting insulins are frequently coupled with NPH insulin.

Long-acting Insulin glargine and insulin detemir are human insulin that has undergone modifications to create a peakless basal insulin level lasting more than 20 hours. This feature helps manage basal glucose levels without causing hypoglycemia. methods for delivering insulin Insulin treatment is typically administered by subcutaneous injection using standard disposable needles and syringes. There are also more practical ways to administer. Subcutaneous injection is made easier by the use of portable, pen-sized injectors. Some are disposable, while others have replacement cartridges. Devices for continuous subcutaneous insulin infusion spare patients from having to administer numerous daily injections and provide them freedom in timing their daily activities. Programmable pumps deliver a consistent 24-hour

basal rate, and manual modifications can be made to the rate of administration to account for variations in the amount of insulin needed (eg, before meals).[23]

The most frequent side effect of using insulin is hypoglycemia, which is brought on by an overactive insulin response. The rapid provision of glucose (sugar or sweets by mouth, glucose by vein, or glucagon (by intramuscular injection)) or glucagon is crucial to preventing the brain damage that may occur as a result of hypoglycemia. The elderly, children under 7, and patients with severe renal illness are especially at risk for the negative consequences of hypoglycemia. The development of antibodies to insulin or noninsulin protein impurities, which results in resistance to the drug's effect or allergic responses, is the most typical kind of insulin-induced immunologic problem. Immunologic problems are rare when highly pure human insulins are used, and normal and NPH insulin's duration significantly lengthens with greater dosage.[24]

According to the Kaufmann B et al. 2021 study, insulin was stable and maintained biological activity across a four-week treatment period in tropical conditions. Circular dichorism analysis of the insulin 3-D structure demonstrated that the conformation of the insulin monomer did not change significantly. Insulin bioactivity of the samples kept at fluctuating temperature during the usage time was equal to that of the samples kept at 2-8 0C, according to measurements of insulin efficiency on the insulin receptor and AKt phosphorylation in hepatic cells.[25] In the current study, after one month of storage at ambient temperature, which varied between 25 and 37 0C, standard insulin's potency lost 15% of its original strength; however, after more than 35 days of storage at the same temperature range, the potency totally reduced. Test insulin maintained 91% of its potency and effectiveness at two months as compared to the negative and positive control insulin series vial groups; while demonstrating therm-stability at ambient temperatures between 25 and 37 0C for 60 days.

Conclusion

The present study was a promising approach due to overcoming of the drawback of thermolability insulin injection at ambient temperature. Recombinant thermostable human soluble insulin could be stored at ambient temperature.

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A patent 881/2021 authorized by ministry of scientific research, Egypt.

Conflict of interest:

There is no conflict of interest.

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Data availability:

Raw data were generated at faculty of pharmacy, Cairo university, Egypt. Derived data supporting the findings of this study are available from the corresponding author Dr.Mohammed Kassab up on request.

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