University Of Petra
Amman-Jordan

First Pass Metabolism of Oral Insulin in Normal and Streptozotocin-Intoxicated Animals

by

Nehal Mohammed Jaber

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MSc Thesis

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Abstract

Several attempts for delivering proteins through the oral route have been reported. However, most of these attempts revealed low bioavailability of the administered proteins. As first pass metabolism might be attributed to such loss in the bioavailability, the current investigation aims to study the first pass metabolism of insulin, as a model protein, delivered orally in normal and STZ-diabetic rats.

The oral insulin formulation adapted in this work combines the advantages of using LMWC in the fabrication of W/O microemulsion. The utilized LMWC was initially prepared and characterized. Later, the oral insulin preparation was evaluated for its biological stability. The nanosized preparation was found to possess a unimodal particle size distribution with a mean diameter of 85 ± 3 nm. *In vivo* studies performed on normal and STZ-diabetic rats confirm that insulin retained its biological activity and stability as evidenced by the remarkable reduction of blood glucose levels post oral administration. Such hypoglycemic effect was sustained for a longer period of time post oral insulin administration compared to subcutaneous administration.

In order to study the first pass metabolism of the current insulin formula, different studies were established. The absorption and permeation of the orally administered insulin was conducted utilizing everted gut sac model and *in situ* intestinal perfusion technique. While the absorption of insulin nanoparticles compared to the free insulin solution was reduced in the everted gut sacs, the *in situ* intestinal perfusion study confirmed the ability of the nanoparticles to be absorbed by the intestine as evident by the significant hypoglycemic effects seen in both normal and diabetic rats.

Since liver is the major organ involved in the metabolism of insulin, *in situ* liver perfusion technique was performed and isolated hepatocytes were cultured from both normal and STZ-diabetic rats in order to investigate insulin's metabolism in liver. The results indicated that hepatic insulin metabolism was decreased in STZ-diabetic rats compared to normal rats. Similarly, insulin degradation was reduced in STZ-diabetic isolated hepatocyte compared to normal hepatocytes. When the *in situ* "liver passed" insulin was re-injected *in vivo* in both normal and diabetic rats, it was found that the insulin collected from diabetic livers was more active than the collected from non-diabetic livers. Such effect can be attributed to the decrease in insulin degradation in diabetic liver and diabetic isolated hepatocytes due to the toxic action of STZ. In addition, the result also confirms the effect of bacitracin on inhibiting insulin metabolism when incubated with hepatic cells *in vitro*.

In conclusion, the current study confirms the ability of the current investigated nanoparticle system with its modifications to enhance the pharmacological response of insulin when administered orally. The first pass metabolism of the absorbed oral insulin was seen variable between normal and STZ-intoxicated rats where the effect of insulin was more enhanced in diabetic animals. This enhancement in activity is mainly due to the reduction of insulin's first pass metabolism in these animals. The toxic effects of STZ on the liver and intestinal function is expected to be responsible of such reduction in metabolism.

To My Loving Husband Mohamed

For Your Constant Support and Understanding.

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Abbreviations

DAG	Diacylglycerol
DDA%	Degree of deacetylation
DSC	Differential Scanning Calorimetry
gm	Gram
GDM	Gestational diabetes
GITe	Glutathione-insulin transhydrogenase
h	Hour
HMWC	High molecular weight of chitosan
HPβCD	Hydroxypropyl-β-cyclodextrin
IDDM	Insulin dependent diabetes mellitus
IDE	Insulin degrading enzyme
IR	Infrared Spectroscopy
IU	International unit
KDa	kilo Dalton
Labrasol®	PEG-8 caprylic/capric glycerides
LMWC	Low molecular weight of chitosan

M.W.	Molecular weight
mg	Milligram
min	Minute
NIDDM	Non-insulin dependent diabetes mellitus
nm	Nanometer
NPs	Nanoparticles
p.s	Particle size
GIT	Gastrointestinal tract
IVC	Inferior vena cava
PEC	Polyelectrolyte complex
PEG	polyethylene glycol
Plurol®	Polyglyceryl-6 dioleate
r.p.m.	Rotating per minute
S.C.	Subcutaneous
SCOS	Surfactant/cosurfactant
SEM	Standard error of mean
STZ	Streptozotocin
W/O	Water in oil

Chapter One

Introduction

1. Introduction

1.1. Oral drug delivery

1.1.1. Oral rout of administration

Oral drug delivery has been known for decades as the most widely utilized route of administration among all the routs that have been employed for the systemic delivery of drug via various pharmaceutical products of different dosage forms. Oral rout has by far received the most attention with respect to research on physiological and drug constraints as well as design and testing of products, also oral rout is perhaps the most preferred to the patient and the clinician alike (Shojaei, 1998). However, oral drug delivery is defined as the method of swallowing a pharmaceutical compound with the intention of releasing it into the GIT of humans and animals (Ghosh & Pfister, 2005; Chugh et al., 2012).

1.1.2. Pharmacokinetics of oral drugs

For any given drug and dose, the plasma concentration of the drug will rise and fall according to the rates of three processes: absorption, distribution, and elimination.

Absorption of a drug refers to the passage of drug molecules from the site of administration into the circulation, with the rate dependent on the physical characteristics of the drug and its formulation (Evans et al., 1992). Drug delivery by oral route may result in only partial absorption and, thus, lower bioavailability, the oral route requires that a drug dissolves in the gastrointestinal fluid and then penetrates the epithelial cells of the intestinal mucosa. Most drugs are absorbed by

passive diffusion across a biologic barrier and into the circulation. The rate of absorption is proportional to the drug concentration gradient across the barrier and the surface area available for absorption at that site, known as Fick's Law. Drugs can be absorbed passively through cells either by lipid diffusion or by aqueous diffusion (Finkel et al., 2009). Lipid diffusion is a process in which the drug dissolves in the lipid components of the cell membranes. This process is facilitated by a high degree of lipid solubility of the drug. Aqueous diffusion occurs by passage through aqueous pores in cell membranes. Because aqueous diffusion is restricted to drugs with low molecular weights, many drugs are too large to be absorbed by this process. A few drugs are absorbed by active transport or by facilitated diffusion. Active transport can transfer drugs against a concentration gradient (Finkel et al., 2009).

Distribution of a drug refers to the process of a drug leaving the blood stream and going into the organs and tissues. Drugs are distributed to organs and tissues via the circulation, diffusing into interstitial fluid and cells from the circulation (Boullata & Armenti, 2010). Most drugs are not uniformly distributed throughout total body water, and some drugs are restricted to the extracellular fluid or plasma compartment. The delivery of drug from blood to the interstitium primarily depends on blood flow, capillary permeability, the degree of binding of the drug to plasma and tissue proteins, and the relative hydrophobicity of the drug.

Drug metabolism and excretion are the two processes responsible for the decline of the plasma drug concentration over time (Alavijeh et al., 2005). Both of these processes contribute to the elimination of active drug from the body, most drug metabolism takes place in the liver, but drug-metabolizing enzymes are found in many other tissues including the gut, kidneys, brain, lungs, and skin (Kanter et al., 2002). The major role of drug-metabolizing enzymes is to inactivate and detoxify

drugs and xenobiotics that can harm the body. Drug metabolites are usually more hydrophilic than is the parent molecule and, therefore, the kidneys more readily excrete them. Drugs that are absorbed from the gut reach the liver via the hepatic portal vein before entering the systemic circulation (Brenner & Stevens, 2013). Many drugs are extensively converted to inactive metabolites during their first pass through the gut wall and liver, and have low bioavailability after oral administration. This phenomenon is called the first-pass effect (Martinez & Amidon, 2002). Drug metabolism can be divided into two phases, each carried out by unique sets of metabolic enzymes. In many cases, phase I enzymatic reactions include oxidative, hydrolytic and reductive reactions, create or unmask a chemical group required for a phase II reaction. In phase II metabolism, drug molecules undergo conjugation reactions with an endogenous substance such as acetate, glucuronate, sulfate, or glycine. Conjugation enzymes, which are present in the liver and other tissues, join various drug molecules with one of these endogenous substances to form water-soluble metabolites that are more easily excreted (Duffus & Worth, 1996; Jancova et al., 2010).

Excretion is the removal of drug from body fluids and occurs primarily in the urine through the kidney. Other routes of excretion from the body include in bile, sweat, saliva, tears, feces, breast milk, and exhaled air (Shargel et al., 2005).

1.1.3. Factors affecting the oral drug delivery

Many factors may affect the delivery of oral drug, and finally affect the rate and extent of oral drug absorption. These factors can be divided into three categories. The first category comprises physiological factors, such as gastrointestinal pH, gastric emptying, small intestinal transit time, bile salt, absorption mechanism, intestinal and

liver metabolism and presence of food. The second category represents physicochemical properties of a drug, including solubility, intestinal permeability, drug pKa, lipophilicity, stability, surface area and particle size. The third category contains dosage form factors, such as solution, capsule, tablet, suspension and formulation additives (Karalis et al., 2008).

1.1.4. Advantages and disadvantages of oral drug delivery

Oral drug delivery offers several advantages, but also disadvantages, depending on the nature of the drug being delivered. The advantages of the oral route for drug delivery include firstly, the cost of oral therapy is generally much lower in comparison to parenteral and other routes of delivery. Secondly, compared to all other possible routes of drug delivery, oral drug administration is the most common and convenient administration method and demonstrates high patient acceptability and compliance. Thirdly, the total surface area of the small intestine in humans is approximately 200 m^2, which represents a large effective surface area for drug absorption. Fourthly, rich blood supply because the vascular surface of the gastrointestinal mucosa ensures rapid absorption and onset of action, as well as the maintenance of sink conditions. Fifthly, prolonged retention of the drug moiety is possible within the GIT, if the appropriate delivery system is used. This allows a lowering of the dosing frequency. Finally, the oral drug delivery offers the potential to achieve zero-order controlled release and the controlled release offers the further advantages of avoiding the peaks (risk of toxicity) and troughs (risk of ineffectiveness) of conventional therapy, reducing the dosing frequency, and increasing patient compliance (Zhang et al., 2002; Patel et al., 2011).

However, oral drug delivery systems also have some disadvantages as variability, the rate and extent of drug absorption from conventional dosage forms are affected by many factors, including fluctuating pH in the stomach and small intestine, the presence or absence of food, esophageal transit and gastric emptying rates, posture, diurnal rhythms, drug interactions and gastrointestinal or other pathology. In addition, a number of patient variables (gender, race, age, and disease state) can also drastically alter the absorption of orally administered drugs. As so many variables influence the availability of the drug at the target site, there is great potential amongst orally administered drugs for bioinequivalence. The other disadvantages are the adverse reactions; the locally irritating or sensitizing drugs must be used with caution in this route. For example, some drugs are gastro-toxic, causing damage to the mucosal lining of the stomach (Jain, 2008). The adverse environmental effects, the nature of the gastrointestinal environment also limits the types of drugs that may be administered via this route. Adverse environmental effects include the high metabolic activity creates a formidable biochemical barrier to the delivery of enzymatically labile drugs. In particular, the oral bioavailability of therapeutic peptides and proteins is very low (typically<1%). Metabolic activity within the GIT is further compounded by first-pass metabolism in the liver, extreme of pH some drugs are acid-labile and are degraded by the highly acidic conditions of the stomach. Delays in gastric emptying rates can prolong the residence time of drugs in the stomach, increasing the potential for acid-mediated degradation. Base-labile drugs are susceptible in the lower GIT, intestinal motility can severely constrain the contact time of a drug moiety with the absorbing surface. The physical barrier of the mucus layer and the binding of drugs to mucus may limit drug diffusion. P-glycoprotein efflux pump, this pump

serves as further barrier to drug absorption, impermeable epithelium (Wang et al., 2005; Hillery et al., 2001).

1.1.5. Evolution of oral drug delivery technology

Oral drug delivery still is the preferred route of administration for drug products. The evolution of oral drug delivery technology may be described by a three-stage course to reach its current level. With every step forward in drug delivery technology, scientists strive to gain more control over the pharmacokinetics of the drug substance with the goal to increase the therapeutic benefit-risk ratio or to improve bioavailability.

The first generation of oral drug delivery systems show unmodified release (Verma & Garg, 2001). This means that they have no controlled delivery technology incorporated and the drug release process starts immediately upon oral intake.

The second generation products consists of those oral drug products that have some sort of controlled delivery technology build-in, designed to change the pharmacokinetic behavior of the drug (Srikanth et al., 2013).

The third generation oral drug delivery systems aims to target the site where the disease is located ("targeted oral drug delivery"). To navigate to this location, the oral drug delivery system needs to interact with disease-specific targets (Basit, 2005). This approach is under investigation since the 90s and is not reach the market place yet.

Today, many scientific challenges and breakthrough technologies are required to generate novel dosage forms raising drug delivery to higher level. Some are self-emulsifying systems, solid self-nanoemulsion, polymeric micelles, spray freezing, pH

controlled systems, and time delayed system, osmotic pumps and prodrugs (Tarun et al., 2011).

New and emerging technologies in oral drug delivery are concentrated in three main points, via the use of mucoadhesives, retention of drug delivery devices in the GIT will improve. Increasing the targeted drug release, via the use of osmotic pumps, colonic specific delivery systems; developing the absorption of poorly absorbed drug moieties, via the use of lipidization strategies and penetration enhancers (Hillery et al., 2001).

1.2. Delivery of protein

Proteins are important to all living organisms; they are the major structural and functional components of cells. Peptides are very similar to proteins (Wilson & Hunt, 2002); technically, all proteins are peptides, but not all peptides are proteins. The difference between a peptide and a protein is that a protein is typically larger, folded, and has biological significance.

Peptides and proteins are highly potent and specific in their physiological activities. The effective and potent action of the proteins and peptides makes them the drugs of choice for the treatment of numerous diseases (Kim, 2013).

A variety of new therapeutic proteins have been developed showing therapeutic benefits in the treatment of ailments like diabetes, rheumatoid arthritis, hepatitis, cancer which offer several advantages over the conventional small molecule drugs. Firstly, proteins often serve a highly specific functions in the body that the chemical compounds cannot mimicked it. Secondly, since the action of proteins is highly specific, there is often less potential for therapeutic protein to cause adverse effects. Thirdly, because the body naturally produces many of the proteins that are used for therapeutic purpose, these agents are often well-tolerated and are less likely to elicit immune responses. Fourthly, for diseases in which a gene is mutated, protein therapeutics can provide an effective replacement for the treatment without the need for gene therapy (Leader et al., 2008; Dulal, 2010).

Delivery of therapeutic proteins has found an important position in therapeutics. Recent advances in pharmaceutical biotechnology have led to an increase in the number of protein products in the market.

As these therapeutic proteins are available in market, in the formulation of these drugs should ensure safety, consistency, potency and effectiveness of delivery systems. Despite the attractive features that protein offers, a large number of them have serious limitations (Nayak, 2014).

The chemical and structural complexities involved demand an effective delivery system in which the physicochemical and biological properties including molecular size, solubility, stability, light sensitivity, moisture, temperature, and biological half-life are duly main considerations (Bandyopadhyay, 2013).

Physical instabilities like denaturation, aggregation, precipitation and adsorption onto surfaces and chemical instabilities as oxidation, hydrolysis, deamidation and disulfide exchange, may occur for a given protein, due to the presence of multiple susceptible sites (Hasija et al., 2013).

The most important challenge to formulations of therapeutic proteins into effective dosage forms is to ensure their stability over their shelf lives. In the GIT, digestive enzymes normally break down proteins (Morishita & Peppas, 2006).

Therefore, formulation of therapeutic proteins is very critical, regardless of the route of administration, protein drug development should start with preformulation studies including physicochemical characterization, solubility and stability determination under various conditions, pH determination, also, the choice of buffer system and pH of vehicle, solvent selection as well as preservation of the formulation (Carpenter & Manning, 2002).

Among the factors that should be considered in the formulation of therapeutic proteins, in order to prevent the degradation pathways. Pharmaceutical formulations

of therapeutic proteins comprise the preservation of their biological activities with an acceptable shelf-life, effective and safe transport at the site of action.

According to the route of administration, the most protein pharmaceuticals are usually formulated as solution or suspension and delivered by invasive routes such as intravenous, intramuscular and subcutaneous administration, which are not well tolerated by patients usually.

Although, the elimination after invasive administration of therapeutic proteins may range from a few minutes to several days, most protein has short half-lives in the blood stream. After administration, unwanted deposition may occur, resulting in the need of frequent administration of high doses to obtain therapeutic efficacy. Both unwanted distribution and repeated dose administration of therapeutic proteins can lead toxic side effects (Jain et al., 2012).

Upon subcutaneous injection, protein bioavailability may be as 100%, but also may be much lower, the fate depending on molecular weight, and site of injection. While proteins over 16 KDa can diffuse through the blood endothelial wall entering blood capillaries at the injected site, or enter the lymphatic system and the systemic circulation, lower molecular proteins are predominantly absorbed in the systemic circulation via, local blood capillaries (Nayak, 2014).

Alternative non-invasive protein delivery routes are currently emerging of greater importance and these include nasal, ophthalmic, buccal, vaginal, transdermal, pulmonary and oral routes (Jain et al., 2013).

Delivery of protein by the nasal route offers virtue of relatively rapid drug absorption, possible bypassing of presystemic clearance and relative ease of administration.

Higher bioavailabilities can be obtained with more advanced delivery systems, especially by adding enhancers (Ozsoy et al., 2009) that modulate the permeability of the epithelium. Endogenous hormones such as LHRH and TRH have been shown to be absorbed nasally in animal and human (Morimoto et al., 1985).

Pulmonary administration is an attractive route of proteins and peptides than other alternative routes of administration. The lungs offer a large surface area for drug absorption, of approximately 80-140 m^2. Several formulations for pulmonary delivery are in various stages of development and various protease inhibitors, surfactants, lipids, liposomes, polymers and absorption enhancers have been tested for their efficacy in improving the systemic availability of protein and macromolecular drugs after pulmonary administration (Andrade et al., 2011).

The buccal mucosa represents a potentially important site for controlled delivery of macromolecular therapeutic agents, such as peptides and protein drugs with some unique advantages such as the avoidance of hepatic first-pass metabolism, acidity and protease activity encountered in the GIT. Another interesting advantage is its tolerance (in comparison with the nasal mucosa and skin) to potential sensitizers (Sudhakar et al., 2006).

The ocular route is the choice for the localized delivery of ophthalmologically active peptide and protein for the treatment of ocular disease. Some of the general approaches that have been found useful in enhancing the ocular absorption such as the use of nanoparticles, liposomes, gels, ocular inserts, or surfactants may also improve the ocular delivery of peptide-based pharmaceuticals (Lee et al., 1985).

During recent years various scientist have developed method for facilitating transdermal delivery of proteins. Chemical enhancers based on biphasic lipid system

showed good effect in the skin of experimental animals (Bandenhorst et al., 2014). Additional approaches to facilitate transdermal delivery of proteins include altering skin characteristics by physical tools such as iontophoresis, sonophoresis, and electroporation. Combination of chemical enhancers and iontophoresis also showed facilitated transdermal delivery of proteins.

Delivering therapeutically active protein and peptides by the oral route has been a challenge for many years. The oral route is unsuitable for the systemic delivery of therapeutic peptides and proteins because of the potential degradation by the strongly acid environment in the stomach and by the proteolytic enzymes in the intestinal tract, as well as presystemic elimination in the liver. For such drugs to be absorbed through the GIT, they must be protected from enzyme and must traverse through the luminal barriers into the blood stream in an unchanged form (Nayak, 2014; Jitendra et al., 2011).

To overcome this problem, a variety of permeation enhancers including mixed bile salts fatty acids micelles, chealators, surfactants, lipids and also using excipients like mucoadhesives polymers and enzymes inhibitors. Another approach is by chemical modification of the proteins and their hydrophobisation or lipidization to improve their enzymatic stability and membrane penetration (Muheem et al., 2014).

Currently only two peptide and protein based drugs (Interferon alpha and human growth hormone) that can be given orally are known to be in clinical developed (Jitendra et al., 2011).

1.3. Metabolism of proteins

Protein is a large, complex molecule that must undergo a series of processes inside the body. During digestion and absorption, protein passes through many organs. Once protein is digested, the body can utilize its nutrients to build and repair many of the cells in the body. The body also uses the calories from protein, which are released during the digestion process for energy when carbohydrates and fats are not available. When there is too much protein in the body, the excess is converted into fat for storage (Erickson, 1990).

Locations of intensive protein or peptide metabolism are gastrointestinal tissue, kidneys, liver, but also blood and other body tissues. Molecular weight determines that major metabolism site as well as the predominant degradation process (Kayser & Warzecha, 2012).

Once protein is chewed and swallowed, hydrochloric acid and pepsin begin protein metabolism in the stomach. HCl causes denaturation of proteins and converts proteins to metaproteins, which are easily digested. It also makes the stomach very acidic with a pH of 1.5. This acidic environment is necessary for HCl to react with pepsinogen to form pepsin so that it can break the central peptide bond in proteins. Rennin is a milk-clotting enzyme that is present in infants to help break down milk protein (Sherman, 1952).

Major factors limiting systemic bioavailability of orally administered protein drugs include acidic degradation and protein metabolism in the GIT (Gavhane & Yadav, 2012).

Metabolism of proteins is completed in the small intestine by proteolytic enzymes present in pancreatic and intestinal juices. The pancreas releases digestive enzymes into the small intestine. In the duodenum, the first section of the small intestine, trypsin breaks down proteins into single amino acids by a process called hydrolysis. During hydrolysis, a water molecule is placed between two amino acids, breaking the bond. Trypsin also activates the enzymes chymotrypsin, carboxypeptidase and elastase that are released into the small intestine for amino acid chain breakdown. While intestinal juice consists of aminopeptidase, tripeptidase and dipeptidase (Whitcomb & Lowe, 2007).

Finally, the end products of protein metabolism in the small intestine are amino acids.

The metabolic activity of the GIT, however, is not limited to orally administered proteins. Parenterally administered proteins and peptides may also be metabolized in the intestinal mucosa following intestinal secretion. At least 20 % of the degradation of endogenous albumin takes place in the GIT (Kontermann, 2011).

The kidneys constitute a major site of metabolism for many smaller sized proteins that undergo glomerular filtration. Glomerular filtration is generally the dominant, rate-limiting step in renal metabolism of protein drugs, with a cut-off value of approximately 60 KDa for molecular weight. In addition, molecular conformation and charge of proteins also contribute to the selectivity of glomerular filtration. For example, cationic macromolecules pass through the capillary wall more readily than neutral macromolecules, while neutral macromolecules pass through more readily than anionic macromolecules (Crommelin et al., 2008).

Various renal processes contribute to the elimination of peptides and proteins. For most substances, glomerular filtration is the dominant, rate-limiting step as

subsequent degradation processes are not saturable under physiologic condition. Hence, the renal contribution to the overall elimination of peptides and proteins is reduced if the metabolic activity for these proteins is high in other body regions, and it becomes negligible in the presence of unspecific degradation throughout the body. In contrast to this, the contribution to total clearance approaches 100 % if the metabolic activity is low in other tissues or if distribution is limited (Meibohm, 2006).

A part from general proteolysis and the kidneys, the liver substantially contributes to the metabolism of peptide and protein drugs. Proteolytic degradation usually starts with endopeptidases that attack in the middle part of the protein, and the resulting oligopeptides are then further degraded by exopeptidases. The ultimate metabolites of proteins, amino acids, and dipeptides, are finally utilized in the endogenous amino acids pool (Meibohm, 2006; Crommelin et al., 2008).

The rate of hepatic metabolism is largely dependent on the specific amino acids sequences in the protein. As proteolytic enzymes in the hepatocytes are mainly responsible for the catabolism of proteins in the liver, intracellular uptake of proteins into hepatocytes is a prerequisite for hepatic protein metabolism. While small peptides may cross the hepatocytes membrane via passive diffusion if they have sufficient hydrophobicity, various carrier-mediated energy dependent membrane transporters and receptor-mediated endocytosis are usually responsible for the uptake of larger peptides and proteins (Freeman & Kim, 1978).

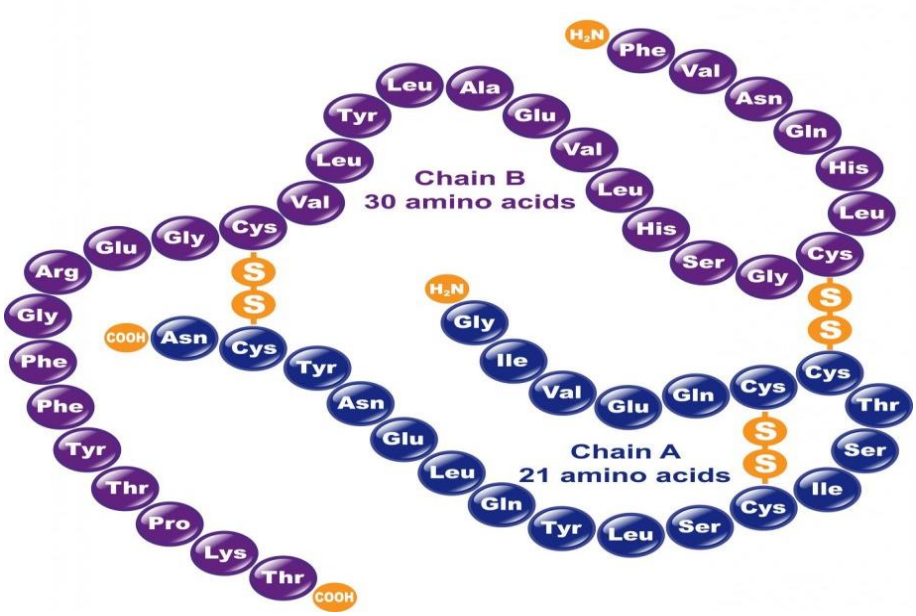
1.4. Oral insulin delivery

1.4.1. Insulin: physicochemical properties and function

Insulin was discovered in 1921 and shortly afterwards was described as a protein. Insulin is a polypeptide hormone, which is synthesized in pancreatic β -cells. Its synthesis involves sequential cleavage of its two precursor molecules preproinsulin and proinsulin (Joshi et al., 2007). Following synthesis, the preproinsulin molecule undergoes rapid enzymatic cleavage to proinsulin, which contains the insulin A and B chains linked by connecting or C-peptide. Proinsulin is packaged into small granules within the Golgi complex, which then migrate towards the cell surface. As the granules mature, proteases split proinsulin into equal amounts of insulin and C-peptide, allowing the insulin molecule, consisting of A and B chains linked by two disulfide bridges, to assume its active configuration. The resulting mature insulin composed of the A chain with 21 amino acids and the B chain with 30 amino acids as shown in (Figure 1.1) and with a molecular weight of 5800 g mol^{-1}. Both chains are held together by two disulphide bonds. Another disulphide bond is internal within the A chain itself (Owens, 2002).

Following biosynthesis, insulin is stored as crystalline zinc-bound hexamers in vesicles within the pancreatic β-cells from which secretion occurs in response to elevated blood glucose levels (Lemaire et al., 2012). Insulin has an isoelectric point (pI) of 5.3 and a charge of -2 to -6 in the pH range 7-11. Another intrinsic property of insulin is its ability to readily associate into dimmers, hexamers and higher-order aggregates. At the low concentrations found in the blood stream (< 10-3 μM), insulin exists as a monomer, which is its biologically active form (Chien, 1996).

The major function of insulin is to counter the concerted actions of a number of hyperglycemia-generating hormones and to maintain low blood glucose levels. The biological actions of insulin are initiated when insulin binds to its cell surface receptor. Insulin is an anabolic hormone and when binding to its receptor begins, many protein activation cascades occur. These include the translocation of the glucose transporter to the plasma membrane and the influx of glucose, glycogen synthesis, glycolysis and fatty acid synthesis. Insulin has been observed as promoting the transport of some amino acids and potassium ions. Insulin also inhibits the liberation of free fatty acids and glycerol from the adipose tissue (Andersen et al., 2006).



(Figure 1.1): Insulin-Primary Structure.

1.4.2. Diabetes Mellitus

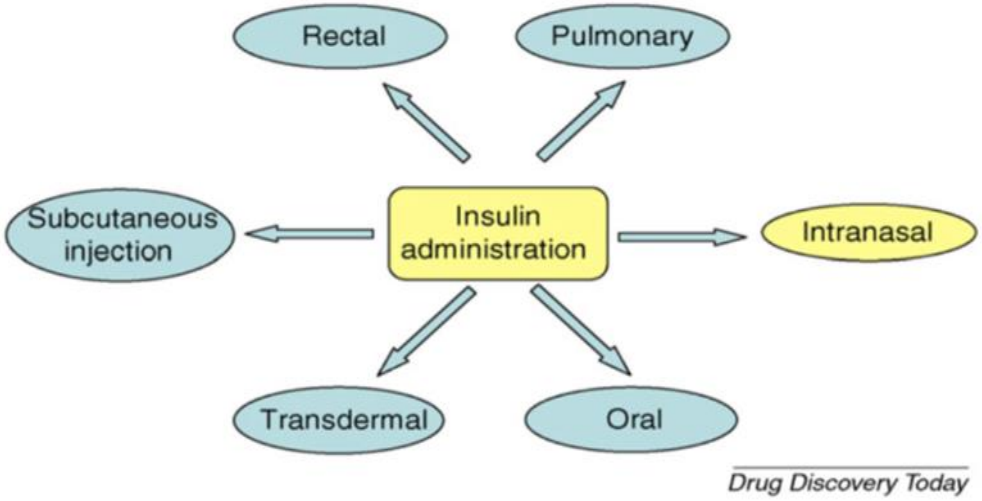
Insulin is used for the treatment of diabetes. The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2009). The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death (Prabhu et al., 2012).

The diabetes is classified into four groups: (i) type 1 diabetes mellitus; (ii) type 2 diabetes mellitus; (iii) diabetes mellitus those due to other specific mechanisms of elevated blood glucose or non-pancreatic diseases; and (iv) gestational diabetes mellitus (Craig et al., 2014). Type 1 diabetes (insulin dependent diabetes mellitus, IDDM) is characterized by destruction of pancreatic ß-cells, or the insulin-producing cells of the body. Type 2 diabetes (non-insulin dependent diabetes mellitus, NIDDM) is characterized by combinations of decreased insulin secretion and decreased insulin sensitivity (insulin resistance). Gestational diabetes (GDM) is defined as any abnormality in glucose levels noted for the first time during pregnancy.

1.4.3. Alternative routes for insulin administration

Since the introduction of insulin therapy 85 years ago, subcutaneous injections have been the only route of delivery of insulin therapy to diabetic patient. During this time, numerous researches have been made to explore alternative routes for systemic insulin administration, but little has changed about the way insulin is administered (Figure 1.2). The traditional and most predictable method for administration of insulin is by subcutaneous injections. To decrease the suffering, the use of supersonic injectors, infusion pump and pens has been adopted (Yaturu, 2013). Insulin pumps are medical devices designed to deliver measured amount of insulin into patient's body in a controlled manner. Advances in insulin-infusion pumps have extended from the earlier, relatively bulky, external continuous subcutaneous insulin-infusion devices, to the present smaller, lighter and more reliable variable-rate pumps, which have improved catheters and inbuilt alarms. Evidence indicates that patients with problems such as frequent, unpredictable hypoglycemia could benefit from this approach. Implantable insulin pumps have also been in development (Tyagi, 2002). Insulin pens more discreet compared with vials and syringes. Insulin pen is a small pen-shaped device with small needle that can deliver exact amount of insulin to patient's body. Insulin pens combine the insulin container and the syringe into a single modular unit and eliminate the inconvenience of carrying insulin vials and syringes are more accurate and less painful (Perfetti, 2010).

The quest for a noninvasive route for delivery of insulin was attempted as early as 1925. Various noninvasive routes have been investigated including transdermal, nasal, pulmonary, rectal, oral, buccal, and vaginal (Kumria & Goomber, 2011).



(Figure 1.2): The main administration routes for insulin (Duan & Mao, 2010)

A transdermal system is a common route of insulin delivery systems being developed. Skin offers the advantages of an easy access and a very large surface area (1-2 m^2) (Paudel et al., 2010). However, it represents an effective barrier that limits penetration of large, hydrophilic polypeptide, like insulin, as the stratum corneum, the outermost layer of the skin constitutes the major barrier. Various methods have been tested overcome the skin barrier and to allow insulin absorption. They can be separated into chemical (liposome and chemical enhancers) and physical methods (mainly iontophoresis and sonophoresis) (Lassmann-Vague & Raccah, 2006). Sintov, A.C. et al. have done the study for transdermal insulin delivery by using topical iodine. The pretreatment of skin with iodine followed by a dermal application of insulin results in reduced glucose level and elevated hormone levels in the plasma. Topical iodine protects the dermally applied insulin by inactivation of endogenous sulfhydryls, which can reduce the disulfide bonds of the hormone. Thus, the effect of iodine is mediated by retaining the potency of the hormone during its penetration via the skin into the circulation (Shah et al., 2010).

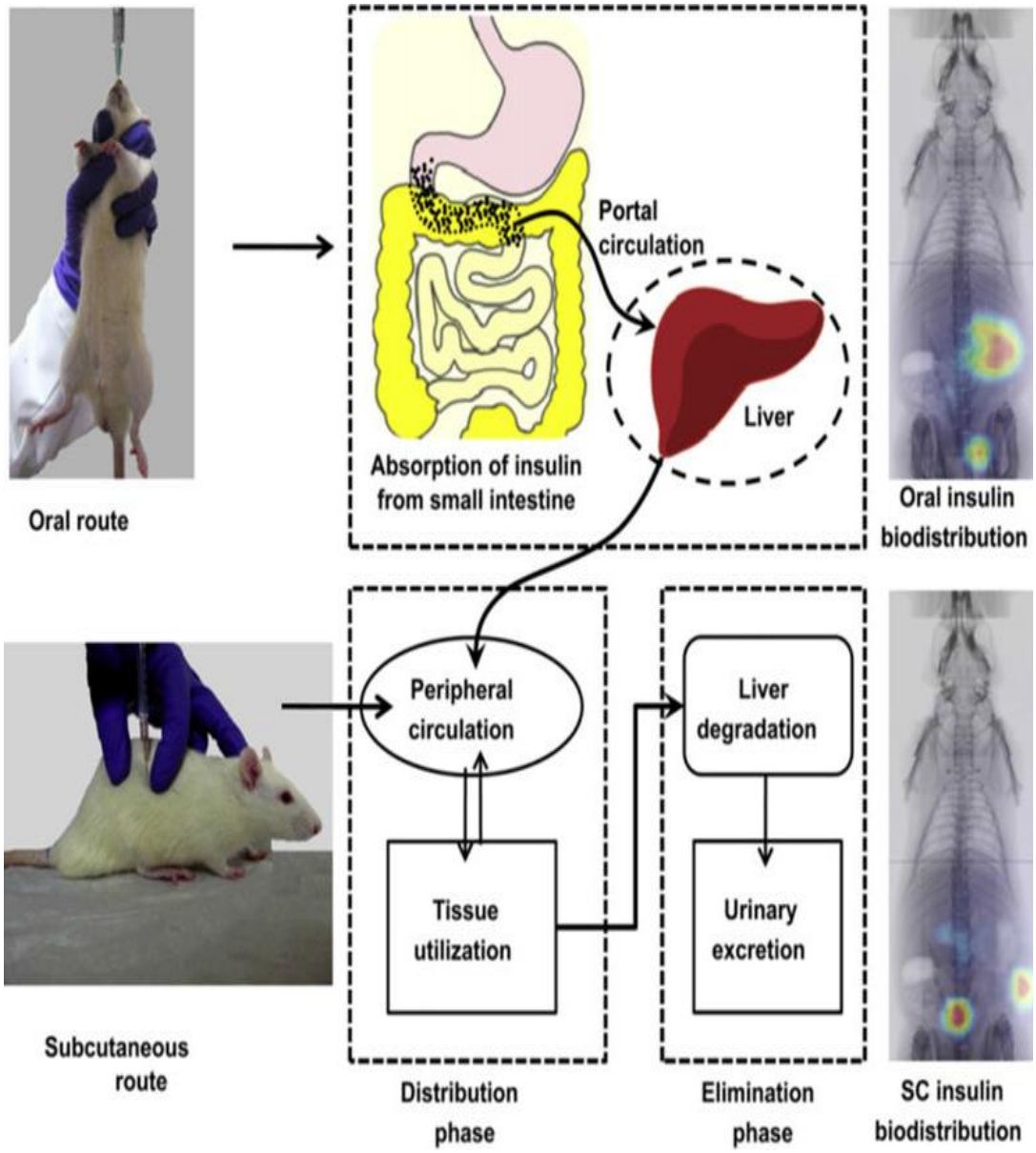
A third investigated process is the transmucosal delivery of insulin into blood stream. There are a number of mucosal surfaces possible for transport: nasal, buccal, pulmonary, oral, vaginal, and rectal. The nasal, pulmonary and oral routes are leading candidates for transmucosal delivery.

Nasal administration has attracted a lot of interest as a highly efficient route for the systemic delivery of insulin. The large surface area available for absorption through nose cause a wide range for insulin reaches the systemic circulation, thereby avoiding the loss of insulin from first-pass hepatic metabolism (Shah et al., 2010). Some barriers limit the intranasal absorption of insulin, low permeability of nasal mucosa to large molecules and the low bioavailability of insulin act as barriers to intranasal absorption. To overcome the various barriers by the nasal route, researchers have studied many extensive ranges of enhancers such as bile salts and derivatives, sodium lauryl sulfate, phospholipids, cyclodextrins, chitosan and enzyme inhibitors. At first chitosan-nanoparticles is seemed to be the safest and most effective as a carrier for the nasal delivery of insulin. It protected insulin from degradation in the nasal cavity and increased intranasal absorption of insulin with its positive charge (Fernández-Urrusuno et al., 1999). However, recent studies showed that insulin-chitosan solution formulation was more effective than the intranasal nanoparticles complex (Dyer et al., 2002).

Pulmonary insulin delivery requires the addition of absorption enhancers to achieve good insulin delivery (Hussain et al., 2003). Recently, Experimental studies investigated that insulin could be efficiently encapsulated in. Liposome mediated pulmonary drug causes enhancement in drug retention time in the lungs and decreases side effects which results increased therapeutic effects. When aerolized insulin

liposomes delivered by the inhalation route in mice caused significantly reduction in plasma glucose levels (Huang & Wang, 2006).

Taking all of this account oral insulin delivery is considered to be the most safest and convenient which delivers the insulin directly into the liver through portal circulation, where it inhibits hepatic glucose production. Hence, by oral delivery largely the natural physiological route of insulin can be mimicked (Figure 1.3). Insulin being a protein, difficulties encountered in oral delivery includes denaturation and degradation by low pH of the stomach and different digestive enzymes in the stomach and small intestine; and the major barrier for absorption is the intestinal epithelium. All these lead to low bioavailability. Different formulations of polymers for insulin delivery such as liposomes, microspheres, microemulsion and nanoparticles (NPs) have been investigated to circumvent these GI barriers (Carino & Mathiowitz, 1999; Chandra et al., 2014).



(Figure 1.3): Schematic diagrams illustrating the absorption, distribution and elimination of insulin following oral or subcutaneous (S.C.) administration (Sonaje et al., 2010).

1.4.4. Strategies for oral delivery of insulin

Different approaches, alone or in combination, have been developed to overcome the barriers of enzymatic degradation and improve the oral bioavailability of insulin like use of permeation enhancers (detergents, fatty acids or bile salts which improve the permeability through the mucus and epithelial layers and open the intercellular tight junctions) (Aungst, 2012), Surfactants and fatty acids affect the transcellular pathway by altering membrane lipid organization and therefore increase the oral absorption of insulin (Wadher et al., 2009), Enzyme inhibitors (Del Curto et al., 2009), enteric coating (Zhao et al., 2007), carrier systems (Chalasani et al., 2007) and chemical modifications of insulin (Shah et al., 2010).

Enzyme inhibitors slow the rate of degradation of insulin, which increases the amount of insulin available for absorption. The addition of proteolytic inhibitor such as diisopropylfluorophosphate (Danforth et al., 1959) or naturally occurring aprotinin (Owens et al., 1988) or inhibitors of insulin-degrading enzyme include 1,10 phenanthroline and bacitracin (Shaji & Patole, 2008) show some decreased in blood glucose level. However, although the effectiveness of enzyme inhibitors is not very high when used alone, they have the potential to be used in combination strategies with other methods to increase oral insulin absorption.

Enteric coating techniques with acrylic polymers were used to create a controlled release from oral insulin system (Foss et al., 2004).

Stefanov et al. have used liposomes prepared from phosphotidylcholine (PC) and cholesterol (CH) for oral insulin delivery. They have reported a significant reduction in blood glucose levels in diabetic rats. Further investigations with liposomes

containing insulin in rats and dogs showed reduction in blood glucose levels (Moupti et al., 1980; Patel et al., 1982).

Peppas et al. investigated oral insulin delivery using hydrogels of poly (methacrylic acid-g-ethylene-glycol). A hypoglycemic effect combined with insulin absorption was observed in rats using a closed loop absorption method. 25IU/kg of human recombinant insulin was incorporated into the polymer and infused in an isolated illeal segment; the bioavailability of insulin was shown to increase to 6.2% compared to the control (Nakamura et al, 2004).

A wide range of biodegradable and conventional polymers has been investigated as possible oral insulin delivery systems (Guo & Gao, 2007; Nakamura et al., 2008; Badwan et al., 2009). These systems should protect the insulin as it passes through the stomach, and were design to release their insulin and successfully transport it across the gastrointestinal lining. Most systems developed to this point either protect the insulin while in transit or aided in the transport of insulin across the cell layer of the upper part of small intestine, none have accomplished both.

It was demonstrated that lectin modified solid lipid nanoparticles containing insulin orally administrated to rats resulted in relative bioavailabilities of between 4.99% and 7.11% (Zhang et al., 2006). Another group successfully maintained plasma glucose level at pre-diabetic levels for 11 hours after oral administration of chitosan and insulin nanoparticles to diabetic rats (Ma et al., 2005). In addition, microparticles composed of poly(methacrylic acid) and poly(ethylene glycol)(PEG) and containing insulin that were orally administrated to type 1 diabetic rats resulted in suppressed post-prandial blood glucose levels (Morishita et al., 2006). The production and characterization of PEG-coated silica nanoparticles (SiNP-PEG) containing insulin for

oral administration shows that the presence of PEG onto the silica nanoparticles did not increase the permeation behavior of insulin through the small intestinal mucosa (Andreani et al., 2014).

1.4.5. Nanotechnology and Nanoparticles

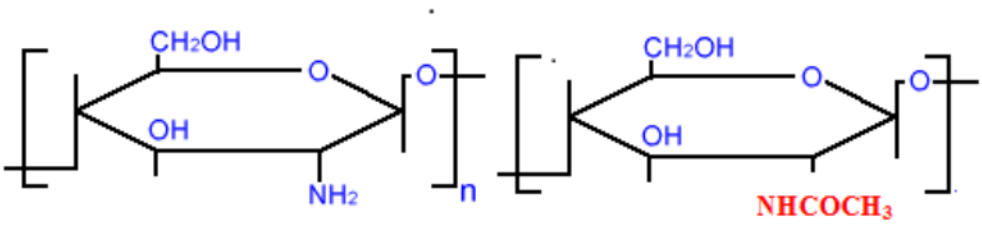
Today, a vast number of investigations have been focused on nanoparticles and their role as drug delivery vehicles. Nanoparticles were first introduced in the mid-seventies by Birrenbach and Speiser (Birrenbach & Speiser, 1976). Our work is related in general to nanotechnology, involved the fabrication of nanoparticles of insulin by utilizing one of biodegradable polymer. Nanoparticles are generally defined as particles between 10nm and 1000 nm (De Jong & Borm, 2008).

Polymeric nanoparticles have the advantages of protecting the protein and peptide drugs, such as insulin, from chemical and enzymatic degradation in the GIT, increasing their stability and absorption across the intestinal epithelium as well as controlling the drug release (Vila et al., 2002). For the conventional medicine, it is well understood the nanosize along with other characteristics does play an important role as evident from the improved bioavailability/pharmacological availability. Owing to the high surface area to volume ratio of NPs, the window of absorption is also high in comparison with microparticles; this is an added advantage in improving the bioavailability of the administered drug. One example for biodegradable polymer is called chitosan (Sung et al., 2012).

1.4.6. What is Chitosan?

Chitosan is a natural polymer obtained by N-deacetylation of chitin. After cellulose, chitin is the second most abundant polysaccharide in nature. It is biologically safe, non-toxic, biocompatible and biodegradable polysaccharide (Sailaja et al., 2010).

Chitosan is a linear copolymer consisting of ß (1-4)-linked 2-amino-2-deoxy–D-glucose (Dglucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (Figure 1.4). Chitosan is a weak poly base due to the large quantities of amino groups on its chain (Sarmento & das Neves, 2012).



(Figure 1.4): Chemical structure of Chitosan.

The physiochemical properties of chitosan polymer can manipulate through two parameters: the molecular weight (M.W.) which is the sum of the weights of the atoms of which it is made) and the degree of deacetylation (DDA %) defined in terms of the percentage of primary amino groups in the polymer backbone. The DDA % of typical commercial chitosan is usually between 70%-99% and the M.W. between 10-1000 kDa (Gao & Wan, 2006).

Both HMWC and LMWC are available. The latter were obtained by the depolymerization of HMWC. This can be carried out by enzymatic (Kittur et al., 2003), physical (Cravotto et al., 2005) or chemical methods (Tian et al., 2004). Industrially, chemical acidic degradation using hydrochloric acid was preferred to prepare LMWC (Varum et al., 2001). This method is usually simple, easy, reproducible and non-random.

Chitosan dissolves easily at low pH due to the protonation of the amino groups, while it is insoluble at higher pH ranges since the amino groups become deprotonated, as the pH approach the pKa of chitosan (6-6.5). The solubility of chitosan depends upon M.W. and DDA % (Marguerite, 2006).

Chitosan with its mucoadhesives and non-toxic properties can act as a significant absorption enhancer by opening the intercellular tight junctions of the epithelia and promoting the paracellular permeation of hydrophilic macromolecules (Junginger & Verhoef, 1998).

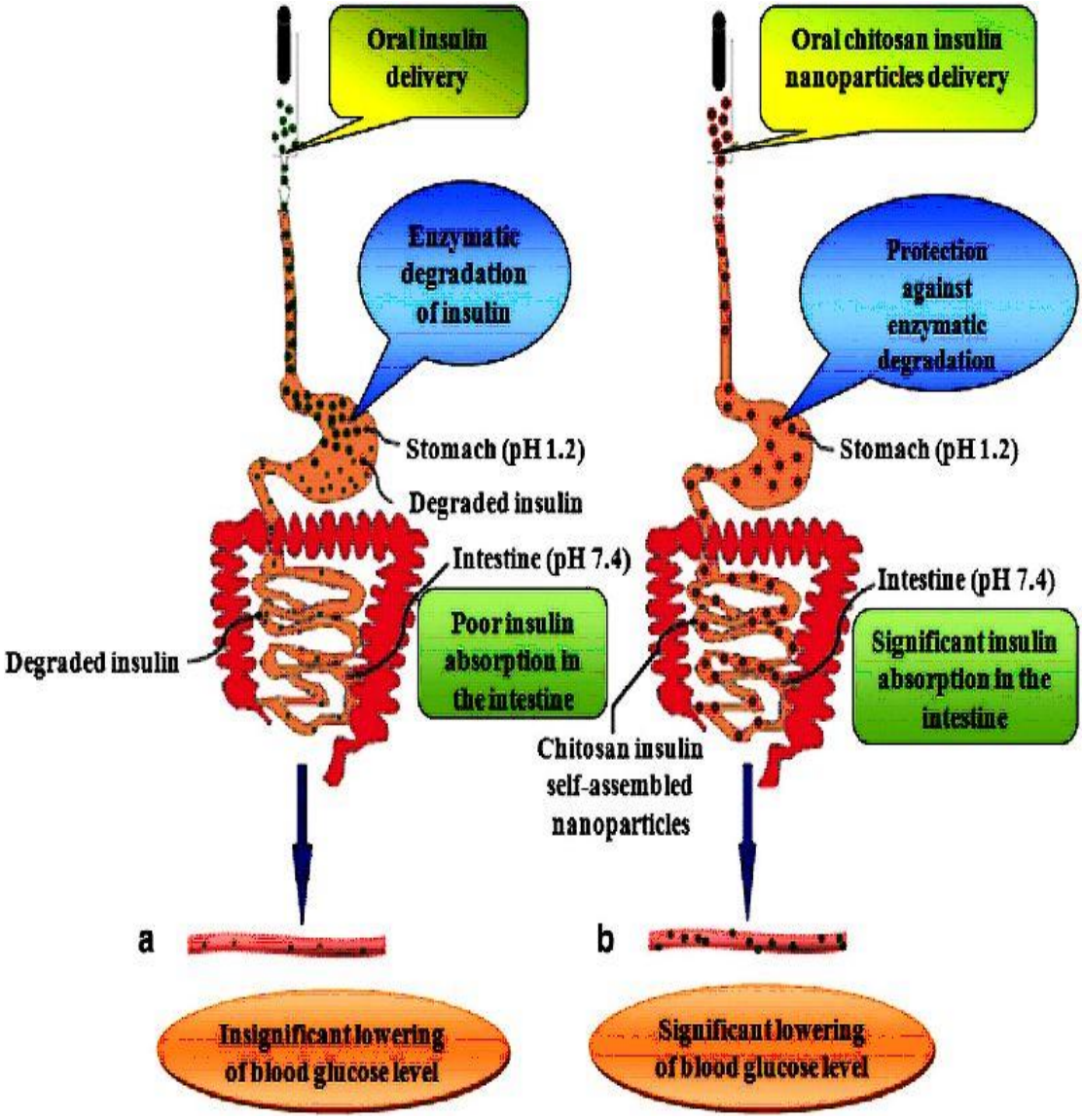
Several methods have been developed during the last two decades for preparation of chitosan nanoparticles, such as emulsion cross-linking, spray drying technique, emulsion coalescence method, reverse micellar method and ionic gelatin method (Zhao et al., 2011).

1.4.7. Method of Insulin-Chitosan nanoparticles formulation

Insulin-chitosan nanoparticles (NPs) have been prepared using ionotropic gelation with tripolyphosphate or even simply polyelectrolyte complexation between insulin and chitosan. The work of Sadeghi et al. showed that trimethyl chitosan and diethylmethyl chitosan nanoparticles prepared by the PEC method had higher insulin loading efficiency and zeta potential than ionotropic gelatin method (Sadeghi et al., 2008). The interaction of chitosan and polyanions leads to the spontaneous formation of nanoparticles in an aqueous environment without the need for heating or the use of organic solvents (Gan & Wang, 2007)

The most important factors that have to be controlled during preparation of PEC are the pH of the solution, temperature, ionic strength and order of mixing. In addition, there are secondary factors, related to the components that have to be considered, such as flexibility of polymers, M.W. and DDA % of chitosan (Berger et al., 2004).

However, these PECs dissociate easily in acidic gastric conditions, because both insulin and chitosan are soluble at lower pH such as that of the stomach. In order to protect the PECs from the gastric environment, some researchers have to use of oily vehicle, where the most promising oily-based formulations W/O microemulsions (Figure 1.5) (Elsayed et al., 2009).



(Figure 1.5): In-vivo efficiency of orally delivered insulin and chitosan/insulin NPs (Mukhopadhyay et al., 2013).

Microemulsion is clear, thermodynamically stable, isotropic liquid mixtures of oil, water and SCOS combination with complex and diverse microstructures (Basheer et al., 2013).

Many studies revealed that the bioavailability of insulin increased when the PEC surface was more lipophilic. Elsayed et al., 2009 stated, "Significant improvement in the oral absorption of insulin can be achieved by rendering the protein hormone more lipophilic through microemulsion constructed from insulin-chitosan in oleic acid".

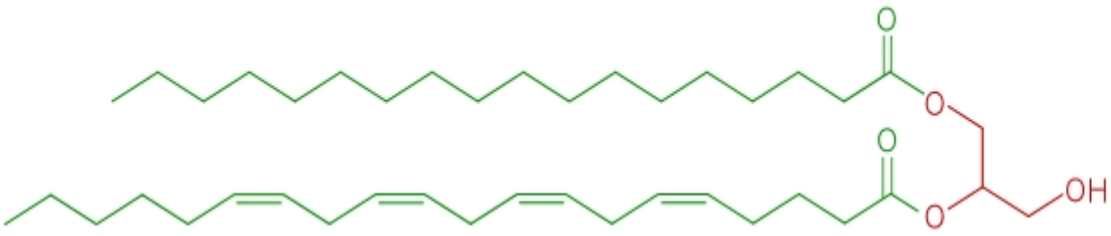
Ability cyclodextrin derivatives to form complexes with variety of peptides are well studied. Complexation enhances the stability of peptide formulation and improves their shelf-time (Sajeesh & Sharma, 2006). Zhang et al showed that insulin encapsulated in CS–alginate nanoparticles was protected from degradation and release under simulated gastrointestinal conditions, on forming complex with hydroxypropyl-β-cyclodextrin (HPβCD) polymer.

Oleic acid is the most common fatty acid in food. Oleic acid has been shown to alter membrane permeability by increasing fluidity of the membrane phospholipids. Furthermore, oleic acid containing microemulsion did not exhibit any membrane damage to the GIT lining when passes in it (Aungst et al., 1996; Muranishi, 1990). Thus, the insulin emulsions containing an unsaturated fatty acid are acceptable for use in oral delivery system. The reason for the use of oleic acid as a carrier is the formation of protective hydrophobic coating layer at the surface of insulin-chitosan nanoparticles. This layer is formed due to the interaction between the free chitosan amine groups and the adjacent carboxylic acid groups of oleic acid as dispersion medium.

Generally, the nanoparticles adopt irregular shapes within the dispersion phase that may cause an increment in its aggregation diameters. Thus, SCOS combination has been added in order to achieve more symmetrical shape for the insulin-loaded chitosan nanoparticles, to decrease the particle size of the microspheres and to improve the bioavailability by various mechanisms including improved drug dissolution, increased intestinal epithelial permeability (Elsayed et al., 2011; DeRosa et al., 2000).

In spite of numerous advantages of microemulsion, a high content of surfactant that can lead to interfacial tension; the co-surfactant is used to lower the interfacial tension because a low interfacial tension is essential for producing microemulsions (Lovelyn & Attama, 2011). Labrasol® as a surfactant and Plurol® Oleique as a cosurfactant were proposed to be used in the formulation of oily-based system for oral delivery of insulin in this study.

Based on previous researches, diacylglycerol (DAG) is defined as is a simple lipid consisting of a glycerol molecule linked through ester bonds to two fatty acids (Figure 1.6) (Carrasco & Mérida, 2007).



(Figure 1.6): Chemical structure of DAG.

The presence of DAGs is related in many cases, to the increase of membrane permeability, thereby increasing the drug uptake and solubilizing, fill the gaps between the surfactant/cosurfactant and solidify the dispersion system therefore increasing the physical stability of insulin-loaded nanoparticles formula (Martins et al., 2007; Yi et al., 2013; Fatouros et al., 2007).

When DAG introduced in large amount, prevent the formulation of microemulsion or break the dispersion system. More specifically, no more than 2.5% is used for the preparation of dispersion system of the present preparation.

1.5. First pass insulin metabolism

1.5.1. The liver is a primary site for insulin metabolism

The liver plays a major role in the metabolism of insulin (Duckworth et al., 1988). Approximately 70% of portal insulin is removed by hepatocytes during first-pass effect before entering the systemic circulation, but this percentage varies widely under different conditions.

Hepatic uptake of insulin is not a static process, but rather is influenced by both physiological and pathophysiological factors (Duckworth et al., 1988). In addition, the hepatic insulin uptake is incompletely understood and involves several different systems and controls.

Since most hepatic uptakes are a receptor-mediated process, at physiological concentrations, uptake is mediated primarily by the insulin receptor with a smaller contribution from nonspecific processes. At higher concentrations, non-receptor processes start to assume greater importance, very high concentrations of insulin (500-2000 μIU/ml) result in a decrease in the fractional uptake although total uptake is increased (Jochen et al., 1989). Prolonged increases in portal insulin levels also result in reduced clearance due to receptor down-regulation.

Hovorka et al., 1993 used a five-compartment model to reflect insulin distribution in systemic plasma, hepatic plasma, and interstitial fluid and insulin binding to the liver and peripheral receptors, and it included receptor-mediated and non-receptor-mediated insulin degradation. The mean residence time of endogenously secreted insulin was 71 min; 62 min found bound to the liver receptor, the time of binding to peripheral receptors was 6 min, and 3 min in blood or interstitial fluid, 80% of the

total insulin in the body was bound to liver receptors. Other tissues as muscle transiently bind and can release insulin back into the blood.

Nutrient intake alters insulin clearance (Hennes et al., 1997). In general, glucose ingestion increases hepatic insulin uptake, the glucose-induced increase in insulin secretion may also decrease hepatic fractional extraction. Under normal physiological conditions increasing doses of glucose (10 g, 25 g, and 100 g) result in increases in insulin secretion (1.8 U, 2.7 U, and 7.2 U) with decreasing hepatic extraction (67%, 53%, 42%). Insulin clearance is reduced by oral but not by intravenous glucose administration (Meier et al., 2007).

Hepatic insulin metabolism and clearance rates are decreased in obesity and diabetes and with increases in other hormones such as Catecholamines and glutathione. In subjects with a more severe degree of Diabetes, decreased hepatic insulin removal is the primary cause of hyperinsulinemia (Bonora et al., 1983).

Despite the potential role of insulin clearance in the etiology of diabetes, little is known about the factors that are independently associated with decreased insulin removal. In the current study, by using the liver perfusion technique and isolated rat hepatocytes we will evaluate the insulin first pass metabolism in diabetic and non-diabetic rat. In addition, we will determine if the high flow rate of perfused insulin one of the factors that associated with the decreased insulin metabolism or not.

Given the importance of the liver in insulin metabolism, it is not surprising that liver disease and liver toxicity may result in a decrease in insulin metabolism, although not all studies agree. Part of this study will be evaluated the STZ toxicity of liver and if there is any decrease in insulin clearance or not. The decreased clearance is due both to reduced hepatic function and to portosystemic shunts, but not to decrease insulin-

degrading activity. The reduced hepatic clearance is also associated with reduced insulin sensitivity, supporting the relationship of insulin degradation to insulin action.

Streptozotocin (STZ) is a naturally occurring nitrosourea, broad-spectrum antibiotic and cytotoxic chemical. It is widely used to induce insulin-dependent diabetes mellitus in experimental animals because of its toxic effects on islet beta cells (Ohno et al., 2000). STZ has various biological actions including the production of acute and chronic cellular injury, carcinogenesis, teratogenesis and mutagenesis (Bolzan & Bianchi, 2002). In addition, it is hepatotoxic, nephrotoxic and causes gastric ulceration.

Zafar et al. 2009 reported that STZ-induced diabetes complication in liver is as destruction of hepatocytes. STZ made alteration in liver enzyme levels and morphological changes.

Streptozotocin action depends proportionally on concentration and time of experiment. STZ shows strong cytotoxic effect on liver at high concentration and after 48 h of STZ administration (Dabros et al., 2007).

The liver is responsible for more than half of the total insulin degradation, with kidney responsible for most of the rest (Duckworth & Kitabchi, 1981). After liver and kidney, the peripheral tissues, fat and muscle play important role in insulin removal, probably degrade the remainder of the insulin in the body, but the absolute contribution of these tissues to insulin turnover is uncertain.

Insulin uptake and degradation occur in adipocytes (Marshal, 1985), fibroblasts (Baldwin et al., 1981), monocytes (Powers et al., 1980), lymphocytes (Sonne & Gliemann, 1980), gastrointestinal cells (Bai et al., 1995), and many other tissues. All

cells that contain insulin receptors and internalization mechanisms can degrade insulin. As in liver, insulin action in adipocytes correlates better with degradation than with delivery (Donner et al., 1982).

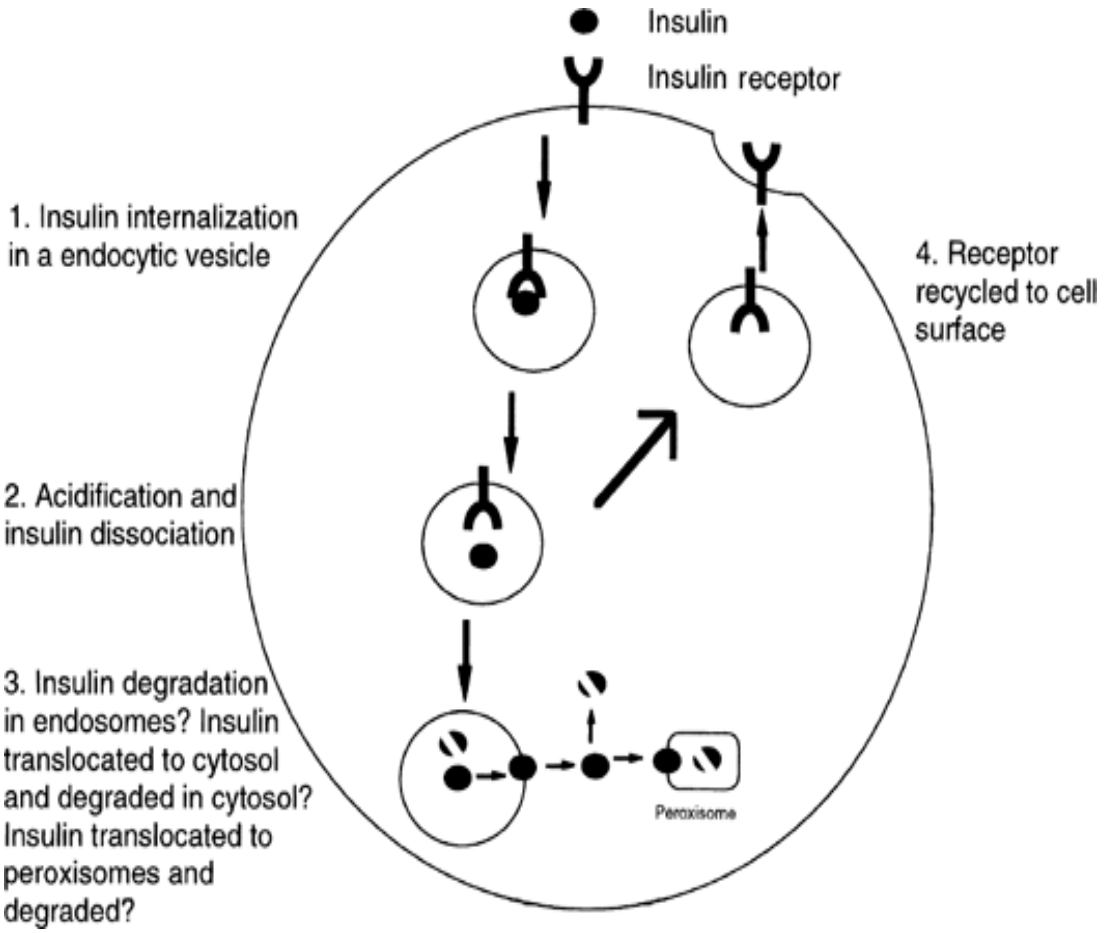
Under normal conditions, almost all insulin is uptake and degraded intracellularly or at least by membrane processes as mentioned below.

1.5.2. Cellular insulin uptake, processing and degradation

The uptake, processing, and degradation of insulin by cells are a complex process with multiple intracellular pathways (Figure 1.7).

The initial step in the action of insulin is binding of insulin to its specific receptor on the plasma membrane of cells. Receptor-bound insulin serves as a reservoir (Duckworth et al., 1998) that can return intact insulin to the blood or deliver it to another intracellular site.

After insulin binds to its receptor, the receptor–insulin complex is internalized in endocytotic vesicle. Internalized insulin can be processed through multiple pathways (Di Guglielmo et al., 1998), resulting in degradation or release from the cell intact (diacytosis or retroendocytosis). Intracellular pathways of insulin processing include degradation of insulin in endocytotic vesicles (Seabright & Smith, 1996) or delivery of intact insulin and degradation products to other subcellular sites. Locations of internalized insulin are cytosol, nucleus, Golgi, or other locations (Smith & Jarett, 1990; Khan et al., 1982). Insulin may be degraded in these sites or later transferred to the lysosomal system for degradation (Ward & Moss, 1985). Ultimately, most internalized insulin, partially degraded insulin, and insulin fragments localize to lysosomes for completion of degradation.



(Figure 1.7): A model for cellular uptake and degradation of insulin (Duckworth et al., 1998).

The biological role of this partially degraded insulin is unknown, although some degradative products of insulin retain receptor binding and biological activity (Duckworth, 1988).

Most receptor-bound insulin is internalized into endosomes where degradation is initiated. Internalization is a property of the insulin receptor (Duckworth et al., 1997). While the concept that different forms of the insulin receptor have different functions and effects on different pathways is attractive and probably true, it seems unlikely that the complexity of insulin-cell interaction and insulin action will be so easily defined.

It may well be that different mechanisms for insulin internalization result in different intracellular processing mechanisms. This area clearly deserves further study.

As the endosomes are acidified, the receptor-insulin complex dissociates, with most of the receptors being recycled to the cell surface and the insulin being degraded inside the cell (LeRoith et al., 2004). Endosomal degradation of insulin is initiated before acidification of the vesicles. Not all of the internalized insulin is degraded in endosomes. Endosomal degradation varies depending on insulin concentration, duration of exposure, and other factors (Seabright & Smith, 1996). Even with controlled conditions, in vitro, the maximum amount typically degraded does not exceed 50%. The remainder of the insulin is delivered to other subcellular compartments including cytosol, nucleus, and lysosomes (Harada et al., 1995). The mechanism whereby insulin reaches these compartments is unknown.

The most likely explanation for this discrepancy is that degradation occurring early after exposure of cells to insulin is nonlysosomal and mostly endosomal, whereas the hormone that escapes endosomal degradation, either intact or partially degraded, is ultimately delivered to lysosomes for complete metabolism. In this system, non-lysosomal degradation is the initial step and lysosomal degradation is the final step.

1.5.3. Enzymes for insulin metabolism

The enzymatic mechanisms for insulin metabolism have not been established, but three systems have been implicated: insulin protease, glutathione-insulin transhydrogenase (GITe), and lysosomal enzymes (Duckworth & Kitabchi, 1981).

Whereas the GITe has previously been suggested as first step in the degradation of insulin, with initial cleavage of the molecule, then A and B chain degradation by

nonspecific proteinases (Duckworth et al., 1988). While it is now clear that this hypothesis is incorrect in detail, the initial degradative step occurs in endosomes. The insulin protease makes two or more cleavages in the B chain. This is followed by reduction of the disulfide bonds by GITe, yielding an intact A chain and several B chain fragments The insulin fragments are then further cleaved probably by multiple proteolytic systems, including lysosomes. A number of careful researches have concluded that lysosomes play little or no role in cellular insulin metabolism.

Insulin protease, also known as insulinase, insulin degrading enzyme (IDE), cleaves the peptide hormone insulin and proceeds through a series of intermediates and that the initial cleavage of insulin is between residues 16 and 17 in the B chain (Duckworth et al., 1979), resulting in a molecule consisting of three peptide chains held together by disulfide bonds.

Most activity of IDE is found in cytosol, small amounts found in other subcellular fractions, including plasma membranes, endosomes, and peroxisomes (Duckworth et al., 1998).

Glutathione-insulin transhydrogenase (GITe) inactivates insulin by splitting the hormone at the disulfide bonds into A and B chains (Varandani, 1972). The enzyme carriers out this reaction by catalyzing sulfhydryl-disulfide interchange.

In previous studies about GIT, The locations of GITe were found on four different organs liver (Tomizawa & Varandani, 1965; Tomizawa, 1962) pancreas (Varandani & Tomizawa, 1966), kidney and heart (Varandani, 1972) of three different species (human, bovine and rat). After purification the GITe's have similar physical and enzymatic properties (Varandani & Nafz, 1970).

Varandani et al. 1974 demonstrated that "The changes in insulin degradation are due to adaptive changes in hepatic GITe level and the concentrations of GITe in rat liver are regulated by insulin through a feedback mechanism".

Further evidence provides that the GITe activity is the primary determination of the rate of hepatic insulin metabolism. It must be assumed, however, that insulinase and lysosomal enzymes are involved in the process of insulin degradation.

Bacitracin is commonly used in studies on insulin action. Bacitracin, as a cyclic polypeptide derived from *Bacillus Licheniformis*, is widely used as an antibiotic and as a proteinases inhibitor to inhibit the degradation of insulin and glucagon in studies on hormone-receptor binding and action (Juul & Jones, 1982). Bacitracin has been used in high concentration (0.6-1.2mM) to inhibit extracellular degradation in order to study the internalization and processing of insulin by hepatocytes (Carpentier et al., 1979).

Also in high concentration of Bacitracin, it was used to inhibit the ability of purified GITe to split insulin into its constituent A and B chains. Kinetic studies indicated that this inhibition was by a complex mechanism that decreased both the Vmax and affinity of the enzyme for insulin (Roth, 1981). In view of its wide usage at high concentrations in studies on insulin binding and action, the current study will examine the inhibitory effects of bacitracin on isolated rat hepatocytes at concentrations currently used in insulin studies.

1.6. Aim and scope

Successful oral delivery of insulin, can greatly improve the efficacy of treatment of diabetes mellitus. Some significant advances have been made in the recent past that has led to better ways of addressing the challenges of oral insulin delivery.

Use of nanoparticles-oily vehicle combination has been proposed as a promising alternative for oral insulin delivery. Nanoparticles dispersion systems effectively address some of the key challenges in insulin delivery. However, better understanding of how the insulin-loaded nanoparticle formulations function is desired for developing better ways to enhance oral bioavailability of insulin.

The overall goal of this work was to evaluate the first pass metabolism of oral insulin in normal and streptozotocin-intoxicated rats. The specific goals of this research are outlined below:

1) Preparation of insulin-loaded nanoparticles formula for oral delivery of insulin.
2) Pharmacological evaluation of oral insulin formula on diabetic and normal rats.
3) Investigation of the mechanisms of absorption and transport of insulin-loaded nanoparticles preparation across the intestine using the everted gut sac model and in situ intestinal perfusion technique.
4) Determination of the level of insulin, which is metabolized in liver using in situ liver perfusion technique and isolated hepatocytes culture on diabetic and normal rats.
5) Examination the inhibitory effect of bacitracin on insulin degradation using isolated hepatocytes model.

Chapter Two

Experimental part

2. Experimental Part

2.1. Chemicals and Instruments

2.1.1. Chemicals

High molecular weight chitosan (HMWC) (M.W.~250 kDa and DDA 99%, Xiamen Xing, Shanghai, China). Oleic acid was obtained from Merck, Germany. Surfactant, Labrasol® (PEG-8 caprylic/capric glycerides, which is a mixture of 30% mono-, di- and triglycerides of saturated C6-C14 fatty acids, 50% of mono- and di-fatty acid esters of polyethylene glycol (PEG 400), and 20% of free PEG 400, HLB 14), and cosurfactant, Plurol Oleique® (polyglyceryl-6 dioleate, HLB 6), were obtained from Gattefosse, France. Diacylglycerol (DAGs) from Merck, Germany. Recombinant human (rh) insulin powder (Batch No. EE-B10-10-000481/00563) was obtained from Biocon Limited, Bangalore, India. Streptozotocin (STZ) was purchased from Calbiochem, Germany. Tris (hydroxymethyl)-aminomethane was obtained from Merck, Germany and hydroxyl propyl ß-cyclo dextrin (HPßCD) from Merck, Germany. Bacitracin was purchased from Sigma–Aldrich, MO, USA. Chloroform was obtained from chloroform spirit BP, medex company, UK. All chemicals were used as received without further purification. Water was double distilled and deionized (measured conductance was less than 2 µS/cm).

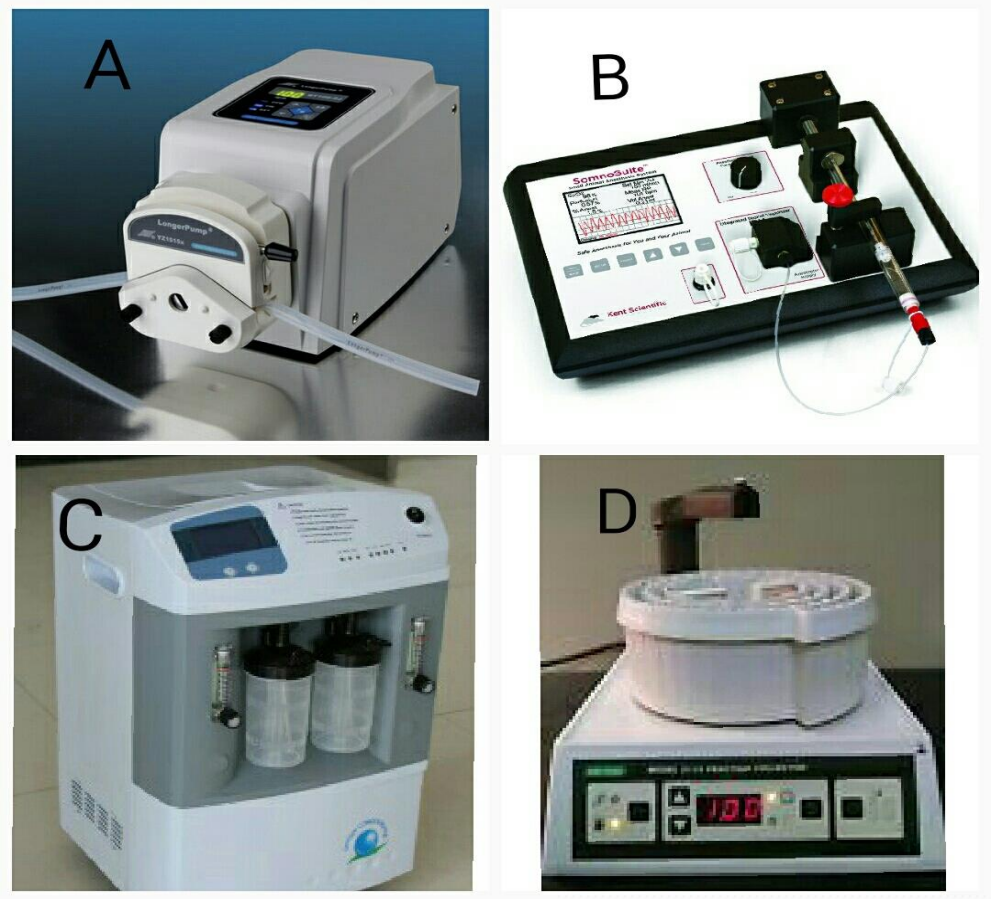
2.1.2. Instruments

The prepared polymers were dried using a Hetopower dry PL 9000 freeze dryer (Thermo Fisher Scientific-Inc, Waltham-MA, USA). Centrifugation was carried out using a Sorvall Super Speed RC2-B centrifuge (Ivan Sorvall-Inc, Norwalk-CT, USA). The pH of the solutions was modulated by using pH/Conductivity Meter (Model number MPC227, Mettler-Toledo International Inc). The Viscosities of samples were measured by using a Sine-wave Vibro-viscometer (Vibro-Viscometer SV-10/SV-100, A&D Company, Japan). The chitosan powder was identified by Nicolet Avatar 360 FT-IR ESP Spectrometer (Nicolet, USA). To agitate the solutions for speeding up reactions or improving mixtures a heating magnetic stirrer was used (ARE F20520162, VELP Scientific-Inc, Italy). Vortex mixers (ZX3 F202A0176, VELP Scientific-Inc, Italy) were used for mixing purpose. Particle size measurements were performed using a Malvern Zetasizer nanoparticles analyzer with 633nm red laser light (Model number ZEN 3600, Malvern nano series, Malvern, UK).

Insulin measurement was performed by insulin electrochemiluminescence immunoassay, using Elecsys® 2010 analyzer (Roche Diagnostics, Mannheim, Germany). While anti-insulin antibodies present in the serum of rats either insulin-treated diabetic rat, even when they are treated with biosynthetic human insulin, and may also be found in the serum of type 1 diabetic rats before insulin administration. These antibodies interfere in competitive and noncompetitive insulin immunoassays. Glucose level was measured by blood glucose meter (GluChec®, D Vision, Ltd, UK).

The *in situ* perfusion apparatus consist of the following components: incubation of Krebs-bicarbonate buffer bottle at 40 °C was performed using water bath (Memmert Company, Germany). The oxy-life oxygen concentrator (JAY-6, Longfian Scitech

Company, China) was used to oxygenate Krebs buffer with carbogen gas (95% O_2 + 5% CO_2) and provide compressed gas to anesthesia instrument. Anesthesia process for rats was carried out using a small animal anesthesia system (SomnoSuite, Kent Scientific Corporation, Torrington-CT, USA). The freshly prepared Krebs buffer was perfused into the liver using low flow rate peristaltic pump (Model number BT100-2J, Langer Instruments Corporation, USA). The outflow perfusate was collected by using fraction collector (Model number 2110, Bio Rad-Inc, California, USA). The surgery was performed using mouse surgical kit. Silicon tubes (part number: 2.4*0.66 and #16, Langer Instruments Corporation, USA) and connectors (Econo pump fitting kit, Bio Rad-Inc, California, USA) were used in connecting the apparatus.



(Figure 2.1): Some of *in situ* perfusion instruments, (A): Low flow rate peristaltic pump, (B): SomnoSuite™ Small Animal Anesthesia System, (C): Oxy-life oxygen concentrator, (D): Bio Rad 2110 Fraction Collector.

2.2. Methods

2.2.1. Preparation and characterization of low molecular weight chitosan (LMWC)

2.2.1.1. Depolymerization of high molecular weight chitosan (LMWC)

Ten grams of high molecular weight chitosan (the degree of deacetylation is 99%) was dissolved in 830 ml of 0.1M HCl. Then 170 ml of concentrated HCl was added to the solution. The solution was heated (100 °C) for different times under reflux to give different molecular weight. After cooling, 3L of ethanol (96%) was added. The precipitated chitosan was then separated from the liquid and washed thoroughly with ethanol until neutral pH was obtained. The chitosan was then freeze-dried for 48 h, weighted and stored at room temperature for further analysis (Elsayed et al., 2009).

2.2.1.2. Determination of viscosity average molecular weight (M.W.)

The average molecular weight of different chitosan samples was identified by viscometric measurements. The intrinsic viscosity was measured by a viscometer and the viscosity average molecular weights for each chitosan samples were calculated using Mark-Houwink equation.

$$[\eta] = k \cdot M^{a}$$

Where [η] is the intrinsic viscosity, M is the viscosity average molecular weight, while the used k and a values were 0.00058 and 0.69 based on previous study (Kasaai, 2007).

The viscometer underwent two-point calibration using purified water and 7.434 mPas standard (Viscosity Standard, Poulten Selfe and Lee, UK) at 25°C. For each molecular weight determination, 2.5 gm of the sample was dissolved in 50mL of water and series of dilution were prepared to give final concentrations l%, 2%, 3%, 5%, and 7% w/v. The viscosity of the solvent and the samples were measured at 25°C using the Sine-wave Vibro viscometer (Model: SV-10/SV-100). Each sample was measured duplicate and an average reading was used for the subsequent calculations. The relative and reduced viscosities were calculated and used in determining the viscosity average molecular weight (Qinna et al., 2015).

2.2.1.3. FT-IR Spectroscopy

Measurements have been performed in the transmission mode, with chitosan contained in potassium bromide (KBr) discs. Potassium bromide was mixed with chitosan in mass ratio 100:1 (200 mg KBr and 2 mg chitosan). KBr was placed in an oven at 300°C for 24 h before mixing. Substances were mixed in agate mortar and pressed to disc form using IR hydraulic press at a pressure of l0 tons for 20 seconds. Discs were dried for 24 hours at 50 °C in order to remove moisture. For every kind of chitosan, three discs were produced. The discs were placed in the diffused reflectance cell and the spectrum were recorded over a wave number that ranges from 400-4000 cm^{-1} at room temperature with accumulation of at least 10 seconds and OPD velocity of 2 cm^{-1} using Fourier transform infrared (FTIR) spectroscopy connected to Omnic software. Duplicate lR measurements were made for each sample (Qinna et al., 2015).

2.2.1.4. Differential Scanning Calorimetry (DSC)

DSC thermogram of all chitosan samples were recorded on differential scanning calorimeter (AT-50WS, Shimadzu Company, Japan). About 5–10 mg of each sample were placed in a pinholed aluminum sample pan with lid and heated in atmospheric air at a rate of 10 °C/min between 30 and 350 °C, under nitrogen purge of 20 ml/min. The instrument was periodically calibrated with a standard sample of indium (m.p. 156.66 °C) (Qinna et al., 2015).

2.2.2. Formulation section

Based on formulation technique used in (Elsayed et al., 2009), the current study will formulate the formula (F1) that used in (Elsayed et al., 2009) and also will add new two materials to the old formula (F1) to yield the new formula (F4). Moreover, formulation the old formula with DAG only (F2) to make a reduction in the particle size. In addition, preparation the old formula with HPßCD only (F3) to maximum benefits protection for the formula (Table 2.1).

(Table 2.1): Composition of different preparation of chitosan-insulin PEC particles dispersed in oily system

Name of formula	Composition
F1	**Oily phase:** Oleic acid 80%, Labrasol 10%, Plurol 10% **Aqueous phase:** 25 mg/ml insulin sol 50%, 25 mg/ml chitosan sol 50%. **(2% loading)**
F2	**Oily phase:** Oleic acid 79%, Labrasol 10%, Plurol 10%, DAG 1% **Aqueous phase:** 25 mg/ml insulin sol 50%, 25 mg/ml chitosan sol 50%. **(2% loading)**
F3	**Oily phase:** Oleic acid 80%, Labrasol 10%, Plurol 10% **Aqueous phase:** 25 mg/ml insulin sol (with HPβCD) 50%, 25 mg/ml chitosan sol 50%. **(2% loading)**
F4	**Oily phase:** Oleic acid 79%, Labrasol 10%, Plurol 10%, DAG 1% **Aqueous phase:** 25 mg/ml insulin sol (with HPβCD) 50%, 25 mg/ml chitosan sol 50%. **(2% loading)**

2.2.2.1. Preparation of insulin-chitosan polyelectrolyte complex (PEC) aqueous phase

The aqueous phase was composed of chitosan and insulin mixture in solution state. The preparation of the aqueous phase was carried out in two stages. The first stage represented the preparation of chitosan solution while the second stage involved preparation of insulin solution.

In order to prepare the chitosan solution, in clean glass vial, the low molecular chitosan (13KDa and 99% DAA) in weight of 125 mg was dissolved in 4 ml deionized water. By using pH meter the pH of the solution was adjusted to 5.5 using 0.2 M of NaOH added drop by drop under stirring using magnetic stirrer and the final volume should be completed to 5 ml using water.

Conversely, the insulin solution was prepared by weighting 25 mg of recombined insulin (rh-insulin) in another clean vial. The insulin powder was dissolved in 250 µl of 0.1 M HCL under gentle hand shaking then 750 µl of 1 M modified Tris (hydroxymethyl)-aminomethane buffer pH 6.95.

The modified Tris buffer was composed of 400 mg of hydroxyl propyl ß-cyclo dextrin (HPßCD) dissolved in 10 ml of 1 M tris buffer.

The polyelectrolyte complex solution was constructed by the addition of 1 ml insulin solution to 1 ml chitosan solution drop wisely in new vial. The equal volumes of the chitosan and insulin were shaked gently for 15 minutes at room temperature.

2.2.2.2. Preparation of the oily phase

The oily phase was composed of oleic acid, Labrasol® (PEG 8 caprylic/capric glycerides) and Plurol Oleique® (polyglyceryl-6-dioleate) as Surfactant/Cosurfactant (SCOS), and diacylglycerol (DAGs).

A mixture of 4 gm of Labrasol® - Plurol Oleique® at (1:1) ratio, 0.2 gm of diacylglycerol and 15.8 gm of Oleic acid was accurately weighed into screw-capped glass tube. The compounds were vortexed at 40 HTZ by using vortex mixer for 30 seconds.

2.2.2.3. Preparation of the insulin-loaded nanoparticles dispersion system

The aqueous phase and an oily phase were mixed together to prepare the nanoparticle dispersion system. By using micropipette, the aqueous phase (400 µl) was added to 20 gm of oily phase then vortexed for 30 seconds. The insulin-loaded nanoparticles formula was held for 15 minutes to equilibrate before measuring their particle size.

2.2.2.4. Characterization of the insulin-loaded nanoparticles dispersion system

The particle size distribution of the nanoparticles prepared with the surfactant system was assessed by photon correlation spectroscopy, using a Malvern Zetasizer Nano-ZS series (Malvern Instruments, UK). Collective 13 readings were performed three times on a sample at 25°C with detection angle of 173°. The instrument built-in software calculated the average and the standard deviation of particle size measurements for each percent of loading.

2.3. Preclinical studies

2.3.1. Animals

Adult male Sprague-Dawley laboratory rats weighing around 200-250 gm were supplied from University of Petra animal house. The animals were kept in air-conditioned environment with temperature of (20-25 °C) and 55%-65% relative humidity. A 12/12 h light and dark schedule was maintained in the animal house and animals were acclimatized to the laboratory conditions.

The experimental protocols were carried out in accordance with the Code of Ethics of the World Medical Association.

2.3.2. Induction of diabetes in rats using streptozotocin (STZ)

Rats were fasted for 12 h before diabetes was induced. The animals were injected by STZ at the dose of 100 mg/kg of the body weight intraperitoneal. STZ induces diabetes within 2 days by destroying the beta cells. STZ was freshly dissolved in 0.1 M citrate buffer (pH 4.5). Diabetes was monitored by measuring glucose concentration in blood samples obtained from the tail vein using a blood glucose meter. Only rats with a basal blood glucose level around (220 mg/dL) were considered as diabetic for our studies.

Diabetic animals and normal control groups were kept in metabolic cages individually, separately, under feeding and metabolism control.

2.3.3. Preparation of Krebs- bicarbonate buffer

A simulated physiological solution, Krebs-bicarbonate buffer was prepared by the mixing the following volume (in ml) of 1 M: NaCl 118, KCl 4.5, $MgSO_4$ 1.6, $NaHCO_3$ 25, KH_2PO_4 1.2, Glucose 5.5 and $CaCL_2$ 2.5, complete the volume to 1 L by distilled water. The buffer was oxygenated with carbon gas (95% O_2 + 5% CO_2). However, $CaCl_2$ was added after oxygenation to prevent $CaCO_3$ formation. The pH was adjusted to 7.2 using 1M NaOH.

2.3.4. Evaluation of pharmacological activity of insulin-loaded nanoparticle preparation

STZ-diabetic and normal rats were used to evaluate the hypoglycemic effects of, freshly formulated, oral insulin-loaded nanoparticles. The rats were considered to be diabetic when the fasted glucose levels exceeded 220 mg/dL after STZ treatment. Rats were fasted for 12 h prior to the experiment with free access to water. The total duration of the experiment after dosing was 18 h. The animals were fasted from food, but had free access to water during the first 9 h, while in the second 9 h the animals had free access to both water and food. Diabetic rats were separated randomly and divided into three groups for the antidiabetic study. Also the normal rats were separated into two groups.

Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**
(Diabetic rats n= 10 rats)	**(Diabetic rats n=10 rats)**	**(Diabetic rats n=10 rats)**	**(Normal rats n= 7 rats)**	**(Normal rats n= 7 rats)**
Oral insulin in a dose (50 IU/Kg)	**SC insulin in a dose (1 IU/Kg)**	**Oily placebo**	**Oral insulin in a dose (50 IU/Kg)**	**SC insulin in a (dose 1 IU/Kg)**

The different groups were received as follows, group 1 (diabetic rats) was received oral insulin formula (F.C. = 7 IU/ml) using gavage needle. Group 2 (diabetic) was injected subcutaneously Rh-insulin solution (F.C. = 1 IU/ml). Group 3 (diabetic) was received intragastrically oral placebo preparation (nanoparticles dispersion without insulin) and used as control. Group 4 (normal rats) was received oral insulin formula (F.C. = 7 IU/ml). Group 5 (normal) was injected subcutaneously with Rh-insulin solution (F.C. = 1 IU/ml).

Blood samples were collected from the tail veins of rats prior to drug administration and at different time intervals (0, 1, 2, 3, 4, 6, 8, 10 and 18 h) and blood glucose level time profiles were constructed after dose administration using blood glucose level at zero time interval as a base line to glucose level in the later blood samples. The blood glucose was then determined by a glucose meter.

2.3.5. Measurement the intestinal absorption of insulin-loaded nanoparticles by everted gut sac model

Rats were sacrificed by chloroform overdose sprinkled to a piece of cotton wool in a glass container equipped with a lid. The abdomen was opened by a midline incision; the whole of the small intestine was then removed by cutting across the upper end of the duodenum and the lower end of the ileum. The small intestine was then washed with normal saline solution (0.9% w/v NaCl) using a syringe equipped with blunt end.

The isolated small intestine was cut into 2 pieces each about 15 cm; approximate diameter of intestine was 0.8 cm. To evert the gut, a stainless steel rod (300 mm long, 1-5 mm diameter) was used to push the ileal end of the gut, into the gut lumen until it

appeared at the duodenal opening of the intestine, and the eversion was completed by rolling the proximal half of the intestine on the rod. The everted intestine was then slipped off the steel rod and placed in saline solution at room temperature in a flat petri dish.

After eversion, the mucosal side came out and serosal side is present inside.

The everted intestine was filled with the oxygenated Krebs buffer solution and the other end was tied off. Then the filled everted intestine was divided into sacs approximately 2-4cm in length (Figure 2.2). The sacs were placed in beaker with Krebs buffer inside water bath.

(Figure 2.2): The eversion of small intestine on a stainless steel rod then filling the everted intestine with the oxygenated Krebs buffer solution. Moreover the filled everted intestine was divided into sacs.

The beaker was oxygenated with 95% O2 and 5% CO2 for 1 min. The sacs were then transferred into two beakers on water bath constant temperature oscillator (SHA-B, Everich Corporation, Miami, USA) at 37°C. The first beaker contains the oral insulin formula (F.C. = 7 IU/ml) and the other beaker contains Rh-insulin solution (F.C. = 1 IU/ml). The sampling can be done at different time intervals to measure insulin levels.

Equal number of sacs was removed from the two beakers at 20, 40 and 60 min. These sacs were emptied and the serosal fluid from the sacs was used for the analysis and measurement of insulin level by Elecsys 2010 analyzer.

Everted gut sac experiment was performed on normal and diabetic rats.

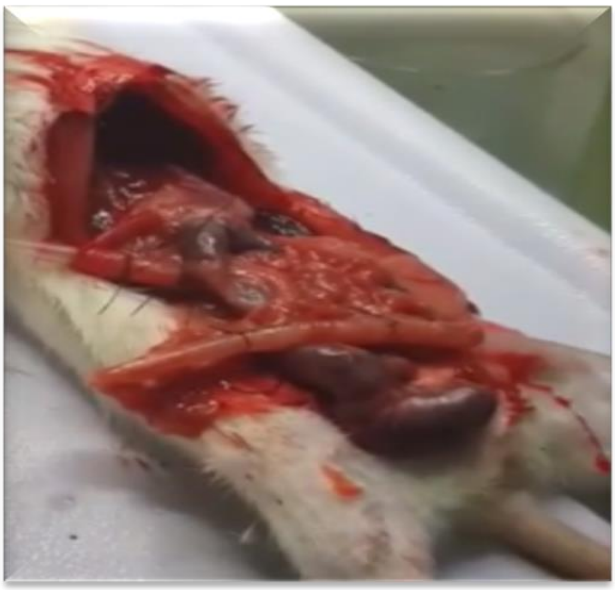
2.3.6. Evaluation the intestinal absorption of insulin-loaded nanoparticles by *in situ* intestinal perfusion technique

After an overnight fasting, normal and diabetic male Sprague-Dawley rats were anesthetized with 2ml halothane using a small animal anesthesia system, then placed on a board under a surgical lamp to maintain body temperature. Upon verification of the loss of pain reflex, a midline abdomen incision of 3-4 cm was made and the small intestine was exposed. The intestine (length 80 ± 5 cm) was isolated and cannulated at both ends with silicon tubing (internal diameter 2 mm). Both cannulas were secured with surgical silk sutures (Figure 2.3). The isolated intestine was perfused with physiological saline (0.9% w/v NaCl) to clean any residual debris until the out flowing became colorless and drops of saline were added to the surgical area then covered with wet gauze to avoid loose of fluid. Care was taken to handle the small intestine gently in order to maintain an intact blood supply. The experiment was

initiated by measuring blood glucose from the tail at zero time of insulin formula pumping. After that, the insulin perfusion preparation was perfused through the isolated intestine; the circulation rate was 3.5 ml/min, controlled by a peristaltic pump to pre-balance. Blood glucose was measured from tail vein by glucometer at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75 and 90 min.

To evaluate the intestinal uptake of insulin and compare the absorbed insulin level between the oral insulin formula (7 IU/ml) and Rh-insulin solution (1 IU/ml), *in situ* intestinal perfusion of insulin solution was performed on normal and diabetic rats. Also blood glucose concentrations were measured at the same time intervals.

The efficacy of the enteral route of insulin administration relative to subcutaneous (s.c.) was estimated. Briefly, insulin solution was administered subcutaneously. The insulin s.c. dose was 250 µl (1 IU/ml). Thus, placebo formula was perfused and the same conditions were performed on normal and diabetic rats in the S.C. experiments (Matsuzawa et al., 1995).

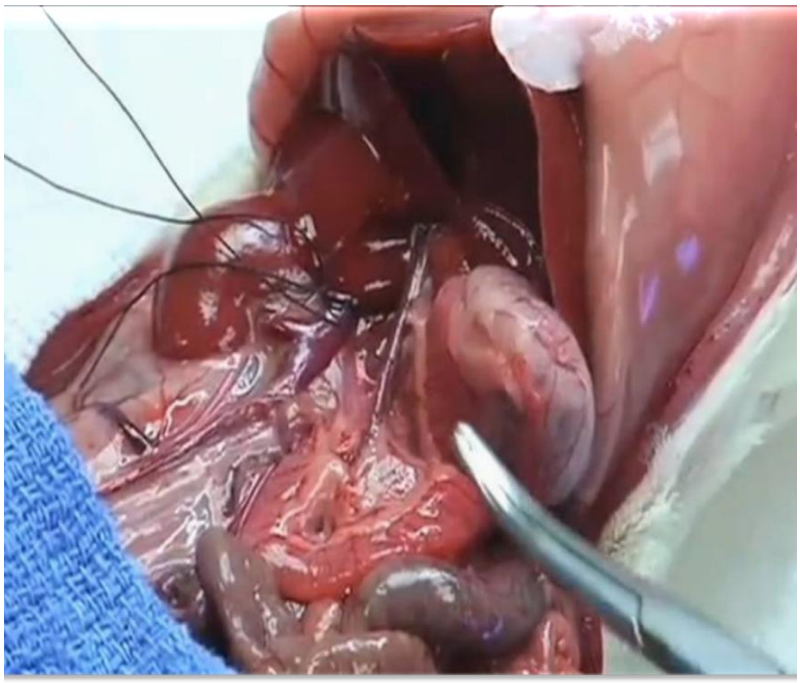


(Figure 2.3): Cannulation of exposed intestine at both ends then the isolated intestine was perfused in circulating system.

2.3.7. Evaluation of hepatic first pass metabolism of insulin by *In situ* liver perfusion method

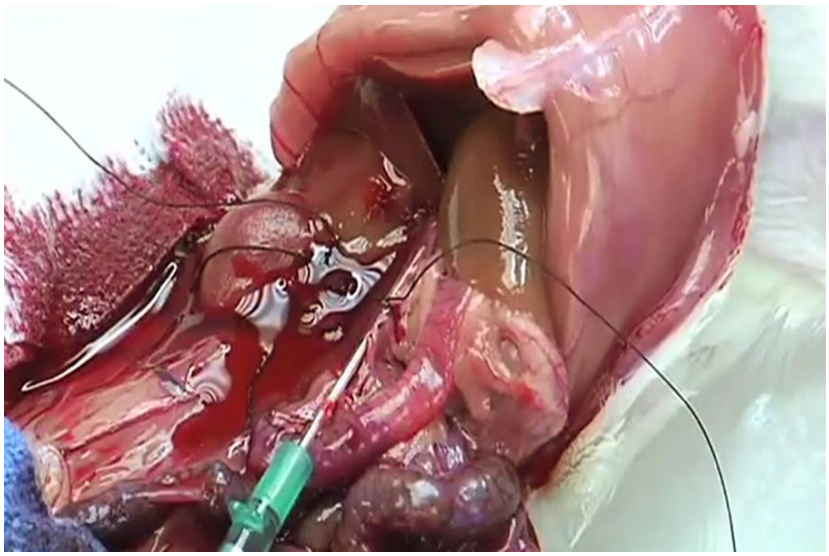
Male Sprague-Dawley rats weighing 200-250 gm were fasted for 24 h before use. *In situ* liver perfusion experiment was conducted on normal and diabetic rats. The apparatus of perfusion were as previously described. Rats were anesthetized with 2ml halothane using a small animal anesthesia system; the rats were restrained in a supine position on aboard which was kept at surface temperature of 37 °C.

A small midline incision was made in the abdomen. The hepatic portal vein was exposed by carefully moving the abdominal contents to the animal's right. Loose ligatures will be placed around the portal vein ensuring exclusion of the hepatic artery and the second ligature around the inferior vena cava (IVC) beside the left kidney (Figure 2.4). The portal vein will be cannulated with 16 GA catheter and perfusion start at a flow rate of 13 ml/min, using peristaltic pump.



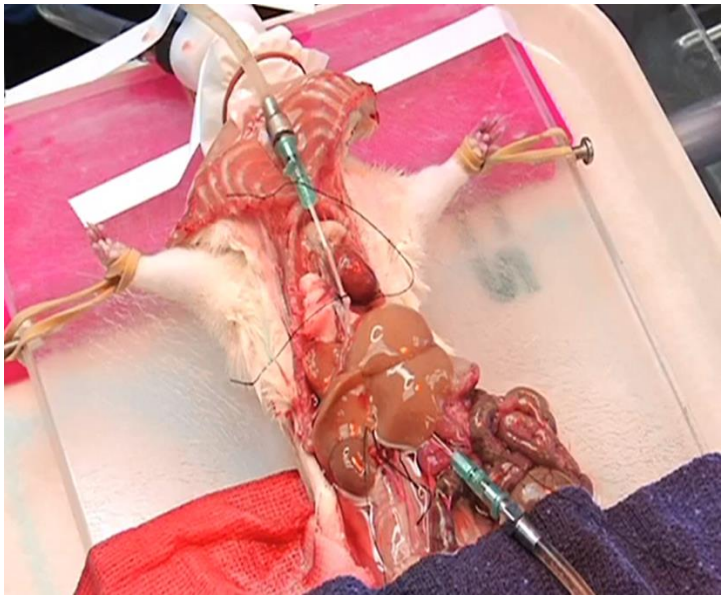
(Figure 2.4): Ligation of the portal vein and the inferior vena cava (IVC) beside the left kidney.

The liver were initially perfused with Krebs- bicarbonate buffer (pH=7.2) for 10 min. When the lobes of liver begin swelling, a cut was made at lower IVC (Figure 2.5).



(Figure 2.5): Cannulation the portal vein with 16 GA catheter and perfusion with Krebs buffer, then cut the lower inferior vena cava.

Then exsanguination of the liver will be facilitated by inserting tubing into the IVC through the right atrium and the ligature around the IVC was tied very well to avoid leakage (Figure 2.6).



(Figure 2.6): Exsanguination of the liver will be facilitated by inserting tubing into the IVC through the right atrium.

150 µl of Rh-insulin solution (F.C. = 2 IU/ml) was injected as a single dose through the perfusion buffer. The total effluent from the liver will be collected by fraction collector at 5 sec intervals, the sample collecting will start at the same time of insulin dose injection. Then samples will be frozen and prepared for measuring the insulin levels and its hypoglycemic effects (Sahin & Rowland, 2000).

2.3.8. Evaluation the effect of different flow rates on insulin metabolism in liver

In order to study the effect of using different flow rates on insulin metabolism, *in situ* liver perfusion technique on normal and diabetic livers was performed. The experiment was carried out with two different flow rates, namely 5 ml/min and 1 ml/min on the same liver. The surgical procedure was the same as that described previously (section 2.3.7.). Briefly, after induction of anesthesia, cannulation and perfusion Krebs-bicarbonate buffer (pH=7.2) for 10 min, the liver was perfused in a single-pass mode with the first 25 ml of Rh-insulin solution (80 mU/ml) at 5 ml/min flow rate. The total outflow was collected via tubing in the first bottle. After washing the liver with perfusion Krebs-bicarbonate buffer for 10 min, the second perfusion of 25 ml insulin solution (80 mU/ml) was started at a flow rate of 1 ml/min. The effluent was pooled in the second bottle to freeze-dry. All operative procedures were completed within 40 min without interruption of flow to the liver.

To obtain a pharmacological action of outflow perfused insulin, the two bottles were freeze-dried and stored at 4°C until used. The freeze-dried insulin powder from each bottle was dissolved in 2 ml distilled water and subcutaneously injected to number of normal rats (n=8). Blood glucose level was measured from tail vein by glucometer at 0, 15, 30, 60, 90 and 120 min.

2.3.9. Isolation and culture of normal and diabetic rat hepatocytes followed by determination the capacity of insulin metabolism in liver

2.3.9.1. Preparation

All buffers are freshly prepared using sterile technique.

1- Prepare Perfusion buffer I by adding the following to Hank's Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}): Mg^{2+} ($MgCl_2$) to 0.9 mM, EDTA to 0.5 mM, and Tris base to 0.5 mM.

2- Prepare Perfusion buffer II by adding to HBSS (with Ca^{2+} and Mg^{2+}): Tris base to 0.5 mM.

3- Prepare Perfusion buffer II plus collagenaseII: Dissolve collagenase II (1000 U) with 300 ml Perfusion buffer II and keep the solution warm in water-bath before perfusion. This solution should be used within 30 min because the activity of collagenase II decreased with the time.

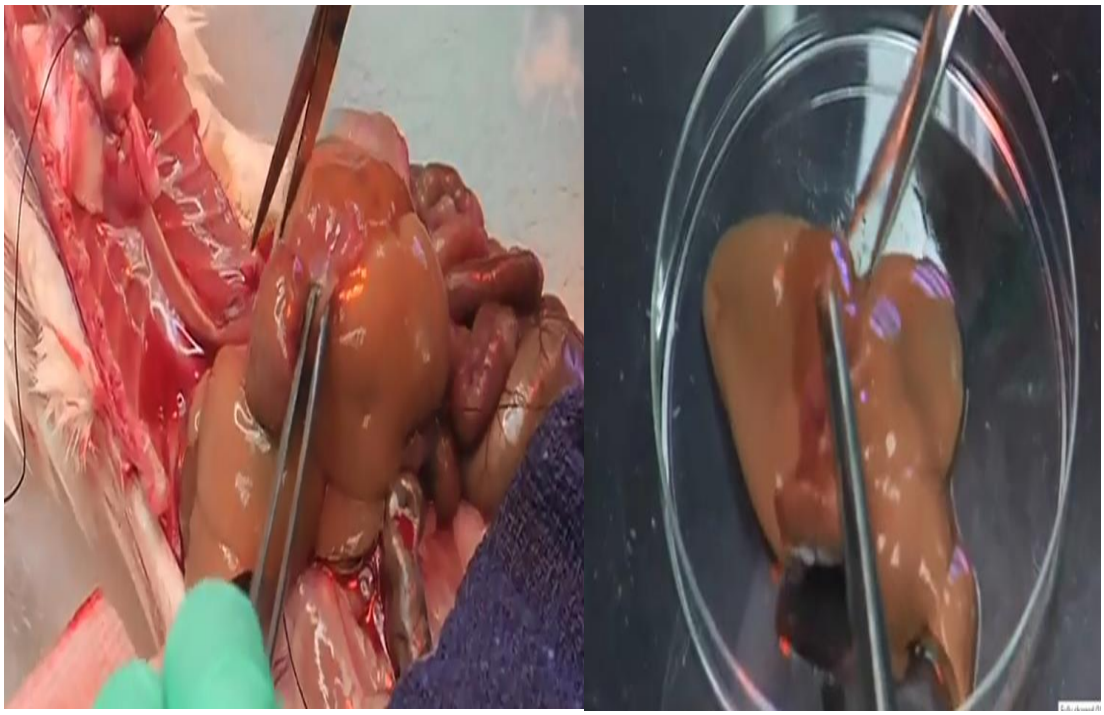
4- Prepare William's complete Medium: Add the following to Williams' Medium E: L-glutamine to 2 mM, fetal bovine serum (FBS); dexamethasone to 100 nM, penicillin to 100 IU/ml and streptomycin to 100 mg/ml.

5- These buffers should be warmed for 30 minutes in the water bath at 42 °C, an optimal temperature corresponding to an outlet temperature at the cannula of 37 °C.

2.3.9.2. Rat Perfusion for Liver Isolation

In situ liver perfusion technique as described above. After anesthesia of adult normal and diabetic rats and the surgery was performed. The perfusate tubing was connected

to the portal vein cannula and initiated the infusion in situ at a low flow rate (10 ml/min) with pre-warmed (37 °C) perfusion Buffer I. The liver should be instantly begun to blanch. Once successful cannulation was confirmed, a cut was made at IVC to allow efflux. The chest of the animal was cut. A second cannula connected to a soft tube was placed into the vena cava above the liver in order to enable a recirculating system; the cannula was fixed with a clamp.The perfusion solution was switched to Perfusion buffer II plus collagenase II, increase the rate of flow to 25 ml/min. The liver was become pale in color. A non-recirculating mode was started until the perfusion system was completely filled with collagenase solution then a recirculating perfusion mode was changed with collagenase solution for 15 minutes. After collagenase perfusion, liver was begun to look mushy. The liver was dissected free and placed in a pre-chilled sterile beaker with 20 ml collagenase solution then was taken to tissue cell culture hood (Figure 2.7).



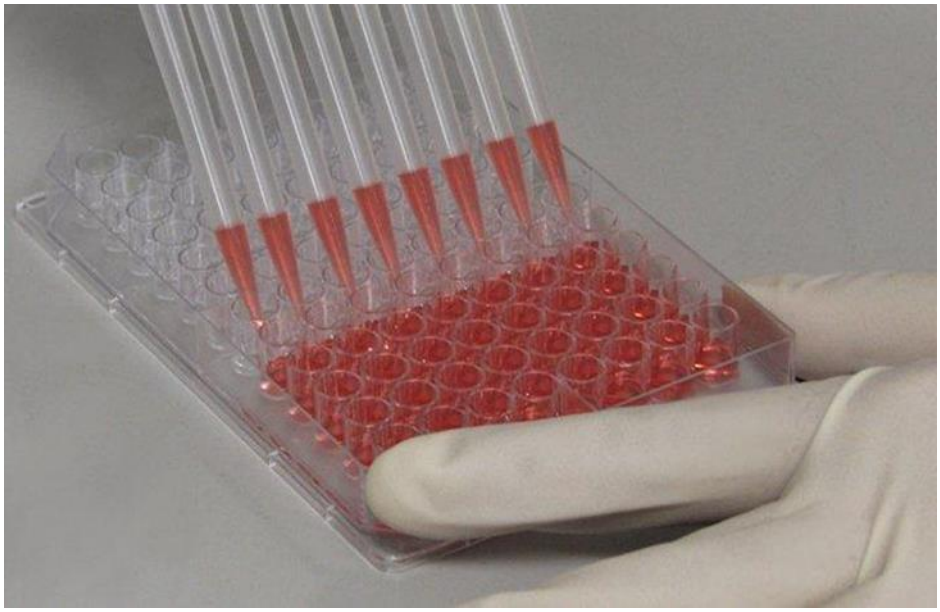
(Figure 2.7): Dissociation of the liver and placing it into sterile Petri dish with collagenase solution.

2.3.9.3. Hepatocyte Cell Isolation

Within the cell culture hood, a cell scraper was used to gently disperse the cells into collagenase solution within a sterile Petri dish. The cell suspension was filtered through gauze into a second Petri dish, the suspension was dispensed into centrifugation tubes (50 ml tubes) in order to remove connective tissues and undigested tissue fragments. The cells were suspended in 40 ml collagenase solution and centrifuged at 100 x g for 3 min at 4 °C. The supernatant was aspirated, and gently cells were re-suspended in 40 ml cold William's complete Medium to wash cells then centrifugation was repeated. For the third time, the supernatant was aspirated and the cells were re-suspended with 40 ml William's complete Medium and centrifuged at 200 x g for 10 min at 4 °C. The cells were counted within the cell suspension using a hemocytometer.

2.3.9.4. Hepatocyte Culture

The cells were diluted with warm William's complete Medium to preferred concentration and plated at a desired volume on cell culture plates (96-wells) (Figure 2.8). The hepatocytes were cultured at 37 °C in a humidified atmosphere of 95% air and 5 % CO_2. The cells were recovered and grown at least overnight prior to experimentation then the cells were used for test within 24 h because this may help preserve the function of critical enzymes (Shen et al., 2012).



(Figure 2.8): The diluted cells were plated at a desired volume on cell culture plates (96-wells).

2.3.9.5. Cell-based insulin degradation assay

Normal and diabetic rat hepatocytes were grown to subconfluence on a 69-well dish. One well should be a cell-free control in each study. The appropriate amount of human insulin (100 nM, 200 nM) was applied to the cell cultures. 100 µl of medium was collected after 15, 30 and 60 min of incubation and the insulin concentration was measured by Elecsys 2010 analyzer.

2.3.9.6. The inhibitory effect of bacitracin on cell-mediated insulin degradation in isolated hepatocytes

Normal and diabetic rat hepatic cells were grown to subconfluence on a 69-well plate. One well should be a cell-free control in each study. Each of concentrations of bacitracin (300 µM, 1 mM) was applied for plate of 100 nM human insulin. Similarly, each of the same amounts of bacitracin was applied on 200 nM insulin. 100 µl of

medium will be collected after 15, 30 and 60 min of incubation and the insulin level was analyzed by Elecsys 2010 analyzer.

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) followed by the Tukey's post-test was used to analyze the differences between groups and differences between time intervals amongst groups using SPSS 17 statistical package, USA. A probability value <0.05 was considered the minimum level of statistical significance. The average particle sizes (in nM) are expressed as means ± SD (standard deviation) while insulin levels are expressed as means ± SEM (standard error of means). Also blood glucose levels are presented as a percentage of control and expressed as means ± SEM (standard error of means).

Chapter Three

Results

3. Results

3.1. Characterization of low molecular weight chitosan (LMWC)

3.1.1. Determination of viscosity average molecular weight (M.W.)

The average of duplicate viscosity measurements was used for the calculation of the inherent and reduced viscosities for each sample. The produced grades of chitosan had differed in molecular weight with same degree of deacetylation under the same depolymerization condition (molarity of acid HCI = 0.1M, temperature= 100 °C, pH>1, stirring strength = 750 r.p.m.) except the duration of reaction as show in the following table:

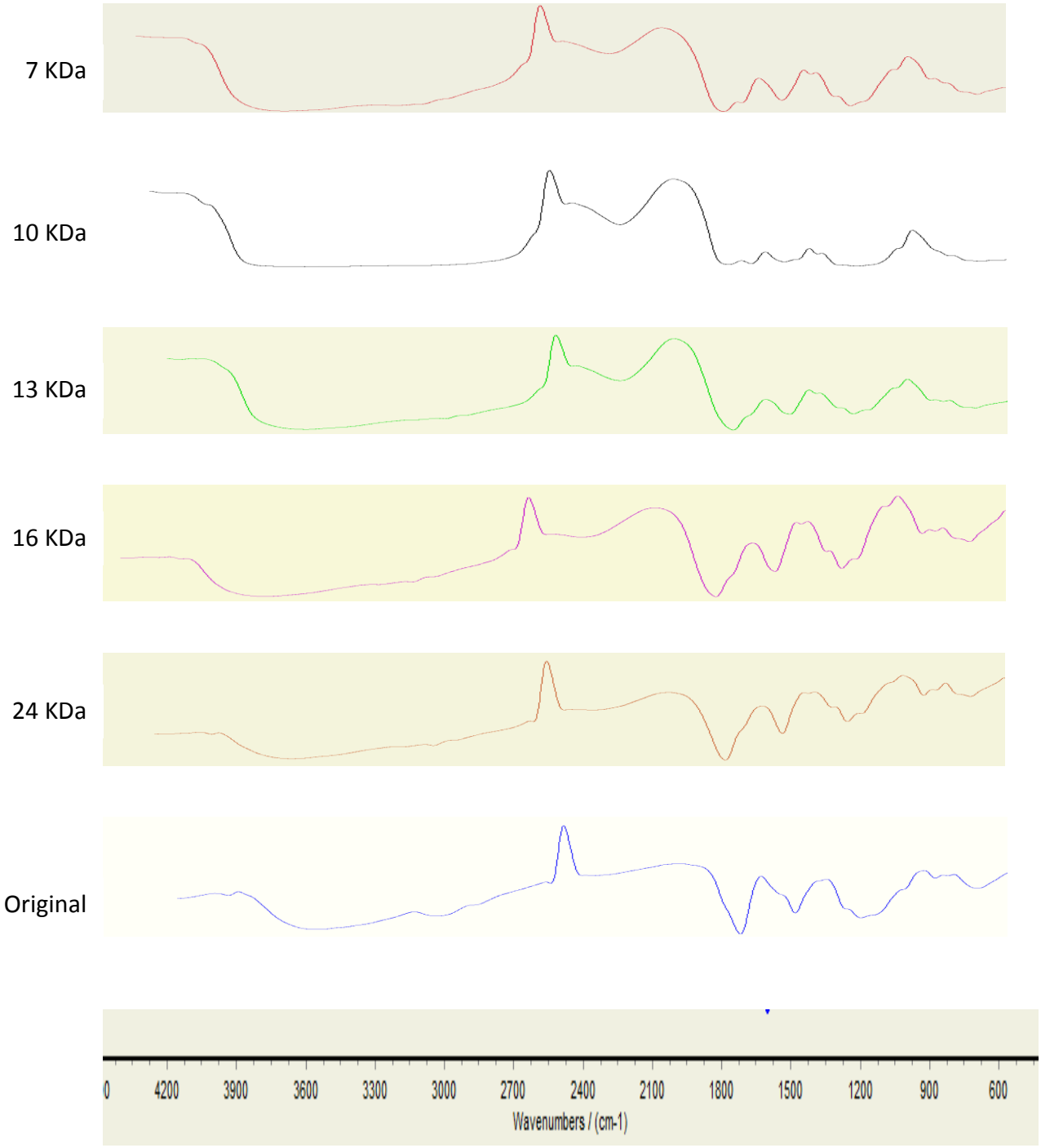
(Table 3.1): The average molecular weight of chitosan produced by acid hydrolysis within different time intervals, calculated using Mark-Houwink Equation.

Time of depolymerization reaction (Hours)	Molecular weights determined (KDa.)
3.5	24
5	16
5.10	13
5.5	10
6	7

From (Table 3.1), it can be seen that the molecular weight of chitosan grades decrease as the time of depolymerization reaction increase.

3.1.2 FT-IR Spectroscopy

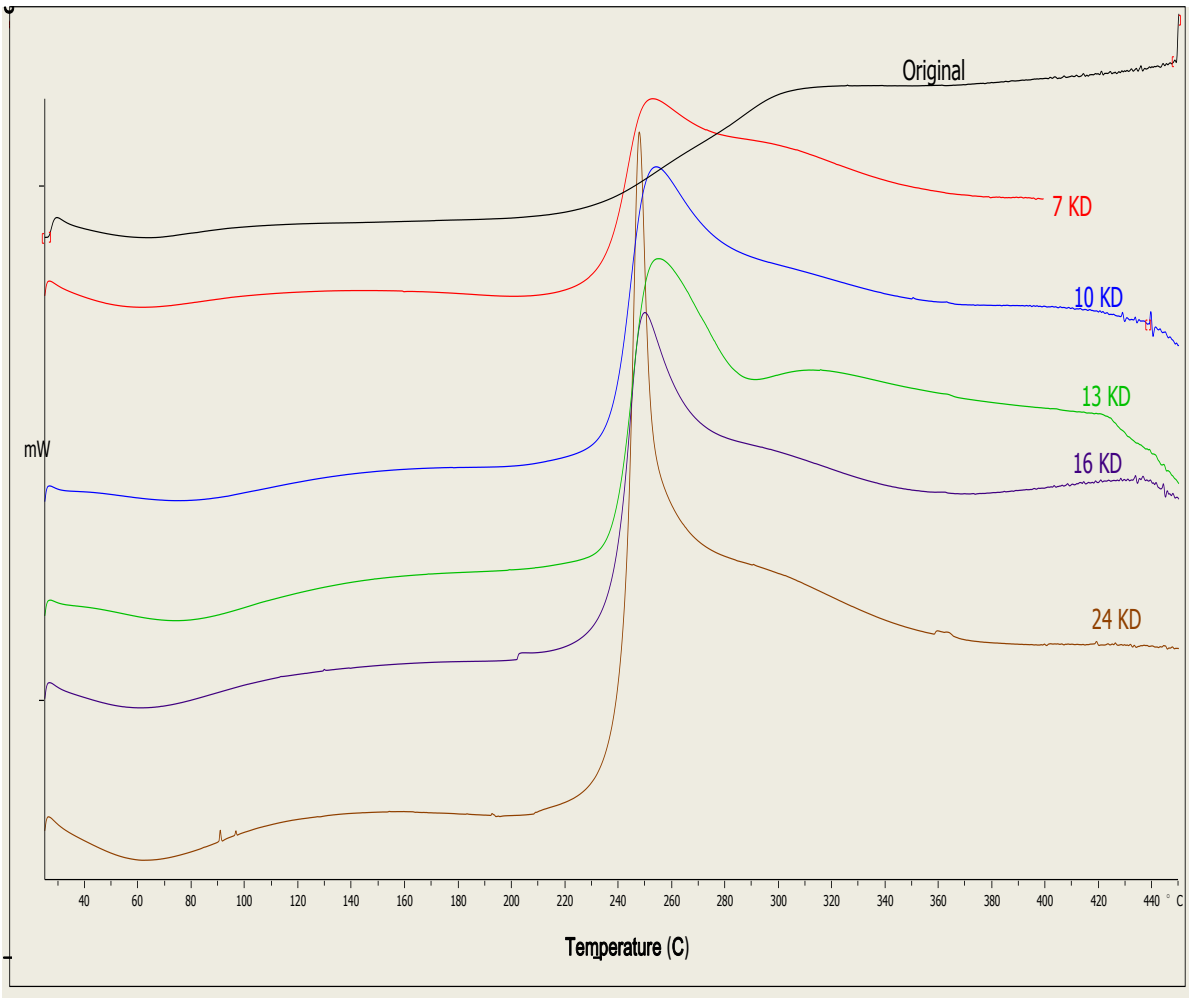
FT-IR spectra of different chitosan grades are shown in (Figure 3.1). All different grades of chitosan showed the same absorption peak 1570 cm^{-1}. The spectral data indicates the absence of carbonyl (C=O –NHR) at 1665 cm^{-1}.



(Figure 3.1): IR spectra for different grades of fully deacetylated chitosan over the frequency range 4000-400 cm^{-1}.

3.1.3. Differential Scanning Calorimetry (DSC)

The analysis of the DSC thermogram for different molecular weight grades of fully deacetylated chitosan showed strong exothermic peak around 240 °C to 260 °C (see Figure 3.2). The exothermic peak at 240-260 °C may be related to the thermal decomposition of chitosan.



(Figure 3.2): DSC thermogram for different grades of fully deacetylated chitosan.

3.2. Characterization of the insulin-loaded nanoparticles dispersion system

3.2.1. Particle Size Determination

Particle size distribution of different insulin-loaded nanoparticles preparations is presented in (Table 3.2). The average diameters of F4 were 85 ± 2.53 nm, while F1 was 108 ± 9 nm.

The addition of the new two materials to the old formula that used in previous work (Elsayed et al., 2009), F2 give 64.5 ± 2.07 nm, which make a reduction in the particle size while F3 give the largest particle size 280 ± 8.51 nm and maximize benefit protection for the formula.

(Table 3.2): Particle size distribution of different preparation of chitosan-insulin PEC particles dispersed in oily system (expressed as mean ± SD).

Formula	Particle size (nm)
F1	108 ± 9
F2	64.5 ± 2.07
F3	280 ± 8.51
F4	85 ± 2.53

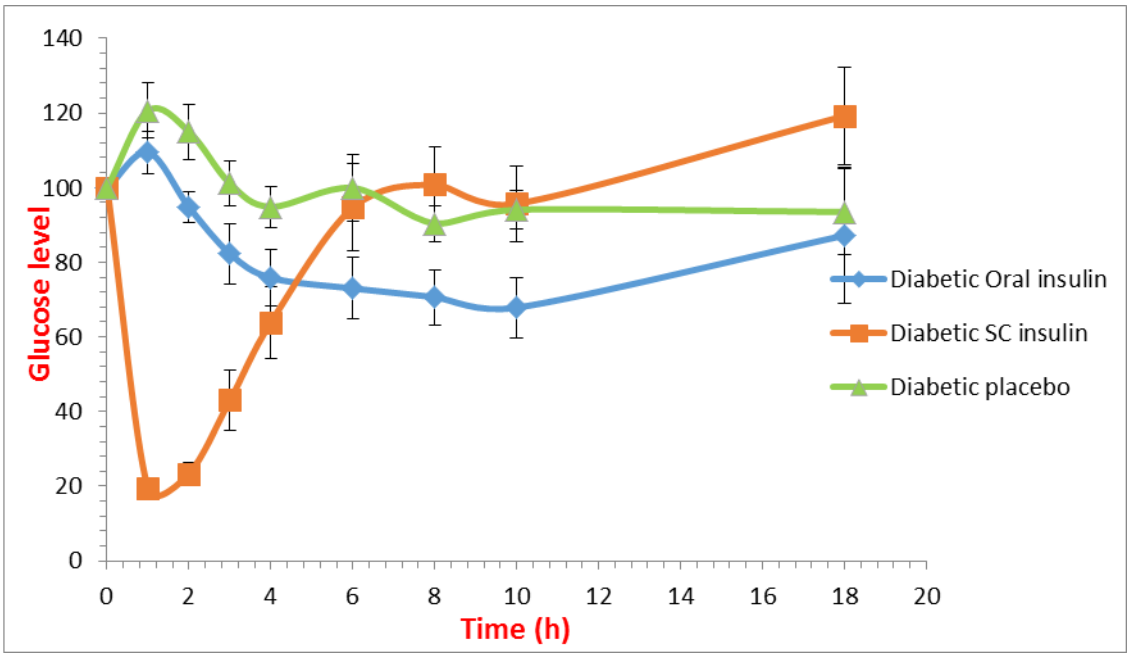
3.3. Evaluation of pharmacological activity of insulin-loaded nanoparticle preparation

The percentage base level blood glucose change of diabetic rats after administration of 50 IU/Kg of insulin-loaded nanoparticles orally showed a decrease in blood glucose of experimental animals to a significant level starting from 2 h reaching a peak change of 68% at 10-hour interval and afterwards blood glucose levels increased. The subcutaneous insulin (1 IU/Kg) on diabetic rats showed an expected effect on blood glucose concentration appeared from the first hour and its action ended after 6 hours. At 1,2,3 h SC insulin significantly dropped down ($p<0.001$). The percentage base level blood glucose change of diabetic rats after administration of placebo nanoparticles showed increment within the first 2 hours of the experiment due to the stress that the rats exposed to it during the placebo administration, and this increment came back to the original level after that. (Figure 3.3)

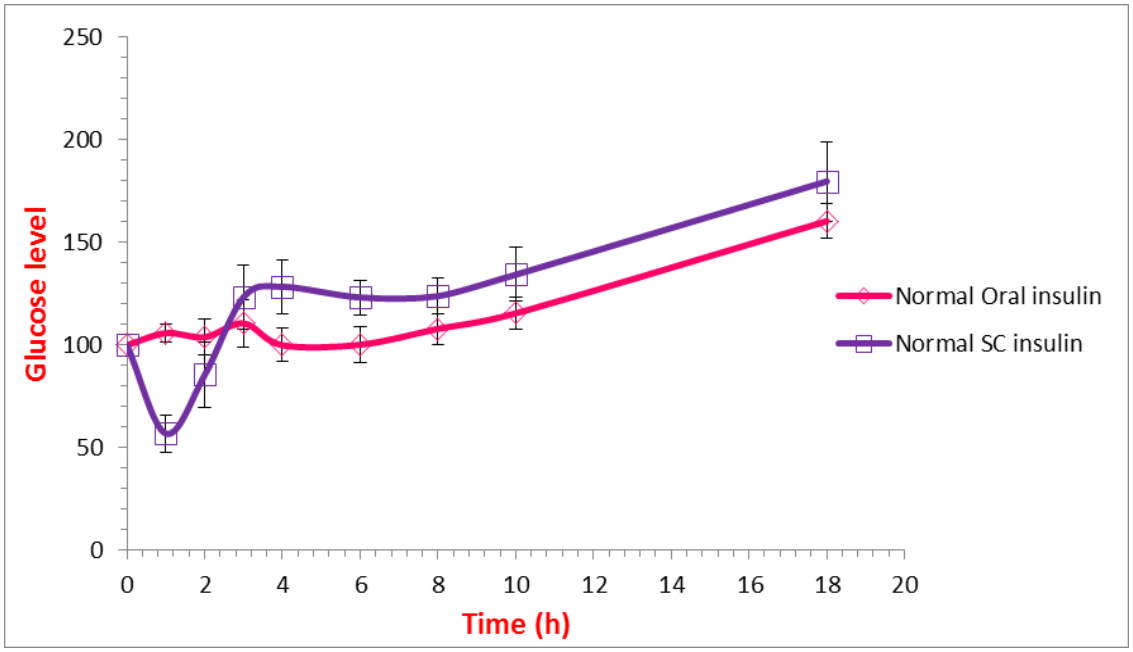
Results indicated there was no evident hypoglycemic effect after the oral administration of the insulin-loaded nanoparticles formula to normal rats. In normal rats, significant reduction on blood glucose level was obtained at the first hour ($p<0.05$), while at 2 hours postdose of subcutaneous injection of 1 IU/ml insulin, the glucose level began to increase, recovering to about 95% of the initial level at 3 hours (Figure 3.4).

The glucose level versus time profiles following the administration of oral insulin formulation to STZ diabetic and normal rats was showed in (Figure 3.5). At time 8, 10, 18 hrs, significant reduced glucose level was obtained in STZ diabetic rat ($p<0.05$).

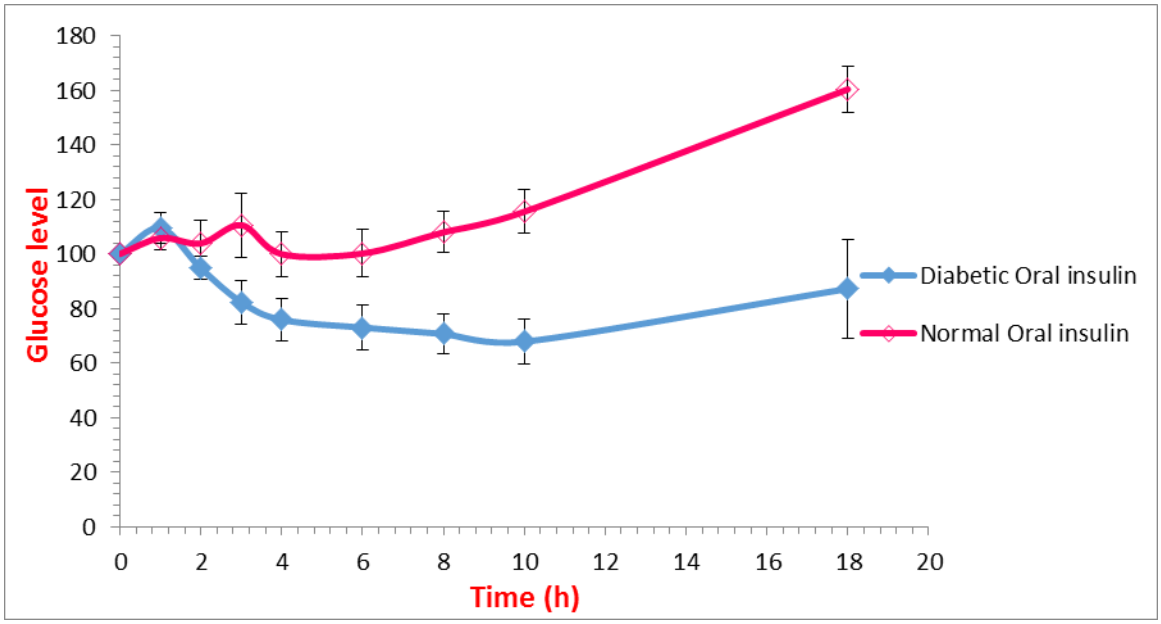
The hypoglycemic effect in normal and diabetic rats after subcutaneous administration of insulin was shown in (Figures 3.6). Importantly, the blood glucose level achieved the largest decreases (to 70% of the initial glucose level) after subcutaneous administration of insulin with 1 IU/kg to diabetic rats. Approximately Significant decreased glucose level was showed at all times except 8 h ($p<0.05$).



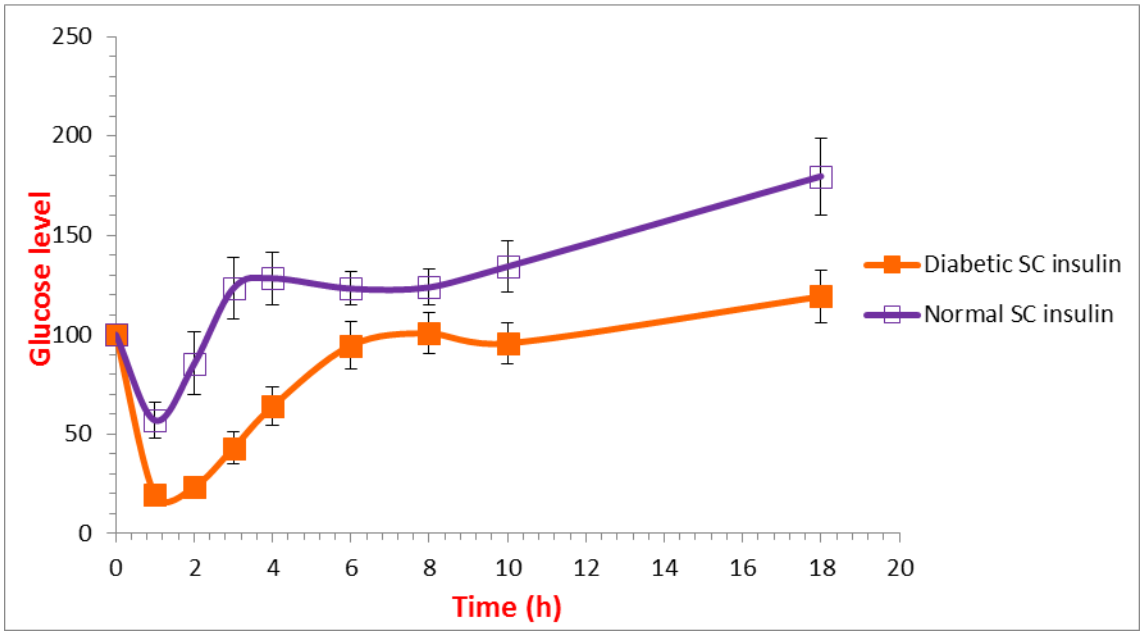
(Figure 3.3): Percentage reduction in blood glucose level of STZ diabetic rats given oral insulin formula (50 IU/Kg, *n*=10) and SC insulin (1 IU/Kg, *n*=10) compared to a placebo group (Data is as mean ± SEM).



(Figure 3.4): Percentage reduction in blood glucose level of normal rats given oral insulin formula (50 IU/Kg, *n*=7) and SC insulin (1 IU/Kg, *n*=7) (Data is as mean ± SEM).



(Figure 3.5): Comparison the percentage reduction in blood glucose level between STZ diabetic and normal rats after oral insulin formula administration (Data is as mean ± SEM).



(Figure 3.6): Comparison the percentage reduction in blood glucose level between STZ diabetic and normal rats after subcutaneous insulin administration (Data is as mean ± SEM).

3.4. Evaluation of the intestinal absorption of insulin-loaded nanoparticles by everted gut sac model

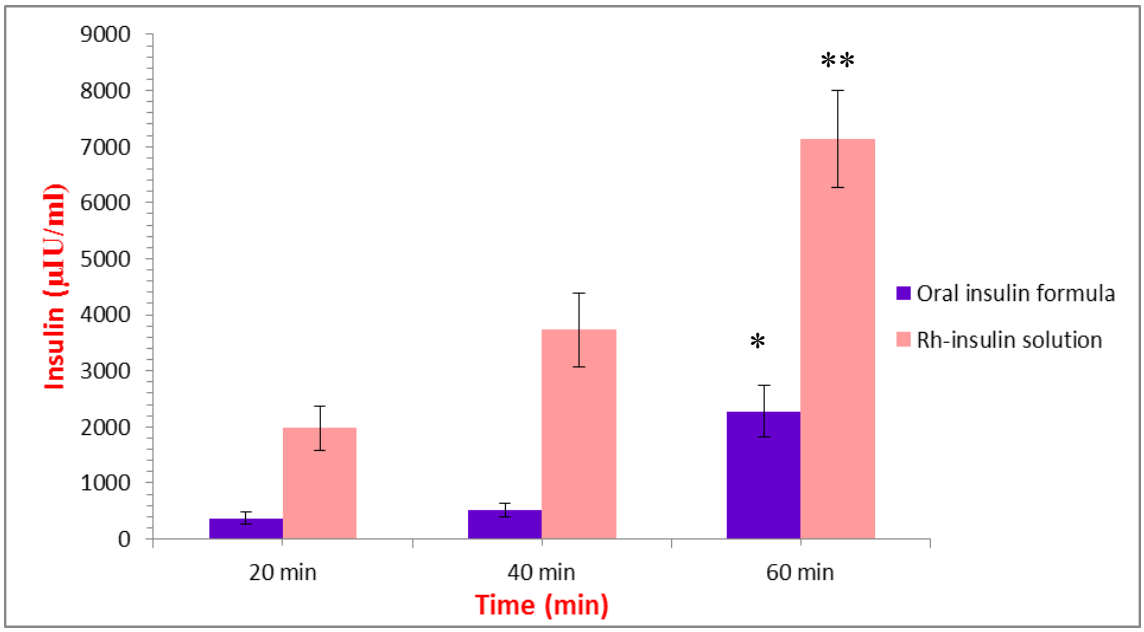
In normal rat, insulin at a concentration of 1 IU/ml was seen absorbed in a time dependent manner in its solution form. After 60 min of incubation, it was noted that the highest concentration of insulin in the serosal part was 7134 µIU/ml and it was highly significant ($p < 0.001$). On the other hand, insulin loaded nanoparticles (7 IU/ml) showed a different trend. It was observed that less amount of insulin was transferred into the serosal area. The difference in absorption between initial two time intervals namely, post 20 and 40 minutes was insignificant ($p > 0.05$). However, after 60 minutes of incubation it was noted that the amount of the absorbed insulin increase significantly ($p < 0.05$) (Figure 3.7).

Intestinal absorption profiles in diabetic rat showed very small absorbed insulin at all interval time with oral insulin formula and the difference in absorption was insignificant ($p > 0.05$), while the uptake profiles with Rh-insulin solution showed larger absorption especially at 40 min ($p < 0.05$). (Figure 3.8)

The sampling is continued for 60 min and found that the concentration of insulin absorbed is more in the standard insulin formula in all cases (normal and diabetic rat). After 20, 40 and 60 min the amount of absorbed insulin were 1976, 3729, 7134 µIU/ml respectively in normal rats and 7388, 19675, 8275 µIU/ml respectively in diabetic rats, where in oral insulin formula the absorbed insulin was 369, 520, 2270 µIU/ml respectively in normal rats and 233, 138, 146 respectively in diabetic rats.

Compared with insulin loaded nanoparticles formula, the gut sacs of normal rat were contained larger insulin amount than gut sacs of diabetic. Significance was obtained at 40, 60 min ($p<0.05$) (Figure 3.9)

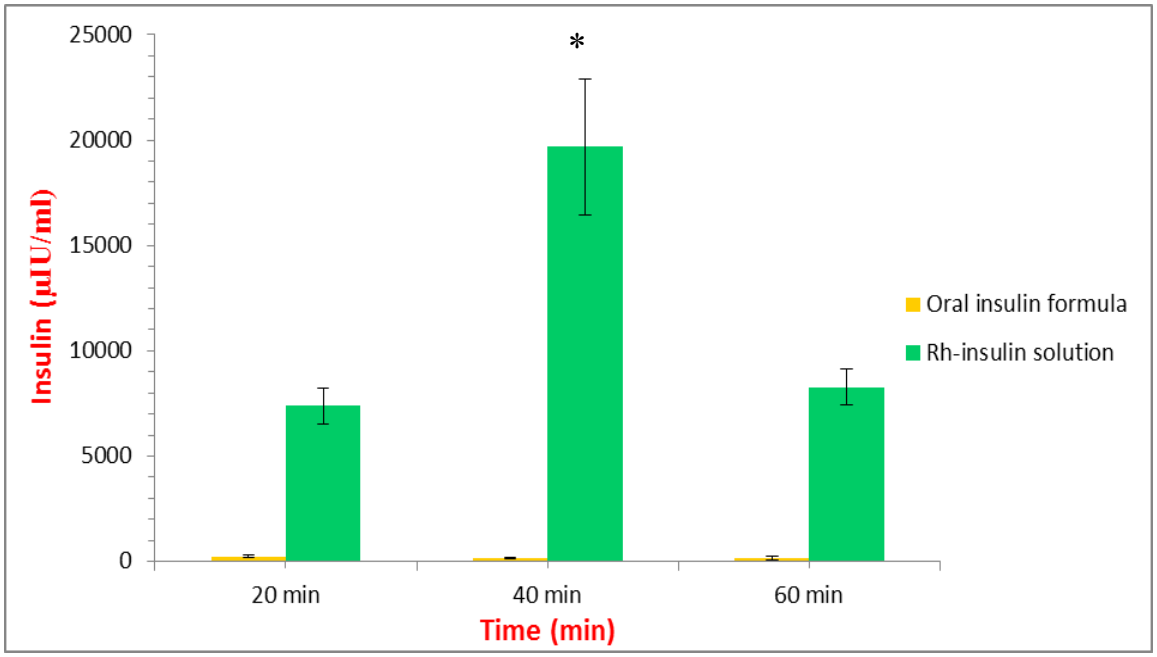
In (Figure 3.10), the largest insulin levels were obtained with gut sacs of diabetic rat placed in Rh-insulin solution when compared with gut sacs of normal rat. The significance amounts were obtained at 20, 40 min ($p<0.05$).



(Figure 3.7): Intestinal absorption of insulin from oral insulin formula ■ and Rh-insulin solution ■ in normal rat after 60 min incubation by everted gut sac experiment. Data are as mean of 10 sacs ± SEM. (*: p<0.05 , **: p < 0.001)

(Table 3.3): Insulin Level (µIU/ml) present in serosal fluid of gut sacs in normal rat for Rh-insulin solution and insulin-loaded nanoparticle formula.

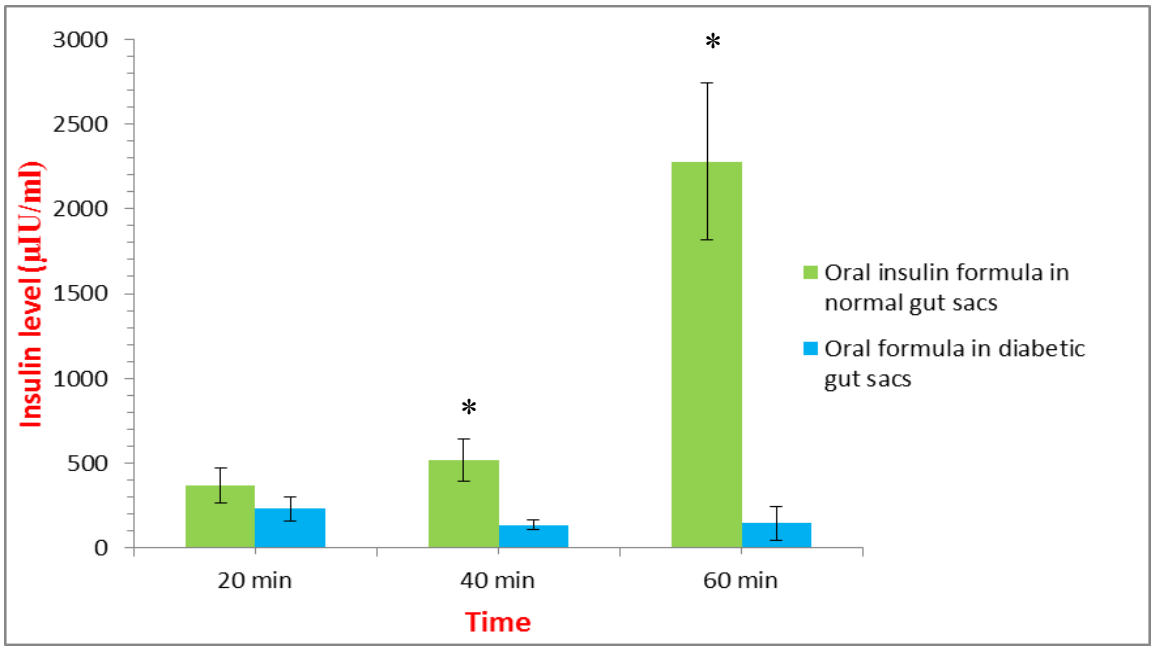
Time	20	40	60
Rh-insulin solution	1976 ± 403	3729 ± 658	7134 ± 866
Oral insulin formula	369 ± 104	520 ± 126	2279 ± 462



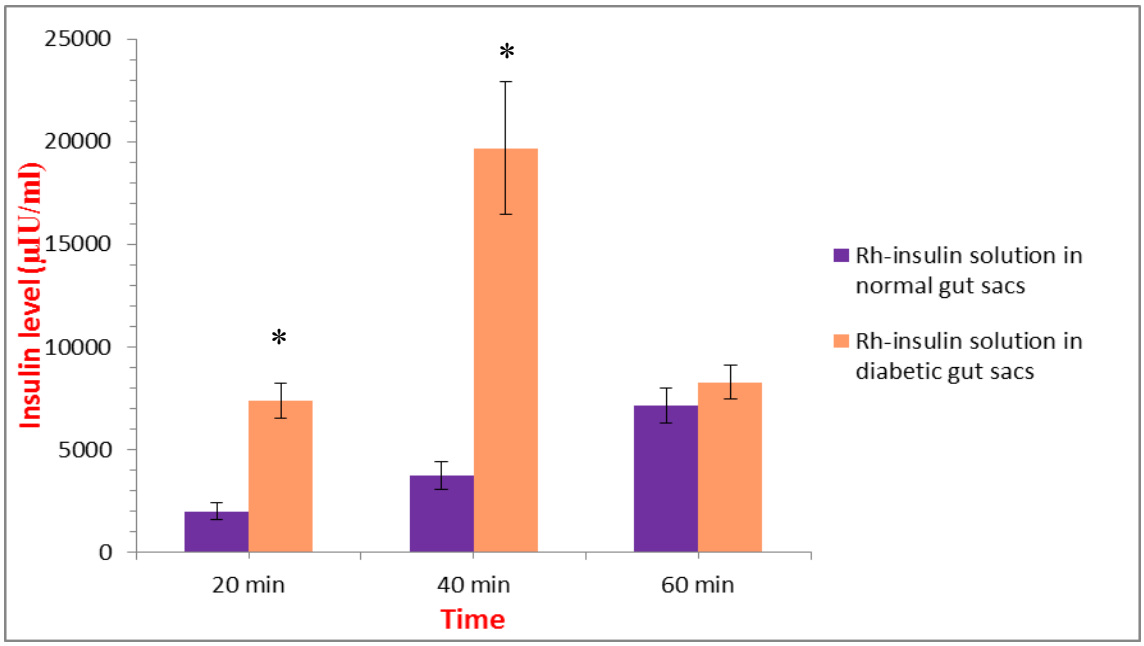
(Figure 3.8): Intestinal absorption of insulin from oral insulin formula and Rh-insulin solution in diabetic rat after 60 min incubation by everted gut sac experiment. Data are as mean of 10 sacs ± SEM. (*: $p < 0.05$)

(Table 3.4): Insulin Level (µIU/ml) present in serosal fluid of gut sacs in diabetic rat for Rh-insulin solution and insulin-loaded nanoparticle formula.

Time	20	40	60
Rh-insulin solution	7388 ± 850	19675 ± 3245	8275 ± 841
Oral insulin formula	233 ± 71	138 ± 30	146 ± 101



(Figure 3.9): Comparison the intestinal absorption profile between normal and diabetic gut sacs after incubation with oral insulin formula. (Data is as mean ± SEM) (*: p<0.05)



(Figure 3.10): Comparison the intestinal absorption profile between normal and diabetic gut sacs after incubation with Rh-insulin solution. (Data is as mean ± SEM) (*: p<0.05)

3.5. Evaluation of the intestinal absorption of insulin-loaded nanoparticles by *in situ* intestinal perfusion technique

To examine the biological effect and intestinal uptake of insulin-loaded nanoparticles formula, the oral insulin formula was administered to full-length intestine. In addition, Rh-insulin solution was perfused to compare the intestinal absorption between the oral preparation and the insulin solution. Moreover, the *in situ* perfusion study was carried out with normal and diabetic rats to evaluate the differences in insulin transfer between healthy and STZ-induced diabetes intestine.

The profiles of percentage of all results are shown in (Figure 3.11-20). The glucose levels of normal rats after intestinal perfusion of Rh-insulin formula presented in (Figure 3.11) and compared it with effect of subcutaneous insulin. The maximum decrease in glucose level after insulin perfusion was obtained at 30-40 min, then the glucose level started to increase again, while the perfusion of placebo with subcutaneous insulin that used as reference group shows continuing decline in glucose level.

On the other hand, (Figure 3.12) shows the same comparison in diabetic rats. Glucose levels after insulin solution perfusion did not change. However, after subcutaneous injection in the diabetic rat decrease in blood glucose was observed.

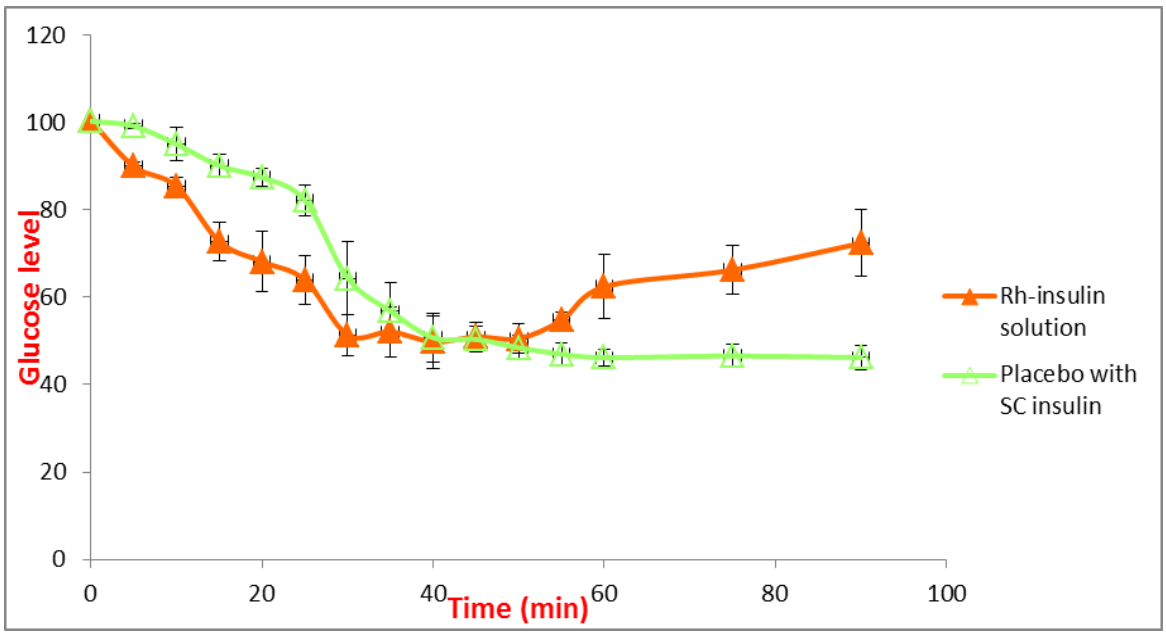
The pharmacological action of insulin after perfusion through intestine was compared between normal and diabetic rats in (Figure 3.13). Significant reduced glucose level in normal rats was obtained from 30 to 60 min ($p<0.05$). The comparison of insulin subcutaneously effect between diabetic and non-diabetic rats was presented in (Figure 3.14). In particular, Placebo perfusion with SC insulin causes a reduction in blood glucose concentrations significantly from 30 to 60 min in normal rats ($p<0.05$).

With insulin-loaded nanoparticles preparation, glucose levels of normal rats were measured after intestinal perfusion and compared with effect of subcutaneous insulin. Approximately 70% of blood glucose level was reduced over 45-55 min and the perfusion of placebo with subcutaneous insulin that used as reference group shows almost the same trend in the decline of glucose level (Figure 3.15).

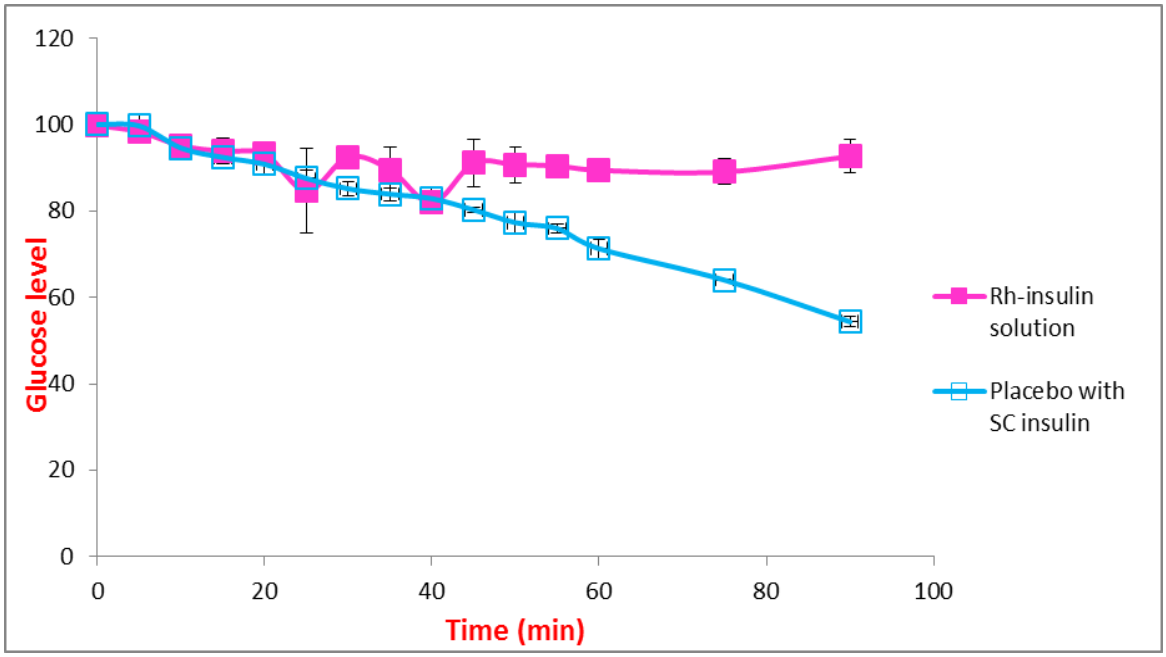
In the case of diabetic rats as shown in (Figure 3.16), hypoglycemic effect after insulin-loaded nanoparticles formula perfusion was larger than the effect after placebo perfusion with insulin subcutaneous injection. All points at 55 min and after are significant ($p<0.05$).

Comparison of glucose concentration in normal and diabetic rats can be obtained from in situ perfusion of oral insulin-loaded formula in (Figure 3.17). Significant glucose level reduction after oral formula perfusion in normal rats was obtained from 15 min to 55 min ($p<0.05$). Moreover, (Figure 3.18) represents the comparison of insulin subcutaneously effect between diabetic and non-diabetic rats. While the significant reduction in glucose level in normal rats was showed at 30 min to 60 min ($p<0.05$).

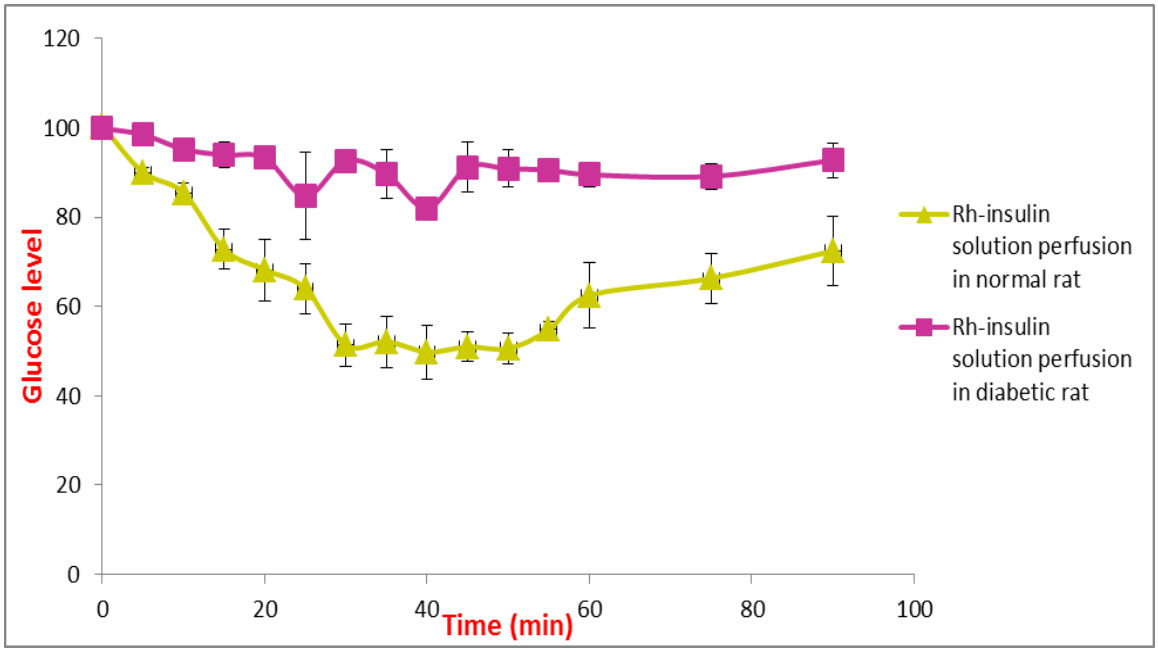
The insulin-loaded nanoparticle formulation was more effective in increasing the intestinal absorption of insulin in normal rats and the hypoglycemic effect of oral formula was significantly greater than standard insulin solution especially at 55 min to 90 min ($p<0.05$) (Figure 3.19). Steady-state glucose concentrations in diabetic rats can be observed from in situ perfusion of Rh-insulin solution, while the most significant hypoglycemic effect was observed after insulin-loaded nanoparticles preparation perfusion in 45 min to 90 min ($p<0.05$) (Figure 3.20).



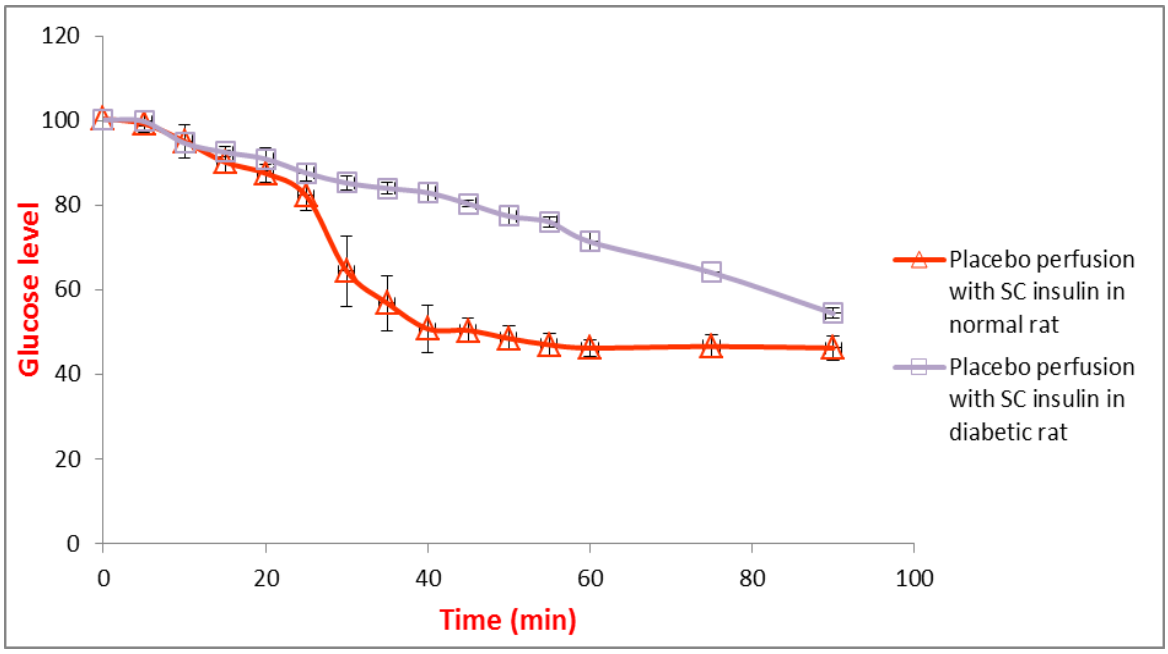
(Figure 3.11): Plot showing percentage of blood glucose levels of normal rats after in situ intestinal perfusion of Rh-insulin solution and placebo with SC insulin. (Data is as mean ± SEM).



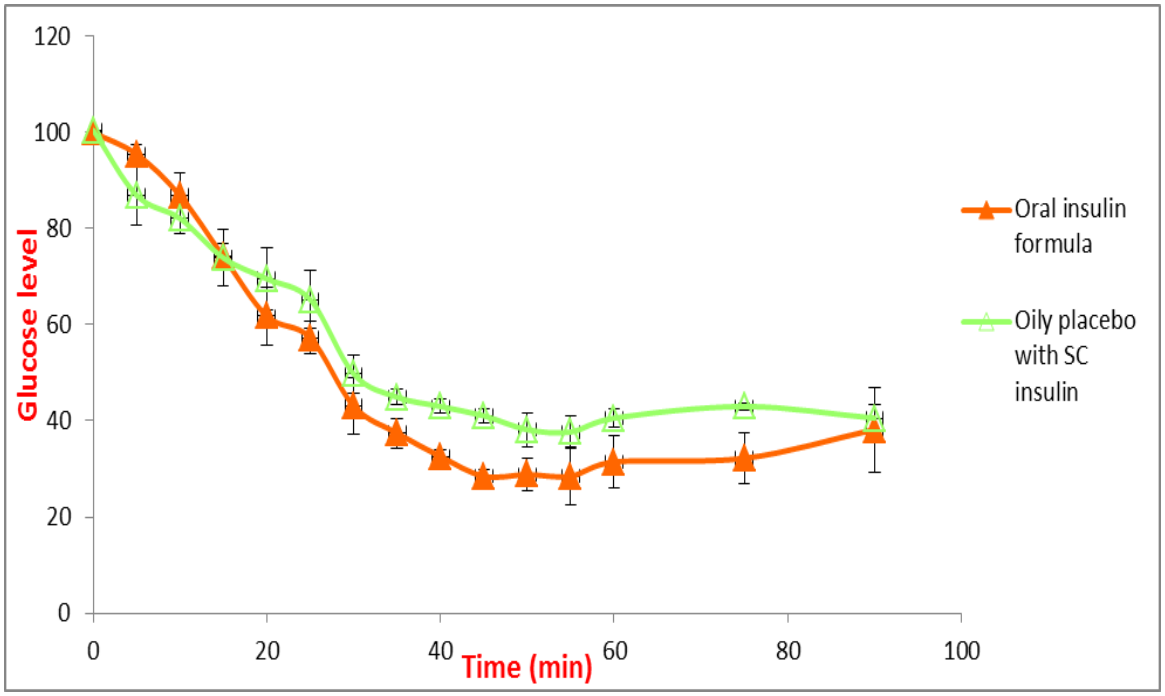
(Figure 3.12): Plot showing percentage of blood glucose levels of diabetic rats after in situ intestinal perfusion of Rh-insulin solution and placebo with SC insulin. (Data is as mean ± SEM).



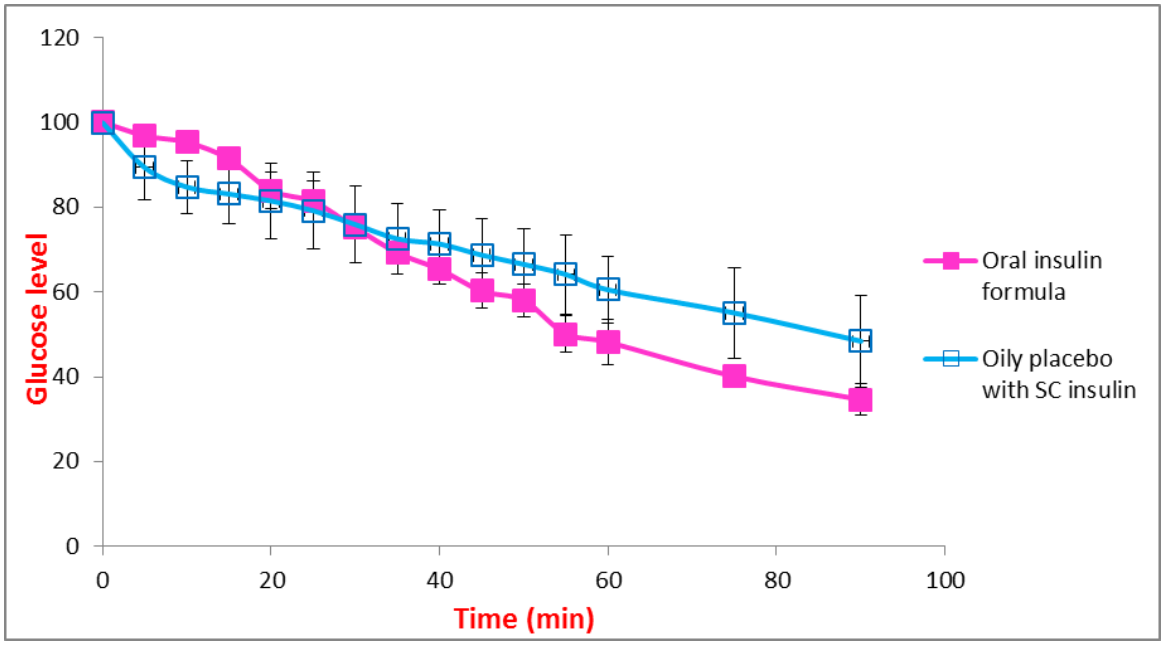
(Figure 3.13): Comparison percentage of blood glucose levels between normal and diabetic rats after in situ Rh-insulin solution perfusion. (Data is as mean ± SEM).



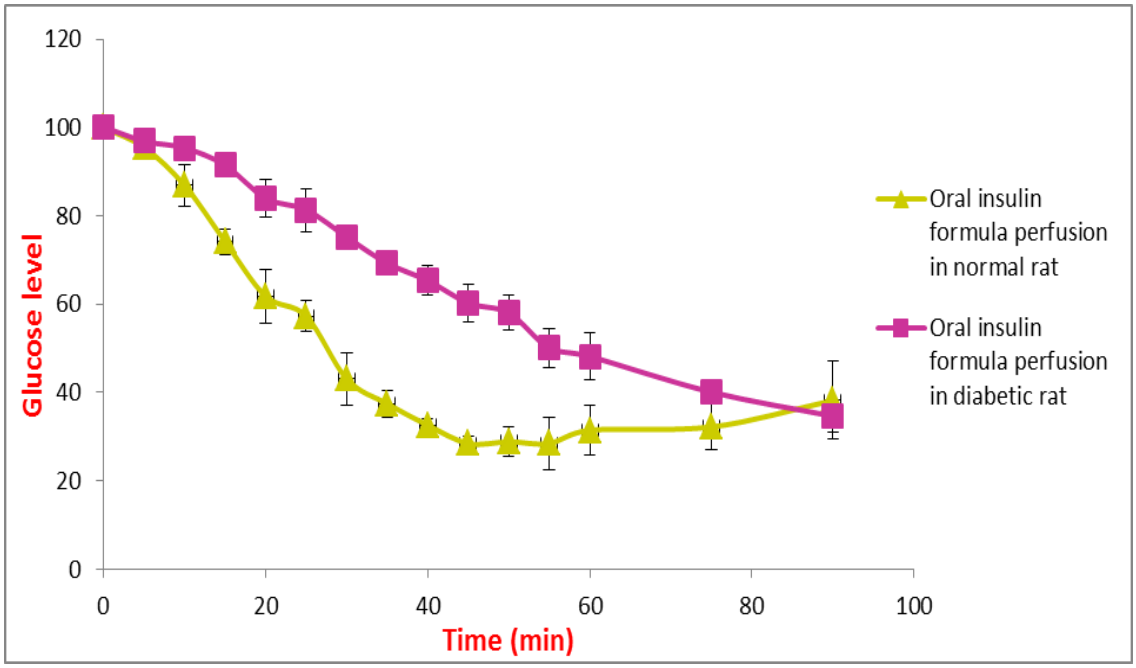
(Figure 3.14): Comparison percentage of blood glucose levels between normal and diabetic rats after in situ placebo perfusion with SC insulin injection (1IU/ml). (Date is as mean ± SEM).



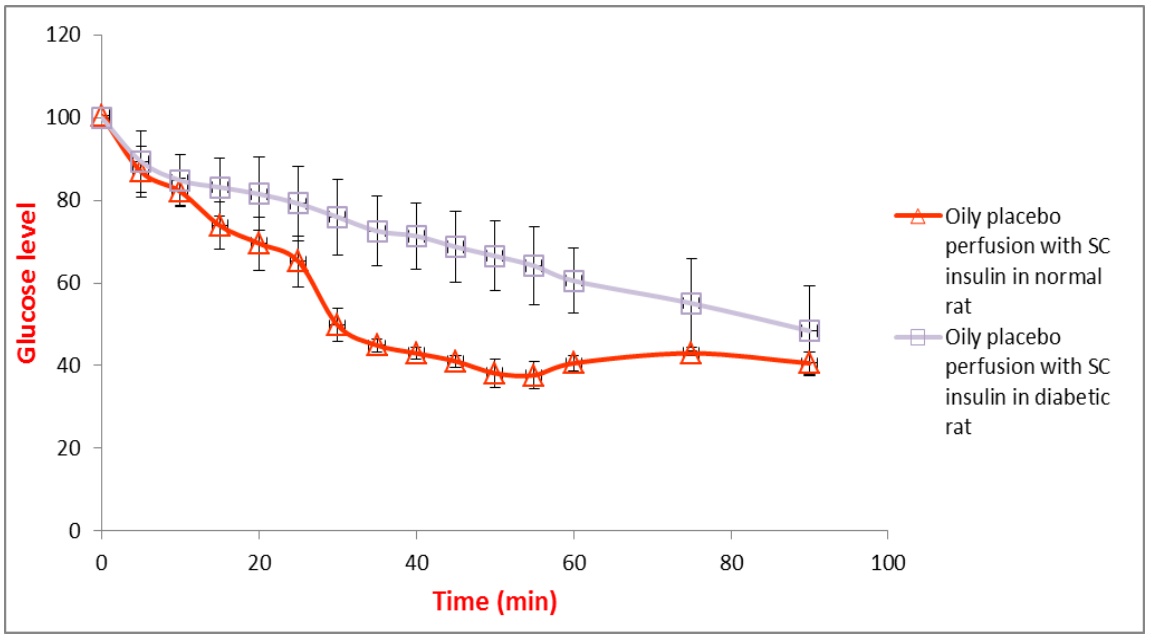
(Figure 3.15): Plot showing percentage of blood glucose levels of normal rats after in situ intestinal perfusion of oral insulin-loaded nanoparticles formula and oily placebo with SC insulin. (Data is as mean ± SEM).



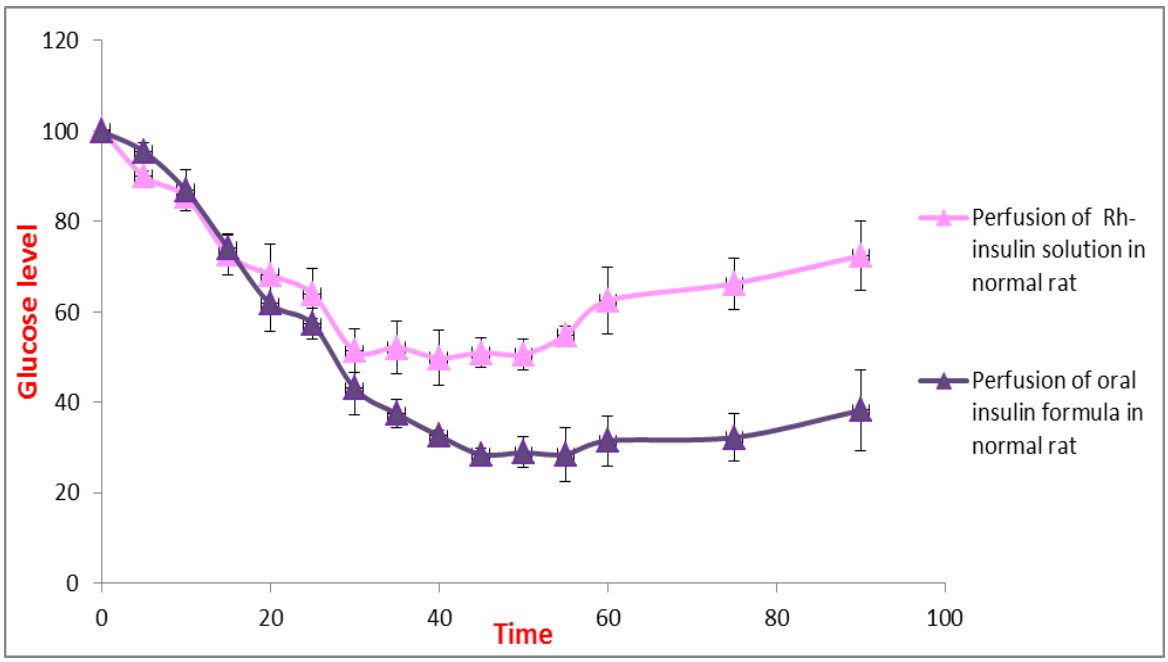
(Figure 3.16): Plot showing percentage of blood glucose levels of diabetic rats after in situ intestinal perfusion of oral insulin-loaded nanoparticles formula and oily placebo with SC insulin. (Data is as mean ± SEM).



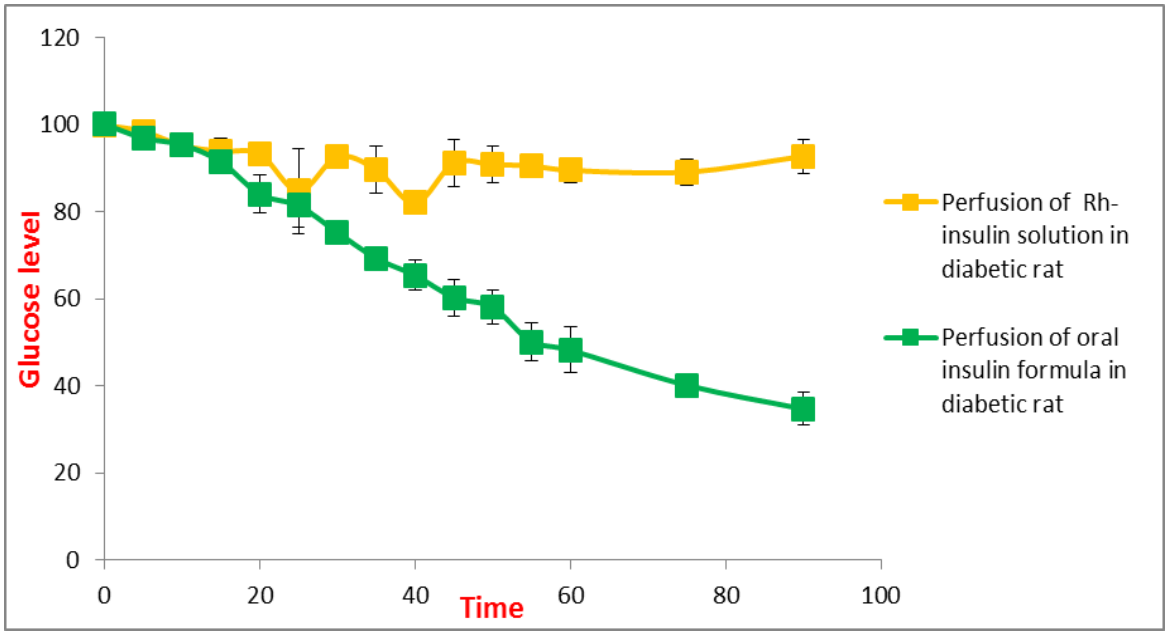
(Figure 3.17): Comparison percentage of blood glucose levels between normal and diabetic rats after in situ oral insulin formula perfusion. (Data is as mean ± SEM).



(Figure 3.18): Comparison percentage of blood glucose levels between normal and diabetic rats after in situ oily placebo perfusion with SC insulin injection (1IU/ml). (Date is as mean ± SEM).



(Figure 3.19): Percentage of glucose level in normal rats after perfusion of oral insulin preparation and compared with perfusion of Rh-insulin solution (Date is as mean ± SEM).

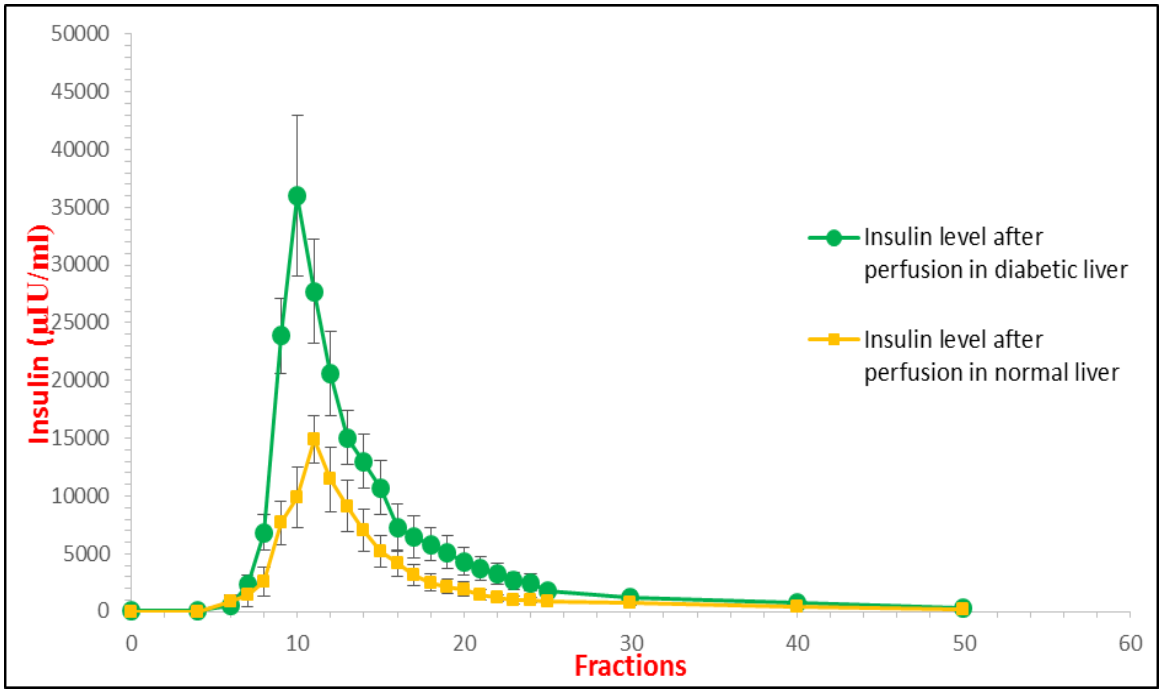


(Figure 3.20): Percentage of glucose level in normal rats after perfusion of oral insulin preparation and compared with perfusion of Rh-insulin solution (Date is as mean ± SEM).

3.6. Evaluation of hepatic first pass metabolism of insulin by *in situ* liver perfusion method

The insulin level (µIU/ml) profile for insulin (2 IU) obtained after bolus administration into the portal vein (PV) under 13 ml/min flow rate is displayed in (Figure 3.21).

Normal rat liver degraded more insulin than diabetic liver. As shown in (Figure 3.21) over 3500 µIU/ml of insulin eluted from diabetic liver after the end of the pulse infusion, while 1500 µIU/ml of insulin eluted only from the normal liver in the same conditions. From fraction 9 to fraction 11 significant amount of insulin was obtained in diabetic liver compared to normal one ($p<0.05$).



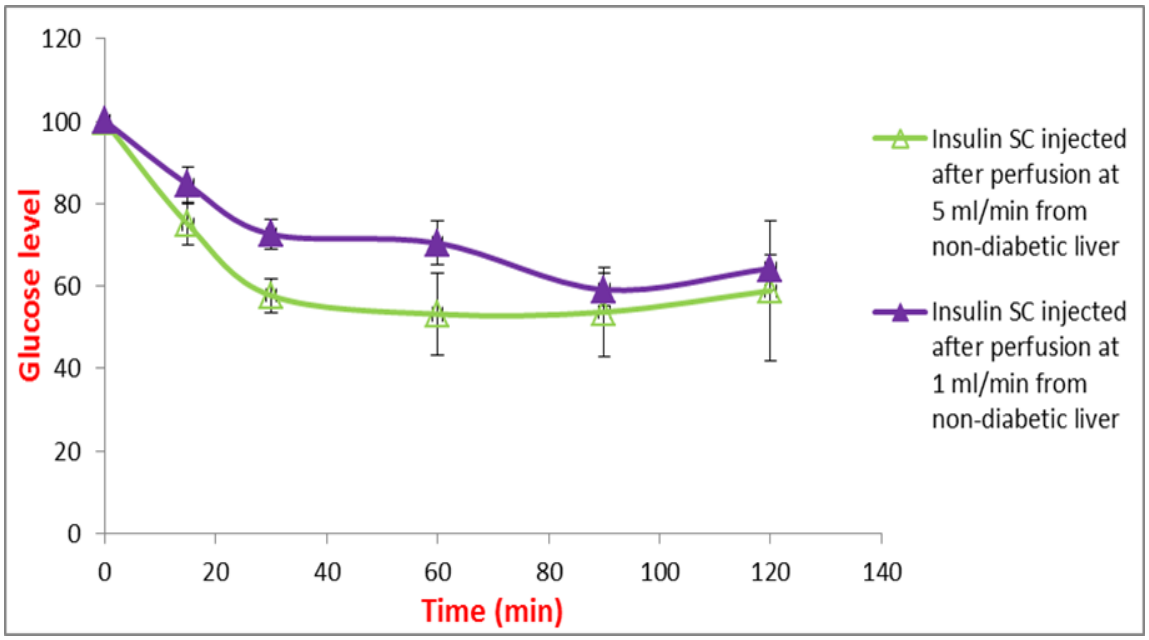
(Figure 3.21): Semilogarithemic plot showing the insulin level (µIU/ml) obtained after bolus injection of insulin into the portal vein. Data are as mean of 8 rats ± SEM.

3.7. Evaluation the effect of different flow rates on insulin metabolism in liver

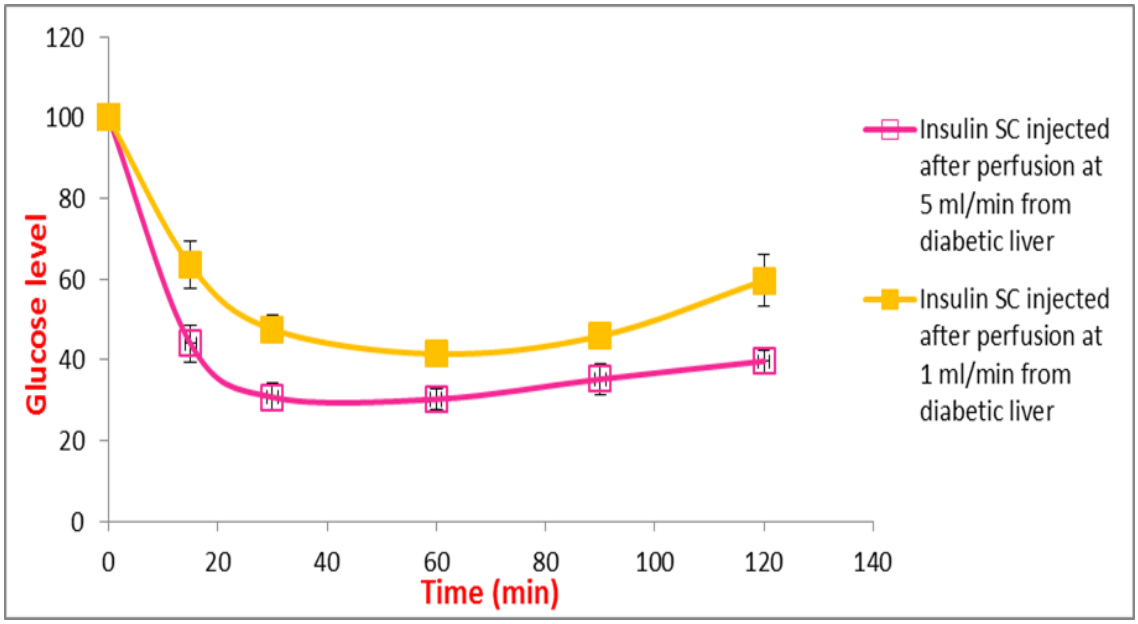
In the present study, significant differences were found between insulin perfusion at 5 ml/min and 1 ml/min flow rates in both normal and diabetic livers ($p < 0.05$). In addition, streptozotocin-treated livers at high flow rate were resulted in the most potent insulin action.

After insulin perfusion at different flow rates and drying the effluent, in normal rats the dissolved insulin administration decreased the blood concentration of glucose at 5 ml/min more than 1 ml/min flow rate (Figure 3.22). Moreover, in diabetic rats the glucose levels were reduced in the same manner as can seen in (Figure 3.23).

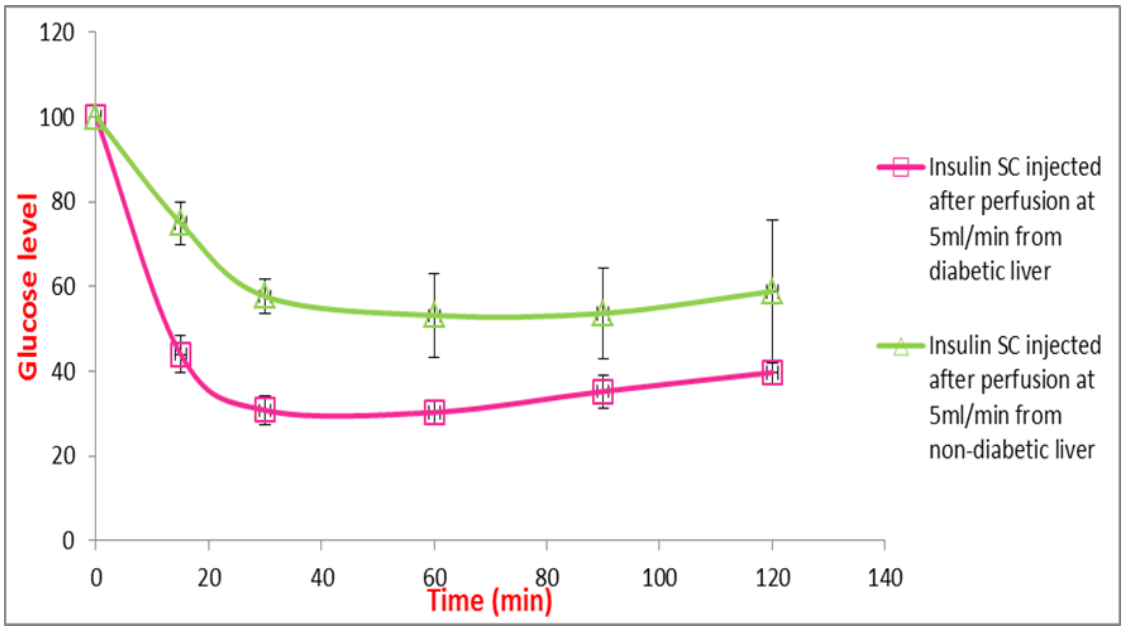
In (Figure 3.24), the maximal blood glucose reduction was evaluated from insulin subcutaneously injected after the perfusion in diabetic liver at 5 ml/min ($p < 0.05$). The observed difference of insulin action between perfusion on diabetic and non-diabetic livers at the low flow rate (1 ml/min) was reported in (Figure 3.25).



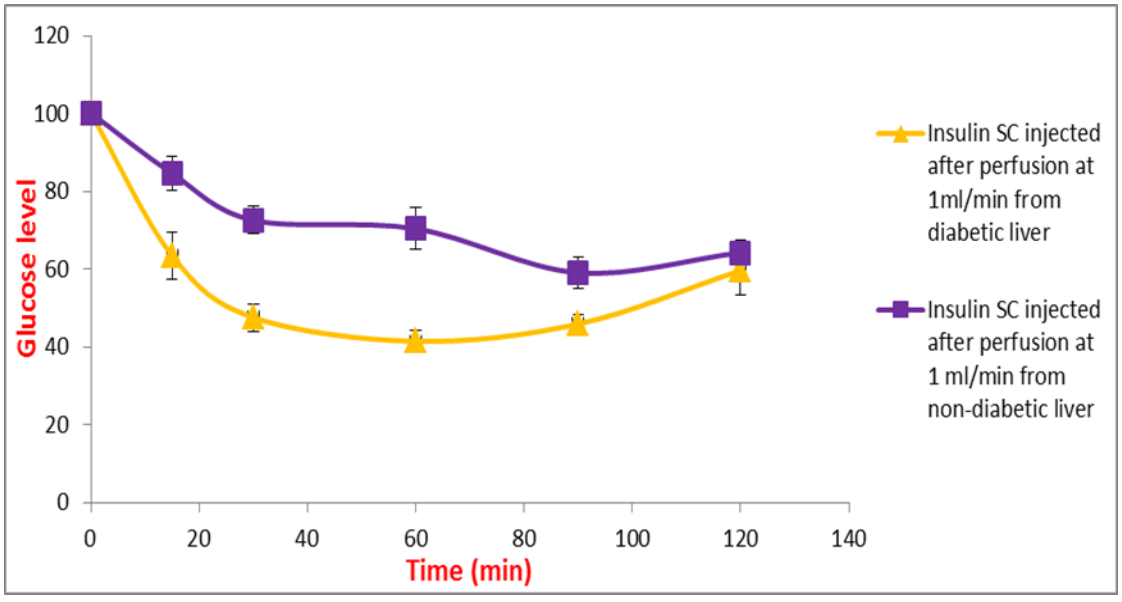
(Figure 3.22): Percentage of glucose levels of normal rats after insulin perfusion in normal liver at different flow rates followed by SC injection (Data are as mean of 8 rats ± SEM).



(Figure 3.23): Percentage of glucose levels of normal rats after insulin perfusion in diabetic liver at different flow rates followed by SC injection (Data are as mean of 8 rats ± SEM).



(Figure 3.24): Comparison the percentage of blood glucose level of normal rats after insulin perfusion at 5 ml/min in normal and diabetic livers followed by SC insulin injection (Data are as mean of 8 rats ± SEM).



(Figure 3.25): Comparison the percentage of blood glucose level of normal rats after insulin perfusion at 1 ml/min in normal and diabetic livers followed by SC insulin injection (Data are as mean of 8 rats ± SEM).

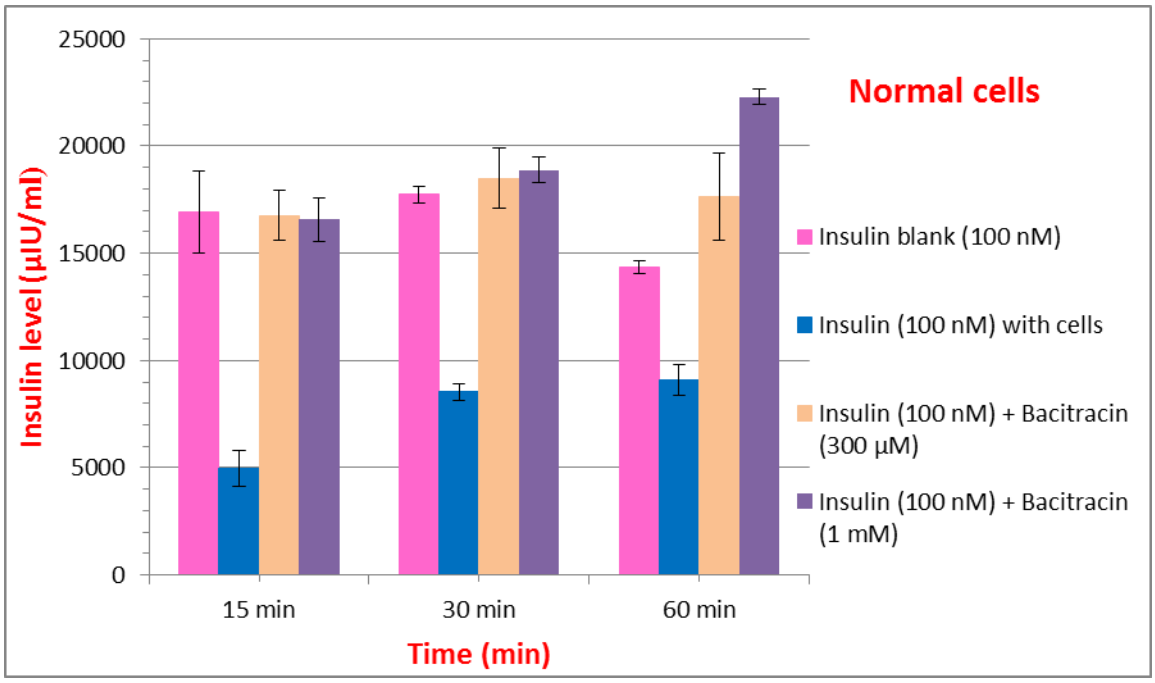
3.8. Determination the capacity of insulin metabolism in liver and evaluation of the inhibitory effect of bacitracin on insulin degradation in isolated hepatocytes

Normal or diabetic isolated hepatocytes were incubated for various periods of time at 37 C° with 100 nM insulin or 200 nM insulin with or without different concentrations of bacitracin (300 µM, 1 mM).

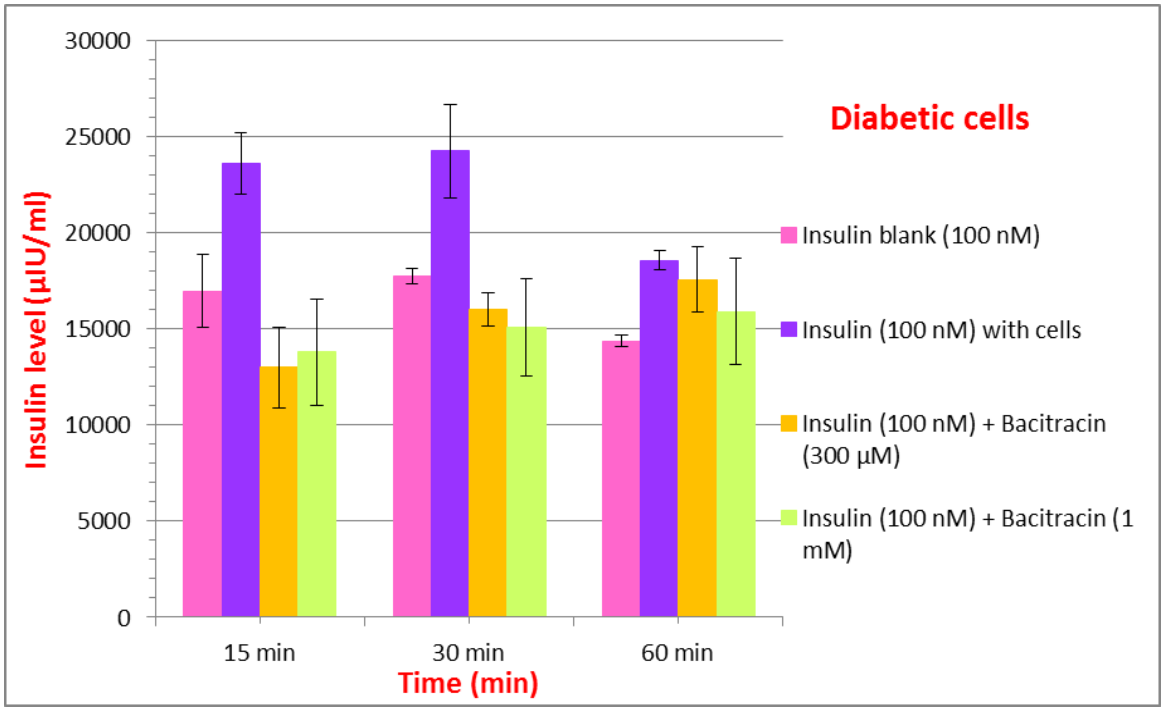
In normal isolated hepatocytes, firstly the addition of 100 nM insulin to intact monolayers (15 min), insulin was significantly degraded by the cells in comparing with insulin blank ($p < 0.05$). Insulin degradation significantly decreased over time ($p < 0.05$). Degradation of insulin in cells was inhibited by bacitracin in two concentrations (300 µM, 1 mM). Almost 80% of inhibition was obtained. (Figure 3.26)

As shown in (Figure 3.27), there was no degradation of 100 nM insulin in diabetic isolated hepatocytes. Insulin level was significantly increased in streptozotocin-intoxicated hepatocytes when compared with insulin blank ($p < 0.05$). In the presence of bacitracin (300 µM, 1 mM), insulin level significantly decreased and returned to the normal level ($p < 0.05$).

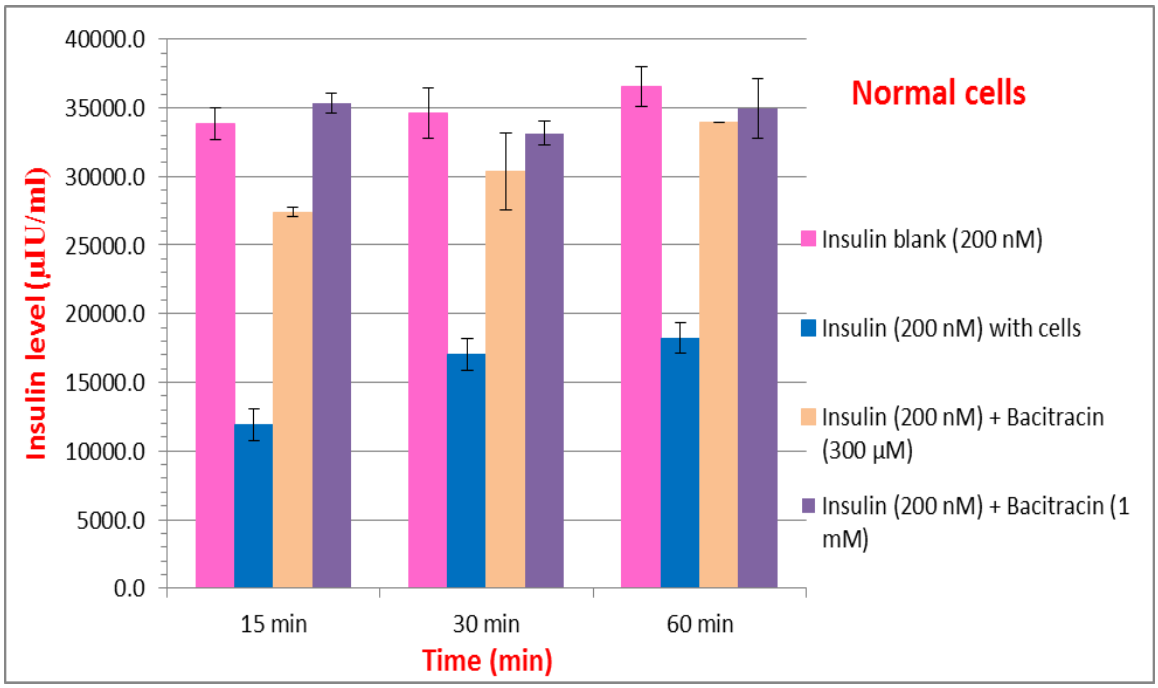
Significant degradation of 200 nM insulin during 60 min incubation with normal hepatocytes at 37 C° is shown by the second column of (Figure 3.28) ($p < 0.05$). Bacitracin (300 µM), produced at least 70% decrease in degradation. 1 mM of bacitracin was decreased the degradation and having the greatest effect (90% inhibition) ($p < 0.05$). (Figure 3.29), it indicates that some reactivity of insulin (200 nM) occurs in isolated diabetic hepatocytes and thus bacitracin can exert an inhibitory effect of insulin reactivity.



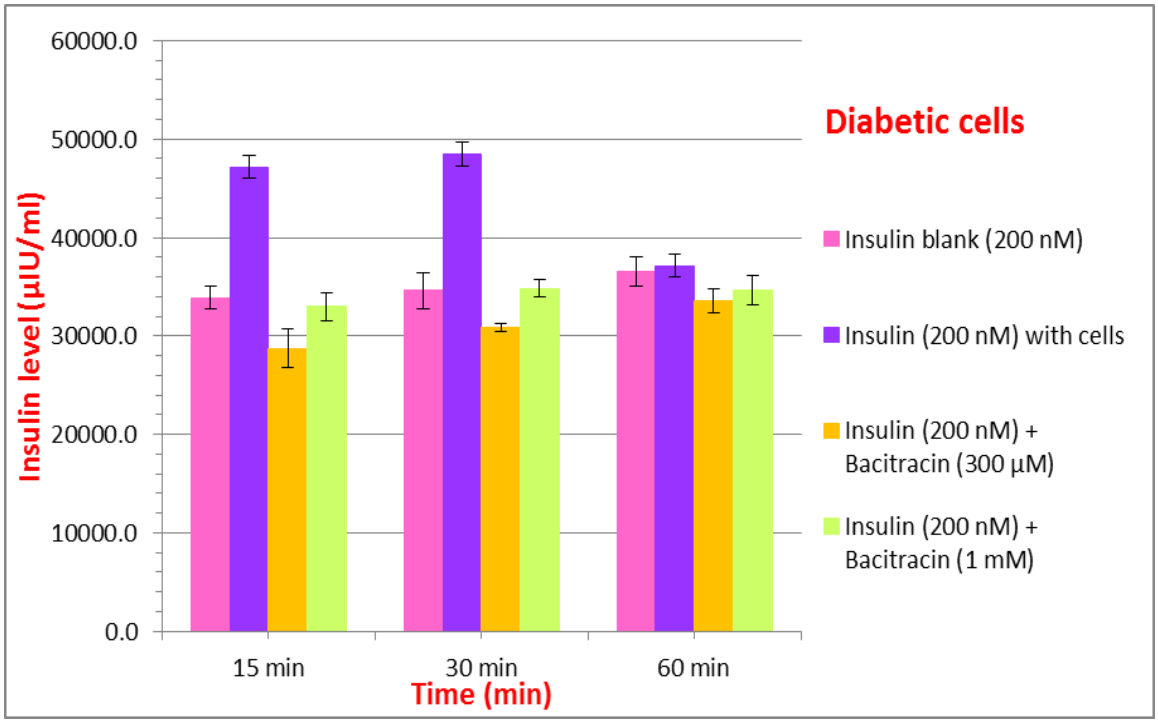
(Figure 3.26): Interaction of 100 nM insulin with normal isolated hepatocytes and the effect of bacitracin (300 µM, 1 mM) on insulin degradation.



(Figure 3.27): Interaction of 100 nM insulin with diabetic isolated hepatocytes and the effect of bacitracin (300 µM, 1 mM) on insulin degradation.



(Figure 3.28): Interaction of 200 nM insulin with normal isolated hepatocytes and the effect of bacitracin (300 µM, 1 mM) on insulin degradation.



(Figure 3.29): Interaction of 200 nM insulin with diabetic isolated hepatocytes and the effect of bacitracin (300 µM, 1 mM) on insulin degradation.

Chapter Four

Discussion

4. Discussion

Insulin therapy is recommended for patients with type 1 diabetes mellitus (Fonseca, 2006). Insulin is often administered parenterally for systemic treatment due to its inherent instability in the GIT and its low permeability across biological membranes, which is a result of its high molecular weight and hydrophilic nature (Bruno et al., 2013). Moreover, approximately 70% of portal insulin is removed by hepatocytes during first-pass metabolism before entering the systemic circulation (Duckworth et al., 1988). Thus, oral delivery systems of insulin are still considered a challenge in the field of drug development.

Despite the numerous reported oral delivery studies, oral bioavailability of insulin is still quite low and normally insufficient for producing an effective systemic effect. For example, Andreani et al. reported the production of PEG-coated silica nanoparticles containing insulin for oral administration did not increase the permeation behavior of insulin through the small intestinal mucosa (Andreani et al., 2014). Moreover, insulin-loaded chitosan/sodium alginate nanoparticles prepared by complex coacervation technique were showed a half of oral bioavailability as compared to subcutaneous insulin (Prusty & Sahu, 2013). In Contrast, Sarmento et al. has formulated insulin-loaded alginate/chitosan nanoparticles and evaluated the reduced blood glucose level as pharmacological activity of in diabetic rats (Sarmento et al., 2007).

Chitosan is biocompatible and biodegradable polymers; in addition; it has permeability enhancing and mucoadhesives properties (Pedro et al., 2009). These

properties made chitosan a polymer of choice for insulin delivery. While chitosan has important functional properties, nevertheless, the high molecular weight, high viscosity and insolubility at physiological pH of chitosan restrict its use *in vivo* (Qinna et al., 2015). However, LMWC can be more beneficial than HMWC due to their higher water solubility, and their ability to form nanoparticles (Lavertu et al., 2006). A recent study by Qinna et al. has shown that W/O nanosized system containing LMWC–insulin PEC achieved the highest glucose reduction with 1.3 KDa LMWC (Qinna et al., 2015). For all the above, expected that using LMWC (13KDa) would be able to entrap insulin and could stabilize insulin within their nanostructure.

Therefore, this research was performed to evaluate the first pass metabolism of insulin when delivered orally in normal and STZ-intoxicated rats since oral insulin may denature or degrade before it reaches its target.

Based on formulation of oral delivery system for insulin was carried on previous study (Elsayed et al., 2009). The adopted nanoparticle system was (W/O) microemulsion that consist from mixing LMWC (13 KDa, 99 DDA%) with insulin (with HPβCD) to form a PEC (aqueous phase) which was then solubilized in the oily phase made from Labrasol® and Plurol Oleique® as Surfactant/Cosurfactant (SCOS), and DAGs dissolved in oleic acid.

In the current study, two new materials were added to the previously reported oral delivery system. Zhang et al. reported that hydroxypropyl-β-cyclodextrin (HPβCD) protect insulin encapsulated in CS–alginate nanoparticles from enzymatic degradation (Zhang et al., 2006). Moreover, HPβCD used to enhance the stability of insulin formulation and improve their shelf-time (Sajeesh & Sharma, 2006). In the preparation, HPβCD was added in the aqueous phase during insulin-chitosan PEC

formation. While the second oily component added was diacylglycerol (DAG). DAG increases membrane permeability during nanoparticle absorption (Yi et al., 2013). Moreover, DAG was found to be capable of filling the gaps between the surfactant/cosurfactant and solidifying the dispersion system (Fatouros et al., 2007). Therefore, adding DAG would be expected to increase the physical stability of insulin-loaded nanoparticles formula. DAGs dissolved in oleic acid during formulation.

Insulin was protected from gastric enzymes by incorporation into lipid-based formulation because oleic acid is not affected neither by the acidic media of the stomach nor proteolytic enzymes of the GIT. By this efficient technique, the insulin in the formula had good protection against the GIT enzymes with nanosize structure that might enhance its permeability across intestinal mucosa.

The first part of the current research involved synthesizing different grades of LMWC with average M.W. of 7, 10, 13, 16 and 24 KDa and quantitatively and qualitatively characterize the different grades of chitosan using Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC) and Viscometer. Furthermore, the construction of insulin-loaded nanoparticles and measuring its diameter in the dispersion system was carried out. The current formula was found to have significant reduction in the particle size of the nanoparticles (85 ± 2.53 nm) when compared with the old formula (108 ± 9 nm). This reduction in the particle size is due to the addition DAGs.

After that, *in vivo* evaluation was required to validate the true performance of an oral delivery system. The hypoglycemic studies on oral insulin-loaded nanoparticles revealed significant hypoglycemic effect in the glucose level of STZ-induced diabetic

rat models, while the hypoglycemic effect of insulin preparation in normal rats was weak **(Figure 3.5)**. The increase of hypoglycemia in STZ-induced diabetic rats might be attributed to the deleterious and toxic actions of STZ on organs, other than pancreas, involved in maintaining normal glucose homeostasis in the body (Qinna & Badwan, 2015). Moreover, hypoglycemic effect of insulin-loaded nanoparticles administered orally was sustained for a longer period than the subcutaneous injection.

Streptozotocin is the most used diabetogenic agent for inducing diabetes in experimental animal models. Further studies with STZ revealed that it produced irreversible damage to pancreatic β cells, triggered intermittent hypoglycemia (Ohno et al., 2000). Streptozotocin is able to pass through any cell membrane that contains glucose transporter (GLUT-2). Once STZ passes the cell membrane, it causes alkylation of DNA. This damage leads to the depletion of cellular NAD+ and ATP and the formation of superoxide radicals. STZ can also liberate nitric oxide inside the cells that inhibits aconitase activity resulting in further DNA damage. Indeed this toxic activity is more pronounced in β cells in pancreas. In addition, STZ-induced diabetes complication in liver is as destruction of hepatocytes, alteration in liver enzyme levels and morphological changes (Zafar et al., 2009).

Comparing the effect of insulin in diabetic with normal rats, the current results are in line with a recent study, which reported that STZ reduces the endogenous insulin secretions due to pancreatic destruction while the exogenous insulin accumulates more in the blood of the diabetic animals. This might be caused by decreasing in kidney and liver functions. Such combination of inefficient metabolism and excretion caused the accumulation of the injected insulin in the blood circulation (Qinna & Badwan, 2015). In other words, STZ is responsible for the loss of normal glucose

homeostasis while normal rats have resisted the reduction of glucose levels due to the presence of normal glucose homeostasis.

Formulation into nanoparticles to facilitate insulin cellular uptake represents the most popular configuration for intracellular delivery. Further studies have shown that insulin in nanoparticulate form was more likely to be delivered across the GIT than in its free soluble form (Elsayed et al., 2011; Sadeghi et al., 2008; Bayet et al., 2008). According to the intestinal first pass metabolism, many studies are involved in evaluation the intestinal metabolism. In the current research, absorption and permeation studies were carried out. Our absorption and permeation studies were *ex vivo* everted gut sac and *in situ* intestinal perfusion.

It was found that the absorbed amount of insulin from Rh-insulin solution into the everted sacs was three-folds the absorbed amount from the insulin-loaded nanoparticles formula at 60 min in normal rats. In diabetic gut sacs, however, little amount of insulin from the oral preparation was absorbed compared to the Rh-insulin solution.

Although nanoparticles dispersion system may increase insulin absorption through intestinal mucosa, the currently tested oral formula did not significantly change the permeation behavior of insulin through the small intestinal mucosa on everted gut sac model. Since such effect was seen in both diabetic and normal rats, it might be attributed to the presence of free insulin particles available for absorption. Alternatively, the reasons for such absorption failure perhaps are large particle size, negative surface charge and disturbance in SCOS ratio or emulsifying agent. Also the nanoparticles are bigger size in comparison to the soluble protein molecules and the fact that a lot of particles may get trapped inside the cells within the cellular

membrane such as the Golgi apparatus or the endoplasmic reticulum being unable to pass across the cells intactly. Moreover, due to the presence of some limiting factors for the use of everted rat intestine studies, such as tissue viability, loss of protein and enzymes elimination.

On the other hand, Matsuzawa et al. in 1995 reported that significant hypoglycemic effect was obtained after *in situ* intestinal perfusion of W/O/W insulin emulsion, and determined the best region for insulin absorption by comparing the biologic effects of the emulsion after administration to various sites in the rat intestine. According to the effectiveness of *in situ* intestinal perfusion technique, *in situ* intestinal perfusion model was performed for testing nanoparticles permeation.

In the current study, results indicate that the single-pass intestinal perfusion *in situ* model, a significant hypoglycemic effect was obtained in both normal and diabetic rats post oral insulin preparation administration, while the administration of insulin solution did not decrease glucose level significantly **(Figure 3.19, 3.20)**. As expected, such results seems more representative than the *ex vivo* results since *in situ* approaches provide experimental conditions closer to what is encountering following oral administration (Hogerle & Winne, 1983). In addition, these techniques maintain an intact blood supply to the intestine, and can be used to estimate the impact of clearance pathways such as enzymes and transporters, that are present in the gut. Moreover, drug permeability, expression of drug metabolizing enzymes and transporters has been shown to vary along the intestinal tract (Ungell et al., 1998).

Evaluation the hepatic first pass metabolism of the current oral insulin preparation was conducted by using *in situ* liver perfusion and cell culture methods. Which the liver plays a major role in the first pass metabolism of insulin (Duckworth et al.,

1988). Approximately 70% of portal insulin is removed by hepatocytes during first-pass effect before entering the systemic circulation. Bonora et al 1983 reported that hepatic insulin metabolism and clearance rates are decreased in diabetes mellitus. Also our results were in line with this assumption. After *in situ* liver perfusion technique was conducted on normal and STZ induced diabetic rats, more insulin was degraded by normal rat liver than diabetic liver **(Figure 3.21).** Similarly, insulin degradation is reduced in STZ-diabetic isolated hepatocyte compared to normal hepatocytes. Indeed, STZ causes liver function altering and destruct liver enzymes (Zafar et al., 2009) which are responsible for decreased insulin degradation in diabetic liver and diabetic isolated hepatocytes.

Concerning the factors that affect the insulin metabolism, *in situ* liver perfusion of insulin at different flow rate (1 ml/min and 5 ml/min) was performed. After insulin perfusion at different flow rates and drying the effluent, the dried effluent dissolved in distilled water and subcutaneously injected into normal rats to evaluate the effect of flow rate on insulin degradation and reveal the change on insulin efficacy and composition. According to our knowledge, this is the first *in situ* liver perfusion of insulin at different flow rate was conducted. The current study shows the high flow rate (5ml/min) of perfused insulin into the liver degraded the insulin less than the low flow rate (1 ml/min), while streptozotocin-treated livers at high flow rate were resulted the less degraded insulin. These results can be explained that the high flow rate, amount of insulin enters the liver rapidly for short time, liver enzymes metabolized so small amount of insulin while the rest of insulin will not be affected so when reinjected into normal rats should give hypoglycemic effect. On the other hand, insulin perfusion at low flow rate resulted in delaying of insulin in the liver, so the liver enzymes take enough time to degrade insulin then did not reduce glucose level

enough. Moreover, due to STZ effect on destroying liver enzymes. When insulin enters the liver at high flow rate, almost insulin will not be affected and then give the most potent hypoglycemic effect.

The first pass metabolism of insulin in liver mainly depends on the enzymatic mechanisms for metabolism, but these mechanisms have not been established. Three systems have been implicated for enzymatic mechanisms: insulin protease, glutathione-insulin transhydrogenase (GITe), and lysosomal enzymes (Duckworth & Kitabchi, 1981)

Further evidence provides that the GITe activity is the primary determination of the rate of hepatic insulin metabolism. It must be assumed, however, that insulin protease and lysosomal enzymes are involved in the process of insulin degradation.

Varandani, 1972 have shown that GITe is major enzyme responsible for hepatic insulin degradation by splitting the hormone at the disulfide bonds into A and B chains. Data of the current study in normal isolated hepatocytes model confirm their findings. While STZ caused alteration on enzymes levels the degradation (Zafar et al., 2009), the effect of GITe in STZ-induced diabetic hepatocytes was weak.

To avoid first pass metabolism, inhibition of this enzyme has been suggested to block internalization and, therefore, to result in an increase in surface-bound hormone. Bacitracin is commonly used in studies on insulin action, is widely used as an antibiotic and as an inhibitor to inhibit the degradation of insulin in studies on hormone-receptor binding and action (Juul & Jones, 1982). It was used to inhibit the ability of purified GITe to split insulin into its constituent A and B chains. Kinetic studies indicated that this inhibition was by a complex mechanism that decreased both the Vmax and affinity of the enzyme for insulin (Roth, 1981). So that, the present

research used bacitracin concentrations currently used in insulin studies and demonstrated that the degradation of insulin in normal and diabetic isolated hepatocytes was inhibited by bacitracin.

Chapter Five

Conclusion and Future work

5. Conclusion and future work

The present study evaluated the first pass metabolism of oral insulin-loaded nanoparticles formula with unimodal particle size (85 ± 2.53 nm) in normal and STZ-intoxicated rats. *In vivo* evaluation revealed significant sustained hypoglycemic effect in the glucose level of STZ-induced diabetic rat model.

However, the intestinal first pass metabolism was tested by absorption studies as *ex vivo* everted gut sac and *in situ* intestinal perfusion. The everted gut experiments showed that insulin in a solution form was absorbed more than insulin formulated in the tested nanoparticle oral delivery systems may due to the presence of free insulin particles available for absorption or enzymes elimination of everted model. On the other hand, the intestinal *in situ* perfusion study produced a significant hypoglycemic effect in both normal and diabetic rats.

In situ liver perfusion technique and cell culture model in both normal and STZ-diabetic rats studied insulin first pass metabolism in liver. The results indicated that hepatic insulin metabolism is decreased in STZ-diabetic rats compared to normal animals. Similarly, insulin degradation is reduced in STZ-diabetic isolated hepatocyte compared to normal hepatocytes. Moreover, When the *in situ* "liver passed" insulin was re-injected *in vivo* in both normal and diabetic rats, it was found that the insulin collected from diabetic livers was more active the collected from non-diabetic livers. Such effect can be attributed to the decrease in insulin degradation in diabetic liver and diabetic isolated hepatocytes due to the toxic action of STZ. Bacitracin as insulin

degradation inhibitor showed that our results were in line with the previously reported studies.

Finally, the current work might suggest some future work such as:

- Elucidate the mechanisms by which the insulin degraded in the hepatocytes.
- Define and measure the degraded components of insulin.
- Examine other different flow rates on insulin perfusion into liver and clarify its influence on insulin metabolism.
- Study how to inhibit insulin degradation inside the liver by tagging insulin with other amino acids.

Chapter Six

References

6. References

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