ORAL INSULIN DELIVERY SYSTEM DEVELOPMENT BY BIOINFORMATICS AND PROTEIN ENGINEERING.

Kassab Mohammed

Instructor of molecular biology, microbiology and immunology, faculty of pharmacy, Cairo university, Egypt.

Corresponding author
Mohammed Kassab
Mobile: +201032579044

e.mail:
Ksabmhmd676@gmail.com

Received: 22/6/2022
Accepted: 11/10/2022

Online ISSN: 2735-3540

ABSTRACT:

Background: *Diabetes mellitus is a chronic and overwhelming disease that is managed by insulin. Subcutaneous insulin injection has several disadvantages such as nerve damage, microbial contamination, thermolabile, and pain. Exogenous insulin is essential for the management of Diabetes mellitus type 1 and has an adjunct role in the management of type 2 diabetes mellitus in which oral hypoglycemic medicines display the leading management role. Pain, Lipodystrophy at the injection site, Nerve damage, Thermolabile and microbial contamination during injection are the principal adverse effects of insulin administered via IV or SC routes.*

The aim of the study: *Development of different oral drug delivery systems by bio-informatics and/peptidomimetics.*

Methodology: *In this study, insulin was prepared by recombinant DNA technology using bioinformatics technology via the addition of cysteine-cysteine beside each other in the alpha-helices of the core of the two subunits of insulin. Protease inhibitor and polymeric adhesive were added with oral film enteric-coated insulin tablets of insulin. The test insulin was tested on animal models and compared with standard subcutaneous insulin for efficacy. The human insulin tablets were prepared by wet granulation technique utilizing different concentrations of starch, sucrose, talc and sodium caroxy methylcellulose. DSC and FTIR spectroscopy were utilized in the drug and the polymer compatibility studies. Evaluation of preformulation properties of active principal ingredient(API) was performed. As well postcompressional parameters as wetting time, disintegration time, in vivo bio-availability, in vitro drug release, water absorption ratio study of the optimized formulation were assessed.*

Results: *In this research, we designed and developed thermostable and acid-stable insulin that can be formulated as an oral drug delivery system and can be taken also by the sublingual route. The efficacy of the test insulin was nearly 60% during human clinical trials phases 1/2.*

Conclusion: *The new thermostable and acid-stable insulin helped to overcome the disadvantages of subcutaneous injections of insulin.*

Keywords*: insulin tablets; acid stable; therm-stable; bio-informatics*

INTRODUCTION:

Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia and sometimes ketoacidosis due to beta-oxidation of fatty acids to provide energy; it results from insulin deficiency and/or insulin

resistance[1]. Approximately 10% of diabetics worldwide are due to type 1 diabetes mellitus[2]. It is characterized by an absolute deficiency of insulin caused by an autoimmune attack on the beta cells of Langerhans of the pancreas[3]. Insulin is essential for the treatment of Diabetes mellitus type 1. Type 2 diabetes has a strong genetic component. It results from a combination of insulin resistance and dysfunctional beta cells. Obesity is the most common cause of insulin resistance[4]. The endocrine pancreas in the adult human consists of nearly 1 million islets of Langerhans interspersed throughout the pancreatic gland[5]. Within the islets, at least four hormone-producing cells are present[6]. Their hormone products include islet amyloid polypeptide (IAPP, or amylin), which modulates appetite, gastric emptying, and glucagon and insulin secretion; glucagon, the hyperglycemic factor that mobilizes glycogen stores; insulin, the storage and anabolic hormone of the body; somatostatin, a universal inhibitor of secretory cells; and pancreatic peptide, a small protein that facilitates digestive processes by a mechanism not yet clarified[7].

The endocrine portion of the pancreas islets of Langerhans consists of different cells that secrete different peptide hormones; Insulin from beta cells[8]; Glucagon from alpha cells[9]; Somatostatin from sigma cells which locally regulates insulin and glucagon secretion and pancreatic polypeptide[10]. Insulin is a small protein, with a molecular weight of about 6000 Daltons. It is composed of two chains held together by disulfide bonds[11]. Insulin has anabolic actions, it increases glucose uptake and storage by many tissues[12] as well as protein production by increasing the uptake of amino acids by cells and increasing ribosomal activity[13].

The standard model of insulin therapy is subcutaneous injection using conventional disposable needles and syringes[14]. Disadvantages of insulin injection include; Pain; Lipodystrophy at the injection site; Nerve damage; Thermolability; microbial contamination during injection[15].

AIM OF THE STUDY:

In this study, we aimed to overcome these drawbacks via the development of new oral delivery systems containing recombinant thermostable and acid-stable modified insulin designed by genetic engineering and peptidomimetics which improved the physicochemical characteristics of insulin.

MATERIAS AND METHODS:

Material:

Starch, sucrose, talc, sodium caroxy methylcellulose, Magnesium stearate, Magnesium silicate, talc, sucrose DC and SBBPI.

All chemical and biochemical materials were purchased from Algomhoria pharmaceutical company, Cairo, Egypt, Alnasr pharmaceutical company, Abo zabal Alkhanka, Qalyobia, Egypt and Merck Millipore and Sigma-Aldrich, Germany.

Ethical statement:

In the present study, we followed All applicable national, international and/or institutional guidelines for the attention and utilization of humans and animals. All processes carried out in study including humans and animals were authorized by the local authorities, Ethical committee for human and animal handling at Cairo university (ECAHCU), at the faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the Weatherall report with approval number P-2-2-2021. All efforts were performed to ablate the number of humans and animals utilized and their suffering during study.

Equipment:

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	Artikel
Refrigerator 5	Whirpool
PH meter electrode	Mettler-toledo, UK
Deep freezer -20	whirlpool
Gyratory shaker	Corning gyratory shaker, Japan
190-1100nm Ultraviolet-visible spectrophotometer	UV1600PC, China
Light(optical) microscope	Amscope 120X-1200X,China

Source of animal models:

They were obtained and legalized by the pharmacology and toxicology department of the faculty of pharmacy, Cairo University, Egypt.

The type of the study:

Screening experimental study.

Source of animal models:

They were obtained and legalized by the pharmacology and toxicology department of the faculty of pharmacy, Cairo University, Egypt.

Inclusion criteria for animal models are:

1. Adult obese animals (rabbits and /or mice)
2. Animal model can be induced by hyperglycemia.
3. Animal blood glucose levels can be easily estimated.
4. Obese animal models.

Exclusion criteria are:

1. Young animals.
2. Pregnant female animals.
3. Animal blood glucose levels can not be easily estimated.
4. Non-obese animals.

Adult obese male rabbits weighing about 2kg, and obese male albino mice weighing between 160-190gm were utilized in the existing study. Mice were acclimatized for one week before the experiment. At a humidity (50%±5), light-dark cycle (12/12 h), and a controlled temperature (25±2 ^{0}C). Mice were provided with a commercially accessible natural diet of chow (Elnasr pharmaceutical and chemical company).

Place and date of the study:

This study was done in the faculty of pharmacy, Cairo University, Egypt in June 2020.

Methodology

Primer for expression of physically stable insulin which was modified using genetic engineering and bioinformatics:

Forward primer:

ACATTGGTGCTACCAGCCTC

Tm=60.04 ^{0}C, Ta=55.04 ^{0}C

Reverse primer:

GCGGGTATCGCTGGTATGAA

Tm=59.97 ^{0}C, Ta=54.97 ^{0}C

Biosynthesis of recombinant thermostable insulin:

Determination and identification of the most flexible regions in the core region of human insulin protein, then applying genetic engineering where 2 residues in these regions were mutated with 2 cysteines beside each other not apart more than 0.2nm

to form disulfide bond which increases the stability of protein using Pymol and in silico analysis pdb 2 max in gomacs software programs. This was followed by the design of physically stable insulin by adding 2 cysteine residues adjacent to each other in the alpha-helices in the core region of the insulin protein. Design of new primer for expression of this modified physically stable insulin using genetic engineering and bioinformatics.

Then synthesis of modified insulin by recombinant DNA technology using *Saccharomyces cerevisiae BJ1824* as expression host. C-terminal was 6x histidine, promoter AUG1, inducer was methanol and PYES2-DEST52 was the expression system vector. Genes of thermostable insulin of interest were cloned using PCR and then sub-cloned into PYES2-DEST52 using Hind III and EcoRI restriction endonucleases II for the digestion of the plasmid, followed by ligation by ligase enzyme. The recombinant plasmid was designated and propagated first in *Escherichia coli* Top 10(Invitrogen, USA), then transformed into *Saccharomyces cerevisiae BJ1824*. For insulin production using galactose as an inducer, YNBG selective medium (0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% galactose) was used for the growth of yeast transformants at 30C^0, followed by maintenance in YPG-rich media (2% bacteriopeptone,1% yeast extract and 2% galactose).

Clarification and purification of thermostable insulin:

Centrifugation was carried out for 3 minutes at 4000 rpm. Clarification of soluble insulin protein precursor from the supernatant of the culture by the precipitation by ammonium sulfate, followed by purification by nickel affinity chromatography. Recombinant fused insulin proteins with polyhistidine-tagged proteins could speedily be purified from the supernatant via Nickel columns using immobilized metal affinity chromatography (the metal-ligand was a nickel-metal ion; while the target bio-molecule was polyhistidine tag fusion protein) on Nickle affinity resins after extraction of them by precipitation (salting out) of 100 ml of the supernatant with 53 ml of a 4.1 M ammonium sulfate saturated solution at 25 C^0 following centrifugation at 4000 rpm for 3 minutes. Before the final formulation the preparations are sterilized by filtration through 0.22-micrometer sterile-grade filters (Whatman-1541-042 filter paper (0.22 micron) purchased from the USA).[16]

Formulation of oral insulin drug delivery systems:

Preparation of film-coated micronized insulin tablets containing bioadhesive (such as sodium carboxy methyl cellulose (1mg/gm tablet) which is an anionic polymer-forming hydrogen bond with mucin and is characterized by high mucoadhesive and low toxicity) and protease inhibitors such as Soybean Bowman-Birk protease inhibitor (SBBPI)(0.5 mg/g tablet) which inhibits both trypsin and chymotrypsin. Tablets of micro-particles of insulin 10 mg/g tablet were prepared by the wet granulation method. Magnesium aluminum silicate 3mg/g tablet was added as an excipient. It was a binder, glidant, and disintegrant. Starch 15.5mg/g tablet as diluent. Magnesium stearate 1mg/g tablet was added as a lubricant agent. All ingredients were passed through 80# mesh sieve. All ingredients were passed through 80# mesh sieve. 80 mesh is a medium size U.S. Mesh size was a0.0075(185µm) with a nominal sieve opening with a typical wire diameter of 0.120mm.the die size ranged from 7-9mm. The film-coated tablets were prepared via the aqueous film coating method (film coating is a single process that involves the deposition of a thin film polymer such as 100-micrometer hydroxypropyl methyl-cellulose phthalate via spraying coating solution onto the tablet beds in a pan coater

followed by immediate drying to form thin, film and enteric coat on the micronized tablets in presence of plasticizer such as polyethylene glycol (200-6000)). The evaluation of biological activity of oral insulin drug delivery systems was done through rabbit animal models induced by hyperglycemia and mouse convulsion method using 2 and 2 dose assay.

Evaluation tests of oral insulin tablets:

These tests were carried out as per British pharmacopeal specifications.

Compatibility study:

We characterized recombinant insulin and different excipients utilized in the preparation of oral tablet formulations by FT-IR(Perkin-Elmer 1600 FTIR spectrophotometer) spectroscopy and DSC (Shimadzu-DSC 50) to see the compatibility. The optimized formulation was blended with 200 mg KBr; then compressed into discs which were scanned at 5mm/sec with a resolution of 1 cm^{-1} at a range of 4000-200 cm^{-1}. Experiments of thermal analysis were carried out utilizing various scanning calorimeter (DSC). We heated the samples of the optimized formulation in hermetically sealed Aluminium pans at a temperature range 0-4000 ^{0}C at a constant rate of 110 ^{0}C/minute under a purge of nitrogen (35 ml/min).

Hardness:

We performed a diametric compression test according to British pharmacopeal technique 2.9.8 utilizing Monsanto hardness tester (USA). A hardness of 2kg/cm^2 was acceptable in case of oral insulin tablets according to standard literature. For 20 tablets we measured the pressure required to break diametrically placed matrix tablet, by a coiled spring.

Friability:

We dedusted, accurately weighed and placed a random sample of the whole tablets corresponding to 6.5 g in the drum of a Roche friability tester. we rotated the drum 100 times and tablets were accurately weighed, dedusted and removed.1% was considered acceptable as a maximum weight loss. In the roche friabilator test apparatus 20 tablets were weighed and put in. The tablets were uncovered to the recurrent shocks and rolling consequent on the falls inside the apparatus. The tablets were dedusted after 100 process. The percentage loss in the weight of the tablets was the determining factor of the friability.

Wetting time:

Two layers of a rectangular absorbent paper (10cm×7.5 cm) fitted into a petri dish and wetted thoroughly with distilled water were used for carrying out the test for wetting time. Then we placed the tablet at the center of the plastic dish and recorded the time required for the water to diffuse from the absorbent paper using stopwatch.

Determination of water absorption ratio:

We kept a piece of tissue paper folded twice in a petri dish (internal diameter 6 cm) incorporating 7 ml of purified water. Then we settled the tablets on the tissue paper and left to wet wholly. The wetted tablets were separated and reweighed.

Disintegration test:

The test was carried out according to British pharmacopoeia 2019 standards. we placed one tablet in each of the six tubes and utilizing distilled water maintained at 37 C^0; then tablets were observed for disintegration. The basket from the fluid was lifted up and observed for the tablets complete disintegration at the end of the time limit.

Weight variation:

From each batch 20 tablets were chosen randomly and their average weights were calculated utilizing digital weighing balance (Essae Teraoka ltd); then percentage weight difference was estimated and checked with British pharmacopoeia 2019 specifications.

Determination of uniformity of drug content:

From each formulation twenty tablets were weighed and powdered; then10mg of the powder was weighed and dissolved in 100 ml of distilled water. we sonicated the mixture for 170 seconds and filtered through Whatman filter paper No. 40.Then the filtrate was diluted with distilled water and the absorbance at 275 nm was estimated due to disulfide photolysis and covalent insulin dityrosine dimerization induced by UV light exposure.[17]

In vitro drug release profile:

Distilled water was used as the dissolution medium (300 ml) at 37 ^{0}C,PH 7.4 and 50 rpm(paddle) in presence of phosphate buffer 6.8.We collected samples at 3, 6, 8, 11, 16, 19, 60, 120, 240 minutes intervals according to European pharmacopoeia specifications 2020 and the withdrawn volumes were replaced by equivalent amounts of the plain dissolution medium.The amount of insulin released was measured using UV spectrophotometer at 275 nm.

Stability study:

It was carried out for optimized formulation. The storage conditions utilized for stability studies were accelerated conditions 40 ^{0}C and room temperature 30 ^{0}C. Optimized formulation tablets were kept, striped and packed in humidity chamber for thirty days on above mention temperature. The parameters that were measured before and after the storage for one month comprised hardness, the percentage friability, disintegration time and drug content.

Screening and bio-assay of insulin using:

I- Rabbit blood sugar method for screening and bio-assay:

Principle: insulin decreases the blood glucose level in rabbits and the decrease in blood glucose level is directly proportional to the dose. In our study we used 10 rabbits, the rabbit weighed approximately 2 kg.

Procedure: rabbits weighing 2 kg are used. A preliminary experiment was carried out by injecting each rabbit of the positive control group with graded doses of standard insulin (0.1-0.5 IU /kg) subcutaneously (S.C); while the test insulin oral tablets comprising graded doses of test insulin (0.1-0.5 IU /kg) insulin were given to the test group via oral route of administration after fasting for 18 hours. Any rabbit which showed convulsions within 5 hours was excluded. The rabbits were then randomly distributed into four groups, fasted for 18 at least 18 hours then a blood sample was taken from the ear vein to determine the initial blood glucose level (BGL). Each group was then injected with a dose of insulin according to the 2 and 2-dose assay and blood samples were taken each hour for 5 hours the samples of each rabbit were pooled and the BGL of the pooled sample was determined. A decrease in the blood glucose level was recorded. Cross over test was carried out the next day. The mean decrease in BGL for each chosen dose was calculated and the relative potency was determined.[18]

II- Mouse convulsion method for bio-assay only using 2 and 2 dose assay technique:

Principle: insulin decreases the blood glucose level in mice. When it reaches a critical level the hypoglycemic convulsion occurs. The percentage of mice showing convulsions is directly proportional to the dose.

Procedure:2 and 2 dose assay techniques were carried out. 100 Mice **weighing 160-190 gm** were fasted for 12-24 hours and kept at a constant temperature of 29-35 C. The standard insulin was injected with graded doses of insulin (0.1-0.5 IU /kg) intraperitoneally (IP); while the test insulin tablets containing graded doses of insulin

(0.1-0.5 IU /kg) were given via oral route of administration and the animals were observed for 1.5 hours. The percentage of animals that died, showed convulsions, or remained on their back for 2-3 seconds when they were turned on their back in each group was determined and the relative potency was calculated. Cross-over tests could not be carried out because the animals might die.[19]

Human evaluation of different oral insulin drug delivery systems via human clinical trials phases 1/2:

3 groups of adult diabetic type1 patients with hyperglycemia greater than 200 mg/dl are included in our study. Each group consisted of 100 subjects:

Group (1) (negative control group) was administrated graded amounts of the placebo by oral route.

Group (2) (positive control group) were administrated graded amounts of the standard insulin (0.2-0.5U/kg) intravenously and subcutaneously.

Group (3) (test group) were administrated graded amounts of the test recombinant oral insulin micronized tablets (0.2-0.3U/kg of insulin injection were equivalent to 50 mg of human insulin tablets). The activity of insulin was estimated by the reduction in blood glucose level during fasting.

In vivo bio-availability study:

Before dosing oral tablets 0.7-0.9ml of samples were withdrawn, and immediately after dosing at 30,60,120,240 minutes. Blood samples were further refrigerated and centrifuged at 4 C within one hour of sampling. Insulin concentrations were determined using HPLC.HPLC analysis was through a reversed phase column utilizing phosphate buffer (PH 4.4) and acetonitrile (660/340, v/v) as mobile phase with a flow rate 0.9ml/min. The limit of UV estimation of insulin concentration in blood was at 275 NM. Area under the curve (AUC) and the % of relative bio-availability were measured. % of relative bio-availability was determined by the following equation:

% Relative bio-availability = (AUC Oral/AUC Intravenous) × (Dose Intravenous/ Dose Oral) ×00%.

The same procedures were performed for the control and the standard groups (groups 1 and 2).

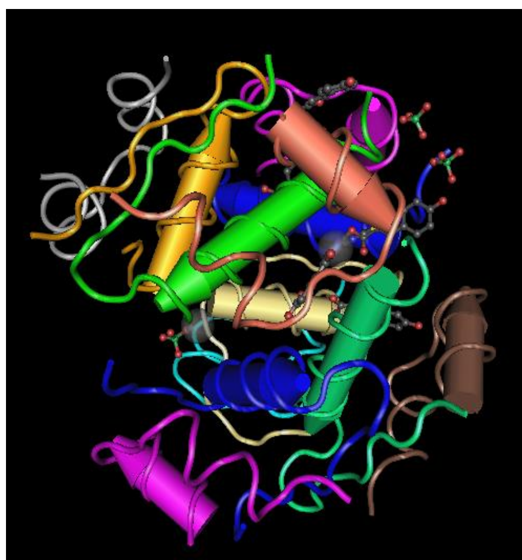
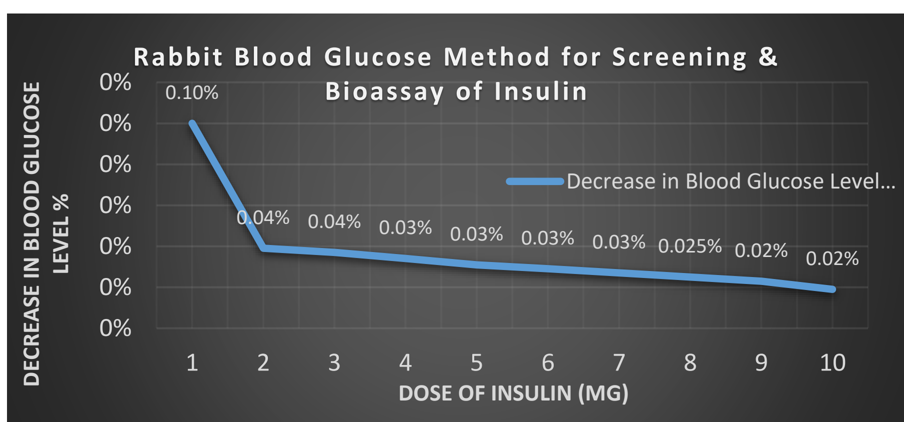


Figure 1. It represents the 3D structure of recombinant thermostable homosapines insulin protein.

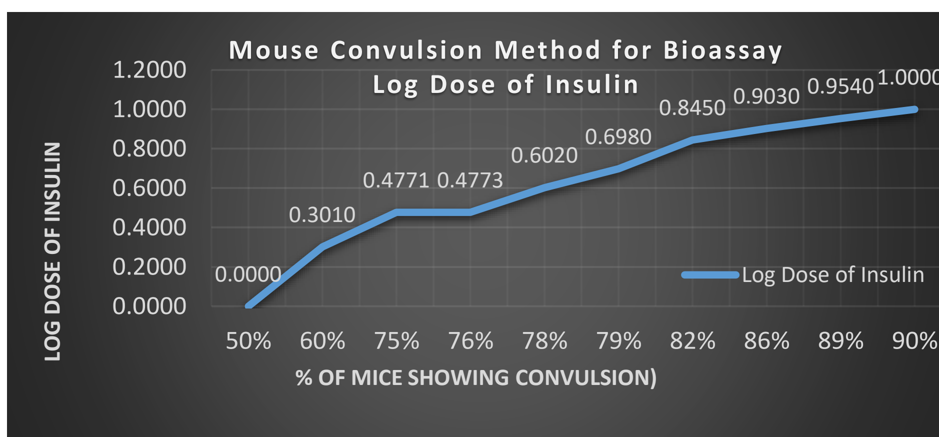
Figure 2. It shows the purification of recombinant thermostable insulin via Nickel columns using immobilized metal affinity chromatography on Nickle affinity resins. The purity of recombinant insulin was approximately 86%.

Statistical analysis:

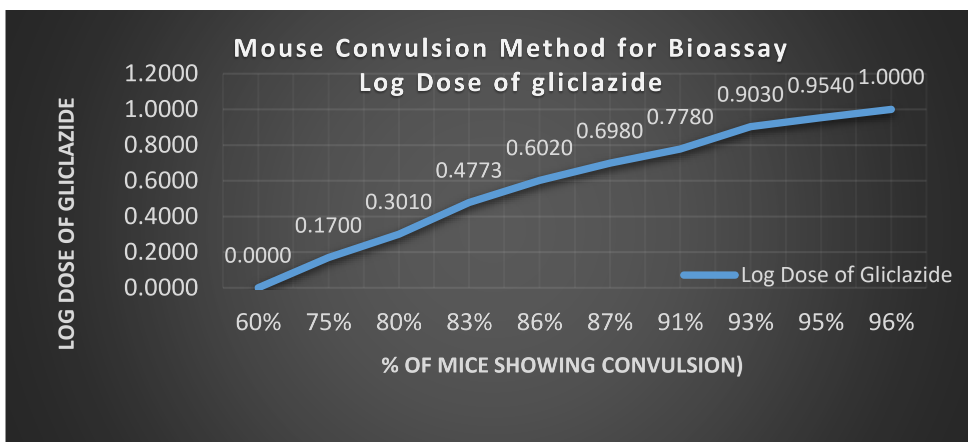
All cultures were conducted in triplets. Their presentation was by means and standard deviation. One-way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.



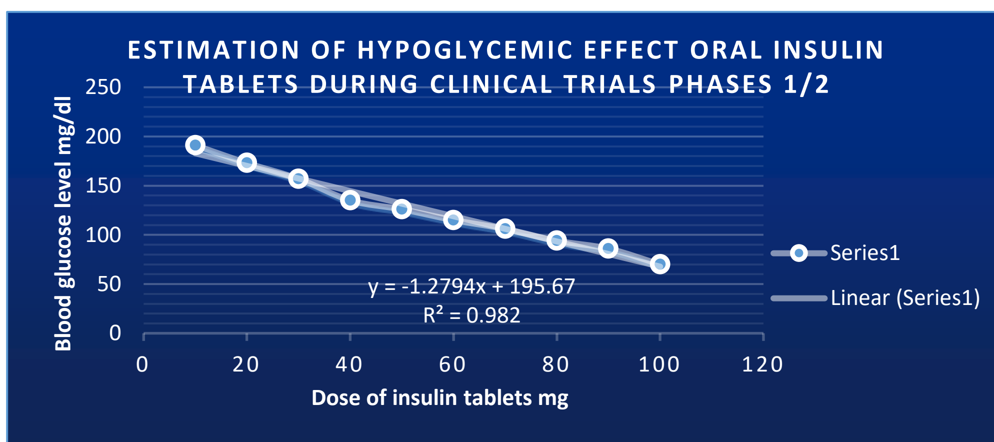
Graph 1. It represents the hypoglycemic effect of recombinant thermostable human insulin via rabbit blood glucose assay.



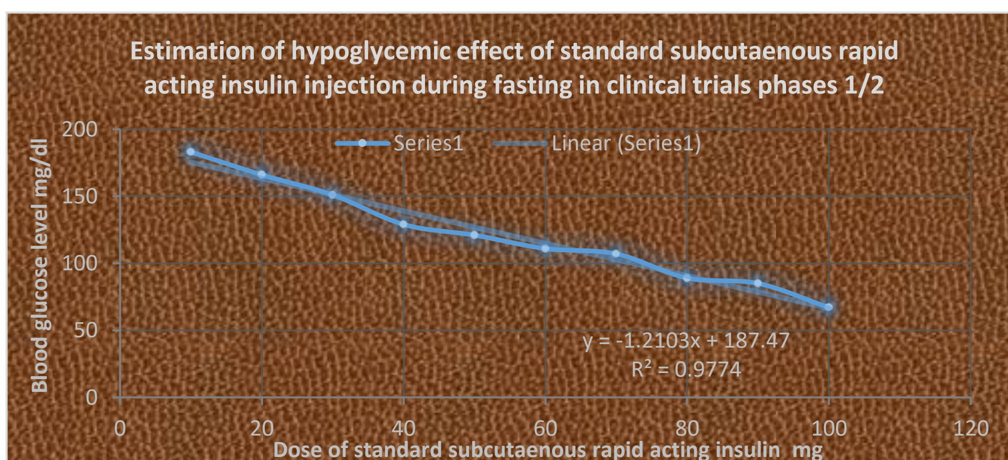
Graph 2. It represents the hypoglycemic effect of recombinant thermostable human insulin via mouse convulsion bio-assay.



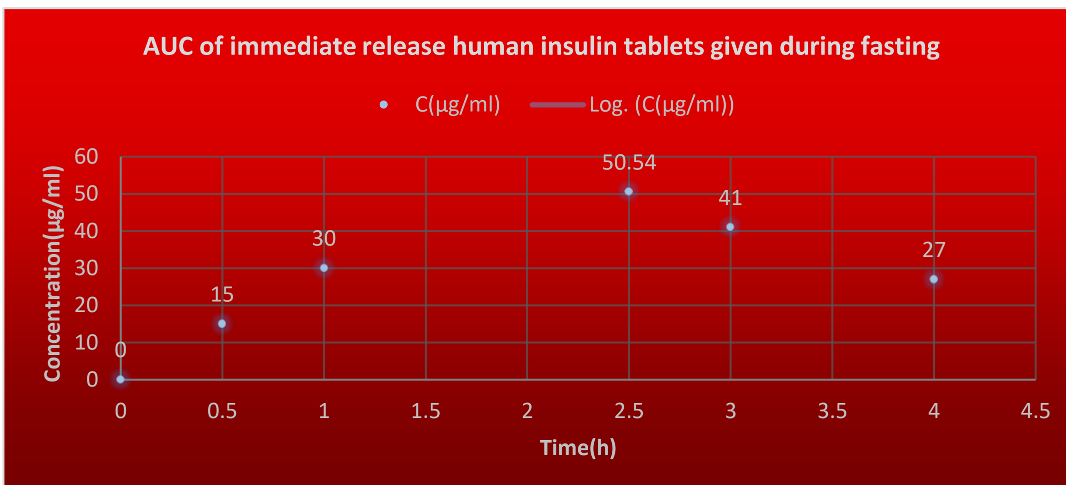
Graph 3. It represents the hypoglycemic effect of standard gliclazide oral hypoglycemic drug via mouse convulsion bio-assay.



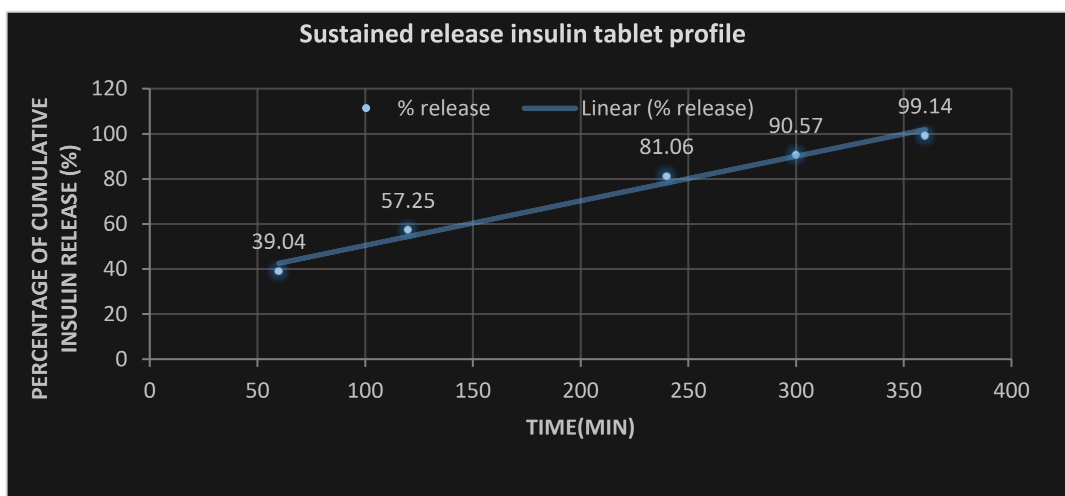
Graph 4. It represents the hypoglycemic effect of oral insulin tablets during human clinical trials phases1/2.



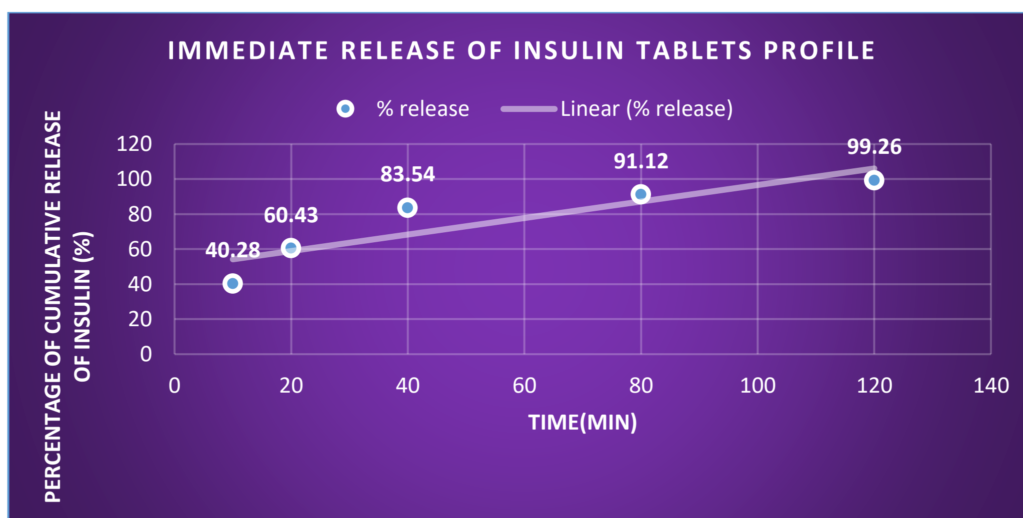
Graph 5. It represents the hypoglycemic effect of standard subcutaneous rapid acting insulin drug delivery system during fasting in human clinical trials phases1/2.



Graph 6. It shows the estimation of area under the curve(AUC) of insulin tablets given during fasting.



Graph 7. It represents sustained insulin release profile.



Graph 8. It represents immediate insulin release profile.

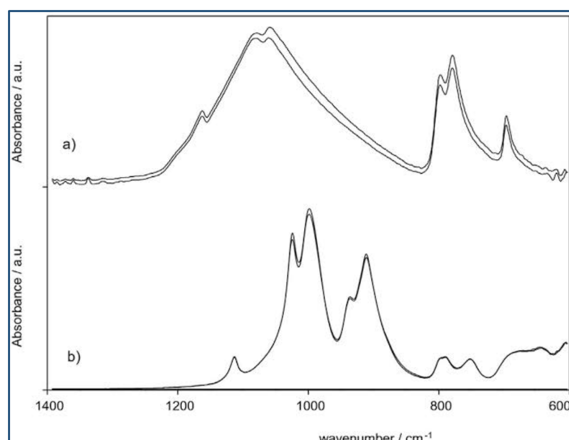


Figure 9. FTIR spectroscopy shows no interaction between recombinant human insulin and excipients.

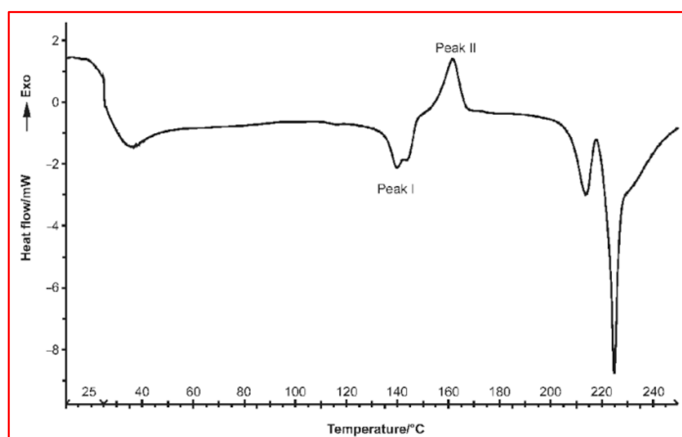


Figure 10. DSC thermal analysis shows no possibility of interaction between recombinant human insulin and excipients.

RESULTS:

In our study we prepared different batches of recombinant human insulin Oral tablets utilizing various ingredients as starch, sucrose DC, talc, SBBPI etc (Table 2).

Table 2. Batch formulation of Oral tablets of insulin F1-F5 by wet granulation technique:

Ingredients(mg/tablet)	F1	F2	F3	F4	F5
Recombinant insulin	10	10	10	10	10
Starch	15	17	18	16	14
SCMC	1	0.5	1.5	0.5	1.5
SBBPI	1	0.5	1.5	1.5	0.5
Sucrose DC	11	12	12	13	10
Talc	2	3	1	3	3
Mg stearate	3	1	2	2	5
Mg silicate	4	3	1	1	3
Total weight(mg)	47	47	47	47	47

No possibility of interaction between recombinant human insulin and excipients was shown by FT-IR and DSC study. The determination of the hardness of the tablets was done and was observed between 1.73 to 1.97 kg/cm^2.The variation of wight of all formulations was estimated which were within the standard limit as per British pharmacopoeia. We found percentage friability in the range of 0.61% to 0.82% which was in the limit of extent. The ratio of water absorption for all formulations was observed between 37.24 to 39.63. The wetting time for all formulations was estimated between 18 to 23 seconds. We subjected the oral tablets for evaluation of in vitro disintegration time. For formulations F1 to F5, in vitro disintegration time was found to be in the range of 8 to 10 minutes. Rapid disintegration time of 8 minutes was observed by the formulation F2. This is because of burst effect and the rapid water uptake from the medium. All formulations percentage drug content was observed between 98.93 to 99.77 of recombinant insulin which was in the unexceptionable extent. The release time for the immediate release insulin tablets ranged from 97.81% to 99.26% at 2 hours at 37 C and 50 rpm but 98.35% to 99.14% at 6 hours at 37 C and 50 rpm for the controlled release tablets. Batch F2 displayed quicker drug release than all the other batches. 98.35 % cumulative drug release in 240 minutes was demonstrated by batch F2 at 37 C and 50 rpm. Batch F2 t50 % was observed to be 180 minutes. Owing to the rapid disintegration time and dissolution profile Batch F2 was well-advised as an optimized formulation. Batch F2 was formulated with 12 mg sucrose DC and 17 mg starch. The optimum storage temperature of insulin oral tablets (batch F1 to F5) was noticed between 2-8 C. We performed in vivo study by taking formulation F2 and the outcome was compared with intravenous insulin injection. At different time intervals the blood samples were withdrawn, then were analyzed for the drug content utilizing HPLC. Tmax and Cmax of recombinant human insulin was determined to be 2.5 hours and 50.54 microgram /ml. percentage relative bio-availability was estimated by equation 1 and was dictated to be 70%. Bio-availability have been improved by insulin oral tablets as was incontestable by results of in vivo study. T max of subcutaneous injection of rapid acting insulin was 1 hour and C max was approximately 480 microgram/ml at an average dose of 0.2-0.3 U/kg. The onset of action of SC insulin was 10 minutes, besides its duration of action was approximately 5 hours and its bio-availability was about 90%. Bio-availability of IV insulin injection was 100%. Gliclazide in our study, as a standard hypoglycemic oral drug showed a biological half life of approximately 8 hours, duration of action of 10-24 hours, C_{MAX} 2.3 microgram /ml, T_{MAX} at 2 hours at a single oral dose 40 mg.

During human clinical trial phases1/2 the bio-availability of oral insulin tablets was approximately 70% while the efficacy reached nearly 60%. The pharmacokinetic profile of insulin micronized tablets during clinical trials showed rapid onset of action, and 3 hours biological half-life. The duration of action was approximately 6 hours. It mimicked the physiology of endogenous insulin secreted by pancreatic beta cells of Langerhans. The majority of insulin catabolism was accounted for in the liver and the kidneys. Nearly 60% of insulin released into the portal vein was degraded by the liver via hepatic insulin protease present inside the hepatocytes and lysosomes and approximately 40% was degraded by the kidneys. There were no risks of weight gain, hypoglycemia, or hyperinsulinemia. When the standard insulin was injected exogenously, the catabolism profile was changed because insulin was no longer delivered directly to the portal vein. The liver had a minor role in insulin degradation (approximately 35%), with the kidney degrading nearly 65%. The renal dysfunction decreased the clearance of

insulin and prolonged its effect. This reduced clearance was detected with both endogenous oral insulin and exogenous insulin administration. Clinically, a progressive decline in exogenous and endogenous oral insulin requirements and an increased hypoglycemia risk resulted from a deterioration in renal function.

Rabbit Blood Glucose Method for Screening & Bioassay of Insulin:

Table 3. represents the results of the Rabbit Blood Glucose assay:

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%

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

Table 4. represents the results of mouse convulsion methods(insulin):

% of Mice Showing	*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

Mouse Convulsion Method for Bioassay (Log Dose of Gliclazide):

Table 5. represents the results of mouse convulsion methods(gliclazide):

% of Mice	*Log Dose of Gliclazide*
60%	0.0000
75%	0.1700
80%	0.3010
83%	0.4773
86%	0.6020
87%	0.6980
91%	0.7780
93%	0.9030
95%	0.9540
96%	1.0000

Estimation of hypoglycemic effect of oral insulin tablets during clinical trials phases 1/2:

Table 6. represents the results of the hypoglycemic effect of oral insulin tablets during human clinical trials phases1/2.

Test insulin dose (mg)	Blood glucose level (mg/dl)
10	191
20	173
30	157
40	135
50	126
60	115
70	106
80	94
90	86
100	70

Table 7. It represents the results of the hypoglycemic effect of standard subcutaneous rapid acting insulin injection during fasting in human clinical trials phases 1/2:

S.C insulin dose (mg)	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 8. It shows the estimation of area under the curve(AUC) of immediate release insulin tablets given during fasting:

Time(h)	C(µg/ml)
0	0
0.5	15
1	30
2.5	50.54
3	41
4	27

Table 9. It represents sustained release insulin tablets profile:

Time (min)	% release
60	39.04
120	57.25
240	81.06
300	90.57
360	99.14

Table 10. It represents immediate release insulin tablets profile:

Time(min)	% release
10	40.28
20	60.43
40	83.54
80	91.12
120	99.26

Formulation parameters:

Table 11. Batch formulation F1-F5 hardness, thickness, percentage Fri-ability, diameter and weight variation:

Batch	Hardness (kg/cm^2)	%Fri-ability	Diameter (mm)	Thickness (mm)	weight variation (mg)
F1	1.97±0.35	0.61±0.02	6.02±0.01	3.3±0.02	47.08±1.9
F2	1.73±0.35	0.67±0.04	6.07±0.02	3.5±0.01	47.64±1.5
F3	1.75±0.36	0.63±0.01	6.04±0.01	3.4±0.04	46.81±1.7
F4	1.83±0.39	0.82±0.03	6.05±0.06	3.5±0.03	47.25±1.2
F5	1.88±0.46	0.75 ±0.01	6.06±0.03	3.6±0.07	48.38±1.4

Table 12. Drug content uniformity, wetting time, water absorption ratio, disintegration time of batch formulation F1-F5:

Batch	Drug content uniformity	Wetting time(sec)	Water absorption ratio	Disintegration time(min)
F1	99.77 ±2.24	21 ±2.80	38.51 ±1.78	9 ±1.68
F2	98.78 ±1.36	22±1.91	38.14 ±2.19	8 ±2.71
F3	98.34 ±0.99	18 ±2.99	39.63 ±2.82	9 ±2.10
F4	98.93 ±1.78	23 ±1.87	37.24 ±1.42	10 ±2.08
F5	99.61 ±2.07	19 ±2.04	39.29 ±1.60	10 ±2.54

Table 13. Comparison of different parameters for stability study of batch F2 between its initial production and after the storage for one month:

Evaluation parameter	Initial	After one month
Drug content	98.78± 1.36	98.57 ±1.32
Hardness	1.73 ±0.35	1.89 ±0.25
Disintegration time(min)	8.00±2.71	9.09 ±2.15
Percentage friability	0.67 ±0.04	0.73 ±0.08

DISCUSSION:

For screening and bioassay of insulin film-coated micronized tablets containing graded doses of insulin from 1 to 10 mg which is physically stable with protease inhibitor and bioadhesive additives, we found after applying that the lowest effective dose which reduced normal rabbit blood glucose from 0.1% to .039 was 2.5 mg of insulin. For bioassay only of film-coated micronized tablets containing graded doses of insulin, the mouse convulsion method using 2 and 2 dose assay was applied to fasting mice for 24 hours. It showed that 75% of mice suffered from convulsion due to the hypoglycemic effect of insulin starting from a dose of 2.8 mg of insulin in comparison to 1.5 mg of gliclazide as a standard hypoglycemic drug. In both experiments hypoglycemia was shown after2- 3 hours and the duration of the effect of insulin was about 6 hours. This suggested that modified insulin by genetic engineering by adding 2 adjacent cysteines in the alpha-helices of the core region of insulin hormone improved its physicochemical properties. Film-coated micronized insulin tablets using genetic engineering and peptidomimetics could overcome a lot of the disadvantages of insulin injection.

In a comparison with a previous study(Oral insulin delivery: existing barriers and current counter strategies 2017, journal of pharmacy and pharmacology, volume 70) conducted in Australia, our study showed that the efficacy of oral insulin delivery systems was nearly 60% and bio-availability was approximately 70% during clinical trials phases1/2, while the previous study showed that efficacy of oral insulin delivery systems did not exceed 60% and bio-availability was less than 70% due to difficulties in the absorption of different oral insulin delivery systems. The insulin oral routes of administration showed reduced levels of systemic insulin, therefore less weight gain and hypoglycemic risks than exogenous insulin administrated subcutaneously. As well as, it was devoid of pain, risk of infection at the injection site, and lipodystrophy.

Oral tablets of insulin manufactured by recombinant DNA technology were successfully prepared to improve its bioavailability, to avoid hepatic first pass metabolism and pre-systemic metabolism in the gastrointestinal tract. There was no possible interactions between the drug and polymers according to FTIR spectroscopy and DSC study. In this work , we embattled different batches of recombinant human insulin oral tablets via wet granulation technique utilizing different ingredients like starch , sucrose DC etc. NO possibility of interaction between excipients and insulin was unconcealed by the FT-IR and DSC study. Starch events as a disintegration agent and a diluent . Sucrose DC events as a sweetener . Many excipients showed water solubility and thus had better a patient acceptability.Our study was prosperous in terms of decreasing cost, manufacturing difficulties and stipulating an effective medication with better patient compliance. Direct reciprocity between the disintegration time and wetting time was present. Batch F2 showed less disintegration than all other formulations. Optimized formulation was well advised to be batch F2. Fri-ability and hardness of batch F2 were too good. In vivo and stability studies were carried out on batch F2. No change occurred after one month as was informed by stability study. Batch F2 demonstrated a good uniformity of the drug content, dissolution profile, disintegration time and boost a good in vivo absorption profile and stability. Bio-availability of insulin has been improved by oral tablets formulation as was indicated via in vivo studies. In a comparison with gliclazide as a standard hypoglycemic drug insulin tablets showed lower biological half life and duration of action than gliclazide, while insulin drug delivery systems showed

less hypoglycemia and weight gain tendency. In another comparison with glimepride(second generation sulfonylurea oral hypoglycemic drug) insulin tablets demonstrated less hypoglycemia and weight gain tendency than glimepride; but glimepride displayed longer duration of action (12-24h) and half life (5h) than insulin drug delivery systems.[20]

Conclusion:

Our study was a promising approach to solve many of the side effects of subcutaneous insulin injection routes such as pain, nerve damage, microbial contaminat-ion, and lipodystrophy at the injection site.

Conflict of interest:

There is no conflict of interest.

Fund:

This study was a patent with registration number 881/2021 approved by the ministry of scientific education and high education in Egypt.

REFERENCES:

1. Parveen Kumar (2020). Kumar, Clark's clinical medicine. Ninth edition, Elsevier Edinburgh London.
2. Caroline S,Zeind Michael G(2018).Applied therapeutics, the clinical use of drugs. Eleventh edition, Wolters Kluwer, London.
3. Trevor Anthony, Katzung Bertram, Kruidering-Hall Marieke (2021). Katzung Trevor pharmacology examination board review. Thirteen editions, Mc Graw Hill Education, New York.
4. Bardal Stan, Waechter Jason, Martin Douglas (2020). Applied pharmacology. Fourth edition, Elsevier Edinburgh, London.
5. Olson James (2020). Clinical pharmacology made ridiculously simple. Seventh edition, Med Master, Miami, United States of America.
6. Levinson Warren(2021).Review of medical microbiology and immunology. fifteen editions, Mc Graw Hill Education, New York.
7. Swanson Larry N, Souney Paul F, Muntnick Alan H, Shargel Leon (2019). Comprehensive Pharmacy Review for NAPLEX. Tenth edition, Wolters Kluwer, London.
8. Fisher Bruce, Champe Pamela, Harvey Richard (2021). Lippincott illustrated reviews microbiology. Sixth edition, Wolters Kluwer, London.
9. Dipro Cecily, Schwing hammer Terry, Dipro Joseph, Well Barbara (2021). pharmacotherapy handbook. Eleventh edition, Mc Graw Hill Education, New York.
10. Golderg Stephen (2020). Clinical physiology is made ridiculously simple. Sixth edition, Med Master, Miami, United States of America.
11. Ahmed Gedawy, Jorge Martinez, Hani Al-Salami, Crispin R Dass (2017). Oral insulin delivery: existing barriers and current counter-strategies. Journal of pharmacy and pharmacology, volume 70, issue 2,p.197-213).
12. Wilson Golder N (2019). Biochemistry and genetics. Eighth Edition, Mc Graw Hill Education, New York.
13. Metting Patricia J (2019). Physiology. Sixteen editions, Mc Graw Hill Education, New York.
14. NS al-Walili (2018). Sublingual human insulin for hyperglycemia in type I diabetes. Journal of J Pak Med Assoc, volume 49,issue 7, p.167.
15. WC Duckworth et al (2017). Degradation products of insulin generated by hepatocytes and by insulin protease. Journal of Biol Chem, volume 4,issue 2,p.33.
16. Nengah et al 2010, Construction of pY-AF vector for expression of thermostable ∝-L-Arabinofuranosidase in *Saccharomyces cerevisiae*. Journal of research gate, volume 31,issue 3, p. 246-267.

17. Manuel correia et al (2012). UV-light exposure of insulin: pharmaceutical implications up on covalent insulin dityrosine dimerization and disulfide bond photolysis. Journal of Plos One 7(12): e50733.

18. Sabrina Ruggeberg et al (2016). The replacement of the rabbit blood sugar bioidentitiy assay by an in vitro test for batch release of insulin glargine drug substance. Journal of Rudiger Hack, volume 3, issue 17, p. 413-427.

19. K Anuradha et al (2004). Investigation of central mechanism of insulin induced hypoglycemic convulsions in mice. Journal of India journal of experimental biology. Volume 42(4):72-368.

20. Ananya Sarkar et al (2011). Pharmaceutical and pharmacological profile of glimepride. Journal of applied pharmaceutical science; 01 (09); 2011: 11-19.

انتاج اقراص انسولين بتقنية المعلوماتية الاحيائية وهندسة البروتينات.

محمد كساب

محاضر علوم مايكروبيولوجي ومناعة طبية كلية الصيدلة جامعة القاهرة

مرض السكر مرض مناعي مزمن ومشكلة مؤرقة للعالم اجمع. ويحدث بسبب نقص افراز هرمون الانسولين كليا او جزئيا من خلايا بيتا لانجر هانز بالبنكرياس المنتجة له .

مرض السكر له انواع اشهرها

النوع الاول الذي يعتمد في علاجه على حقن الانسولين مدى الحياة والنوع الثاني يعتمد بشكل اساسي على تناول اقراص من ادوية مخفضة لنسبة السكر وجزئيا على حقن الانسولين في مكافحته. اشهراعراض مرض السكر هي التبول كثيرا وكثرة الفجع وكثرة الظمأ.

حقن الانسولين لها اثار جانبية تتمثل في الم عن موضع الحقن و امكانية حدوث عدوى ميكروبية وعدم انتظام الدهون بشكل مثالي عند موضع الحقن وزيادة الوزن والانخفاض الحاد المفاجئ في نسبة السكر في الدم عن المعدل الطبيعي عند تناول جرعة زائدة من حقن الانسولين بالاضافة لزيادة تركيز هرمون الانسولين في الدم.تمكنا من خلال بحثنا انتاج اقراص انسولين للتغلب على هذه الاثار الجانبية لحقن الانسولين التي تستعمل تحت الجلد او بالحقن الوريدي وهذه الاقراص ممتدة المفعول باضافة مادة كاربوكسي ميثيل سيليلوزالصوديوم ولاتتكسر بفعل حموضة المعدة ولا الانزيمات الهاضمة لاننا انتجنا الانسولين بتقنية المعلوماتية الاحيائية في تصميم انسولين معدل وراثيا وثابت حراريا وفيزيائيا وقادر على تحمل حموضة المعدة ومقاومة الانزيمات المحللة الهاضمة بالجهاز الهضمي وعدم التكسر بانزيمات الكبد الايضية وتجنبه التأثير الكبدي الاولي عند مروره داخل الكبد بعد تناوله مباشرة عن طريق الفم بالاضافة قمنا باضافة مادة مانعة للانزيمات المحللة الهاضمة للبروتينات مثل ابروتينين وغيرها لضمان عدم تكسر الانسولين بواسطة الانزيمات المحللة الهاضمة للبروتينات داخل تجويف القناة الهضمية بالانسان. قمنا باستعمال الهندسة الوراثية بعمل طفرة جينية لانتاج جين انسولين قادر على تكوين رابطة كبريتية ثنائية قوية واستطاعنا من خلال هندسة البروتينات من اضافة هذه الرابطة الكبريتية داخل جزئ الانسولين مما اكسبه الثبات الحراري والفيزيائي وامكانية الاحتفاظ به خارج الثلاجة لمدة طويلة وعدم التكسير بفعل حموضة المعدة او الانزيمات المحللة الهاضمة بالقناة الهضمية او التحلل بواسطة انزيمات الكبد خلال مروره بالكبد في مرحلة التأثير الاولي للكبد بعد تناول اقراص الانسولين مباشرة بواسطة الفم وعن طريق هذه الرابطة الكبريتية استطاعنا التغلب على مشكلة الانسولين الاساسية من كونه هرمون ببيتيدي وتمكنا من اطالة مفعوله ايضا بفضل هذه الرابطة الجديدة المضافة عن طريق عمل طفرة من نوع طفرات عديد الشكل النيكلوتيدي الاحادي حيث استبدلنا حمضيين امينين متجاورين لايبعدان عن بعضهما لمسافة اكثر من ا نانوميتر مثل الجليسين من المنطقة المرنة بالكور داخل هرمون الانسولين بحمضين امينين من السيستيين الذي يتميز بانه ثابت حراريا وفيزيائيا وقادرين على تكوين رابطة كبيريتية ثنائية قوية مع بعضهما بعكس الجليسين الغير ثابت حراريا ولا فيزيائيا وغير قادرعلى تكوين نفس الرابطة القوية.وقمنا باضافة الرابطة الكبريتية في منطقة الكور المسئولة عن الخواص الفيزيائية لهرمون الانسولين بحيث لايفقد الانسولين فاعليته الحيوية.نجحنا بانتاج الانسولين المعدل وراثيا عن طريق استعمال تقنية المادة الوراثية الدي ان ايه المهجنة وتمكنا من عمل بادئة امامية من عديدات النيكلوتيدات عددها حوالي عشرون نيكلوتيدة ومثلها لكن بادئة عكسية بغرض تصنيع هرمون الانسولين المعدل وراثيا وادخلنا هاتين البادئتين للبي سي ار وقمنا بعمل استنساخ للجين المكون للانسولين ثم ادخلناه داخل خلية فطرالسكاروميسيز لانتاج جزئ انسولين مترابط في شكل رابطة واحدة وليس وحدتين كما حدث بالدراسات السابقة عندما تم تصنيع الانسولين داخل خلية بكتيرية مثل الاشرشيا كولاي ثم يتم ربط وحدتي الانسولين برابطة كبريتية ثنائية ليكتمل تكون جزئ الانسولين وبذلك تمكنا من توفير الوقت والجهد في انتاج الانسولين المعدل وراثيا .

قمنا باختبار اقراص الانسولين على حيوانات تجارب كالفئران والارانب وعلى بشر خلال مرحلة المحاولات العلاجية الاولى والثانية وثبتت فاعلية اقراص الانسولين بنسبة تصل تقريبا ل 60% بالمقارنة بحقن الانسولين الوريدية وتحت الجلد ونأمل ونتوقع من خلال الابحاث القادمة زيادة هذه النسبة لتصل لثمانين بالمائة من خلال تطوير اقراص الانسولين وزيادة امتصاصه واطالة مفعوله باضافة مواد اخرى تساعده على ذلك.