Subcutaneous and Oral Delivery of Insulin

BY

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A thesis submitted in partial fulfillment of the requirements of the University of Greenwich for the Degree of Doctor of Philosophy

April, 2015

DECLARATION

"I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others".

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ABSTRACT

Subcutaneous and Oral Delivery of Insulin

The aim of this project was to prepare, optimize, characterize and compare a subcutaneous/oral delivery system for insulin.

The effect of various low molecular weight chitosans (LMWCs) on the stability of insulin, using USP HPLC methods, was investigated. Insulin was found to be stable in a polyelectrolyte complex (PEC) consisting of insulin and LMWC in the presence of Tris-buffer at pH 6.5. In the presence of LMWC, the stability of insulin increased with decreasing molecular weight of LMWC; 13 kDa LMWC was the most efficient molecular weight for enhancing the physical and chemical stability of insulin. The bioactivity of insulin in the PEC was assessed using enzyme-linked immunosorbent assay (ELISA) testing; results showed that insulin is still functionally active in the presence of chitosan. Solubilization of the PEC in a reverse micelle formulation (RMF) and administration to diabetic rats resulted in an oral delivery system for insulin with acceptable bioactivity.

The effect of reduced (GSH) and oxidized (GSSG) glutathione on the bioactivity of insulin was studied. A PEC of insulin with low molecular weight chitosan (13 kDa) was prepared and characterized. The PEC was then solubilized, in the presence and absence of GSH and GSSG, in a reverse micelle consisting of oleic acid and two surfactants (labrasol and plurol). The in vitro and in vivo performances of the reverse micelle formulations (RMFs) were evaluated in rats. At pH 6.5 the association efficiency of the PEC was 76.2%. In vitro insulin release from the RMs was negligible at pH 1.2 and was markedly increased at pH 6.8. The hypoglycemic activity of insulin in the PEC was reduced when administered via the subcutaneous route, regardless of the GSH content. On the other hand, the presence of GSSG significantly enhanced hypoglycaemia. When the RMF was administered via the oral route, the presence of GSH had no effect on the hypoglycemic activity of insulin compared with the GSH free system. However, the presence of GSSG in the oral preparation increased the hypoglycemic activity of insulin; probably by inhibiting insulin degradation, thereby prolonging its effect. Thus,

incorporation of GSSG in the RMF reduces blood glucose levels in rats and protects insulin from degradation.

The effect of glucosamine HCl (GlcN·HCl) on the bioactivity of insulin, administered via subcutaneous (SC) and oral routes, in rats was also investigated. The oral insulin delivery system (IC-RM) was prepared by solubilizing insulin-chitosan (13 kDa) polyelectrolyte complex (IC-PEC) in a RM system consisting of oleic acid, PEG-8 caprylic/capric glycerides and polyglycerol-6-dioleate. The blood glucose levels were measured using a blood glucose meter. The results revealed that the extent of hypoglycemic activity of SC insulin was GlcN·HCl dose dependent when they were administered simultaneously. A significant reduction in blood glucose level ($p < 0.05$) was found for the insulin:GlcN·HCl at mass ratios of 1:10 and 1:20, whereas lower ratios (e.g. 1:1 and 1:4) showed no significant reduction. Furthermore, enhancement of the action of SC insulin was achieved by oral administration of GlcN·HCl for five consecutive days prior to insulin injection ($p < 0.05$). For oral insulin administration via the IC-RM system, the presence of GlcN-HCl increased the hypoglycemic activity of insulin ($p < 0.05$). The relative pharmacological availabilities (PA) were 6.7% and 5.4% in the presence and absence of GlcN·HCl (i.e. the increase in the relative PA was about 23% due to the incorporation of GlcN·HCl in the IC-RM system), respectively. The aforementioned findings offer an opportunity to incorporate GlcN·HCl in oral insulin delivery systems in order to enhance a reduction in blood glucose levels.

ACKNOWLEDGEMENTS

Firstly, I wish to express my gratitude to my academic supervisors Prof. B. Z. Chowdhry and Prof. S. A. Leharne for the valuable guidance and advice they have provided; I have been extremely lucky to have supervisors who cared so much about my work.

My deepest, gratitude for Dr Adnan A. Badwan for his support, continuous encouragement and for giving me the opportunity to undertake a PhD.

Special thanks also to my colleagues Dr Al Omari, Dr Mayyas Al-Remawi, Dr Nidal Qinna, and Nawzat Al-Jbour for their help and support during my research program.

I would like also to convey my thanks to the Jordanian Pharmaceutical Manufacturing Company for providing financial support and laboratory facilities without which this endeavour would not have been possible.

Lastly, I would like to thank my family, my mother for her continuous prayers, my sister for her support, my beautiful daughters Nahida and Haneen and my son Mohammed, and most of all for my loving, supportive, encouraging and patient husband, Ahmad.

Thank you so much.

Dedication

This thesis is dedicated to my Mum and to my family who are always there for me when I need them.

"Intellectual growth should commence at birth and cease only at death."

- *Albert Einstein*

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Abbreviations

Symbol	***Description***
AAC	*- area above the curve*
AE	*-association efficiency*
AFGF	*-acidic fibroblast growth factor*
Ag	*-antigen*
AUC	*-area under the curve*
Asn	*-asparagine*
ATP	*-adenosine triphosphate*
CH	*-chitosan*
CMC	*-critical micelle concentration*
CYP	*-cytochrome p450*
DA	*-degree of acetylation*
DD	*-degree of deacetylation*
DLS	*-dynamic light scattering*
DM	*-diabetes mellitus*
DME	*-drug metabolism enzyme*
DNA	*-deoxyribonucleic acid*
ELISA	*- enzyme-linked immunosorbent assay*
EMA	*-European Medicines Agency*
FTIR	*- Fourier-transform infrared*

GCSF	*- granulocyte colony-stimulating factor*
GI	*-gastrointestinal*
GIT	*-gastrointestinal tract*
GlcN	*-glucosamine*
Gln	*-glutamine*
GSH	*-glutathione reduced form*
GSSG	*-glutathione oxidized form*
GST	*-glutathione-s-transferase*
HLB	*-hydrophilic-lipophilic balance*
HMWP	*-high molecular weight proteins*
HPLC	*-high performance liquid chromatography*
ICH	*-international conference on harmonization*
IDDM	*-insulin-dependent diabetes mellitus*
IDE	*-insulin degrading enzyme*
IEF	*-isoelectric focusing*
IRS	*-insulin receptor substrate*
GLUT	*-glucose transporter*
LD	*-lethal dose*
LMC	*-low molecular chitosan*
LMWC	*-low molecular weight chitosan*
MAPK	*-mitogen-activated protein kinase*
MW	*-molecular weight*

N	*-native state*
NIDDM	*-non insulin-dependent diabetes mellitus*
NQ01	*- NAD(P)H-quinone-1-reductase*
PA	*-pharmacological activity*
PK	*- pharmacokinetics*
PDI	*-protein disulfide isomerase*
PEC	*-polyelectrolyte*
PEG	*- polyethylene glycol*
pI	*-isoelectric point*
PI3K	*-phosphatidylinositol-3-kinase*
PLA	*-polylactide*
PLGA	*-poly (lactide-co-glycolide)*
PP	*-polypeptide*
rh	*-recombinant human*
RH-HPLC	*-reversed phase high performance liquid chromatography*
RMs	*-reverse micelles*
SC	*-subcutaneous*
SDS-PAGE	*-sodium dodecyl sulfate-polyacrylamide*
SGF	*-simulated gastric fluid*
STZ	*-streptozocin*
TJ	*-tight junctions*
TMC	*-trimethyl chitosan*

TRIS	*- hydroxymethyl amino methane*
U	*-unfolded state*
UEC	*- university ethics committee*
UGT	*-UDP-glucuronosyltransferase*
US FDA	*-United States Food and Drug Administration*
W-Blot	*-western blot*

Overview of Thesis

The text is divided into four chapters, which may be summarised individually as follows.

Chapter one consists of an introduction to biological pharmaceuticals, their importance and the need for improved formulation. The barriers to oral delivery for peptides/proteins and the existing platform technologies for their delivery are described. Protein formulations and stability (including chemical and physical degradation) are also described. RP-HPLC, ELISA and FT-IR techniques are discussed as tools in monitoring proteins.

Chapter two details the influence of complexing LMWCs of different molecular weights (1.3-30 kDa) with insulin on its physicochemical and biological stabilities. Insulin content was monitored and examined as well as the formation of high molecular weight proteins (HMWPs) of insulin. Furthermore the system is evaluated for bioactivity using ELISA and in vivo activity using rats.

In chapter three, with the objective of improving the bioactivity of orally administered insulin, the effects of reduced and oxidized forms of glutathione (GSH and GSSG) on the bioactivity of subcutaneously (SC) administered insulin is reported. Furthermore, the hypoglycemic effects of an orally administered reverse micelle formulation containing GSH and GSSG are also examined.

Chapter four describes the effect of glucosamine on insulin bioactivity. Such an effect is evaluated when glucosamine was injected SC before insulin or with insulin as a mixture. Further, the effect of glucosamine was evaluated when incorporated in a reverse micelle formulation with insulin as an oral delivery system. Pharmacological activity was assessed and is reported.

Finally, chapter five summarises the research work performed and outlines the main conclusions. It also provides brief recommendations for future research relating to the proposed insulin delivery system.

Publications

The following peer-reviewed research manuscripts have been published as a result of the research reported in this thesis.

(1) **Zakieh I. Al-Kurdi**, Babur Z. Chowdhry, Stephen A. Leharne, Mahmoud M. H. Al Omari and Adnan A. Badwan; Low Molecular Weight Chitosan–Insulin Polyelectrolyte Complex: Characterization and Stability Studies, Mar. Drugs, **13**, 1765-1784, 2015.

(2) **Zakieh I. Al-Kurdi**, Babur Z. Chowdhry, Stephen A. Leharne, Nidal A. Qinna, Mahmoud M.H. Al Omari and Adnan A. Badwan; Influence of Glutathione on the Bioactivity of Subcutaneously or Orally Administered Insulin to Rats, Protein & Peptide Letters, **22 (6)**, 489-496, 2015.

(3) **Zakieh I. Al-Kurdi**, Babur Z. Chowdhry, Stephen A. Leharne, Nidal A. Qinna, Mahmoud M.H. Al Omari and Adnan A. Badwan; Influence of glucosamine on the bioactivity of subcutaneously and orally delivered insulin, Drug Design, Development and Therapy, **9**, 1-10, 2015.

(4) **Zakieh I. Al-Kurdi**, Babur Z. Chowdhry, Stephen A. Leharne, Nidal A. Qinna, Mahmoud M.H. Al Omari and Adnan A. Badwan; Advances in Oral Insulin Delivery, 2nd Mena Regulatory Conference on Bioequivalence, Biowaver, Bioanalysis and Biosimilar. Organized by the International Pharmaceutical Research Centre (IPRC) and American Association of Pharmaceutical Scientists (AAPS); Amman, Jordan (15-17, September, 2015).

CHAPTER 1: INTRODUCTION

1.1. General Background

As a consequence of the advances in biotechnology, recombinant deoxyribonucleic acid (DNA) technology, protein engineering and analytical separation methodologies, the production of a variety of peptides and proteins, on a commercially viable scale, is now possible resulting in the potential to treat a range of disease states (Stolnik et al., 2009). Proteins are biopolymers comprising a specific number, identity and sequence of amino acid monomers which, in specific solvents/milieu, adopt structural motifs that provides these molecules with physiological activity. Smaller chains of amino acids are termed peptides or polypeptides (PPs) (Ganong et al., 2013).

From 1989 to 2012, the number of marketed biotechnology products grew from ~13 to ~210, while worldwide product sales increased to US$163 billion. This accounted for 71% of the worldwide revenue generated by top-selling pharmaceuticals in 2012, and an increase of 353% compared to 2001 (US$ 36 billion) (Tufts CSDD Impact Report, No Author, 2013). Such changes are also affecting pharmaceutical/biotechnological development pipelines. Over 900 biotechnology products are currently in development, targeting a wide range of therapeutic areas (PhRMA, 2013) with new therapies based upon proteins, monoclonal antibodies (MAbs) and other biological accounting for 30% of new drug approvals (Rader, 2013).

Currently these new therapeutic peptide/protein compounds are mainly administered by intravenous, intramuscular and subcutaneous injections of liquid formulations as this provides the fastest way towards commercialization and are accepted as the most widely used routes of administration. Improved formulation of PPs and proteins and equally effective alternative delivery systems are required in order to increase their use in clinical medicine. However, their structural complexity (high molecular weight and hydrophilicity) together with their biophysical properties (low bioavailability) makes their formulation and delivery challenging.

1.2. Different Delivery Technologies for PPs and Proteins

Several strategies have been developed to address the in vivo delivery of PPs and proteins (Table 1.1).

Table 1.1 Comparison of different delivery technologies for PPs and proteins (Mitragotri et al., 2014).

Method	Advantages	Limitations
Microparticles	*Controlled release can be achieved. *Delivery is possible using subcutaneous injections (Stolnik et al., 2009); example: darbepoetin alfa	* Burst release can occur, which cause potential for local toxicity or adverse events (Mitragotri et al. 2014)
Nanoparticles	*Targeted delivery: small size allows enhanced permeation (Sykes et al., 2014). Paclitaxel nanoparticle injection is available in the market.	*Non-specific uptake in reticuloendothelial system (RES) organ permeation (Sykes et al., 2014) *Immunotoxicity can occur
Pumps	*Precise control over rates of delivery especially insulin. *Long duration of delivery	*Implanted devices are invasive (Schaepelynck et al., 2011) *Infection can occur with patch pumps
Transdermal delivery	*Painless and sustained delivery *High patient compliance (Prausnitz et al., 2008), e.g., parathyroid hormone	*Low bioavailability *Some devices are bulky
Jet injections	*Allows a needle-free approach *Broad applicability *Works with injectable formulation *Rapid systemic absorption (Mitragotri et al., 2014)	*Cause occasional pain *Inconsistent delivery of drug
Pulmonary delivery	*High bioavailability *Rapid systemic uptake *Ease of use (Zisser et al., 2012) Example: inhaled insulin	*Some devices are bulky *Potential for local toxicity

Oral delivery	*Ease of use *High patient compliance	*Low bioavailability *Enzyme degradation in the stomach *Interference in absorption rates from food
Other mucosal routes (vaginal, nasal and	*Non-invasive *Ease of use	*Low bioavailability *Enzymatic degradation *Variable absorption

1.3. Barriers to Oral Delivery of PPs and Proteins

1.3.1. Enzymatic Barriers

The major enzymatic barriers to the absorption of PPs and proteins are the pancreatic enzymes, peptidases, nucleases, lipases and esterases that are secreted in considerable quantities into the gastrointestinal lumen and rapidly hydrolyse PPs and proteins. In the case of peptidases, they work in a coordinated fashion, whereby the action of the pancreatic enzyme is augmented by those in the brush borders of the intestinal cells. The sloughing-off of the mucosal cells into the lumen also furnishes a mixture of enzymes that are a threat to PPs and proteins; enzymes secreted in the GIT are summarized in Table 1.2 (Woody et al., 2009).

Table 1.2 Enzymes secreted in the GIT and their function (Lee et al., 1991).

Enzyme	**Location**	**Function**
Aspartic peptidase (pepsin)	Stomach	Responsible for fragmentation of large proteins into smaller PPs
Pancreatic peptidases (chymotrypsin, trypsin, elastase, carboxypeptidase A and carboxypeptidase B)	Small intestine	Catalyse degradation of PPs at specific amino residues
Cellular peptidases (e.g. aminopeptidase A and N, diaminopeptidase I, endopeptidase 24.11)	Brush border and cytosol of enterocytes	Brush border proteases are mainly involved in hydrolysis of tri-and tetrapeptidases Cytosolic proteases preferentially digest dipeptides

1.3.2. Gastrointestinal Mucus Gel Barrier

Between the gastrointestinal lumen and the epithelial cells there is a viscous solution, known as mucus, which lubricates the epithelia, helping in the passage of substances and particles through the digestive tract, and forms a protective layer against microbial infection, dehydration and changing luminal conditions. Although mucus is a complex of aqueous solutions of proteins, lipids, ions, carbohydrates, etc., a family of glycoproteins, the gel-forming mucins, is largely responsible for the viscoelastic and adhesive properties of mucus. In the mucus, large oligomeric glycoproteins are organized into entangled networks, this network is a formidable chemical and physical barrier that not only protects the underlying epithelia but also limit the usefulness of orally administered drugs (Perez et al., 2009).

1.3.3. The Absorption Barrier

Drugs are absorbed at the intestinal epithelia via several pathways, as illustrated in (Figure 1.1) as follows:

A. transcellular and this include:
   -passive diffusion
   -transport systems (active carrier mediated transport)
   -endocytosis
B. paracellular transport,
C. efflux transport.

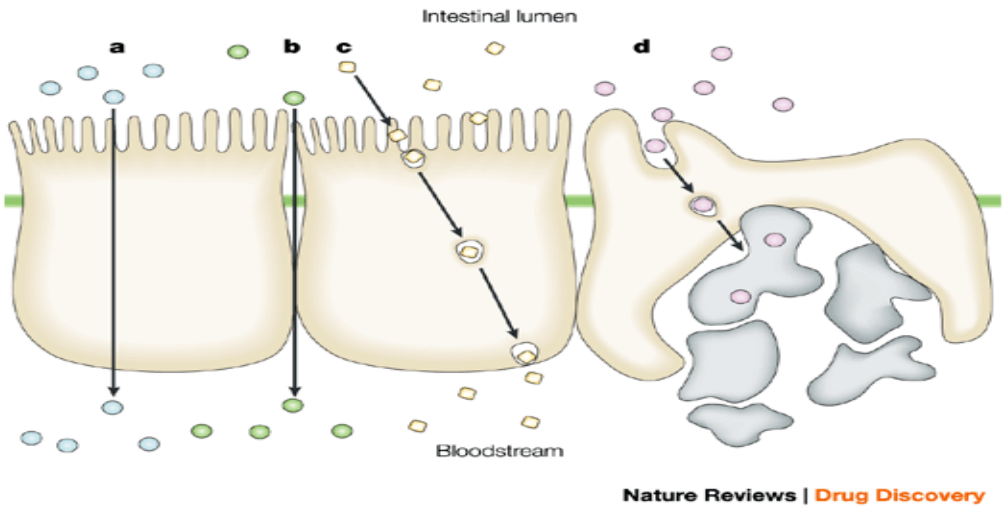


Figure 1.1. Absorption pathways of drugs across intestinal epithelium.

a | Transcellular pathway. **b** | Paracellular pathway **c** | Transcytosis and receptor-mediated endocytosis. **d** | Absorption into the lymphatic circulation via M-cells of Peyer's patches (www.nature.com).

Transcellular absorption of drugs depends on their physicochemical properties, including molecular weight, lipophilicity and hydrogen-binding potential (Borchard et al., 2009). Whereas paracellular absorption is characterized by intercellular junctions at the luminal side of epithelial cells, so called tight junctions (TJ) (Harder et al., 2008).

Table 1.3 Summarize the barriers of different nature and their effects on protein and PP drug absorption.

Table 1.3 Oral absorption barriers and their bearing on PP drug absorption from the GIT.

Barrier Nature	Location and Description	Effect on Protein PP Drug Absorption
Chemical	Acidic environment in stomach (pH 1.2-3.0) and alkaline environment in intestine (pH 6.5-8.0)	pH-induced oxidation, deamidation or hydrolysis
Enzymatic	Luminally secreted, membrane-bound, and cytosolic proteolytic enzymes throughout the length of GIT; and microbial flora present in the colon	Proteolytic degradation in the lumen and during absorption via enterocytes Breakdown of PP as part of their metabolic activity
Physical	Unstirred aqueous boundary layer and viscous mucus layer covering the surface of GI epithelial cell lining and the lipid bilayer of epithelial cell membrane Intercellular spaces (mean pore radii of 0.8, 0.3 and 0.3 nm in the duodenum, ileum, and colon, respectively) gated by closely fitting tight junctions (TJs) on the apical side of epithelial cells P-glycoprotein present on the epithelial cell membrane (Mizuno et al., 2003)	Decreased diffusion to reach absorptive epithelial cell membrane. Inhibits absorption of hydrophilic and charged PP drugs through the cell (trans-cellular transport) TJs prevent passage of PP macromolecules through the intercellular spaces (paracellular transport) Promotes apically polarized efflux to remove permeated drug molecules (Mizuno et al., 2003)

1.4. Existing Platform Technologies for Protein Delivery via the Oral Route

1.4.1. Chemical Modification of Protein Prodrugs/Analogues

The pro-drug/analogue approach can be defined as the conversion of proteins into derivatives (pro-drugs and analogues) by incorporation of sufficient chemical

modifications so as to produce oral activity (Oliyai et al., 1996). Chemical modification (such as masking or blocking polar amide bonds and terminal amino and carboxyl groups) primarily brings about an alteration in the physico-chemical properties of drugs such as lipophilicity, charge, molecular size, chemical stability, etc., which are known to affect their membrane permeability, enzymatic properties and affinity for the carrier systems used to deliver prodrugs orally (Mizuno et al., 2003).

Pro-drugs have the ability to be converted quantitatively to the parent protein *in vivo* by a spontaneous or unspecified plasma enzyme-catalyzed reaction after absorption (Bundgaard et al., 1991). Modified proteins that lack such an ability are considered to be new proteins rather than pro-drugs and the approach is known as an analogue approach (Bundgaard et al., 1991).

Pegylation, the coupling of a protein with polyethylene glycol (PEG) polymer, has been shown to improve biopharmaceutical and clinical properties (including enhanced solubility, sustained absorption, reduced immunogenicity, decrease dosing requirements due to increased circulation time and reduced toxicity). Pegylation is now being explored for developing orally effective molecules. A PEG-insulin complex has been developed by the Nobex Corporation for peroral administration. The success of PEG modified proteins is due to their non-toxic, non-immunogenic and highly water soluble nature. PEG polymer is approved for use by the United States Food and Drug Administration (US-FDA) (Charles et al., 2000).

1.4.2. Absorption Enhancers

Absorption enhancers are defined as "formulation components that act upon different drug absorption pathways with a defined mechanism of action to improve the permeation of poorly permeable drugs" (Swenson et al., 1992).

The concept underlying permeation and absorption enhancers is to improve trans-cellular and para-cellular transport of proteins through the intestine wall.

Mechanisms involved in the improvement of intestinal permeation by absorption enhancers involve (Chao et al., 1999):

1. interactions of absorption enhancers with membrane lipid/protein which leads to membrane perturbation followed by an increase in permeability e.g. bile acids, surfactants and mid-chain length fatty acids including caproates (C6), caprylates (C8) and laureates (C12) (fatty acids with aliphatic chains of 6-12 carbon atoms).
2. the capability of bile salts to form complexes with calcium, which has been shown to be responsible for opening and loosening tight junctions and thereby enhancing para-cellular permeation.

Most absorption enhancers disrupt the intestinal barrier. However, the possibility of manifestations of rapid reversibility and the transient nature of the damage caused by absorption enhancers are key to their acceptability. Extended disruption of the barrier function of the GI cell layer covering the intestine may result in loss of control of water and ion movement causing diarrhoea as well as allowing non-specific absorption of toxins and other chemicals that could lead to other side-effects (Swenson et al., 1992).

Other absorption enhancers involve mucolytic agents since proteins of a molecular mass greater than 5 kDa can hardly permeate the mucus layer covering the GI epithelial cell lining. Use of mucolytic agents, e.g., the addition of 2% *N*-acetylcysteine led to an approximately 4-fold increase in the amount of a model protein, with a molecular mass of 12.4 kDa that could permeate a porcine mucus layer within 5 hr (Bernkop-Schnurch et al., 1996).

1.4.3. Enzyme Inhibitors

Rapid luminal enzymatic degradation can be regarded as a bottle neck accounting for the low bioavailability of proteins. The enzymatic degradation of protein drugs is caused by proteases and peptidases that are secreted or located throughout the length of the GIT. Hence, the use of protease inhibitors is gaining considerable interest. In the case of insulin (trypsin and chymotrypsin being the most important intestinal enzymes) the use of

inhibitors such as a protinin (a pancreatic inhibitor or soybean tryspin inhibitor) were found to affect the intestinal absorption and degradation of insulin (Mustata et al., 2006).

Because of the side-effects of these agents and the need for nutritive proteins, the use of these inhibitors is questionable.

1.4.4. Multifunctional Mucoadhesive Polymers

Several polymers have been tested in the past few years including polyacrylates, polycarbophil (Ghandehari et al., 2008) as well as chitosan (Tiyaboonchai et al., 2003 & Wong et al., 2009) to promote absorption of proteins. These have been shown to enhance membrane permeability. Different mechanisms have been proposed for this:

1. the mucoadhesive properties of these polymers enhance the contact between the formulation and mucosal surface and so increase the residence time at the site of drug absorption.
2. shielding enzymatic attacks via:
    - inhibition of proteolytic enzymes present in the GIT, which bind to essential enzyme cofactors(calcium and zinc) in the enzyme system causing loss of enzyme activity.
    - direct interaction of polymers with enzymes reduces the free enzyme concentration and sometimes denatures enzymes (Walker et al., 1999).

1.4.5. Particulate Carrier Delivery System

Various particulate carriers for oral protein delivery have been applied to protect protein from acidic and enzymatic degradation in the harsh environment of the GI tract. These particulate systems could also provide enhanced delivery of drugs across the epithelial mucosa (Toorisaka et al., 2003), control release rate, and target drug delivery to specific sites in the intestine (Stolnik et al., 1995). Some of the delivery systems that have been used with success are as follows.

1. Matrix carrier systems

   Nanoparticles and microparticles are solid colloidal particles with diameters ranging from 1-1000 nm and 4 to 5 um, respectively. Polymers used to form nano/micro particles can be both synthetic and natural. Currently the most prominent materials are biodegradable polyesters e.g. polylactide (PLA) (Vila et al., 2002), poly(lactide-co-glycolide) (PLGA) (Vila et al., 2002) and chitosan (Azevedo et al., 2011).

   There are two types of nanoparticles depending on the preparation process: nanospheres and nanocapsules. Nanospheres have a monolithic-type structure (matrix) in which drugs are absorbed or adsorbed onto their surfaces. Nanocapsules exhibit a membrane-wall structure and drugs are entrapped in the core or adsorbed onto their exterior (Allemann et al., 1993). Depending on the biodegradable material used, particle size distribution and degradation kinetics, various delivery profiles and therapeutic applications may be achieved.

2. Nanovesicles

   Surfactants and amphiphilic polymers/copolymers form colloidal dispersions of molecular aggregates or vesicles (Li et al., 2012). Lipid-based vesicles are solid-lipid nanocarriers (SLN), micelles, lipid microspheres and liposomes (LIP) while surfactant based are termed niosomes (Li et al., 2012). Liposomes provide well proven and extensively investigated particulate carrier systems that have been employed for potential site specific drug delivery. Liposomes consist of one or more phospholipid bilayers separated by internal aqueous compartments (Oussoren et al., 2000). The most studied phospholipid used in liposomes is phosphatidycholine (or lecithin). Liposomes have the ability to entrap proteins. The attractiveness in the application of liposomes lies in their compatibility with cellular systems and low toxicity. The major disadvantages of liposomes are their poor chemical and physical stability and the rapid un-controlled release of the drug from the formulation (Li et al., 2010). The surface coating of liposomes with chitosan, for example, can help to overcome their instability problems in acidic and bile salts containing GI fluids. Oral administration of chitosan-coated bioadhesive liposomes containing insulin to

normal rats has been shown to result in a marked reduction in blood glucose levels in comparison to uncoated liposomes (Wu et al., 2004). Similarly, niosomes or nonionic surfactant vesicles can be used as drug carriers. Niosomes are vesicles resulting from the self-assembly of nonionic surfactant molecules, such as polyoxyethylene alkyl ethers. The stability of niosomes and protection of insulin against enzymatic degradation has been reported (Varshosaz et al., 2003).

The polymer or copolymers used to form micelles should (i) spontaneously self-assemble in water, (ii) enhance drug solubility (iii) provide high loading efficiency (iv) remain stable in GI tract, (v) be biocompatible and non-toxic, and (vi) cheap and easily accessible (Alli et al., 2013).

3. Liquid emulsions

   Liquid emulsions are another category of delivery systems that have been investigated as an oral delivery system for proteins. This carrier system allows a reduction in side-effects but is thermodynamically unstable. Therefore, emulsions tend to agglomerate or even break down and the drug is rapidly released once it reaches the blood stream. Water-in-oil microemulsions have been shown to enhance oral bioavailability of proteins (Bilati et al., 2005).

4. Colonic drug delivery systems

   Since the colon is a less hostile acidic environment and has lower concentrations of proteolytic enzymes than the stomach, colon targeted delivery is currently of significant interest. Design of a colonic drug delivery system can be based upon a site-specific chemical signal, e.g. the pH difference between the small and large intestine. Methacrylic acid copolymers (Eudragit) and cellulose derivatives (HPMC) are used for such purposes; or the design can be based upon a physical signal e.g. the presence of microbial enzymes (bacterial glycosidases). Copolymers of styrene can be used for such purposes (Malik et al., 2007).

Figure 1.2. shows a diagrammatic representation of different barriers to protein and peptide drug absorption from the intestinal tract and technologies to overcome such barriers.

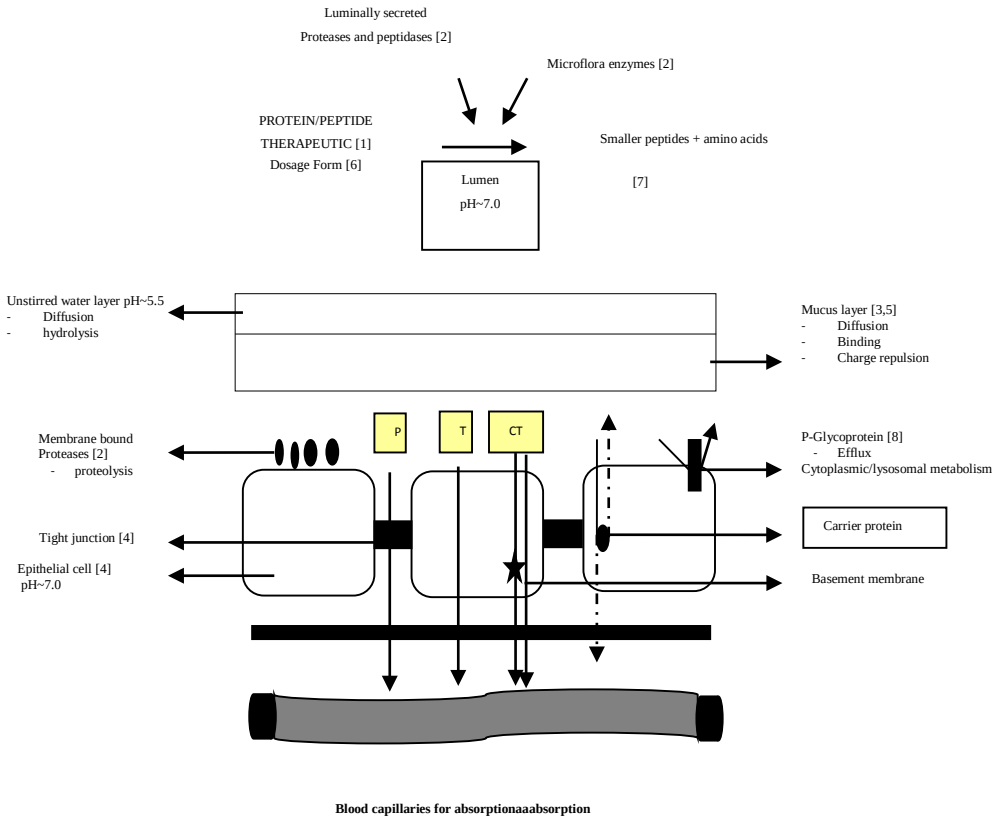


Figure 1.2. Diagrammatic representation of different barriers to protein and peptide drug absorption from the intestinal tract (Mizuno et al., 2003). The text boxes show the pathways for drug absorption: P; paracellular; T, transcellular; CT, carrier mediated transport. Target sites for different absorption enhancement strategies are indicated by numerals in parenthesis: 1, pro-drugs/analogues; 2, protease inhibitors; 3, mucolytic agents; 4, paracellular and transcellular absorption enhancers; 5, mucoadesive polymers; 6, dosage form modifications; 7, pH modulation for enzymatic activity minima and 8, p-glycoprotein inhibitors.

1.5. Protein Formulation

Formulation components may alter the environment of a protein, resulting in either an increase or decrease in protein stability. The following are the main factors affecting formulations.

1. Protein concentration is very important since protein aggregation is generally concentration dependent. It has been suggested that increasing the protein

concentration to greater than 0.02 mg/mL may potentially facilitate protein aggregation (Ruddon et al., 1997).

However, the effect of protein concentration on its aggregation properties may depend on the mechanism of aggregation and the experimental conditions used. The formation of bovine insulin fibrils (aggregates) increases with increasing protein concentration from 0.75 to 5% during storage at pH 2.5 (Brange et al., 1997). In contrast, bovine insulin at 0.1 mg/mL (pH 7.4) aggregates more readily than that at 0.6 mg/mL during shaking. This is due to the formation of insulin hexamers at 0.6 mg/mL which are less susceptible to hydrophobic surface induced aggregation than insulin monomers (Sluzky et al., 1992). In some cases, protein concentration may affect chemical degradation. Increasing insulin concentration increases the formation of covalent insulin polymers in neutral solution at 37 or 45°C (Brange et al., 1992A), further increasing aggregation.

2. Formulation pH

Control of pH is an important part of the formulation process, because of the pH dependence of protein solubility as well as physical and chemical stability. Usually proteins are stable in a narrow pH range such as pH 6.5 – 7.0 for recombinant factor VIII (Fatouros et al., 1997). Formulation pH may affect both the physical and chemical stability of a protein; such as protein aggregation. For example, at pH 2.5, bovine insulin forms a significant amount of fibrils within a few hours at 37°C. The monomeric form has the highest tendency to form fibrils due to its large exposed hydrophobic surface area as compared to other forms (Ruddon et al., 1997). As the pH increases from 2 to 7, monomeric insulin gradually converts to insulin dimers and hexamers. Different chemical degradation mechanisms may be facilitated at different pH values.

3. Nature and concentration of excipient(s)

Excipients can have different effects on the chemical and physical stability of proteins. The stabilizing effects of these excipients can vary significantly. A summary of the key factors regarding excipients is presented in Table 1.4

Table 1.4 Key factors regarding excipient stabilization of proteins.

Excipient	Key factors
Stabilizing buffer	- Phosphate buffer has been shown to stabilize certain proteins (Won et al., 1998). - Both buffer species and concentration may affect the physical and chemical stability of proteins and so a balanced buffer concentration should be selected to maximize protein stability (Chan et al., 1996).
Sugars and polyols	- Sucrose and trehalose are mostly used as stabilizers (Pikal et al., 1991). - Different sugars or polyols may stabilize a protein to a similar or different degree (Pikal et al., 1991). - Sugars and polyols can also protect proteins from chemical degradation (Li et al., 1996). - Stabilizing effect of sugars depend on their concentration (Herman et al., 1996). - Reducing sugars should be avoided due to their interaction with amino groups in proteins via the Maillard reaction (Chuyen et al., 1998).
Surfactants	- Nonionic surfactants are generally preferred in protein stabilization (Bam et al., 1995). - Low concentrations of non-ionic surfactants are often sufficient to prevent or reduce protein aggregation due to their relatively low critical micelle concentration (Bam et al., 1995). - Non-ionic surfactant can also inhibit chemical degradation in protein - Ionic surfactants are usually not used because they can bind to both polar and non-polar groups in proteins and cause denaturation (Giancola et al., 1997).
Salts	- Salts may stabilize, destabilize or have no effect on protein stability depending on type and concentration of salt, nature of ionic interactions and the charge in a protein (Kohn et al., 1997).

Polymers	- Polymers stabilize proteins by one or more of these properties: surface activity, preferential exclusion, steric hindrance of protein-protein interactions and increasing viscosity thus limiting protein structured movement. - Polymers may inhibit chemical degradation in proteins - The polymers used as stabilizers should be stable against chemical and/or enzymatic degradation so that they can retain their stabilizing effect during storage.
Metal ions	- Metal ions, such as calcium and zinc, can be used as protein stabilizers as they bind to a protein and make the overall protein structure more rigid, compact and stable (Dunn et al., 2005).

1.6. Protein Instability

One of the most challenging problems in the development of liquid protein pharmaceuticals is their physical and chemical instability. According to the US-FDA, EMEA and ICH a pharmaceutical product is considered as stable as long as it degrades by no more than 10% in 2 years (EMA Q6B, 1999). However, for proteins, the term stability needs to be defined more accurately.

1.6.1. Physical Degradation

Physical stability is generally defined as the ability of a protein to retain at least its tertiary structure, which is crucial for biological activity.

The physical degradation of proteins includes:

- conformational changes
- undesirable adsorption to surfaces
- denaturation
- precipitation and aggregation (Wang et al., 1999).

Protein aggregation is a major instability issue in all phases of drug development. Protein aggregates may exhibit no activity or reduced activity, decreased solubility and altered immunogenicity. The presence of any insoluble aggregates in a protein pharmaceutical is generally not accepted for product release, and should be tested for and monitored over the shelf-life of the therapeutic agent (ICH Q6B, 1999). The physical stability of a protein is expressed as the difference in free energy, ΔG_u, between the native and the denatured states. For protein molecules in solution a temperature dependent equilibrium composition exists with protein molecules in either of these two states, as long as unfolding is reversible. At low temperatures ΔG_u is positive, indicating that the native form of the protein is stabilised. As the temperature is increased, $\Delta G_u$is reduced to zero and then to negative values at which point the denatured form of the protein becomes thermodynamically favoured. The reduction in ΔG_u is driven by entropy. The enthalpy of denaturation is endothermic and becomes increasingly endothermic as the temperature of an aqueous protein solution is raised because the heat capacity change of denaturation is

also positive. For a reversible system the native form of the protein should be recoverable by cooling. However in many cases, this reversibility is not observed because of the subsequent aggregation of the denatured protein molecules. Thus unfolding is made irreversible by aggregation:

Therefore, any stress that decreases ΔG_u and increases K (the equilibrium constant for the reaction) will cause an accumulation of irreversibly, inactivated forms of the protein. Such stresses may include alterations in pH, protein concentration and temperature (Arakawa et al., 2002).

1.6.2. Chemical Degradation

Chemical stability (Table 1.5) typically involves the integrity of the amino acid sequence (primary structure) and the reactivity of the amino acid side chains (R groups). Chemically, the protein surface is highly heterogeneous and contains reactive groups. Long term exposure of these groups to environmental stress causes various chemical alterations. The chemical stability, for instance, of a pharmaceutical protein can be impaired by proteolysis. Such a degradation pathway leads to two or more products of smaller molecular weight which should be characterized. It is indeed not only necessary to recover a high amount of active protein from the pharmaceutical but even low amounts of the degraded protein must also be physiologically safe, since degradation products may be therapeutically active or cause unpredictable side-effects such as toxicity or antigenicity.

Table 1.5 Major chemical degradation mechanisms for proteins.

Chemical Degradation	Main Points	Reference
1. Deamidation	• Deamidation is the most common degradation pathway in proteins. • Asn and Gln are the two amino acids susceptible to deamidation. • Rate, mechanism and location of deamidation are pH dependent; deamidation is much reduced approaching neutral pH. • The sequence, conformation and flexibility of the protein is important for the catalytic effect of appropriate residues or side chains in close proximity to Asn or Gln.	(Daniel et al., 1996) (Brange et al.,1992 B) (Cleland et al., 1993)
2. Oxidation	• The side chains of His, Met, Cys, Trp and Tyr residues are potential sites of oxidation. • The most easily oxidizable sites are thio groups on Met and Cys. • Oxidation at Met residues is not as significant as deamidation and hydrolysis. • Formulation pH may affect the rate of oxidation by changing the oxidation potential of oxidant, the affinity of binding (catalytic metal ions and ionizable amino acids) and stability of oxidation intermediates.	(Daniel et al., 1996) (Powell et al.,1996) (Wang et al., 1999)
3. Disulfide bond breakage and formation	• Disulfide bonds are often critical in controlling both protein activity and stability. • Free Cys residues in proteins can be oxidized easily to form disulfide bond linkage or cause thio-disulfide exchange, causing protein aggregation or polymerization.	(Evans et al., 1986) (Wang et al., 1996)
4. Hydrolysis	• Amino acids, the components of proteins, are subject to acid and base hydrolysis. • The Asp-Y bond may be at least 100 times more labile than other peptide bonds in dilute acid to hydrolysis. • In many cases, hydrolysis is a continuation after deamidation of Asn residue. Insulin first forms Asp derivatives at Asn^{A-21} and or Asn^{B-3}. Depending on the solution pH during storage, these derivatives are further hydrolysed and the rate of hydrolysis at B3 position is independent of the insulin strength between 40 and 100 IU/mL.	(Powell et al., 1996) (Brange et al.,1992)

Other chemical degradation pathways described in the literature (Wang et al., 1999) include isomerization, deglycosylation and the Maillard reaction.

1.7. Analytical Techniques for Monitoring Protein Instability

The choice of an accurate analytical technique for monitoring protein stability is of importance, since certain methods might not be able to discriminate between denatured and native protein. A simple spectrometric method, for instance, does not always allow discrimination between the monomeric form of the protein and its aggregates, whereas high performance liquid chromatography (HPLC) might separate these species and thus provide more accurate qualitative data (Blanco et al., 1997). However, HPLC cannot exclusively quantify the amount of active antigen, as is the case with enzyme-linked immunosorbent assay (ELISA) techniques (Johansen et al., 1999).

Nowadays, Fourier-transform infrared spectroscopy (FTIR) has become a popular, non-invasive method, as it is able to characterize the secondary structure of entrapped proteins (Jorgensen et al., 2003).

Table 1.6 lists a number of analytical testing methods for proteins and the detection ability of such methods.

1.7.1. Revered –Phase High Performance Liquid Chromatography (RP-HPLC)

This method takes advantage of the hydrophobic properties of proteins. The functional groups on the column matrix contain between one and up to eighteen carbon atoms in a hydrocarbon chain. The longer the chain, the more hydrophobic is the matrix. The hydrophobic patches of proteins interact with the hydrophobic chromatographic matrix. Proteins are then eluted from the matrix by increasing the hydrophobic nature of the mobile phase. Acetonitrile is a commonly used solvent, although other organic solvents such as methanol may also be employed. The solvent is made acidic by the addition of trifluoroacetic acid, since proteins are increasingly soluble as pH values become further removed from their isoelectric point (pI) values. A gradient with

increasing concentration of hydrophobic solvent is passed through the column. Different proteins have different hydrophobicities and are eluted from the column depending on the "hydrophobic potential" of the solvent.

This technique can be very powerful. It may detect the addition of a single oxygen atom to the protein, as when a methionyl residue is oxidized, or when the hydrolysis of an amide moiety on a glutamyl or asparginyl residue occurs. Disulfide bond formation or shuffling also changes the hydrophobic characteristic of the protein. Hence, RP-HPLC can be used not only to assess the homogeneity of the protein, but also to follow degradation pathways occurring during long term storage.

Reversed-phase chromatography of proteolytic digests of recombinant proteins may serve to identify a protein. Enzymatic digestion yields unique peptides that elute at different retention times or different organic solvent concentrations. Moreover, the map, or chromatogram, of peptides arising from enzymatic digestion of one protein is quite different from the map obtained from another protein. Several different proteases, such as trypsin, chymotrypsin and other endoproteinases, are used for such identity tests (Arakawa et al., 2002).

Table 1.6 Analytical testing methods and their detection ability for proteins.

Method	**Size**	**Charge**	**1º Structure**	**2º/3º Structure**	**Purity**	**Potency**
HPLC						
Size exclusion	+++	-	-	++	++	-
Ion exchange	-	++++	+++	-	+++	-
Reverse phase	+++	+/-	+++	++	+++	-
Electrophoresis						
(SDS-PAGE)	+++	-	+++	-	+++	-
Isoelectric Focusing (IEF)	-	+++	+	++	+++	+
Western Blot (W-Blot)	+++	-	++	+++	+++	-
Assays						
Immunoassays	-	-	+/-	+/-	-	++
ELISA	-	-	++	+++	-	++
In vivo assay	-	-	+++	++++	+/-	+++++

1.7.2. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA provides a means to quantitatively measure extremely small amounts of proteins in biological fluids and serves as a tool for analyzing specific proteins during purification. This procedure takes advantage of the fact that plastic surfaces are able to adsorb low but detectable amounts of proteins. This is a solid phase assay.

Therefore, antibodies against a certain desired protein are allowed to adsorb to the surface of microtitration plates. Each plate may contain up to 96 wells so that multiple samples can be assayed. After incubating the antibodies in the wells of the plates for a specific period of time, excess antibody is removed and residual protein binding sites on the plastic are blocked by incubation with an inert protein. During the ELISA assay, a sample solution containing the protein of interest is incubated in the wells and the protein (Ag) is captured by the antibodies coating the well surface. Excess sample is removed and other antibodies which now have an enzyme (E) linked to them are added to react with the bound antigen.

The format described in Figure 1.3 is called a sandwich assay since the antigen of interest is located between the antibody on the titre well surface and the antibody containing the linked enzyme. A suitable substrate is added and the enzyme linked to the antibody-antigen-antibody complex converts this compound to a coloured product. The amount of product obtained is proportional to the enzyme adsorbed in the well of the plate. A standard curve can be prepared if known concentrations of antigen are tested in the system, and the amount of antigen in the unknown sample can be estimated from this standard curve. A number of enzymes can be used in ELISA. However, the most commonly used are horseradish peroxides and alkaline phosphates. A variety of substrates for each enzyme are available which yield coloured products when catalysis occurs by the linked enzyme. Absorbance of the coloured product solution is measured on plate readers; instruments which rapidly measure the absorbance in all 96 wells of the microtitration plate, and data processing can be automated for rapid throughput of information. The above ELISA format is only one of many different methods. For

example, the microtitration wells may be coated directly with the antigen rather than having a specific antibody attached to the surface. Quantitation is made by comparison with known quantities of antigen used to coat individual wells (Arakawa et al., 2002).

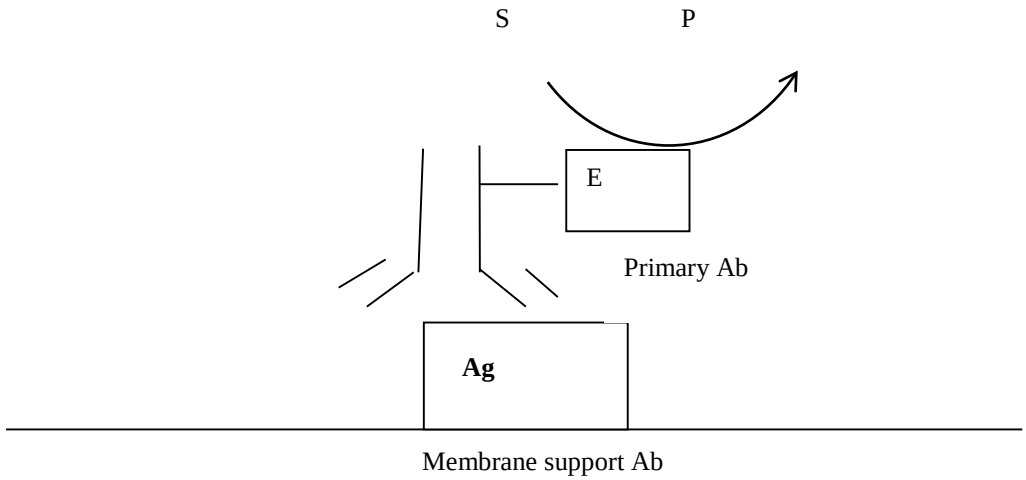


Figure 1.3. Common ELISA format system used to detect antigens (Ag), on membranes. (Abbreviations used: Ab, antibody; E, enzyme, such as horseradish peroxidase or alkaline phosphatise; S, substrate; P, product, either coloured and insoluble or chemiluminescent.)

1.7.3. Fourier-Transform Spectroscopy (FTIR)

Infrared spectroscopy can give information on the secondary structure of a protein in the aqueous and solid state. Conventional infrared spectroscopy measurements can be performed to obtain complementary information on the protein structure. A disadvantage of FTIR for protein formulation studies is the interference of the large absorption peak of water (Jorgensen et al., 2003).

1.8. Insulin as Drug of Choice for an Oral Delivery System

1.8.1. Need for Insulin

Diabetes Mellitus (DM) is defined as a group of metabolic disorders characterized by high blood glucose levels (hyperglycaemia). The incidence of diabetes is rising; the number of affected patients worldwide is expected to increase from over 280 million

adults at the end of 2014 to over 400 million by 2030. The total annual costs associated with the treatment of diabetes and its complications amount to US$500 billion (Shaw et al., 2010).

Two main types of primary diabetes mellitus are identified; a comparison is presented in Table 1.7. Type I diabetes (Insulin Dependent Diabetes Mellitus, IDDM), accounts for 10% of all cases of diabetes. It results from a deficiency in insulin which regulates blood glucose level. Type II diabetes (Non-Insulin Dependent Diabetes Mellitus NIDDM) is associated with obesity and lack of physical activity. Patients with type NIDDM develop insulin resistance. Other types of DM are type III diabetes which refers to specific types of diabetes caused by rare genetic defects in pancreatic islet insulin secreting cell function and genetic defects in insulin action and type IV which refers to diabetes related to pregnancy. Diabetes may also occur secondarily to general disease of the pancreas (Owens et al., 2003).

Insulin replacement therapy provides the most effective means for glycemic control. Replicating physiological insulin secretion as a means of restoring normal metabolism, minimizes complications, and has thus become the essential goal of diabetes treatment (Subhashini et al., 2013).

Modes of administration of insulin include subcutaneous injection using syringes, pump or by repeated-use insulin pens with needles. Amongst alternative routes for insulin administration, the oral route is potentially the most convenient.

Table 1.7. Comparison of Type I and Type II diabetes (Owens et al., 2003).

Type I	Type II
Childhood and adolescence onset	Late middle-age elderly onset
Thin	Obese
Ketoacidosis common	Ketoacidosis rare
Sever insulin deficiency	Relative deficiency and end –organ resistance
Islet-cell antibodies	No islet –cell antibodies
Autoimmune mechanism	No autoimmune mechanism
Genetic predisposition associated with HLA-DR genotype	Polygenic inheritance

1.8.2. History of Insulin

Langerhans identified the eponymous islets in the pancreas in the 1860's but did not understand their function. The link between the islets of Langerhans and diabetes was suggested by de Mayer in 1909 and by Sharpey-Schaffer in 1917, but it was Banting and Best who proved this association in 1921. After ligating the pancreatic duct in dogs, which resulted in atrophy of the exocrine pancreas, these investigators used acid ethanol to extract from the remaining tissue an islet cell factor that had potent hypoglycaemic activity. The factor was named insulin, and it was quickly learned that bovine and porcine islets contained insulin that was active in humans. Within a year, insulin was in widespread use for the treatment of diabetes and proved to be lifesaving (Rosenfeld et al., 2002).

1.8.3. Insulin Chemistry and Structure

Insulin is a hormone consisting of 2 polypeptides (PP) chains having 51 amino acids; the A-chain has 21 while the B-chain has 30 amino acids, which are linked by two disulphide bridges that connect A7 to B7 and A20 to B19. A third intra-chain disulfide bridge

connects residues 6 and 11 of the A chain (Champe et al., 2008). The structure of insulin is illustrated in Figure 1.4.A (www.biotopic.co.uk).

Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulfide bonds, both ends of the A chain and the C-terminal residues of the B chain. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species and insulin from one animal is very likely to be biologically active in other species (Chien et al., 1996). While the A chain forms two nearly anti-parallel α helices (A2 to A8 and A13 to A20) the B chain forms a single α helix (B9 to B19) followed by a turn and a β strand (B21 and B30), Figure 1.4.B (www.biotopics.com.uk).

Insulin molecules exist as a monomer only at low concentration (<0.1μM or ~0.6 μg/mL). At higher concentrations, which are often found in pharmaceutical preparations, insulin dimerizes (association dimer). At pH 4-8 and in the presence of Zn^{2+} ions, three dimers assemble further at concentrations greater than 10 μM to form a hexamer (Hvidt et al., 1991).

Insulin is produced and stored in the body as a hexamer, however, in the circulation it is maintained as a monomeric molecule which is the active form (Binder et al., 1984). The hexamer is an inactive form with long-term stability, which serves as a way to keep the highly reactive insulin protected, yet readily available. The hexamer-monomer conversion is one of the central aspects of insulin formulations for injection (Binder et al., 1984).

1.8.4. Insulin Secretion

In the resting cell with normal/low adenosine triphosphate (ATP) levels, potassium diffuses down its concentration gradient through ATP-gated potassium channels, maintaining the intracellular potential at fully polarized negative level. Insulin release is minimal. When glucose is available, ATP production increases, potassium channels close,

and depolarization of the cell results. The resulting depolarization of the membrane activates voltage-gated calcium channels and allows more calcium to enter the cell. Calcium mediates fusion of insulin secretory vesicles with plasma membrane, leading to insulin secretion (Figure 1.5; Bilous et al., 2014).

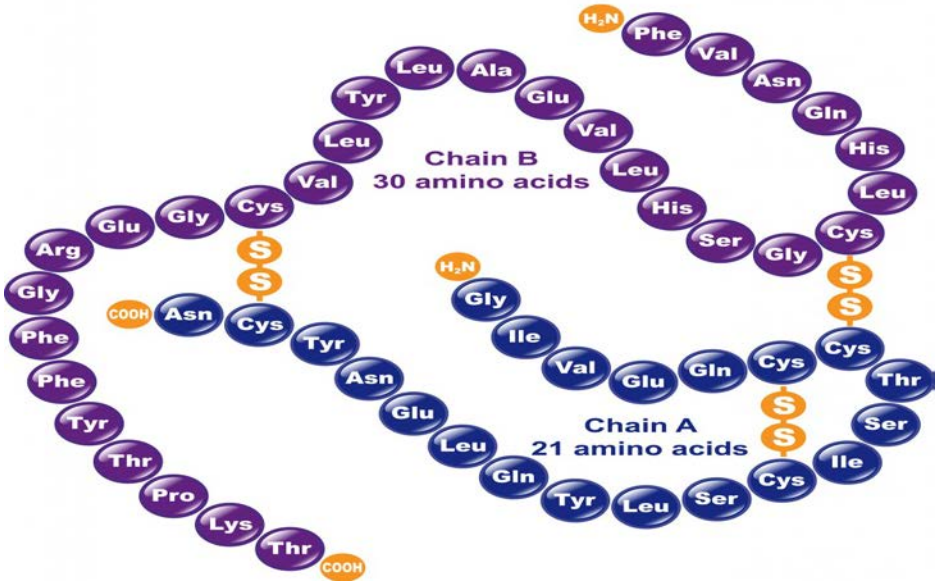


Figure 1.4.A. Primary structures of insulin. (www.biotopics.com.uk, A)

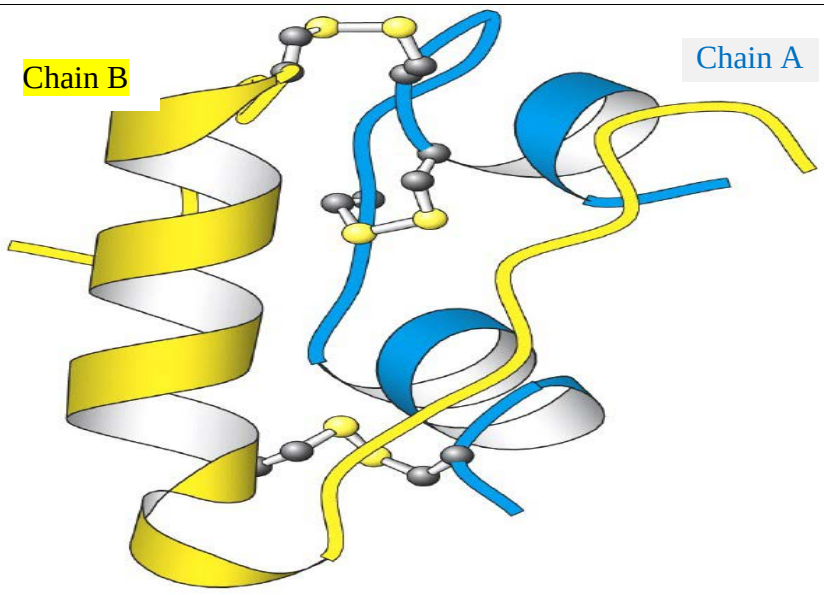


Figure 1.4. B. Secondary structures of insulin. (www.biotopics.com.uk, B)

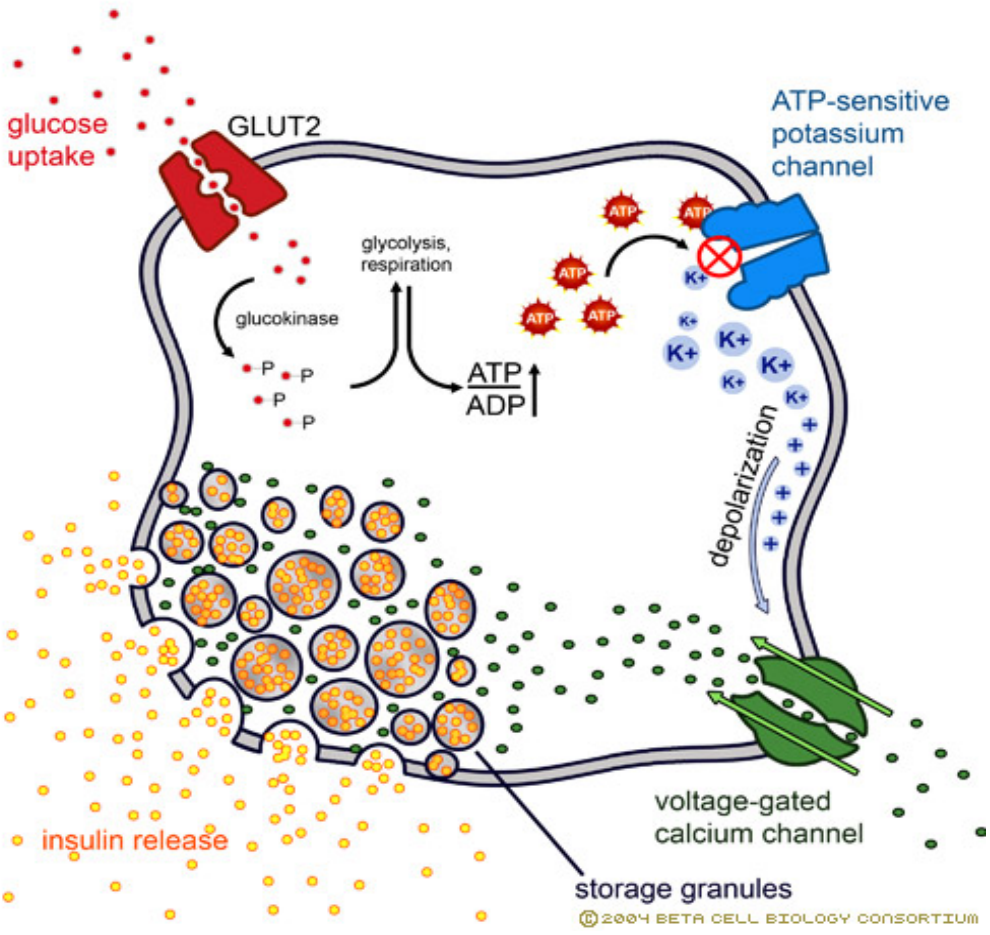


Figure 1.5. Insulin secretion. (www.betacell.org)

1.8.5. Insulin Receptor

Insulin receptors are found on the membranes of most tissues. The full insulin receptor consists of two covalently linked heterodimers, each containing an extracellular α subunit, which constitutes the recognition site, and a β subunit that penetrates through the plasma membrane and contains a tyrosine kinase.

The binding of an insulin molecule to the α subunits at the outside surface of the cell activates the receptor and causes phosphorylation of the β subunits, thus activating the catalytic activity of the receptor. The activated receptor then phosphorylates a number of other intracellular proteins and converts them to the active state. The first proteins to

be phosphorylated by the activated receptor tyrosine kinases are insulin receptor substrate -1 through-6 (IRS-1 to IRS-6).

Tyrosine phosphorylated IRS then displays binding sites for other signalling partners. Among them, PI3K, phosphatidylinositol-3-kinase, which has major role in insulin function, mainly via the activation of the Akt/ PKB. Activated Akt induces glycogen synthesis through inhibition of GSK-3; protein synthesis via mTOR. Insulin stimulate glucose uptake in the muscle and adipocytes via translocation of GLUT4 vesicles to the plasma membrane. GLUT4 translocation involves the PI3K/ Akt pathway. Insulin signalling has also growth and mitogenic effects, which is mediated by the activation of the Ras/MAPK pathway. (Bilous et al., 2014).

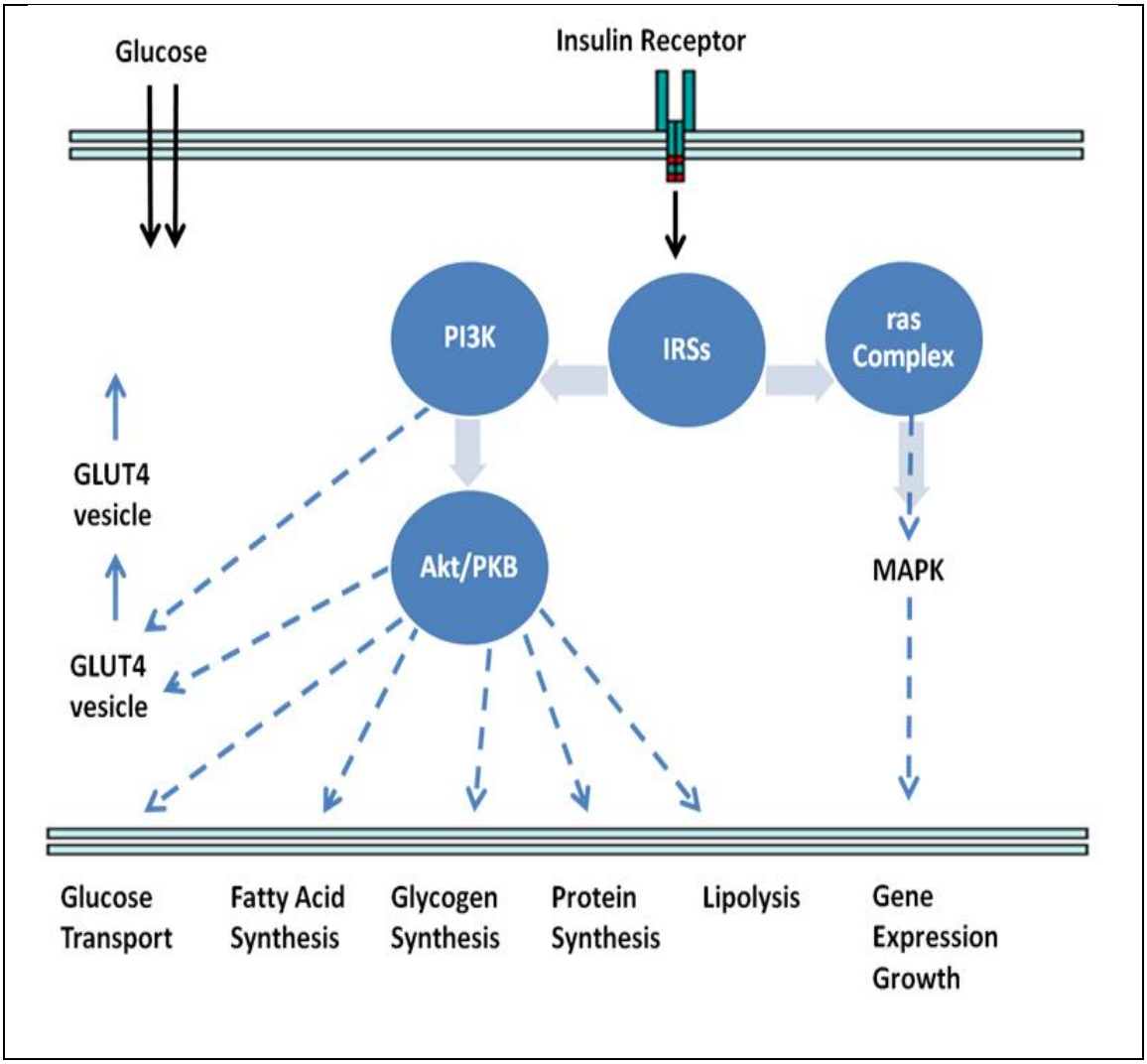


Figure 1.6. Insulin receptor signalling.

1.8.6. Metabolic Action of Insulin

Insulin is an anabolic hormone, causing cells to take up energy substrates at times of excess. The action of insulin on muscle and fat cells is countered by catabolic hormones like the glucagons and growth hormone.

A summary of the effects of insulin on carbohydrate, fat and protein metabolism in liver, muscle and adipose tissue is presented in Table 1.8. (Bilous et al., 2014).

Table 1.8. Summary of the effects of insulin.

Type of metabolism	**Liver cells**	**Fat cell**	**Muscle**
Carbohydrate metabolism	⇓ gluconeogenesis	⇑ glucose intake	⇑ glucose uptake
	⇓ glycogenolysis	⇑ glycerol synthesis	⇑ glycolysis
	⇑ glycolysis		⇑ glycogenesis
	⇑ glycogenesis		
Fat Metabolism	⇑ lipogenesis	⇑ synthesis of triglyceride	
	⇓ lipolysis	⇑ fatty acid synthesis	
		⇓ lipolysis	
Protein metabolism	⇓ protein breakdown		⇑ amino acid uptake
			⇑ protein synthesis

1.8.7. Insulin Therapies: Current and Future Trends

1- Commercial Available Preparation

The subcutaneous (S.C) injections of insulin have been considered the main treatment for insulin replacement after its discovery.

The following insulin preparations have been developed and are available commercially for glycaemic control in insulin-dependent diabetic patients, summarized in Table 1.9.

Table 1.9. Hypoglycemic activity and duration of activity of commercial insulin products (Ferri, 2013).

	Insulin Preparation		Hypoglycaemic Activity		
	Generic Name	**Trade Name**	**Onset**	**Peak (hr)**	**Duration (hr)**
Rapid acting	Insulin Aspart	Novolog®	5-10 min	1-3	3-5
	Insulin Lispro	Humalog®	<15 min	0.5-1.5	2-4
	Insulin Glulisine	Apidra ®	<15 min	0.5-1.5	1-2.5
Short acting	Regular	Humulin®R Novolin®R	0.5-1 hr	2-3	3-6
Intermediate acting	NPH	Humulin®N Novolin®N	2-4 hr	4-10	10-16
Long acting	Insulin Glargine	Lantus®	1 hr	No peak	24
	Detemir	Levemir®	1-2 hr	No peak	6-23
Combinations	70% NPH 30% Regular	Humulin® 70/30 Novolin® 70/30	0.5-1 hr	2-3 & 4-10	10-16
	70% Aspart Protamine + 30% Aspart	Novolog® Mix 70/30	5-10 min	1-3 & 4-10	10-16
	50% Lispro Protamine + 50% Lispro	Humalog® 50/50	< 15 min	0.5-1.5 & 4-10	10-16

2- Non-Invasive Insulin Delivery System

Insulin delivery by a non-invasive route is an area of current interest in diabetes mellitus treatment. Research approaches for non-invasive insulin delivery are summarized in Table 1.10.

Table 1.10 Potential non-invasive insulin delivery options (Cavaiola et al., 2014, Yatura. 2013 & Shah et al. 2010).

Delivery	Potentials
Oral Route **1. Enteric** **2. Buccal**	Oral enteric insulin delivery has limited bioavailability. Insulin is too large and hydrophilic to readily cross the intestinal mucosa. Polypeptides undergo extensive enzymatic and chemical degradation. Only around 0.5% of a dose of oral insulin reaches the systemic circulation. Ongoing phase I and II clinical trials with new formulation suggest a bioavailability of 5%, which may result in an acceptable glucose lowering effect.
	Liquid aerosol insulin is sprayed into the buccal cavity without entering the airways. A liquid formulation of human recombinant insulin with added enhancers, stabilizers, and a non-chlorofluorocarbon propellant delivered via a metered dose spray device inhaler is in clinical trials. New system called Oral-Lyn which uses a metered dose spray device Rapid Mist is in Clinical Trail III. Other systems uses a film with a small size, thickness and shape, allowing a uniform drug delivery through buccal, sublingual or enteral route.
Pulmonary Inhalers	High permeability and large surface area provide a favourable framework for protein/drug uptake. Very rapid absorption of insulin after inhalation mimics time-activity profile of fast-acting insulin; appropriate for pre-meal delivery. Appears comparable to subcutaneous insulin on glycaemic parameters for both type 1 and type 2 diabetic patients. One product Afreeza® was introduced by Sanofi after USFDA approval, June/2014.
Transdermal **1. Iontophoresis** **2. Microneedles** **3.Transferosomes**	 -Electrical current used to enhance transdermal insulin delivery; proof-of-principle from animal studies; human studies needed. -Transdermal microneedles, needles that are fabricated on a microscale are used to increase the transdermal insulin delivery. -Elastic, flexible and deformable vesicle which allows to squeeze itself in order to pass though the pores of the skin are used. Transfersulin consists of encapsulation of insulin into transferosome which is good approach to overcome S.C. insulin injection.
Nasal	Nasal administration of certain proteins (e.g., insulin, and calcitonin) is now well established. Permeability enhancers are generally required to augment insulin bioavailability; insulin bioavailability is typically in the range of 8–15% with enhancers. Nasal irritation is common (e.g., with lecithin, bile salts, or laureth-9 as enhancers). Nasal tolerance and high rates of treatment failure are major limitations.

	Two technologies have reached Phase II, one is Nasulin, a nasal spray developed by CPEX. The other is developed by Nastech Pharmaceutical.

3 - Oral Insulin Delivery Systems

Oral routes have largely been studied as a novel alternative to SC insulin injections. This route can be considered the most patient-friendly way of insulin administration and offers better patient compliance. Numerous delivery systems for oral insulin delivery have been actively developed, especially by pharmaceutical companies, in the hope of making them clinically useful. Although most of the development work still remains in the development stage, many have progressed beyond the proof-of-concept stage to clinical trials. These technologies are summarized in Table 1.11 (Yaturu, S., 2013, Shah et al., 2010 & Heiemann et al., 2009)

Table 1.11 Insulin oral delivery technologies under development by industry.

Company	Systems	Product name	Outcomes for absorption
Diabetology	Enteric coated capsule	Capsulin	Allow absorption in the small intestine
Emisphere	Carrier molecules	Eligen®	Increase membrane permeability
BioSante	Calcium phosphate Nanoparticles	BioOral™	Protect proteins from acidic degradation and improve membrane permeability
Generex	Spray device andaerosol part icles	Oral-Lyn™	Penetrate the buccal epithelium
NOBEX/ Biocon	Amphiphilic oligomers	HIM2	Resist enzyme digestion and increase membrane permeation I
Apollo Life Science	Nanoparticles	Oradel™	Protect proteins from enzyme digestion in the stomach and facilitate the transport of proteins in the intestine
Eli-Lilly	Oral formulation	AI-401	Protect proteins from enzyme digestion

Provalis PLC	Lipid-based microemulsion	Macrulin™	Protect proteins from proteolysis or acidic degradation, and enhance the protein absorption in GIT
Endorex	Polymerized-liposomes	Orasome™	Protect proteins from the stomach and upper GIT
Oramed Pharma	Enteric Coated Capsule	0801	Protect insulin from enzyme and proof successful absorption.
Merrion & Novo Nordisk	Enteric Coated Tablet	NN1953	Using absorption enhancer which activate micelle formation facilitating transport and absorption.

1.8.8. Insulin Degradation

It is well documented that every tissue and cell that responds to insulin also can degrade the insulin molecule. Two enzymes that degrade insulin have been studied extensively: one that interchanges the disulphide bonds in the insulin molecule (called glutathione-insulin transhydrogenase (GIT) or protein disulphide isomerase (PDI)) (Wroblewski et al., 1992). The second enzyme called insulin degrading enzyme (IDE) (Shii et al., 1985). GIT utilize thiol of low molecular weight such as glutathione as well as high molecular weight sulfhydryl containing protein such as insulin (Varandani et al., 1975). The interaction between insulin and GSH catalysed by enzyme GIT is reversible, interaction:

INSULIN + GSH  GSSG + A Chain + B Chain

Oxidized glutathione “GSSG” was found to be a linear competitive inhibitor vs. both GSH and insulin (Varandani et al., 1975**).**

1.8.9. Insulin Resistance

In the early 1990s, Marshall and Traxinger remarked that the development of insulin resistance requires three key components: glucose, insulin and glutamine (Michalski et al., 2010)

Increasing the concentration of extracellular glucose and glucosamine, or facilitating

glucose transport by over-expressing glucose transporters (Gluts), results in insulin resistance; a hallmark of type II diabetes (Hart et al., 2006)

Resistance to insulin exerts dramatic and deleterious effects at the level of insulin target tissues, including pancreatic beta-cells, the liver, adipocytes and skeletal muscle. Coupled with glucose toxicity, insulin resistance is responsible for complications such as cardiovascular disorders, retinopathies, nephropathies and erectile dysfunction in diabetics (Michalski et al., 2010)

At the biochemical level, insulin resistance and glucose toxicity induce the production of reactive oxygen species (ROS), the glycation of proteins and an enhancement of intracellular protein *O*–GlcNAcylation (Michalski et al., 2010)

Insulin resistance inhibits glucose uptake and glycogen synthesis in skeletal muscle and adipocytes, and it has been proposed that this resistance to insulin is an adaptation by the organism in response to a nutrient excess (Michalski et al., 2010)**,** the phenomenon of insulin resistance and the corresponding decrease in glucose uptake are correlated with a defect in the translocation of the glucose transporter GLUT4 to the plasma membrane (Quon et al., 1997).

Virkama et.al demonstrated that infusion of GlcN to normal rats induces insulin resistance in several insulin sensitive tissues including fast- and slow-twitch skeletal muscles, the heart, liver, adipose tissue, and the submandibular gland. The magnitude of insulin resistance exhibited tissue specificity and was greatest in the liver and lowest in the fast-twitch glycolytic abdominis muscle. These data raise the possibility that over activity of HBP may contribute to glucose toxicity not only in skeletal muscle but also in the heart and in the liver (Virkama et al., 1997).

1.9. The Development of an Oral Delivery System

1.9.1. Description

In aqueous solution, molecules having either polar or charged groups and nonpolar regions (amphiphilic molecules) form aggregates called micelles, aggregates form only when the concentration of the amphiphilic molecule reaches a given concentration called critical micelle concentration (cmc). Amphiphilic molecules can form micelles not only

in water but also in nonpolar organic solvents, in such cases, micelle aggregates are called reverse micelles. Oral delivery system for insulin is prepared in which polyelectrolyte complex (PEC) prepared from low molecular weight chitosan and insulin drug is solubilized in a reverse micelle made from PEG-8 caprylic/capric glycerides and glycerol-6-dioleate as emulsifying agents and dispersed in oleic acid Schematic representation of the reverse micelle is shown in Figure 1.8. This delivery system has the potential to offer the opportunity to permit the oral delivery of insulin (Elsayed et al., 2009).

1.9.2. System Components

1- Chitosan: Multifunctional Polymeric Excipient

Chitosan (poly(*N*-acetylglucosamine)) is a partially deacetylated chitin which is one of the most abundant polysaccharides in nature; second only to cellulose. It has a sugar backbone consisting of β-1, 4 linked glucosamine with a high degree of *N*-acetylation (70-90% *N*- acetylglucosamine and 10-30% D-glucose units), a structure very similar to that of cellulose; the only difference being the replacement of the hydroxyl by amino groups (Figure 1.8) (Soares et al., 2012).

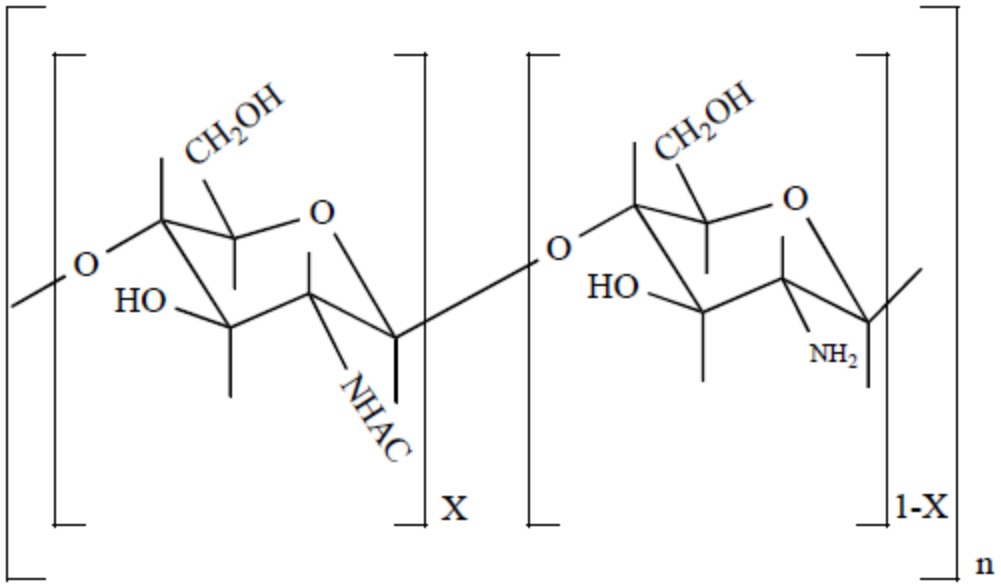


Figure 1.7. Chemical structure of chitosan polymer. X= degree of acetylation and n= number of sugar units (Ogawa et al., 2004).

Physicochemical Properties of Chitosan

Chitosan is broadly classified as chitosan oligomer which composes of about 12 monomer units and chitosan polymer with more than 12 monomer units (Jon et al.,2007). Chitosan is considered a weak base due to the presence of a primary amine group with pKa values range from 6.2 to 7. Thus, it undergoes the typical neutralization reactions of basic compounds. Chitosan is insoluble in neutral or alkaline aqueous media and at physiological pH of 7.4 (Hejazi et al., 2003). Chitosan in solution exists in an extended conformation due to the repelling effect of each positively charged deacetylated unit on the neighbouring glucosamine units (Singla et al., 2001).

Biological Properties of Chitosan

Chitosan, being a natural polymer, is biodegradable and biocompatible. It is degraded *in-vivo* by lysozymes producing N-acetyl-glucosamine (George et al., 2006). Chitosan has a low level of toxicity where oral LD_{50} value is found to be in excess of 16 g/kg body weight of mouse (George et al., 2006). Chitosan has shown to have a high capacity to adhere to the mucosa owing to ionic interaction between positively charged amino groups in chitosan and negatively charged gel layers (George et al., 2006).

2- Oleic Acid: Permeation Enhancers

Oleic acid is a monounsaturated ω-9 fatty acid found in various animal and vegetable sources. It has the formula $C_{18}H_{34}O_2$ (CH_3 $(CH_2)_7CH=CH(CH_2)_7COOH$).

Physicochemical Properties of Oleic Acid

Oleic acid is a colourless to pale yellow, oily liquid with a lard-like odour and taste. It has a, specific gravity of 0.889 to 0.985. It congeals at a temperature below 10ºC, pure oleic acid solidifies at 4ºC, at atmospheric pressure it decomposes when heated at 80ºC to 100ºC, on exposure to air it gradually absorbs oxygen, darkens and develops a rancid odour. It is practically insoluble in water, miscible with alcohol, chloroform, ether, benzene and fixed and volatile oils (Swinyard et al., 1985).

Biological Properties of Oleic Acid

Oleic acid, as long chain fatty acids, are effective as intestinal permeation enhancers, but because of their lower water solubility they have to be combined with emulsifying agents (Suzuki et al., 1998).

3- PEG-8 Caprylic/capric glycerides (Labrasol®) and Polyglycerol-6-dioleate (Oleique CG®): Emulsifying Agents.

PEG-8 Caprylic/capric glycerides (Labrasol®): a non-ionic water dispersible surfactant composed of well-characterised polyethylene glycol (PEG) esters, glyceride

fraction and free PEG. Work as wetting agent improving the solubility of active pharmaceutical ingredients. Able to self-emulsify on contact with aqueous media forming microemulsion (www.gattefosse.com 296).

Polyglycerol-6-dioleate (Plurol Oleique CG®): a water insoluble surfactant composed of a mixture of polyglyceryl diesters of mainly oleic acid. Use in self-emulsifying systems to obtain a microemulsion. It works as absorption enhancer due to the long chain fatty acids present in its composition (www.gattefosse.com 166).

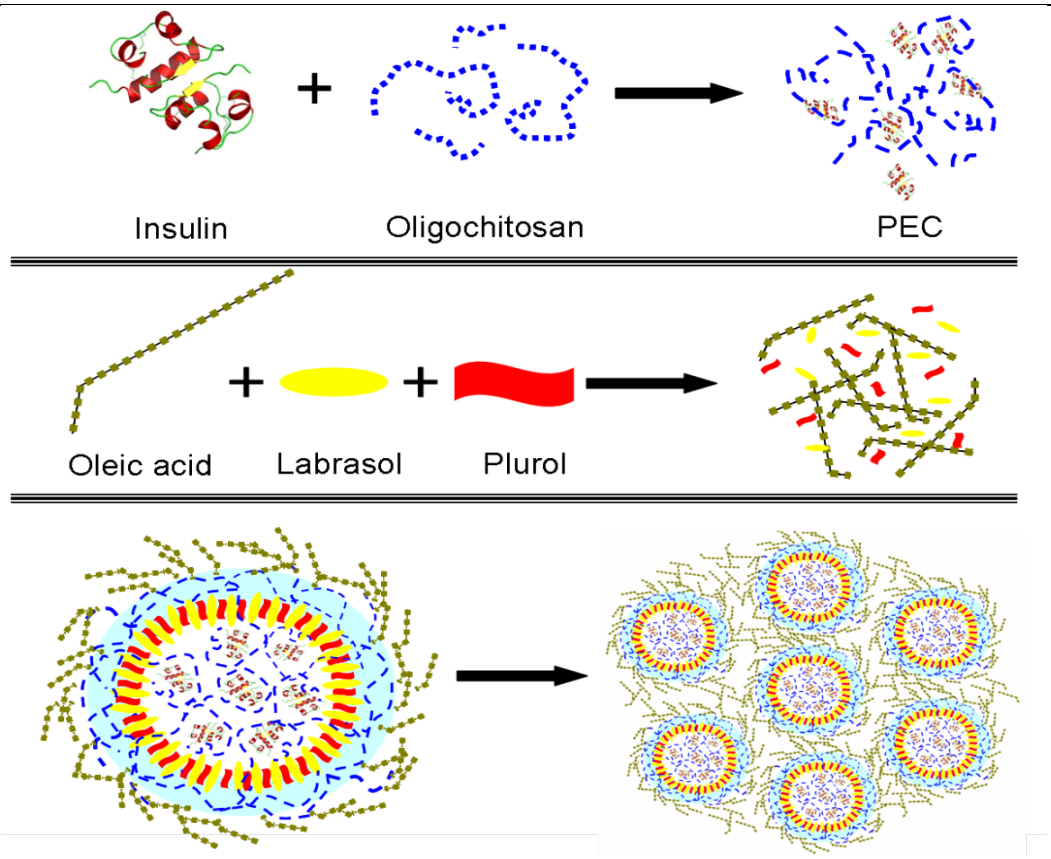


Figure 1.8. Schematic representation of insulin nanoencapsulated in the reverse micelle.

1.9.3. System Advantages

Low molecular weight chitosan (LMWC) interacts weakly and non-covalently with insulin, increasing the lipophilicity of the nanoparticles; by interacting chitosan with oleic acid a lipophilic non-inverting vesicular system is obtained. This system is expected to:

1. temporarily alter the drug shape non-covalently by engulfing it in a lipophilic nano-structure, and, because of the lipophilicity of the nanoparticles there is the potential for them to pass into the blood stream. The oleic acid which acts as an absorption enhancer will enhance this process.
2. stabilize the drug molecule in a conformation that reduces acid and enzymatic degradation, which are highly likely in the digestive tract prior to its absorption into the blood stream.

1.10. Objectives of the Project

Although high molecular weight chitosan have already been proposed as vehicles for protein delivery, however, due to their high molecular weight, low solubility is resulted in physiological solutions. The use of LMWC (<20 kDa) which are water soluble is attracting more attention as a substitute for high molecular weight chitosan.

Firstly, the main objective of this report was to experimentally investigate the interaction between LMWC and insulin in terms of chemical and physical stability. Specific aims related to this objective are:

- Optimize analytical methods to qualify insulin and to determine the formation of HMWP.
- Study the effect of different formulation parameters on insulin stability.

Secondly, the other objective is to carry out physicochemical characterization and in vivo evaluation of PEC nanoparticles prepared. Specific aims related to this objective are:

- Characterize the formulation of PEC and providing results related to morphology, particle size, and association efficiency (AE).
- Carry out in vivo testing to evaluate the effect of different formulation parameters on glucose level as response.

Thirdly, to evaluate the oral delivery systems for insulin. Specific aims in this objective are to:

- evaluate the system regarding insulin release and protection against enzymatic attack.
- assess the chemical and physical stability of insulin in the system.
- carry out *in vivo* evaluation of the system.

Fourthly, evaluating the effect of glutathione, oxidized and reduced form (GSH and GSSG) on the bioactivity of subcutaneously or orally delivered insulin to rats. Specific aims related to this objective are to evaluate:

- the time interval needed between GSH injection and insulin injection to detect GSH effect.
- the effect of GSH and GSSG on SC insulin bioactivity
- the effect of different concentration of GSH on SC insulin bioactivity.
- evaluate the effect of GSH and GSSG on oral insulin bioactivity

Finally, evaluate the effect of glucosamine on the bioactivity of subcutaneously or orally delivered insulin to rats. Specific aims related to this objective are to evaluate the effect of:

- different doses of SC GlcN on SC insulin
- GlcN on insulin when administered simultaneously.
- continuous oral GlcN administration on insulin bioactivity.
- GlcN on insulin bioactivity when given in reverse micelles together with insulin.

CHAPTER 2: Low Molecular Weight Chitosan–Insulin Polyelectrolyte Complex; Characterization and Stability Studies

2.1. Introduction

One of the most challenging problems in the development of liquid peptide/protein pharmaceuticals is their physical and chemical instability. Most peptides and proteins are formulated so that they can be administered clinically by parenteral injections, as this is the fastest route towards commercialization. However, stabilization of peptides and proteins in a designated delivery system against degradation, particularly in the gastrointestinal tract (GIT), is a prerequisite for oral delivery. This can be carried out by using several excipients such as salts, amino acids, surfactants, polyhydric alcohols, and carbohydrates (Hiroyuki et al., 2009). The latter include chitosans, which are composed of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units (Aranaz et al., 2010). Chitosans are non-toxic, degradable, and biocompatible polymers that exhibit different characteristics. This allows them to be used as excipients for protein formulations intended for *in vivo* delivery of biopharmaceuticals (Junginger et al., 2009).

The presence of free NH_2 groups allows chitosan to form polyelectrolyte complexes (PECs) with negatively charged moieties. For example, PECs containing chitosan, alkyl-chitosan, and PEG-grafted alkyl-chitosan have been shown to improve insulin delivery (Mao et al., 2005 &Sadeghi et al., 2008). Such PEC nanoparticles showed a pH-dependent stabilization of insulin (Berger et al., 2004).

Schatz (Schatz et al., 2009) synthesized a partially *N*-sulfated chitosan. Upon acidification, nanoparticles were formed by electrostatic interactions between the non-sulfated protonated amine groups of chitosan and the negatively charged *N*-sulfated chitosan amines. These PECs can be used for encapsulation of macromolecules (Schatz et al., 2009).

However, the main function of such PECs is to increase protein stability towards harsh conditions in the GIT or unfavorable storage conditions and to protect them against physical and chemical instabilities. PEC nanoparticles prepared by Mao *et al*. using chitosan, trimethyl-chitosan, PEGylated-trimethyl-chitosan, and insulin were unaffected by lyophilization and PECs were shown to protect insulin from degradation even at temperatures as high as 50 °C for 6 h (Mao et al., 2006). LMWCs have been used to prepare the PECs employing poly-γ-glutamic acid, which was used as a carrier for insulin, followed by enteric coating or layering with calcium alginate in order to allow oral administration (Lin et al., 2007). Jintapattanakit *et al*. found that PECs prepared using trimethyl-chitosan (100 kDa) and PEG-graft-trimethyl-chitosan copolymer improve the stability of oral insulin (Jintapattanakit et al., 2007). Also, Song (Song et al., 2014) reported an oral insulin delivery system based upon ultrathin nanofilm encapsulation technology. The proposed system can be used to load a high amount of insulin (90%) using chitosan (l50–190 kDa, 75% deacetylation) (Song et al., 2014). Additionally, it has

also been reported that the inclusion of chitosan in lipid nanoparticles enhances the physical stability of insulin by protecting against proteolysis (Tozaki et al., 1997).

A novel system based on solubilization of the insulin–chitosan PEC in RM system made from PEG-8 caprylic/capric glycerides and glycerol-6-dioleate as emulsifying agents and dispersed in oleic acid, has been patented by Badwan *et al.* (Badwan et al., 2007 & Elsayed et al. 2009). The function of this solubilized PEC is to reduce the size of particles intended for oral delivery of insulin (Assaf et al., 2011).

Generally, studies have been undertaken by using high molecular weight chitosans, and in the vast majority of cases the chemical stability of insulin was not evaluated in term of insulin degradation and formation of its degradation products. The objective of the work reported herein was to investigate the influence of complexing LMWCs of different molecular weights (1.3–30 kDa) with insulin on its physicochemical and biological stabilities. Insulin content was monitored and examined as well as the formation of high molecular weight proteins (HMWPs) of insulin. Furthermore, the system was evaluated for bioactivity.

2.2. Experimental Section

2.2.1. Materials

USP human insulin RS (26.4 USP insulin human units/mg, lot No. J0J250) was purchased from USP Convention (Rockville, MD, USA). Recombinant human (rh) insulin of pharmaceutical grade standardized by using USP insulin human RS (potency 99.4 %) was purchased from Biocon (Bangalore, India). Purified water and LMWCs of different molecular weights (1.3, 6, 13, 18, and 30 kDa) with >95% degree of deacetylation (DDA) were provided by the Jordanian Pharmaceutical Manufacturing Company (Naor, Jordan). LMWCs may be considered as derivative of chitosan with lower molecular weight, obtained by depolymerization of high molecular weight chitosan HCl (≈ 250 kDa and

DDA 95%, Xiamen Xing, Shanghi, China). Oleic acid of pharmaceutical grade (purity >99%) was purchased from Merck (Darmstadt, Germany). Labrasol® (PEG-8 caprylic/capric glycerides) and Plurol®Oleique CG (polyglycerol-6-dioleate) were purchased from Gattefosse (Saint-Priest, Lyon, France). Streptozotocin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical/HPLC grade and purchased from Merck.

2.2.2. Determination of Molecular Weight of LMWCs

The average molecular weight of LMWCs was determined by viscosity measurements (Vibro viscometer, SV-10 Japan) in water as previously published (Elsayed et al., 2009).

2.2.3. Characterization of LMWC, IC-PEC and IC-RMs

The LMWC was characterized by FT-IR (Thermo scientific Nicolet Avatar 360 FT-IR ESP Spectrometer; Madison, WI, USA) using a KBr pellet. The spectra were obtained in the 400 -4000 cm^{-1} range at a resolution of 1 cm^{-1} with a minimum of 16 scans per spectrum. All measurements were undertaken at room temperature.

The film of LMWC, obtained after freeze-drying on glass Petri dishes for 24 h, was examined visually and photographed using a Sony Cybershot digital camera (Tokyo,

Japan). Furthermore, the morphology of the LMWC was ascertained using a FQuanta 200 Scanning Electron Microscopy (Hillsboro, OR, USA) (Qinna et al., 2015).

2.2.4. Preparation of I-LMWC PEC

LMWC (1.3, 6, 13, 18, and 30 kDa) solutions of the required concentration (0.3–7 mg/mL) were prepared by dissolving LMWC powder in water, adjusting the pH to 5.5 with 0.1 N NaOH, and then making up to the required volume with water.

Insulin solutions (7 mg insulin/mL) were prepared by dissolving insulin powder in 1 mL of 0.1 N HCl, neutralized with 0.1 N NaOH and made up to a volume of 10 mL with water or 1 M Tris (hydroxylmethyl-aminomethane) buffer, pH 6.5.

I-LMWC PEC was prepared by mixing equal volumes of LMWC and insulin solutions in a glass vial under gentle magnetic stirring and incubating for 10 min at room temperature. I-LMWC PEC preparations contained 3.5 mg insulin/mL and different concentrations of LMWC (0.0–3.5 mg/mL).

2.2.5. Preparation of I-LMWC Reverse Micelle

A reverse micelle (RM) system was prepared based on a previous study (Badwan et al., 2007). A surfactant mixture of Labrasol® and Plurol® Oleique CG was prepared at a 1/1 (w/w) ratio by mixing the constituents using magnetic stirring for 5 min. Oleic acid (80 g) and surfactant mixture (20 g) were mixed together for 5 min; 8 mL of I-LMWC PEC (3.5/1.5), which contained 3.5 mg insulin and 1.5 mg 13 kDa LMWC/mL, was added to the mixture and mixed using a magnetic stirrer (250 rpm for 5 min).

2.2.6. Extraction Procedures

For the I-LMWC PEC system, equal volumes of the sample and a solvent mixture of 0.01 N HCl and methanol (2/3, v/v) were mixed together.

For the reverse micelle system, 2 g of the sample and 4 mL of the solvent mixture were vortexed vigorously for 2 min, centrifuged at 4000 rpm for 15 min, and the aqueous phase collected. The samples were then analyzed by HPLC to determine the insulin, A-21 desamido insulin, and HMWP content (3 replicates); the RSD values were < 5%, < 0.5%, and < 3%, respectively.

2.2.7. HPLC Determination of Insulin

Quantitative determination of insulin was based on the USP assay method (United State Pharmacopeia, 2013). The HPLC system consisted of a TSP 1000 pump system, a TSP 1000 UV-VIS detector, and a TSP AS 3000 auto-sampler (TSP, USA). A C18 (L1) column (particle size 5 µm), dimensions of 4.6 × 150 mm (Thermo column from Thermo Fisher Scientific Inc., Rockford, IL, USA), maintained at 40 °C during analysis, was used as the stationary phase, together with a Lichrospher 100 RP-18, 5 µm particle size guard column (Merck, Germany). Elution was performed isocratically (flow rate 1 mL/min) using sulfate buffer pH 2.3-acetonitrile (73/27, v/v) as the mobile phase and UV detection at 214 nm. The injection volume was 20 µL. The resolution, R, between insulin and A-21 desamido insulin was >2, the tailing factor for insulin peak was <1.8, and RSD was <1.6%.

The resolution solution was prepared by dissolving 1.5 mg of insulin in 1 mL of 0.01 N HCl, followed by incubation of the solution at room temperature for not less than 3 days. Equipment control, data acquisition, and integration were undertaken using a ChromQuest work station.

2.2.8. Size Exclusion HPLC Determination of High Molecular Weight Proteins (HMWPs)

The method used was based on the USP limit test for HMWPs (United State Pharmacopeia, 2013). A column containing dihdroxypropane bound to silica packing material (L20) with a particle size of 5 μm and dimensions of 7.8 × 300 mm was used as the stationary phase (Waters insulin HMWP column, Dublin, Ireland). Elution was performed isocratically (flow rate 0.5 mL/min) using a mixture of an arginine solution (1 mg/mL), acetonitrile, and glacial acetic acid (65/20/15, v/v) as the mobile phase and UV detection at 276 nm. The injection volume was 100 μL.

Resolution solution was prepared by dissolving 4 mg of insulin containing not less than 0.4% HMWPs in 1 mL of 0.01 N HCl (insulin containing the indicated fraction of HMWPs was prepared by allowing insulin powder to stand at room temperature for about 5 days) (Unites State Pharmacopeia, 2013).

2.2.9. HPLC Method Validation

The two USP HPLC methods (insulin assay and limit test for HMWPs) were validated according to USP (Unites State Pharmacopeia <1226>, 2013) and the International Conference of Harmonization (ICH) guidelines (EMA, 1995). Standard insulin solutions of different concentrations (0.9, 1.75, 3.5, 7.0, and 10.0 mg/mL) were used to assess the linearity of the calibration plot (3 replicates). The precision of the assay was determined

by analyzing samples of I-LMWC PEC preparations at three different insulin concentrations (1.0, 3.5, and 10.0 mg/mL). For the assessment of the inter-day variation, samples were analyzed in triplicate (n = 3) on three different days. For the intra-day variation, they were analyzed 5 times (n = 5) on the same day. Accuracy was assessed by analyzing samples of I-CCS PEC preparations at the target concentrations of insulin of 3.5 mg/mL. Specificity was verified by analyzing the matrix, *i.e.*, LMWC PEC, in the absence of insulin using the extraction method stated above. Additionally, a resolution solution containing insulin and A-21 desamido insulin was prepared and injected. The detection limit (DL) and quantitation limit (QL) for the HPLC method were determined based on the standard deviation of the response and the slope of the calibration curve, as follows:

DL= 3.3 σ/S, where σ is the standard deviation and S is the slope of the calibration curve.

QL= 10 σ /S where σ is the standard deviation and S is the slope of the calibration curve.

The stability of solutions of insulin (0.9 mg/mL) was analyzed by HPLC at 0, 12, and 24 h of storage at room temperature.

2.2.10. Stability of I-LMWC PEC

The initial experiments were designed to incubate the I-LMWC PEC samples at 40 °C; however, as long periods of time (>2 months) were required to obtain indicative results, the experimental design was changed and the samples were incubated at a higher temperature (50 °C). Samples of I-LMWC PEC preparations, using LMWCs of different

molecular weights and concentrations, were incubated at 50 °C for different periods of time. Samples were withdrawn at pre-determined intervals (0, 12, 24, 36, 48, and 72 h) and tested for insulin, A-21 desamido insulin, and HMWP content and the physical stability of I-LMWC PEC preparations. In addition, the stability of insulin at different concentrations (0.9–10 mg/mL) in water and Tris-buffer at pH 6.5 was investigated at 50 °C.

2.2.11. Immunological Bioactivity of Insulin

Enzyme-linked immunosorbent assay (ELISA) was used to assess the immunological stability of insulin following formulation. Active insulin ELISA DSL-10-1600 micro-titration kits (Diagnostic Systems Laboratories Inc., USA) were used. Insulin concentration was measured using an enzymatically amplified “one-step” sandwich–type immunoassay. The samples were incubated with an anti-insulin antibody in micro-titration wells that had been coated with another anti-insulin antibody. Insulin was extracted from the I-LMWC PEC (3.5/1.5 mg/mL) preparations of different molecular weight (1.3, 6, 13, 18, and 30 kDa), as described above, and then assayed according to the instructions of the manufacturer. The results were obtained by reading the optical density at 450 nm with background wavelength correction at 620 nm using a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA).

2.2.12. Characterization of I-LMWC PEC

Particle size measurements were undertaken by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern, UK). Replicate measurements (n = 6) were carried out at 25 °C using a detection angle of 90° for each sample.

Zeta potential measurements (n = 3) were also carried out using the Zetasizer Nano ZS instrument at 25 °C using folded capillary cells integrated with gold electrodes. Samples measurement were carried out directly with no further treatment.

Association Efficiency I-LMWC PECs were centrifuged at 14,000 rpm for 30 min at room temperature. The quantity of insulin in the supernatants was measured using HPLC and the insulin association efficiency was calculated accordingly [14] as follows:

Association efficiency =

$$\frac{\text{Total amount of insulin - free insulin}}{\text{Total amount of insulin}} \times 100\%$$

. The number of replicates for each experiment was 3.

2.2.13. In Vitro Evaluation of I-LMWC PEC and I-LMWC RM

2.2.13.1. *In Vitro* Release

The *in vitro* release studies of insulin from I-LMWC RM were performed by incubating in a simulated gastric medium at pH 1.2 and a simulated intestinal medium, pH 6.8, (1% bile salt v/w) at 37 °C under continuous shaking at 50 rpm. Samples were withdrawn at specific time intervals (1, 2, 3, 4, 5, and 6 h), centrifuged, and analyzed for insulin release using the USP HPLC method (Unites State Pharmacopeia, 2013).

2.2.13.2. *In Vitro* Evaluation of the Protective Effect of RMs

A sample of 5 mL simulated gastric fluid (SGF) with pepsin (pH 1.2) was added to a 1 mL sample of free insulin solution, I-LMWC PEC solution, and a 2.0 g sample of I-LMWC PEC solubilized in the RM system. The samples were incubated for 1 h at 37 °C while shaking at 100 strokes/min. A 1.5 g sample of the RM was mixed with 5 mL of extraction solution of 0.010 M HCl and methanol (2/3, v/v), vortexed for 3 min, and a 100 µL sample of the aqueous layer was analyzed for insulin content by HPLC (Unites State Pharmacopeia, 2013). For insulin and I-LMWC PEC solutions, 100 µL samples were withdrawn after 1 h and analyzed for insulin content.

2.2.14. *In Vivo* Pharmacological Activity

2.2.14.1. Animal Handling

Adult male Sprague Dawley rats (200-250 g) were purchased from Yarmouk University, Irbid, Jordan and accommodated at Petra University Animal House Unit, Amman, Jordan, under standard temperature, humidity and photoperiod light cycles. All rats were acclimatized for 10 days before experimenting day and received standard chow and tap water *ad libitum*. Animal care and use were performed in compliance with guidelines of the Federation of European Laboratory Animal Science Association (FELASA) and European Union (Council Directive 86/609/EEC). The study protocol (SUG/13/45) was revised and approved by the Ethical Committee of The Jordanian Pharmaceutical Manufacturing Company (JPM), Naor, Jordan.

2.2.14.2. Induction of Diabetes Using Streptozotocin (STZ)

Diabetes was induced in rats by intraperitoneal injection of two doses of 80 mg STZ/kg over two days. STZ solution was freshly prepared by dissolving in 0.1 M citrate buffer pH 4.5. Only fasted rats (18 h, drinking water *ad libitum*) with a basal blood glucose level above 200 mg/dL were considered diabetic. Blood samples were collected directly from

each rat's tail and blood glucose measurements (in mg/dL) were performed using a blood glucose meter (Gluco Dr. All Medicines, Korea). Only rats with a basal blood glucose level above 200 mg/dL were considered diabetic.

2.2.14.3. Experimental Design of Oral Testing

STZ diabetic rats (n = 10) were randomized into different groups. Following initial blood glucose determinations, one group was injected with 2 IU/kg insulin and served as a positive control for insulin bioactivity. The other groups were given single an oral dose administration (50 IU/kg) of a blank control (same RM components, but without insulin), oral insulin solution, and I-LMWC RM. Blood sampling for glucose measurements proceeded during the experiments at specific time intervals (1, 2, 3, 4, 5, 6, 8, 12, and 18 h) post insulin administration.

2.3. Results and Discussion

2.3.1. Characterization of LMWC

Samples of LMWC were prepared by acid hydrolysis and characterized by different techniques (Qinna et al., 2015). The full deacetylation of 13 kDa LMWC was confirmed using FT-IR by the absence of an amide I band at 1650 cm^{-1}, while the band corresponding to free amino acid (amide II) occurred at 1574 cm^{-1} (Figure 2.1.A). The surface morphology (Figure 2.1.B) showed that the film of 13 kDa LMWC is a thick and rigid fiber, whilst the scanning electron microscope image (Figure 2.1.C) showed that the fully deacetylated LMWC has a rough and irregular surface (Qinna et al., 2015).

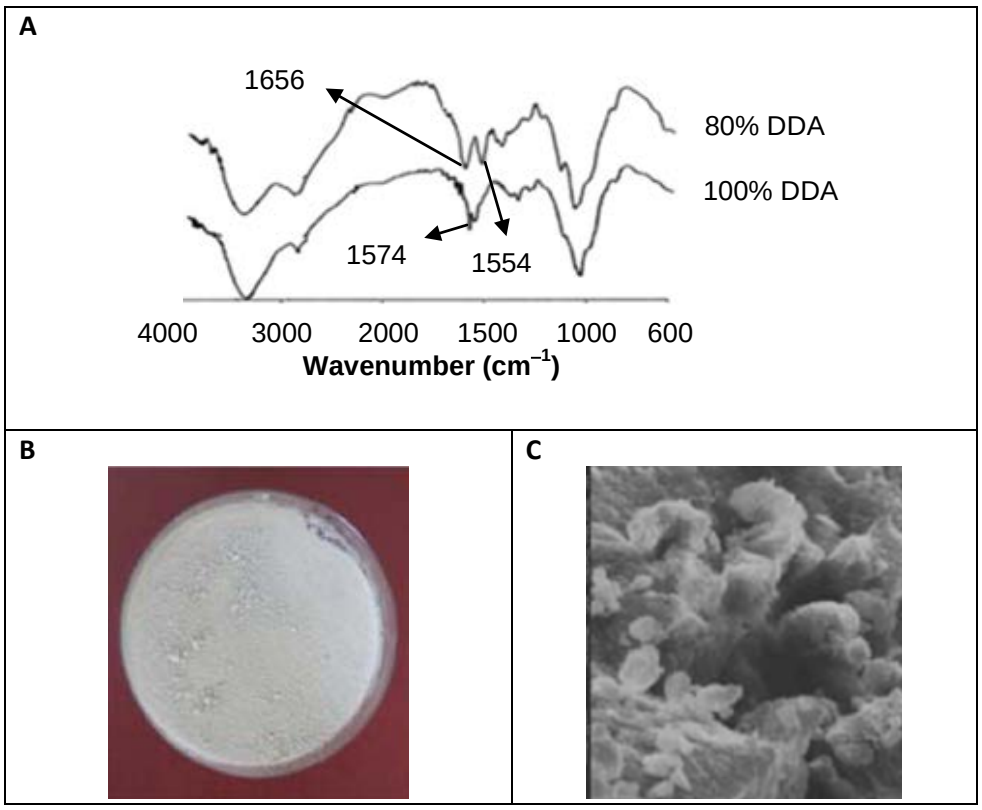


Figure 2.1 Characterization of 13 kDa LMWC with 100% DDA.

Notes: (**A**) **FT-**IR spectra for 13 kDa LMWC with 80% and 100% DDA over the frequency range 4000–400 cm^{-1}, (**B**) Surface image, and (**C**) SEM capture of dry film of 13 kDa LMWC with 100% DDA, 4000×magnification.

Abbreviations: LMWC, Low molecular weight chitosan; DDA, degree of deacetylation; SEM, scanning electron microscope.

2.3.2. HPLC Methods Validation

Insulin (I), A-21 desamido insulin, and HMWPs were determined by using the assay and limit of HMWP tests as stated in the human insulin USP monograph (Unites State Pharmacopeia, 2013). The insulin assay method was adopted for evaluating insulin stability in bulk insulin powder and in injectables (Smith et al., 1985). Additionally, the method was found to be convenient for evaluating the stability of insulin in other delivery systems (Zhang et al., 2004).

In the present work, the suitability of USP HPLC methods for the determination of insulin and HMWP in I-LMWC PEC systems was examined. Practically, insulin may be recovered either by an extraction-based method with organic solvents or by hydrolysis of the carriers (PEC or RM) with an alkaline reagent (Bilati et al., 2005). In the current work, different extraction solvents and procedures to extract insulin were evaluated. Aqueous solutions (0.01 or 0.1 N HCl) and methanol at different ratios (1/1, 2/1, 1/2, 2/3, and 3/2 v/v) were used to obtain the optimal ratio of the extraction solvent mixtures. Extraction of insulin from I-LMWC PEC preparations with a mixture of 0.01 M HCl and methanol at a ratio of 2/3 (v/v) gave acceptable recoveries for insulin (>98%); moreover, the stability of insulin in such a mixture was retained for 24 h.

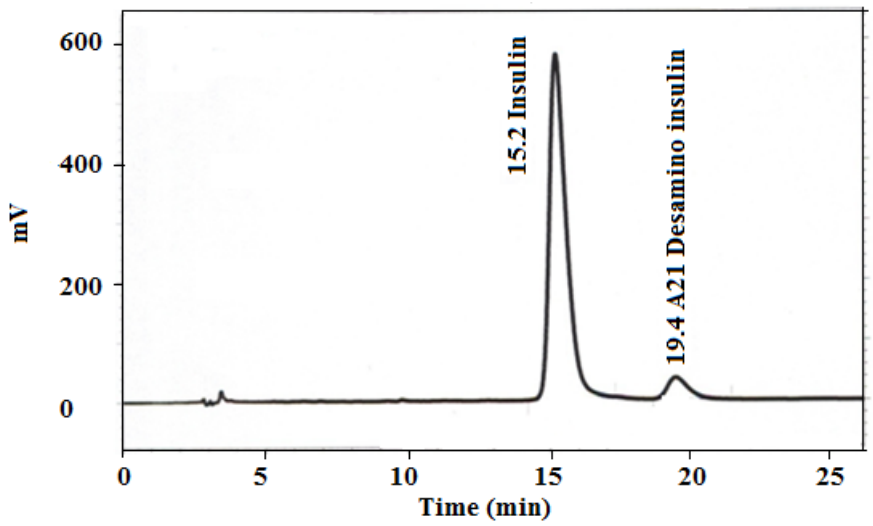
Furthermore, the USP HPLC method parameters used for the assessment of the stability of bulk insulin, such as the ratio of mobile phase components and temperature (United State Pharmacopeia, 2013), were evaluated for the PEC and RM systems. The ratio of mobile phase components (aqueous to acetonitrile) was found to be critical as a high acetonitrile content and a decrease in column temperature (e.g., to 35 °C) led to the precipitation of sulfate salt and subsequently affected the HPLC system parameters (e.g., retention time of insulin and resolution).

Validation of the USP HPLC method for insulin showed a linear response for signal output *versus* insulin concentration over the concentration range of 0.9–10 mg insulin/mL with an R^2 value >0.995; such results are in line with the acceptable verification limits (ORA, 2013). The intra- and inter-day relative standard deviation (RSD) values were less than 2%, indicating good precision. No interfering peaks from the components of the delivery systems were detected. The resolution factor between insulin and A-21 desamido insulin was >2.0, indicating that the method is specific. The method sensitivity was proved by low detection limit (DL) (0.02 mg/mL) and quantitation limit (QL) (0.08

mg/mL) values. Thus, the isocratic HPLC method developed herein is, analytically, advantageous in comparison with the published gradient methods (Sarmento et al., 2006 & Iwasa et al., 2009). The results of validation of the HPLC method confirmed the applicability of the USP HPLC method for the analysis of preparations other than injectables, such as polyelectrolyte systems. Consequently, the USP method can be considered as stable according to the investigated PEC and RM delivery systems.

The data in Figure 2.2 shows representative HPLC outputs for both assay and limit of HMWP tests, where the peaks corresponding to insulin, A-21 desamido insulin (Figure 2.2.A), and HMWPs (Figure 2.2.B) are well resolved. The results of HPLC method validation are summarized in Table 2.1; all the validation parameters are within acceptable limits (ORA, 2013).

(**A**)



(**B**)

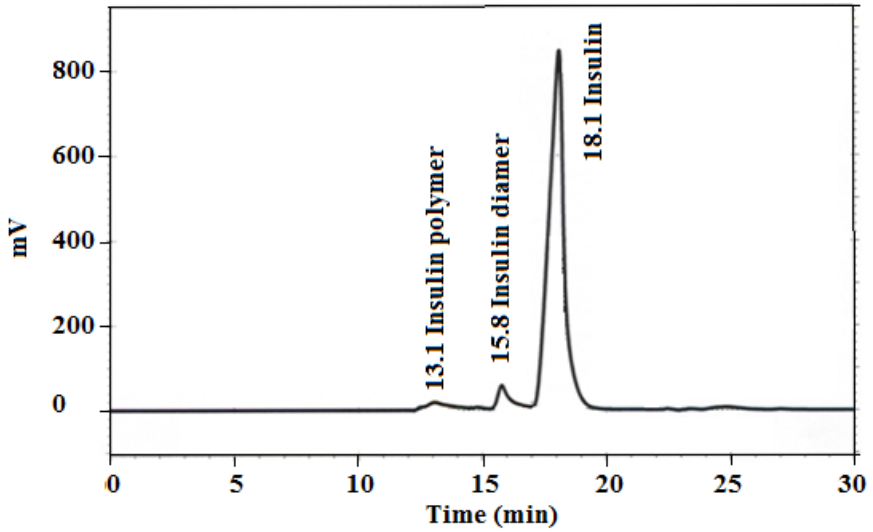


Figure 2.2. Representative HPLC chromatograms for (**A**) insulin assay (1.5 mg/mL) and (**B**) high molecular weight proteins (HMWPs) limit test (4.0 mg/mL).

Table 2.1 HPLC method verification results for insulin assay.

Analytical Parameter	Result	Limit
Linearity Range		
- 0.9–10.0 mg/mL	R^2 = 0.9998	> 0.995
Specificity		
- Interference	No interference from formulation components and related compounds	No interference
Precision (%)		
- Inter-day RSD (n = 3 × 3)	0.8–1.1	< 2.0 %
- Intra-day RSD (n = 5)	0.7–0.9	< 2.0%
Recovery (%)	98.3 ± 0.9	95.0-105.0 %
DL (mg/mL)	0.02	
QL (mg/mL)	0.08	
Stability of Solution		
- Decrease in assay at ambient condition	2.5% decrease in assay after 24 h	
System Suitability		
- Resolution between insulin and A-21 desamido insulin	3.7	> 2.0
- Tailing factor for insulin peak	<1.8	< 2.0
- RSD of replicate injections	<1.6%	< 2.0

DL and QL: detection and quantitation limits, RSD: relative standard deviation.

2.3.3. Effect of the Molecular Weight and Concentration of LMWC on Insulin Stability

Prior to studying the effect of the molecular weight of LMWC on the stability of insulin, different parameters including insulin concentration (0.9–10.0 mg/mL) and solvent (water, phosphate buffer pH 6.5, and Tris-buffer of pH 6.5), were investigated at 50 °C for 72 h. It was found that the stability of insulin increases when the concentration of

insulin is increased; these results agree well with reported data (Hansen et al., 1991, Hvidt et al., 1991, Sluzky et al., 1992 & Dathe et al., 1990). However, it appears from the results of the present study that a concentration of 7 mg/mL is sufficient for maintaining insulin stability at pH ≈ 6.0. Furthermore, Tris-buffer (an organic buffer) improved the stability of insulin even at low concentrations of insulin (0.9 and 1.75 mg/mL) by inhibiting the re-aggregation and precipitation of insulin. Also, at neutral pH, the conversion of monomeric insulin to form dimeric, tetrameric, and eventually hexameric insulin seems to stabilize insulin (Ugwu et al., 2004). At insulin concentrations of 0.9–10 mg/mL, the fractional content of the degradation products A-21 desamido insulin and dimer did not exceed 0.4% after incubation at 50 °C for 72 h. On the other hand, other HMWPs were not detected over the investigated range of insulin concentration. In neutral solutions, the insulin molecules are associated mainly into non-covalent, Zn^{2+}-containing hexamers (Brange et al., 1990). The observation that dimer formation is independent of insulin concentration indicates that the intermolecular chemical reaction occurs mainly within the hexameric units and not between the hexamers in solution (Brange et al., 1992A). This might be due to the fact that hexamers are less susceptible to degradation (Hansen et al., 1991).

The stability of I-LMWC PECs prepared by using LMWCs of different molecular weights (1.3–30 kDa) at 50 °C is shown in Figure 2.3.A. The kinetic parameters for insulin degradation, as shown in Table 2.2, indicate a noticeable effect of molecular weight of LMWC on the extent of insulin degradation in the I-LMWC PEC preparations (*i.e.*, stability decreases with increasing molecular weight of LMWC).

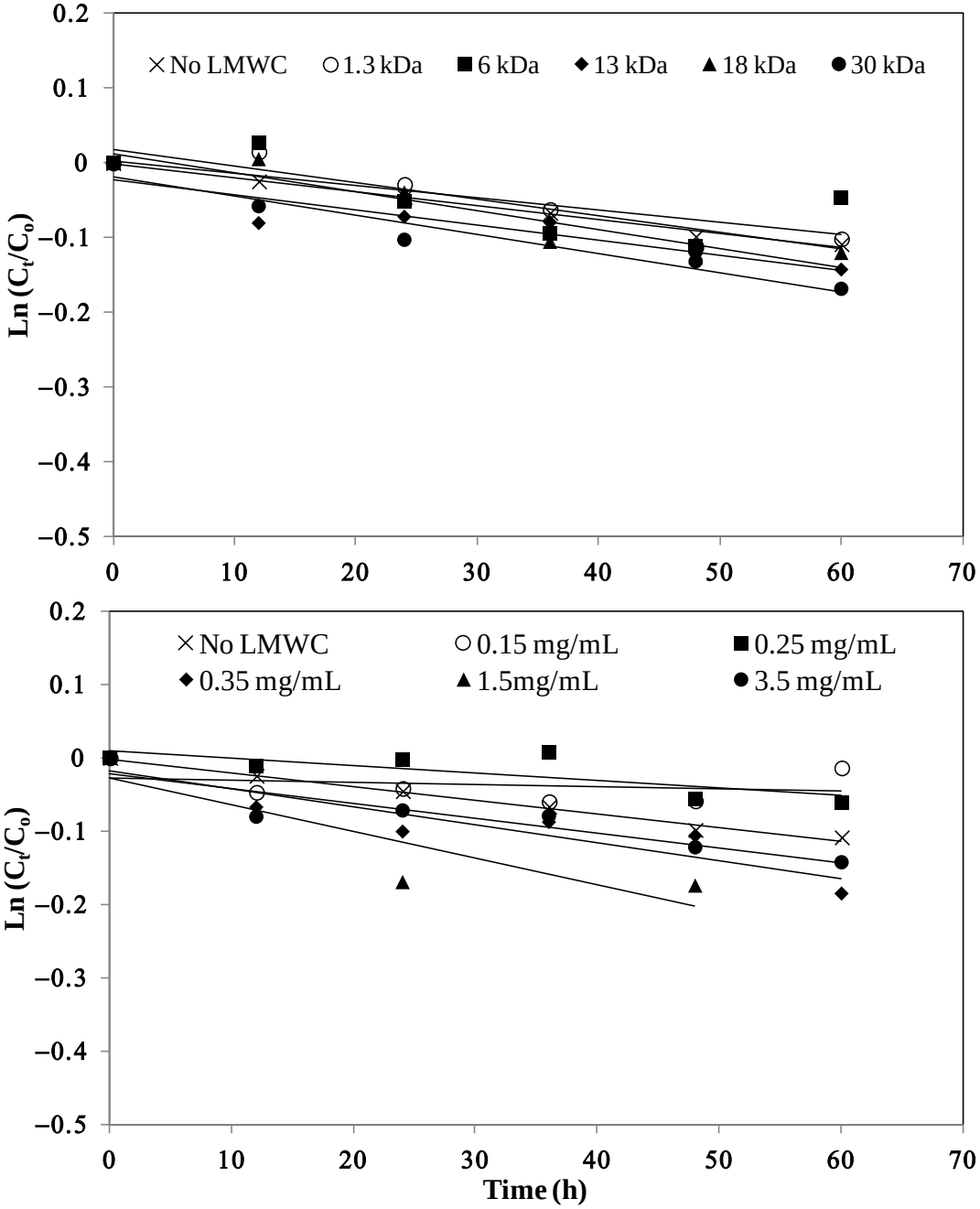


Figure 2.3 Stability of insulin in the presence of (**A**) different LMWCs (1.5 mg/mL) and (**B**) different concentrations of 13 kDa LMWC at 50 °C. C_o is the initial insulin concentration and C_t is the insulin concentration at different periods of incubation time. The values of Ln (C_t/C_o) are the mean of 3 replicates for each experiment (RSDs < 5%).

Table 2.2 First-order kinetic parameters for insulin–LMWC polyelectrolyte complex (I-LMWC PEC) degradation in the presence of different LMWCs and different concentrations of 13 kDa LMWC at 50 °C.

LMWC Molecular Weight (kDa) *,+	**Insulin/LMWC Molar Ratio**	***k* (h^{-1}) × 10^3**	**t_{90} (h)**	**t_{50} (h)**	**R^2**
0.0	-	1.2	88	587	0.2065
1.3	1/4.5	2.2	48	315	0.8780
6	1/1	1.6	64	423	0.4835
13	1/0.45	2.0	52	344	0.8428
18	1/0.3	2.5	42	274	0.8831
30	1/0.2	2.6	41	272	0.9388
[13 kDa LMWC] (mg/mL) +					
0.0	-	1.2	88	587	0.2065
0.15	1/0.02	1.3	81	533	0.9200
0.25	1/0.03	1.0	102	675	0.5871
0.35	1/0.045	2.4	43	283	0.8388
1.5	1/0.2	3.6	29	191	0.7704
3.5	1/0.45	2.0	52	344	0.8428

k: 1st order rate constant, t_{90} and t_{50}: shelf-life for 90% and 50% intact potencies.

+ [Insulin] is 3.5 mg/mL.

***** [LMWC] is 3.5 mg/mL.

The effect of 13 kDa LMWC concentration on the stability of I-LMWC PECs at 50 °C is shown in the data in Figure 2.3.B. The results revealed a noticeable effect of increasing LMWC concentration on the degradation of insulin (Table 2.2).

In addition, monitoring the physical stability of I-LMWC PEC indicated that the formation of a physically stable system depends upon the molecular weight of LMWC.

For example, LMWCs of shorter chains formed soluble complexes, while turbidity and precipitation were observed with higher molecular weight LMWCs (e.g., 30 kDa). This can be explained by the fact that complex formation between insulin and LMWC is mainly governed by kinetic factors which leads to preferential binding with the shorter chains due to their flexibility (Mao et al. 2006). On the other hand, the chemical stability of insulin was affected to the same extent in the presence of different molecular weight LMWCs (Figure 2.3.A). This may be attributed to the ratio of ionized groups of insulin (7.0×10^{20}) to chitosan (5.3×10^{21}), which was fixed at about 1/8 in the preparation of different I-LMWC PECs by changing the molar ratio of insulin: LMWC 1/0.2–1/4.5 (Table 2.2). The number of ionizable groups was calculated on the basis of previously published work (Assaf et al., 2011).

Furthermore, changing the concentration of 13 kDa LMWC showed a significant effect on insulin stability (Figure 2.3.B). Insulin instability was observed mainly at high concentrations of LMWC (3.5 mg/mL), while at lower concentrations the effect is less. This may be explained by the changes in LMWC structure and particle size with concentration. With increasing concentration of LMWC, the structure becomes more helical, leading to the formation of aggregates and a concomitant increase in particle size (see Section 2.4). At high concentration, such aggregates may prevent LMWC from protecting insulin (Figure 2.3.B).

By monitoring the degradation of insulin as a function of molecular weight of LMWC, the fraction of A-21 desamido insulin (0.2% initial) increases when the molecular weight of LMWC is increased after 72 h incubation at 50°C (0.9, 1.1, 1.2, 1.4, and 1.6% for 1.3, 6, 13, 18, and 30 kDa LMWC, respectively). While the results showed an increase in the content of insulin dimer (from 0.1% to 0.6%–0.7%) regardless of the molecular weight of LMWC. This may be attributed to the fixed ratio of ionized groups of insulin to

LMWC. The amino groups of LMWC have the capacity to react with insulin by intermolecular aminolysis, resulting in transamidation between the molecules. Such a reaction can result in dimer formation (Brange et al., 1992A).

However, the formation of other HMWPs increases as the molecular weight of the LMWCs increases (1% for 1.3 kDa and 3%–4% for 6–30 kDa LMWCs). The formation of dimers and polymers can be explained by the ability of free NH_2 groups to react with the carbonyl groups of insulin (available in Asn at A-21 and B-3, and Gln available at A-5, A-15, and B-4) by intermolecular aminolysis, resulting in transamidation or Schiff base-mediated reactions between molecules forming HMWPs and, in parallel, dimer formation, probably as a result of disulfide interchange (Brange et al., 1992 A). It is worth mentioning that the impact of HMWP formation on the quality and therapeutic usefulness of the pharmaceutical preparation is attributed to safety rather than efficacy. Preparation efficacy will not be affected by a decrease in the content of insulin. However, some of the immunological side effects associated with insulin therapy may be due to the presence of covalent aggregates of insulin in the therapeutic preparations, and specific antibodies against dimers have been identified in 30% of insulin-treated diabetic patients (Robbins et al., 1987). Dimer levels of 2% generated a highly hypersensitive response (Ratner et al., 1990). Accordingly, the content of HMWPs should be kept as low as possible. The acceptable USP limit does not allow more than 3.0% HMWPs in insulin pharmaceutical preparations. This indicates that during the development of insulin delivery systems, optimization of the system is essential to prevent the formation of HMWPs.

2.3.4. Effect of the Molecular Weight of LMWC on Immunological Bioactivity of Insulin

The ELISA method used for insulin determination was found to be linear in the range of 0–100 µIU/mL with an R^2 value of 0.868 and DL of 0.26 µIU/mL. Method recovery at the recommended concentration (by the kit supplier), 50 µIU/mL, was carried out; the fraction recovered was 92.7%, with an RSD of 6.1% (average of 3 samples). Monitoring of insulin solutions incubated at 50 °C indicated a loss in bioactivity of 40% after an 8 h incubation, which indicates that the method is specific and loss in bioactivity of insulin can be detected using ELISA. The ELISA results indicated that insulin in the different I-LMWC-PECs prepared with LMWC of different molecular weights (1.3–30 kDa) retained its bioactivity (assay > 85%) (Table 2.3). The ELISA method has very high sensitivity and specificity as it depends on the reaction of the predominant protein with a specific antibody to form a complex (Mansur et al., 2005). Therefore, ELISA antigenicity is considered to be an appropriate means for detection of changes of insulin antigenic activity (Gander et al., 1995). The surface structure of insulin was assessed by antibodies that bind to the epitopes on the insulin. The ELISA results indicated that insulin in the PEC had retained its bioactivity. ELISA results were comparable with HPLC results, which were used to assess the integrity of insulin and confirmed that the receptor-binding epitopes on insulin were maintained after complexation with LMWC (Cleland et al., 1997).

Table 2.3 The immunological bioactivity of insulin-LMWC polyelectrolyte (I-LMWC PEC) of different molecular weight after incubation at 50 °C for 72 h.

LMWC Molecular Weight (kDa)	Bioactivity (%)	RSD
No LMWC	58.9 *	10.3
1.3	91.1	3.1
6	92.3	4.7
13	102.9	6.7
18	94.6	6.1
30	97.1	5.7

* After 8 h of incubation, RSD: relative standard deviation.

The aforementioned stability results show a profound impact of molecular weight (*i.e.*, chain length), concentration (*i.e.*, charge ratio), pH, and buffer type of the LMWC on the development of a stable oral delivery systems for insulin. In the present work, the 13 kDa LMWC can be considered a suitable candidate to prepare the PEC and the RM systems. It showed an optimal formulation with respect to chemical and physical stability. Furthermore, it produced a suitable vehicle with optimal interfacial surface tension and nano-size particles (Assaf et al., 2011), in addition to the absence of precipitation (Mao et al., 2006). Subsequently, characterization and further *in vitro* and *in vivo* investigation of the bioactivity study were undertaken for the delivery systems prepared using the 13 kDa LMWC.

2.3.5. Characterization of I-LMWC PEC and I-LMWC RM Systems

The particle size results for I-LMWC PECs prepared using the same insulin concentration (3.5 mg/mL) and different concentrations of 13 kDa LMWC (1–5 mg/mL) were compared with the particle size results for insulin and LMWC alone. The particle size of

insulin in solution was found to be around 5.0 nm, which indicates that it is present in its hexameric form (Petersen et al., 2002). However, the particle size of LMWC is concentration dependent. When the LMWC concentration is low, LMWC particles are present in a more extended form; however, above a certain concentration aggregates start to form and the particle size starts to increase; similar results have been previously reported (Tsaih et al, 1997).

At low concentration of LMWC (1 mg/mL) the size distribution intensity of I-LMWC PECs was 6.38 ± 0.26 nm (higher than the size of insulin), and only one peak was present when measuring particle size. This means that all the LMWC molecules interact with insulin molecules to form a particle larger than insulin. Increasing the LMWC concentration to 3.5 and 5 mg/mL resulted in a particle size of 6.9 ± 0.17 nm; however, another peak started to appear in the region of around 100 ± 20 nm, which represents the size of the free LMWC and aggregated LMWC in the sample (*i.e.*, LMWC un-reacted with insulin). The results of mean particle size are shown in Figure 2.4.A. Since LMWC will react with insulin at one site only, an excess amount of LMWC will be free in the sample that may also aggregate and give a particle size of around 100 nm. The mean particle size of the RMs was 300 ± 19 nm.

The effect of LMWC concentration on the zeta potential of I-LMWC PEC (3.5 mg insulin/mL) was investigated. LMWC (1–5 mg/mL) without associated insulin shows a positive zeta-potential of +15 - +25 mV (Figure 2.4.A). On the other hand, the presence of insulin (with a zeta-potential of −27 mV) decreases the zeta-potential value to about +5 mV. The ionized groups of amino acid residues of insulin will be attracted to the positively charged LMWC via coulombic forces (Verma et al., 2013). The zeta potential of the complex is positive, which means that insulin is encapsulated in the polymer, projecting positively charged chains towards the external aqueous medium. Although the

zeta potential value of the complex is positive, the surface charge of the complex will be decreased when compared to LMWC alone, which is expected. This facilitates absorption of the PEC to the biolipid membranes of the GIT (Borchard et al., 1996).

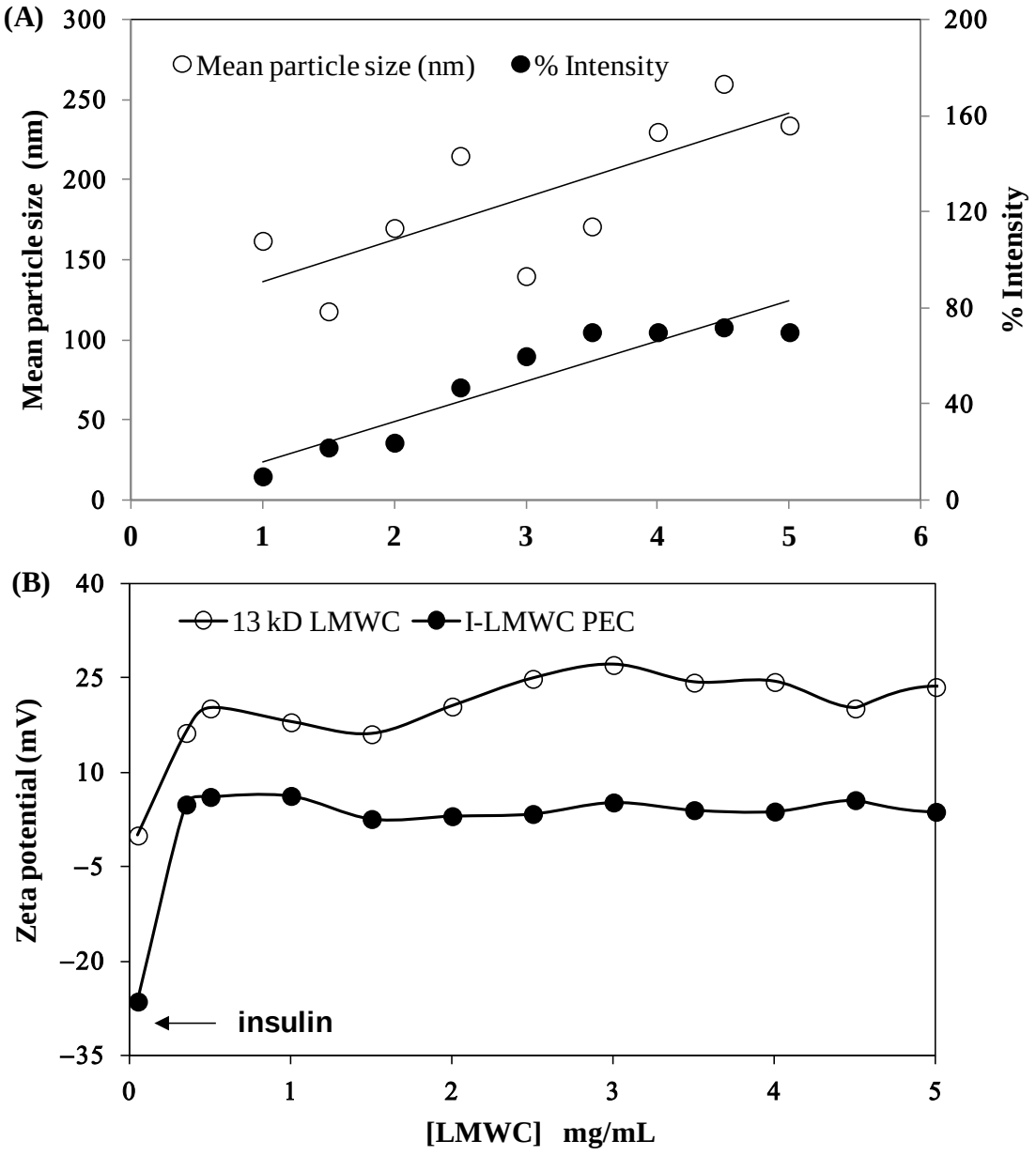


Figure 2.4 Effect of 13 kDa LMWC concentration on (**A**) the particle size of major peaks at 100–250 nm and (**B**) the zeta potential of the insulin-LMWC polyelectrolyte (I-LMWC PEC). The number of replicates is 6 and 3, respectively.

The data for the effect of 13 kDa LMWC concentration and pH of the I-LMWC PEC on insulin association efficiency (AE) are presented in Table 2.4. It is well known that the formation of PEC is pH dependent (Mao et al, 2006 & Ma et al., 2002). The investigated pH range (6.5–7.0) in the present work is close to the pK_a of chitosan (about 6.5) and to the isoelectric point (pI) of insulin (about 6.4). In this pH range, electrostatic, hydrophobic interactions as well as hydrogen bonding may be involved in PEC formation, as neutral and ionized chitosan species exist in an almost equal proportion (Lee et al., 2013).

Table 2.4 Effect of 13 kDa LMWC concentration and pH on insulin association efficiency (AE).

LMWC Concentration (mg/mL)	**pH_f**	**AE ± RSD (%)**
0.7	6.5	63.8 ± 2.1
3.0	6.5	76.2 ± 3.2
0.7	6.7	22.7 ± 2.8
3.0	7.0	8.6 ± 4.6

Number of replicates is 3 for each experiment.

In the present work, the PEC using 13 kDa LMWC was prepared at different pH values of 6.5, 6.7, and 7.0. However, lower and high pH values were not considered ($8 < pH < 5$), where insulin degrades rapidly (Mao et al., 2006 & Anderson et al., 1995). A change in pH from 6.5 to 7.0 results in a significant decrease in the value of the AE (from 76.5% to 8.6%) (Table 2.4). This may indicate that the PEC begins to precipitate at pH values higher than 6.5 as a result of a decrease in the solubility of the 13 kDa LMWC above its pk_a value (about 6.5) (Mao et al., 2006 & Kabanov et al., 1994). Such precipitation was observed for aqueous solutions of 13 kDa LMWC following pH adjustment to 7.0. This noticeable precipitation is probably due to the formation of neutral LMWC. However, the AE was

improved by using higher concentrations of LMWC. Such behavior was previously observed by Wu *et al.* (Wu et al., 2005).

2.3.6. *In Vitro* Evaluation of I-LMWC PEC and I-LMWC RM

2.3.6.1. *In Vitro* Release

The results for the *in vitro* release of insulin from the I-LMWC RM system showed a negligible release at pH 1.2 (<10% at 6 h), while at pH 6.8 the release is markedly increased (80% of the encapsulated amount at 6 h). Furthermore, the release profile at pH 6.8 was gradual and free of any detectable burst effects which may occur very rapidly, within first five minutes (Sarmento et al., 2006), as shown in the data in Figure 2.5.

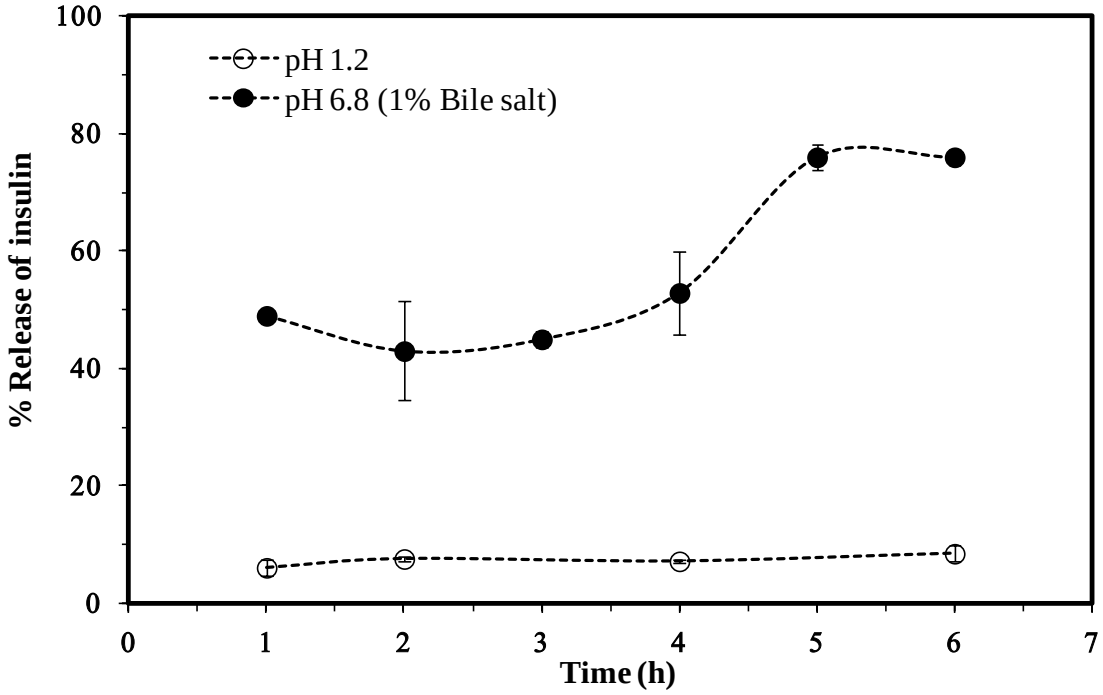


Figure 2.5 A cumulative release of insulin from insulin–LMWC reverse micelles (I-LMWC RMs) using 13 kDa LMWC.

2.3.6.2. *In Vitro* Evaluation of the Protective Effect

The protective ability of the RMs under conditions simulating the gastric environment was evaluated and compared with free insulin and I-LMWC PEC. Free insulin and I-LMWC PEC were found to be completely degraded during incubation with pepsin, while in the RM about 90% of insulin was recovered after incubation with simulated gastric fluid with pepsin. These results emphasize the importance of the RM system in the protection of insulin from degradation by pepsin.

2.3.7. Biological Activity

The data in Figure 2.6 illustrate the changes in blood glucose levels of rats after oral administration of I-LMWC PEC after solubilization in the reverse micelles. A decrease in plasma glucose level was observed. The results are significantly different when compared to a blank control ($p < 0.05$). The reverse micelle preparation resulted in minimum glucose levels, about 70% after 3 h, and the reduction in glucose levels was maintained over a prolonged period of time. It is worth mentioning that insulin and I-LMWC PEC are completely degraded under conditions simulating the gastric environment, as reported previously (Elsayed et al., 2009). However, the decrease in blood glucose levels (Figure 2.6) may be attributed to the improved stability of insulin in the reverse micelle preparation against degradation at gastric pH values and in the presence of enzymes in the GIT system.

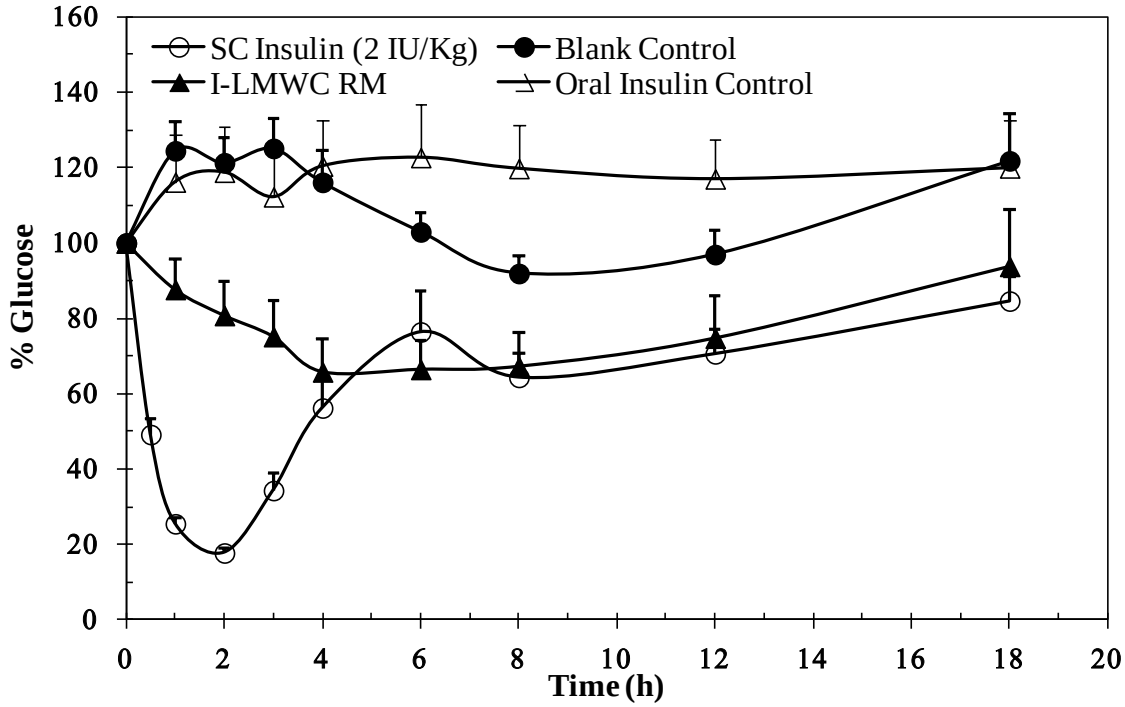


Figure 2.6 Blood glucose levels *versus* time profile after a single oral administration of 50 IU/kg insulin-LMWC reverse micelle (I-LMWC RM) and a subcutaneous administration (SC) of 2 IU/kg insulin to STZ-diabetic rats The results are expressed as mean ± SD (n = 10 for each group). Blank Control is RM containing only LMWC with no insulin, Oral insulin control is insulin 2 IU/Kg given orally.

4. Conclusions

The insulin USP compendium method for insulin analysis and HMWPs can be used to evaluate insulin stability in a delivery system containing insulin as well as I-LMWC PEC. Insulin stability was evaluated for insulin content, formation of HMWPs, and biological activity. In the literature, minimal studies are available covering these three elements, especially formation of HMWPs, which are very important in evaluating the suitability of the delivery system. Extraction procedures must be carefully carried out in order not to affect insulin results. Retention time optimization is important and currently insulin analysis can be executed within 20–30 min with such a convenient HPLC method. Based

on stability studies, solutions of 3.5 mg/mL insulin and 1.5 mg/mL 13 kDa LMWC constitute the optimum stability formulation of the I-LMWC PEC for orally delivered insulin. Our experimental design allowed for the optimization of insulin formulation by determining parameters that affect the chemical and physical stability of insulin. The I-LMWC PEC preparation method has the advantage of not necessitating sonication and use of organic solvents during preparation, thereby minimizing possible degradation of insulin. The I-LMWC PEC system has been characterized in terms of insulin stability, where the study revealed that complexation with LMWC improves the stability of insulin. Both ELISA and *in vivo* studies confirmed the bioactivity of loaded insulin in the delivery system examined.

CHAPTER 3: Influence of Glutathione on the Bioactivity of Subcutaneously or Orally Administered Insulin to Rats

3.1. Introduction

Restoration of the normal metabolic *milieu* and thus minimizing the risk of complications caused by diabetes by replicating physiological insulin secretion, has become an essential target for the treatment of diabetes (Owens et al., 2003). Insulin replacement therapy offers the most effective means for glycemic control in patients with type I diabetes mellitus. Several means of insulin administration have been used including subcutaneous injections (SC), insulin pumps and repeat-use insulin pens. Alternative delivery routes including vaginal, rectal, oral (e.g. buccal, gastro-intestinal and sublingual), nasal and pulmonary have also been investigated (Shah et al., 2010). Amongst the foregoing methods of insulin delivery, the oral route is considered to be the most feasible and convenient in order to improve compliance among diabetic patients (Shah et al., 2010). However, the delivery of insulin via the oral route is a challenge primarily due to its poor bioavailability as a result of enzymatic degradation in the gastrointestinal tract and its low cross-intestinal epithelium permeability (Wong 2010).

Oral bioavailability of insulin is less than 1% (Renukuntla et al., 2013). Consequently, the primary objective in developing oral insulin formulations is to improve its bioavailability. It is worth mentioning that oral administration of insulin mimics - in part - its physiological pathway. Such a pathway generates a difference in concentration of insulin between the portal vein and the general circulation. This allows externally delivered insulin to act in a similar manner to endogenous insulin (Arbit et al., 2009). Various approaches have been undertaken in order to improve insulin oral bioavailability

by e.g. the addition of permeation/absorption enhancers, enzyme inhibitors, or by chemical modification (Mesiha et al., 1994, Morishita et al., 1992 & Asada et al., 1995).

The use of chitosan as a biopharmaceutical drug carrier is regarded as a promising approach because it is biocompatible, non-immunogenic, non-toxic, biodegradable, a permeation enhancer and has mucoadhesive property (Mukhopadhyay et al., 2012). Thus, interest in using natural materials such as chitosan as part of drug development has increased in the past two decades (Wong, 2009). Chitosan and modified chitosans have been widely used in nanoparticle preparations for the oral delivery of insulin (Mukhopadhyay et al., 2012, Wong, 2009, Wotiski et al., 2009 & Damge et al., 2007). Such nanoparticles can be prepared by ionic gelation, microemulsion, self-assembly by solvent evaporation, use of PECs or reverse micelles (Nagpal et al., 2010).

A novel oral delivery system suitable for proteins based on a reverse micelle (microemulsion) system, using insulin as a model drug has been previously reported (Badwan et al., 2007). The proposed delivery system was synthesized by forming a PEC between low molecular weight chitosan (LMWC) and insulin. The PEC was solubilized in the micelle using oleic acid as the continuous phase and a mixture of PEG 8 caprylic/capric glyceride and glycerol-6-dioleate as a surfactant-co-surfactant system. *In vivo* studies showed that this process results in nanoparticles which were effective in reducing glycaemia following oral administration to fasted diabetic rats.

It has been reported that GSH has an important role in the degradation and regulation of insulin (Hird, 1962 & Ammon et al., 1989). In diabetes, decreased cellular GSH content is a common finding that is mainly associated with increased oxidative stress (Ammon et al., 1980). GSH depletion affects peripheral insulin action as the hormone stimulates glucose transport and metabolism in adipocytes and skeletal muscles. This occurs by

inducing the translocation of glucose transporter (GLUT4) from internal membrane pools to the plasma membrane; consequently GSH depletion occurs, modulating either metabolic insulin signaling or GLUT4 protein expression (Khamaisi et al., 2000). It has been reported that exogenous GSH potentiates glucose-induced insulin secretion from rat pancreatic islets in a dose-related manner (Ammon et al., 1986); however, GSH does not appear to initiate but rather modulates the insulin secretory action of glucose since no effect on the secretion of insulin in the presence of non-stimulatory glucose was noted (Ammon et al., 1986). Direct molecular interactions between insulin and GSH have also been reported *in vitro* (Du et al., 2012). GSH is capable of converting insulin into A and B chains by reduction of disulfide bridges, which serves as an electron donor. In this process, GSH is converted to its oxidized form GSSG (Hird, 1962). GSH participates in many cellular reactions as an antioxidant defense system by scavenging free radicals and reactive species. Also, by regulating intracellular redox status and signal transduction and gene expression (Wu et al., 2004).

Herein, the effects of reduced and oxidized forms of glutathione (GSH and GSSG) on the bioactivity of subcutaneously administered insulin is evaluated with the objective being to improve the bioactivity of orally administered insulin. Furthermore, the hypoglycemic effects of orally administered RMFs containing GSH, GSSG are also reported.

3.2. Materials and Methods

3.2.1. Materials

Recombinant human (rh) insulin (99.4 %, Biocon, Bangalore, India), reduced glutathione (98.5%, Sigma-Aldrich Corp., St. Louis, MO, USA), oxidized glutathione (98%, Across, Germany) and chitosan HCl (~250 kDa, 95% degree of deacetylation (DDA), Xiamen Xiang, Shanghai, China) were used as received. Low molecular weight chitosan of

molecular weight 13 kDa and DDA of 99% was obtained by depolymerization of chitosan HCl (Varum et al., 2001). Oleic acid (> 99%) was purchased from Merck KGaA, Darmstadt, Germany). Labrasol® (PEG-8 caprylic/capric glycerides), Plurol® Oleique CG (Polyglycerol-6-dioleate) (Gattefosse, France) and Calbiochem® Streptozotocin (Merck Millipore Company, MA, USA) were of analytical/HPLC grade. Purified water was provided by the Jordanian Pharmaceutical Manufacturing (JPM) Company.

3.2.2. Preparation of Insulin Solution

Insulin solutions (1 IU/mL) were prepared by dissolving 70 mg of insulin powder in 1mL of 0.1 N HCl, neutralized with 0.1N NaOH and made up to a volume of 10 mL with 1M Tris (hydroxymethyl aminomethane) buffer (pH 6.5). The 1 mL solutions were then diluted to 100 mL volume with water.

3.2.3. Preparation of Insulin-Chitosan Polyelectrolyte Complex (IC-PEC)

The IC-PEC was prepared following a general procedure previously reported by Elsayed *et al* (Elsayed et al., 2009). LMWC solution (3 mg/mL) was prepared by dissolving LMWC powder in water; the pH was adjusted to 5.5 with 0.1N NaOH, and then made up to a volume of 10 mL with water. Insulin solution (7 mg/mL) was prepared by dissolving insulin powder in 1 mL of 0.1 N HCl, neutralized with 0.1N NaOH and made up to a volume of 10 mL with 1M Tris (hydroxymethyl aminomethane) buffer (pH 6.5). The IC-PEC was then prepared by mixing equal volumes of LMWC and insulin solutions in a glass vial under gentle magnetic stirring and incubated for 10 min at room temperature.

Different IC-PECs were also prepared following the same procedure, but with different concentrations of GSH (1.4, 2.1 and 6.3 mg/mL). Stock solutions of GSH (14, 21 and 63 mg/mL) were initially prepared in water; 1 mL of each solution was separately mixed

with 4 mL of insulin solution (7 mg/mL) and then mixed with an equal volume of LMWC solution. Another IC-PEC was also prepared following the same procedure, but at a GSSG concentration of 2.1 mg/mL. Thereafter, 1 mL of GSSG solution was separately mixed with 4 mL of insulin solution (7 mg/mL) and then mixed with an equal volume of LMWC.

3.2.4. Preparation of Insulin-Chitosan Reverse Micelles (IC-RMs)

The IC-RMs were prepared as previously described in Section 2.2.5.

3.2.5. Particle Size and Zeta Potential Determinations

The hydrodynamic diameters of insulin (7 mg/mL, at pH 6.5), LMWC (3 mg/mL, at pH 5.5), IC-PEC and IC-RM were analyzed by dynamic light scattering (DLS). Each analysis was performed in triplicate at 25°C (the RSDs being in the range of 3-10%) with an angle of detection of 90°. The zeta potential was determined by laser Doppler velocimetry (LDV). DLS and LDV analyses were performed using a Zetasizer 3000 HS (Malvern Instrument, Malvern, UK).

3.2.6. Evaluation *of In Vivo* Pharmacological Activity

3.2.6.1. Animal Handling

As described in section 2.2.14.1, Chapter 2.

3.2.6.2. Induction of Diabetes Using Streptozotocin (STZ)

As described in section 2.2.14.2, Chapter 2

3.2.6.3. Experimental Design of Subcutaneous (SC) Testing

Non-diabetic fasted rats were randomized into groups (n = 10). For all experiments, a group of rats was injected subcutaneously with 1 IU insulin/kg and used as a control group. Other groups were injected with GSH or GSSG as described later. The initial blood glucose level (at 0 h) was determined for each rat just before non-glutathione control samples, GSH or GSSG injection. Later, blood glucose determination was performed at 0.5, 1, 2, 3 and 4 h post insulin (1 IU/kg) SC injection.

In order to evaluate effect of GSH on bioactivity of insulin solution administered SC, the effect of the time interval between GSH (50 mg/kg) and insulin (1 IU/kg) injections was investigated. Different groups of rats were injected with insulin at 10, 20 and 30 min after SC injection with GSH. Changes in blood glucose levels were compared with non-GSH and non-insulin treated rats.

The effect of GSH at doses of 5, 25 and 50 mg/kg on the insulin bioactivity was investigated. Rats were injected with GSH at 30 min prior to SC insulin administration and changes in blood glucose levels were compared to the non-glutathione treated rats. SC injection was undertaken at the same place for both GSH and insulin.

Control experiments were performed in the same manner to compare the effect of GSSG and GSH (both at 50 mg/kg) on the bioactivity of the injected insulin.

3.2.6.4. Experimental Design of Oral Testing

STZ diabetic rats (n=10) were randomized into different groups. Following initial blood glucose determinations, one group was injected with oral placebo (same RM components, but without insulin and GSH or GSSG) and served as a negative control. Another group was injected with 1 IU/kg insulin and served as a positive control for insulin bioactivity. The other groups received different oral preparations of 50 IU/kg insulin formulated with

different concentrations of GSH, namely: 1.4, 2.1 and 6.3 mg/mL, using a stainless steel oral gavage needle. Blood sampling for glucose measurements preceded during the experiments as described earlier at specific time intervals (1, 2, 3, 4, 5, 6, 8 and 10 h) post-insulin administration.

In a separate set of experiments, the effect of GSH and GSSG incorporated RMFs, both at 2.1 mg/mL, were compared to non-glutathione control samples (0 mg GSH/mL, 0 mg GSSG/mL). This served as a positive control for insulin bioactivity and as an oral placebo (same as the reverse micelle components (RMF), with GSH or GSSG but without insulin).

3.2.7. Data and Statistical Analysis

Blood glucose levels are expressed as the mean percentage of the baseline glucose levels and plotted against time to evaluate the cumulative hypoglycemic effect over time. Glucose level profiles were quantified by the area above the curve (AAC) following the trapezoidal rule.

The pharmacological activity (PA) values were calculated as a relative measure of cumulative reduction in glucose blood levels compared to 100% bioactivity of the control insulin administered subcutaneously at a dose of 1 IU/kg.

All data are expressed as mean values ± standard error of means (SEM). One way analysis of variance (ANOVA) followed by Tukey's *post hoc* test form multiple comparisons were used for statistical evaluation (SPSS 17, Chicago, IL, USA). P values <0.05 were considered as being significant.

3.3. Results

3.3.1. Preparation, Characterization and *In Vitro* Performance of IC-PEC and IC-RM

As illustrated in the data in Table 3.1, the hydrodynamic diameter of the IC-PEC particles, obtained by mixing solutions of insulin and LMWC (13 kDa), was found to be 118 ±10 nm at pH 6.5. The encapsulation of the prepared IC-PEC, by mixing it into an oily phase consisting of oleic acid and surfactants (labrasol and plurol), produced an IC-RM system with a hydrodynamic diameter of 295 ±19 nm. On the other hand, zeta potential measurements of insulin (7 mg/mL, pH 6.5), LMWC (3 mg/mL, pH 5.5), IC-PEC (pH 6.5), and IC-RM (acidic nature) were −26.4, +20.5, +3.1 and +200.7 mV, respectively (Table 3.1).

Table 3.1 The particle size and zeta potential for insulin-chitosan polyelectrolyte and its reverse micelles.

System	Particle size (nm)	Zeta potential (mV)
Insulin	4.5 ± 1	−26.4
LMWC	70 ± 12	+20.5
IC-PEC	118 ± 20	+3.1
IC-RM	295 ± 19	+200.7

LMWC, low molecular weight chitosan; IC-PEC, insulin-chitosan polyelectrolyte; IC-RM, insulin-chitosan reverse micelle. Results are the mean of triplicate measurements.

3.3.2. Effect of GSH and GSSG on SC Insulin Bioactivity

The effects of the time interval between GSH injection (50 mg/kg) and SC insulin injection (1 IU/kg) on the bioactivity of insulin is shown in the data in Figure 3.1. The hypoglycemic activity of SC insulin was affected by the presence of GSH regardless of

the time interval. Such an effect was clearly observed when GSH was injected 30 min prior to insulin. This time interval was therefore selected for further investigation.

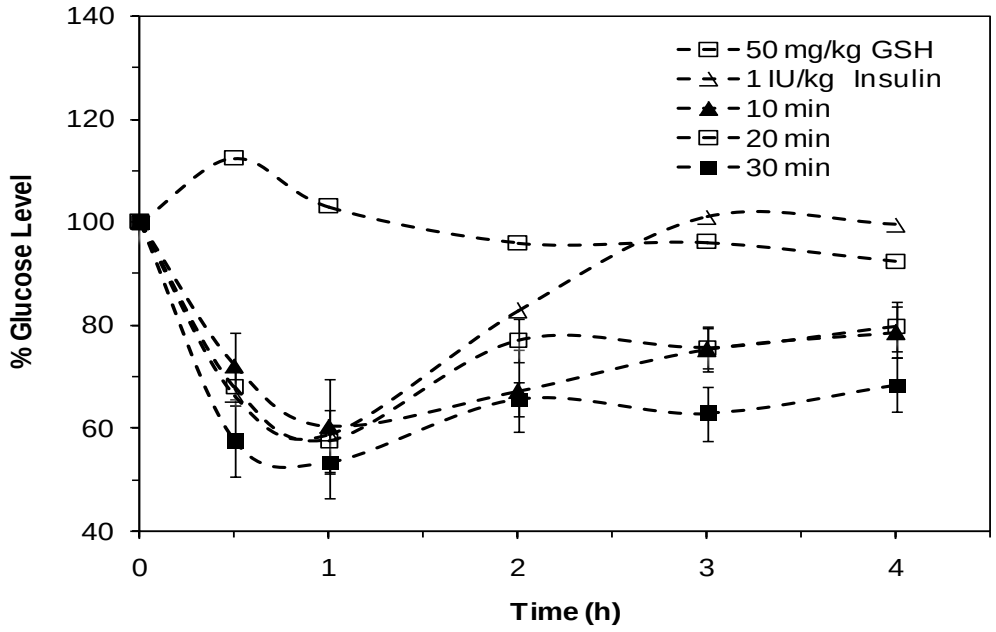


Figure 3.1 Percentage blood glucose levels in non-diabetic rats after subcutaneous (SC) administration of 50 mg/kg of reduced glutathione (GSH) at different time intervals prior to SC insulin injection (1 IU/kg). Glucose measurements were performed at different time intervals (0 – 4 h). Each data point represents the mean ± SEM (n = 10).

The effect of different doses of SC GSH (0, 5, 25 and 50 mg/kg) on the bioactivity of SC insulin (1 IU/kg) is shown in the data in Figure 3.2. The hypoglycemic activity of SC insulin, in the presence of GSH, was significantly affected in a dose-dependent manner, where reductions in insulin activity were noted ($p < 0.01$). Rats treated with 50 mg GSH/kg showed the maximum reduction in activity and this was statistically significant compared to the other GSH doses as revealed by Tukey's multiple comparison tests ($p < 0.05$).

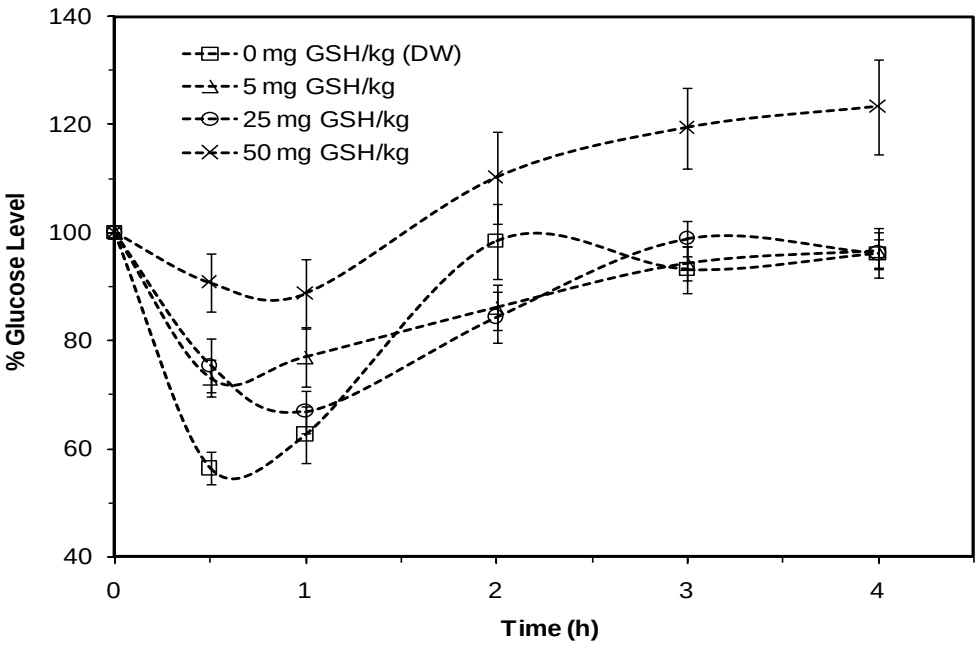


Figure 3.2 Percentage blood glucose levels in non-diabetic rats after subcutaneous (SC) administration of different doses of reduced glutathione (GSH) prior to SC insulin injection (1 IU/kg). Glucose measurements were performed at different time intervals (0 – 4 h). Each data point represents the mean ± SEM (n = 10). GSH significantly reduced the activity of insulin in a dose dependent manner ($p < 0.05$).

The effects of GSH and GSSG, both at a dose of 50 mg/kg, on the bioactivity of SC insulin (1 IU/kg) is shown in Figure 3.3, and their corresponding pharmacological parameters are listed in Table 3.2. The presence of GSH depleted the action of insulin (relative bioactivity of 91.6%) while GSSG significantly enhanced hypoglycemia (relative bioactivity 110.7%) by further reducing the glucose levels ($p < 0.05$). Compared to non-glutathione tested rats, the enhancement of hypoglycemia by 50 mg/kg GSSG was evident 1 h post-insulin administration.

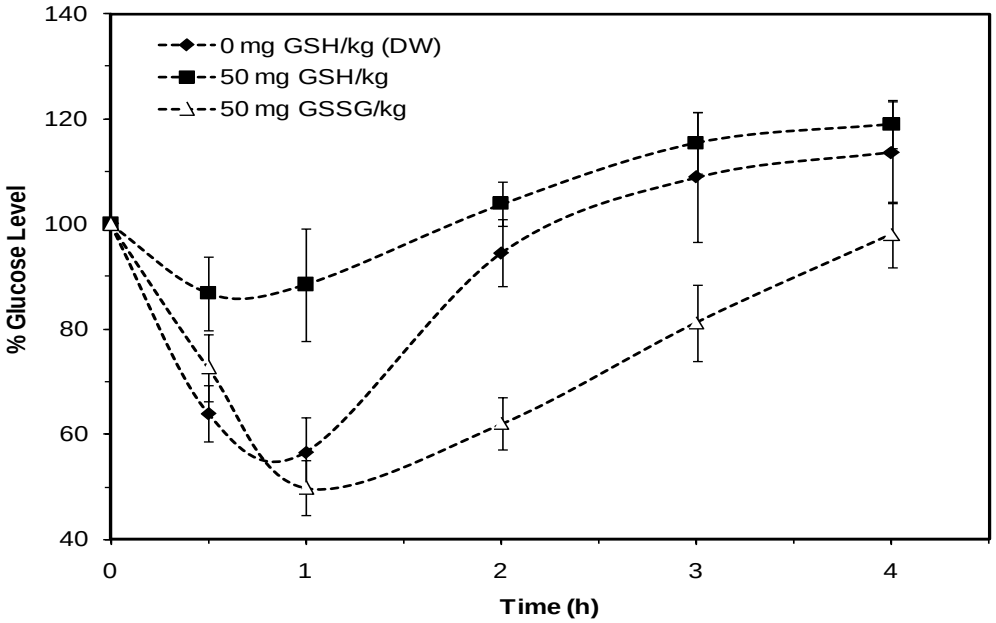


Figure 3.3 Percentage blood glucose levels in non-diabetic rats after subcutaneous (SC) administration of 50 mg/kg of reduced (GSH) or oxidized glutathione (GSSG) prior to SC insulin injection (1 IU/kg). Glucose measurements were performed at different time intervals (0 – 4 h). Each data point represents the mean±SEM (n = 10). GSSG administration significantly enhanced hypoglycemia ($p < 0.05$).

Table 3.2 The relative bioactivity of insulin in the presence of reduced and oxidized glutathione following subcutaneous (SC) and oral administration.

Route*	**GSH/GSSG concentration**	**AAC±SEM (% glucose * hr)**	**RBA (%)**
Subcutaneous	0 mg GSH/kg	640±44	-
	50 mg GSH/kg	586±25	91.6
	50 mg GSSG/kg	709±65	110.7
Oral (RM)	0 mg GSH/mL	380±29	5.9
	2.1 mg GSH/mL	368±21	5.8
	2.1 GSSG/mL	478±31	7.5

GSH and GSSG: reduced and oxidized glutathione, respectively. AAC, area above the curve; SEM, standard error of means. Relative bioactivity (RBA) is calculated with respect to SC system at 0 mg GSH/mL. *Insulin doses were 1 IU/kg and 50 IU/kg for SC and oral administrations, respectively.

3.3.3. Effect of GSH and GSSG on Oral Insulin Bioactivity

To our knowledge the incorporation of GSH or GSSG in orally delivered insulin systems has not been reported. The effect of incorporating GSH at 1.4, 2.1 or 6.3 mg/mL in the IC-RM preparation on blood glucose levels is shown in Figure 3.4. The results indicated that concurrent oral administration of GSH with insulin had no effect on the hypoglycemic activity of insulin ($p > 0.05$). However, the activity of insulin was not significantly ($p > 0.05$) reduced when rats were administered oral IC-RM preparations containing the highest concentration of GSH (6.3 mg/mL).

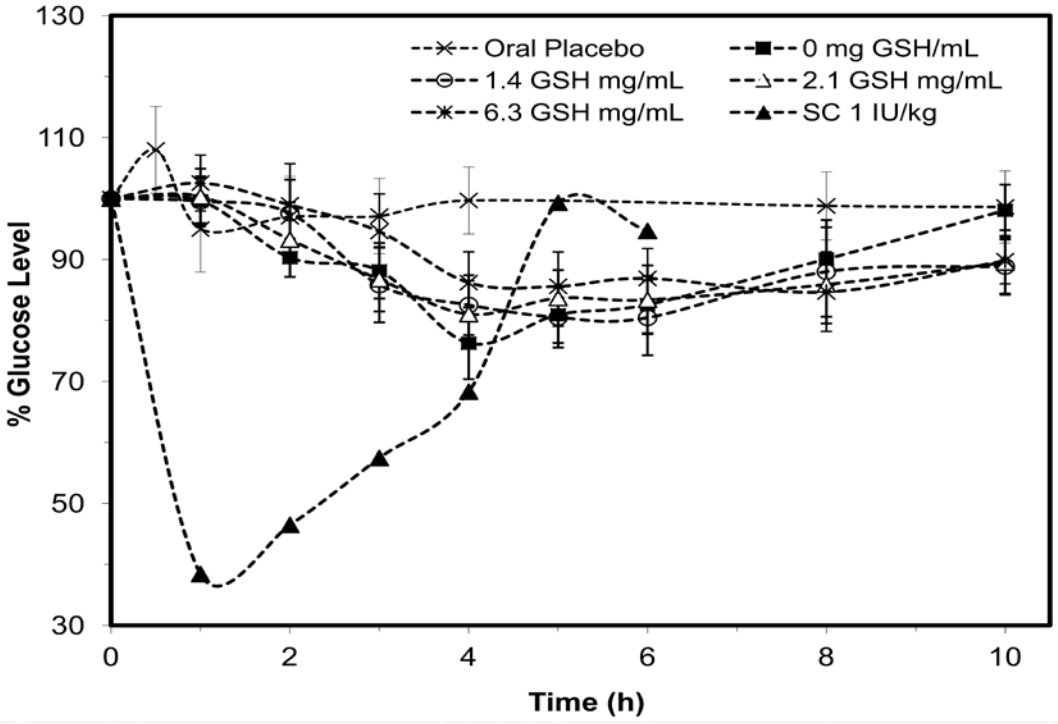


Figure 3.4 Percentage blood glucose levels in diabetic rats after oral administration of insulin-chitosan reverse micelle (IC-RM) containing 50 IU insulin/mL and different concentrations of reduced glutathione (GSH) in comparison with SC insulin injections (1 IU/kg). Glucose measurements were performed at different time intervals (0 – 10 h). Each data point represents the mean±SEM (n = 10). The reduction in insulin activity was insignificant with GSH containing preparations ($p > 0.05$).

A comparison of the effects of incorporating GSH and GSSG, both at 2.1 mg/mL, in the IC-RM preparation are illustrated in Figure 3.5, and their corresponding pharmacological parameters are summarized in Table 3.2. Compared to the non-glutathione treated rats, incorporation of GSH in the oral insulin preparations did not induce any significant changes in the blood glucose profile ($p > 0.05$). The relative bioactivity values were 5.9 and 5.8% for the non-glutathione control and GSH containing samples, respectively. Conversely, it was found that incorporating GSSG in the IC-RM system induced a

consistent and significant reduction of blood glucose levels (relative bioactivity of 7.5%) compared to GSH and non-glutathione treated rats ($p < 0.05$).

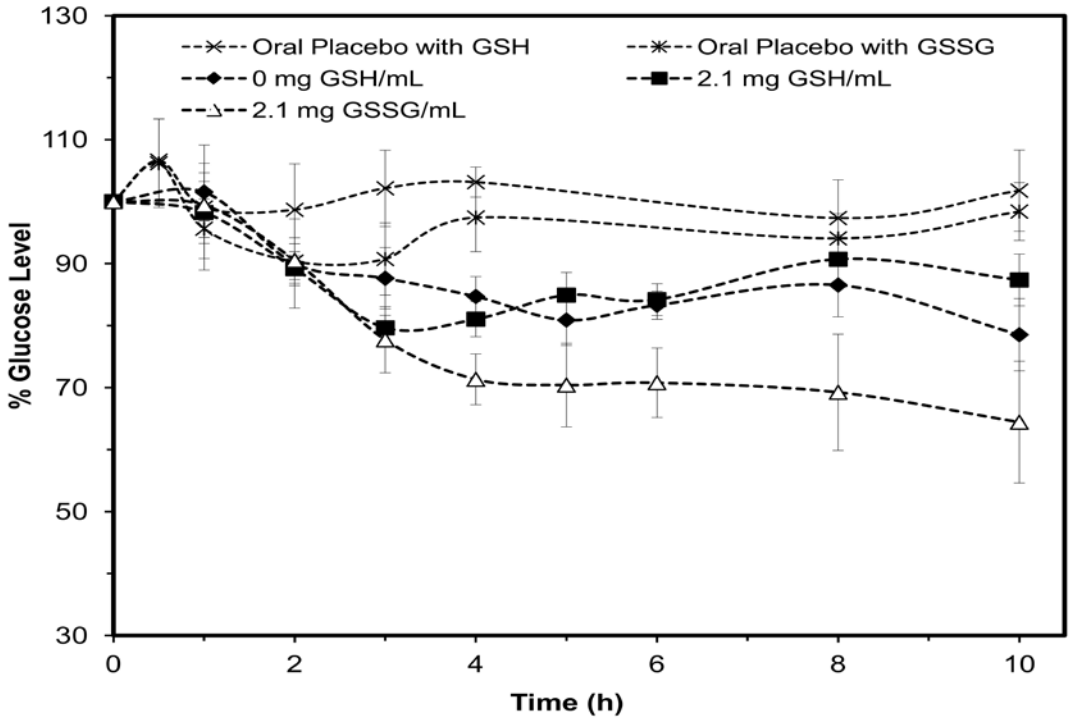


Figure 3.5 Percentage blood glucose levels in diabetic rats after oral administration of insulin-chitosan reverse micelles (IC-RM) containing 50 IU insulin/mL and 2.1 mg/kg of reduced (GSH) or oxidized glutathione (GSSG). Glucose measurements were performed at different time intervals (0 – 10 h). Each data point represents the mean±SEM (n = 10). GSH ($p > 0.05$), GSSG ($p < 0.05$) compared to glutathione free preparations.

3.4. DISCUSSION

3.4.1. Preparation, Characterization and *In Vitro* Performance of IC-PEC and IC-RM

In the present work, in which an oral insulin delivery system was studied, soluble LMWC was used to prepare nanosize polyelectrolyte complexes. These were formed by the interaction of positively charged amine groups of LMWC with the carboxylic groups in

insulin by gentle mixing, while avoiding any harsh conditions which may degrade the peptide (Mao et al., 2006). The prepared complex (IC-PEC) was further solubilized in a reverse micelle. The reverse micelles produced aggregated in nanoparticle vesicles with a narrow size distribution with a hydrodynamic diameter of 295 ±19 nm compared to a hydrodynamic diameter of 118 nm before aggregation. It was previously reported that such solubilization of LMWC in the core of the reverse micelles resulted in a size reduction from about over 2000 nm to 300 nm range (Assaf et al., 2011). As the excess ionized amine groups (NH_3^+)of free chitosan will penetrate through the reverse micelle membrane and interact with the external phase of oleic acid, this results in tightening the reverse micelle membrane at the interphase, thus not allowing any gastrointestinal acid or enzyme to diffuse into the micelle (Assaf et al., 2011).

When LMWC, which has a zeta potential in the vicinity of + 20.5 mV, was mixed with insulin, which has a zeta potential in the vicinity of -26.4 mV, the resulting zeta potential decreased. The complex formed between LMWC and insulin at pH 6.5 was found to have a zeta potential close to +3.1 mV. This indicates that the charge on the LMWC was partially neutralized by the charges on the amino acids in insulin. When this complex was solubilized in the reverse micelles, a highly positive zeta potential in the vicinity of +200.7 mV was obtained. Instrument limitation of reading such oil sample do exist and so measurement may not be as accurate as expected. However, such a high value guarantees the stability of the nanosize vesicles with minimum tendency to flocculate.

3.4.2. Effect of GSH and GSSG on Insulin's Bioactivity

GSH is a tripeptide with a γ-peptide linkage between the amine group of cysteine, which is attached by a normal peptide linkage to glycine, and the carboxyl group of the glutamate side chain. Cysteine is chemically active due to the presence of a thiol group

(–SH) which is critical for the biological functions of enzymes (Dean et al., 2011). The thiol can be oxidized to disulfide. This facilitates the reducing properties of GSH, whereby cysteine is oxidized to form disulfide GSSG (Dean et al., 2011). GSH is considered to function as the major intracellular thiol-disulfide redox buffer (Schafer et al., 2001).

Administration of SC insulin alone 30 min after SC administration of GSH guarantees the presence of GSH in the circulation prior to insulin injection. This arises due to the long half-life of GSH (90 min) (Baudouim-Cornu et al., 2012) compared to the relatively short half-life of insulin (5 min) (Matthews et al., 1985). A decrease in insulin activity was detected even when a low concentration of GSH (5 mg/kg) was tested. The decrease in insulin activity was dependent on the concentration of GSH (Figure 3.2).

Although GSH is a physiological constituent of blood plasma, its concentration in blood is far less than its intracellular concentration. It is evident that changes in GSH concentration and GSH/GSSG ratio can influence the rate of insulin degradation (Ammon et al., 1989).

The initial degradation of insulin takes place in endosomes with two or more cleavages in the B-chain by an insulin-degrading enzyme (IDE) (Seabright et al., 1996). This is followed by reduction of disulfide bonds by the enzyme glutathione insulin transhydrogenase (GIT) which has been also recognized as a protein disulfide isomerase (PDI) (Seabright et al., 1996 & Chandler et al., 1975) utilizing insulin and GSH as substrate and cosubstrate, respectively.

The reaction of insulin, regardless of the delivery method, with GSH is expected to yield multiple reaction products. The action of GIT results in reduced chain A and chain B together with GSSG (Chandler et al., 1975). It is worth mentioning that the rate of such a

reaction is a function of GSH concentration where the rate increases with increasing GSH concentration (Du et al., 2012, Chandler et al., 1975 & Cordes et al., 2011). Such findings are in-line with our results where rats treated with 50 mg GSH/kg showed the maximum reduction in activity of insulin (Figure 3.2). This was statistically significant compared to other doses of GSH, as also reported by Du *et al*. (Du et al., 2012).

On the other hand, S.C. administration of GSSG increased the hypoglycemic activity of insulin (Figure 3.3). This result indicates that the degradation reaction of insulin was inhibited. It has been reported that GSSG affects IDE function via glutathionylation of one or more of its cysteines (Cordes et al., 2011); additionally, GSSG has a competitive inhibition effect compared to GSH (Chandler et al., 1975).

The difference in activity of GSH and GSSG on insulin bioactivity administered SC is clearly shown by calculating the area above the curve (AAC) and the fractional pharmacological activity (PA) (Table 3.2). Using the same concentrations of GSH and GSSG, AAC was decreased by 10 % with GSH and increased by 10 % with GSSG. The presence of GSH depleted the action of insulin (relative bioactivity 91.6%) while GSSG significantly enhanced hypoglycemia (relative bioactivity 110.7%) by further reducing the glucose levels ($p < 0.05$). Compared to non-glutathione tested rats, the enhancement of hypoglycemia by 50 mg/kg GSSG was evident 1 h post-insulin administration.

The biological activity of IC-RM administered orally to STZ diabetics rats revealed a hypoglycemic effect. A significant decrease in glucose was achieved at 4 h and maintained for 8 h (Figure 3.4). The relative bioactivity (BA) was calculated to be 5.9 % based on a S.C. insulin standard at a dose of 1 IU/kg. These results clearly indicate that insulin absorption was enhanced by the IC-PEC dispersion in reverse micelles. Incorporating different concentration of GSH within RM along with insulin did not have

any effect on insulin activity. So it is evident that even high concentrations of GSH (6.3 mg/mL) did not have any degradation effect on insulin and RM system retained the activity of insulin and protected from degradation.

Nanoparticles of GSH-chitosan conjugates were prepared in order to improve GSH stability against oxidation. This conjugate resulted from the formation of an amide bond between the chitosan (NH_2) and the glycine carboxylic group (COOH) of glutathione (Koo et al., 2011 & Kafedjiiski et al., 2005). In the present work, the effect of incorporating non-conjugated GSH in the reverse micelles on insulin stability was investigated. The micellar delivery system avoided the effect of pepsin and protected insulin from degradation, minimizing the effect of IDE on insulin. Additionally, the micellar system protected insulin from degradation in hepatic cells. Accordingly, insulin activity is retained in the presence of different concentrations of GSH, as shown in the data in Figure 3.4. On the other hand, the results of the oral administration of IC-RM containing GSSG indicated that the hypoglycaemic activity was increased as shown in the data in Figure 3.5. This increase might be attributed to competitive inhibition of GSSG against GIT. The relative PA was increased by 7.5% in the GSSG treated animals compared to the non-treated group of animals. It is evident that the inclusion of GSH in the RMs would not jeopardize the activity of insulin; however, when GSSG was solubilized with IC-PEC in the RMs and administered to rats, the hypoglycemic activity of insulin was increased.

3.5. CONCLUSIONS

Formation of a PEC between insulin and LMWC was successfully prepared and solubilized in a reverse micelle (RM)to produce nanoparticles suitable for the oral delivery of insulin. This novel delivery system was used to show that co-solubilization of

GSSG with insulin in the RMs and could effectively reduce blood glucose levels in rats *in vivo* mainly through protection of insulin against degradation. Further studies are needed to verify the actual mechanism as to how GSSG solubilized in a RMF increases the bioactivity of insulin.

CHAPTER 4: Influence of Glucosamine on the Bioactivity of Insulin Delivered Subcutaneously and in an Oral Nano-Delivery System

4.1. Introduction

Glucosamine (2-amino-2-deoxy-D-glucose; GlcN) is an endogenous amino monosaccharide synthesized in the body via the hexosamine biosynthetic pathway (HBP) (Groves et al., 2013).

GlcN can also be obtained from exogenous sources in which it is phosphorylated to glucosamine-6-phosphate by hexokinase, which then by-passes glutamine:fructose-6-phosphate amidotransferase to HBP (Chatham et al., 2010). Salts of GlcN (chloride or sulphate) differ in their pharmacokinetic parameters; however, body exposure to either salt is the same as when ingested; both salts dissociate fully yielding free GlcN, and the bioavailability of GlcN derived from either source is expected to be the same (Alghazadeh-Habashi et al., 2011). In addition, extracellular GlcN is transported into muscle and adipose tissue via specific glucose transporters (GLUT1, 2 and 4). Whereas GLUT1 and GLUT4 have similar apparent affinities for glucose and GlcN, GLUT2 has a 20-fold higher affinity for GlcN than for glucose (Mueckler et al., 2013).

GlcN is one of the most widely used over the counter dietary supplements for the management of osteoarthritis (Bruno et al., 2005). The therapeutic effect of GlcN in osteoarthritis is due to its immunomodulatory activity (Nakamura et al., 2011), and via

inhibition of the COX-2 enzyme (Park et al., 2013). GlcN has also been shown to display antioxidant activity (Xing et al., 2006), increase the biosynthesis of glutathione (Greenen et al., 2013 & Gupta et al., 2010) and affect transcription regulation in response to stress (FOXO4 induction) (Ho et al. 2010).

Marshall et al. (Marshall et al., 1991) have examined the effect of GlcN on glucose metabolism and insulin secretion. They hypothesized that insulin resistance, resulting from chronic hyperglycemia, might be related to the increased flux of metabolites through the HBP. The effect of increasing GlcN concentrations on glucose transport and glycogen synthesis, in muscle and adipose tissue has also been investigated (Nelson et al., 2000). GlcN inhibits the glucose transporter system, GLUT4, further limiting glucose entry into cells and reduces glucose storage via glycogen. Infusion of GlcN to normal rats induces insulin resistance in several insulin sensitive tissues including muscle, heart, liver and adipose tissue (Virkamaki et al., 1997). In human clinical studies (Pouwels et al., 2001), a large amount of GlcN (7.2-9.7 g, 1.6 μmol/min/kg-5.0 μmol/min/kg) was infused over a 5 h period with no change in blood glucose levels. Reginster et al. studied the effect of GlcN sulfate supplementation (1.5 g/day) given to participants for a period of 3 years (Reginster et al., 2001). The study showed that blood glucose values were slightly lower than baseline values. A recent study by Jae et al., described a pharmaceutical formulation comprising GlcN·HCl as an active ingredient for the treatment of diabetes in order to lower blood sugar levels. The proposed formulation can be administered (0.1-1.0 g/kg) orally, transdermally, subcutaneously, intravenously or intramuscularly (Jae et al., 2006).

The co-administration of GlcN, highly used over the counter product, with hypoglycemic drugs in diabetic patients worth attention. Recently, it has been shown that the co-administration of GlcN with paracetamol (Alkhawaja et al., 2014) and statin (Al-Akkam

et al., 2013) may inhibit the metabolism of these drugs by lowering the concentration of paracetamol metabolites (paracetamol mercapurate and paracetamol cysteine), which may subsequently influence the side effects of these drugs.

Using chitosan and modified chitosan to deliver insulin via the oral route by nanoparticles has been widely investigated (Mukhopadhyay et al., 2012). Badwan et al. described a novel system based on solubilization of insulin-chitosan PEC in a reverse micelle (RM) system synthesized using PEG-8 caprylic/capric glycerides and glycerol-6-dioleate as emulsifying agents and dispersed in oleic acid (Badwan et al., 2007). RM system characterization, factors affecting the physic-chemical stability of this system and its bioactivity were investigated (Elsayed et al., 2007, Assaf et al., 2011 & Al Kurdi et al., 2015).

However, to the best of our knowledge, the effect of GlcN on the pharmacological activity of orally delivered insulin has not been reported in the literature. In the current work, an oral insulin delivery system (IC-RM) was prepared by solubilizing IC-PEC in a RM system consisting of oleic acid, PEG-8 caprylic/capric glycerides and polyglycerol-6-dioleate and then characterized by different techniques. Furthermore, the effect of incorporating GlcN in subcutaneous (SC) and in IC-RM oral preparations administrated to rats on the bioactivity of insulin was also investigated.

4.2. Materials and Methods

4.2.1. Materials

USP human insulin RS (26.4 USP insulin human units/mg, lot No. J0J250) was purchased from USP Convention (Rockville, MD, USA). Recombinant human (rh) insulin of pharmaceutical grade (99.4%), standardized by using USP insulin human RS and GlcN·HCl (99.1%) were purchased from Biocon (Electronic City, Bangalore, India).

Chitosan HCl (250 kDa, 95% degree of deacetylation (DDA)), was obtained from Xiamen Xiang (Shanghi, China). Low molecular weight chitosan (LMWC), 13 kDa, of 99% DDA was obtained by the depolymerization of chitosan according to a previously published method (Varum et al., 2001). Vegetable oleic acid was purchased from Merck KGaA (Bundesland Hesse, Darmstadt, Germany). Labrasol® (PEG-8 caprylic/capric glycerides) and Plurol® Oleique CG (polyglycerol-6-dioleate) were purchased from Gattefosse (Saint-Priest, Lyon, France). Streptozotocin (>98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Jordanian Pharmaceutical Manufacturing (JPM) Company (Naor, Jordan) provided purified water. All other chemicals were of analytical grade.

4.2.2. Preparation of Insulin Solution

Insulin, 35 mg, was dissolved in 1 mL of 0.1N HCl, neutralized with 0.1N NaOH and the volume made up to 10 mL with Tris (hydroxymethyl aminomethane) buffer (pH 6.5). Then 1 mL of this solution was diluted to 100 mL with water to obtain a solution at a concentration of 0.035 mg/mL of insulin (equivalent to 1 insulin unit (IU)/mL).

4.2.3. Preparation of GlcN Solutions

Appropriate amounts of GlcN HCl were separately dissolved in water to obtain solutions at concentrations of 12.5, 25 and 50 mg/mL.

4.2.4. Preparation of Insulin-GlcN·HCl Mixture Solutions

Equal volumes of insulin solution (2 IU/mL), prepared following the above-mentioned procedure, and GclN HCl solutions at concentrations of 0.07, 0.28, 0.70 and 1.32 mg/mL were gently mixed using a magnetic stirrer. The solutions obtained contained insulin at a concentration of 1 IU/mL and different insulin:GlcN HCl mass ratios of 1:1, 1:4, 1:10 and 1:20, respectively.

4.2.5. Preparation of Insulin-Chitosan Reverse Micelles (IC-RM)

IC-PEC and IC-RM systems were prepared following the method reported by Elsayed et al., (Elsayed et al., 2009). 30 mg of LMWC powder was dissolved in water, the pH was adjusted to 5.5 with 0.1N NaOH and the volume made up to 10 mL with water. 70 mg of insulin powder was dissolved in 0.1N HCl, neutralized with 0.1N NaOH and the volume adjusted to 10 mL with 1M Tris (hydroxylmethyl aminomethane) buffer, pH 6.5. The IC-PEC was prepared by gently mixing equal volumes of LMWC and insulin solutions in a glass vial using a magnetic stirrer. The prepared IC-PEC contained 100 IU/mL of insulin and 1.5 mg LMWC/mL.

The same procedure was used to prepare another IC-PEC containing GlcN·HCl. 420 mg GlcN·HCl was initially dissolved in the insulin solution prior to mixing with LMWC solution. The prepared IC-PEC_{GlcN} contained 21 mg GlcN·HCl/mL.

In order to prepare the IC-RMs, 80 g of oleic acid and 20 g of surfactant mixture were mixed together for 5 min; 8 mL of IC-PEC was added to the mixture of oleic acid and surfactant and mixed using a magnetic stirrer (250 rpm for 5 min). A surfactant mixture of Labrasol® and Plurol® Oleique CG was prepared at a mass ratio of 1:1 by mixing the constituents using a magnetic stirring for 5 min. The two IC-RM systems prepared contained 6.7 IU/mL of insulin with and without 2.8 mg GlcN·HCl/mL, respectively.

4.2.6. *In vivo* Pharmacological Activity Evaluation

4.2.6.1. Animal Handling

As described in section 2.2.14.1.

4.2.6.2. Induction of Diabetes Using Streptozotocin (STZ)

As described in section 2.2.14.2, Chapter 2.

4.2.6.3. SC Experimental Design

For all experiments, a group of rats was injected SC with 1 IU/kg insulin (control group).

Non-diabetic fasted rats were randomized into groups (n = 10 rats per group). The initial blood glucose level at 0 h was determined for each rat just before sample injection and at time intervals of 0.5, 1, 2, 3 and 4 h post SC injection.

GlcN·HCl solutions, which represent doses of 0, 50, 100 and 200 mg/kg, were individually injected SC 30 min prior to injection of insulin solution (I IU/kg) and changes in blood glucose levels were monitored.

In other experiments, the effect of simultaneous SC administration of insulin and GlcN·HCl on insulin bioactivity was investigated by injection of insulin-GlcN·HCl solutions, which contained 1 IU/kg insulin and different insulin:GlcN·HCl mass ratios of 1:0, 1:1, 1:4, 1:10 and 1:20.

Furthermore, rats with free access to food were offered either water or GlcN·HCl solution at a concentration of 25 mg/mL for 5 consecutive days in the feeding bottles ed libitum. At day 6 the fasted rats were injected with insulin solution (1 IU/mL) and changes in blood glucose levels were monitored.

4.2.6.4. Oral Experimental Design

STZ diabetic rats (n=10) were randomized into two groups. One group received IC-RMs and the other group received IC-RMs containing 2.8 mg GlcN·HCl/mL. This group of animals received the samples orally using a stainless steel oral gavage needle. Blood sampling for glucose measurements were undertaken at time intervals of 0, 1, 2, 3, 4, 5, 6, 8 and 10 h post sample administration.

4.2.6.5. Data and Statistical Analysis

Blood glucose levels are expressed as the mean percentage of the baseline glucose levels, and plotted against time to evaluate the cumulative hypoglycemic effect over time. Glucose level profiles were quantified by the area above the curve (AAC) following the trapezoidal rule. All data are expressed as mean values ± standard error of means (SEM). One way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test form multiple comparisons were used for statistical evaluation (SPSS 17, Chicago, IL, USA). P values < 0.05 were considered significant.

Figure 4.1 shows the scheme for IC-PEC and IC-RM preparations, administration and monitoring glucose levels in rats.

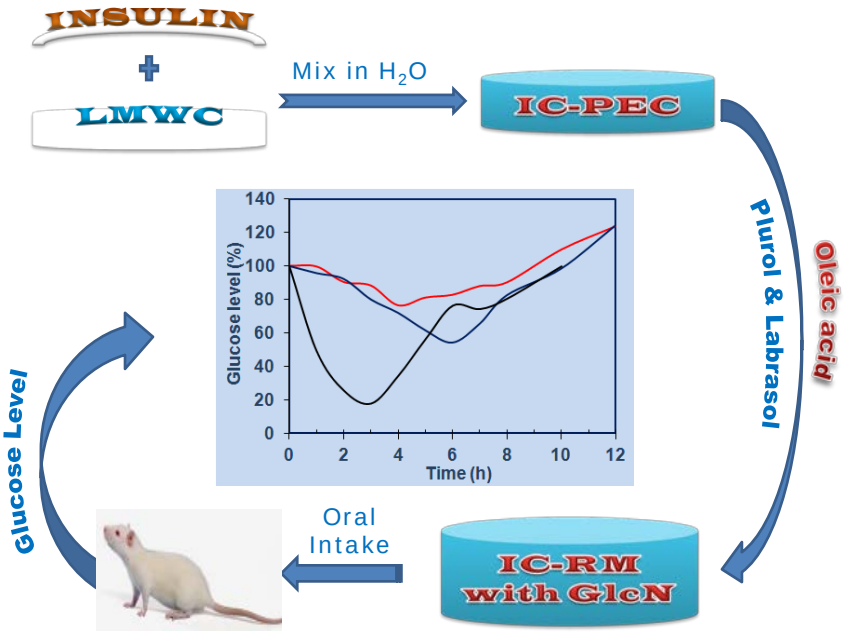


Figure 4.1 Scheme of present work procedures including IC-PEC and IC-RM system preparations, administration and glucose level measurement.

Abbreviations: LMWC, Low molecular weight chitosan; IC, insulin-LMWC; PEC, polyelectrolyte complex; RM, reverse micelle; GlcN, glucosamine; Labrasol, PEG-8 caprylic/capric glycerides; Plurol, polyglycerol-6-dioleate.

4.3. Results

4.3.1. Preparation and Characterization of IC-PEC and IC-RM

In previous reports (Al-Kurdi et al., 2015 & Qinna et al., 2015), the preparation of LMWCs of different molecular weights and %DDA and their effects on the physicochemical and bioactivity properties of oral insulin delivery system were investigated. In the current work, 13 kDa LMWC was selected because this molecular weight of chitosan gave an optimal oral formulation with respect to its physical and chemical stability (Al-Kurdi et al., 2015).The IC-PEC and IC-RM systems were prepared following the scheme in Figure 4.1 and characterized. The results as discussed in section 3.3.1.

4.3.2. Effect of GlcN·HCl SC Administration on Insulin Bioactivity

The presence of GlcN·HCl prior to SC injection of insulin (1 IU/kg) significantly enhanced the action of insulin by reducing the blood glucose levels of the tested rats ($p < 0.01$). In the *post-hoc* analysis, the reduction in blood glucose levels was confirmed to be significant at GlcN·HCl doses of 50, 100 and 200 mg/kg. Therefore, a dose dependent effect of GlcN·HCl on enhancing the bioactivity of insulin could be inferred. The

maximum glucose reduction was also obtained in rats pre-treated with 200 mg GlcN·HCl/kg where the glucose level reached 47% (Figure 4.2).

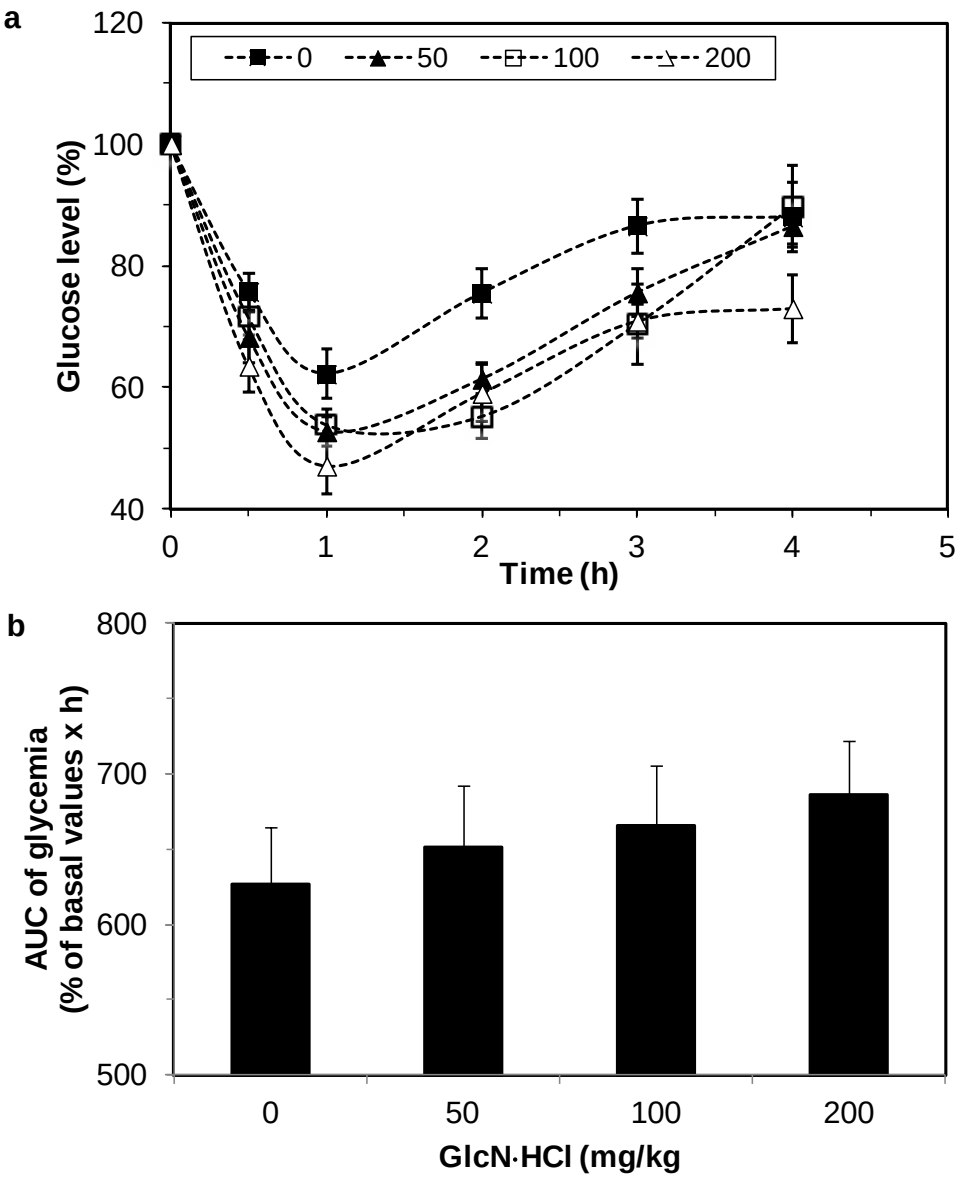


Figure 4.2 Effect of GlcN·HCl SC administration on insulin bioactivity.

Notes: GlcN·HCl solution was SC injected with doses of 0, 50,100 and 200 mg/kg prior to SC insulin (1 IU/kg) administration. The reduction in blood glucose levels was confirmed to be significant as P values were < 0.01.

Abbreviations: GlcN, glucosamine; SC, subcutaneous; IU, insulin unit.

4.3.3. Effect of Simultaneous SC Insulin-GlcN·HCl Administration on Insulin Bioactivity

Blood glucose levels of fasted rats administered insulin-GlcN·HCl solutions at different insulin-GlcN·HCl mass ratios (1:0, 1:1, 1:4, 1:10 and 1:20) are presented in Figure 4.3. The hypoglycemic activity of insulin in the presence of GlcN·HCl was retained and the onset of action was rapid and similar to that of free insulin. The maximum reduction in glucose levels was detected 30-60 min after insulin injection in all tested groups of animals. The hypoglycemic effects of 1:1 and 1:4 insulin-GlcN·HCl solutions were comparable to those of free insulin with a small increase in glucose levels at all time intervals except 0.5 h ($p > 0.05$). However, mixtures prepared at mass ratios of 1:10 and 1:20 induced significant reductions in the blood glucose levels of the tested rats. Such reductions were confirmed statistically significant compared to the free insulin group at 0.5 and 4 h time intervals, as revealed by Turkey's multiple comparison test ($p < 0.05$).

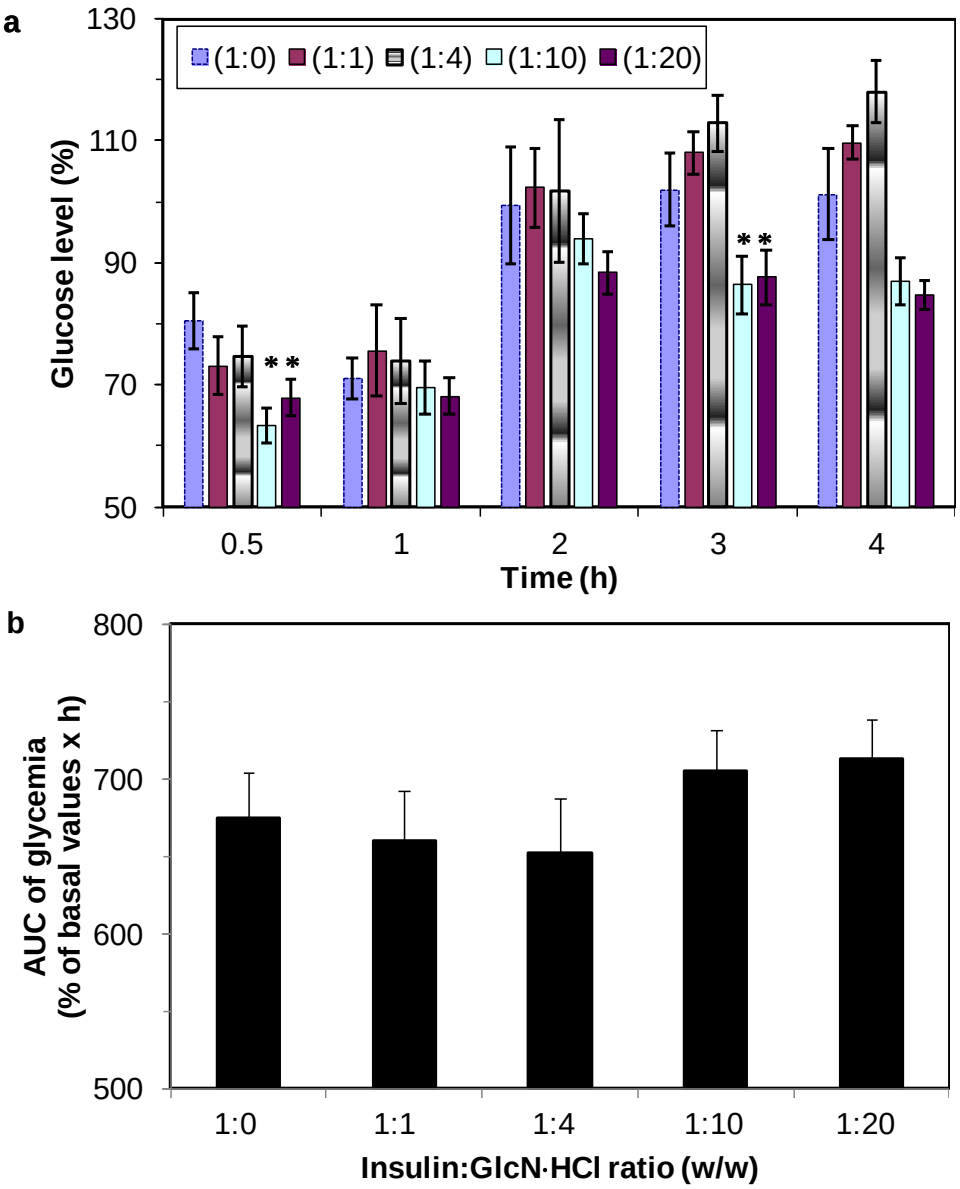


Figure 4.3 Effect of simultaneous SC insulin-GlcN·HCl administration on insulin bioactivity.

Notes: Insulin-GlcN·HCl mixture solutions of mass ratio 1:0, 1:1, 1:4, 1:10 and 1:20 were SC injected (1 IU/kg) to fasted rats. The mixtures prepared at mass ratios of 1:10 and 1:20 (indicated by *) induced significant reductions in the blood glucose levels of the tested rats compared to the free insulin group at 0.5 and 4 h time intervals ($p < 0.05$).

Abbreviations: GlcN, glucosamine; SC, subcutaneous; IU, insulin unit.

4.3.4. Effect of continuous oral GlcN·HCl administration on insulin bioactivity

Feeding rats *ad libitum* with 25 mg GlcN·HCl/mL for 5 days enhanced the action of SC insulin (1 IU/kg), as shown in the data in Figure 4.4. Blood glucose levels post SC insulin administration were significantly reduced when rats were continuously fed with GlcN·HCl compared to rats offered deionized water in the feeding bottles ($p = 0.028$). By comparing the effect at different time intervals, the reduction was revealed to be significant at 1 and 3 h post insulin administration ($p < 0.05$).

The minimum glucose level in case of GlcN fed rats reached 66.2±5.0 % compared to 69.7±2.9 % glucose level of water fed rats, such difference was not significant ($p> 0.05$). However, the minimum glucose level for GlcN fed group was reached 1 h post insulin administration (T_{min}) compared to 0.5 h for water group. Moreover, due to such action of GlcN on reducing glucose level and shifting (T_{min}), the calculated area above the curve (AAC) for GlcN (658.7) was found significantly different ($p< 0.05$) from the calculated AAC for water fed group (619.8).

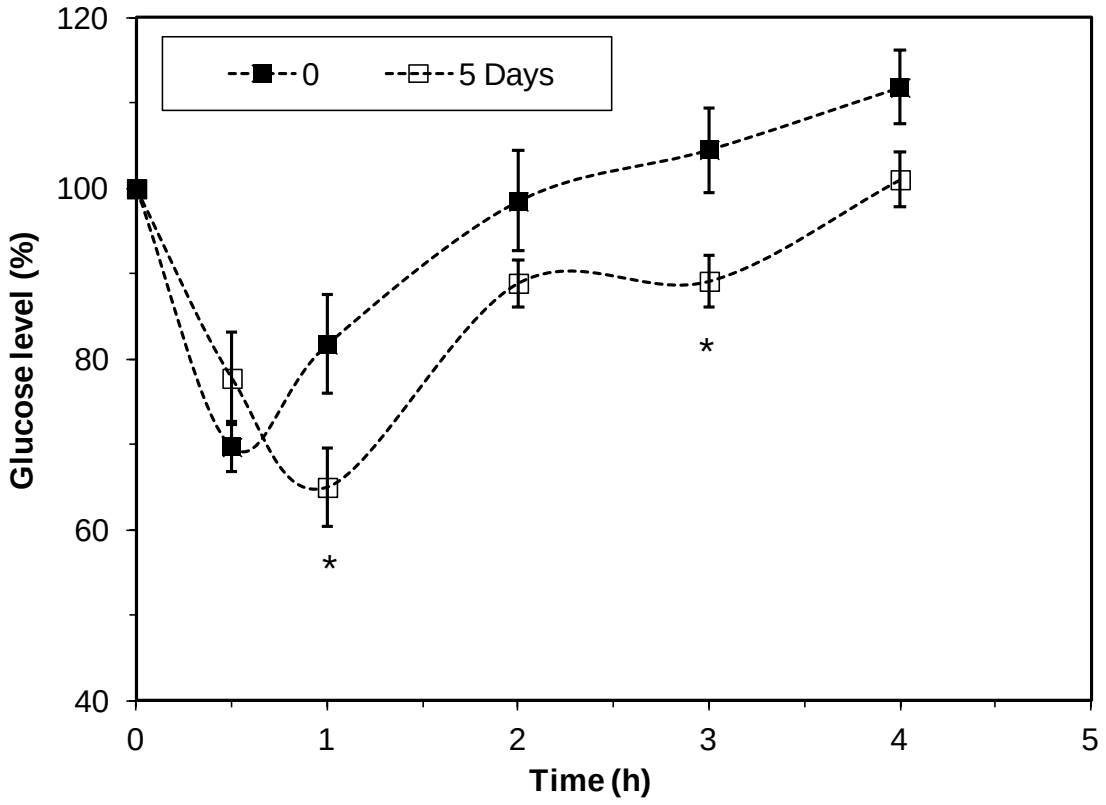


Figure 4.4 Effect of continuous oral GlcN·HCl administration on insulin bioactivity.

Notes: Rats were fed with **GlcN·HCl** (25 mg GlcN·HCl/mL) for 5 days. Blood glucose levels post SC insulin (I IU/kg) administration were significantly reduced when compared to rats offered water ($p = 0.028$).

Abbreviations: GlcN, glucosamine; SC, subcutaneous; IU, insulin unit.

4.3.5. Effect of GlcN·HCl in IC-RM Bioactivity

The effect of incorporating GlcN·HCl in IC-RM preparations on the bioactivity of insulin in diabetic rats is shown in the data in Figure 4.5 and the corresponding pharmacodynamic parameters for glucose levels are shown in the data in Table 4.1. The results obtained indicate that the hypoglycemic activity of IC-RM containing GlcN·HCl was higher

compared with IC-RM containing no GlcN·HCl and that such a difference is significant ($p < 0.05$).

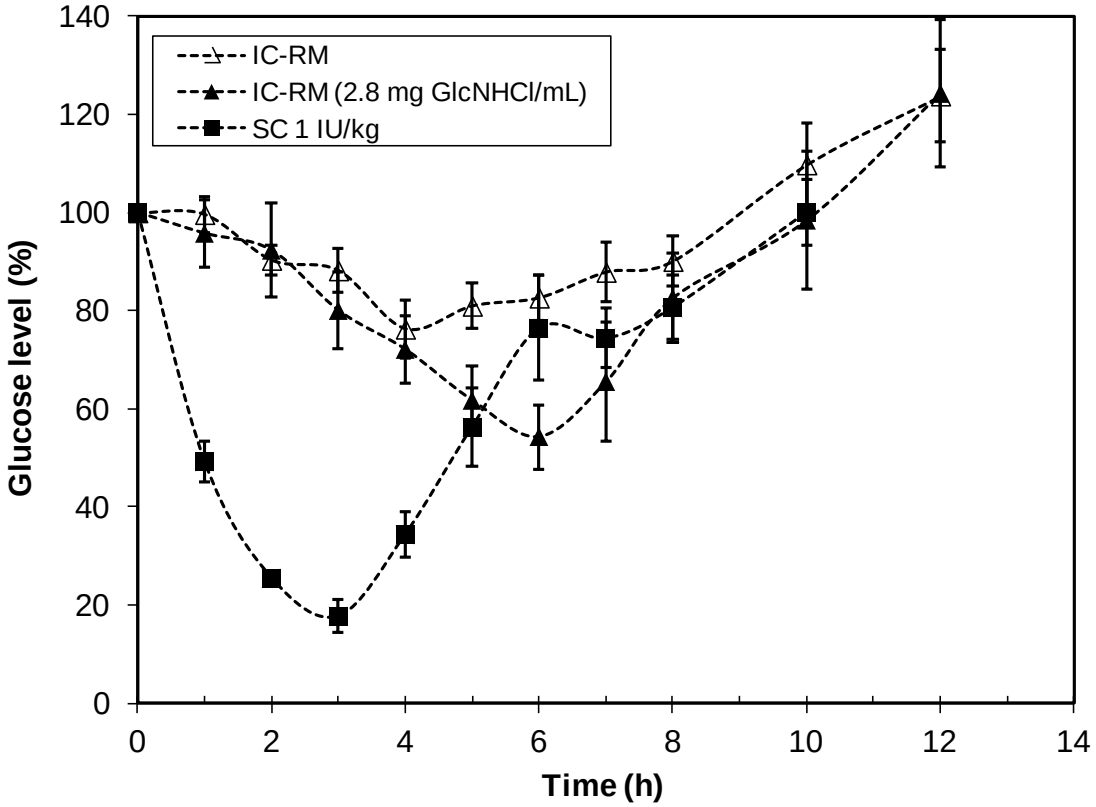


Figure 4.5 Effect of GlcN·HCl on IC-RM bioactivity in diabetic rats.

Notes: The hypoglycemic activity of IC-RM containing GlcN·HCl was higher compared with IC-RM containing no GlcN·HCl and the difference is significant ($p < 0.05$).

Abbreviations: GlcN, glucosamine; SC, subcutaneous; IU, insulin unit; IC, insulin-LMWC; PEC, polyelectrolyte complex; RM, reverse micelle; LMWC, Low molecular weight chitosan.

Table 4.1 Pharmacodynamic parameters for oral IC-RMs in the presence and absence of 2.8 GlcN·HCl mg/mL. The concentration of insulin in the IC-RMs was 50 IU/mL.

$C_{GlcN·HCl}$ (mg/mL)	AAC 0-12 (% glucose*hr)	RSD	PA (%)	C_{min} (% basal glucose)	T_{min} (% glucose level)
0.0	380.2	36.3	5.4	80.9 ± 4.6	4
2.1	466.7	42.1	6.7	54.3 ± 5.1	6

Abbreviations: IC, insulin low molecular weight chitosan; RMs, reverse micelles; IU, insulin unit; GlcN, glucosamine; C, concentration; AAC, area under the curve; PA, pharmacological availability; T, time; hr, time needed to get lowest glucose level (hr).

4.4. Discussion

GlcN is a widely used dietary supplement that is described as efficacious and safe for many individuals with osteoarthritis especially of the knees (Simon et al., 2011).

As previously stated *in vitro* studies by Marshal et al. showed that exogenous GlcN could increase the activity of HBP, a metabolic process that is believed to function as a nutrient sensor modulating insulin resistance (Marshal et al., 1991). Increasing tissue levels of GlcN also impair insulin secretion (Uldry et al., 2002). To assess the biological importance of these studies it is important to compare the GlcN concentrations used *in vitro* with those expected *in vivo*. Interference with glucose metabolism occurs only at concentrations comparable with those of GlcN ≈ 6.6 mmol; a concentration that is several hundred fold greater than plasma concentrations that occur during oral GlcN therapy in humans (Schneeman et al., 2004). At these concentrations (<10 μmol/L) GlcN neither

augments the HBP nor does it reduce mediated glucose uptake (Marshall et al., 2004 & Heart et al., 2000).

In this study the SC co-administration of insulin and GlcN yielded different results depending on the ratios of insulin:GLcN used (Figure 4.3). At ratios of 1:1 and 1:4, the hypoglycemic effect was similar to insulin as no significant difference was obtained. However, at ratios of 1:10 and 1:20, the hypoglycemic effect was higher than insulin alone. GlcN can reduce blood glucose levels (Reginster et al., 2001 & Jae et al., 2006); the mechanism is not well understood. However, it might be related to the role of ATP depletion (Hresko et al., 1998). The effect is only observed at 2 and 3 hr, thus supporting the role of ATP depletion in enhancing the hypoglycemic effect of insulin (Chatham et al., 2010, Alghazadeh-Habashi et al., 2011 & Mueckler et al., 2013), and that GlcN as a COX-2 inhibitor will prevent insulin degradation (Park et al., 2013).

When GlcN·HCl was administered SC 30 min before insulin, the reduction in the level of glucose was greater when compared with insulin alone (Figure 4.2). The peak level of GlcN·HCl is around 2 hr and the maximum hypoglycemic effect occurs between 1-2 hr, taking into account that GlcN·HCl was administered 30 min before insulin. GlcN·HCl at a dose of 100 mg/kg showed a different hypoglycemic peak effect compared to other (50 and 200 mg/kg) concentrations. The time at minimum glucose level for doses of 50mg/Kg and 200 mg/Kg was at 1.0 hr, similar to a dose of insulin alone, while for GlcN·HCl at a dose of 100 mg/kg a minimum glucose level was obtained after 1.5 hr as GlcN is rapidly absorbed. After 6 hr the GlcN·HCl level is minimal as plasma GlcN is eliminated (Song et al., 2012), i.e., the least effect on insulin activity. The decrease in blood glucose level is mostly likely due to ATP depletion. Such an effect leads to a

decrease in insulin degradation which explains the lower glucose levels at 1, 2, 3 and 4 hr.

Insulin is metabolized in the liver by the oxidoreductase behaviour of a protein-disulfide isomerase, also known as glutathione-insulin transhydrogenase enzyme. This enzyme breaks the disulfide bonds of insulin, thereby causing its degradation (Chandler et al., 1975). As the enzyme is an oxidoreductase and depends on NADH to degrade insulin, it might be inhibited by ATP/NADH depletion state as was noted in cytochrome P450 and GlcN (Yao et al., 2012).

In this study blood glucose levels tended to decrease compared to the control group in the group fed with 100 mg GlcN·HCl/kg for 5 days. A high fraction of GlcN·HCl taken orally (≈ 90%) is rapidly absorbed (Setnikar et al., 2001) and the peak concentration occurs about 2 hr after oral administration (Song et al., 2012). Orally administered GlcN·HCl has only 26% of the bioavailability of the intravenously administered form of GlcN·HCl (Setnikar et al., 1993) in humans and in rats the comparable figure is 20% (Aghazadeh-Habashi et al., 2002). Data on pharmacokinetics, bioavailability and metabolism of GlcN in rats (Aghazadeh-Habashi et al., 2002) are similar to those reported for human (Setnikar et al., 2001). Plasma GlcN is eliminated in about 10 hr post-dosing. Pharmacokinetic parameters for GlcN, after multiple doses for 7 days, indicated no significant accumulation effects (Song et al., 2012). This may be explained by the fact that the absorption of orally administered GlcN is limited, which is due to its dependence on facilitated transport and pre-systemic loss brought by the gut microbiota and so the liver is not exposed to high concentrations of GlcN in the portal venous blood even when it is consumed at high concentrations. So feeding of GlcN·HCl for 5 days is not increasing plasma GlcN·HCl levels (Hirayama et al., 2007 & Ibrahim et al., 2012).

Based on such facts, we can explain why the hypoglycemic effect was not more pronounced than that of a single dose of GlcN·HCl. It seems, as anticipated, that GlcN homeostasis is responsible for preventing the accumulation of GlcN.

Encapsulation of GlcN·HCl in nanoparticles using chitosan as a polymer together with insulin for oral delivery was not investigated. We solubilized GlcN·HCl with IC-PEC inside reverse micelles in order to evaluate its effect on insulin administered orally. The use of the micellar delivery system avoids the effects of pepsin and protects insulin from degradation, minimizing the effect of degrading enzymes on insulin. Therefore, part of insulin activity is retained. In the presence of GlcN·HCl the hypoglycemic effect of insulin encapsulated in the RMs is higher that obtained using insulin alone. The relative pharmacological bioactivity was calculated to be 6.7% compared to a value of 5.4% obtained for the group of rats treated with the RMs, which did not contain GlcN·HCl. The pharmacological bioactivity obtained for insulin delivered via the micellar system reflects the fact that insulin activity is partially retained. A higher value is obtained when GlcN is encapsulated with insulin in RMs. This result may be because when GlcN·HCl is given orally together with insulin it will enter the hepatic portal system and so it is carried through the portal vein into the liver, where most drug metabolism occurs. The biological activity of GlcNHCl on drug metabolism enzymes (DMEs) has been reported (Yao et al., 2012 & Nam et al., 2007). Total cytochrome P450 content in liver decreased with rats fed with GlcN·HCl. Modulation of DME activity by GlcN may originate from indirect action on the immune system as this alters DME expression (Sherry et al., 2010).

4.5. Conclusions

LMWC was prepared and characterized. Oral nano delivery system was prepared by solubilizing IC-PEC in reverse micelle system. The effect of SC administration of GlcN

on insulin bioavailability was evaluated; the results showed a dose dependent effect of GlcN. The co-solubilization of GlcN with insulin in the RMs effectively reduced blood glucose levels in rats *in vivo*. Further studies needed to verify the actual mechanism.

CHAPTER 5: Overall Conclusions and Future Work

5.1. Overall Conclusion

It is evident from the work presented that polyelectrolyte complex (PEC) formation between insulin and low molecular weight chitosan (LMWC) is very important step in the preparation of oral delivery system of insulin. PEC solubilized in reverse micelle system administered orally to diabetic rats, results in an oral delivery system with acceptable bioactivity. The aim of the work reported herein was to investigate the effect of the molecular weight and concentration of chitosan on the stability of insulin using HPLC methods. The HPLC method was verified and proven to be linear, specific and stability indicating. Insulin was found to be stable in a polyelectrolyte complex (PEC) consisting of insulin and LMWC in the presence of Tris-buffer at pH 6.5. In the presence of LMWC, the stability of insulin increased with decreasing molecular weight of OCS; 13 kDa chitosan was the most efficient molecular weight for enhancing the physical and chemical stability of insulin. PEC of insulin with low molecular weight chitosan (13 kDa) was prepared and characterized. Association efficiency was around 70 % at pH 6.5. Positive zeta potential values were obtained which can explain the entrapment of insulin in chitosan. Reverse micelle (RM) system proves to have protective effect under condition simulating gastric environment. At pH 6.8, around 80 % of insulin was released over 6 hr and further good bioactivity.

Glutathione is the most important antioxidant, preventing damage to important cellular components caused by reactive oxygen species. The experiments reported in Chapter 3 investigated the effect of reduced (GSH) and oxidized (GSSG) glutathione on the bioactivity of insulin was studied. A polyelectrolyte complex (PEC) of insulin with low molecular weight chitosan (13 kDa) was prepared and characterized. The PEC was then solubilized, in the presence and absence of GSH and GSSG, in a reverse micelle consisting of oleic acid and two surfactants (labrasol and plurol). The *in vitro* and *in vivo* performances of the reverse micelle formulations (RMFs) were evaluated in rats. At pH 6.5 the association efficiency of the PEC was 76.2%. *In vitro* insulin release from the RMs was negligible at pH 1.2 and was markedly increased at pH 6.8. The hypoglycemic activity of insulin in the PEC was reduced when administered via the subcutaneous route, regardless of the GSH content. On the other hand, the presence of GSSG significantly enhanced hypoglycemia. When the RMF was administered via the oral route, the presence of GSH had no effect on the hypoglycemic activity of insulin compared with the GSH free system. However, the presence of GSSG in the oral preparation increased the hypoglycemic activity of insulin; probably by inhibiting insulin degradation, thereby prolonging its effect. Thus, incorporation of GSSG in the RMF reduces blood glucose levels in rats and protects insulin from degradation.

Glucosamine is an amino sugar and an important factor in the synthesis of glycosylated proteins and lipids. It is one of the most common dietary supplement used by adult. The experiments carried out in chapter 4 investigated the effect of Glucosamine (GlcN) on the bioactivity of insulin was studied. A polyelectrolyte complex (PEC) of insulin with low molecular weight chitosan (13 kDa) was prepared. The PEC was then solubilized, in the presence and absence of GlcN, in a reverse micelle formulation (RMF) consisting of oleic acid and two surfactants (labrasol and plurol). When GlcN was given subcutaneous (SC)

30 minutes prior SC administration of insulin, a dose dependent effect of GlcN on enhancing the hypoglycemic activity of insulin was obtained. Additionally, when insulin was administered in solution with GlcN at different mass ratio, different effect was obtained. At 1:1 and 1:4 showed slight increase in glucose level ($p > 0.05$), however, 1:10 and 1:20 induced significant reduction in blood glucose level ($p < 0.05$). Enhancement of the action of SC injected insulin was obtained after GlcN was administered orally for five days. When the RMF was administered via the oral route, the presence of GlcN in the RMF increased the hypoglycemic activity of insulin. Thus, incorporation of GlcN in RMF along with insulin enhances the reduction of blood glucose levels in rats.

5.2. Future Work

- **Insulin-low molecular weight chitosan polyelectrolyte complex (PEC)-** Polyelectrolyte complexes are a promising approach for protein delivery. The presence of free NH_2 groups allows chitosan to form complexes with negatively charged moieties such as insulin. Solubilizing the PEC in a reverse micelle system allowed insulin to be delivered orally. The system protects insulin from the harsh conditions of the body and facilitates absorption throughout the gastrointestinal tract.

  The outcome of the presented work could be improved to allow:

  - Higher association efficiency between insulin and low molecular weight chitosan.
  - Improve the chemical stability of insulin.
  - Improve oral insulin bioavailability.

- **Glutathione effect on insulin activity-** Glutathione a tripeptide substance acts as an important antioxidant in most living organisms. The effect of glutathione on insulin activity is clear. However, the mechanism of action of glutathione on insulin needs further investigation. Such investigations may provide more detailed

information that could support optimizing the oral delivery system for insulin with glutathione.

- **Glucosamine effect on insulin activity-**Glucosamine (GlcN) has been investigated extensively for its action in osteoarthritis. Combinations of GlcN with other drugs are available. In treating patients for osteoarthritis, GlcN HCl is combined with Diacerein. In mice, GlcN 500 mg/kg and low doses of cyclosporine are used for immunosuppressive treatment (Kwon et al. 2013). In rats, the combination of GlcN HCl and methotrexate might decrease the dose of methotrexate. The dose dependent action of GlcN has already been proved. The dose of GLcN that can be combined with insulin to lower insulin dose requirements to maintain normal glycaemia needs further investigation. The suggested dose in animals could be correlated to that necessary in humans. Once this dose is determined, the same concept can be investigated with other drugs; the objective being to reduce the dose of the drug and reduce side effects.

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