Design and Evaluation of Oral Delivery Systems for Biologically Active Macromolecules - Insulin

THESIS

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CERTIFICATE

This is to certify that the thesis entitled "**Design and Evaluation of Oral Delivery Systems for Biologically Active Macromolecules - Insulin**" which is submitted for award of Ph.D. degree of the institute, embodies original work done by him under my supervision.

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SUMMARY

Delivering protein and peptides by the oral route is extremely challenging due to very nature of the digestive system designed to break down these polypeptides into amino acids prior to absorption and very poor permeability of gut wall for hydrophilic large molecules like proteins and peptides. Variety of approaches have emerged in the recent past for designing oral delivery systems for therapeutic proteins and peptides, although a clinically viable solution to this long standing problem still alludes the scientific community. These approaches come from such diverse research disciplines as biomaterials, conjugation chemistry, nanotechnology, cell biology, and employ different methodologies for solving the same problem. Some of these strategies have distinguishing beneficial characteristics that make them good candidates for oral protein delivery. Interfacing different strategies to combine the benefits of novel approaches is an interesting new possibility. Despite extensive research in this area, a successful oral delivery systems of proteins and peptides especially large macromolecules, like insulin is yet to see the successful commercial product.

Diabetes mellitus is occurring in epidemic proportions in many developing and newly industrialized countries. Globally, it is now one of the most common non-communicable diseases and is the fourth or fifth leading cause of death in most developed countries. The global burden of diabetes is estimated to rise from about 118 million in 1995 to 220 million in 2010 and 300 million in 2025.

Oral delivery formulation for insulin is highly desirable from a patient compliance point of view and other considerations discussed above. However, only a small portion of insulin administered orally reaches the blood stream, mainly due to extensive degradation of the protein in the gastrointestinal tract. Further, large size and hydrophilicity of the molecule greatly limits its transport across the intestinal epithelium. No specific transport mechanism is present for the passage of insulin cross the intestinal cell monolayer. Insulin molecules that are unable to cross the intestinal barrier thus get further exposed to the intestinal proteolytic activity, this in turn reduces the bioavailability of the protein. Owing to these factors, less than 0.5% of the orally administered insulin typically reaches the systemic circulation.
In the present work, better oral delivery systems for insulin were envisaged to be designed,

developed and evaluated based on following principles.

(a) Stabilization of insulin in oral delivery formulations.
(b) Improvement over degradation of insulin in the presence of proteolytic enzymes.
(c) Design of hydrophobic delivery systems to enhance oral absorption.

The overall goal of this work was to evaluate the effectiveness of hydrophobic delivery systems in addressing some key problems in oral protein delivery and to design a system based on integrating the hydrophobic delivery systems with other effective strategies for oral protein delivery. The specific goals of this research are outlined below:

(1) Design and development of hydrophobic nanopartilculate delivery system containing stearic acid, α-tocopherol acetate and soya phosphatidyl choline (SPC) in presence of stabilizers such as zinc and hydroxypropyl β cyclodextrin to improve oral bioavailability of Human insulin.

(2) Design and development of self microemulsifying delivery system containing dispersions of Human insulin with egg yolk to improve the oral bioavailability and stability of insulin.

(3) Design and development of self microemulsifying delivery system containing dispersions of Human insulin with sodium caseinate to improve the oral bioavailability and stability of insulin.

As a necessary to support the whole work, analytical method for estimation of Human insulin was developed by modification of current official USP method for assay of Human insulin. The method was validated as per ICH guidelines and by recovery studies. For in-vivo studies, pre-validated diagnostic kits for estimation of plasma glucose and serum Human insulin were used by drawing the calibration curve.

Stearic acid nanoparticle formulation was found to possess good physical and chemical

stability upon long term storage at 2-8°C. Zinc and hydroxyproyl β cyclodextrin has been shown to improve the stability of Human insulin in the formulation. The innovative process for the preparation of nanoparticles was designed and optimized with excellent entrapment efficiency. The stearic acid and hydroxyproyl β cyclodextrin content was optimised to have desired physicochemical properties of formulation such as particle size, in-vitro release and stability in presence of proteolytic enzyme. The duration of in-vitro insulin release was found to get extended beyond 3 hours from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The oral dose of 20IU/kg of nanoparticle formulation was found to be comparable with 2 IU/kg subcutaneous injection of Human insulin injection in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma glucose % glucose reduction. Cmax for Human insulin and extent of plasma glucose reduction was lower with oral formulation as compared to subcutaneous injection of Human insulin solution, but the serum Human insulin concentration of the latter diminished after 6 hour of injection, the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose and 10 IU/kg dose of nanoparticle formulation was 9.48% and 4.60% respectively.

The developed self microemulsifying formulation containing dispersion of Human insulin – egg yolk was found to possess good physical and chemical stability upon long term storage at 2-8°C. The use of egg yolk has been shown to improve the stability of Human insulin in the formulation. The nanometer particle size of the formulation was achieved by unique process of dispersion formation with lyophilization and then dispersing in SMEDDS. The particle size of SMEDDS solution was optimized by varying the surfactant and co-surfactant concentration. The particle size was found to increase after dispersion of Human insulin-egg yolk in SMEDDS. The duration of in-vitro insulin release was found to get extended beyond 3 hours from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0\text{-}12h}$ for Human insulin serum

versus time profile and $AAC_{0\text{-}12h}$ for plasma glucose % glucose reduction of 20 IU/kg dose of InsEY-M2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished after 6 hour of injection the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsEY-M2 formulation was 6.87 %.

Self microemulsifying formulation containing dispersion of Human insulin – sodium caseinate was also found to possess good physical and chemical stability at long term storage at 2-8°C. The use of sodium caseinate in presence of zinc has been shown to improve the stability of Human insulin in the formulation The nanometer particle size of the formulation was achieved by unique process of dispersion formation by addition of zinc chloride and calcium chloride as complexing agent to Human insulin solution in presence of hydroxypropyl β cyclodextrin followed by lyophilization and then dispersing in SMEDDS. The duration of in-vitro insulin release was found to get extended beyond 3 hours from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has been shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma glucose % glucose reduction of 20 IU/kg dose of InsEY-M2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter was diminished after 6 hour of injection the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsSC-M2 formulation was 6.92%.

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LIST OF SYMBOLS AND ABBREVIATIONS

A°	Amstrong unit
ACE inhibitor	Angiotensin converting enzyme inhibitor
$AUC_{0\text{-}12h}$	Area under the curve from 0 to 12 hour time interval
$AAC_{0\text{-}12h}$	Area above the curve from 0 to 12 hour time interval
ATP	Adinosine triphosphate
cm	Centimeter
CMC-BBI	Carboxymethylcelluose-Bowman Brik inhibitor
CMC-Ela	Carboxymethylcelluose-elastinal
CEM	Extra cellular matrix
C_{max}	Maximum plasma concentration
CMP	Caseinmacropeptide
DG	Diglycerides
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunoassay
EDTA	Ethylenediaminetetraacetic acid
FPG	Fasting plasma glucose
FPE	Fluid phase endocytosis
FA	Fatty acid
GI tract	Gastrointestinal tract
GIT	Gastrointestinal tract
GC-Na	Sodium glycocholate
g	Grams
GMP	Glycomacropeptide
HBSS	Hank's buffered salt solution
HP-β CD	Hydroxypropyl beta cyclodextrin
HMWT	High molecular weight
HPLC	High pressure liquid chromatography
HC	Hydrolyzed casein
HbA_{1c}	Glycosylated haemoglobin
h	Hour

InsSA	Stearic acid based hydrophobic nanoparticles
InsSC	Human insulin-sodium casinate dispersion
InsEY	Human insulin-egg yolk dispersion
InsSC-SMEDDS	Human insulin-sodium caseinate dispersion in self microemulsifying solution
InsEY-SMEDDS	Human insulin-sodium caseinate dispersion in self microemulsifying solution
IRS-1	Insulin receptor substrate
IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose
IU/kg	International units per kilogram
IU/ml	International units per mililitre
IM	Intramuscular
IV	Intravenous
HCl	Hydrochloric acid
kV	Kilovolts
kD	Kilo Dalton
kHz	Kilo Hertz
K_e	Elimination rate constant
LM	N-Lauryl-beta-D-maltopyranoside
LC	Liquid chromatography
LL (95% CI)	Lower level at 95% confidence interval
m^2	Square meter
mmol/l	Milimoles per litre
min	Minutes
MW	Molecular weight
M cells	Microfold cells
MRP	Multi drug resistance associated protein
MDR	Multi drug resistance
M cells	Microfold cells
mm	Milimeter

MAGUK	Membrane associated guanylate kinase
mM	Milimoles
mg/ml	Miligram per mililitre
mm	Micrometer
mIU/l	Mili international units per litre
mIU/l . h-1	Mili international units per litre per hour
mg	Miligrams
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium bicarbonate
nm	Nanometer
OGTT	Oral glucose tolerance test
PEPT-1	Oligopeptide transporter-1
PTR3	Peptide transporter protein 3
PHT1	Peptide histidine transporter
PCP-Cys	Polycarbophil-cysteine
PDA detector	Photodiode array detector
RME	Receptor mediated endocytosis
rpm	Rotations per minute
RSD	Relative standard deviation
r	Correlation coefficient
sec	Seconds
SPC	Soya phosphatidyl choline
SEM	Scanning electron microscopy
SMEDDS	Self microemulsifying solution
SD	Standard deviation
TG	Triglycerides
TC-Na	Sodium taurocholate
TFA	Trifluroacetic acid
T_{max}	Time to achieve maximum plasma concentration
$T_{1/2}$	Half life
TMB	3.3',5,5'-tetramethylbenzidine

UV	Ulltaviolet
UL (95% CI)	Upper level at 95% confidence interval
ZO-1	Zonula occludens -1
ZO-2	Zonula occludens -2
ZO-3	Zonula occludens -3
µl	Microlitre
°C	Degree celcius

CHAPTER 1

INTRODUCTION

DESIGN AND EVALUATION OF ORAL DELIVERY SYSTEMS FOR BIOLOGICALLY ACTIVE MACROMOLECULES - INSULIN

1.1 INREODUCTION:

Biological macromolecule is the one that is found in living organisms. Biological macromolecules include molecules such as carbohydrates, proteins and nucleic acids. Lipids are also biological macromolecules. They are essential for all forms of life to be able to survive.

Majority of the biologically active macromolecules such as insulin, heparin, calcitonin, interferons, Human growth hormone, eptifibatide, etc., are required to be administered parenterally in few micrograms to milligram quantities to achieve the desired therapeutic effect. Conventional means for delivering these biologically active macromolecules to animals and Human beings are often severely limited by chemical and physical barriers imposed by the body.

In recent years, a spurt of research and development has been conducted involving a wide range of protein therapeutics for various conditions. Most of the proteins have been successfully applied in therapy, mainly as parenterals. An oral dosage form is the preferred form of delivery because of ease of administration, patient compliance and cost. Major hurdles must be overcome before the oral delivery of a protein becomes a reality. Among the proteins, oral delivery of insulin has received the widest attention, yet no currently available oral insulin preparation exists [1].

Insulin therapy is at the heart of protein delivery research. Insulin is a protein of immense importance because of its role in the treatment of diabetes, which is growing into epidemic proportions in many developing countries. The options currently available for administration of insulin are mostly limited to insulin injections through needles though other routes of administration such as pulmonary, dermal, buccal and oral are currently being investigated. Oral route remains to be a preferred route of administration due to ease, cost and effectiveness of oral dosage forms. Oral administration is also linked to increase in patient compliance and overall increase in efficacy of the treatment.

However, the enzymatic instability of insulin in the GI tract and its inability to transverse the intestinal barrier severely reduce the fraction of orally administered insulin that reaches the blood stream (bioavailability).

In this work we have developed oral insulin formulations based on hydrophobic nanoparticles and self microemulsifying delivery systems. The oral formulations developed in the scope of this project are shown to be promising candidates for oral insulin administration. The success of these formulations for insulin is due to the ability of the carriers to protect insulin from degradation in the harsh conditions encountered in the gastrointestinal tract. These formulations also exhibit potential to enhance the oral absorption of insulin.

The objectives of this work are given in Chapter 1. Drug profile for Human insulin and analytical method development is presented in Chapter 2 and Chapter 3 respectively. Formulation design, optimization and experimental investigations along with in-vivo studies on streptozotocin induced diabetic rats for stearic acid hydrophobic nanoparticles, Human insulin –egg yolk dispersions in self microemulsifying delivery system and Human insulin – sodium caseinate dispersions in self microemulsifying delivery system are presented and discussed under Chapter 5, Chapter 6 and Chapter 7, respectively. In Chapter 2 an overview of diabetes treatment and insulin therapy is presented with focus on oral insulin delivery.

1.2 BACKGROUND:

Major innovations in the drug delivery field have surfaced in recent years. Oral administration of therapeutic agents is the preferred means of delivering drugs because of ease of administration, low cost and high patient compliance. However, formulating a drug for oral delivery is a complicated process. Poor intrinsic protein permeability as a result of large molecular weight, degradation by proteolytic enzymes in the stomach and in the small intestine and chemical instability are some of the major hurdles for developing effective formulations for delivery of peptides and proteins. Although there are several success stories in the development and commercialization of oral dosage forms for small molecules, very few oral delivery systems have been developed for proteins and peptides. The oral formulation of cyclosporin [2] is one of the very few examples of successful development of oral formulations for peptide drugs.

In this chapter we will discuss clinical significance of insulin oral delivery and novel methods for administration of insulin for the treatment of diabetes. Since its initial administration to Humans in 1922, insulin has been the cornerstone of Type 1 diabetes. Conventionally, insulin is administered by subcutaneous injections which mimic, as close as possible, secretion of insulin by healthy pancreas. However, due to compliance related issues and other complications, more acceptable delivery systems are highly desirable. An overview of diabetes mellitus and novel ways of treating this disease are included here. Special attention is given to oral insulin delivery which is the focus of this work.

1.3 Diabetes Mellitus:

Diabetes is characterized by the body's inability to produce or properly use insulin. Diabetes mellitus is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [3]. Diabetes mellitus is occurring in epidemic proportions in many developing and newly industrialized countries [4]. Globally, it is now one of the most common non-communicable diseases and is the fourth or fifth leading cause of death in most developed countries [5]. The global burden of diabetes is estimated to rise from about 118 million in 1995 to 220 million in 2010 and 300 million in 2025 [6,7].

The etiological types of diabetes are designated as Type 1, Type 2 diabetes and other specific types of diabetes resulting from genetic defects of β- cell function, genetic defects in insulin action, endocrinopathies (resulting from over secretion of insulin antagonizing hormones), infections and drug- or chemically induced diabetes [3]. Further, in nearly 3 to 5 % of all pregnancies, women develop gestational diabetes [8].

Type 1 diabetes can be classified as autoimmune diabetes mellitus or idiopathic Type 1 diabetes. Autoimmune diabetes mellitus is characterized by the autoimmune mediated destruction of the insulin secreting pancreatic β-cells [3]. Individuals suffering from this form of Type 1 diabetes typically become dependent on insulin for survival and are at a risk of ketoacidosis, a condition resulting from extremely high levels of blood glucose (over 249 mg/dl) wherein the body begins to burn fat and muscle for energy which causes release of ketone bodies in the bloodstream. Idiopathic Type 1 diabetes is a diabetes of unknown origin. Some of the patients suffering from this disease have permanent insulin deficiency and are prone to ketoacidosis, but this form of diabetes shows no evidence of autoimmunity [3,9].

Type 2 is the most common form of diabetes and is characterized by disorders of insulin action and insulin secretion, either of which may be the predominant feature [3]. Individuals suffering from this form of diabetes are typically resistant to the action of insulin [3,10]. The individuals suffering from this form of diabetes are at increased risk of developing macrovascular and microvascular complications. If untreated, Type 2 diabetes can cause serious complications, including kidney failure, blindness, heart attack, lower-limb amputation [11]. Fortunately, in many cases, Type 2 diabetes can be adequately controlled through a combination of proper nutrition and exercise. However, some people with Type 2 diabetes do require oral medications or insulin injections.

To determine if a patient is normal, pre-diabetic or diabetic, health care providers conduct fasting plasma glucose test (FPG) or an oral glucose tolerance test (OGTT) [12]. Either test can be used to diagnose pre-diabetes or diabetes. Pre-diabetes, characterized by a glucose level between that of a healthy individual and a diabetic patient, is also called impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) [13].

The FPG test is easier, faster and less expensive to perform. With the FPG test, a fasting blood glucose level between 100 and 125 mg/dl indicates prediabetes. A person with a fasting blood glucose level of 126 mg/dl or higher is considered diabetic [3,12].

In the OGTT test, a person's blood glucose level is measured after a fast and two hour after drinking a glucose-rich beverage (2h-PG value). Two-hour blood glucose level of 140-199 mg/dl signals pre-diabetes, whereas the two-hour blood glucose level of >200 mg/dl, signals diabetes [3,12]. Although the OGTT (which consists of an FPG and 2h-PG value) is recognized as a valid way to diagnose diabetes, the use of the test for diagnostic purposes in clinical practice is limited because of its inconvenience, less reproducibility, greater cost [12, 14].

1.4 Insulin and Insulin Therapy:

In 1889, von Mehring and Minkowski showed that legation of the pancreas in dog caused diabetes [15]. This lead to an intensive search for the active component that the pancreas was producing that was involved in preventing the development of diabetes. In 1922 Banting and Best successfully extracted insulin. For more than a decade following its discovery, insulin was used in the form of crude extracts of the pancreas of cow, pig or sheep for diabetes treatment [16]. Insulin was first purified by crystallization in the presence of zinc in 1934 [17]. Sanger in 1955 elucidated the primary structure of insulin [18]. Although the amino acid sequence of insulin varies among different species, certain sequences are highly conserved [19]. The sequences porcine insulin and Human insulin are almost identical, differing by only one amino acid whereas bovine insulin differs by three amino acids from the Human analog. However, none of the variations in the amino acid sequence are at sites crucial for the activity and function of insulin [19].

When insulin is synthesized by the beta cells of the pancreas, it is produced as a large preprohormone. This preprohormone has a molecular weight of about 11,500. It is cleaved within the cisternae of the endoplasmic reticulum of the β-cells to form proinsulin which has a molecular weight of about 9,000. This molecule then splits into two pieces: insulin, with a molecular weight of 5,808 in Humans and C-peptide, before being secreted outside the cells through the secretory granules. The secreted insulin has 31 amino acid long B-chain and the 20 amino acid long A-chain which are locked in their relative conformation by two disulfide

bonds. The cleaved and secreted insulin is 51 amino acids long and has a hydrodynamic radius of about 20 Å [20].

The plasma glucose level is maintained within a very narrow range of 3.5- 7.0 mmol/L in spite of wide fluctuations introduced by food intake, exercise and physiological disturbances [21]. This glucose homeostasis is achieved by regulating release and inhibition of glucagons and insulin, both secreted by the pancreatic cells.

Increase in blood glucose triggers secretion of insulin from the pancreas. Insulin secreted from the pancreas is infused via the portal vein to the liver, where it leads to an increase in the storage of glucose with a concomitant decrease in hepatic glucose release to the circulation [22]. Insulin circulates in the blood with plasma half life of about 6 min, so that it is almost entirely cleared from the circulation within 10-15 min. The freely circulating insulin then acts on several peripheral tissues including muscle, liver and fat tissue by binding to the specific insulin receptors on cells.

An Insulin receptor is a hetero-tetramer with a molecular weight of about 300,000. The two alpha subunits lie entirely outside of the cell membrane and the two beta subunits penetrate through the cell membrane. Insulin binding to the alpha subunits on the outside of the cell triggers autophosphorylation of the beta subunits on several tyrosine residues protruding into the cytoplasm.

The activated beta subunits then phosphorylate and activate IRS-1. IRS-1 is an enzyme and a key mediator of insulin's biological activity. Following this, various intracellular regulators are recruited to IRS-1 and this initiates a regulatory cascade of signals with each molecule binding to IRS-1 activating its own complex cascade [23]. This finally leads to increase in the permeability of cell membranes to glucose and glucose uptake increases. This increased uptake of glucose due to insulin binding occurs in the muscle cells, adipose cells and other types of cells in the body constituting about 80 percent of all the cells. In addition to its role in uptake of glucose, insulin also stimulates conversion of carbohydrate or proteins to fat (lipogenesis) and increases amino acid transport into cells. It also stimulates growth, DNA synthesis and cell replication.

In the absence of curative therapy for the treatment of diabetes, insulin replacement therapy is required for all people with Type 1 diabetes [24]. Benefits of insulin therapy in patients with Type 2 diabetes are also well recognized now [25-27]. Intensive glucose control delays the onset and retards the progression of microvascular and macrovascular diseases in patients with Type 2 diabetes [28]. Insulin therapy is, therefore, central to management of patients with diabetes [29,30].

Recent developments of improved injection devices, such as insulin pens and very sharp needles, have reduced the pain associated with the injection therapy to a considerable extent. But even with these developments, injection therapy still requires the handling of a device and is associated with pain. This often results in low patience compliance to the therapy. Clinical studies have shown that because of the non-compliance related issues, even on insulin treatment, a significant percentage of patients fail to attain lasting glycemic control [22]. Further, this route of insulin administration has associated side effects, such as hyperinsulinemia and localized deposition of insulin that lead to local hypertrophy and fat deposits at injection sites [31]. Thus, there is great interest today in developing formulations that deliver insulin through other routes of administration. These alternative routes of insulin administration are discussed in the following section.

1.5 Clinical significance of the oral delivery of insulin:

Physiological insulin that is secreted by the pancreas enters portal circulation and inhibits hepatic glucose production. It undergoes metabolism in the liver to a significant extent (~ 50%). The ratio of plasma insulin in portal circulation versus that in peripheral circulation is two. The physiological hypoglycaemic effect of insulin is a result of the absence of hepatic glucose production that is enhanced by the increase in glucose use caused by lower insulin levels in peripheral circulation. When insulin is injected subcutaneously, the plasma insulin concentration in portal circulation and in peripheral circulation is almost equal. The hypoglycemic effect of insulin is a result of its action on peripheral tissues. Oral delivery of insulin can mimic the physiological fate of insulin and may provide better glucose homeostatis. This also will lessen incidences of peripheral hyperinsulinaemia, which is linked to neuropathy, retinoendopathy and so forth [1].

1.6 Alternative Routes of Insulin Administration:

Recent advances in alternative routes of administration as an approach to improve insulin therapy are discussed here. Investigated delivery systems for insulin therapy mainly include transdermal, nasal, buccal, pulmonary and oral delivery systems.

1.6.1 Transdermal delivery:

Large, hydrophilic macromolecules such as insulin cannot efficiently permeate the skin. This skin impermeability is mainly due to the intercellular lipid layer of the stratum corneum [32]. Thus, the delivery approaches targeted at improving the transdermal delivery of drugs involve breaking down or removing the lipid barrier by chemical, electrical or physical methods [33]. Iontophoresis, low-frequency ultrasound [34], or chemical agents acting as vesicles are some of the methodologies investigated. In Iontophoresis, low-level electric current is used to enhance the permeability of the drug across the stratum corneum and the permeability of insulin depends on the net charge of the insulin molecule [35,36]. However the potential of this methodology has only been demonstrated in animal studies and the amount of insulin transferred across the skin was barely enough to meet basal insulin requirement [33].

Low-frequency ultrasound (20-150 kHz) has been shown to be more effective by causing several-fold increase in macromolecular permeability [37]. However, even with this technique, insulin delivery rates may not be sufficient for physiological replacement of insulin [38]. Combined use of iontophoresis and ultrasound has been proposed as an approach for increasing macromolecular transfer across the skin barrier [39]. Skin pretreatment with iodine followed by a dermal application of insulin solution has been shown to reduce glucose and elevate hormone levels in plasma. It has been proposed that topical iodine protects the dermally applied insulin by inactivation of endogenous sulfhydryls which can reduce the disulfide bonds of hormone. [40]

1.6.2 Intranasal Delivery:

The nasal mucosa is characterized by large surface area for drug absorption (about 150 cm^2), high coverage density of the epithelium by microvilli and a rich, vascularized subepithelium

[41]. The drugs entering through this route directly enter the systemic circulation, thus avoiding hepatic first-pass metabolism. Nasal absorption also provides for rapid attainment of therapeutic blood levels and quicker onset of pharmacological activity [42]. The main barriers to absorption include the very active mucociliary clearance, enzyme activity, low permeability and low pH of the nasal epithelium. In addition, little information is available on the influence of nasal pathology on the efficacy of insulin administration [41,43]. Small molecular weight molecules such as oxytocin (MW 1,007) and calcitonin (MW 3,431) have been successfully delivered via the nasal route [44], but reproducible and reliable intranasal delivery methodology for insulin has not been developed.

The observed low bioavailability of insulin administered via the intranasal route often necessitates use of absorption enhancers. However many of these enhancers have been known to cause damage to the nasal mucosa. Use of lecithin, bile salts or laureth-9 as absorption enhancers for nasal absorption enhancement caused nasal irritation in patients [45,46]. In one of the study intranasal microemulsion spray has been shown to improve the bioavailability of insulin in diabetic rabbits with about 35% drop in plasma glucose levels [47]. Even with the use of absorption enhancers, reproducible and reliable insulin delivery has not been achieved.

Potential damage to the nasal mucosa and nasal irritation, especially in the long-term treatment regimen and high rates of treatment failure are the main obstacles in clinical application of intranasal insulin delivery [33,38].

1.6.3 Buccal Delivery:

Buccal mucosa offers an interesting portal of administration for both systemic and local delivery of therapeutic agents. The oral mucosa consists of a non-keratinized area (sublingual and buccal mucosa) and the keratinized area (the gum or gingiva, the palatal mucosa and the inner side of the lips). The nonkeratinized regions are generally more permeable as compared to the keratinized areas [48]. The buccal cavity has a large surface area for absorption (100–200 cm^2), is richly vascularized and has little proteolytic activity. It has fewer proteolytic enzymes than the gastrointestinal, nasal, vaginal and rectal administration areas [49]. The predominant peptidases found in the buccal mucosa are aminopeptidases. The proteolytic enzymes such as trypsin, chymotrypsin and pepsin, which are present in the gastric and

intestinal fluids, are largely absent [48,50]. The factors limiting insulin absorption via the buccal route are the relatively thick multi-layered buccal barrier, the high rate of mucus turnover and the proteolytic degradation. Strategies investigated to improve absorption of insulin include use of absorption enhancers, bioadhesive systems and enzyme inhibitors [48,52-55].

Recently, Generex (Toronto, Canada) demonstrated efficacy of a buccal insulin delivery system based on an oral insulin spray which administers insulin in the form of a high velocity, fine particle aerosol directly into the patient's mouth. The particles consist of mixed micelles made from absorption enhancers that encapsulate insulin [56]. The fast moving, fine particles are able to penetrate a thin membrane that guards the surface area in direct contact with the blood circulation. Combined with the effect of absorption enhancers, the penetrating particles cause a rapid increase in the uptake of insulin into the systemic circulation [57]. The administered insulin appears in the circulation within 10 min of application. Bioavailability of 7 to 8% was determined in a proof-of concept study involving Type 2 diabetic patients and the insulin peak was reached in about 30 min after application. The subcutaneously injected insulin reached the absorption peak in about 60 min [56]. More clinical and toxicological data is required to establish the efficacy of this system.

1.6.4 Pulmonary Delivery:

The respiratory tract offers a large area for drug absorption, a thin epithelial membrane and a rich vasculature. It also lacks effective mucociliary clearance and is highly immunotolerant. In addition, this route of administration also eliminates the first-pass hepatic metabolism [58]. This makes the lungs an attractive option for drug delivery [41,59,60]. Inhalers are typically used for pulmonary insulin delivery, which enables the drug to reach the deep-lung, from where it can be quickly absorbed into the circulation. The factors that need to be controlled for optimizing insulin delivery include the type of propellant used, the air flow speed, the losses within the device, the particle size and drug deposition into the throat and bronchial tubes [38]. Particle size and velocity greatly influence the deep-lung deposition of the inhaled protein [59].

Several pulmonary insulin delivery systems including Exubera®, jointly developed by Nektar Therapeutics, Pfizer and Aventis, the AERx®, developed by Aradigm Corporation

and Novo Nordisk and AIR® developed by Alkermes and Eli Lilly, are in development and in phase III testing. One of these devices, Exubera is currently marketed but requires large doses of insulin to achieve therapeutic effect. In some studies however, it was observed that patients treated with inhaled insulin had increased insulin antibody levels, but these levels thus far have not been related to any significant clinical change [38,58]. Adverse effects of the treatment on pulmonary function are also a cause of concern [61]. Safety and tolerability issues need to be addressed for this treatment to be clinically applicable [62].

Delivery of insulin to the deep lung presents unique challenges to the body's mucosal defense system. Inhaled insulin whether formulated as a powder or liquid, has been shown to be more immunogenic than comparator insulin given by subcutaneous route [63].

Of all the routes of delivering drugs, the oral route is the most desirable. This route of administration results in higher patient compliance than any other route of administration. If successful, this delivery system will solve the current non-compliance related problems associated with insulin injections. The attractiveness of this delivery system has led to several attempts to develop oral insulin formulations [64,65]. Another great advantage of this route of administration is that insulin delivered via the oral route mimics more closely the endogenous secretion of insulin. Insulin absorbed from the intestinal epithelium reaches the liver through the portal vein and can have a direct effect on the hepatic glucose production. This mode of distribution is highly appropriate, since the liver plays an important role in maintaining glucose homeostasis in the bloodstream, taking up and storing the energy in carbohydrates in the form of glycogen. Parenterally administered insulin mainly targets the peripheral tissue rather than the liver with pharmacokinetics that do not replicate the normal dynamics of endogenous insulin release [21]. A detailed description of the challenges associated with oral administration of proteins and attempted solutions are included in Section 1.11

1.7 Oral Delivery of Proteins:

Oral delivery formulation for insulin is highly desirable from a patient compliance point of view and other considerations discussed above. However, only a small portion of insulin administered orally reaches the blood stream, mainly due to extensive degradation of the protein in the gastrointestinal tract. Further, large size and hydrophilicity of the molecule

greatly limits its transport across the intestinal epithelium. No specific transport mechanism is present for the passage of insulin cross the intestinal cell monolayer. Insulin molecules that are unable to cross the intestinal barrier thus get further exposed to the intestinal proteolytic activity, this in turn reduces the bioavailability of the protein. Owing to these factors, less than 0.5% of the orally administered insulin typically reaches the systemic circulation [66].

For many years researchers have tried to find a solution to these problems and in effect increase the oral bioavailability of insulin. The investigated approaches include use of permeation enhancers, protease inhibitors [67-69], or concomitant administration of permeation enhancers and protease inhibitors, chitosan coatings to stabilize the protein and improve cellular permeability [70], entrapment of insulin within microparticles [71-73] and protein modification to make it resistant to proteolytic attack and more permeable across the epithelium [74-76]. Some of these promising approaches are discussed in detail in Section 1.10. The physiology of the GI tract in relation to oral protein delivery is discussed in the Section 1.8

1.7.1 Physiology of the Gastrointestinal Tract:

To maintain a constant state of homeostasis, the body needs to constantly replace nutrient molecules and replenish water and electrolytes that are lost through urine, sweat and other means. The digestive system helps achieve this by continually supplying the body with energy-rich nutrients, water and electrolytes from the external environment. In most cases, the digestive tract itself does not directly regulate the uptake and transfer of these components based on the body's requirement, but optimizes the digestion and absorption of the ingested food. This is achieved by the movement of the ingested food through the GI tract, digestion of the ingested food by secretion of the digestive juices, absorption of the digested components, water and electrolytes and movement of absorbed components away from the digestive tract by blood circulation.

The process of digestion converts complex food matter into simpler and smaller fragments that can be absorbed by the cells lining the GI tract to be utilized for various bodily functions. The digestive juices are emptied into the lumen of the digestive tract by the exocrine glands via the tubular channels formed by the duct cells. Specific enzymes in these secretions digest the energy rich foodstuff which can be primarily classified into carbohydrates, fats and

proteins. Proteins of therapeutic value, such as insulin, are broken down into smaller peptide fragments and finally into individual amino acids rendering them biologically inactive. The specific physiology of the stomach and intestine in relation to oral proteins delivery are discussed below.

1.7.2 Gastric and Intestinal Environment:

Degradation of proteins during their transit from mouth, the pharynx and the esophagus is minimal. The secretions of the salivary glands contain mucus, amylase and lysozyme. Digestion in the mouth is limited to initiation of polysaccharides hydrolysis into disaccharides by amylase. No absorption of food material occurs in the mouth. The secretions of esophagus are entirely mucoid in character and maintain a well lubricated esophageal lumen. Mucus secreted by the esophagus protects the esophageal wall from damage due to food. The entire movement of food through the pharynx and esophagus takes between 6-10 sec [66].

After traveling through the esophagus the food reaches the stomach where it is stored and digestion of the ingested food begins. Based on anatomical and histological characteristics, the stomach is divided into the fundus (part of stomach that lies above the esophageal opening), the body (middle portion) and the antrum (lowermost part of the stomach characterized by thick muscles). The three regions coordinate and control the motility function of stomach. The digestive juices of the stomach are mainly secreted by gastric (or oxyntic) glands. These glands are responsible for secretion of hydrochloric acid and pepsinogen and mucus along with other components [77].

Other exocrine glands present in the gastric mucosa are the pyloric glands. These mainly secrete mucus and some pepsinogen. The oxyntic glands are mainly present in on the inside surface of the body on the fundus (oxyntic mucosa) whereas the pyloric glands are present in the pyloric gland area lining the antral surface. Pepsinogen, the inactive precursor of pepsin, is converted into pepsin by the HCl secreted by the oxyntic glands. This then leads to autocatalytic conversion of pepsinogen into pepsin, which is the main digestive enzyme of the stomach. Pepsin is active at low pH but is rapidly inactivated above pH 5.0. Pepsin acts at specific amide bonds within protein molecules and convert them into smaller peptide

fragments. It is most efficient in cleaving amino acid linkages involving the aromatic amino acids, phenylalanine, tryptophan and tyrosine [78].

No absorption of foodstuff takes place through the stomach. Food components digested in the stomach are still large fragments and hence cannot get absorbed through the gastric mucosa. Most of the digestion and absorption occurs in the small intestine. The small intestine is divided into three sections: the duodenum, the jejunum and the ileum. The small intestine secretions themselves do not contribute significantly to the process of digestion. Although the intestinal cells do secrete digestive enzymes (such as aminopeptidase), they mainly act in the brush border of the epithelial cells. The exocrine glands in the small intestine mainly secrete mucus that lines the inside of the intestinal wall. Protein digestion activity of the small intestine mainly comes from the pancreatic secretions [66].

The gastric contents, on emptying into the upper portion of the small intestine (duodenum), mix with the juices in the small intestine and also with the secretions of the pancreas. The secretions of the pancreas come from the exocrine pancreatic acinar cells. These cells form sac-like structures with the duct cells forming channels leading to the pancreatic duct. The secretions of the pancreas mix with the bile duct coming from the liver before mixing into the intestinal contents [66].

The acinar cells are responsible for the secretion of proteolytic enzymes (protein digestion), pancreatic amylase (carbohydrate digestion) and pancreatic lipase (fat digestion). The duct cells secrete large amounts of sodium bicarbonate ($NaHCO_3$) that neutralizes the acidity of the contents emptied by the stomach. The proteolytic enzyme secretions from the pancreas contain trypsinogen, chymotrypsinogen and procarboxypeptidase. Trypsinogen is converted by an autocatalytic reaction to its active form, trypsin, by an enzyme called enterokinase present in the wall of the duodenum. Trypsin then converts chymotrypsin and procarboxypeptidase into their active analogues. Trypsin is the most abundant of the three enzymes. Each of these enzymes acts on specific amino acid linkages and convert the peptide fragments (formed by pepsin digestion) into small peptides and amino acids. Trypsin cleaves the peptide bonds where the carboxyl group comes from arginine or lysine residue, whereas chymotrypsin specifically breaks the peptide bonds where the carboxyl group is donated by tyrosine, phenylalanine, tryptophan, methionine, or leucine. Due to the high specificity of

trypsin for amino acid linkages, it is commonly used as a reagent in unambiguous cleavage of proteins [66,79,80].

1.7.3 The Intestinal Epithelial Cell Barrier:

The epithelial tissue consists of cells specialized in exchange of materials between the external environment and the internal host milieu. Since these cells form the boundary between the external and the internal environment, the transport across these cells is highly regulated and passage of only selective components is allowed. Most of the absorption of nutrients is completed in the duodenum and the jejunum. The small intestinal absorptive epithelium is remarkably well adapted for absorption of nutrients from the lumen. The inner surface of the intestine forms macroscopic circular folds, called valvulae conniventes that increase the surface area for absorption threefold. Each of these folds has microscopic fingerlike projections called villi that result in a 10- fold increase in the surface area. The villus surface is covered by the absorptive epithelial cells along with some mucous cells. The epithelial cells form hairlike projections on the luminal side called the microvilli that further increase the absorptive area 20-fold. Enzymatic secretions of the small intestine are active within these brush borders. Thus these macroscopic and microscopic projections together increase the surface area nearly 600-folds compared to a hollow cylinder of similar dimensions or about 250 m^2 [66].

Nutrients, such as amino acids and sugars, enter the circulation by crossing the epithelium covering the villi. Each villus contains a blunt-ended lymphatic capillary bed and a blunt-ended lymphatic vessel called the central lacteal. It also has a network of capillaries which absorb the molecules transported across the epithelial cells. The intestinal capillaries converge into venules and the hepatic portal vein that take the absorbed molecules to the liver. Fats, instead of being transported into the capillaries, are absorbed into the lymphatic vessels that rapidly flow into the blood via the thoracic duct [66].

The small intestinal epithelium is highly differentiated and consists of six distinct cell types: the enterocytes or absorptive cells, mucin producing goblet cells, endocrine cells, paneth cells, M cells and tuft and cup cells [81]. Epithelial cells are the most common type of cells in the intestinal cell monolayer. The epithelial cells are organized as polarized monolayer, which provides a permeability barrier between two distinct environments. Disruption of this

highly regulated polarized cell barrier can cause passage of toxic luminal contents to the systemic circulation.

The epithelial cells are circumferentially tied to one another by intercellular junctional complexes. These include the tight junctions or zonula occludens, zonula adherens and desmosomes [82,83]. The tight junctions, which appear as a series of fusion points involving the outer leaflets of the plasma membranes, attach the adjacent cells to one another at the apical region of the cells. A century ago, these complexes were thought to be absolute and unregulated barriers in the paracellular space [83]. Physiological studies of the past several decades have shown the tight junctions to be dynamic structures, able to respond to extracellular environmental changes. They are composed of the transmembrane proteins occludin and claudin and cytoplasmic plaque proteins zonula occludens ZO-1, ZO-2, ZO-3, cingulin and 7H6 [83].

Next to the tight junction lies the adherens junction, in which cadherins act as adhesion receptors. The cadherins are a group of functionally related glycoproteins responsible for the calcium-dependent cell-to-cell adhesion.

The transcellular pathway is limited to those molecules which have specific mechanisms of active or facilitated transport, such as receptor mediated endocytosis, wherein the molecules recognize specific receptors presented on the cell surface and get endocytosed in the form of endocytotic vesicles. The absorption of large and hydrophilic macromolecules is almost exclusively limited to the paracellular pathway, which consists of aqueous pores created by the cellular tight junctions [84]. However, under normal conditions, this pathway is restricted to molecules with molecular radii less than 11 Å [85].

The development of efficacious delivery systems based on well-regulated modulation of the tight junctions by absorption enhancers has not been very successful, mainly due to lack of comprehensive understanding of the function of the tight junctions. Some drug delivery approaches based on this approach are discussed in section 1.9.1.1

1.7.4 Changing the intestinal milieu for improved absorption:

As pointed out earlier, the reduced bioavailability of proteins from the oral route is mainly due to their extensive degradation in the gastric and intestinal fluids and to some extent, in the brush border of the epithelial cells and also due to their limited permeability across the intestinal epithelial barrier. Thus, inhibition of the degradative functions of the proteolytic enzymes or an overall increase in the paracellular or transcellular permeability of the epithelium may result in significant increase in the bioavailability of orally administered proteins. Some approaches based on this methodology are highlighted here.

1.7.5 Challenges associated with oral delivery of insulin:

The various challenges associated with the oral delivery of proteins usually are evaluated by determining the fate of the protein in the gastrointestinal tract (GIT). The greatest barriers to the oral delivery of proteins lie in the GIT. Dietary proteins do normally cross the intestinal epithelium intact, but must be broken down to free amino acids which are then absorbed. The main challenges reported are enzymatic degradation, lack of sufficient permeability of proteins through the GIT and manufacturing / dosage form stability of proteins. This explains why typical oral bioavailability of proteins is usually less than 1-2%. Overcoming these barriers is the focus of efforts to develop oral insulin delivery system.

The successful design of an oral delivery system therefore requires an integrated knowledge of the protein drug entity, anatomy and physiology of GIT, manufacturing/dosage form stability and the constraints to the delivery offered by oral route of administration.

This chapter mainly contains (i) Anatomy and physiology of GIT pertaining to oral protein delivery with emphasize on oral drug absorption, transport & disposition (physical barriers); (ii) enzymatic barriers to oral protein delivery; (iii) Barriers due to manufacturing/dosage form stability of proteins; (iv) An overview of importance of oral delivery of proteins vs. traditional injectable dosage forms; (v) An overview of diabetes mellitus and treatment regimes. Lastly on the basis of these five aspects, objective of the present research and development endeavour to design oral delivery system for insulin has been presented.

1.8 Anatomy and physiology of GIT pertaining to oral protein drug delivery system (physical barriers):

The absorption of drugs from the GIT is one of the important determinants for oral bioavailability. Intestinal epithelium is a major barrier to the absorption of hydrophilic macromolecules such as peptides & proteins, as they can not diffuse across the cells through the lipid bilayer cell membrane.

1.8.1 Morphology of small intestine:

The small intestine in an average adult Human is about 280 cm long and 4 cm in diameter. It is strategically located at the interface between the systemic circulation and the environment and plays a major role as a selective permeability barrier. It permits the absorption of nutrients such as, sugars, amino acids, peptides, lipids and vitamins [86,87] and limits the absorption of xenobiotics, digestive enzymes and bacteria. To accomplish these varied functions the small intestine comprises structures and cells with unique characteristics. Drug and nutrient absorption takes place in the intestinal mucosa.

The intestinal mucosa consists of three layers: the muscularis mucosa, the lamina propria and the epithelial cell layer (Figure 1.1).

The muscularis mucosa is located at the boundary between the mucosa and the submucosa. It is a continuous sheet of smooth muscle, 3-10 cells thick, composed of inner circular and outer longitudinal layers. Contraction of the muscularis mucosa may help the emptying of the crypt luminal contents [88]. The lamina propria is the connective tissue inside the villus and surrounding the crypt (Figure 1.1) and contains numerous defensive cells such as, lymphocytes, plasma cells and macrophages, which interact with foreign substances that enter this layer from the gastrointestinal tract. The lamina propria also contains eosinophils, mast cells, blood and lymph vessels and fibroblasts. In addition to carrying out several immunologic functions the lamina propria provides structural support to the epithelial cell layer. And through its capillaries the lamina propria nourishes the epithelial cells and allows the transport of absorbed substances to the systemic circulation. The epithelial cell layer is a monolayer of heterogeneous cells, which include, enterocytes or absorptive cells, undifferentiated (crypt) cells, endocrine cells, goblet cells, which secret mucin. The lamina

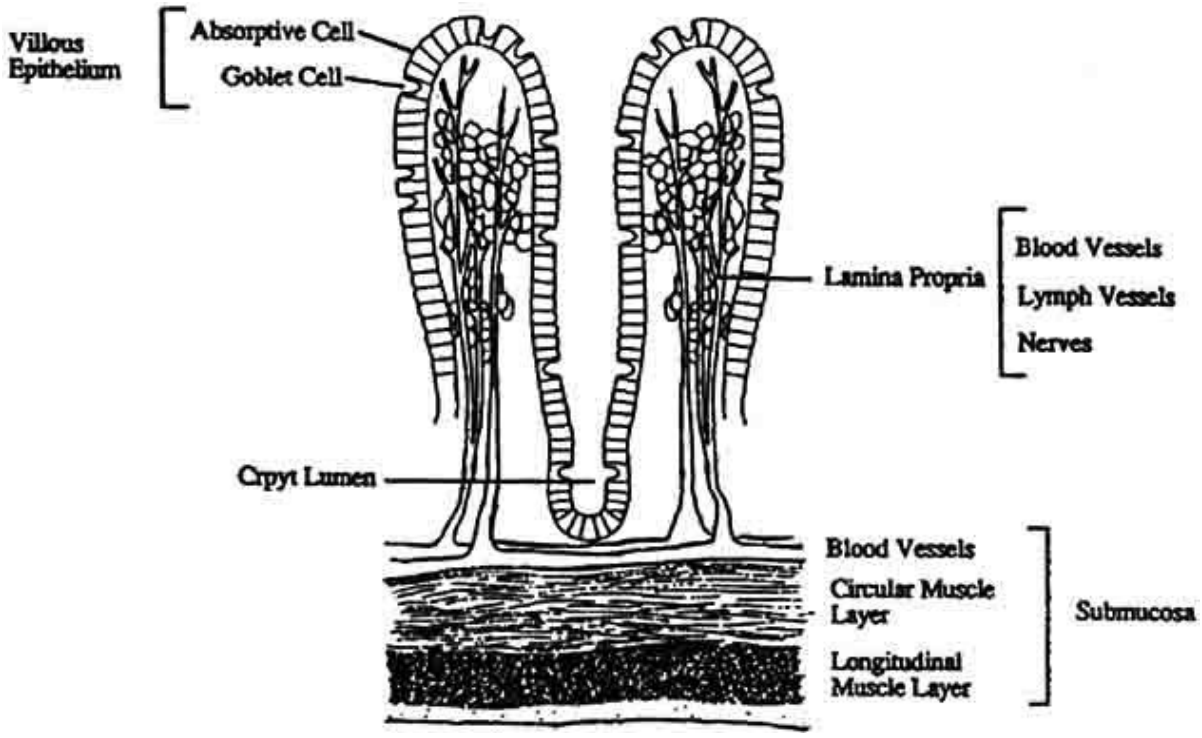

Figure 1.1: Schematic representation of the intestinal mucosa [89]

propria also contains other cell types, paneth cells, tuft cells and cup cells, whose functions are not known. In certain parts of the intestine (Payer's patches) the epithelial cell layer contains M (microfold) cells, which sample antigens from the intestinal lumen to the lymph [90,91]. All these cells originate in the crypts of Lieberkuhn as undifferentiated cells, which undergo differentiation as they migrate upward along the crypt–villus axis. As undifferentiated crypt cells move up, they differentiate into absorptive, goblet, paneth, M, tuft, or cup cells. The epithelial cell layer consists mainly of absorptive and undifferentiated crypt cells. The absorptive cells are highly polarized, columnar cells whose main function is absorption. The apical surface is characterized by closely packed microvilli about 0.5-1.5 mm in length and 0.1 mm in width, depending on the species [91]. The thickness of the apical membrane, 10-11 nm, is larger than that of most eukaryotic plasma membranes [91]. The reason for this peculiar feature may be related to the unique biochemical composition of these membranes. A specialized structure of the apical membrane is the glycocalyx or fuzzy coat, a fine filamentous surface coat directly connected to the outer leaflet of the apical membrane. The glycocalyx consists of weakly acidic sulfated mucopolysaccharides

synthesized and secreted by the cells to which they are connected and is histochemically different from the mucus of the goblet cells [92]. The apical membrane has a high (1.7:1) protein-to-lipid molar ratio and an abundance of glycolipids and cholesterol [93]. The basolateral membrane of the absorption cells differs from the apical membrane in morphology, biochemical composition and function [94]. For instance, it is about 7 nm wide and contains Na^{+}-K^{+}-ATPase. The basolateral domain of neighboring cells is usually separated by spaces, whose width depends on the degree of hydration and position on the crypt-villus axis. Intestinal epithelial cells are joined at intercellular junctional complexes. These junctional complexes, which are approximately 0.5-2 nm wide consists of three components; tight junctions, intermediate junctions and spot desmosomes [90].

The tight junctions, located at the apical end of the lateral membrane of adjacent cells, eliminate the intercellular space over a variable distance [90]. The tight junctions of ileal absorptive cells are deeper and denser than tight junctions of the jejunum. The depth and density of the tight junctions between absorptive cells are greater than those of undifferentiated crypt cells and goblet cells. Compared to the intermediate junctions and spot desmosomes, the tight junctions are the most significant diffusion barrier component of the junctional complexes

1.9 Absorption Pathways:

The intestinal mucosa is a selective permeability barrier. Drugs administered into intestinal lumen can cross the mucosal epithelium by the paracellular and transcellular routes (Figure 1.2) [95]. In a general sense, the transport pathways can be divided into a) passive paracellular transport, b) passive transcellular transport, c) carrier mediated transcellular transport and d) vesicular transport.

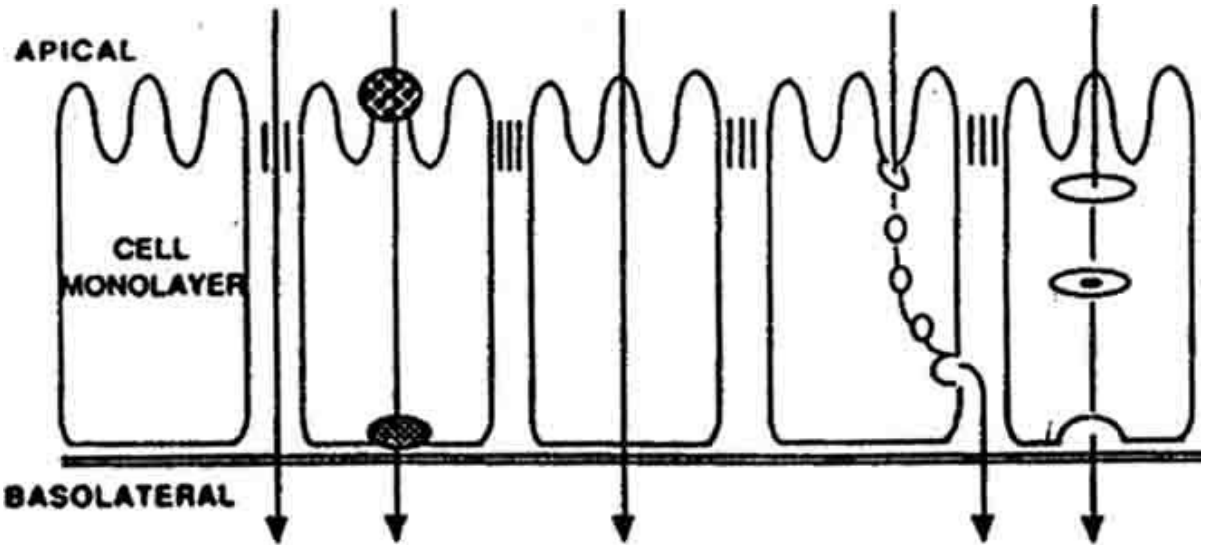

Figure 1.2: Diagram of pathways and mechanisms that mediate transepithelial transport. From left to right, paracellular diffusion, carrier mediated transport, passive transcellular diffusion, vesicular transport and lipid pathway. [95].

1.9.1 Passive paracellular transport:

The paracellular pathway is an aqueous, extracellular route across the epithelium. The driving forces for passive paracellular diffusion are the electrochemical potential gradients derived from differences in concentration, electrical potential and hydrostatic pressure between the two sides of the epithelium. The main barrier to passive paracellular diffusion is the tight junction. In general, the transepithelial permeation of hydrophilic compounds occurs mainly through the paracellular route.

The intercellular junctions can be divided into three different regions: (1) tight junctions (zonula occludens, (2) adherens junctions (zonula adherens) and (3) desmosomes. The intercellular junctions form an 80 nm long torturous path between the two adjacent cells that runs the entire lateral side of the cell as discovered by transmission electron microscope studies.

1.9.1.1 Tight junctions:

At the most apical junction of the cells, the tight junctions or zonula occludens functions to bring adjacent cells into close apposition. This is defined as gate function of the tight junction. The components of intercellular junctions are depicted in Figure 1.3. Tight

junctions cause cell surface polarity that produces the fence function and restricts free diffusion of lipids and proteins from the apical plasma membrane to the basolateral surface.

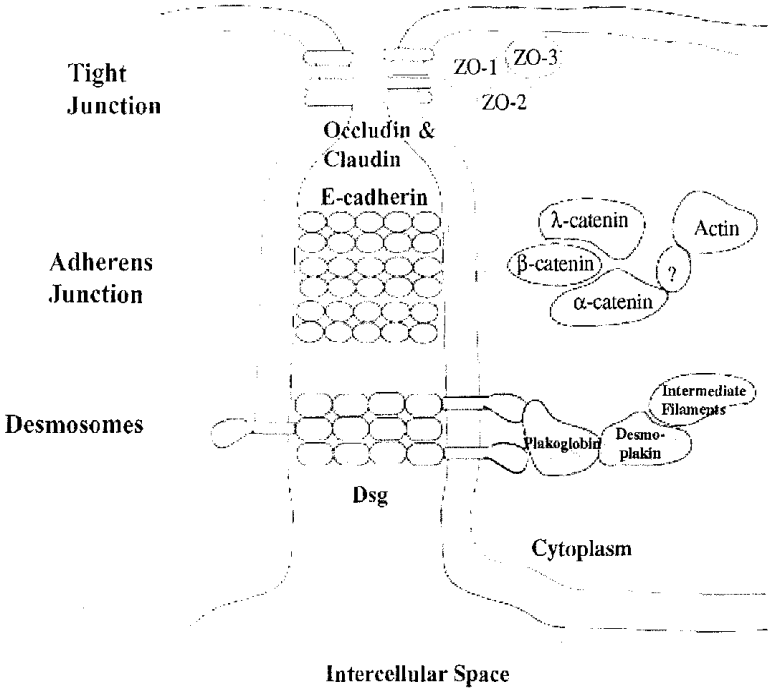

Figure 1.3: Tight junction, Adherens junction and desmosomes [97]

Tight junctions constitute the main barrier to paracellular diffusion. The diameters of the tight junction pores are approximately 4-8 Å and 10-15 Å in Humans and animals, respectively [96].

Thus, paracellular permeation of drug through the intercellular junctions depends on the pore size of the tight junction. The smallest pores are found on the villus tips and the region in the cryptus contains the largest pores.

Because in Humans the paracellular route will not allow the passage of molecules with diameters greater than ~8 Å, this route is unlikely to play an important role in the absorption of most pharmaceutical compounds, this pathway is of little importance for most drugs because of the small surface area of the tight junctions, which accounts for 0.01% of the total surface area (i.e. cell membrane plus tight junctions) [84,97].

However, evidence showing that the diameter of tight junctions can be increased by cellular regulatory processes [98], has fuelled efforts to increase the paracellular permeability of poorly absorbed compounds through co-administration of agents that open tight junctions [99-101].

The integrity of the tight junctions is calcium dependent and removal of calcium causes a rearrangement of the tight junction proteins. A number of cytokines and growth factors have also been shown to decrease the barrier function of the tight junction.

Occludin and claudin proteins have been found at the tight junctions; they are involved in both gate and fence functions. Both these proteins have four transmembrane domains, two extracellular domains that form loops and a cytoplasmic carboxyl tails. The extracelluar loops play a vital role in creating cell-cell contact. The hyperphosphorylated form of occluding is the main form located in the tight junction. There are three scaffolding proteins associated with the tight junctions: ZO-1, ZO-2 and ZO-3. These proteins belong to the membrane associated guanine kinase (MAGUK) family. ZO-1 stabilizes the tight junction by interacting with occluding and claudin, cross-linking them to the actin cytoskeleton.

At present, the potential application of modulation the tight junction permeability is limited by safety concerns. With the possible exception of a few specific cases, this situation will most likely change very slowly in the foreseeable future.

1.9.1.2 Adherens junctions:

Immediately below the tight junction is an area known as the zonula adherens or adherens junction, which is responsible for cell-cell adhesion. The paracellular path has the tight junction and intercellular junction working in series to make up the resistance across this pathway. This resistance is the reciprocal of the permeability, which is dependent upon size and charge in the paracellular path.

Cell-cell adhesion within zonula adherens is controlled by E-cadherin. (120 kD glycoprotein). E-cadherins are calcium dependent molecules that interacts in a homotypic fashion.

1.9.1.3 Desmosomes:

The last region of the paracellular pathway is desmosome, which is located nearest the basolateral membrane surface of the enterocyte. This region appears to be less critical for the function of the paracellular path than two regions situated nearer the apical membrane.

1.9.2 Passive transcellular transport:

A drug with appropriate physicochemical characteristics can transverse through the cell by passive diffusion. In the case of peptides or peptidomimetics, their physicochemical properties may not be suitable for permeation through the cell membrane via the transcellular pathway. The drug molecule must travel through the lipid bilayers that make up the membranes. The bilayer consist of four regions: (1) the outermost region, which has a large number of water molecules and is accountable for the interactions with the other proteins and membranes; (2) the next region, which contains the polar head groups, causing this region to have the highest molecular density and making it most difficult region for diffusion; (3) the third, region which contains the nonpolar tails that form the barrier to penetration based on limitations on molecular size and shape; and (4) the inner region, which is the most hydrophobic and acts as the hydrophobic barrier. The resistance across the transcellular path can be visualized as resistors in a series, where the apical and basolateral membranes act as the two resistors. These membranes form the rate limiting barriers to the flow of molecules [102-105].

1.9.3 Carrier-Mediated Transport:

The small intestinal mucosa expresses large numbers of absorption transporters, which are responsible for the absorption of nutrients and vitamins. In addition to transporting vitamins and nutrients, these transporters have been shown to mediate the absorption of some drugs. Previous reviews have discussed the involvement of transporters on intestinal drug absorption [105-108]. Transporters for di-/tripeptides (PEPT1), large neutral amino acids, bile acids, nucleosides and monocarboxylic acids have received a great deal of attention for their perceived potential to deliver drugs across the intestinal mucosa [108-113]. Recent studies have reported the presence of two additional peptide transporters, PTR3 and PHT1, in

Human and rat intestine. The full potential impact in drug absorption of these transporters and others not yet known, remains to be established. Influx transporters can increase intestinal drug absorption whereas efflux transporters can have the opposite effect. Influx transporters can bind compounds that are dissolved in the intestinal fluid and translocate them across the apical membrane of enterocytes, thus facilitating the drug absorption process. Of these, PEPT1 mediates the transport of peptidomimetic drugs such as, angiotensin converting enzyme (ACE) inhibitors, β- lactam antibiotics and renin inhibitors [109-114]. Compounds that are substrates for these transporters exhibit intestinal absorption higher than expected from their diffusion across cell membranes.

1.9.4 Carrier-Limited Transport (Apical Efflux):

In contrast to the role of absorption transporters, which can enhance intestinal drug absorption, other transporters may have the opposite effect. Efflux transporters mediate the extrusion of compounds from the cell cytoplasm to the intestinal lumen through a process known as apical efflux. Two families of transporters that belong to the ATP binding cassette (ABC) super family of transporters mediate apical drug efflux. They are the multidrug resistance (MDR) and multidrug resistance-associated protein (MRP) families [115-117]. P glycoprotein (Pgp), the most studied member of the apical efflux transporters, is the product of the MDR1 gene. Pgp has 2 subunits with 6 trans-membrane domains and 2 ATP binding sites [118,119]. Located on the apical membrane of normal enterocytes, it has been shown to limit intestinal absorption of a large number of drugs [120-125]. More recently, at least 6 MRP transporters have been discovered. Of these, three (MRP1, MRP2/cMOAT and MRP3) have been found in Human duodenum whereas MRP1, MRP3 and MRP5 are found in Human colon [126]. Their location on the apical membrane of enterocytes and colonocytes, together with their efflux function, makes these transport a potentially formidable barrier to gastrointestinal drug absorption. So far, this family of transporters has been shown to export glutathione S conjugates and organic anions [127-129]. The extent to which they are involved in intestinal drug transport will probably be identified as they become more fully characterized. The MRP family of transporters is emerging as potentially important in determining drug absorption and excretion. The MRP protein that has been most actively studied is MRP2/cMOAT.

1.9.5 Vesicular Transport:

While the action of intestinal enzymes limits the extent to which a compound can permeate the intestinal mucosa, enterocytes also possess vesicular transport processes that can facilitate drug absorption. These include fluid-phase endocytosis (pinocytosis), receptor-mediated endocytosis (RME) and transcytosis. Fluid-phase endocytosis (FPE) is a process by which solute molecules dissolved in the luminal fluid are incorporated by bulk transport into the fluid-phase of endocytic vesicles. This process starts when the plasma membrane forms invaginations that pinch off to form vesicles or pinosomes, which migrate inwardly. Molecules dissolved in the vesicle are transported to endosomes (prelysosomal vesicles), which subsequently fuse with lysosomes. There is evidence that the mucosal uptake of some peptides and proteins involves fluid-phase endocytosis [130]. Receptor-mediated endocytosis is generally of importance only for the mucosal permeation of macromolecules, but not for small molecules. It involves the binding of the macromolecule to a binding site (receptor) on the membrane followed by clustering of the receptor ligand complex into clathrin-coated pits. After endocytosis, the fate of the elements of the receptor-ligand complex is determined through a process called sorting. Usually, the sorting process results in the destruction of the ligand in the lysosomes while the receptor can either undergo lysosomal destruction or recycling back to the cell membrane [131-133]. The ligand dissolved in the endocytic vesicle, following FPE or RME, can by-pass the lysosomes and undergo release across the basolateral membrane [134,135]. This process, known as transcytosis, may result in the intestinal absorption of molecules unable to permeate the cell membrane by simple diffusion.

1.9.6 Lymphatic system and absorption pathways:

The lymphatic system consists of lymph, lymphatic pathways, such as lymphatic capillary, lymphatic vessel, lymphatic duct etc. and some lymphatic organs including lymph node, thymus and spleen. The major function of the lymphatic system is to maintain the body's water balance to the normal level as blood vessels do. This system plays an important role in helping to defend the tissues against infection by filtering particles from the lymph and by supporting the activities of lymphocytes, which furnish immunity, or resistance, to the specific disease causing agents. Also it is well known that the lymphatic absorption of a drug after intestinal administration provides an advantage over the portal blood route for the

possible avoidance of liver pre-systemic metabolism (hepatic first pass effect). Research into lymphatic targeting has recently attracted increasing interest not only for providing a preferential anticancer chemotherapy, but for improving oral absorption of macromolecules drugs, or achieving mucosal immunity [136].

The lymphatic comprise a one-way transport system for fluid and proteins by collecting them from the interstitial space and returning them to the blood circulation. As the blood travels from branching arteries down to the smallest capillaries, plasma fluid and proteins are forced out into the interstitial space. Most of these exudates get reabsorbed into the post-capillary venule, but because of osmotic forces resulting from protein extravagation, there is small net fluid flux out of the vasculature. This excess fluid is convicted through the interstitium and into the initial lymphatics, which are freely permeable to macomolecules and thus serve primary role in maintaining osmotic and hydrostatic pressure within the tissue space. The net fluid efflux and therefore the net flow rate of lymph, is approximately 100-500 times less than the flow rate of the blood [137].

The intestinal lymph, which contains a high amount of fat resorbed directly from the intestine. The lymph is turbid, milky emulsion and is often referred as ‘chyle’ [21,22].

1.9.6.1 Organization of lymphatic system:

There are five main categories of conduct in the lymphatic system: the capillaries, collecting vessels, lymph nodes, trunk and ducts. Their sizes range from 10 μm to 2 mm in diameter. Lymph forms when interstitial fluid moves into the lymphatic capillaries. It then drains from the capillaries into the collecting vessels, which pass through a least one usually several clusters of lymph nodes. Collecting vessels booth enters and exits the nodal vasculature. These vessels then drain into larger trunks, which in turn lead into the ducts. Finally, the ducts return the lymph back into the bloodstream, completing the circuit of fluid transport [137].

Lymphatic capillaries are 10-60 μm in diameter and are comprised of one endothelial cell layer, typically made up of one or two nonfenestrated, highly attenuated cells in cross-section [138]. They have a discontinuous or absent basement membrane and, with the exception of the initial lymphatics in bat’s wing are noncontractile [139].

Lymph nodes function as filters and reservoirs (i.e., long residence time) and are ideal as 'incubators' for white blood cells (and sometimes tumour cells), which proliferate and gain access to the blood through high endothelial venules while in the lymph nodes. Furthermore, the white cells in the nodes may phagocytose molecules and particles; this contributes to lymph node protein dilution along with fluid exchange with the lymph node vasculature vessels [140-142].

The lymphatic trunks are the largest vessels that drain lymph from the final set of lymph nodes into the ducts. The exceptions are the lymphatics from the intestinal, hepatic and lumbar areas; these drain directly into the sac like structure at the distal end of the thoracic duct (the cisterna chyle). The thoracic duct is the final branch of the lymphatic system and enters the lower region of the chest by passing through the aortic opening of the diaphragm [143-147].

1.9.6.2 Solute uptake:

Although the endothelial cell junctions of the initial lymphatics are not tight and considered freely permeable to most proteins, the physicochemical properties of the extracellular matrix (ECM) can affect interstitial solute transport. Since the solute must travel at least some distance through the interstitium before entering the lymphatics, the interstitial resistance to molecular transport greatly affects the apparent lymphatic uptake rate [148].

The size, shape, charge and lipophilicity of a molecules affect its uptake rate into the lymphatics may actually reflect its interstitial hindrance prior to lymphatic uptake. Indeed interstitial transport can not be easily decoupled from lymphatic uptake and the path through the extracellular matrix to the lymphatics should be considered when interpreting uptake data for proteins, colloids, drugs or drug carriers [143,145,149].

Size is one of the most important determinants of lymphatic uptake and lymph node retention. Molecules that are smaller than 10 nm are preferentially reabsorbed into the blood capillaries. While the optimal size for lymphatic uptake is between 10 and 100 nm. Larger the particles or molecules, more the selectivity for uptake into the lymphatic system and slower the uptake. For example, liposomes of 30-60 nm were found to have faster rates than

those of 400 nm, but the smaller ones also showed higher level in the blood circulation [150]. The upper size for lymphatic uptake has not been strictly defined. Particles upto 1 µm have been taken up by the lymphatics following interstitial injection [151]; but above 100 nm, a percentage of injected solute will remain trapped in the interstitial spaces for longer period of time and thus lower uptake efficiencies.

The composition of the molecule or particle is also important in determining uptake and lymph node retention. Colloids and lipids seem to have high uptake efficiencies. Lipophilicity has also been associated with uptake efficiency. Depending on the size, charge, method of preparation and composition, various molecules such as monoclonal antibodies, peptide drugs and anticancer agents may be encapsulated into carriers and optimally targeted to lymphnodes. Further more the carriers can be coated (with polyethylene glycol) to improve lymph node retention by avoiding white cell phagocytosis; such as stealth liposomes [152-154].

Other than intercellular pathways, there is ample evidence for transendothelial pathways for solute and lipid transport in the initial lymphatics. This pathway may be especially important in organs where open junctions in the initial lymphatics are seldom seen, such as the lung [155], intestine [156] and diaphragm [157]. After food uptake, lymphatic endothelial cells may be able to phagocytose particulate matter and 7-8 µm transendothelial channels were seen following milk lipid adsorption [158].

1.9.6.3 Lymphatic drug transport:

The ability of to efficiently deliver highly lipophilic drug molecules, especially in combination with lipophilic drug delivery systems has also led to a renewed interest in intestinal lymphatic drug transport. Highly lipophilic drug molecules, however, may associate with lymph lipoproteins in the enterocyte and gain access to the mesenteric (intestinal) lymphatics, effectively by passing the liver and gaining access to the systemic circulation via the thoracic lymph duct.

The extreme high drug concentration attainable in the lymph (up to 1000 × higher than plasma concentrations) offers drug delivery advantages in addition to reduce first pass metabolism for lymphatic transported drugs, including, specific delivery to lymph resident B

and T lymphocytes (and therefore application in, e.g. interferon and immunomodulator therapy).

Lipid digestion and absorption involves three main sequential steps; (1) dispersion of fat globules into a coarse emulsion of high surface area, (2) enzymatic hydrolysis of the fatty acid glyceryl esters at the oil/water interface and (3) dispersion of the digestion products into an absorbable form [149]. Digestion of formulation derived or dietary lipids (predominantly in the form of triglycerides, TG) begins in the stomach where preduodenal lingual and gastric lipases secreted by the salivary gland and gastric mucosa, respectively, initiate the hydrolysis of TG at the sn-3 position to the corresponding diglyceride (DG) and fatty acid (FA). Liberation of these amphiphilic lipid digestion products, in combination with the shear produced by antral contractions, retropulsion and gastric emptying, facilitates the formation of a crude emulsion that empties into the duodenum [158,159].

The presence of lipid in the duodenum stimulates the secretion of pancreatic fluids and bile slats and biliary lipids from the gall bladder which markedly alter both chemical and physical properties of the TG emulsion. Biliary lipids such as phospholipid and cholesterol adsorb onto the surface of the crude emulsion, leading to the production of a more stable (in colloidal terms) emulsion, with a smaller particle size. The reduction in droplet size provides an increase in the surface area available for binding of pancreatic lipase / co-lipase complex, an interfacial enzyme system that preferentially acts at the surface of the emulsified TG droplets, leading to the quantitative production of one molecule of 20 monoglyceride (MG) and two FA molecules for each TG [160]. Miceller solubilization greatly enhances the luminal solubility (up to 1000 fold) of the lipid digestion products and also facilitates their passage across the UWL thereby providing a concentration gradient for absorption [159,161-163].

Transport of lipid digestion products across the apical membrane of the enterocyte appears to occur via both passive transport and via specific membrane bound carrier proteins. Following absorption into enterocyte, the chain length of the absorbed lipid dictates its subsequent intracellular processing. As a guide, short and medium-chain lipids (chain length < 12 carbons), generally diffuse across the enterocyte and directly enter the capillaries that supply the portal vein. In contrast, long chain lipids (>12 carbons) migrate from the absorptive site

to the endoplasmic reticulum where re-esterification and assembly into intestinal lipoprotein carriers (chylomicrons) occurs prior to secretion into the mesenteric lymph [164-170].

Specificity for access to the intestinal lymph (as opposed to the portal blood) is provided by differences in endothelial architecture between lymph and blood vessels. Whereas the vascular endothelial barrier features tight inter-endothelial junctions and a continuous basal lamina, the lymphatic vessels are characterised by either discontinuous or absent basal lamina and relatively wide inter-endothelial junctional distances. The transcelluar access of small, lipophilic molecules into either the lymph or the blood is therefore relatively unimpeded and under these circumstances absorption into the blood predominates, due to significantly higher blood flow compared to lymph flow aiding the mass transport process. However for either large (>10 000 Da) or extremely hydrophilic compounds or large colloidal structures, transcellular transport into the blood is significantly hindered and paracellular access across the more 'leaky' lymphatic endothelium is favoured [171].

1.9.6.4 Approaches to enhance intestinal lymphatic transport of peptides and proteins:

The apparent requirement for drug association with intestinal derived lipoproteins as a prerequisite for drug transport via the intestinal lymphatics has led to the common use of lipids and lipid based formulations to enhance intestinal lymphatic transport [172-175]. The basic premise underlying the design of these formulations is that the digestion and absorption of co-administered lipids stimulates lipid turnover through the enterocyte, enhancing chylomycron synthesis and thereby increasing the lipoprotein-based lipid sink into which drugs may partition.

Lipid-based formulation may also enhance drug absorption generically via improvement in dissolution and solubilization within the intestinal milieu, a reduction in gastric emptying rate and increases in mucosal permeability and therefore, have the potential to both enhance the overall extent of absorption as well as increase the proportion of what is absorbed, being transported to the systemic circulation via the intestinal lymph [176-178].

The common lipid properties, namely degree of saturation, fatty acid chain length and lipid class have a greater impact to enhance lymphatic transport.

In general, the degree of fatty acid unsaturation has been shown to have a large effect on the rate of absorption and partitioning of lipids between portal blood and intestinal lymph and lipids with increasing lipid unsaturation appears to produce large size lymph lipoproteins and preferentially promote lymphatic lipid transport [179-184].

The majority of fatty acids with chain length s of 14 and above were found to be recovered in thoracic lymph, where as a larger portion of shorter lipids were absorbed directly into the blood [185-187]. Sylven and Borgstrom demonstrated that the lymphatic transport of exogenously administered cholesterol increased in a linear fashion after co-administration with triglycerides of increasing fatty acid chain length. Lymphatic transport of cholesterol was increased by co-administration with oleic acid, linoleic acid and stearic acid [188].

A shorter lag time was associated with administration in fatty acid vehicle when compared with triglyceride vehicle as a result of the shorter time required for the synthesis of chylomicron from fatty acid vehicle when compared with TG vehicle that required additional pre-absorptive digestion.

1.9.7 Biochemical Barrier:

The biochemical component of the mucosal barrier consists of drug metabolizing enzymes. Enzymes found within the intestine are from two sources, mammalian and bacteria-associated. The mammalian enzymes are located within the lumen and in the enterocytes. Enzymes from the microflora within the ileum and colon have also been identified. This discussion will focus on degradation by mammalian enzymes.

1.9.8 Metabolizing enzymes:

Within the lumen of the stomach, a mixture of hydrochloric acid and proteolytic pepsin is the first metabolic barrier that a peptide drug will encounter. Subsequently, the hydrolysis of acidic proteins occurs at pH 2-5; this especially the case for peptides containing aspartate residues [189]. Larger proteins are quite susceptible to this gastric proteolysis, while smaller peptides are unaffected by this mixture.

Fricker and Drewe describe the luminal enzymes of the upper small intestine as second barrier. Trypsin, chymotripsin, elastase and carboxypeptidase A and B are positioned in the lumen of the duodenum. Their highest activity occurs at pH 8. These enzymes degrade 30-40% of large proteins within the duodenum to small peptides within 10 min [189]. Small peptides have been shown to be stable against these pancreatic proteases.

The proximal small intestine shows the greatest metabolic activity due to its large surface area and the plethora of intestinal enzymes and transporters [190]. Phase I and II enzymes have also been identified in the intestine. The most notable Phase I enzymes are those of CYP superfamily. The P450 enzymes are also present in the intestinal walls in concentration approximately 20 times less than those seen within liver [191,192]. The highest activity of P450 enzymes is displayed in the proximal part of the gastrointestinal tract and their activity decreases distally. The greatest concentration of P450 enzymes is found in the villus tips of the upper and middle third of the intestine allowing extensive metabolism. The major factor that influences the route dependent metabolism is the residence time of the drug within the enterocyte. The residence time can be lengthened by binding in the cytoplasm, the activity of efflux pumps and limited blood flow or, conversely, shortened by basolateral clearance and basolateral transporters.

Conjugating enzymes are also referred as “Phase II metabolizers” are also found in intestine. Glucoronyltransferase, N-acyltransferase, sulfotransferase and glutathione-*S*-transferase show high activity for intestinal Phase II enzymes. Conjugates that are formed by these enzymes within cell are reported to be substrates of the multidrug resistance-associated protein family (MRP) of transporters and are excreted into the lumen.

1.9.9 Chemical Barriers:

The chemical structure of a drug determines its solubility and permeability profiles. In turn the concentration at the intestinal lumen and the permeability of the drug across the intestinal mucosa are responsible for the rate and extent of absorption. Unfavourable physicochemical properties have been a limiting factor in the oral absorption of peptides and peptidomimetics [193]. The structural factors involved in the permeation of peptides will be described here.

1.9.10 Hydrogen-Bonding Potential:

Hydrogen-bonding potential has been shown to be an important factor in the permeation of peptides. The energy needed to desolvate or hydrogen-bonding the polar amide bonds in the peptide to allow it to enter and transverse the cell membrane is the principle behind the concept of hydrogen-bonding potential. Burton *et al* have reported partition coefficients of model peptides in n-octanol/hanks' balanced salt solution (HBSS), isooctane/HBSS and heptane/ethylene glycol systems. The later system of heptane/ethylene glycol correlates well with the hydrogen-bonding potential and provides a simpler and more direct measurement [194-197].

1.9.11 Other properties:

Physicochemical properties of the peptide are also important determinants in the passage of drugs via the paracellular path. Size, charge and hydrophobicity are the factors influencing paracellular permeation. A change in the hydrophilicity of a peptide may later its route of permeation; as the hydrophilicity of peptide decreases, its lipophilicity increases, causing a shift in permeation of the peptide from the paracellular to transcellular route. Molecules with radii larger than 11°A are unable to penetrate the tight junctions [198]. Studies of Caco-2 cells confirm that drug permeation via the paracellular path is size-dependent and this highlights the sieving abilities of the intercellular junctions [193].

Although the paracellular path is negatively charged, the effect of charge on paracelluar permeation is not well understood. One study suggest that a positive net charge on a peptide produces the best paracellular permeation, but another study suggest that a -1 or -2 charge is most effective in paracellular transport. It has also been suggested that the effect of charge is negligible on the molecular size of the peptide increases [193].

1.10 Oral protein & peptide drug delivery:

Delivering therapeutically active proteins and peptides by any route other than the invasive methods has been a challenge and a goal for many decades [199]. Among the alternate routes that have been tried with varying degree of success are oral, buccal [200], intranasal [201], pulmonary [202], transdemal [203], ocular [204] and rectal [205] approaches. In addition to

high level of patient compliance and long term compliance, oral delivery also benefits the patients through avoidance of pain and discomfort associated with injections; greater convenience. In addition, growing body of data suggests that for certain polypeptides. Such as insulin, the oral delivery route is more physiological [206,207]. Thus oral delivery would be an ideal route if appropriate oral dosage forms of therapeutic peptides and proteins were available.

In spite of the obstacles to oral delivery, substantial evidence suggests that pharmaceutical polypeptides are absorbed through the intestinal mucosa, although in minute amounts [208]. Small amount of polypeptide drugs can be absorbed by the action of specific peptide transporters in the intestinal mucosa cells [209]. This suggests that properly formulated proteins or peptide drugs may be administered by the oral route with retention of sufficient biological activity for their therapeutic use.

Designing and formulating a polypeptide drug for delivery through the GI tract requires a multitude of strategies [210,211]. The dosage form must initially stabilize the drug while making it easy to take orally [212]. It must then protect the polypeptide from the extreme acidity and action of pepsin in the stomach [213]. When the drug reaches the intestine, the formulation must incorporate some means for limiting drug degradation by the plethora of enzymes that ere present in the intestinal lumen [214]. In addition, the polypeptide and/or its formulation must facilitate both aqueous solubility at near neutral pH and lipid layer penetration in order for the protein to transverse the intestinal membrane and then the basal membrane for entry into the blood stream [215]. To accomplish this pharmaceutical excipients that promote absorption may be required [216]. Finally, when the modified peptide enters the systemic circulation, the structural modification may add to the functionality of the drug, e.g., extending its half life in the circulation. However, any structural changes that may have been employed to enhance oral bioavailability must not interfere with receptors binding and uptake at the site of biological activity.

1.10.1 Formulation approaches:

Oral delivery of peptides has been an ongoing challenge. Nearly every oral dosage form used for delivery of conventional small-molecule drugs has been used to explore oral delivery of

polypeptides. Except for cases where the polypeptide has been chemically modified or where proprietary absorption enhancers have been used, the results have been disappointing.

1.10.2 Enzyme Inhibitors:

Researchers have evaluated the use of protease inhibitors with the aim of slowing the rate of degradation of proteins and peptides in GI tract. The hypothesis was that a slow rate of degradation would increase the amount of protein and peptide drug availability for absorption. For example, enzyme degradation of insulin is known to be mediated by the serine proteases trypsin, α-chymoptripsin and thiol metalloprotease insulin-degrading enzymes [217]. The rate of degradation was found to be ~10 times higher in the presence of α-chymotripsin when compared with that in the presence of trypsin [4]. The stability of insulin has been evaluated in the presence of excipients that inhibit these enzymes [218,219]. Representative inhibitors if trypsin and α-chymotripsin include pancreatic inhibitor and soyabean trypsin inhibitor [220], FK-448 [221], camostatmesylate [222] and aprotinin [223]. Inhibitors of insulin-degrading enzyme include 1,10 phenanthroline, p-choromeribenzoate [224] and bacitracin [225]. Ziv *et al* [226] reported the use of a combination of an enhancer, sodium cholate and a protease inhibitor to achieve a 1-% increase in rat intestinal insulin absorption.

Another approach to enzyme is to manipulate the pH to inactive local digestive enzymes. Lee *et al*. conducted studies demonstrating that oral absorption properties of salmon calcitonin can be modulated by changing intestinal pH. Reducing the intestinal pH in the GI tract, increased absorption of the intact peptide [227]. A sufficient amount of a pH-lowering buffer that lowers local intestinal pH to values below 4.5 can deactivate trypsin, chymotripsin and elastase.

1.10.3 Absorption enhancers:

Permeation enhancers improve the absorption of protein and peptides by increasing their paracellular and transcellular transport. An increase in paracellular transport is mediated by modulating the tight junctions of the cells and an increase in transcellular transport is associated with an increase in the fluidity of the cell membrane. Paracellular permeation enhancers include calcium chelators, bile salts and fatty acids. Calcium chelators, such as

ethylenediaminetetraacetic acid (EDTA) [228], act by inducing calcium depletion, thereby creating global changes in the cells, including disruption of actin filaments, disruption of adherent junctions and diminished cell adhesion [229]. Zonula occludens toxin (ZOT) acts specifically on the actin filaments of tight junctions [230] and chitosan triggers opening of the tight junction between the cells [231].

Transcellular permeation enhancers, include surfactants, medium chain fatty acids, non-ionic surfactants [232], sodium cholate and their bile slats [233] and many other surfactants.

A novel approach using functional peptides for improving intestinal absorption of insulin has also been studied. Co-administration of oligoarginine with insulin showed increased insulin absorption without causing detectable damage to cellular integrity [234].

1.10.4 Nanoparticles:

Nanoparticles have been studied in recent decades as particulate carriers to deliver the protein and peptides drugs orally [235,236]. This approach is supported by the literature, which state that particles in the nanosize range are absorbed intact by the intestinal epithelium, especially Peyer's patches and travel to site such as the livery, the spleen and other tissues [237-239]. The proteins and peptides encapsulated in the nanoparticles are less sensitive to enzyme degradation through their association with polymers. The extended release of protein and peptide drug from the particles could have pharmacological and clinical significance.

The factors affecting uptake include the particle size of the particulate, the surface charge of the particles, the influence of surface ligands and the dynamic nature of particle interaction in the gut. In one example, after intraduodenal administration of chitosan nanoparticles in rats, particles were detected in both epithelial cells and Peyer's patches. Chitosan naoparticles seemed to be taken up and transported by adsorptive transcytosis [240,241], while polystyrene nanoparticles uptake was probably mediated by nonadsorptive transcytosis. The encapsulated insulin was released from the nanocapsules over a period of approximately 6 hour, was shown to be effective orally and had 11.4% if the efficacy of intraperitoneally delivered insulin [242].

Utility of vitamin B12 carrier system for oral delivery of conjugated peptides/proteins and enhancement of nanoparticles has been shown enhance the oral absorption of insulin. The pharmacological availability of oral insulin relative to subcutaneous injection reported to be about 29.4% [243].

One problem in using nanoparticles for peptide and protein delivery is the erratic nature of nanoparticle absorption. For, example, the proportion of intact particles reaching the systemic circulation was estimated to be generally below 5% [244]. Considering the generally low encapsulation efficiency of the protein in the particulates, the overall oral bioavailability of proteins and peptides is not significant.

1.10.5 Emulsions:

Liquid emulsions have been used to deliver proteins and peptide orally. Emulsions are thought to protect the drug from chemical and enzymatic breakdown in the intestinal lumen. Drug absorption enhancement is dependent on the type of emulsifying agent, particle size of the dispersed phase, pH, solubility of drug, type of lipid phase used, etc. Water-in-oil microemulsions have been shown to enhance oral bioavailability of proteins and peptides [245,246]. The lipid phase of microemulsion composed of medium chain fatty acid triglycerides increased the bioavailability of muramyl dipeptide analog [247]. Water-in-oil microemulsion formulations have been developed for oral insulin delivery.

1.10.6 Micelle formulations:

A micelle system can be either water-based or oil-based. The use of micelle formulation for poorly water soluble drugs for systemic delivery has been well recognized. In recent years, the effective development of self-emulsifying microemulsions or mixed micelle-based lipid formulations products, such a Sandimune Neoral (cyclosporine), Norvit (ritonavir) and Fortovase (saquinavir), has substantially increased interest in the application of lipid-based micelle formulation to improve oral delivery of poorly water-soluble drugs as well as proteins and peptide drugs [248].

Modi *et al* patented a mixed micellar for administering insulin to the buccal mucosa using a metered dose inhaler [249]. The formulation includes a micellar proteonic pharmaceutical

agent, lauryl sulphate, salicylate, edentate and at least one absorption-enhancing compound such as lecithin, hyaluronic acid, glycolic acid, oleic acid, linolenic acid, or glycerine.

1.10.7 Liposomes:

Liposomes have also been studied as a way to deliver peptides and proteins orally. Liposomes are prone to the combined degrading effects of the acidic pH of the stomach, bile salts and pancreatic lipase upon oral administration. Compared to parenteral route, there have been fewer attempts to develop oral formulations to deliver the protein and peptides using liposome system. Attempts have been made to improve the stability of liposomes either by incorporating polymers at the liposome surface or by using GI-resistant lipids [250-257]. The efficacy of liposomes coated with muco-adhesive polymers such as chitosan in improving oral uptake of insulin has also been studied [258-262].

1.10.8 Chemical modification:

Chemically modified proteins and peptides offer some significant advantages over native proteins. Proteins are inherently unstable to digestive enzymes; however chemical modifications can be made that inhibit enzyme attack. Such modifications include prodrug approaches and permanent modifications [263]. Permanent modifications [264], if conducted in the appropriate functional group, may increase the stability and oral bioavailability of a peptide or protein without compromising its biological activity. Various permanent modifications have been studied, especially with proteins. These include glycosylation, pegylation [265], cross-linking and other polymer conjugation. So far, two of the most successful examples in protein drug delivery using chemical modification are calcitonin and insulin.

Another approach to improve the transcellular delivery of therapeutic proteins exploited is the receptor-mediated endocytosis using insulin-transferrin conjugates to improve oral bioavailability in diabetic rats [266].

1.11 Oral Delivery of Insulin – Challenges:

The various challenges associated with the oral delivery of proteins usually are evaluated by determining the fate of the protein in the gastrointestinal tract (GIT). The main challenges reported are enzymatic degradation and a lack of sufficient insulin permeability through the GIT. The enzymatic barrier and epithelial barrier for proteins have been reviewed in detail elsewhere [267,268]. The following sections focus on the details of these barriers with respect to insulin.

1.11.1 Enzymatic degradation of insulin:

Upon ingestion, insulin is subjected to acid-catalyzed degradation in the stomach, luminal degradation in the intestine and intracellular degradation. The pancreatic enzymes that degrade insulin are trypsin and a-chymotrypsin [269,270]. The rate of degradation was found to be; 10 times higher in the presence of a chymotrypsin when compared with that in the presence of trypsin [271]. The cytosolic enzyme that degrades insulin is insulin-degrading enzyme (IDE) [272]. Insulin is not subject to enzymatic degradation by brush-border enzymes. The rate of degradation of insulin also depends on its associated state in solution. Insulin is a monomer at low concentration (0.1 mM) and dimerizes in a pH range of 4–8 at higher concentrations. At concentrations greater than 2 mM, the hexamer is formed at neutral pH [273]. The associated state affects the rate of degradation of insulin. In the presence of bile salts, the rate of degradation may increase close to six times [274]. This correlated with complete dissociation of insulin into a monomeric form that was verified by circular dichroism spectroscopy.

Several studies support using protease inhibitors as a protective cover to prevent the degradation of insulin by digestive enzymes, which are mostly located in the upper part of the intestine. More in-vitro experiments to evaluate the potential for cytotoxicity or other gastrointestinal side effects are required to be seen. Most studies cited show increased permeability with little or no toxic effect.

In the 1980s, Ziv and his colleagues studied bile acid (sodium cholate) as a protease inhibitor, along with aprotinin (a trypsin inhibitor), in an insulin-containing solution. This solution was injected into rats' ileums. The results suggest that the combination of both

inhibitors could improve insulin absorption in the intestinal lumen of rats [275]. Interestingly, acarbose, an intestinal alpha glucosidase inhibitor, also showed some positive effects in diabetic animals [276].

A potent and specific inhibitor, 4-(4-isopropylpiper-adinocarnonyl) phenyl 1, 2, 3, 4-tetrahydro-1-naphthoate methanesulphonate (FK-448) of chymotrypsin improved intestinal absorption of insulin and resulted in a reduction in blood glucose in rats and dogs [277]. Fujii's study, which used soybean trypsin inhibitors in the ileum, confirmed the earlier findings of Ziv *et al.* and Kidron *et al.* [278]. There is no evidence that soybean trypsin inhibitor enhanced insulin absorption in the ascending colon, however. Further attempts to increase the hypoglycemic levels in diabetic animals were made by introducing enzyme inhibitors and protease inhibitors (aprotinin or the Bowman–Birk inhibitor) to prevent the degradation of insulin in the intestine by pancreatic enzymes [279,280].

In one study, the use of a trypsin inhibitor (protease inhibitor) caused greater hypoglycemic effect in a gelatin microspheres formulation containing insulin [281]. The study suggested that aprotinin or bacitracin (protease inhibitors) caused a significant and prolonged plasma glucose reduction in diabetic rats [282].

Furthermore, another study suggested that the coadministration of aprotinin and soybean trypsin inhibitor (protease inhibitors) had little effect on the absorption of insulin when compared with aprotinin alone. The same study also suggested the use of a combination of camostat mesilate and bacitracin (protease inhibitors). This combinational strategy was more effective in reducing plasma glucose levels than any other combination previously used by the same authors [283].

A study by Damage *et al*. reported that poly (alkyl cyanoacrylate) insulin nanocapsules dispersed in an oily medium containing poloxamer 188 and deoxycholic acid (surfactants) reduces fasted gylcemia in diabetic rats [284].

Qi and Ping studied the oral coadministration of insulin enteric microspheres with sodium N-(8-[2-hydroxybenzoyl] amino) caprylate (SNAC). The study confirmed that microsphere preparation facilitates insulin absorption in rats because of SNAC's enzyme inhibiting properties, which increase insulin permeability [285]. In this study, insulin was solubilized in the form of anhydrous reverse micelles. Ethylene–diaminetetraacetic acid (EDTA) was

administered before the insulin oil solution was given to rats. A decrease in glucose levels, which primarily resulted from EDTA's enzyme inhibiting properties, was observed [286].

A success with the coadministration of trypsin and chymotrypsin (protease inhibitors) that did not alter any physiological digestive process also was reported [287].

Morishita *et al* used microspheres for insulin delivery in rats [288]. Their study showed that L-microspheres carrying insulin and aprotinin enhanced insulin absorption. Four years later, Hosny investigated the use of sodium cholate as an effective enzyme inhibitor in different doses and found that smaller doses of sodium cholate were more effective in reducing plasma glucose levels than higher doses [289].

The issue of insulin stability in the presence of enzyme inhibitors should be addressed. If the incorporated enzyme inhibitors are not absorbed, then nutrition, or the digestion of protein, may be disturbed [290]. If the enzyme inhibitors are absorbed, it may lead to systemic intoxication [291].

A novel strategy was developed in which the enzyme inhibitors (Bowman–Birk inhibitor and elastinal) were used as covalently attached inhibitors (Bowman–Birk inhibitor–carboxymethylcellulose and carboxymethylcellulose–elastinal). These were used as effective inhibitors in an oral insulin formulation and were focused only on drug delivery systems. They apparently did not cause any damage to intestinal cellular components. The result was a record decrease in blood glucose levels for more than 72 hour [292].

Tozaki and his colleagues used azopolymer-coated pellets containing 12.5 international units of insulin and camostat mesilate (a protease inhibitor) to produce a decrease in plasma glucose levels in rats [293]. Other scientists studied the *in-vitro* oral insulin delivery approach, using chicken ovomucoid and duck ovomucoid as enzyme inhibitors, to evaluate the preparation technique and dissolution stability of the polyethylacrylate-based microparticulate delivery system of insulin [294].

The glycoprotein ovomucoid from duck egg white was used later to immobilize the peptide drug (probably insulin) in a polymeric hydrogel formulation. Glycoprotein ovomucoid prevents the degradation of peptide drugs (insulin) by the proteolytic enzymes in the GIT

[295]. Finally, a double-liposomal formulation containing insulin and aprotinin effectively reduced plasma glucose levels in normal rats [296].

The results from *in situ* closed small and large intestinal loops in rats suggest that protease inhibitors could increase insulin absorption more effectively in the large intestine than in the small intestine. The protease inhibitors that improved insulin absorption in the intestine were, in order of increasing improvement, leupeptin, sodium glycocholate, bacitracin, bestatin and cystatin [297].

Side effects such as systemic intoxication and disturbed digestion of food proteins are regularly associated with the use of insulin with enzyme inhibitors. These side effects have prompted caution in the use of enzyme inhibitors in oral insulin formulations. The abovementioned side effects can be reversed, however, if oral insulin formulations are designed to contain an inhibitor that releases insulin and an inhibiting agent at the same time and limit its action to a certain area within the intestine.

1.11.2 Absorption enhancers and promoters:

As early as the 1970s, nonionic surfactant cetomacrogols were successfully used to enhance insulin's membrane permeability in rats. The formulation was injected directly into diabetic rats' jejunums [298]. In an early attempt to achieve success with enhancers, a combination of Brij® (fatty acid–polyoxyalkylene fatty ether) and stearic acid was used to improve the bioavailability of an insulin formulation [299]. Later in the mid-1980s, studies by Touitou and Rubinstein further supported using polymeric-coated gelatin capsules containing sodium laurate in an oily medium to enhance insulin absorption [300]. The scientists published their results after applying for a US patent in 1985 based on similar findings. Their study arguably represents the earliest success by any group using absorption enhancers and promoters.

Another study showed that the nonionic Brij® surfactant–hydroxypropyl cellulose system was not effective in reducing plasma glucose levels in rabbits when compared with the salicylate–cellulose system. Two absorption promoters (Brij® 35 and sodium salicylate) and two carriers (cellulose and hydroxypropyl cellulose) were used in the study [301].

In a separate study, sodium 5-methoxysalicylic acid was found to reduce plasma glucose levels in rats [302].

Bile salts have been used to influence the absorption of insulin through palmitic acid emulsion formulations. Nishikata *et al.* reported that the addition of different bile salts could enhance the reduction of plasma glucose levels, depending upon the type of bile salt used in the insulin–palmitic acid emulsion formulation [303].

In another study, soft gelatin capsules containing insulin and sodium salicylate as absorption enhancers were coated with a mixture of various Eudragit grades for use in rats. The administration of capsules significantly increased the hypoglycemic effect in rats [304]. An attempt was made to modulate the epithelial structures by introducing zonula occludens toxin (ZOT) as an enhancer in an oral insulin formulation. ZOT produced a multifold increase in intestinal permeability and reduced plasma glucose levels in diabetic rats [305,306].

The author of a review article about the use of absorption-enhancing agents elaborated on the positive outcome of using such agents with insulin for oral administration. The article discussed significant morphological changes in the cellular components of the gastrointestinal epithelium. The author also stressed that the study's limited success resulted from the use of a particular agent and its effect on the cell's morphology [307]

Studies of several low-molecular-weight absorption enhancers, including various bile salts, sodium salicylate and ZOT, produced positive results. Although these agents sometimes damage intestinal tissue, one study confirmed that the damaging effect of bile acids does not occur consistently [308]. Furthermore, studies to ensure the safe use of bile acids and other acids as absorption enhancers in different types of insulin formulations have not yet been completed.

A study conducted in Japan involving *in situ* intestinal experiments on rats indicates that a water-in-oil-in-water emulsion containing docosahexaenoic acid may improve insulin absorption without causing any serious damage to the intestinal epithelial cells [309].

Few studies discussed in this review article have raised questions about the toxicity and long-term clinical application of permeation enhancers in oral insulin formulations. Surface-acting agents act as permeation enhancers by causing exfoliation of the intestinal epithelium and may compromise its barrier functions [310].

Eley and Triumalashetty evaluated the suitability of several alkylglycosides as permeability enhancers. The study proved that most alkylglycosides are unsuitable because they cause varying degrees of cell membrane component solubilization. The cell membranes were badly disrupted and unable to recover fully all cell components above the concentration range of 0.01–0.1% used in the study [311].

One study conducted in 1996 tested sodium glycocholate (GC-Na), sodium taurocholate (TC-Na), sodium deoxycholate, EDTA, sodium salicylate, sodium caprate (Cap-Na), N-lauryl-beta-D-maltopyranoside (LM), diethyl maleate and mixed micelles as absorption enhancers at a concentration of 20 mM to determine whether they caused intestinal damage in rats. Only GC-Na, TC-Na and LM, which have low levels of toxicity at that concentration, promoted the absorption of phenol red and were effective absorption enhancers [312].

Intestinal *in situ* experiments also were conducted in rats using Labrasol (Gattefosse, NJ), a novel emulsifier and oral enhancing agent and insulin. The results showed an increase in insulin absorption resulting from the enhancing effect of Labrasol® [313].

A study published by Hosny *et al.* proved that sodium salicylate is a successful absorption promoter that reduced plasma glucose levels in diabetic dogs [314]

A chemical agent used in an oral insulin formulation to enhance or promote intestinal permeability should be nontoxic. Although such an agent may greatly increase intestinal mucosal permeability in *in-vitro* and *in situ* animal studies, the increase does not ensure improved the oral bioavailability of a drug. Moreover, although chemical agents that enhance or promote intestinal permeability should be nontoxic, they may damage intestinal mucosa or the epithelium.

One of the main drawbacks of using permeation enhancers is that they attack the lipid structure's surface. An essential criterion is knowledge of the mechanism of absorption enhancement. The results described previously indicate the importance of optimizing the amount of absorption-enhancing agents in oral insulin formulation development in animal models. So far, not much data from studies of absorption enhancers and promoters in Humans exist.

1.11.3 Immobilization of inhibitors:

Polymer inhibitor conjugates, carboxymethylcellulose– Bowman Birk inhibitor (CMC–BBI) and carboxymethylcellulose–elastinal (CMC-Ela), have been shown to offer in-vitro protection against trypsin, chymotrypsin and elastase [314] These conjugates when combined with a polycarbophil–cysteine (PCP Cys) conjugate demonstrated a 20–40% reduction in basal glucose levels for more than 80 h [315].However, in these studies, questions remain regarding the amount of insulin absorbed and the factors affecting the long term decrease in glucose level.

1.11.4 Use of polymer systems alone:

Both nondegradable and biodegradable polymers by themselves have been used to prepare nanospheres [316-319] that were intended for intact uptake by the GIT. This approach is supported by literature that states microspheres in the nanosize range are absorbed intact by the intestinal epithelium and travel to sites such as the liver, the spleen and other tissues [320,321]. If the nanospheres accumulate in the liver, they could act as minor depots of insulin. The extended release of insulin could decrease the elevated hepatic glucose production in diabetic patients. A nondegradable system consisting of microspheres of isobutyl 2 cyanoacrylate loaded with insulin at 25 IU/kg when administered to streptozotocin-induced diabetic rats produced a 50% reduction in the fasted serum glucose level compared with a 60% reduction in serum glucose when the same dose was administered by subcutaneous route [322]. Results showed that the same microspheres were not effective when administered in fed rats [323]. The use of biodegradable microspheres is proposed to circumvent the possible accumulation of nondegradable microspheres in the tissues that may lead to harmful effects. A biodegradable system consisting of a mixture of poly (fumaric anhydride) and poly (lactide-co-glycolide) was used to prepare nanospheres (<1 μm) of insulin. Results showed that, upon administration to rats, the nanospheres crossed the intestinal tract intact and appeared in the liver. When these nanospheres were given to fasted rats concurrently with a glucose load, a decrease in plasma concentrations was not observed for as long as 5.5 h [324]. Insulin delivery with polymeric devises and inter-polymer complexes of the graft copolymers has been summarised in detail to improve oral absorption of insulin [325].

1.11.5 Intestinal transport of insulin:

Evidence of active transport for insulin was negative [326]. Morpho-cytochemical and biochemical evidence for insulin absorption was demonstrated in rat GIT [327]. This result was achieved by direct instillation of a solution of insulin into various parts of the GIT, followed by visualization visualization with gold markers and immunoassay of the insulin in blood. No evidence exists for the transport of insulin by the paracellular route. Researchers found that insulin is adsorbed to the apical plasma membrane and is internalized by endocytosis. It then reaches the basolateral plasma membrane via the endosomal pathway of small vesicles and is secreted into the interstitial space. Whether the internalization is a result of the presence of insulin receptors on the surface of the epithelial cells is unclear. The presence of insulin receptors has been demonstrated in enterocytes on both the apical and basolateral sides.

1.11.6 Dosage form stability issues:

The activity of proteins depends on the three-dimensional molecular structure. The dosage form development of proteins may expose the proteins to harsh conditions that may alter their structure. This will have implications in the efficacy and immunogenic response to the proteins. During dosage form development, proteins might be subjected to physical and chemical degradation. Physical degradation involves modification of the native structure of a protein to a higher-order structure, which may be a result of adsorption, aggregation, unfolding, or precipitation. Chemical degradation usually involves bond cleavage and leads to the formation of a new product. Chemical degradation is preceded by a physical process such as unfolding, which exposes the hidden residues to chemical reactions. The processes involved in chemical degradation are deamidation, oxidation, disulfide exchange and hydrolysis. The stability of insulin preparations has been documented in detail [328]. and research data on the solid-state stability of proteins in dosage forms have been reviewed recently [329]. Proteins must be characterized for change in conformation, size, shape, surface properties and bioactivity upon formulation processing. Changes in conformation, size and shape can be observed by the use of spectrophotometric techniques, X-ray diffraction, differential scanning calorimetry, light scattering, electrophoresis, ultracentrifugation and gel filtration. Changes in surface properties can be detected with the use of electrophoretic and chromatographic techniques and changes in the bioactivity of

proteins can be observed by bioavailability studies. Selection of a particular technique is based on the sensitivity of the technique, the system under study and the availability of equipment. The interference by formulation excipients also may be a factor when selecting the characterization technique. Theory about selected techniques used for the characterization of proteins has been reviewed [330,331]. Some examples of characterization of insulin are discussed in the following paragraphs. Size-exclusion chromatography with reversed phase–high performance liquid chromatography (HPLC) was used to determine the formation of covalent insulin dimers with trace amounts of high molecular weight transformation products after microencapsulating insulin in a mixture of poly(DL-lactide-co-glycolide) and poly (L-lactide) [332]. Differential scanning calorimetry was used to differentiate denaturation endotherms of amorphous and crystalline insulin [333]. X-ray diffractograms of insulin have been obtained with mixtures of lactose and mannitol to evaluate the effect of spray-drying on the crystalline changes of insulin [334].

1.11.7 Patented technologies for oral insulin delivery:

There appears to be few patented technologies extensively studied but still yet to find the ways to a commercial product for insulin oral delivery. Patented technologies and corresponding details are summarized Table 1.1.

Table 1.1: List of patented technologies for oral insulin delivery [65]

Technology	Comments	Source
Capsulin™	The Capuslin delivery technologies are based on a mixture of permeation enhancers and solubilizers that can be generally regarded as safe.	www.diabelogy.co.uk
Eligen™	For this technology, a multitude of data demonstrating the efficacy for oral peptide delivery is available. Eligen technology can be regarded as safe.	www.emisphere.com
Nobex™	Conjugated drug–polymer molecules are created by attaching low-molecular weight polymers at specific sites. Biocon uses Nobex technology in its oral insulin programme.	www.biocon.com
Orade™	Insulin is protected from the harsh environment in the stomach and its permeation is improved by small molecule transporters	www.apollolifesciences.com
Oramed Technology	Oramed announced the start of its phase I clinical trial for oral insulin in 2007.	www.aramedpharma.com
Thiomers™	Thiomers are polymers that display thiol groups. Such modifications lead to an improvement of the muco-adhesive, cohesive and permeation-enhancing properties.	www.thiomatrix.com

1.12 Conclusion:

Delivering protein and peptides by oral route is extremely challenging. The very nature of the digestive system is designed to break down these polypepides into amino acids prior to absorption. A variety of approaches have emerged in the recent past for designing oral delivery systems for therapeutic proteins and peptides, though a clinically viable solution to

this long standing problem still alludes the scientific community. These approaches come from such diverse research disciplines as biomaterials, conjugation chemistry, nanotechnology, cell biology and employ different methodologies [49] for solving the same problem. Some of these strategies have distinguishing beneficial characteristics that make them good candidates for oral protein delivery. Interfacing different strategies to combine the benefits of novel approaches is an interesting new possibility. Despite extensive research in this area including emergence of many different patented technologies, a successful oral delivery of proteins and peptides especially for large macromolecules like insulin is yet to see the successful commercial product.

1.13 Objectives of the present research and development endeavor:

Insulin is a protein of immense importance because of its role in the treatment of diabetes. Oral route of administration remains to be a preferred route of administration due to ease, cost and effectiveness of oral dosage forms. Oral administration is also linked to increase in patient compliance and overall increase in efficacy of the treatment. However instability of insulin in the gastrointestinal tract, in the formulations and inability to transverse the intestinal barrier severely reduce the fraction of orally administered insulin that reaches the blood stream (bioavailability). The challenges in developing oral protein formulations largely remain unaddressed and a universally acceptable solution to these challenges still eludes the scientific community. Some significant advances have been made in the recent past, which has led to better ways of addressing these challenges of oral protein delivery.

The objective of this work was to study different approaches to evaluate improvement in the stability and oral absorption of insulin. Better oral delivery systems for insulin were envisaged, developed and evaluated based on following principles.

(a) Stabilization of insulin in oral delivery formulations.
(b) Improvement over degradation of insulin in the presence of proteolytic enzymes.
(c) Design of hydrophobic delivery systems to enhance oral absorption.

The overall goal of this work was to evaluate the effectiveness of hydrophobic delivery systems in addressing some key problems in oral protein delivery and to design a system

based on integrating the hydrophobic delivery systems with other effective strategies for oral protein delivery. The specific goals of this research are outlined below:

(l) Design and development of hydrophobic nanoparticulate delivery system containing stearic acid, α-tocopherol acetate and soya phosphatidyl choline (SPC) in presence of stabilizers such as zinc and hydroxypropyl β cyclodextrin to improve the stability and oral bioavailability of insulin.

(2) Design and development of self microemulsifying delivery system containing dispersions of Human insulin with egg yolk to improve the stability and oral bioavailability of insulin.

(3) Design and development of self microemulsifying delivery system containing dispersions of Human insulin with sodium caseinate to improve the stability and oral bioavailability of insulin.

In all the approaches formulations were designed and optimized by varying the key components for each of the delivery systems. Also formulations were evaluated for in-vitro stability of insulin in presence of proteolytic enzyme. In-vivo studies in streptozotocin induced diabetic rats were performed to evaluate oral absorption of Human insulin and antidiabetic effect of various formulations developed. All the animal experiments were carried out with approval of Animal Ethics Committee.

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CHAPTER 2

DRUG PROFILE – HUMAN INSULIN

2.0 DRUG PROFILE:

2.1 Insulin:

Insulin is a hormone produced by the beta cells of the islets of Langerhans of the pancreas and consists of 2 chains of amino acids, the A and B chains, connected by 2 disulfide bridges. The precursor of insulin in the pancreas is proinsulin which is a single polypeptide chain incorporating both the A and B chains of insulin connected by a peptide termed the C-peptide (or connecting-peptide).

The commercially available insulins are bovine, porcine or Human. The amino acid sequence of bovine insulin differs from Human insulin with three substitutions of alanine, valine and alanine at positions 30, 10 and 8 of the B chain, whereas porcine insulin differs from Human insulin by one amino acid only —alanine instead of threonine at position 30, This site is not a very critical one concerned with the binding of insulin receptor [1]. Structural changes between insulins from different species contribute to their antigenic potential in man. Thus bovine insulin is more immunogenic than porcine insulin.

Small amounts of Human insulin can be extracted from cadaveric pancreas or by a process of chemical synthesis. But these are not suitable for large scale production. Thus commercial insulin is prepared by two methods. Semisynthetic insulin (commonly known as emp insulin-enzyme modified porcine) is produced by substitution of alanine residue of porcine insulin. The other method utilizes the recombinant DNA technology (crb insulin—chain, recombinant DNA, bacteria). The genes coding for A and B subunits of the Human insulin molecule are linked via a methionine codon to an E. coli plasmid which produces the coded insulin in large quantities, The subunits are extracted and split from its chimeric position by cyanogen bromide. The separated chains are sulfated, purified and combined to form insulin molecules which are further purified [1].

Human insulin is less immunogenic (produces less circulating anti-insulin antibodies). Fineberg et al in a study conducted in adults showed that detectable insulin antibodies were present in only 44% of patients receiving Human insulin compared to 60% using purified porcine insulin [2]. Similar observations have been shown in children with type 1 diabetes mellitus [3]. Several studies have indicated no detectable change in antibody concentration on switching from porcine to Human insulin or vice versa [4,5,6].

2.2 Characteristics of insulin:

Insulins are categorized by differences in: onset (how quickly they act), peak (how long it takes to achieve maximum impact), duration (how long they last), and route of delivery (subcutaneous or intravenous) [7].

There are three main groups of insulins: Fast-acting, Intermediate-acting and Long-acting insulin.

2.2.1 Fast acting insulin:

Fast acting insulins are absorbed quickly from your fat tissue (subcutaneous) into the bloodstream to control the blood sugar during meals and snacks and to correct high blood sugars. It includes,

2.2.1.1 Rapid acting insulin analogs:

Insulin aspart, insulin lispro and insulin glulisine have an onset of action of 5 to 15 min, peak effect in 1 to 2 hour and duration of action that lasts 4 to 6 hour. With all doses, large and small, the onset of action and the time to peak effect is similar, The duration of insulin action is, however, affected by the dose – so a few units may last 4 hour or less, while 25 or 30 units may last 5 to 6 hour. As a general rule, assume that these insulins have duration of action of 4 hour [8].

Insulin aspart (marketed by Novo Nordisk as "NovoLog/NovoRapid") is a fast acting insulin analog with aspartic acid substituted for proline at position B28 [9-12]. It has the empirical formula $C_{256}H_{387}N_{65}O_{79}S_6$ and a molecular weight of 5831. This analogue has increased charge repulsion, which prevents the formation of hexamers, to create a faster acting insulin. The sequence was inserted into the yeast genome and the yeast expressed the insulin analogue, which was then harvested from a bioreactor. Insulin aspart is used in CSII pumps, Flexpen and Novopen delivery devices for subcutaneous injection [13]. It is also used immediately before meals and controls postprandial blood glucose concentrations at least as well as regular Human insulin and may cause fewer hypoglycaemic episodes. A meta-

analysis involving 42 studies of insulin lispro or insulin aspart versus regular insulin found that there was evidence of a minor benefit of the analogues in improving HbA_{1c} values in adult patients with type 1 diabetes; no superiority could be shown in patients with type 2 diabetes[14].

Insulin lispro (marketed by Lilly as "Humalog") is a first insulin analogue in which the B28 and B29 amino acid residues of Human insulin are replaced with lysine and proline. This modification does not alter receptor binding, but blocks the formation of insulin dimer and hexamers. This allowed larger amounts of active monomeric insulin to be available for postprandial (after meal) injections. It has the empirical formula $C_{257}H_{389}N_{65}O_{77}S_6$ and a molecular weight of 5813. It is available as a rapidly acting alternative to soluble insulin and as an intermediate-acting complex with protamine [15-20].

Insulin glulisine is another insulin analogue, with asparagine at position B3 replaced by lysine and lysine at B29 replaced by glutamic acid. Chemically, it is 3B-lysine-29B-glutamic acid-Human insulin, has the empirical formula $C_{258}H_{384}N_{64}O_{78}S_6$ and a molecular weight of 5823. It was developed by Sanofi-Aventis and sold under the trade name Apidra®. It also has a rapid onset and short duration of action [21]

2.2.1.2 Regular Human insulin:

Regular Human insulin (Actrapid® and Humulin®) which has an onset of action of 1/2 hour to 1 hour, peak effect in 2 to 4 hour and duration of action of 6 to 8 hour. Larger the dose of regular insulin, faster the onset of action, but longer the time to peak effect and longer the duration of the effect [22].

2.2.2 Intermediate-acting insulin:

Intermediate acting insulin looks cloudy. They have either protamine or zinc added to delay their action. They are absorbed more slowly and lasts longer and mainly used to control the blood sugar overnight, while fasting and in between meals. It includes,

2.2.2.1 NPH Human insulin:

Isophane-porcine-Human insulin (Humulin® NPH) has an onset of insulin effect after 1 to 2 hour, a peak effect after 4 to 6 hour and duration of action for more than 12 hour. Very small doses will have an early peak effect and shorter duration of action, while higher doses will have a longer time to peak effect and prolonged duration [23].

2.2.2.2 Pre-mixed insulin:

Mixed insulin (NovoMix®30, Humalog® Mix 25, Mixtard® 30/70, Mixtard® 20/80) is cloudy in appearance. It is a combination of either rapid onset-fast acting insulin or a short acting insulin and intermediate acting insulin. Advantage of it is that, two types of insulin can be given in one injection. When it shows 30/70 then it means 30% of short acting insulin is mixed with 70% of intermediate acting insulin [24].

2.2.3 Long-acting insulin:

Long acting insulins is absorbed slowly, has a minimal peak effect and a stable plateau effect that lasts most of the day and used to control the blood sugar overnight, while fasting and between meals. It includes,

2.2.3.1 Long acting insulin analogs:

Insulin glargine and insulin detemir have an onset of insulin effect in 1.5-2 hour. The insulin effect plateaus over the next few hour and is followed by a relatively flat duration of action that lasts 12-24 hour for insulin detemir and 24 hour for insulin glargine [8].

Insulin glargine, marketed by Sanofi-Aventis under the name Lantus®, is a long-acting basal insulin analog, usually given once or twice daily. Insulin glargine has the empirical formula $C_{267}H_{408}N_{72}O_{77}S_6$ and a molecular weight of 6063. It is available as a solution at pH 4; on subcutaneous injection and neutralisation by tissue buffering processes, microprecipitates are formed that slowly release insulin glargine over 24 hour with no pronounced peak in concentration or in metabolic activity. Controlled studies have reported insulin glargine to be more effective than Human isophane insulin in producing glycaemic

control as part of a basal-bolus regimen and to be associated with fewer hypoglycaemic episodes [25-29].

Insulin detemir (NN-304) is another long-acting insulin analogue maketed by Novo Nordisk (Levemir®). It is an insulin analogue in which fatty acid (myristic acid) is bound to lysine amino acid at position B29. Insulin detemir has the empirical formula $C_{267}H_{402}N_{64}O_{76}S_6$ and a molecular weight of 5913. It is quickly resorbed after which in the blood it binds to albumin through the fat acid at position B29. This allows insulin detemir to bind reversibly to albumin, producing slow absorption and a prolonged and consistent metabolic effect for up to 24 hour. It appears to be at least as effective as isophane insulin in maintaining overall glycaemic control but with less intra-patient variability, a similar or lower risk of hypoglycaemia and less body-weight gain [30,31].

2.3 Insulin Human:

2.3.1 Chemical structure:

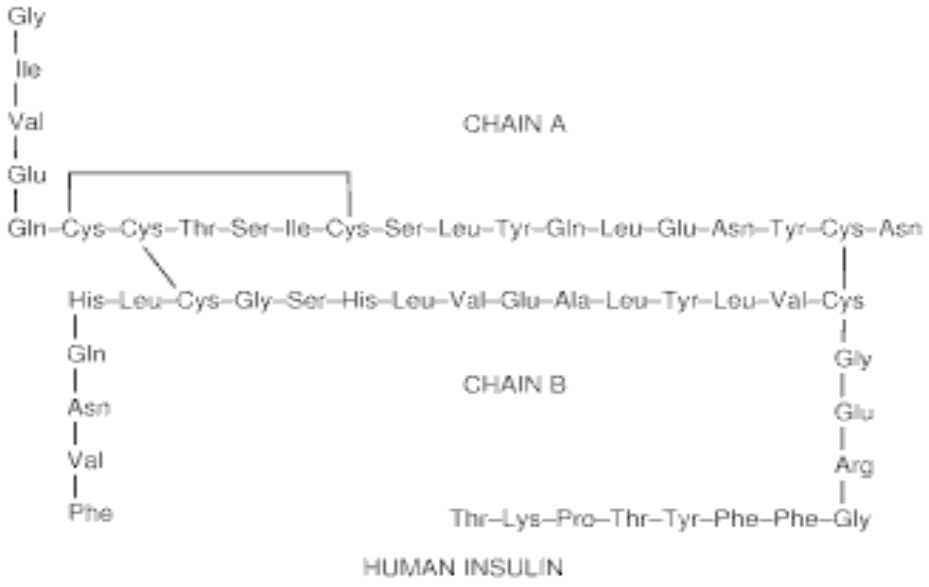

Figure 2.1: Schematic structure of Human insulin [32]

Human insulin consists of two chains, A and B, of amino acids, joined together by two disulphide bonds. The schematic structure of Human insulin is presented in Figure 2.1. Insulin is synthesised from a single chain precursor named proinsulin. On conversion of

Human proinsulin to insulin, 4 basic amino acids and the remaining connector or C peptide is removed by proteolysis. The resultant insulin molecule has 2 chains. The acidic or A-chain with glycine at the amino terminal residue and the basic or B-chain consisting of 30 amino acids with phenylalanine at the amino terminus [32]. Human insulin differs from bovine insulin at positions A8, A10 and B30, where it has threonine, isoleucine and threonine, respectively.

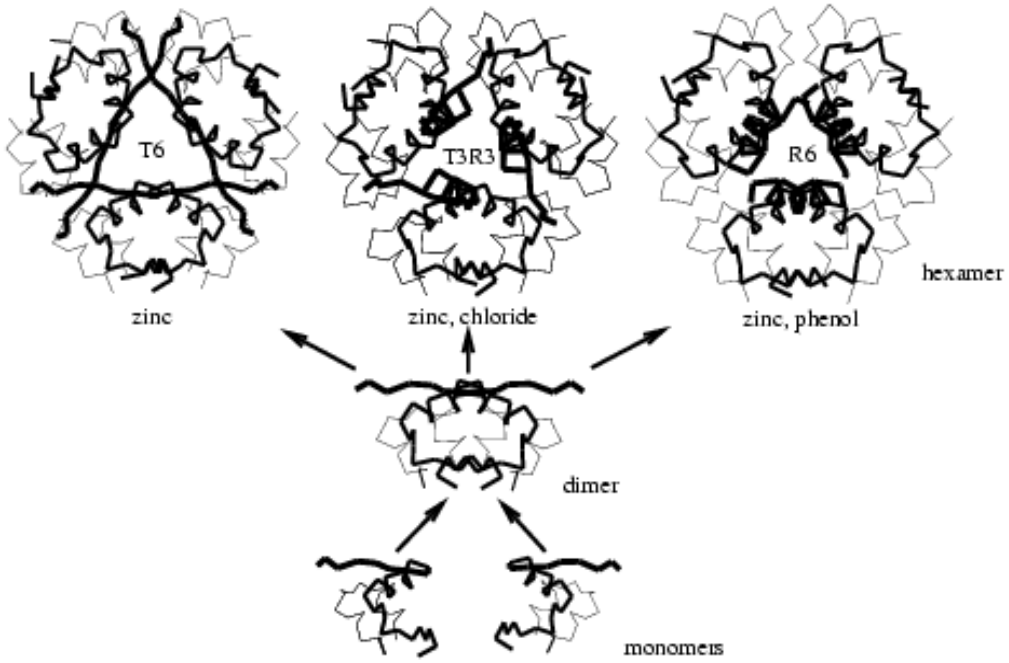

Figure 2.2: Aggregation of insulin in various conformations [33]

In a solution free of metal ions, insulin exists as a mixture of monomer, dimer, tetramer, hexamer and higher aggregates, proportions depending on the concentration [33]. The various aggregation confirmations of insulin are depicted in Figure 2.2. In the presence of zinc ions, as in the B-cells of the Islets of Langerhans, the hexamer species prevails. It has been found that other divalent metal ions (e.g. Ni^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+}) can play the same role in the hexamerization of insulin [34,35]. The insulin hexamer is stable in aqueous solution between pH 5 and 8. Because both polar and non-polar residues are buried between the dimers, the packing of dimers into a hexamer is much looser than that of monomers into a dimer. Six B13 glutamic acid residues come close together in the core of the hexamer, causing electrostatic repulsion which must be reduced in order for hexamerization to take place. The coordination of zinc in the core balances the charges and is the driving force in

hexamer formation in insulin [36]. The biologically active form of the hormone is the monomer.

2.3.2 Physicochemical properties of Human insulin:

Description: White or almost white crystalline powder.

Molecular formula: $C_{257}H_{383}N_{65}O_{77}S_{6.}$

Molecular weight: 5807.6

Isoelectric pH: 5.3 – 5.35 [37].

Solubility: Slightly soluble in water. Soluble in dilute solution of mineral acids and with degradation in solutions of alkali hydroxide. Practically insoluble in alcohol, chloroform and ether. [32,38,39].

Potency: Its potency is calculated on the dried basis, is not less than 27.5 USP Insulin Human Units in each mg. One USP Insulin Human Units is equivalent to 0.0347 mg of pure Insulin Human [32].

Purity: The proinsulin content of Insulin Human derived from pork, is not more than 10 ppm. The host cell derived proteins content of Insulin Human derived from a recombinant DNA process is not more than 10 ppm [32].

Category: Antidiabetic agent [32]

Insulin function: The actions of insulin are a) membrane transport of glucose, amino acids and certain ions, b) increased storage of glycogen, c) formation of triglycerides and d) stimulation of DNA, RNA and protein synthesis [40]

Packaging and storage: Insulin is stored at -10°C to -25°C in tight container, protected from light [32].

2.3.3 Identification (USP 2008):

The retention time of the major peak in the chromatogram of the Assay preparation corresponds to that in the chromatogram of Standard preparation, as obtained in the assay [32].

2.3.4 Official methods of analysis:

Pharmacopoeias (United State Pharmacopoeia, 2008, European Pharmacopoeia, 2009) have reported liquid chromatographic methods for the analysis of Human insulin in pure form and in pharmaceutical formulations. The method recommend use of mobile phase of sodium phosphate buffer pH 2.3 : acetonitrile (74:26) at a flow rate of 1 ml/min., using 40°C column temperature, on 4.6 mm x 150 mm column with L1 packing and detection on 214 nm UV detector [32,41].

2.3.5 Indications:

a) Diabetes mellitus.
b) Complications of diabetes mellitus, e.g. hyperglycemic ketoacidotic coma and hyperglycemic hyperosmolar non-ketotic coma.
c) Hyperkalaemia.
d) Insulin hypoglycemia can be used as a test of anterior pituitary function and to test completeness of vagotomy in reducing gastric secretion. [42]

2.3.6 Pharmacokinetics:

2.3.6.1 Absorption by route of exposure:

Insulin must be injected SC, IM or IV. It is absorbed into the blood and peak plasma insulin concentration with subcutaneous insulin occurs at 60 - 90 min. Absorption is slower if there is peripheral vascular disease or smoking and faster if the patient is vasodilated, e.g. by a hot bath or ultraviolet exposure or exercise.

Any changes in mode of administration either accidentally (e.g. accidental IM or IV injection) or deliberately (e.g. constant subcutaneous insulin infusion) may potentiate the

absorption and action of insulin, leading to hypoglycaemia. Severe hypoglycaemia may occur during constant infusion and several deaths have been reported [43].

Absorption of insulin from injection site affected by insulin lipodystrophy is very unpredictable and rapid absorption may lead to hypoglycaemia.

2.3.6.2 Distribution:

A fraction of endogenous or exogenous insulin in plasma may be associated with certain proteins but the bulk appears to circulate in blood and lymph as the free hormone. The volume of distribution of insulin approximates the volume of extracellular fluid. Insulin is inactivated in the liver and kidneys (about 40% in a single passage). About 10% appear in the urine [44].

2.3.6.3 Biological half-life by route of exposure:

The plasma half-life is 10 minutes for intravenous injection, 4 hour for subcutaneous injection and 2 hour for intramuscular injection [44].

2.3.6.4 Metabolism:

Metabolism occurs mainly in the liver and kidneys; 10% of the dose appears in the urine. Insulin is normally filtered at the glomeruli and then completely reabsorbed or destroyed at the proximal tubule. In patients with impaired renal tubular function, urinary clearance approaches glomerular filtration rates. 50% of insulin that reaches the liver via the portal vein is destroyed in a single passage, never reaching the general circulation. Proteolytic degradation of insulin occurs both at cell surfaces and in the lysosomes. A proteolytic enzyme that degrades insulin has been purified from muscle. An enzyme, glutathione insulin trans-hydrogenase, which utilises reduced glutathione to reduce disulfide bridges of insulin and produce separate chains, has been implicated [44].

Severe impairment of renal function appears to affect the rate of disappearance of circulating insulin to a greater extent.

2.3.6.5 Elimination:

About 10% of the drug appears in urine.

2.3.7 Toxicodynamics:

Insulin in overdose causes hypoglycaemia. Hypoglycaemia deprives the brain of substrate glucose upon which it is almost exclusively dependent for its oxidative metabolism. During insulin coma, oxygen consumption in the Human brain decreases by nearly half.

A prolonged period of hypoglycaemia causes irreversible damage to the brain as evidenced in experimental animals by histological changes in the cortex, basal ganglia and rostral parts of the medulla. Convulsions, coma, mental retardation, hemiparesis, ataxia, incontinence, aphasia, choreiform movements and parkinsonism may occur in man [42].

2.3.8 Pharmacodynamics:

Insulin binds to a receptor on the surface of the target cell and probably also enters the cell in this state. The receptors vary in number inversely with the insulin concentration to which they are exposed [45].

The receptor becomes phosphorylated on addition of insulin and ATP. The cellular mechanism of action of insulin after combination with the receptor is uncertain; the complex may activate a "second messenger" which in turn causes the release of third messenger Ca^{++} ions. Insulin also has a membrane effect in increasing glucose uptake and utilization, especially by muscle and adipose tissue. Its effects include the following [45]:

a) Reduction in blood sugar due to increased glucose uptake in the peripheral tissues which convert it to glycogen or fat and reduction of hepatic output (diminished breakdown of glycogen and diminished gluconeogenesis). When blood glucose falls below renal threshold (180 mg/100 ml or 10 mmol/l) glycosuria ceases as does the osmotic diuresis of water and electrolytes. Polyuria and excessive thirst are thus

alleviated. If blood glucose falls much below normal levels, appetite is stimulated [45].

b) Other metabolic effects: Insulin stimulates the transit of amino acids and potassium into the cells. Insulin regulates utilization of carbohydrate and energy products and enhances protein synthesis [45].

c) Insulin increases concentrations of the active form of the enzyme pyruvate dehydrogenase. Hence pyruvate is oxidized or converted to fat and is unavailable for glucose formation. In addition to enhanced synthesis of fat, insulin increases the activity of membrane bound lipoprotein lipase which makes fatty acids derived from circulating lipoproteins available to the cell [45].

2.3.9 Toxicity – Human data:

In most normal adults, 0.1 - 0.2 Units/kg intravenously is sufficient to cause profound hypoglycaemia. However, in insulin dependent diabetics, it is not possible to indicate the amount of insulin necessary to cause toxicity because the level of hypoglycaemia and its duration are the important factors. For example, patients who have taken 80 to 500 times of the normal dose taken for suicidal purposes have recovered [46].

2.3.10 Carcinogenicity:

Development of cancer at the site of long term insulin injection has been reported [47].

2.3.11 Teratogenicity:

Congenital malformations occurred in 17 of 117 babies born to diabetic mothers taking insulin at the time of conception [48].

2.3.12 Mutagenicity:

Human insulin is not mutagenic in bacterial or mammalian cells [49].

2.3.13 Interactions:

Alcohol, beta blockers, salicylates, oxytetracycline and monoamine oxidase inhibitors potentiate the hypoglycaemic effects of insulin. Bezafibrate and clofibrate may improve glucose tolerance and have an additive effect. Corticosteroids, corticotrophin, diazoxide, diuretics like bumetanide, furosemide and thiazides and oral contraceptives antagonise the effects of insulin. Lithium may occasionally impair glucose tolerance.

2.3.14 Main adverse effects:

a) Hypoglycaemia - symptoms are directly related to duration and depth of hypoglycaemia. Initial sympathetic over activity is followed by signs of neuroglycopenia.
b) Non specific local reactions at site of injection e.g. pain, oedema.
c) Allergic reactions.
d) Lipoatrophy or induration and hypertrophy at the site of injection are associated with chronic use.
e) Insulin resistance [42].

2.4 Insulin formulations:

Insulin formulations having pharmacopoeial monograph are summarized below,

2.4.1 Insulin Injection:

This may be prepared by dissolving crystalline insulin containing not less than 23 units/mg in water for injection containing a suitable substance to render the injection iso-osmotic with blood; hydrochloric acid to adjust the pH to 3 to 3.5; and a suitable bactericide. The USP specifies sterile, acidified or neutral solution of insulin USP containing 40, 80, 100 or 500 units per ml as well as 1.4 - 1.8% w/v of glycerol and 0.1 - 0.25% (w/v) of phenol or cresol. pH of acidified injection is 2.5 - 3.5 and pH of neutral injection is 7.0 - 7.8 [32,42].

Insulin injection is a colourless injection or straw coloured liquid practically free from solid matter which deposits on standing. Insulin injection contains not more than 40 μg zinc/100 units of insulin. Sterilised by filtration and kept in multidose containers [42].

2.4.2 Neutral insulin BP:

Sterile buffered solution of bovine or porcine insulin of potency not less than 23 units/mg; pH 6.6 – 8, colourless liquid. It contains not more than 20 μg zinc/100 units of insulin and a suitable bactericide. Available in multidose containers [42].

2.4.3 Insulin Zinc Suspension BP:

Sterile buffered suspension of mammalian insulin in the form of a complex obtained by addition of zinc chloride. Insulin is in a form insoluble in water. Prepared by mixing 3 volumes of insulin zinc suspension (amorphous) and 7 volumes of insulin zinc suspension (crystalline). It contains 40, 80, or 100 units/ml of insulin. White suspension is available in multidose containers, pH 6.9 - 7.5. Complies with a test for prolongation of insulin effect [42].

2.4.4 Insulin Zinc Suspension BP (Amorphous):

Sterile buffered suspension of mammalian insulin in the form of a complex obtained by addition of zinc chloride. It is prepared from crystalline insulin containing not less than 23 units and is almost colourless suspension in which the particles have no uniform shape and rarely exceed 2 μm in dimension. Suspension has pH of 6.9-7.5 and is iso-osmotic with blood. The preparation contains 40 and 80 units/ml [42].

2.4.5 Insulin zinc suspension (crystalline) BP:

Sterile buffered suspension of bovine insulin to which zinc chloride is added. Crystalline form is insoluble in water. Prepared from crystalline insulin containing not more than 23 units/mg of insulin. White or almost colourless suspension. Particles are mainly crystalline. Majority of crystals have a maximum diameter greater than 10 μm. Suspension has pH 6.9 - 7.5 and is iso-osmotic with blood. Preparation contains 40 and 80 units/ml [42].

2.4.6 USP - Sterile suspension of insulin:

USP describes a sterile suspension of insulin USP containing suitable bactericide. The suspension contains insulin 40, 80 or 100, sodium acetate 0.15 - 0.17%, sodium chloride 0.65 - 0.75%, methyl hydroxy benzoate 0.09-0.11% and 120 - 250 μg of zinc for each 100 units of insulin, pH 7.2-7.5 [32,42].

2.4.7 Biphasic Insulin BP:

Sterile buffered suspension of crystals of insulin containing not less than 23 units/mg in a solution of insulin of similar potency. It is a white suspension having pH 6.6-7.2 and iso-osmotic with blood. It contains 27.5 - 37.5 μg zinc for each 100 units of insulin and quarter of the total insulin is in soluble form. It is available in multidose glass container [42].

2.4.8 Globin zinc Insulin BP:

Sterile preparation of mammalian insulin in the form of a complex obtained by addition of suitable globin and zinc chloride [42].

USP specification: Insulin modified by addition of zinc and globin obtained from beef blood, 40, 80, 100 units/ml. It is colourless liquid with pH 3-3.8 and iso-osmotic with blood. Each 100 units of insulin also contain 3.6–4 mg of globin and 250-350 μg zinc. Also contains phenol, glycerol and cresol and is available in multidose container [32].

2.4.9 Isophane Insulin:

Sterile buffered suspension of insulin in the form of a complex obtained by addition of suitable protamine. It is prepared from crystalline insulin and contains for each 100 units of insulin, 300-600 μg protamine sulphate and not more than 40 μg zinc, a suitable bactericide and sodium phosphate as buffering agent. Suspension has a pH of 6.9-7.5 and is iso-osmotic with blood [42].

USP specification: Sterile suspension of zinc insulin crystalline and protamine sulphate in buffered water for injection. Solid phase contain crystals of insulin protamine and zinc; 40, 80, 100 units/ml. It contains glycerol, meta-cresol, phenol sodium phosphate and zinc [32].

2.4.10 Protamine Zinc Insulin:

Sterile buffered suspension of mammalian insulin to which protamine and zinc chloride are added. White suspension having pH 6.9-7.5 and iso-osmotic with blood. It contains for each 100 units of insulin, 1-1.7 mg of protamine sulphate and zinc chloride equivalent to 200 μg of zinc, 10 -11 mg of sodium phosphate [42].

USP specification: Buffered sterile suspension to which zinc chloride and protamine sulphate are added. The preparation contains insulin 40, 80, 100 units/ml, glycerol, cresol, phenol, sodium phosphate for each 100 units of insulin in addition to protamine and zinc [32].

2.5 Therapeutic dosage (Insulin Injection):

Adults:
Blood sugar < 16.5 mmol/l (300 mg): 20 units.
Blood sugar 11 - 16.5 mmol/l (200 - 300 mg): 10 units [42]

Children:
Dose is adjusted according to the usual monitoring of blood and/or urine glucose. Daily dose increments should be 4 units. When stabilised, two-thirds of the daily dose is generally given 30 minutes before breakfast and one-third 30 minutes before the evening meal.

If only one injection per day is required, 10 - 14 units of intermediate-acting insulin can be given. Dose increment is 4 units given on alternate days. Soluble or neutral insulin may be added or special mixed insulins can be used according to the patient's response [42].

2.6 Stability and storage:

Both the Ph. Eur. 6.0 and the USP 31 recommend that insulin preparations be stored in a refrigerator at 2°C to 8°C and not be allowed to freeze. The Ph. Eur. 6.0 directs that insulin

preparations should be protected from light and the USP 31 that they should be protected from sunlight. It is recognized that patients may not follow such stringent storage guidelines and most manufacturers of commercial insulin preparations consider that storage by the patient at a temperature of up to 25°C would be acceptable for up to one month. Patients should still be advised not to expose their vials or cartridges to excessive heat or sunlight. It is advisable to shake suspensions gently before a dose is withdrawn [32,41].

Insulin in powder form should be stored in airtight containers and protected from light. Storage at a low temperature is also recommended. The Ph. Eur. 6.0 advises storage at a temperature of -20°C for bovine and porcine insulin and at -18°C or below for Human Insulin and for Insulin Aspart and Insulin Lispro; the USP 31 requires storage at -10°C to -25 °C for all types of insulin. [32,41,42,49].

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CHAPTER 3

DEVELOPMENT OF ANALYTICAL METHODS

3.1 INTRODUCTION:

Analysis is an important component of formulation development. Insulin being the most studied macromolecule, there exist an official monographs for Human insulin and its injectable preparations. The USP method reported in the monograph is based on isocratic elution using HPLC equipped with UV detector [1]. The liquid chromatograph equipped with a 214-nm detector and a L1 column of 4.6 mm x 15 cm. The isocratic system consists of mobile phase as a mixture of buffer pH 2.3 and acetonitrile (74:26). The column temperature is maintained at 40° and the flow rate is 1 ml per minute. However the USP chromatographic method for assay could not be applied as such for the intended project work due to different nature of the formulations studied. In the present study, the above reported USP method was modified to support various studies like, insulin entrapment efficiency, in-vitro release study, in-vitro stability study in presence of proteolytic enzymes.

3.2 ANALYTICAL METHOD DEVELOPMENT FOR THE QUANTITATIVE ESTIMATION OF HUMAN INSULIN IN PURE FORM AND IN ORAL FORMULATIONS:

3.2.1 Materials:

Human insulin (Akzo Nobel) was obtained from Shreya Health Care Pvt. Ltd., Mumbai, India. Chromatographic grade ethanol and acetonitrile were purchased from Merck, India. Analytical grade sodium sulphate anhydrous, phosphoric acid, hydrochloric acid and ethanol amine were purchased from Merck, Mumbai, India.

3.2.2 Equipments:

Agilent 1200 series RRLC® gradient HPLC system equipped with auto-injector, column oven, UV detector and Cromeleon® software was used.

3.2.3 Method development

Different solvent systems by varying the proportion between pH 2.3 buffer and acetonitrile were used to develop gradient system for quantitative estimation of Human insulin in the

developed formulations. The final decision on the suitability of a solvent system for mobile phase was based on minimizing the interference from the excipients employed with increased sensitivity for Human insulin detection.

3.2.4 Experimental:

3.2.4.1 Preparation of standard curve:

Standard preparation:

An accurately weighed quantity of Human insulin standard was dissolved in a mixture of 0.01 N HCl and ethanol 99% (4:1) to obtain a solution having a known concentration of about 0.5 to 0.6 IU/ml. The solution was filtered through 0.22 µm disc syringe filter (Ultipore® N66, Pall life sciences) and injected 4 µl onto HPLC.

Preparation of buffer solution:

The buffer solution pH 2.3 was prepared by dissolving 28.4 g of anhydrous sodium sulfate in 1000 ml of water followed by addition of 2.7 ml of phosphoric acid into the solution and pH adjusted with ethanolamine to 2.3. A reversed phase column C18, 1.8 µm, 4.6 x 50 mm, Eclipse XBD was used. The gradient system consisted of mobile phase A, mixture of buffer and acetonitrile (82:18) and mobile phase B, mixture of buffer and acetonitrile (50:50). A gradient was run as follow (Table 3.1).

Table 3.1: Gradient composition for mobile phase

Time	Mobile phase A (%)	Mobile phase B (%)
0	74.4	25.6
8.6	74.4	25.6
14	36	64
15.2	36	64
15.4	74.4	25.6
20	74.4	25.6

The flow rate was 1 ml/min with column oven temperature of 40°C and detection wavelength of 214 nm.

A stock solution of Human insulin was prepared by dissolving 12 mg of drug in 100 ml of 0.01N HCl to get final concentration of 3 IU/ml. From the stock solution, concentration of 0.42 IU/ml, 0.54 IU/ml, 0.60 IU/ml, 0.72 IU/ml and 0.78 IU/ml were made in series by suitable dilution in 10 ml volumetric flask for the purpose of calibration curve and their respective response (peak area) was measured. The results are listed in Table 3.2. The absorbance characteristics, accuracy, precision and other validation parameters of the proposed method are given in Table 3.4. The prepared dilutions were injected serially in increasing order of concentration. The obtained peaks were integrated and the peak area was calculated for each. The results are listed in Table 3.2 and the results of regression analysis are presented in Table 3.3. The stability of the Human insulin solutions during analysis was also investigated by analyzing the samples at different time intervals on the same day. The validation parameters for the proposed method are given in Table 3.4. Chromatogram parameters such as, retention time and asymmetry factor were also evaluated to ensure system suitability of the method.

3.2.5 Method validation:

Following procedures were employed to determine various validation parameters of the method developed and the results are presented in Table 3.4.

3.2.5.1 Accuracy and precision:

To determine accuracy and precision of the developed method two different (5 in each case) concentrations of Human insulin (0.42 and 0.74 IU/ml) standard and test solution were analyzed as per the procedure enlisted in the previous section (preparation of standard curve).

The intermediate precision of the developed method was confirmed by changing the analyst for analyzing standard and test solution of the drug (0.42 -0.74 IU/ml) in triplicate and by varying the instrument using Dionex® HPLC system equipped with UVD l70U detector using Cromeleon® software. The percentage relative standard deviation (% RSD) for the assay results was determined.

3.2.5.2 Linearity:

Three separate series of solutions of the drug, 0.42 - 0.74 IU/ml, were prepared from the stock solution and analyzed.

3.2.5.3 Specificity:

The specificity of the method was evaluated by analyzing diluents, placebo, resolution solution, reference solution and test solution.

3.2.5.4 LOQ and LOD:

The limit of detection and quantitation were calculated on the basis of linearity curve.

3.2.5.5 Robustness:

The robustness of the developed method was also confirmed by making small changes in the composition of mobile phase (upto 2%) on retention time and selectivity was determined.

3.2.5.6 Estimation of Human insulin from commercial injectable formulation by the proposed Method:

For estimation of drug content from the commercially available injectable solution of Human insulin (Huminsulin-R® 40IU/ml) from Indian market, the same proposed method was employed. The resulting solution was filtered through 0.22 µm disc syringe filter. (Ultipore® N66, Pall life sciences) and suitably diluted to get final concentration within the limits of linearity for the proposed method. The drug content per ml of injectable Human insulin solution was calculated (on an average concentration basis) from the peak area values. The results are tabulated in Table 3.5.

3.2.5.7 System suitability solution:

An accurately weighed quantity of Human insulin standard was dissolved in a 0.01 N HCl to obtain a solution having a known concentration of 1.5 mg/ml. The solution was allowed to stand at room temperature for not less than 3 days to obtain a solution containing not less than 5% of A-21 desamido insulin and injected 20 µl onto HPLC.

3.2.5.8 Recovery Studies:

To keep an additional check on accuracy of the developed assay method and to study the interference of formulation additives, analytical recovery experiments were performed by adding known amount of pure drug to placebo formulations of present project work. The percent analytical recovery values calculated by comparing concentration obtained from the spiked samples with actual added concentrations are also listed in Table 3.6. The placebo formulations spiked with known concentration of insulin were dissolved in a mixture of 0.01 N HCl and ethanol (99%) with solvent ratio of 4:1**,** respectively. The solution was sonicated for 8 to 10 sec at 20°C temperature. Then the volume was adjusted to get the Human insulin concentration of 0.5 to. 0.6 IU/ ml with a mixture of 0.01 N HCl and ethanol (3:1). The insulin solution was filtered through 0.22 µm syringe disc filter (Ultipar®N66, Pall life Sciences) and injected 4 µl onto HPLC.

3.2.6 Results and discussion:

3.2.6.1 Method development:

In case of the proposed method, mobile phase investigated was buffer pH 2.3 : acetonitrile at various proportions. The ratio of buffer pH 2.3 and acetonitrile (82:18) for mobile phase improved the peak parameters for insulin. The flow rate selection was based on the peak parameters (height, asymmetry, tailing, baseline drift, run time).

3.2.6.2 Calibration curve:

A typical chromatogram for Human insulin obtained using reverse phase C-18 column with mobile phase composition gradient elution pattern as described in previous section at 1

ml/min flow rate is shown in Figure 3.1. The wavelength of detection was fixed at 214 nm. The statistical analysis [2] of data obtained for the estimation of Human insulin in pure solution indicated high level of precision of this method. The calibration curve peak area (mAU.sec) vs. concentration (IU/ml) was found to be linear. Values obtained for the calibration curve are presented along with standard deviation, coefficient of variance in Table 3.2. Statistical calculations were done at 5% level of significance. The low values of standard deviation, standard error and coefficient of variation established the precision of the proposed method. The linear Regression equation obtained for the proposed LC method was Y = 131.79.X - 0.2675, where Y = peak area in mAU.sec; X = concentration in IU/ml. The retention time and asymmetry factor were found to be 7.323 ± 0.028 min. and 1.05 ± 0.12 min., respectively (Table 3.4). The drug solution was found to be stable for a period of 6 hour at room temperature in the solvent system used.

3.2.6.3 Validation of the developed method:

The developed liquid chromatographic method was validated according to the standard procedures [2] and the results obtained are tabulated in Table 3.5. The linearity range studied for Human insulin solution was 0.42 - 0.74 IU/ml. The limit of detection and limit of quantitation was 0.0093 IU/ml and 0.0283 IU/ml, respectively. In the validation tables (Table 3.4) for the developed methods, the accuracy is reported in terms of percentage relative error and precision in terms of % RSD. The low values of these parameters reflected the excellent measurement accuracy and precision of the proposed methods for estimation of Human insulin. The intermediate precision (%RSD) of the estimation of Human insulin standard and test solution (0.42 and 0.74 IU/ml) in triplicate by different analysts and on different instruments was 0.433. The variation of the relative composition of the mobile phase (greater than or equal to 1%) effected a change in retention time of the analyte peak. However variations in the pH of buffer showed effect on both retention time as well as peak parameters. No internal standard was used as no extraction step was involved in estimation of Human insulin from the formulation. Further, the accuracy of the experimental results established no need of internal standards for the suggested methods.

The method is considered to be stability indicating as it is found to be specific to insulin peak with no interference observed from placebo and the major insulin degradent, A-21 desamido insulin. The peak purity of insulin peak and A-21 desamido insulin peak were found to be

well above 99.0%, as estimated from the spectrum (200 nm – 400 nm) of eluting peak using PDA detector. The results of peak purity of freshly prepared insulin standard, degraded insulin standard (as per USP 31) and insulin test samples freshly prepared from formulations developed are presented in Table 3.7. The chromatographs representing peak purity are presented in Figure 3.6 to 3.11.

3.2.6.4 Recovery studies:

The proposed liquid chromatographic method could be employed successfully for the estimation of Human insulin in the formulations developed in this project work and analysis of reference pure drug solution with low values of standard deviation. The results (presented in Table 3.6) further established the precision of the proposed methods and therefore suggested the non-interference from the formulation matrix present in the studied formulations. The accuracy of the results of estimation was further tested by recovery experiments. The average recovery was found to vary between 98.82 and 99.54 %.

3.3 ANALYTICAL METHOD FOR THE QUANTITATIVE ESTIMATION OF HUMAN INSULIN IN SERUM AND IN ORAL FORMULATIONS:

3.3.1 Materials:

Human insulin (Akzo Nobel) was obtained from Shreya Health Care Pvt. Ltd., Mumbai, India; Mercodia® Insulin ELISA kit for estimation of human insulin was obtained from Mercodia AB, Sweden.

3.3.2 Equipments:

BioRad®, Model 680 microplate reader and Microplate Analyst 3.0.2 software was used.

3.3.3 Contents of the Mercodia® Insulin ELISA kit:

a. Coated plate
b. Calibrators; 3, 10, 30, 100 and 200 mIU/l (recombinant human insulin)

c. Calibrator-0
d. Enzyme Conjugate (peroxidase conjugated mouse monoclonal anti-insulin)
e. Enzyme Conjugate buffer
f. Wash buffer
g. Substrate TMB
h. Stop solution (0.5M H_2SO4)

3.3.4 Method:

Mercodia® Insulin ELISA is a solid phase two-step enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by the reaction with 3.3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

Mercodia® Insulin ELISA kit for estimation of human insulin content came as pre-calibrated kit against 1st International Reference Preparation 33/304.

3.3.5 Experimental:

3.3.5.1 Test procedure:

a) Sufficient microplate wells were prepared to accommodate Calibrators (0, 3, 10, 30, 100, 200 mIU/l) and samples in duplicate.
b) 25 µl each of calibrators and samples were pipetted into appropriate wells followed by addition of 100 µl of Enzyme Conjugate to each well.
c) Microplate was incubated on a plate shaker for 1 hour at room temperature (18-25ºC).
d) After incubation, microplate was washed 6 times with automatic plate washer and each well was filled completely with 350 µl Wash Buffer. The liquid was discarded and the procedure was repeated 5 times. After final wash, microplate was inverted and tapped firmly against absorbent paper.

e) 200 µl Substrate TMB was then added into each well and incubated for 15 min at room temperature (18-25ºC).
f) After incubation, 50 µl of Stop solution was added to each well and the microplate was kept on a shaker for approximately 5 sec to ensure mixing.
g) Optical density was measured at 450 nm within 30 min and results were calculated.

3.3.5.2 Calculation of results:

Data Reduction:

Computerized data reduction of absorbance for the calibrators (3-200 mIU/l) versus the concentration using cubic spline regression was performed using Microplate Analyst® 3.0.2 software to obtain the concentration of insulin.

3.3.5.3 Calibration:

Mercodia® Insulin ELISA kit was calibrated against 1st International Reference Preparation 66/304.

3.3.6 Method validation:

The method was not considered for validation as the Mercodia® Insulin ELISA kit came as precalibrated kit against 1st International Reference Preparation 33/304 and was ready to use for estimation of human insulin content in the samples. Detection limit, precision, specificity, recovery and hook effect were reported along with the ELISA kit.

Freeze-thaw stability of the serum samples was performed by spiking the known amount of human insulin solution into the blank serum samples of rat at 10, 75 and 150 mIU/l concentration. The spiked samples were stored at -50°C for two days and then brought to room temperature just before sample treatment and analysis.

3.3.6.1 Linearity:

Two separate series of solutions of drug, 0, 3, 10, 30, 100, 200 mIU/l were prepared by spiking blank serum samples of rat with Human insulin solution and analyzed to draw a linearity curve.

3.3.7 Estimation of Human insulin in serum samples by the proposed method:

The serum samples from in-vivo studies were analyzed for the insulin content by using ELISA technique. 25 µl each of serum samples and Calibrators were pipetted out into appropriate wells and 100 µl of enzyme conjugate solution was added to each well followed by incubation on a plate shaker (700-900 rpm) for 1 hour at room temperature (l8-25°C). Then plates were washed by adding 350 µl of wash buffer into each well followed by complete aspiration, the washing process was repeated 5 times. After final wash, plate was inverted and tapped against absorbent paper. 200 µl of Substrate TMB was added to each well and the plate was incubated for 15 min at room temperature (l8-25°C). After incubation, 50 µl of stop solution was added into each well and plate was placed on shaker for approximately 5 sec to ensure mixing. Optical density at 450 nm was measured within 30 min on microplate reader (BioRad®, Model 680) and human insulin concentration was calculated using Microplate Analyst 3.0.2 software. The results of the serum insulin concentrations are reported in section 4.5.2.1, 5.5.2.1 and 6.5.2.1 (Chapter 4, 5 and 6 respectively).

3.3.8 Biological activity evaluation of Human insulin from formulations developed:

Biological activity of Human insulin in the formulations developed was assessed using ELISA technique. Insulin loaded formulations (equal to 3 units of human insulin) were dissolved in a mixture of phosphate buffer pH 7.4 and ethanol 99% with solvent ratio of 4:1. The solution was mixed for 5 min and then sonicated for 15 sec and volume adjusted to 5 ml and filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). Second dilution was made by diluting 50 µl of filtrate to 200 ml with phosphate buffer pH 7.4 to final concentration of about 150 mIU/l. An aliquot of sample was withdrawn from the solution and insulin content was analyzed by ELISA as per standard protocol. Results were obtained by reading the optical density at 450 nm using plate reader (BioRad®, model 680 microplate

reader) and results were calculated using Microplate Analyst 3.0.2 software. The results of the serum insulin concentrations are reported in section 4.5.1.8, 4.5.1.9, 5.5.1.8, 5.5.1.9, 6.5.1.8 and 6.5.1.9 (Chapter 4, 5 and 6 respectively).

3.3.9 Results and discussion:

3.3.9.1 Detection limit reported:

The detection reported limit was < 1 mIU/l.

3.3.9.2 Recovery reported:

The recovery upon addition reported was 94-113% (mean 104%).

3.3.9.3 Hook effect reported:

Samples with a concentration of up to 30000 mIU/l can be measured without giving falsely low results.

3.3.9.4 Precision reported for ELISA kit:

Each sample was analyzed in 6-replicates on six different occasions and reported values are presented in Table 3.8.

3.3.9.5 Specificity reported:

The cross-reactions have been reported with the ELISA kit for estimation of human insulin content. The list of cross-reactants along with their maximum percent reactivity (reported) is listed in Table 3.9

3.3.9.6 Freeze-thaw stability study:

Human insulin serum samples were able to withstand the freeze-thaw cycle of 2 days where serum samples were stored at -50°C for 2 days followed by bringing them to room

temperature just before analysis. The Human insulin recovery from the samples was found to be ranging between 92.64% to 102.1% (Table 3.10).

3.3.9.7 Linearity:

Linearity curve, absorbance vs. concentration for various concentrations of insulin was found to be linear. Values obtained for the linearity curve are presented in Table 3.11 and a typical linearity plot is presented in Figure 3.12. The linear regression equation obtained for the proposed ELISA method was Y = 63.298 × -2.450, where Y = absorbance at 450 nm; X = concentration of Human insulin in mIU/l.

Table 3.2:

Calibration curve points of the proposed HPLC method in estimation of standard solution of Human insulin.

Solution concentration (IU/ml)	**Mean area value* (mAU.sec)**	**C.V. %**
0.42	55.39820 ± 0.16278	0.294
0.54	70.78140 ± 0.18387	0.260
0.60	78.29342 ± 0.15589	0.199
0.72	94.73528 ± 0.15744	0.166
0.78	102.72619 ± 0.14108	0.137

* : Average of three determinations with standard deviation.

C.V. : Coefficient of variance

Table 3.3: Results of least square regression analysis of data for the estimation of standard solution of Human insulin by proposed HPLC method

Statistical parameter	**Observed values**
Regression equation	Y = 131.79 × -0.2675
Correlation coefficient (r)	0.9997

Table 3.4: Validation report for the determination of Human insulin from its standard solutions by proposed HPLC method

Analytical parameter	**Observations**
Accuracy (%)	99.89 (range: 99.31 – 100.46)
Precision (%RSD)	0.424
Linearity	0.42 – 0.78 IU/ml
Specificity	No influence to the main peak observed from blank, placebo.
Limit of Detection	0.0093 IU/ml
Limit of Quantitation	0.0283 IU/ml
Ruggedness (%RSD)	0.433
Retention time (min)	7.323 ± 0.028
Asymmetric factor	1.05 ± 0.12

Table 3.5: Results of the assay of commercial human insulin formulation by proposed HPLC method

Sample	Label claim	Mean	C.V. %
Huminsulin-R® Injection	40 IU/ml	98.5 %	0.18 %

C.V. : Coefficient of variance

Table 3.6: Results of the assay of developed human insulin formulations by proposed HPLC method

Placebo formulations	Theoretical amount of human insulin spiked	% Recovery*****
InsSA-2 Placebo	2 IU/ml	99.54 ± 0.98
InsEY-M2 Placebo	2 IU/ml	98.96 ± 1.02
InsSC-M3 Placebo	2 IU/ml	98.82 ± 0.95

***** Mean in % and S.D. for three determinations.

Table 3.7: Peak purity of Human insulin and A-21 desamido insulin peaks

Sample	% Peak purity for insulin peak	% Peak purity for A-21 desamido insulin peak
Freshly prepared insulin standard	99.89	Not detected
Insulin standard solution stored at room temperature for 3 days (system suitability)	99.98	99.96
Test sample prepared from InsSA-2	99.96	Not detected
Test sample prepared from InsEY-M2	99.78	Not detected
Test sample prepared from InsSC-M3	99.88	Not detected

Table 3.8: Precision reported for Mecodia® Insulin ELISA kit

Sample	Mean value (mIU/l)	Coefficient of variation		
		Within assay %	Between assay %	Total assay %
1	11	3.4	3.6	5.0
2	36	4.0	2.6	4.7
3	80	2.8	2.8	4.0
4	154	3.2	2.9	4.4

Table 3.9: Specificity reported for Mecodia® Insulin ELISA kit

Cross reactant proteins	**Maximum % interference**
C-peptide	< 0.01% (by weight)
Proinsulin	< 0.01 % (by weight)
Proinsulin des	(31-32) < 0.5%
Proinsulin split	(32-33) < 0.5%
Proinsulin des	(64-65) 98%
Proinsulin split	(65-66) 56%
Insulin lispro (Humalog®, Eli Lilly)	< 0.006%
Insulin aspart	< 0.006%
Rat insulin	0.7%

Table 3.10: Freeze-thaw stability of serum samples

Sample	**Actual concentration (mIU/l)**	**Recovery after one freeze-thaw cycle**		
		Mean*	**Coefficient of variance (%)**	**Analytical recovery (%)**
Analysis in serum				
Sample-1	10	9.264 ± 0.629	6.78	92.64
Sample-2	75	76.615 ± 3.188	4.16	102.1
Sample-3	150	149.213 ± 4.533	3.04	99.4

***** Mean in mIU/l and S.D. for three duplicate determinations.

Table 3.11: Results of least square regression analysis of data for the estimation of serum Human insulin by ELISA method

Statistical parameter	**Observed values**
Regression equation	Y = 63.298 × -2.450
Correlation coefficient (r)	0.998

Figure 3.1: Resolution between human insulin peak and A-21 desamido insulin (system suitability)

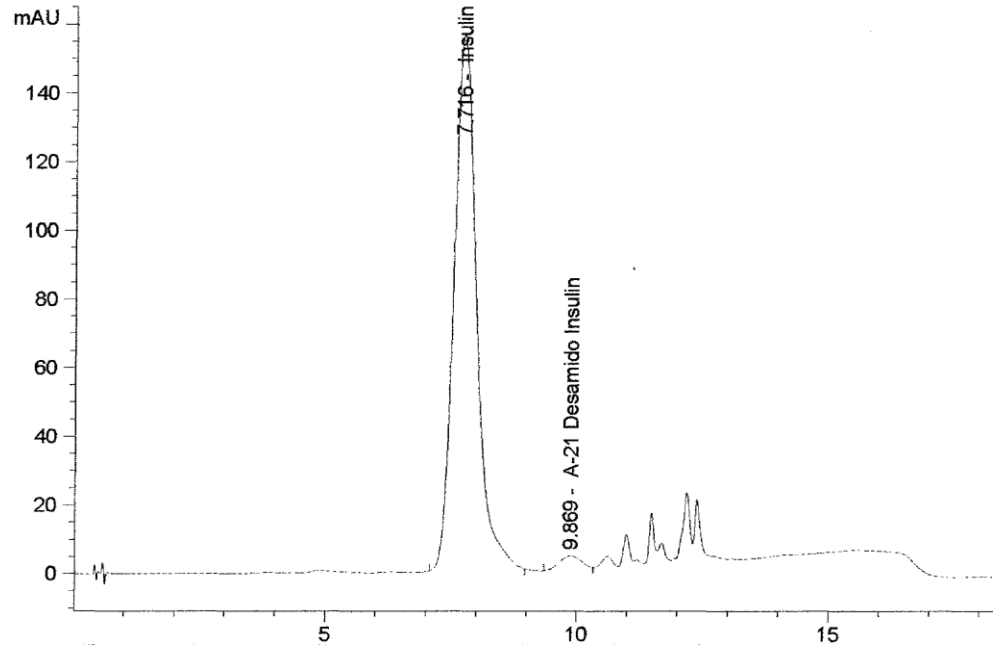


Figure 3.2: Blank solution

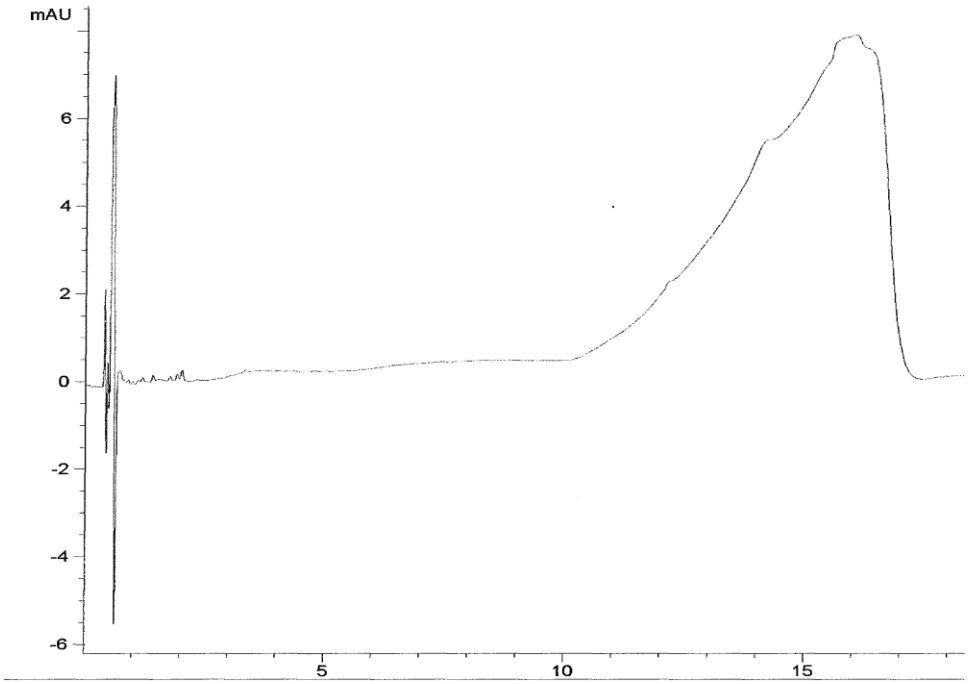


Figure 3.3: Chromatogram for InsSA-2 Placebo.

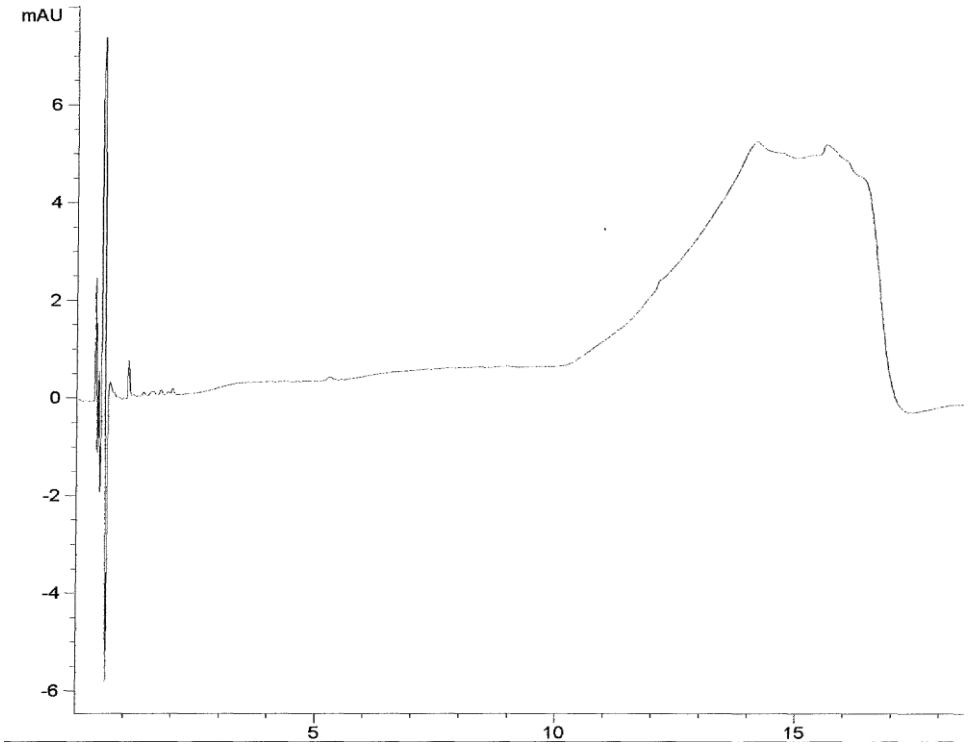


Figure 3.4: Chromatogram for InsEY-M2 Placebo.

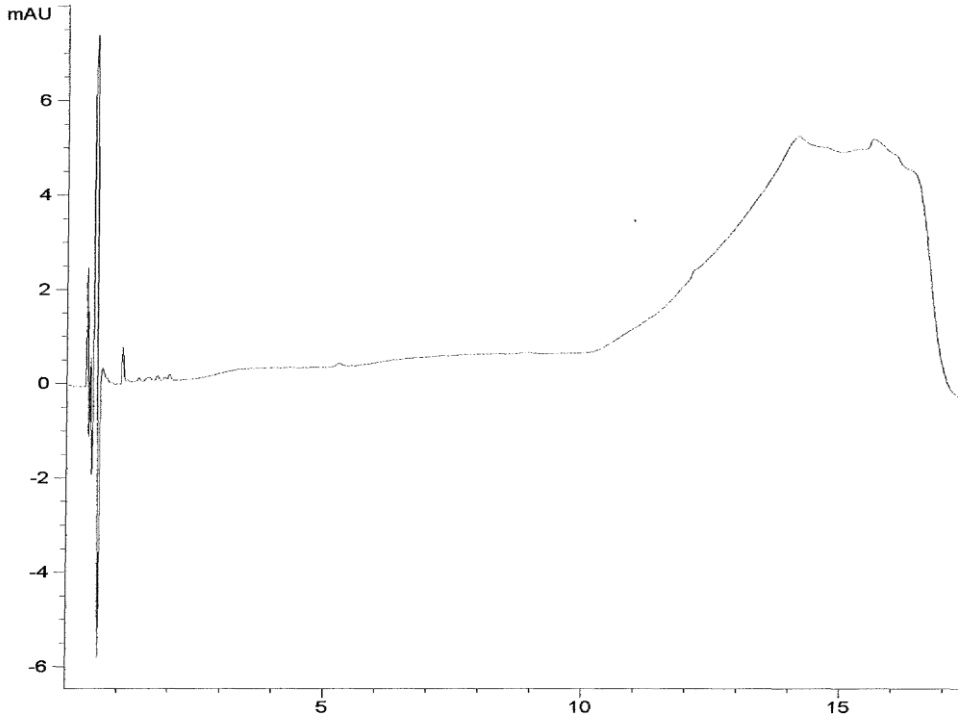


Figure 3.5: Chromatogram for InsSC-M2 Placebo

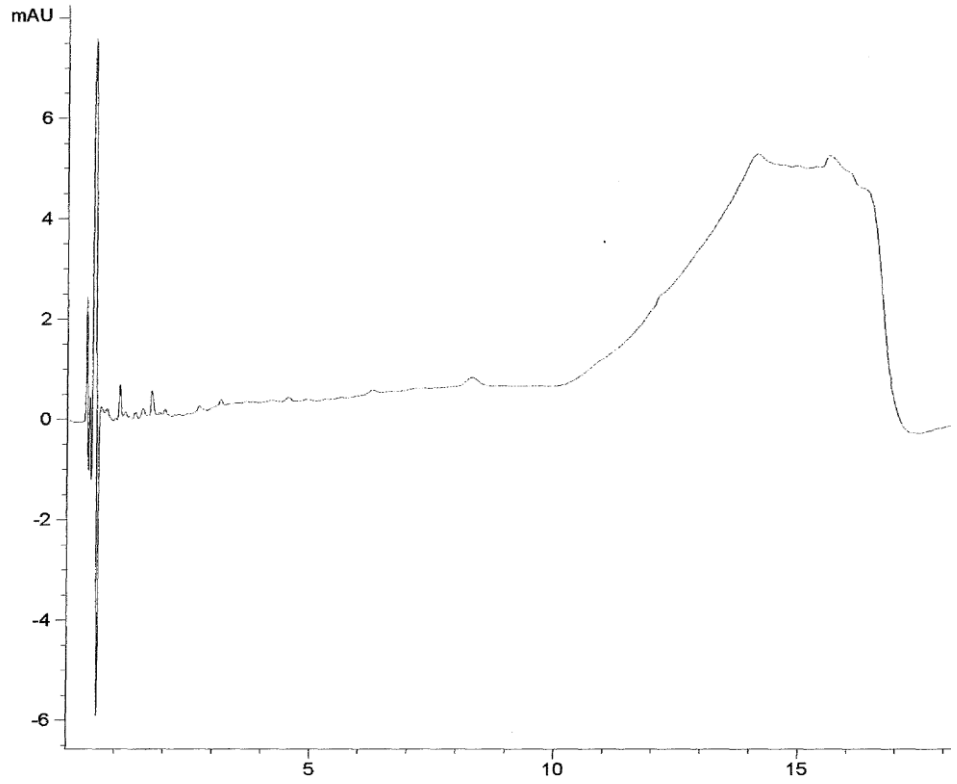
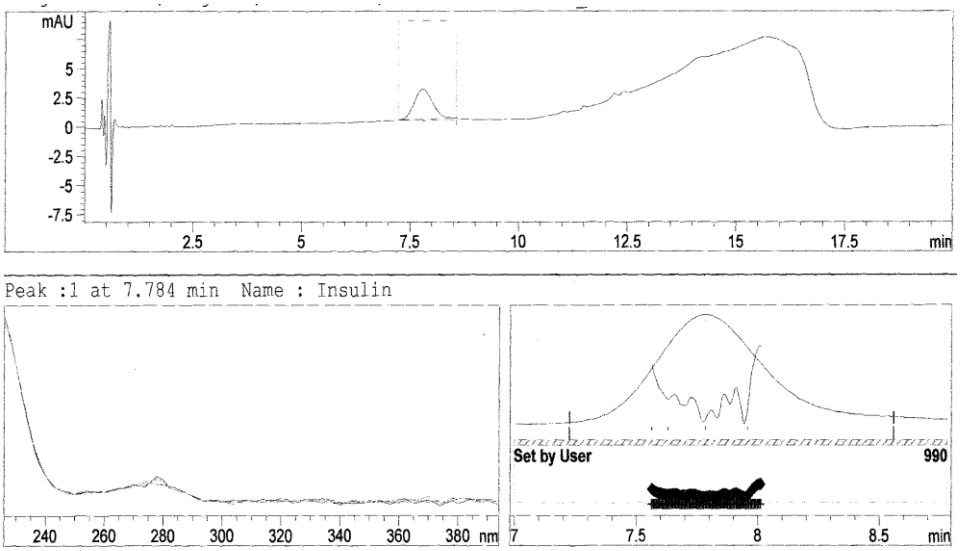


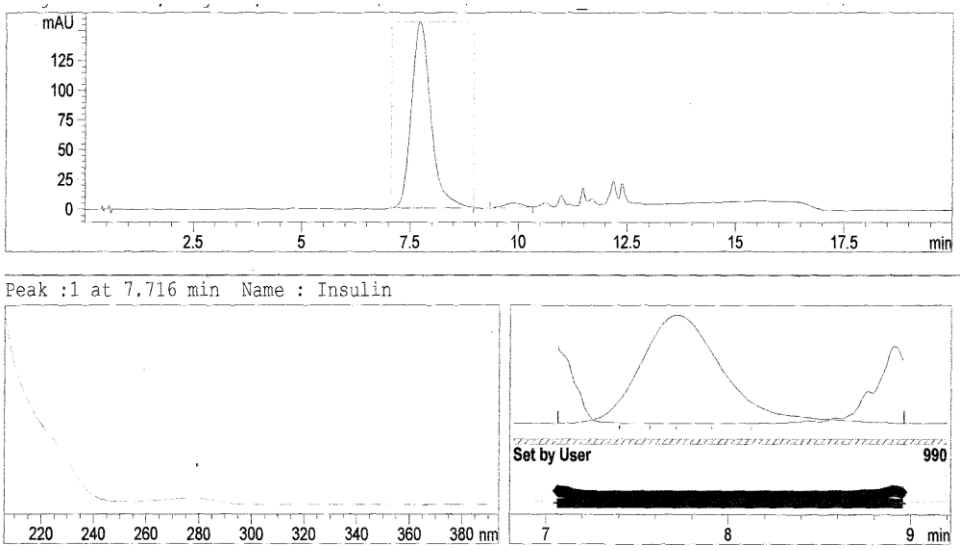
Figure 3.6: Chromatogram for peak purity of freshly prepared insulin standard



-> The purity factor is within the threshold limit. <-

Purity factor : 998.977 (68 of 68 spectra are within the threshold limit.)

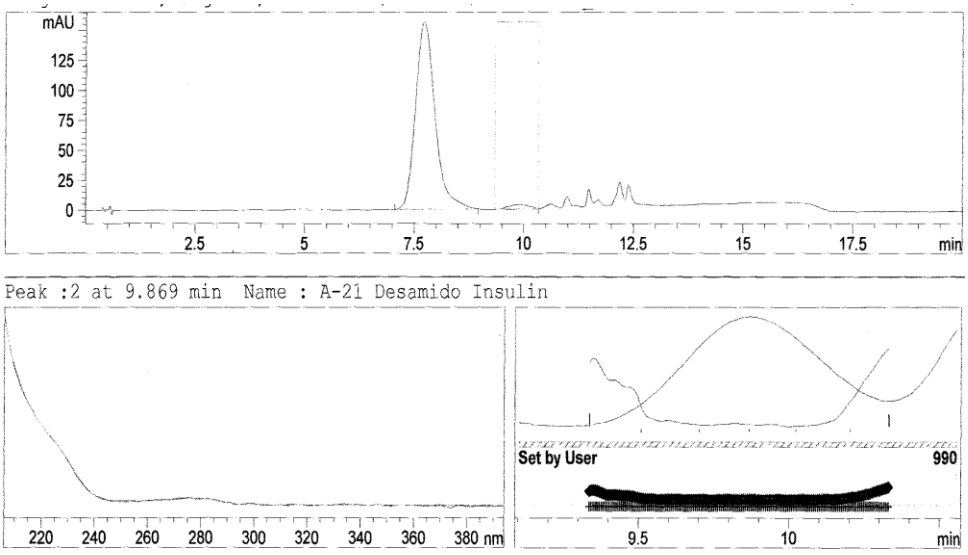
Figure 3.7: Chromatogram for peak purity of insulin from insulin standard solution stored at room temperature for 3 days (system suitability)



-> The purity factor is within the threshold limit. <-

Purity factor : 999.867 (285 of 285 spectra are within the threshold limit.)

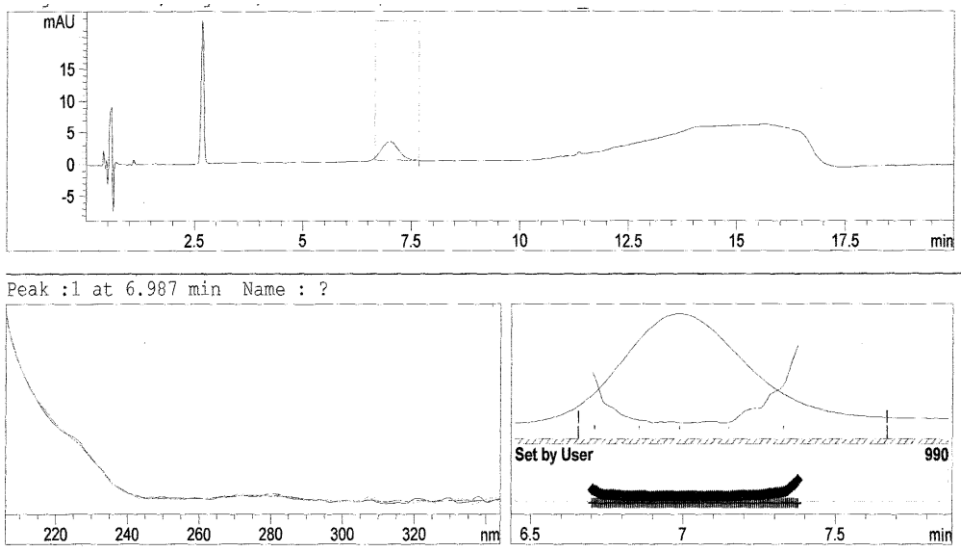
Figure 3.8: Chromatogram for peak purity of A-21 desamido insulin from insulin standard solution stored at room temperature for 3 days (system suitability)



-> The purity factor is within the threshold limit. <-

Purity factor : 999.605 (150 of 150 spectra are within the threshold limit.)

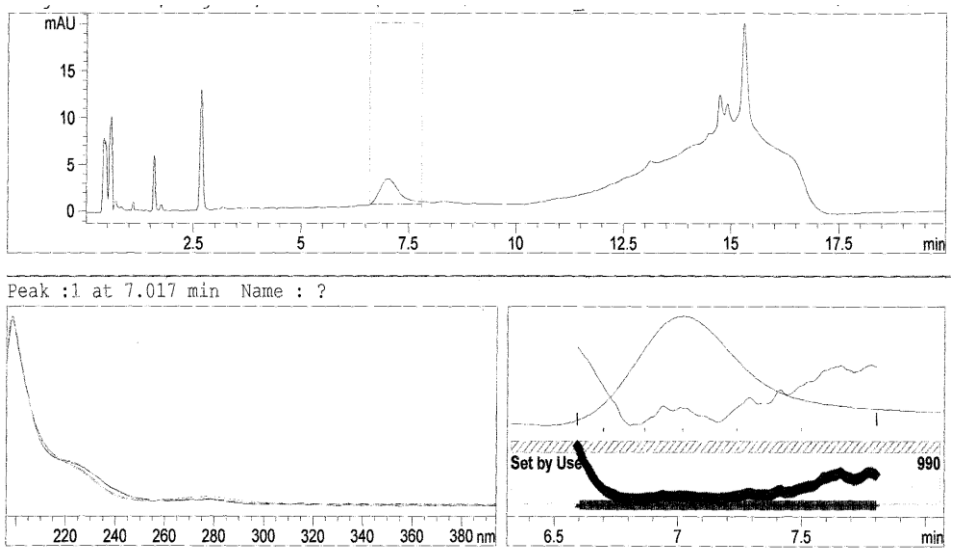
Figure 3.9: Chromatogram for peak purity of insulin from #InsSA-2 formulation



-> The purity factor is within the threshold limit. <-

Purity factor : 999.680 (102 of 102 spectra are within the threshold limit.)

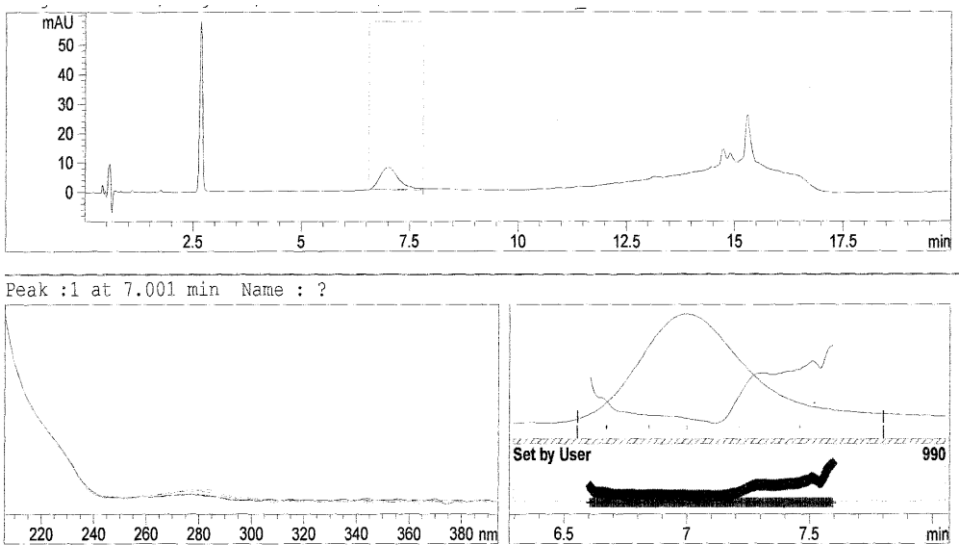
Figure 3.10: Chromatogram for peak purity of insulin from #InsEY-M2 formulation



-> The purity factor is within the threshold limit. <-

Purity factor : 997.842 (180 of 182 spectra are within the threshold limit.)

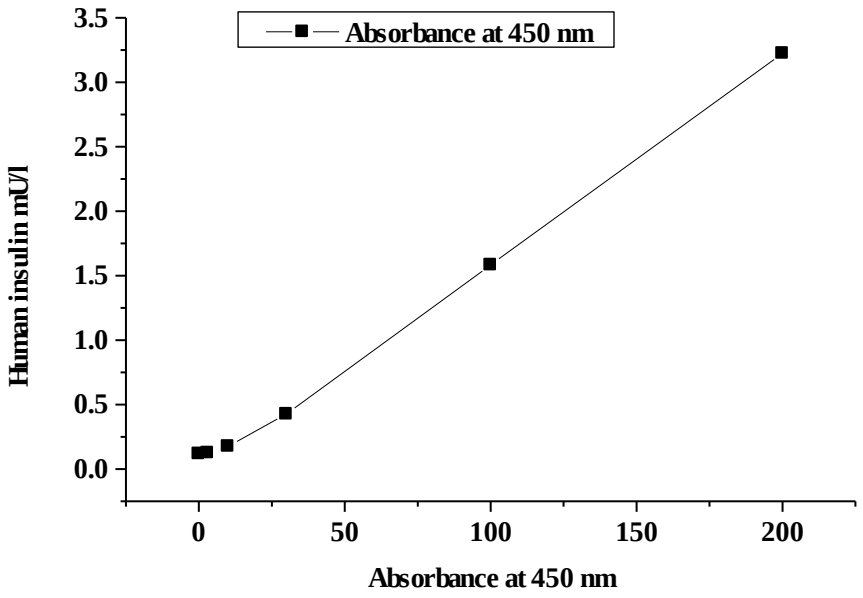
Figure 3.11: Chromatogram for peak purity of insulin from #InsSC-M2 formulation



-> The purity factor is within the threshold limit. <-

Purity factor : 998.851 (149 of 149 spectra are within the threshold limit.)

Figure 3.12: Linearity curve for estimation of serum human insulin content by ELISA



3.4 CONCLUSION:

The proposed method for estimation of Human insulin in pure form and in formulations was accurate, precise, rugged and reproducible.

3.5 REFERENCES:

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CHAPTER 4

STEARIC ACID BASED HYDROPHOBIC NANOPARTICLES

4. STEARIC ACID BASED HYDROPHOBIC NANOPARTICLES:

4.1 INTRODUCTION:

Transport of therapeutic agents such as proteins across the intestinal gut wall may take place via various pathways [1]. The transport can occur primarily through the cell membrane of the enterocytes (transcellular transport) or via the tight junctions between the cells (paracellular transport) or via the lymphatic system. The transcellular passive diffusion pathway is mostly limited to drugs that are non-polar, lipid soluble and not charged at the physiological pH of the small intestinal lumen. The octanol-water system is typically used as a reference system for biological partitioning in drug design work. Drug lipophilicity is estimated by determining the drug's octanol-water partition coefficient.

Insulin has low lipophilicity with an octanol-water partition coefficient of about 0.0215 [1]. Further, the iso-electric point of insulin is around 5 [2] and insulin is negatively charged at the neutral pH of the small intestine. Thus, entry into the cell membrane is unfavorable.

Transport of a drug across the intestinal epithelium is greatly influenced by its physiochemical characteristics. Typically, peptides that have molecular weight greater than 500, are charged at the intestinal pH and tend to form hydrogen bond, diffuse poorly across the epithelial barrier via the transcellular passive diffusion pathway [3]. Paracellular transport, as described previously, is size dependent and hence the paracellular diffusion of structurally complex proteins, such as insulin is negligible [4]. Thus, any enhancement in the paracellular transport of a protein will require improved control over the opening of the tight junctions. Various methods based on this rationale have been explored to improve the transport of therapeutic proteins across the intestinal epithelium. A common method has been the use of permeation enhancers, like detergents, fatty acids or bile salts, which open the intercellular tight junctions and hence increase the transport of insulin across the intestinal membrane [5].

Various permeation enhancers have been investigated for their ability to increase the transport of large molecules across the intestinal cell monolayer. Most of these enhancers act by opening the tight junctions between the cells [6].

One of the absorption enhancer that is relatively well-studied and used clinically in Japan with no reports of side effects is sodium caprate [6-8]. It is the sodium salt of capric acid, a 10-carbon saturated fatty acid that constitutes 2-3% of the fatty acids in dairy products [9]. Sodium caprate facilitates transport via the transcellular route through membrane perturbation and via the paracellular route through the opening of the tight junctions, which is mediated by the contraction of calmodulin-dependent actin micro filaments, due to elevation of intracellular calcium ion levels [7,8].

Approaches to enhance the intestinal lymphatic transport of peptides and proteins have also been investigated for their ability to enhance the oral absorption of proteins. The ability to efficiently deliver highly lipophilic drug molecules, especially in combination with lipophilic drug delivery systems has led to a renewed interest in intestinal lymphatic drug transport [10]. The common lipid properties, namely degree of saturation, fatty acid chain length and lipid class have a greater impact to enhance lymphatic transport. The majority of fatty acids with chain lengths of 14 and above were found to be recovered in thoracic lymph, where as a larger portion of shorter lipids were absorbed directly into the blood [11-13]. Sylven and Borgstrom, demonstrated that the lymphatic transport of cholesterol was increased by coadministration with oleic acid, linoleic acid and stearic acid [14].

Uptake of inert particles has been shown to occur transcellularly through normal enterocytes and Peyer's patches via M-cells and to lesser extent across paracellular pathway [15]. An example of this approach was the development of water in oil microemulsion containing insulin sprayed on to an inert carrier system [16]. Further the lipidic components were in close approximation to those found in chylomicrons.

Fatty acids have also been shown to enhance the permeability of peptide drugs. The mechanism whereby the permeability of peptide drugs was enhanced by the fatty acid is thought to be an association of fatty acid with the membrane and disorder in the membrane’s interior by interaction of these fatty acids with polar head group of phospholipids [17-19].

Physical and chemical instability of protein drug is another major hurdle to formulate effective dosage form. The activity of proteins depends on the three-dimensional molecular structure. Formulation of protein drug into dosage form may expose the protein drug to harsh conditions that may alter their structure. This will have implications on the efficacy and

immunogenic response to the proteins. During dosage form development, proteins might be subjected to physical and chemical degradation. Physical degradation involves modification of the native structure of a protein to a higher-order structure, which may be a result of adsorption, aggregation, unfolding, or precipitation. Chemical degradation usually involves bond cleavage and leads to the formation of a new product. Chemical degradation is preceded by a physical process such as unfolding, which exposes the hidden residues to chemical reactions. The processes involved in chemical degradation are deamidation, oxidation, disulfide exchange and hydrolysis. The stability of insulin preparations has been documented in detail [20,21].

The tendency of insulin to undergo structural transformation resulting in aggregation and formation of insoluble insulin fibrils has been one of the most intriguing and widely studied phenomena in relation to insulin stability. Although the exact mechanism of fibril formation is still obscure, it is now clear that the initial step is an exposure of certain hydrophobic residues, normally buried in the three-dimensional structure, to the surface of the insulin monomer [22]. Chemical deterioration of insulin during storage of pharmaceutical preparations is mainly due to two categories of chemical reactions, hydrolysis and intermolecular transformation reactions leading to insulin HMWT products. The predominant hydrolysis reaction is deamidation of Asn residues which in acid solution takes place at residue A21, in neutral medium at residue B3.

Cyclodextrin represent a unique and effective strategy for stabilization of proteins against aggregation, thermal denaturation and degradation [23]. Proteins are mostly hydrophilic and too bulky to be wholly included into a beta cyclodextrin cavity.

In this study, hydrophobic nanoparticulate system with combined use of stearic acid, α-tocopherol acetate and soya phosphatidyl choline (SPC) in presence of zinc and hydroxypropyl beta cyclodextrin (HP-β CD) was investigated to improve the oral absorption and bioavailability of insulin.

In this work we have demonstrated oral absorption of insulin in the form of hydrophobic nanoparticles as a part of complex formulation system to collectively improve the stability and oral bioavailability of insulin when administered to streptozotocin induced diabetic rats.

The process for the preparation of nanoparticles greatly influence the encapsulation efficiency and stability of the protein drug, especially when protein molecule comes in contact with hydrophobic surfaces, air-water interfaces, shear stress, temperature and organic solvents. In addition, size of the nanoparticles also influences the oral absorption [24,25]. In this work process was optimized to produce well controlled particle size and high encapsulation efficiency of insulin in the hydrophobic nanoparticles.

Lyophilization technique for the preparation of insulin nanoparticles was designed to improve the stability and encapsulation efficiency of insulin in the hydrophobic nanoparticles.

In this study, different ways and approaches including process and formulation components were investigated by considering all of the above factors to stabilize and improve the oral absorption of insulin.

4.2 MATERIALS AND METHODS:

Human insulin (Recombinant DNA origin, Akzo Nobel), Tris hydroxymethylaminomethane (Tris buffer, Merck India), m-Cresol (Merck), Hydrochloric acid 36%, HCI (Merck), Sodium hydroxide pellets, (S.D. Fine Chem), Zinc chloride, anhydrous (S.D. Fine Chem), Hydroxypropyl β cyclodextrin (Roquette), t-Butanol (Merck India), Stearic acid (Cognis), Soya phosphatidylcholine, SPC (Lipoid AG), α Tocopherol acetate (Sigma Aldrich), α-Chymotripsin (HiMedia, Mumbai).

4.2.1 Preparation of Tris buffer pH 7.4:

Tris buffer solution pH 7.4 (10 mM) was prepared by dissolving 1.211 g of Tris (hydroxymethyl)aminomethane in 1000 ml distilled water to get final buffer concentration of 10 mM. pH was adjusted to 7.4 using 0.01N HCl or 0.1N NaOH solution.

4.2.2 Preparation of Human insulin solutions:

Human insulin solutions of different concentrations (25 IU/ml, 50 IU/ml and 100 IU/ml) were prepared by suspending Human insulin crystals in 20 ml of Tris buffer pH 7.4 (10mM)

containing 0.8 mg/ml m-cresol and clear solution was prepared with drop by drop addition of 10 %v/v HCl until pH reached 2 ± 0.05. After formation of a clear solution, pH of the insulin solution was again raised to pH 7.4 ± 0.05 with drop wise addition of 1N NaOH under continuous stirring with the help of glass rod. Final volume of the solution was adjusted to 25 ml with Tris buffer pH 7.4 containing 0.8 mg/ml m-cresol to get Human insulin concentrations of 25 to 100 IU/ml. These neutral solutions of insulin were stored at 2-8°C temperature.

4.2.3 Preparation of Hydroxypropyl β cyclodextrin (HP-β CD) solutions:

HP-β CD solutions of different concentrations (12 mg/ml, 24 mg/ml and 48 mg/ml) were prepared by dissolving HP-β CD in 45 ml of Tris buffer pH 7.4 under stirring for 15 to 20 min in a beaker to get clear solution. The pH of the solution was adjusted to 7.4 ± 0.5 using 1N NaOH. Final volume of the solution was made up to 50 ml with Tris buffer pH 7.4 to obtain a solution of 12 to 48 mg/ml

4.2.4 Preparation of Zinc chloride solution:

Zinc chloride solutions at concentration of 0.2 mg/ml, 0.4 mg/ml and 0.8 mg/ml were prepared by dissolving zinc chloride in purified water followed by sonication in water bath at 25°C for 10 min to form a clear solution.

4.2.5 Preparation of Stearic acid solution:

Stearic acid solutions of different concentrations (250 mg/ml and 500 mg/ml) were prepared by dissolving stearic acid in 190 ml t-butanol followed by addition of *α*-tocopherol acetate (40 mg/ml) and SPC (50 mg/ml). The t-butanol solution was made clear by warming on water bath at 40°C. The solution was then cooled to room temperature and final volume of the solution was made up to 200 ml with t-butanol to obtain stearic acid solution of 250 mg/ml and 500 mg/ml. The concentration of *α*-tocopherol acetate and SPC was 40 mg/ml and 50 mg/ml respectively.

4.2.6 Preparation of hydrophobic nanoparticles of insulin (InsSA):

Formulations were prepared by mixing different respective concentrations and volumes of each individual solution of Human insulin, HP-β CD, zinc chloride and stearic acid. The details for these different formulation compositions prepared are presented in Table 4.1 and Table 4.2. and the process utilized for the preparation of these nanoparticle formulations is summarized below.

Human insulin solution was mixed with HP- β CD solution at a ratio of 0.5:1 in a beaker and stirred for 10 to 15 min using magnetic stirrer without any vortex formation. Then zinc chloride solution was added in the form of micro-droplets to the above insulin-HP-β CD solution under continuous stirring at a ratio of 1:5 respectively. After complete addition of zinc chloride solution, additional stirring was done for 10-15 min to obtain an opalescent solution of insulin.

Stearic acid solution was mixed with purified water (9 %v/v) and pH of the stearic acid solution was adjusted to 7.4 ± 0.05 using 1N NaOH.

The above insulin - HP-β CD - zinc opalescent solution was mixed with above stearic acid solution at a ratio of 0.45:1 in a beaker under stirring for 5 min. The resulting opalescent solution was transferred to tubular glass vials and frozen at -40°C for 3 hour and lyophilized for 38 hour at condenser temperature of -40°C and pressure of 112 x 10^{-3} mbar. After complete drying vacuum was broken with nitrogen gas to purge the vials with nitrogen and capped with butyl stopper and aluminum seals. Vials were stored at 2-8°C.

The process flow diagram for preparation of hydrophobic nanoparticles is presented in Figure 4.1.

Figure 4.1: Flow diagram for the preparation of hydrophobic nanoparticles of insulin (InsSA)

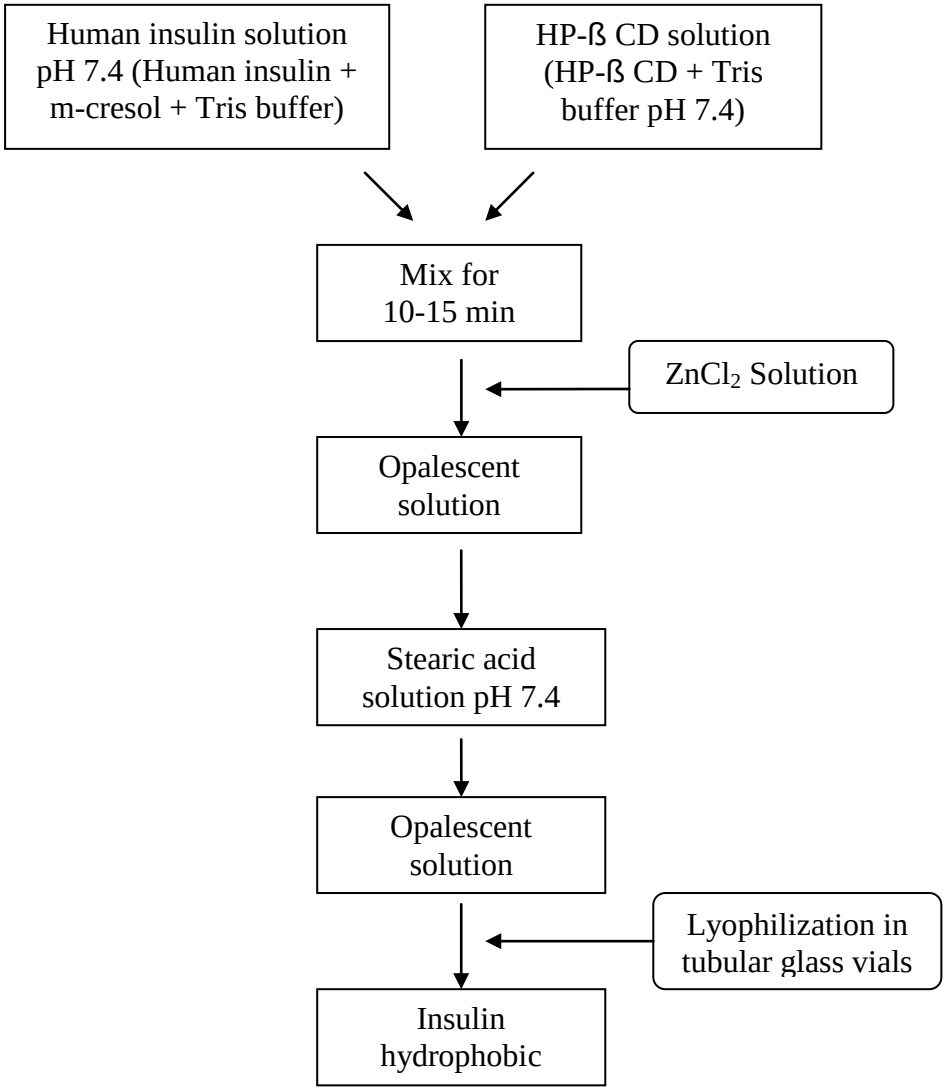

Table 4.1: Formulations of InsSA with varying amount of Human insulin

Components	**Formulations (quantity in mg)**			
	InsSA-P**	**InsSA-1**	**InsSA-2**	**InsSA-3**
Human insulin[#]	-	12.5	25	50
m-Cresol	10	10	10	10
HP-β CD	600	600	600	600
Zinc chloride*****	3	1.5	3	6
Stearic acid	500	500	500	500
α-Tocopherol	40	40	40	40
SPC	50	50	50	50
Purified water	qs	qs	qs	qs
t-Butanol	qs	qs	qs	qs

qs: Quantity sufficient

***** A constant ratio between insulin and zinc chloride was maintained.

****** Placebo formulation of InsSA-2

Theoretical potency of Human insulin : 25 IU/mg

Table 4.2: Formulations of InsSA with varying amount of HP-β CD, zinc chloride and stearic acid

Component	Formulations (quantity in mg)					
	InsSA-CD1	**InsSA-CD2**	**InsSA-Z1**	**InsSA-Z2**	**InsSA-S1**	**InsSA-S2**
Human insulin[#]	25	25	25	25	25	25
m-Cresol	10	10	10	10	10	10
HP-β CD	300	1200	600	600	600	600
Zinc chloride	3	3	1.5	4.5	3	3
Stearic acid	500	500	500	500	250	1000
α-Tocopherol	40	40	40	40	40	40
SPC	50	50	50	50	50	50
Purified water	qs	qs	qs	qs	qs	qs
t-Butanol	qs	qs	qs	qs	qs	qs

qs: quantity sufficient

Theoretical potency of Human insulin : 25 IU/mg

4.3 NANOPARTICLES CHARACTERIZATION:

Developed formulations were subjected to following physicochemical characterization studies.

4.3.1 Appearance:

Visual appearance of the various Human insulin nanoparticulate formulations after lyophilization was noted and compared.

4.3.2 Particle size:

The particle size of the various nanoparticulate formulations was measured using dynamic laser light scattering instrument (Brookhaven Instruments BI 90 Particle sizer, Holtsvilee, New York) after suitable dilution with 0.22 µm filtered Tris buffer pH 5.5 solution.

4.3.3 Scanning electron microscopy:

The morphology of the Human insulin nanoparticles was viewed using a conventional scanning electron microscope (JSM 5400, JOEL, Japan) at an accelerating voltage of 15kV. Nanoparticle samples were dispersed in 0.22 µm filtered Tris buffer pH 5.5 solution. One drop of the nanoparticle suspension was placed on a graphite surface. After air drying, the sample was coated with gold using ION Sputter.

4.3.4 Insulin entrapment efficiency:

The amount of Human insulin loaded into the nanoparticles was estimated as follows. Human insulin nanoparticles (equal to 3 IU of Human insulin) after lyophilization was dispersed in 4 ml of 0.01N HCl and mixed for 5 min on vortex mixer (Remi® India). The mixture was ultracentrifuged for 20 min at 22,000 rpm in order to isolate entrapped insulin from un-entrapped insulin. The supernatant was removed and nanoparticle sediment was washed with distilled water. Sediment was dissolved in a mixture of 0.01N HCl and ethanol (99%) with solvent ratio of 4:1 respectively. The solution was sonicated for 10 to 15 sec at 20°C temperature. Then volume of the solution was adjusted to 5 ml and filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The filtered solution was quantitatively analyzed for Human insulin by HPLC (Section 3.2). The entrapment efficiency was calculated using following eq. (1)

EE (%) = (Amount of drug in the sediment / Amount of drug added) × 100

4.3.5 Residual water content:

Residual water content of the nanoparticles was determined using Karl Fischer auto-titration (Metler®). 50 mg of nanoparticles after lyophilization was added and dispersed into anhydrous methanol and auto-titrated with Karl Fischer reagent.

4.3.6 In-vitro insulin release study:

The release rate of Human insulin from the nanoparticles were determined by suspending a weighed amount of lyophilized nanoparticles (equal to 100 IU of Human insulin) in 50 ml, pH 7.4 phosphate buffer USP. During the experiment (4 hour) samples were shaken horizontally in a constant temperature shaker (Remi® India) at 37 ± 1°C and 50 stokes per minute. At scheduled time intervals, 1 ml of sample was removed and replaced with 1 ml of fresh release medium. The sample was ultracentrifuged at 22,000 rpm at 20°C for 15 min, then the supernatant was collected and quantitatively analyzed for Human insulin content by HPLC (Section 3.2)

4.3.7 In-vitro stability of the lnsulin nanoparticles in presence of proteolytic enzymes:

The protection of insulin nanoparticles was evaluated in presence of α-chymotrypsin with specific activity of 51 U/mg protein at pH 7.8 (optimal pH for enzyme activity) and 37 ± 10°C. The nanoparticles were suspended in 0.05M phosphate buffer pH 7.8 to which α-chymotrypsin had been added. α-Chymotrypsin was also added to insulin solution (as control). The final concentration of the insulin and the enzyme were 15 U/ml and 26 U/ml respectively. Samples were removed after 30 min and 0.5 ml of 0.1 % TFA was added to inactivate traces of enzyme present. Subsequently, 0.5 ml of ethanol 99% was added to each tube and sonicated for 10 to 15 sec. Then the volume was adjusted with a mixture of 0.01N HCl and ethanol (4:1) to get the Human Insulin concentration of 0.5 to 0.6 IU/ml. The insulin solution was filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The filtered solution was quantitatively analyzed for Human insulin content by HPLC (Section 3.2).

4.3.8 Biological activity evaluation of entrapped Human insulin:

Biological activity of entrapped Human insulin in the nanoparticles was assessed using ELISA technique. Insulin loaded nanoparticles (equal to 3 units of human insulin) were dissolved in a mixture of phosphate buffer pH 7.4 and ethanol 99% with solvent ratio of 4:1. The solution was mixed for 5 min and then sonicated for 15 sec and volume adjusted to 5 ml using phosphate buffer pH 7.4 and filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The solution was further diluted using 50 µl of filtrate to 200 ml with phosphate buffer pH 7.4 to final concentration of about 150 mIU/l. An aliquot of sample was withdrawn from the solution and insulin content was analyzed by ELISA as per standard protocol. The optical density of the resultant solution was measured using micoplate reader (BioRad®, model 680 microplate reader) at 450 nm and the final concentration of the solution was calculated using Microplate Analyst 3.0.2 software (Section 3.3.8).

4.3.9 Stability studies:

Stability studies were performed for Human insulin nanoparticles (Lyophilized powder packed in glass vials purged with nitrogen) by storing them at 2-8°C temperature. The samples were analyzed at defined time interval for the insulin content by using ELISA technique as described in Section 3.3.8.

4.4 IN-VIVO STUDIES:

4.4.1 Animals:

Male Wistar rats (200-280 g) were bought at Raj Biotech (India) and housed in a light and temperature controlled environment. The in-vivo studies were performed with approval from Animal Ethics Committee. Urine was collected and its volume was recorded daily. Food and water consumption and weight of the rats was also monitored on a regular basis.

4.4.2 Induction of diabetes mellitus:

To induce diabetes mellitus, rats were injected with 40 mg/kg of streptozotocin (STZ) in citrate buffer (pH 4.5) via intraperitoneal route. The buffer was sterilized by filtration (0.22

µm Ultipor®N66 filter membranes). Blood glucose levels were measured before and on days 2, 3 and 4 after STZ injection by separating the plasma (anticoagulant: sodium fluoride solution 2 mg/ml. The plasma samples were analyzed for glucose content by glucose oxygenase method (Reagent: Pinnacle Biotechnology Ltd, Mumbai) and measuring the optical density at 505 nm (Star® 21 Autoanalyzer). Rats were considered diabetic and included in the study when plasma glucose levels were near and above 300 mg/dl.

4.4.3 Dosing and sampling:

The study was performed on day 4 following STZ injection after overnight fast of 8 hour. Water was allowed ad libitum. Diabetic rats were divided into four groups, each containing 8 rats. **Group-1:** Placebo nanoparticles (Batch: #InsSA-2P equal to 20 IU/kg of insulin) dispersed in 0.5 ml purified water was administered intragastrically to each diabetic rat as a control; **Group-2:** Human insulin injection (Huminsulin-R® 40 IU/ml Injection, Elly Lili) was administered subcutaneously to each diabetic rat at a dose level of 2 IU/kg. This group served as reference standard; **Group 3:** Human insulin nanoparticles (Batch: #InsSA-2) dispersed in 0.5 ml purified water (equal to 10 IU/kg of Human insulin) was administered intragastrically to each diabetic rat; **Group 4:** Human insulin nanoparticles (Batch: #InsSA-2) dispersed in 0.5 ml purified water (equal to 20 IU/kg of Human insulin) was administered intragastrically to each diabetic rat.

After administration of the dosage forms, 0.5 ml of purified water was administered. Prior to and at specified time intervals over 12 hour period blood samples (about 100 µl for glucose estimation and about 100 µl for serum human insulin estimation) were collected from retroorbital vein in capillary tubes for serum Human Insulin estimation and fluoride oxalate impregnated capillary tubes for glucose estimation. During collection of the blood samples, animals were anesthetized by ether inhalation. The samples were centrifuged for 10 min at room temperature, serum and plasma were separated for Human Insulin estimation by ELISA technique and Glucose estimation by glucose oxygenase method respectively. The plasma samples were tested immediately for glucose estimation while serum samples were stored at -50°C for Human insulin estimation.

4.4.4 Human insulin estimation:

The serum samples were analyzed for the insulin content using ELISA technique (Section 3.3.7). 25 µl each of serum samples and Calibrators were pipetted out into appropriate wells and 100 µl of enzyme conjugate solution was added to each well followed by incubation on a plate shaker (700-900 rpm) for 1 hour at room temperature (l8-25°C). Then plates were washed by adding 350 µl of wash buffer into each well followed by complete aspiration, the washing process was repeated 5 times. After final wash, plate was inverted and tapped against absorbent paper. 200 µl of Substrate TMB was added to each well and the plate was incubated for 15 min at room temperature (l8-25°C). After incubation, 50 µl of 'stop solution' was added into each well and plate was placed on shaker for approximately 5 sec to ensure mixing. Optical density at 450 nm was measured within 30 min on microplate reader (BioRad®, Model 680) and human insulin concentration was calculated using Microplate Analysis 3.0.2 software.

4.4.5 Glucose estimation:

10 µl of plasma sample was mixed with 1 ml of glucose oxygenase reagent solution and incubated at 37°C for 10 min. After incubation sample was again mixed. Glucose standard and distilled water as a blank were treated in same manner as the plasma sample and the absorbance of glucose standard and sample against reagent blank was measured at 505 nm within 30 min.

The plasma samples were mixed with glucose oxygenase reagent and the samples were incubated at 37°C for 10 min for colour development. Optical density of the samples was measured at 505 nm. The glucose concentration was auto-calculated using Star® 21 glucose autoanalyzer.

4.4.6 Treatment of data:

4.4.6.1 Treatment of Human insulin data:

Human insulin serum concentrations were estimated prior to dosing and at specified time interval after dosing. Pharmacokinetic parameters such as C_{max}, T_{max}, $T_{l/2}$ and AUC_{0-12} were

estimated from the serum Human insulin concentration versus time profile. Semilogarithmic plot of the serum Human insulin concentration versus time was constructed for estimation of pharmacokinetic parameters such as elimination rate constant K_e and plasma half life $T_{l/2}$. In addition, relative oral bioavailability of Human insulin was estimated for oral administration of nanoparticles as compared to subcutaneous injection. The relative oral bioavailability was calculated as follow,

$$\% \text{ Relative oral bioavailability} = (AUC_{Oral} / Dose_{Oal}) \times (Dose_{Sc.Inj.} / AUC_{Sc.inj.}) \times 100$$

4.4.6.2 Treatment of Glucose data:

Blood glucose concentrations were determined in triplicate prior to dosing and the mean concentration was considered as 100 percent level. All following concentration-time data were expressed as a fraction of the base line, considering the fact that blood glucose concentrations over 12 hour following intragastric administration of placebo nanoparticles of InsSA -2 to diabetic rats (control groups) were not significantly different from the base line (assuming a flat base line). The mean ± S.D. of each concentration-time point in each treatment group was calculated and compared.

4.5.6.3 Pharmacodynamic analysis:

The mean of percent blood glucose concentrations at each time point was subtracted from 100 percent and the area above the percent blood glucose-time curve ($AAC_{0\text{-}12h}$) were estimated by the trapezoidal rule reported by Touitou and Rubinstein [26].

4.5 RESULTS AND DISCUSSION:

4.5.1 Insulin nanoparticles characterization:

4.5.1.1 Appearance:

Visual appearance of the various formulations developed was noted and the observations are presented in Table 4.3. After lyophilization, a white, extremely porous, fragile and light weight cake was observed. The extreme porous cake formation after lyophilization can be attributed to the use of t-butanol as a solvent.

t-Butanol is reported to be versatile lyophilization medium as it has very high vapor pressure (26.8 mm Hg at 20°C) and high freezing point (24°C) [27]. During freeze drying, needle shaped crystals with high surface area are formed and sublimation of t-butyl alcohol takes heat away. This results in the maintenance of low product temperature which prevents the product from reaching the collapse temperature. All these effects help in acceleration of the sublimation rate and extreme porous cake formation after lyophilization. Table 4.4, represents the physical properties of water and t-butanol [27-29].

Table 4.3: Physical appearance of InsSA Human insulin nanoparticles

Formulation	**Appearance**
InsSA-2P	White, highly porous cake after lyophilization
InsSA-1	White, highly porous cake after lyophilization
InsSA-2	White, highly porous cake after lyophilization
InsSA-3	White, highly porous cake after lyophilization
InsSA-CD1	White, highly porous cake after lyophilization
InsSA-CD2	White, highly porous cake after lyophilization
InsSA-Z1	White, highly porous cake after lyophilization
InsSA-Z2	White, highly porous cake after lyophilization
InsSA-S1	White, highly porous cake after lyophilization
InsSA-S2	White, highly porous cake after lyophilization

Table 4.4: Physical properties of water and t-butanol [27-29]

Solvent	**Melting point °C**	**Boiling point °C**	**Vapor pressure at 25°C (kPa)**
Water	0	100	3.16
t-Butanol	25	82	5.49

4.5.1.2 Particle size:

Particle size of the human insulin nanoparticles as a function of addition time of zinc chloride solution into Insulin + HP-β CD solution was evaluated and the results are presented in Table 4.5. When zinc chloride solution was added to Human insulin + HP-β CD solution over 5

min with the help of fine needle syringe, slightly larger particle size with little wide distribution range (0.802 ± 0.181 µm) was observed. When zinc chloride solution addition time was extended over 10, 15 and 30 min, slight reduction in mean diameter was observed with little narrow distribution range. The mean diameter of the human insulin nanoparticles did not display large variation with addition time over 15 min. The mean particle size was about 0.526 ± 0.0.071 µm.

Table 4.5: Effect of addition time for zinc chloride solution on particle size of InsSA nanoparticles

Formulation	**$ZnCl_2$ solution addition time (min)**	**$ZnCl_2$ solution addition rate (ml/min)**	**Mean diameter (µm)***
InsSA-2	5	~ 1.50	0.802 ± 0.181
	10	~ 0.75	0.618 ± 0.092
	15	~ 0.50	0.526 ± 0.071
	30	~ 0.25	0.543 ± 0.072

***** Mean and S.D. for three determinations

Particle size of the human insulin nanoparticles as a function of concentration of stearic acid in the final Human insulin nanoparticles was evaluated and the results are presented in Table 4.6. When stearic acid concentration in the nanoparticles was higher, larger particle size with wide distribution range (1.286 ± 0.469 µm) was observed. When stearic acid concentration was lower, the reduction in particle size with narrow distribution was observed. The mean diameter of the human insulin nanoparticles did not display large variation with stearic acid concentration at 250 mg. The mean particle size was about 0.511 ± 0.082 µm. This could be explained on the basis of limited solubility of stearic acid in t-butanol : water mixture and observed turbidity of the t-butanol : water solution after addition of higher amounts of stearic acid before addition of human insulin phase. At higher concentration excess stearic acid may be micro-precipitating in the t-butanol : water solution.

Table 4.6: Effect of stearic acid content on particle size of InsSA nanoparticles

Formulation	Stearic acid content	Mean diameter (µm)*
InsSA-S1	Low	0.554 ± 0.095
InsSA-2	Optimum	0.511 ± 0.082
InsSA-S2	High	1.186 ± 0.269

***** Mean and S.D. for three determinations

The size of the nanoparticles can be affected by many factors such as the preparation technique, the lipids used and the composition of the solution. The small particle size of the nanoparticles observed could be due to the design of the process of forming nanoparticles. The process involves ionic interaction between insulin and zinc ions in a controlled manner in presence of HP-β CD in solution form. It is well known from the literature that insulin molecules form hexamer in presence of certain concentration range of zinc ion [30]. Insulin hexamer is a large molecular association having six monomer units of insulin and this could be acting as a nucleation for the formation of nanoparticles as a first step. This can also be supported by the opalescent appearance of the insulin solution after addition of zinc chloride solution. At excess concentration of zinc, insulin precipitates to insulin-zinc crystals [31]. The rate of addition of zinc chloride solution into insulin + HP-β CD solution was found to influence the final particle size of the nanoparticles. The addition of insulin - zinc opalescent solution into previously saturated t-butanol with water (9 %v/v), maintained the opalescent appearance of the final solution before lyophilization. When insulin - zinc solution was added to pure t-butanol, micro-precipitation of the particles was observed and this could be due to salting out effect on the protein in pure t-butanol.

4.5.1.3 Scanning electron microscopy:

SEM images of the final Human insulin nanoparticles formulation (#InsSA-2) is presented in Figure 4.2. The SEM images confirm that the nanoparticles are circular in shape and well dispersed and separated on the surface.

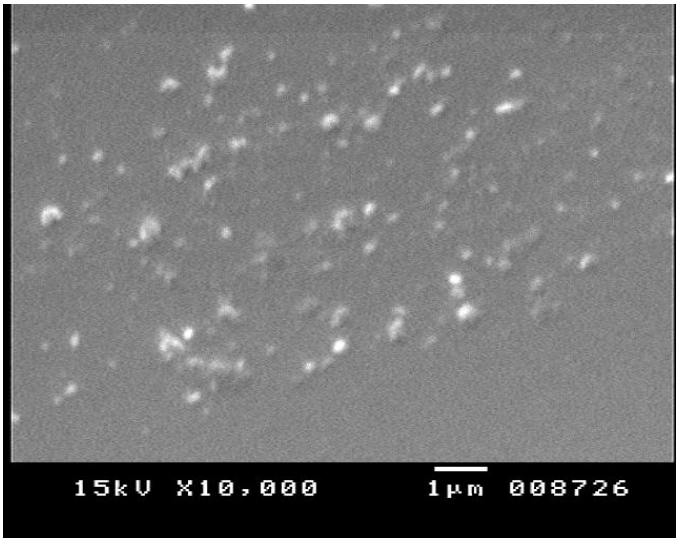

Figure 4.2: SEM image of InsSA nanoparticles

4.5.1.4 Insulin entrapment efficiency:

The entrapment efficiency of the nanoparticles was found to be satisfactorily high with an entrapment of 95.7 ± 1.2 %. The satisfactorily high entrapment efficiency could be attributed to the process of making the nanoparticles. It involved nucleation of insulin molecules by addition of zinc chloride solution in a controlled manner followed by entrapment into lipophilic coat using water miscible organic solvent, t-butanol followed by freezing and lyophilization to get the nanoparticles.

The entrapment efficiency of Human insulin in nanoparticles as a function of preparation technique and amount of hydrophobic coat deposition over insulin are tabulated in Table 4.7. Each formulation was tested in triplicate and average is reported.

Tablet 4.7: Insulin entrapment efficiency of the InsSA nanoparticles

Formulation	Composition variable	Entrapment efficiency* (%)
InsSA-2	Optimum composition	95.7 ± 1.2
InsSA-CD1	Low HP-β CD content	96.1 ± 1.4
InsSA-CD2	High HP-β CD content	88.7 ± 1.7
InsSA-Z1	Low zinc content	95.5 ± 1.5
InsSA-Z2	High zinc content	96.0 ± 0.9
InsSA-S1	Low stearic acid content	77.8 ± 1.9
InsSA-S2	High stearic acid content	96.3 ± 0.7

***** Mean in % and S.D. for three determinations

4.5.1.5 Residual water content:

Water content of the various Human insulin nanoparticle formulations were estimated after lyophilization by autotitration using Karl Fischer reagent. Each sample was analyzed in duplicate and the water content values obtained are reported in Table 4.8. Water content variation from 7.6 to 10.2 % w/w was observed when compared among various nanoparticle formulations.

Tablet 4.8: Residual water content of the InsSA nanoparticles

Formulation	Composition variable	Residual water content (% w/w)
InsSA-2	Optimum composition	7.6
InsSA-CD1	Low HP-β CD content	8.0
InsSA-CD2	High HP-β CD content	10.2
InsSA-Z1	Low zinc content	7.9
InsSA-Z2	High zinc content	8.6
InsSA-S1	Low stearic acid content	8.9
InsSA-S2	High stearic acid content	7.8

4.5.1.6 In-vitro release study:

Human insulin release profile from the nanoparticles are shown in Figure 4.3. Each is generally characterized by slow release rate. No burst effect was observed. The results are presented in Table 4.9. The possible explanation for the slow release rate in the pH 7.4 phosphate buffer, USP could be due to deposition of hydrophobic coat over insulin-zinc-HP β CD. Degradation of insulin observed over the extended time intervals indicating the instability of human insulin to the experimental conditions. The optimized formulation (InsSA-2) showed about 19% of insulin released after 2 hour while burst effect was observed high HP-β CD content and low stearic acid content. It could be due to more leaching of insulin from the nanoparticles with reduced hydrophobicity of the nanoparticles.

Table 4.9: In-vitro release of human insulin from the InsSA nanoparticles

Formulation	**Composition variable**	**% release of human insulin***			
		0 hour	**1 hour**	**2 hour**	**3 hour**
InsSA-2	Optimum composition	0	12 ± 2.5	19 ± 2.0	18 ± 2.0
InsSA-CD1	Low HP-β CD content	0	8 ± 2.1	15 ± 1.2	16 ± 2.0
InsSA-CD2	High HP-β CD content	0	23 ± 1.5	38 ± 2.6	36 ± 1.5
InsSA-Z1	Low zinc content	0	12 ± 1.2	19 ± 2.6	20 ± 2.6
InsSA-Z2	High zinc content	0	11 ± 3.0	18 ± 2.0	18 ± 2.0
InsSA-S1	Low stearic acid content	0	35 ± 3.0	42 ± 3.5	40 ± 2.5
InsSA-S2	High stearic acid content	0	8 ± 2.0	15 ± 3.0	16 ± 1.5

***** Mean in % and S.D. for three determinations.

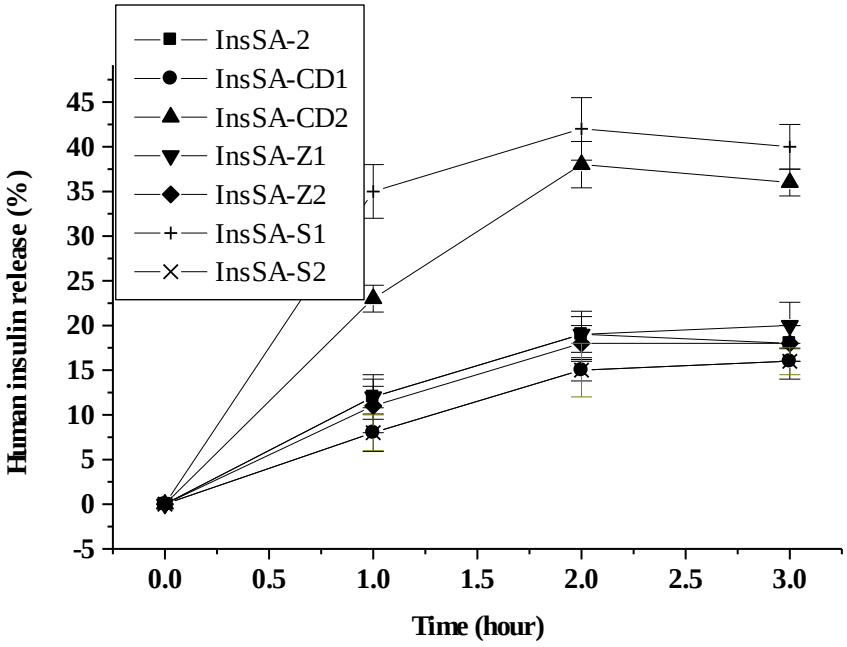

Figure 4.3: In-vitro release of human insulin from the InSA nanoparticles

4.5.1.7 In-vitro stability of Human insulin nanoparticles in presence of proteolytic enzymes:

The stability of Human insulin solution and Human insulin nanoparticles in presence of proteolytic enzyme is depicted in Table 4.10 and Figure 4.4. It can be seen that when insulin solution was incubated with α-chymotripsin, ≤ 20% of Human insulin remained undegraded after 30 min. Human insulin nanoparticles (InsSA-2) incubated in the same medium, ≥ 80% of entrapped insulin remained undegraded over the same period. Each sample was analyzed in triplicate.

The protection against enzymatic degradation found to be reduced with lower concentration of stearic acid and could be due to higher in-vitro release of insulin from the formulation.

Nanoparticles formulations with high content of HP-β CD showed different behavior irrespective of their in-vitro human insulin release behavior, ≥ 68 % of entrapped insulin remained undegraded over the same period. Stabilization effect of HP-β CD on insulin in presence of proteolytic enzyme was observed.

Table 4.10: In-vitro stability of InsSA nanoparticles in presence of α-chymotripsin

Formulation	Composition variable	% undegraded Human insulin* after 30 min
Human insulin crystals	-	18 ± 3
InsSA-2	Optimum composition	83 ± 2
InsSA-CD1	Low HP-β CD content	72 ± 3
InsSA-CD2	High HP-β CD content	68 ± 4
InsSA-Z1	Low zinc content	78 ± 2
InsSA-Z2	High zinc content	80 ± 3
InsSA-S1	Low stearic acid content	54 ± 5
InsSA-S2	High stearic acid content	86 ± 2

***** Mean in % and S.D. for three determinations.

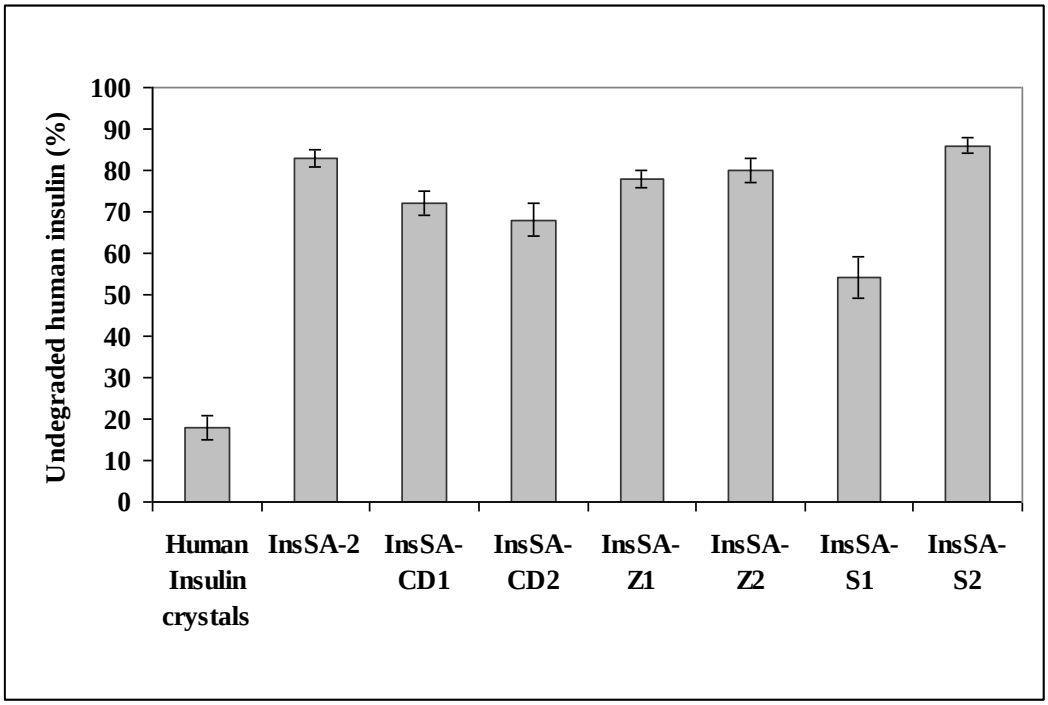

Figure 4.4: In-vitro stability of InsSA nanoparticles in presence of α-chymotripsin after 30 min

4.5.1.8 Biological activity evaluation of entrapped Human insulin:

Biological activity of entrapped Human insulin in the nanoparticles was estimated using ELISA technique. The results obtained are presented in Table 4.11 and Figure 4.5. It can be seen that biological activity of Human insulin from the nanoparticles was ≥ 95.2%. Samples

were analyzed in triplicate. There was no considerable loss of biological activity observed indicating the nanoparticle formulation components and the process of making nanoparticles preserved the integrity of three dimensional structure of Human insulin. Proteins are fragile molecules with liable bonds and reactive side chains, disruption of this complex structure can lead to loss of biological activity. ELISA Human insulin assay measures biologically active insulin with high degree of specificity, using a pair of mouse monoclonal antibodies. The full biological activity of a protein is dependent on preserving the integrity of its three dimensional structure. ELISA results suggest that these nanoparticles are capable of preserving biological activity of entrapped insulin in presence of hydrophobic components (stearic acid, α-tocopherol acetate) and polar organic solvent (t-butanol).

Table 4.11: Biologically active human insulin content in the InsSA nanoparticles

Formulation	**Composition variable**	**% of biologically active human insulin***
InsSA-2	Optimum composition	96.1 ± 0.8
InsSA-CD1	Low HP-β CD content	89.4 ± 0.7
InsSA-CD2	High HP-β CD content	98.2± 0.4
InsSA-Z1	Low zinc content	90.2 ± 0.6
InsSA-Z2	High zinc content	95.4 ± 0.5
InsSA-S1	Low stearic acid content	93.8 ± 0.7
InsSA-S2	High stearic acid content	94.6 ± 0.5

***** Mean in % and S.D. for three determinations.

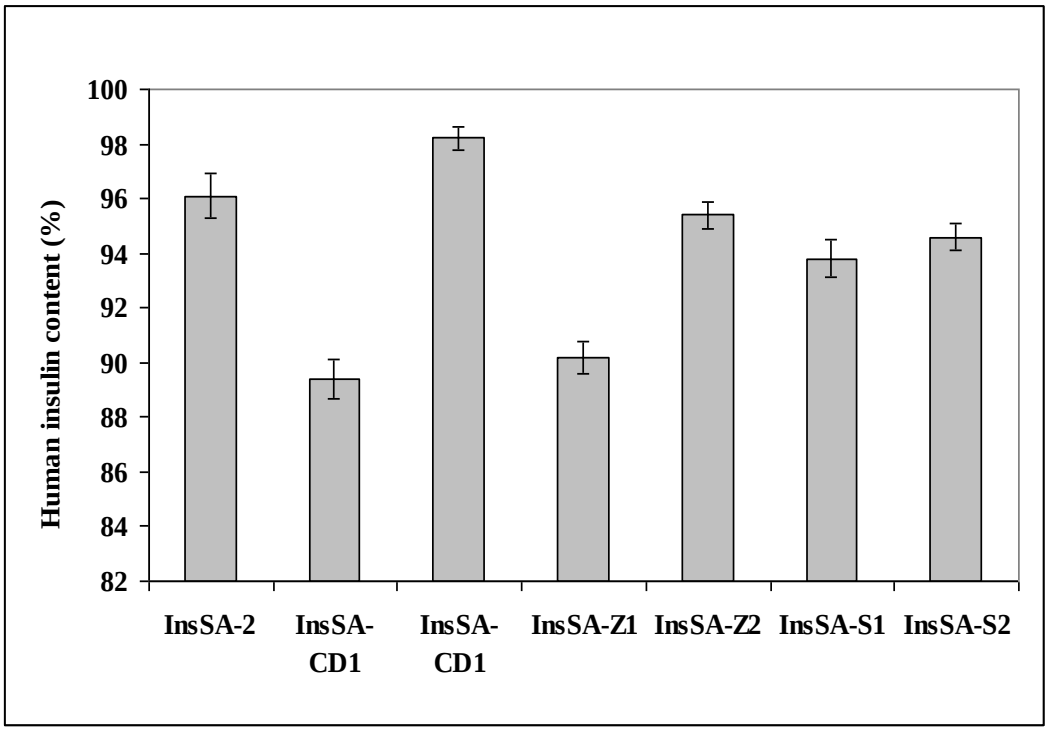

Figure 4.5: Biologically active human insulin content in the InsSA nanoparticles

4.5.1.9 Stability data:

Human insulin nanoparticles stored in nitrogen flushed vials at 2-8°C were tested for biologically active Human insulin content and particle size analysis by dynamic laser light scattering at 0, 3, 6 and 12 month time interval. The data obtained is presented in Table 4.12. No considerable loss of biological activity of Human insulin was observed over the stipulated time interval, indicating Human insulin was stable in the nanoparticle formulation. Particle size estimation revealed marginal rise in the mean diameter. The satisfactory stability of the optimized formulation was observed. The lyophilized nanoparticles were stored in glass vial purged with nitrogen. In addition presence of zinc and HP-β CD in the formulation looks to be stabilizing human insulin even in presence of hydrophobic environment of stearic acid.

Table 4.12: Stability study results for InsSA nanoparticles stored at 2-8°C

Formulation	**% human insulin content***			
InsSA-2	Initial	3 Month	6 Month	12 Month
	96.1 ± 0.8	95.2 ± 0.7	93.8 ± 0.6	90.1 ± 0.7
	Mean particle size of the nanoparticles (µm)			
	0.526 ± 0.071	0.526 ± 0.071	0.622 ± 0.114	0.695 ± 0.124

***** Mean in % and S.D. for three determinations.

4.5.2 In-vivo studies:

4.6.3.1 Human Insulin estimation:

Human insulin nanoparticles (Group-3, Batch: #InsSA-2 at 10 IU/kg and Group-4, Batch: #InsSA-2 at 20 IU/kg) showed significantly high serum Human insulin concentration levels over the 4 to 12 hour period as compared to the placebo control (Group-1). Figure 4.6 shows the mean serum Human insulin concentration profile versus time for each group. The pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-t}, $T_{1/2}$ were estimated for each group and the values observed are reported in Table 4.17. Approximate dose proportionality was observed between the dose range of 10-20 IU/kg for AUC_{0-12h} as evaluated. Area under human insulin concentration curves, AUC_{0-12h} calculated from these curves was found to have a satisfactory correlation with dose of insulin ranging 10-20 IU/kg. AUC_{0-12h} of both the formulations depicted in Figure 4.8 show that the bioavailability of 20 IU/kg dose of nanoparticle formulation was almost similar to subcutaneous injection of 10 IU/kg insulin while the serum Human insulin concentration of the latter diminished after 6 hour of injection, the former seemed to be continuing its absorption and hypoglycemic activity (Figure 4.7). The relative oral bioavailability for 20 IU/kg dose and 10 IU/kg dose of nanoparticle formulation was 9.48% and 4.60% respectively.

Table 4.13: Mean serum human insulin concentration for Placebo InsSA nanoparticles (Batch: #InsSA-P), (Group-1); N=7

Time (hour)	Serum human insulin concentration (mIU/l)*			
	Mean	**S.D.**	**UL (95% CI)**	**LL (95% CI)**
0	2.006	0.964	2.720	1.292
0.5	3.686	1.218	4.588	2.784
1	4.326	1.519	5.451	3.201
2	4.022	1.576	5.190	2.854
4	4.993	2.059	6.518	3.468
6	4.245	1.350	5.245	3.245
9	4.362	0.769	4.932	3.792
12	3.599	1.241	4.518	2.680

***** Average of seven determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.14: Mean serum Human insulin concentration for subcutaneous human insulin injection (Group-2); N=8

Time (hour)	Serum human insulin concentration (mIU/l)*			
	Mean	**S.D.**	**UL (95% CI)**	**LL (95% CI)**
0	2.120	0.992	2.897	1.523
0.5	77.953	18.59	90.835	65.071
1	104.754	24.787	121.931	87.577
2	56.512	13.633	65.959	47.065
4	11.996	3.454	14.389	9.603
6	3.347	0.421	3.639	3.055
9	2.667	0.681	3.139	2.195
12	-	-	-	-

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.15: Mean serum human insulin concentration for oral InsSA nanoparticles (Batch: #InsSA-2), Dose: 10 IU/kg (Group-3); N=8

Time (hour)	Serum human insulin concentration (mIU/l)*			
	Mean	**S.D.**	**UL (95% CI)**	**LL (95% CI)**
0	2.253	2.051	3.674	0.832
0.5	4.735	4.737	8.018	1.452
1	5.480	4.453	8.566	2.394
2	13.921	9.609	20.580	7.262
4	18.725	10.865	26.254	11.196
6	9.763	6.617	14.348	5.178
9	5.727	4.588	8.906	2.548
12	4.647	3.979	7.404	1.890

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.16: Mean serum Human insulin concentration for oral InsSA nanoparticles (Batch: #InsSA-2), Dose: 20 IU/kg (Group-4); N=8

Time (hour)	Serum human insulin concentration (mIU/l)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	1.918	0.809	2.479	1.357
0.5	7.466	6.304	11.834	3.098
1	9.504	7.208	14.499	4.509
2	22.344	15.378	33.000	11.688
4	33.560	20.837	47.999	19.121
6	19.974	12.867	28.890	11.058
9	10.737	7.969	16.259	5.215
12	5.724	5.000	9.189	2.259

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

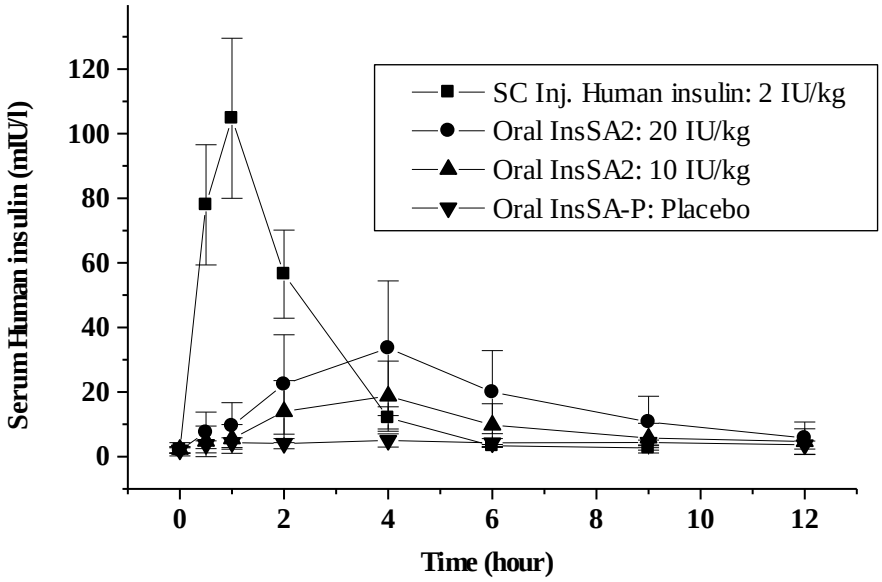

Figure 4.6: Comparative serum human insulin concentration versus time profile for Oral InsSA nanoparticles

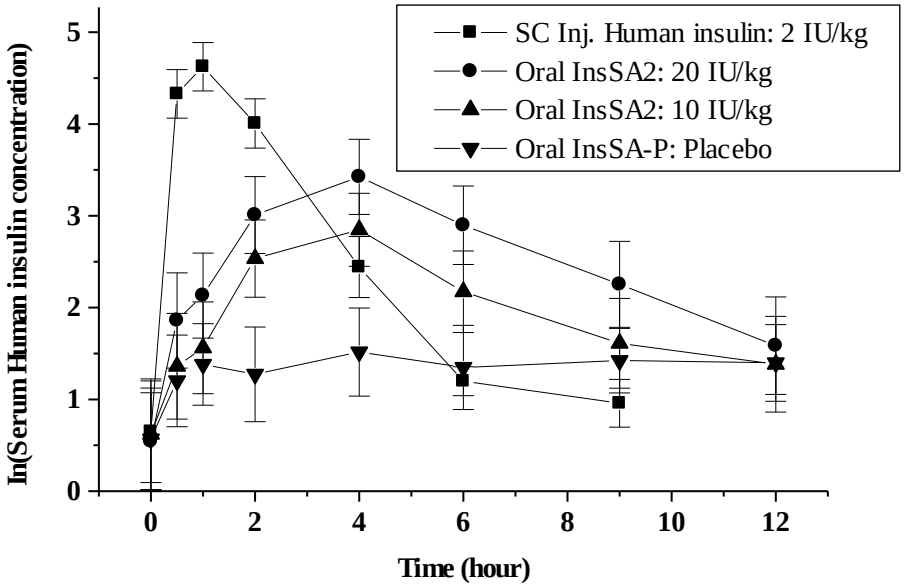

Figure 4.7: Semilogarithmic curve for Human insulin concentration versus time for Oral InsSA nanoparticles

Table 4.17: Pharmacokinetic parameters calculated from serum Human insulin concentration versus time profile for InsSA nanoparticles

Parameter	**Formulation results***		
	Subcutaneous injection of human insulin solution, 2 IU/kg *	**Oral, InsSA-2 nanoparticles, 20 IU/kg ****	**Oral InsSA-2 nanoparticles, 10 IU/kg ****
C_{max} (mIU/l)	104.7 ± 24.0	33.5 ± 18.4	18.7 ± 9.7
T_{max} (h)	1 ± 0.3	4 ± 0.2	4 ± 0.3
AUC_{0-12h} (mIU/l . h-1)	189.2 ± 40.8	178.4 ± 106.8	86.9 ± 53.0
K_e	0.754 ± 0.076	0.903 ±0.027	0.798 ± 0.025
$T_{1/2}$ (h)	0.927 ± 0.085	0.767 ± 0.022	0.869 ± 0.027
Relative oral bioavailability (%)	-	9.48	4.60

***** Mean and S.D. for seven determinations

****** Mean and S.D. for eight determinations

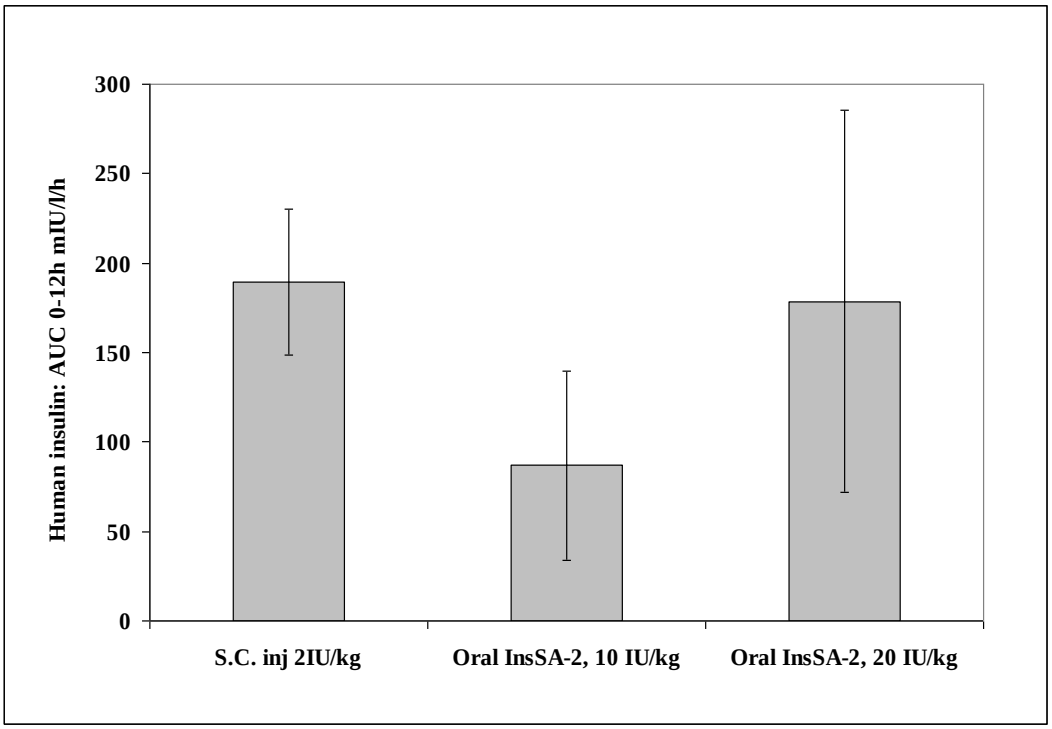

Figure 4.8: Comparison of serum Human insulin AUC_{0-12h} for intragastrically administered oral formulation InsSA-2 at 10 IU/kg and 20 IU/kg with subcutaneous injection of Human insulin injection (2 IU/kg)

4.5.2.2 Glucose estimation:

Human insulin nanoparticles (Batch: #InsSA-2; Group-3 and Group-4) significantly reduced the blood glucose levels over the period of 4 to 12 hour compared to the placebo control (Group-1). Figure 4.9 shows the mean plasma % glucose fraction from the base line versus time profile for each group. Hypoglycemic effect of the 20 IU/kg dose (Table 4.21) was higher than 10 IU/kg dose (Table 4.20) and hypoglycemic effect for 20 IU/kg dose was almost similar to that with 2 IU/kg subcutaneous dose (Tablet 4.19) with respect to the extent of reduction in glucose concentration from base line (~ 56% for 20 IU/kg - intragastrically administered Human insulin nanoparticles and ~78.6 % of baseline for 2 IU/kg subcutaneously injected Human Insulin solution). Area above glucose concentration curves, $AAC_{0\text{-}12h}$ calculated from these curves were found to have a satisfactory correlation with dose of insulin ranging 10 to 20 IU/kg (Figure 4.10), the $AAC_{0\text{-}12h}$ was found to be almost double with 20 IU/kg dose as compared to 10 IU/kg dose. $AAC_{0\text{-}12h}$ of both the formulations depicted in Figure 4.8 show that the effect of 20 IU/kg dose of nanoparticle formulation was almost similar to subcutaneous injection of 2 IU/kg insulin (Figure 4.10), while the effect of the latter diminished 6 h after injection), the former seemed to be continuing its hypoglycemic activity.

Table 4.18: Mean plasma Glucose concentration for Placebo InsSA (Group-1) N= 7

Time (hour)	**Mean plasma glucose concentration (mg/dl)***			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	314.4	28.9	335.8	293.0
0.5	319.2	36.4	346.2	292.2
1	325.1	19.0	339.2	311.0
2	354.2	29.0	375.7	332.7
4	334.0	36.3	360.9	307.1
6	357.5	38.1	385.7	329.3
9	337.1	22.5	353.8	320.4
12	366.1	23.8	383.7	348.5

***** Average of seven determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.19: Mean plasma Glucose concentration for subcutaneous human insulin injection (Group-2) N= 8

Time (hour)	Mean plasma glucose concentration (mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	340.5	60.9	382.7	298.3
0.5	135.7	67.9	182.8	88.6
1	72.0	46.5	104.2	39.8
2	108.2	33.2	131.2	85.2
4	199.4	55.6	237.9	160.9
6	311.1	44.7	342.1	280.1
9	353.6	14.9	363.9	343.3
12	374.6	17.1	386.4	362.8

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.20: Mean plasma Glucose concentration for oral InsSA nanoparticles, Batch: #InsSA-2, Dose: 10 IU/kg (Group-3) N= 8

Time (hour)	Mean plasma glucose concentration (mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	391.9	44.2	422.5	361.3
0.5	392.1	46.3	424.2	360.0
1	368.1	43.1	398.0	338.2
2	342.2	38.5	368.9	315.5
4	291.7	53.7	328.9	254.5
6	323.7	39.4	351.0	296.4
9	363.9	42.1	393.1	334.7
12	374.4	40	402.1	346.7

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 4.21: Mean plasma Glucose concentration for oral InsSA nanoparticles, Batch: #InsSA-2, Dose: 20 IU/kg (Group-4) N= 8

Time (hour)	Mean plasma glucose concentration (mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	373.4	39.1	400.5	346.3
0.5	359.5	40.6	387.6	331.4
1	322.7	50.3	357.6	287.8
2	284.0	62.4	327.2	240.8
4	163.9	59.9	205.4	122.4
6	257.6	81.9	314.4	200.8
9	311.8	63.7	355.9	267.7
12	327.9	40.7	356.1	299.7

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

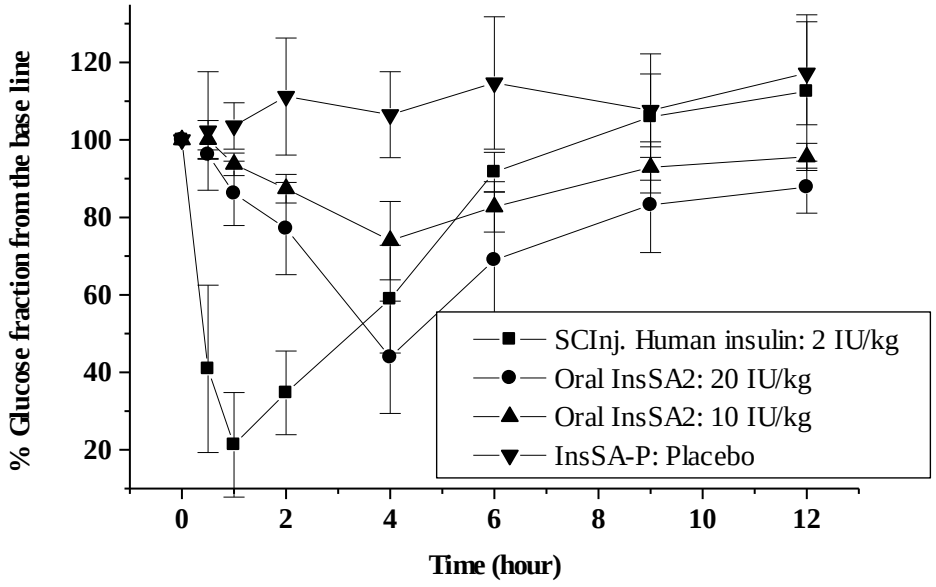

Figure 4.9: Mean plasma % glucose fraction of the base line versus time profile for InSA nanoparticles

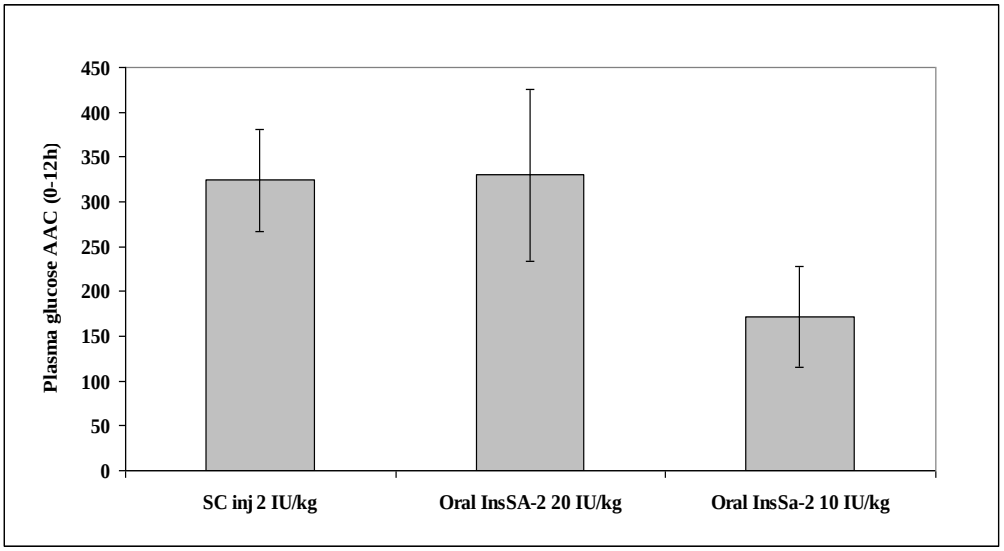

Figure 4.10: Comparison of area above the plasma % glucose concentration versus time profile (% plasma glucose.h), $AAC_{0\text{-}12h}$, for subcutaneous injection and oral InsSA nanoparticles.

4.6 CONCLUSION:

Insulin when formulated in presence of hydrophobic materials has been known to possess physical (agglomeration) and chemical instability and loss of its biological activity. In the present work, the issues of improvement in stability and oral bioavailability of insulin in the form of hydrophobic nanoparticles formulation has been addressed. The formulation developed with use of stearic acid, α-tocopherol acetate and soya phosphatidyl choline nanoparticles was found to possess good physical and chemical stability at long term storage at 2-8°C and retaining the biological activity of insulin in the formulation. The use of zinc and hydroxyproyl β cyclodextrin has been shown to improve the stability of Human insulin in the formulation. The formulation has also been shown to retain the biological activity of insulin as estimated by ELISA technique.

The particle size (0.511 ± 0.082 μm) of the formulation was achieved by unique process of ionic interactions, solvent diffusion followed by lyophilization using mixture of t-butanol and water as a solvent system with excellent entrapment efficiency (95.7 ± 1.2) . The rate of addition of zinc chloride solution into insulin – HP β CD solution was found to influence the

particle size of the nanoparticles, slow addition resulted into fine particle size. The particle size of the nanoparticles was also found to increase with increasing stearic acid content.

The duration of in-vitro insulin release was found to get extended beyond 3 hour from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C.

The nanoparticle formulation was found to protect Human insulin from proteolytic enzyme degradation in-vitro. Reducing concentration of stearic acid content found to reduce the enzymatic protection probably due to higher in-vitro release of insulin. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. ELISA technique allowed detecting the Human insulin absorbed from formulation in the rat. The oral dose of 20IU/kg of nanoparticle formulation was found to be comparable with 2 IU/kg subcutaneous injection of Human insulin injection in terms of AUC 0-12h for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma glucose % glucose reduction. C_{max} for Human insulin and extent of plasma glucose reduction was lower with oral formulation as compared to subcutaneous injection of Human insulin solution, but the serum Human insulin concentration of the latter diminished after 6 h of injection, the oral formulation seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose and 10 IU/kg dose of nanoparticle formulation was 9.48% and 4.60% respectively.

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CHAPTER 5

INSULIN-EGG YOLK DISPERSIONS IN SELF-MICROEMULSIFYING SYSTEM

5. INSULIN-EGG YOLK DISPERSIONS IN SELF-MICROEMULSIFYING SYSTEM

5.1 INTRODUCTION:

Liquid emulsions have been used to deliver proteins and peptide orally. Emulsions are thought to protect the drug from chemical and enzymatic breakdown in the intestinal lumen. Drug absorption enhancement is dependent on the type of emulsifying agent, particle size of the dispersed phase, pH, solubility of drug, type of lipid phase used, etc. Water-in-oil microemulsions have been shown to enhance oral bioavailability of proteins and peptides [1,2]. The lipid phase of micro emulsion composed of medium chain fatty acid triglycerides increased the bioavailability of muramyl dipeptide analog [3].

In recent years, the effective development of self-emulsifying microemulsions or mixed micelle-based lipid formulations products, such a Sandimmune Neoral® (cyclosporine) [4], Norvit® (ritonavir) [5] and Fortovase® (saquinavir) [6], has substantially increased interest in the application of lipid-based micelle formulation to improve oral delivery of poorly water soluble drugs as well as proteins and peptide drugs [7]. However stability of the protein in presence of hydrophobic components and entrapment of hydrophilic molecules like proteins are first limiting factors of effective oral delivery for proteins like insulin followed by selection and ability of the lipid components to enhance oral absorption of proteins by transcellular and lymphatic transport.

Egg yolk is one of the components of egg which mainly contains proteins, carbohydrates, lipids, minerals and water. The composition of the hen egg is presented in Table 5.1. It has excellent emulsifying properties and the phospholipids extracted from the egg yolk are mainly been utilized as emulsifier for making emulsions and in liposome preparations [8]. Effect of egg yolk as penetration enhancer and to improve the stability of protein drugs is relatively less studied. Especially for insulin no reference was found from the literature search for use as penetration enhancer or to improve the stability of formulations for oral delivery.

Table 5.1: Chemical composition of Hen egg (Average values, g/egg) [8]

	Water	**Proteins**	**Free sugar**	**Oligosaccharides**	**Lipids**	**Mineral**	**Total**
Egg yolk	9.1	3.1	0.1	0.1	5.8	0.3	18.5
Egg white	28.9	3.5	0.1	0.2	-	0.2	32.9
Egg shell and membrane	0.1	0.4	-	-	-	5.9	6.4
Total	38.1	7.0	0.2	0.3	5.8	6.4	57.8

Table 5.2: Inorganic element content of hen egg [9]

Inorganic element	**Egg yolk (mg/egg)**	**Egg white (mg/egg)**	**Egg shell (mg/egg)**
Na+	13	53	-
Mg++	24	3	20
P	110	6	20
S	3	64	Trace
Cl^-	23	51	-
K+	21	55	-
Ca++	27	4	2210
Fe+++	2	Trace	Trace
Total	223	236	2250

Contrary to the egg white, egg yolk is richer in lipids than proteins. The egg yolk contains 15.7-16.6% protein, 31.8-35.5% lipid, 0.2-1.0% carbohydrate and 1.1% ash in water [10].

Lipids, the main components of the egg yolk, comprise about 60% of the egg yolk based on dry weight. The lipids include triglycerides, phospholipids, cholesterol, cerebroside and some other minor lipids. The major fatty acids are oleic, palmitic, linoleic and stearic acid

The major fatty acids in egg yolk triglycerides are oleic acid (C18:1) and palmitic acid (C16:0). Linoleic acid (C18:2) and stearic acid (C18:0) [8]. Fatty acids like myristic acid

(C14:0) and others are found only in trace amounts. The fatty acid composition of hen egg is presented in Table 5.3.

The major components of egg yolk phospholipids are phosphatidylcholine and phosphatidylethanolamine. Lysophosphatidylcholine and lysophosphatidylethanolamine are also present. Phosphatidylcholine is a precursor of acetylcholine which functions as a neurotransmitter.

Almost all of the sterol found in egg yolk is cholesterol. Cholesterol contributes about 1.6% of raw egg yolk and about 5% of egg yolk lipids. Free cholesterol is about 84% of the total cholesterol with the remaining 16% being cholesterol ester [11].

Cerebrosides are classified as glycolipids. They are composed of a sugar (galactose or sucrose), sphingosine and a nitrogen-containing base. Two cerebrosides (ovophrenosin and ovokerasin), which differ in their fatty acid content, have been separated from egg yolk [13]. Ovophrenosin is dextrorotatory where as ovokerasin is levorotatory.

Table 5.3: Fatty acid composition of hen egg [12]

Fatty acid	**Amount (mg/100g egg)**	**Proportion (%)**
Palmitic acid	2030 ± 215	23.7 ± 0.4
Stearic acid	670 ± 66	7.8 ± 0.1
Arachidic acid	13 ± 1	0.2 ± 0.1
Total saturated fatty acids	2772 ± 284	32.4 ± 0.4
Palmitoleic acid	253 ± 14	3.0 ± 0.1
Oleic acid	3687 ± 230	43.1 ± 1.2
Vaccinic acid	194 ± 18	2.3 ± 0.1
Eicosaenoic acid	23 ± 3	0.3 ± 0.1
Total monounsaturated fatty acids	4258 ± 272	49.8 ± 1.3
Linoleic acid	1141 ± 168	13.3 ± 0.8
Arachidonic acid	170 ± 21	2.0 ± 0.1
Adrenic acid	16 ± 3	0.2 ± 0.1
4,7,10,13,16-Docosapentaenoic acid	31 ± 10	0.4 ± 0.1
Total omega-6 fatty acids	1385 ± 206	16.1 ± 1.0
Linolenic acid	41 ± 2	0.5 ± 0.1
Ecosapentaenoic acid	-	-
7,10,13,16,19-Docosapentaenoic acid	11 ± 1	0.1 0.1
4,7,10,13,16,19-Docosapentaenoic acid	73 ± 10	0.9 ± 0.1
Total omega-3 fatty acids	126 ± 14	1.5 ± 0.1

In this study, we tried to evaluate the combined use of egg yolk and self microemulsifying system to improve the oral bioavailability and stability of insulin.

The process for the preparation of delivery system greatly influence the encapsulation efficiency and stability of the protein drug, especially when protein molecule comes in contact with hydrophobic surfaces, air-water interfaces, shear stress, temperature and organic solvents. In addition, size of the particles has been shown to have influence on the oral absorption [14-16]. In this work, process was optimized to produce well controlled particle size and high encapsulation efficiency of insulin in the self microemulsifying system.

Lyophilization technique for the preparation of insulin-egg yolk dispersions was designed to improve to the stability and encapsulation efficiency of insulin in the self microemulsifying delivery vehicle.

In this study, different ways and approaches including process and formulation components were investigated by considering all above factors to stabilize and improve the oral absorption of insulin.

5.2 MATERIALS AND METHODS:

Human insulin (Recombinant DNA origin, Akzo Nobel), Tris hydroxymethylaminomethane (Tris buffer, Merck), m-Cresol (Merck), Hydrochloric acid 36%, (Merck), Sodium hydroxide pellets, (Merck), Zinc oxide, anhydrous (Merck), Egg yolk (Hen), Maisine® 35-1 (Monoglyceride of lineloic acid; Gattefosse), Cremophore® RH 40 (BASF), α-Tocopherol acetate (Sigma Aldrich), Polysorbate 80 (ICI India), Propylene glycol, Transcutol® (Gattefosse) , Glycerin (Indian Glycol).

5.2.1 Preparation of Tris buffer pH 7.4:

Tris buffer solution pH 7.4 (10 mM) was prepared by dissolving 1.211g of Tris (hydroxymethyl)aminomethane in 1000 ml distilled water to get final buffer concentration of 10 mM. pH was adjusted to 7.4 using 0.01N HCl or 0.1N NaOH solution.

5.2.2 Preparation of Human insulin solution - 200 IU/ml:

Human insulin crystals (equal to 200 IU/ml) were suspended in 20 ml purified water. m-Cresol, glycerin and zinc oxide were added to this insulin suspension to get final solution concentration of 1.76 mg/ml, 16mg/ml and 0.051 mg/ml, respectively. The pH of the solution was raised to 2.5 ± 0.05 using 10 %v/v HCl under slow stirring to completely dissolve Human insulin. After formation of clear solution, pH was raised to 7.4 ± 0.05 using 1N NaOH. Tris buffer (50 mg/ml) pH 7.4 ± 0.05 solution was added to get final buffer concentration of 1 mg/ml. Final volume of the solution was adjusted to 25 ml using purified water to get Human insulin concentration of 200 IU/ml.

5.2.3 Preparation of Human insulin - Egg yolk dispersion (InsEY):

Yolk from the chicken eggs was separated by decantation and used for preparation of solid dispersion. Human insulin solution (200 IU/ml) of 2.5 ml, 5 ml and 10 ml was added to 4 g solids of egg yolk (equivalent to 8.5 g of egg yolk liquid), respectively to get final Human insulin content of 0.125 IU/mg, 0.250 IU/mg and 0.500 IU/mg. The resultant dispersion was mixed on a magnetic stirrer (Remi®, India) for 10 to 15 min. The dispersion was transferred to tubular glass vials and frozen at -40°C for 3 hour and lyophilized for 38 hour at condenser temperature of -40°C and pressure of 112 x 10^{-3} mbar. After complete drying vacuum was broken with nitrogen gas to purge the vials with nitrogen and capped with butyl stopper and aluminum seals. Vials were stored at 2-8°C. The composition of insulin-egg yolk dispersions with varying insulin content are presented in Table 5.4.

Table 5.4: Composition of insulin-egg yolk dispersions (InsEY)

Ingredients	**Formulations**			
	InsEY-P*	**InsEY-1**	**InsEY-2**	**InsEY-3**
Human insulin solution, 200 IU/ml	-	2.5 ml	5 ml	10 ml
Egg yolk (equivalent to solids)	4000 mg	4000 mg	4000 mg	4000 mg

***** Placebo dispersion of # InsEY-2

5.2.4 Preparation of Self microemulsifying solution (SMEDDS):

Self microemulsifying solution was prepared by mixing different percentage by weight of each ingredient. The final composition was optimized based on the ability of the solution to form microemulsion by varying the type of surfactant and co-surfactant and their percent content with respect to total weight of the final solution. The visual appearance of the solution after addition of 1 ml of final solution into 250 ml purified water was noted and compared for different formulations.

Self microemulsifying solutions was prepared by mixing Maisine® 35-1, 27.2 %w/v; α-Tocopherol acetate, 1.0 %w/v, Cremophore® RH 40, 36.8 %; Transcutol®, 11.4 % w/v; and Propylene glycol, 23.6 %w/v. The mixture was stirred for 30 min at room temperature to get clear solution (Table 5.5 and 5.6).

Table 5.5 Formulations of SMEDDS with varying qualitative composition

Components	**Formulation composition (% w/v)**		
	SMEDDS-1	**SMEDDS-2**	**SMEDDS-3**
Maisine® 35-1	27.2	27.2	27.2
α-Tocopherol acetate	1.0	1.0	1.0
Cremophore® RH 40	36.8	-	36.8
Transcutol®	11.4	11.4	11.4
Propylene glycol	23.6	23.6	-
Polysorbate 80	-	36.8	-
Ethanol 99%	-	-	-
Glycerin	-	-	23.6

Table 5.6 Formulations of SMEDDS with varying concentration of surfactant and co-surfactant

Components	**Formulation composition (% w/v)**			
	SMEDDS-4	**SMEDDS-5**	**SMEDDS-6**	**SMEDDS-7**
Maisine® 35-1	27.2	27.2	27.2	27.2
α-Tocopherol acetate	1.0	1.0	1.0	1.0
Cremophore® RH 40	33.2	40.4	36.8	36.8
Transcutol®	11.4	11.4	10.2	12.6
Propylene glycol	27.2	20.0	24.8	22.4

5.2.5 Preparation of Self microemulsifying solution containing insulin-egg yolk (InsEY -SMEDDS):

Lyophilized Human insulin-Egg yolk powder was dispersed in the above optimized microemulsion solution (SMEDDS-1) to get 40 IU/ml Human insulin content and mixed for 2 hour at room temperature (NMT 25°C) to get opaque dispersion. The solutions were stored

at 10-15°C till use in a glass vial (Table 5.7). The Flow diagram for the preparation of Human insulin – egg yolk dispersions in SMEDDS (InsEY-SMEDDS) is presented in Figure 5.1.

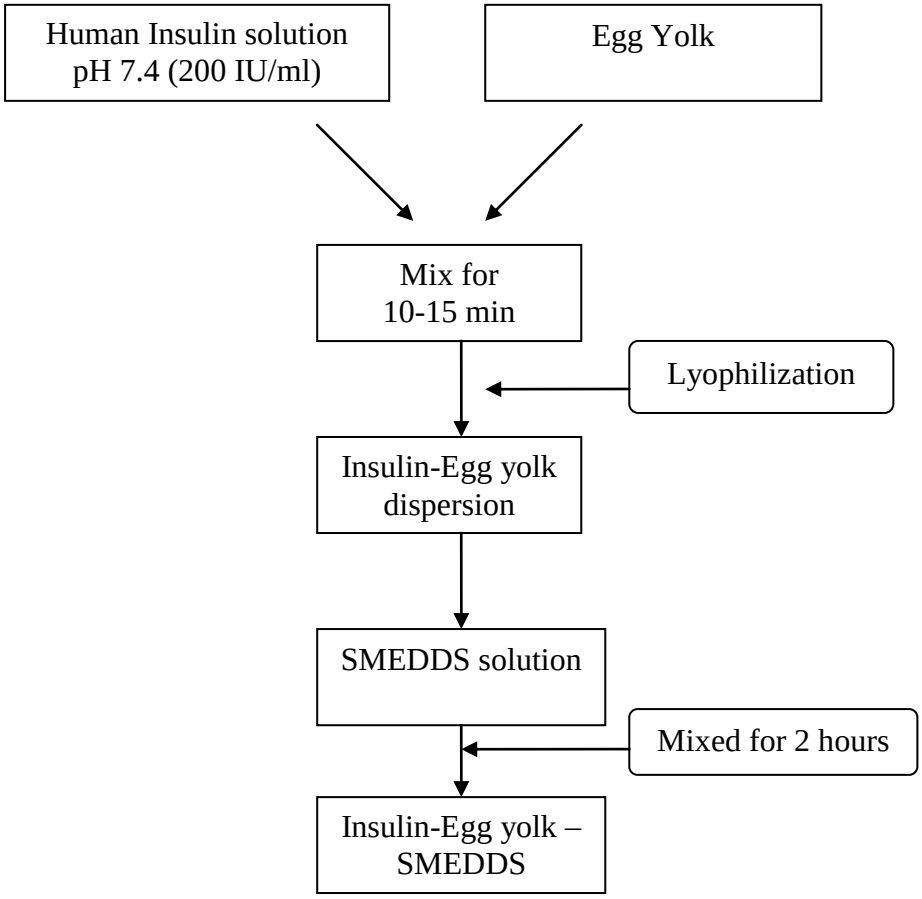
The compositions of various self microemulsifying solution formulations with varying concentration of insulin are presented in Table 5.7.

Table 5.7: Composition of Human insulin – egg yolk dispersions in SMEDDS (InsEY-SMEDDS)

Ingredients	**Formulations**			
	InsEY-MP*	**InsEY-M1**	**InsEY-M2**	**InsEY-M3**
Human insulin- egg yolk dispersion equivalent to IU of insulin	InsEY-P	40 IU/ml (InsEY-1)	80 IU/ml (InsEY-2)	120 IU/ml (InsEY-3)
Self microemulsifying solution (SMEDDS-1)	qs	qs	qs	qs

***** Placebo formulation of InsEY-M2
qs: Quantity sufficient

Figure 5.1: Flow diagram for the preparation of Human insulin – egg yolk dispersions in SMEDDS (InsEY-SMEDDS)



5.3 FORMULATION CHARACTERIZATION:

Developed formulations were subjected to following physicochemical characterization studies.

5.3.1 Appearance:

Visual appearance of the various InsEY dispersions, SMEDDS solutions and InsEY-SMEDDS dispersions were noted and compared. The samples were observed as such and after dispersing (each separately) in purified water at concentration of 0.1 %w/v for InsEY dispersion and 0.4 % v/v for SMEDDS and InsEY –SMEDDS dispersion.

5.3.2 Particle size:

The particle size of the various InsEY-SMEDDS formulations was measured using dynamic laser light scattering instrument (Brookhaven Instruments BI 90 Particle sizer, Holtsvilee, New York) after suitable dilution with 0.22 µm filtered Tris buffer pH 5.5 solution.

5.4.3 Scanning electron microscopy:

The morphology of the InsEY-SMEDDS dispersion was viewed using a conventional scanning electron microscope (JSM 5400, JOEL, Japan) at an accelerating voltage of 15kV. InsEY-SMEDDS samples were dispersed in 0.22 µm filtered Tris buffer pH 5.5 solution. One drop of the nanoparticle suspension was placed on a graphite surface. After air drying, the sample was coated with gold using ION Sputter.

5.3.4 Insulin entrapment efficiency:

The amount of Human insulin loaded into InsEY and InsEY -SMEDDS was estimated as follows. InsEY after lyophilization or lnsEY -SMEDDS (equal to 3 IU of Human insulin) was dispersed in 4 ml of 0.01N HCl and mixed for 5 min on vortex mixer (Remi® India) and sonicated for 10 to 15 sec at 20°C temperature. Then volume of the solution was adjusted to 5 ml and filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The filtered solution was quantitatively analyzed for Human insulin by HPLC (Section 3.2). The entrapment efficiency was calculated using following eq. (1)

EE (%) = (Amount of drug in the sample / Amount of drug added) × 100

5.3.5 Residual water content:

Residual water content of the InsEY sample was determined using Karl Fischer auto-titration (Metler®). 50 mg of InsEY after lyophilization was added and dispersed into anhydrous methanol and auto-titrated with Karl Fischer reagent.

5.3.6 In-vitro insulin release study:

The release rate of Human insulin from InsEY-SMEDDS was determined by suspending the dispersions (equal to 100 IU of Human insulin) in 50 ml, pH 7.4 phosphate buffer USP. During the experiment (4 hour) samples were shaken horizontally in a constant temperature shaker (Remi®, India) at 37 ± 1°C and 50 stokes per min. At scheduled time intervals, 1 ml of sample was removed and replaced with 1 ml of fresh release medium. The sample was ultracentrifuged at 22,000 rpm at 20°C for 15 min., then the supernatant was collected and quantitatively analyzed for Human insulin content by HPLC (Section 3.2)

5.3.7 In-vitro stability of insulin from InsEY-SMEDDS in presence of proteolytic enzymes:

The protection of InsEY-SMEDDS was evaluated in presence of α-chymotrypsin with specific activity of 51 U/mg protein at pH 7.8 (optimal pH for enzyme activity) and 37 ± 1°C. The InsEY-SMEDDS dispersions were suspended in 0.05M phosphate buffer pH 7.8 to which α-chymotrypsin had been added. α-Chymotrypsin was also added to insulin solution (as control). The final concentration of the insulin and the enzyme were 15 U/ml and 26 U/ml respectively. Samples were removed after 30 min and 0.5 ml of 0.1 % TFA was added to inactivate traces of enzyme present. Subsequently, 0.5 ml of ethanol 99% was added to each tube and sonicated for 10 to 15 sec. Then the volume was adjusted using a mixture of 0.01N HCl and ethanol (4:1) to get the Human insulin concentration of 0.5 to 0.6 IU/ml. The insulin solution was filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The filtered solution was quantitatively analyzed for Human insulin content by HPLC (Section 3.2).

5.3.8 Biological activity evaluation of entrapped Human insulin:

Biological activity of entrapped Human insulin in the InsEY-SMEDDS was assessed using ELISA technique. InsEY-SMEDDS (equal to 3 units of Human insulin) was dissolved in a mixture of phosphate buffer pH 7.4 and ethanol 99% with solvent ratio of 4:1. The solution was mixed for 5 min and then sonicated for 15 sec and volume adjusted to 5 ml using phosphate buffer pH 7.4 and filtered through 0.22 µm syringe disc filter (Ultipor®N66, Pall life Sciences). The solution was further diluted using 50 µl of filtrate to 200 ml with

phosphate buffer pH 7.4 to final concentration of about 150 mIU/l. An aliquot of sample was withdrawn from the solution and insulin content was analyzed by ELISA as per standard protocol. The optical density of the resultant solution was measured using micoplate reader (BioRad®, model 680 microplate reader) at 450 nm and the final concentration of the solution was calculated using Microplate Analyst 3.0.2 software (Section 3.3.8).

5.3.9 Stability studies:

Stability studies were performed for InsEY and InsEY –SMEDDS dispersions by storing them at 2-8°C temperature. The samples were analyzed at defined time interval for the insulin content by using ELISA technique as described in Section 3.3.8.

5.4 IN-VIVO STUDIES:

5.4.1 Animals

Male Wister rats (200-280 g) were bought at Raj Biotech (India) and housed in a light and temperature controlled environment. The in-vivo studies were performed with approval from Animal Ethics Committee. Urine was collected and its volume was recorded daily. Food and water consumption and weight of the rats was also monitored on a regular basis.

5.4.2 Induction of diabetes mellitus:

To induce diabetes mellitus, rats were injected with 40 mg/kg of streptozotocin (STZ) in citrate buffer (pH 4.5) via intraperitoneal route. The buffer was sterilized by filtration (0.22 µm filter membranes). Blood glucose levels were measured before and on days 2, 3 and 4 after STZ injection by separating the plasma (anticoagulant: sodium fluoride solution 2 mg/ml. The plasma samples were analyzed for glucose content by glucose oxygenase method (Reagent: Pinnacle Biotechnology Ltd, Mumbai) and measuring the optical density at 505 nm (Star® 21 Autoanalyzer). Rats were considered diabetic and included in the study when plasma glucose levels were near and above 300 mg/dl.

5.5.3 Dosing and sampling:

The study was performed on day 4 following STZ injection after overnight fast of 8 hour. Water was allowed ad libitum. Diabetic rats were divided into three groups, each containing 8 rats. Group-1: Placebo of InsEY- M2 (Batch: #InsEY-MP, equal to 20 IU/kg of insulin) was administered intragastrically to each diabetic rat as a control; Group-2: Human insulin injection (Huminsulin-R® 40IU/ml Injection, Elli Lily) was administered subcutaneously to each diabetic rat at a dose level of 2 IU/kg. This group served as reference standard; Group 3: InsEY-SMEDDS (Batch: #InsEY-M2 equal to 20 IU/kg of Human insulin) was administered intragastrically to each diabetic rat.

After administration of the dosage forms, 0.5 ml of purified water was administered. Prior to and at specified time intervals over 12 hour period blood samples (about 100 µl for glucose estimation and about 100 µl for serum human insulin estimation) were collected from retroorbital vein in capillary tubes for serum Human Insulin estimation and fluoride oxalate impregnated capillary tubes for glucose estimation. During collection of the blood samples, animals were anesthetized by ether inhalation. The samples were centrifuged for 10 min at room temperature, serum and plasma were separated for Human Insulin estimation by ELISA technique and Glucose estimation by glucose oxygenase method respectively. The plasma samples were tested immediately for glucose estimation while serum samples were stored at -50°C for Human insulin estimation.

5.4.4 Human insulin estimation:

The serum samples were analyzed for the insulin content using ELISA technique. 25 µl each of serum samples and Calibrators were pipetted out into appropriate wells and 100 µl of enzyme conjugate solution was added to each well followed by incubation on a plate shaker (700-900 rpm) for 1 hour at room temperature (l8-25°C). The plates were washed by adding 350 µl of wash buffer into each well followed by complete aspiration, the washing process was repeated 5 times. After final wash, plate was inverted and tapped against absorbent paper. 200 µl of Substrate TMB was added to each well and the plate was incubated for 15 min at room temperature (l8-25°C). After incubation, 50 µl of ‘stop solution’ was added into each well and plate was placed on shaker for approximately 5 sec to ensure mixing. Optical density at 450 nm was measured within 30 min on microplate reader (BioRad®, Model 680)

and human insulin concentration was calculated using Microplate Analysis 3.0.2 software (Chapter 3).

5.4.5 Glucose estimation:

10 µl of plasma sample was mixed with 1 ml of glucose oxygenase reagent solution and incubated at 37°C for 10 min. After incubation sample was again mixed. Glucose standard and distilled water as a blank were treated in same manner as the plasma sample and the absorbance of glucose standard and sample against reagent blank was measured at 505 nm within 30 min.

The plasma samples were mixed with glucose oxygenase reagent and the samples were incubated at 37°C for 10 min for colour development. Optical density of the samples was measured at 505 nm. The glucose concentration was auto-calculated using Star® 21 glucose autoanalyzer.

5.4.6Treatment of data:

5.4.6.1 Treatment of Human insulin data:

Human insulin serum concentrations were estimated prior to dosing and at specified time interval after dosing. Pharmacokinetic parameters such as C_{max}, T_{max}, $T_{l/2}$ and AUC_{0-12} were estimated from the serum Human insulin concentration versus time profile. Semilogarithmic plot of the serum Human insulin concentration versus time was constructed for estimation of pharmacokinetic parameters such as elimination rate constant K_e and plasma half life $T_{l/2}$. In addition, relative oral bioavailability of Human insulin was estimated for oral administration of nanoparticles as compared to subcutaneous injection. The relative oral bioavailability was calculated as follow,

$$\% \text{ Relative oral bioavailability} = (AUC_{Oral} / Dose_{Oal}) \times (Dose_{Sc.Inj.} / AUC_{Sc.inj.}) \times 100$$

5.4.6.2 Treatment of Glucose data:

Blood glucose concentrations were determined in triplicate prior to dosing and the mean concentration was considered as 100 percent level. All following concentration-time data were expressed as a fraction of the base line, considering the fact that blood glucose concentrations over 12 hour following intragastric administration of placebo of InsEY-SMEDDS dispersion to diabetic rats (control groups) were not significantly different from the base line (assuming a flat base line). The mean ± S.D. of each concentration-time point in each treatment group was calculated and compared.

5.4.6.3 Pharmacodynamic analysis:

The mean of percent blood glucose concentrations at each time point was subtracted from 100 percent and the area above the percent blood glucose-time curve ($AAC_{0\text{-}12h}$) were estimated by the trapezoidal rule reported by Touitou and Rubinstein [14].

5.5 RESULTS AND DISCUSSION:

5.5.1 Formulation characterization:

5.5.1.1 Appearance:

Visual appearance of the formulations developed was noted and the observations for InsEY, SMEDDS and InsEY -SMEDDS are presented in Table 5.8 and 5.9

Table 5.8: Physical appearance of the insulin-egg yolk dispersions

Formulation	Appearance (as such)	Appearance after dispersion in purified water
InsEY-1	Pale yellow cake	Pale yellow opaque liquid dispersion
InsEY-2	Pale yellow cake	Pale yellow opaque liquid dispersion
InsEY-3	Pale yellow cake	Pale yellow opaque liquid dispersion

Table 5.9: Physical appearance of InsEY -SMEDDS dispersions

Formulation	Variable	Appearance (as such)	Appearance after dispersion in purified water
SMEDDS-1	Optimum formulation	Light brown yellow colour liquid	++ Translucent solution
SMEDDS-2	With polysorbate 80	Light brown yellow colour liquid	+++ White turbid solution
SMEDDS-3	With glycerin	Light brown yellow colour liquid	+ White turbid solution
SMEDDS-4	With reduced amount of cremophore RH 40	Light brown yellow colour liquid	+ Translucent solution
SMEDDS-5	With increased amount of cremophore RH 40	Light brown yellow colour liquid	++ Translucent solution
SMEDDS-6	With reduced amount of transcutol	Light brown yellow colour liquid	+ Translucent solution
SMEDDS-7	With increased amount of transcutol	Light brown yellow colour liquid	++ Translucent solution

+ : represents the intensity.

5.5.1.2 Human insulin - Egg Yolk dispersion (InsEY):

A pale yellow coloured cake formation was observed after lyophilization of a mixture of Human insulin solution and egg yolk. When it was dispersed in purified water a pale yellow opaque solution was observed. This could be due to the emulsifying property of the egg yolk.

5.5.1.3 Self microemulsifying solution (SMEDDS):

The composition of self microemulsifying solution was optimized to get microemulsion which would form the translucent/opalescent solution after dispersing it into water (1 ml of SMEDDS in 250 ml of water). The effect of varying qualitative and quantitative composition change on the appearance after dispersing it in water was noted and compared in Table 5.9.

There was considerable impact on the appearance of microemulsion solution in water when the concentration of surfactant, Cremophore RH 40 (30.2 to 40.4 %w/v) and Transcutol (10.2 to 12.6% w/v) was varied. The translucent appearance was reduced to near translucent indicating the minimum amounts of surfactant and co-surfactant required to form self microemulsifying solution.

The change in the type of the surfactant to polysorbate 80 and diluent to glycerin resulted into turbid appearance after dispersion into water. The effect of these variations was directly related to increase in particle size. The results are presented under Section 5.5.1.4

5.5.1.4 Particle size:

Particle size of the SMEDDS solution and InsEY-SMEDDS dispersion in water was measured. For SMEDDS solution (SMEDDS-1) very fine particle size was observed with mean diameter of 0.387 ± 0.052 µm. The self microemulsifying solution after addition into water resulted into formation of nanoemulsion. When InsEY was dispersed into this SMEDDS system and added into water, the particle size increased to 1.023 ± 0.316 µm. It could be due to formation of dispersed particles of 1nsEY into SMEDDS system and the presence of phospholipids and variety of fatty acids in the egg yolk.

Particle size of the SMEDDS solution as a function of surfactant and co-surfactant concentration was evaluated and the results are presented in Table 5.10. When the surfactant concentration was reduced, increase in particles size 0.926 ± 0.251 µm was observed. When the concentration of surfactant was increased above the optimum level, no significant impact on the particle size was observed.

Formulation SMEDDS-1 was finalized for further studies based on its translucent appearance and smaller particles size when dispersed in purified water.

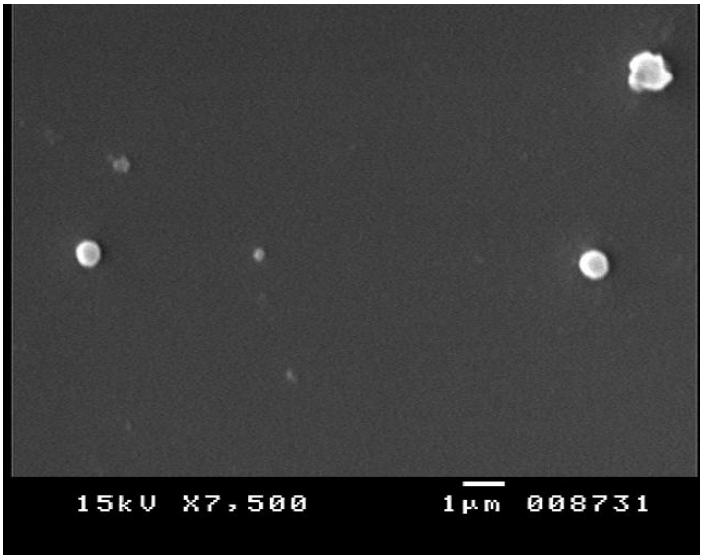
Table 5.10: Particle size of the SMEDDS solution after dispersion in water

Formulation	Variable	Mean diameter (µm)*
SMEDDS-1	Optimum formulation	0.387 ± 0.052
SMEDDS-2	With polysorbate 80	2.147 ± 0.748
SMEDDS-3	With glycerin	1.847 ± 0.548
SMEDDS-4	With reduced amount of cremophore RH 40	0.926 ± 0.251
SMEDDS-5	With increased amount of cremophore RH 40	0.375 ± 0.045
SMEDDS-6	With reduced amount of transcutol	0.687 ± 0.072
SMEDDS-7	With increased amount of transcutol	0.390 ± 0.063

***** Mean and S.D. for three determinations

5.5.1.5 Scanning electron microscopy:

SEM images of the final InsEY-SMEDDS formulation (#InsEY-M2) are presented in Figure 5.2. The SEM images confirm that the solid spherical particles of about 0.854 ± 0.110 µm are well dispersed and separated on the surface.



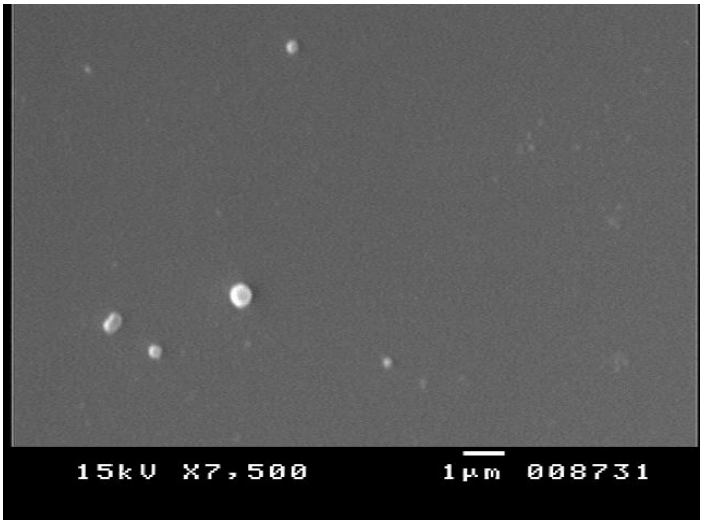


Figure 5.2: SEM images of InsEY-SMEDDS formulation

5.5.1.6 Insulin entrapment efficiency:

The entrapment efficiency of the InsEY and InsEY-SMEDDS were found to be satisfactory. The % entrapment found was 98.2 ± 0.9 %. This could be attributed to the process of making Human insulin-egg yolk dispersion by lyophilization.

The entrapment efficiency of Human insulin in InEY and InsEY-SMEDDS dispersions as a function of insulin content in the InsEY dispersions are tabulated in Table 5.11. Each formulation was tested in triplicate and average is reported.

Tablet 5.11: Insulin content of the InEY and InsEY-SMEDDS dispersions

Formulation	**Composition variable**	**Content (%)***
InsEY-1	0.25 IU/mg insulin concentration	95.7 ± 1.2
InsEY-2	0.5 IU/mg insulin concentration	96.1 ± 1.4
InsEY-3	1.0 IU/mg insulin concentration	97.7 ± 1.7
InsEY-M1	40 IU/mg insulin concentration	95.5 ± 1.5
InsEY-M2	80 IU/mg insulin concentration	98.2 ± 0.9
InsEY-M3	120 IU/mg insulin concentration	97.2 ± 1.9

***** Mean and S.D. for three determinations

5.5.1.7 Residual water content:

Water content of the various InsEY dispersions was estimated after lyophilization by autotitration using Karl Fischer reagent. Each sample was analyzed in duplicate and the water content values obtained are reported in Table 5.12. Water content variation from 6.1 to 8.4 % w/w was observed when compared among various nanoparticle formulations.

Tablet 5.12: Residual water content of the InsEY dispersions

Formulation	Composition variable	Water content (%)
InsEY-1	0.25 IU/mg insulin concentration	7.6
InsEY-2	0.5 IU/mg insulin concentration	6.6
InsEY-3	1.0 IU/mg insulin concentration	8.1

5.5.1.8 In-vitro release study:

Human insulin release profile from the InsEY-SMEDDS formulations are shown in Figure 5.3 and values observed are reported in Table 5.13. The release profile indicates initial burst effect followed by gradual rise in the level of insulin. The initial burst effect could be attributed to physical dispersion of insulin in egg yolk solids which releases the insulin while forming spherical particles when InsEY-SMED is added to pH 7.4 phosphate buffer, there after little slower release of insulin is observed, which could be due to the entrapment of insulin in the hydrophobic component such as phospholipids of egg yolk and SMEDDS microemulsion. The selected concentration of insulin does not affect the release behavior.

Table 5.13: In-vitro release of Human insulin from the InsEY-SMEDDS dispersions

Formulation	**Composition variable**	**% release of Human insulin***			
		0 hour	**0.5 hour**	**1 hour**	**2 hour**
InsEY-M1	40 IU/mg insulin concentration	0	38 ± 2.0	48 ± 2.5	56 ± 2.0
InsEY-M2	80 IU/mg insulin concentration	0	36 ± 3.5	49 ± 3.0	55 ± 3.0
InsEY-M3	120 IU/mg insulin concentration	0	42 ± 2.6	52 ± 3.6	60 ± 2.5

***** Mean in % and S.D. for three determinations

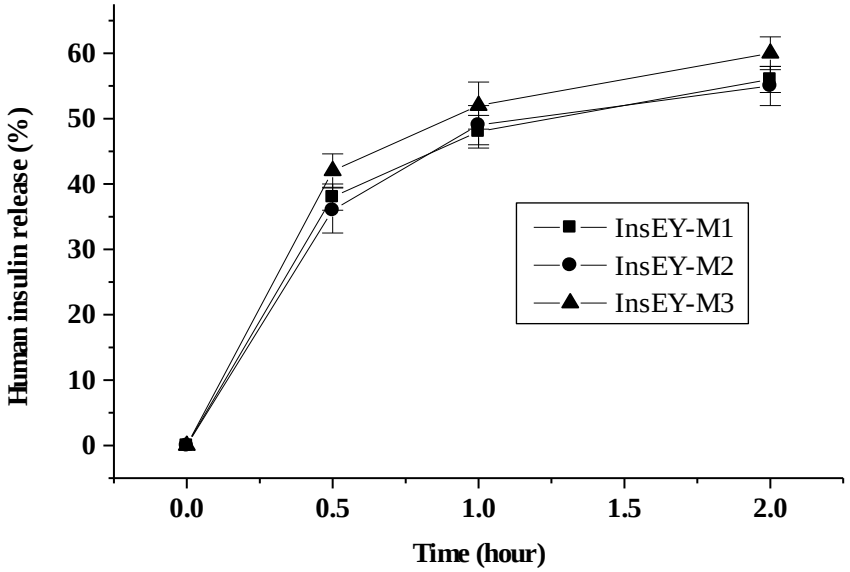


Figure 5.3: In-vitro release of Human insulin from the InsEY-SMEDDS dispersions

5.5.1.9 In-vitro stability of InsEY-SMEDDS dispersions in presence of proteolytic enzymes:

The stability of Human insulin solution and InsEY-SMEDDS formulations in presence of proteolytic enzyme is depicted in Table 5.14 and Figure 5.4. It can be seen that when insulin solution were incubated with α-chymotripsin, ≤ 15 % of Human insulin remained undegraded after 30 min. InsEY-SMEDDS incubated in the same medium, ≥ 54% of entrapped insulin remained undegraded over the same period. Each sample was analyzed in triplicate.

Table 5.14: In-vitro stability of InsEY-SMEDDS dispersions in presence of α-chymotripsin after 30 min

Formulation	Composition variable	% undegraded Human insulin* after 30 min
Human insulin crystals	-	17 ± 2
InsEY-M1	40 IU/mg insulin concentration	48 ± 3
InsEY-M2	80 IU/mg insulin concentration	51 ± 2
InsEY-M3	120 IU/mg insulin concentration	47 ± 3

*****Mean in % and S.D. for three determinations

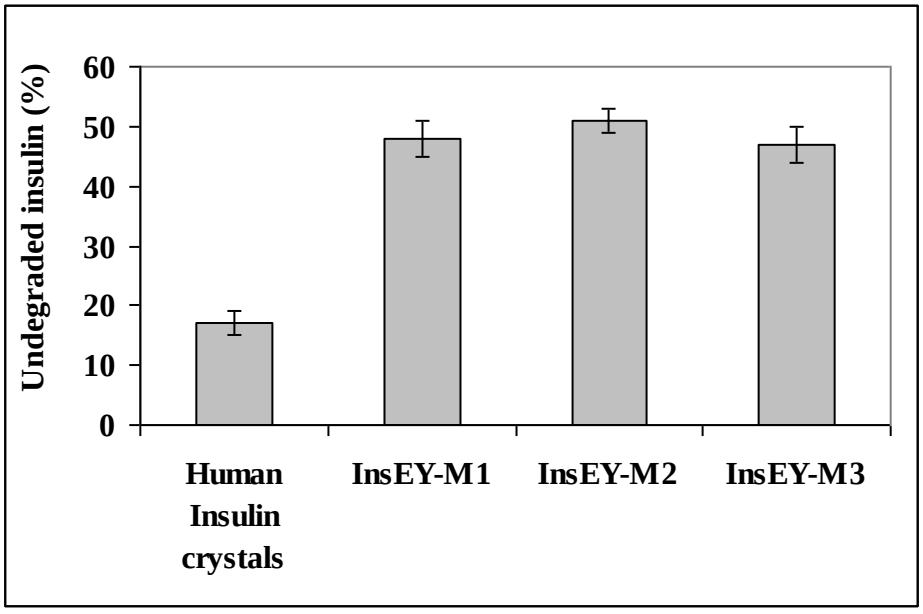


Figure 5.4: In-vitro stability of InsEY-SMEDDS dispersion in presence of α-chymotripsin after 30 min

5.5.1.10 Biological activity evaluation of entrapped Human insulin:

Biological activity of entrapped Human insulin in the InsEY-SMEDDS formulations was estimated using ELISA technique (Table 5.15 and Figure 5.5). It can be seen that biological activity of Human insulin from the InsEY-SMEDDS was ≥ 97.2% of the insulin content in the formulation. Samples were analyzed in triplicate. There was no considerable loss of biological activity observed indicating the InsEY-SMEDDS formulation components and the process of making the formulation preserved the integrity of the three dimensional structure

of Human insulin. Proteins are fragile molecules with liable bonds and reactive side chains, disruption of this complex structure can lead to loss of biological activity. ELISA Human insulin assay measures biologically active insulin with high degree of specificity, using a pair of mouse monoclonal antibodies. The full biological activity of a protein is dependent on preserving the integrity of its three dimensional structure. ELISA results suggest that these InsEY-SMEDDS dispersions are capable of preserving biological activity of entrapped insulin in presence of hydrophobic components soft egg yolk and SMEDDS solution.

Tablet 5.15: Biologically active Human insulin content in InsEY-SMEDDS formulations:

Formulation	**Composition variable**	**% of biologically active Human insulin**
InsEY-M1	40 IU/mg insulin concentration	98.1 ± 0.8
InsEY-M2	80 IU/mg insulin concentration	97.2 ± 0.7
InsEY-M3	120 IU/mg insulin concentration	97.8 ± 0.4

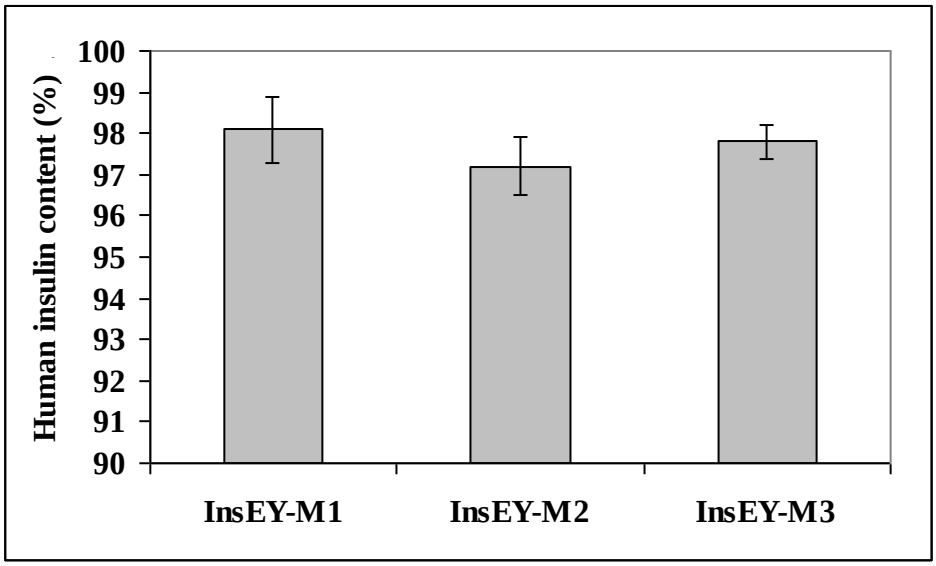


Figure 5.5: Biologically active Human insulin content in the InsEY-SMEDDS dispersions

5.5.1.11 Stability data:

InsEY and InsEY -SMEDDS dispersions were stored in nitrogen flushed vials at 2-8°C. The samples were tested for biologically active Human insulin content at 0, 1, 3 and 6 month time interval. The data obtained is presented in Table 5.16. No considerable loss of biological activity of Human insulin was observed over the stipulated time interval, indicating the stabilization of Human insulin in egg yolk.

Tablet 5.16: Stability of InsEY and InsEY-SMEDDS dispersions at 2-8°C

Formulation	Human insulin content (%)*			
	Initial	1 Month	3 Month	6 Month
InsEY-M2	98.5 ± 0.3	96.8 ± 0.5	94.2 ± 0.7	91.4 ± 0.6
InsEY-2	98.2 ± 0.5	97.4 ± 0.5	96.1 ± 0.4	93.2 ± 0.6

***** Mean in % and S.D. for three determinations.

5.5.2 In-vivo Studies:

5.5.2.1 Human insulin estimation:

Human insulin, InsEY-SMEDDS formulation (Group-3, Batch: #InsEY-M2 at 20 IU/kg) showed significantly high serum Human insulin concentration levels over the period of 4 to 12 hour as compared to the placebo control (Group-1, Batch: InsEY-MP). Figure 5.6 and 5.7 shows the serum Human insulin concentration profile versus time for each group. The pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-t}, $T_{1/2}$ were estimated for both group (Group-2 and Group-3) and the values obtained are reported in Table 5.20. AUC_{0-12h} of both the formulations depicted in Figure 5.8 show that the bioavailability of 20 IU/kg dose of InsEY-M2 formulation was slightly lower than subcutaneous injection of 2 IU/kg insulin (Figure 5.8), while the serum Human insulin concentration of the latter diminished 6 hour after injection, the oral InsEY-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose dose of InsEY-SMEDDS formulation was 6.87%.

Table 5.17: Mean serum Human insulin concentration for Placebo InsEY-SMEDDS (Batch: #InsEY-MP), (Group-1); N= 8

Time (hour)	Serum Human insulin concentration (mIU/l)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	2.452	0.870	3.055	1.849
0.5	3.855	1.119	4.630	3.080
1	4.409	1.341	5.338	3.480
2	3.420	1.209	4.258	2.582
4	3.167	1.005	3.863	2.471
6	3.935	1.195	4.763	3.107
9	2.769	0.703	3.256	2.282
12	3.002	0.818	3.569	2.435

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 5.18: Mean serum Human insulin concentration for subcutaneous Human insulin injection, 2 IU/kg (Group-2) N= 8

Time (hour)	Serum Human insulin concentration (mIU/l)			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	2.617	0.955	3.279	1.955
0.5	68.219	23.394	84.430	52.008
1	91.553	31.192	113.168	69.938
2	49.552	17.156	61.441	37.663
4	10.786	4.270	13.475	7.827
6	3.618	0.405	3.899	3.337
9	3.153	0.656	3.608	2.698
12	2.857	0.616	3.284	2.430

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 5.19: Mean serum Human insulin concentration for oral InsEY-SMEDDS dispersion (Batch: #InsEY-M2), Dose: 20 IU/kg (Group-3); N=8

Time (hour)	Serum Human insulin concentration (mIU/l)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	2.422	0.778	2.961	1.883
0.5	10.366	7.723	15.718	5.014
1	11.105	7.771	16.490	5.720
2	22.084	14.803	32.342	11.826
4	22.330	13.953	31.999	12.661
6	12.247	8.050	17.825	6.669
9	5.471	4.383	8.508	2.434
12	3.295	3.068	5.421	1.169

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

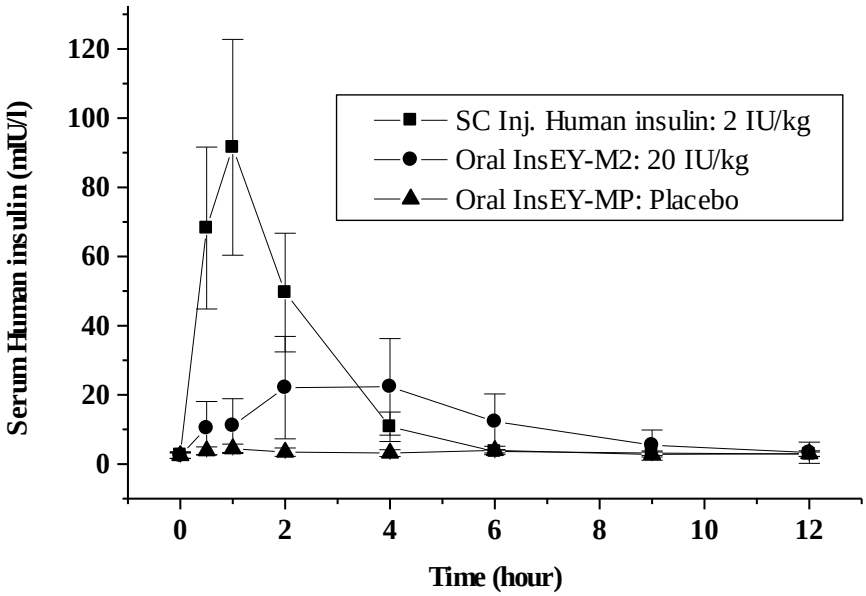


Figure 5.6: Serum Human insulin concentration versus time profile for Oral InsEY-SMEDDS dispersion

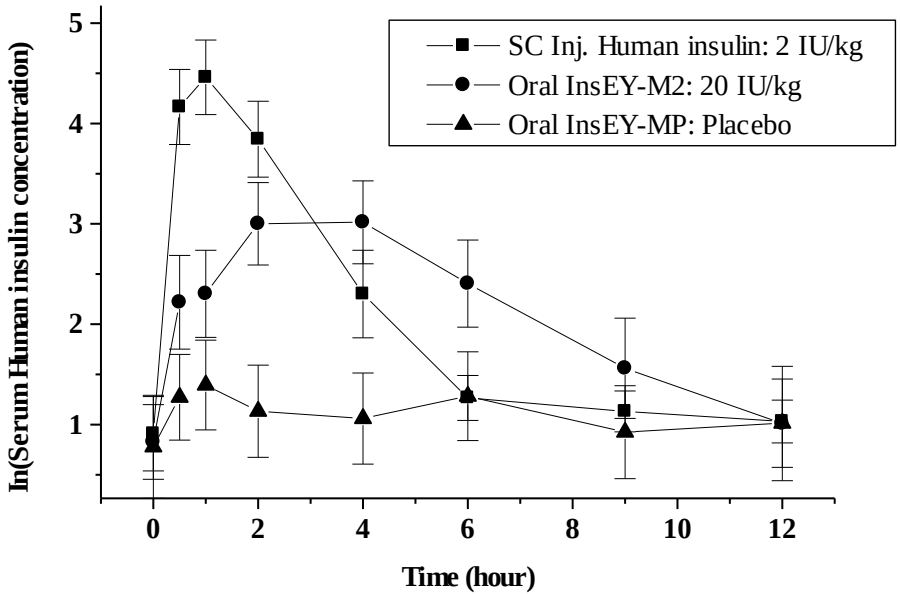


Figure 5.7: Semilogarithmic plot of Serum Human insulin concentration versus time for Oral InsEY-SMEDDS dispersion

Tablet 5.20: Pharmacokinetic parameters calculated from serum Human insulin concentration profile versus time profile for Oral InsEY-SMEDDS dispersion

Parameter	**Formulation**	
	Subcutaneous injection of Human insulin solution, 2 IU/kg*	**Oral, InsEY-M2 20 IU/kg***
C_{max} (mIU/l)	91.5 ± 30.9	22.3 ± 12.3
T_{max} (h)	1 ± 0.3	4 ± 0.2
AUC_{0-12h} (mIU/l . h-1)	163.6 ± 64.9	112.4 ± 83.6
K_e	0.794 ± 0.042	0.786 ± 0.035
$T_{1/2}$ (h)	0.872 ± 0.047	0.881 ± 0.039
Relative oral bioavailability (%)	-	6.87

***** Mean and S.D. for eight determinations

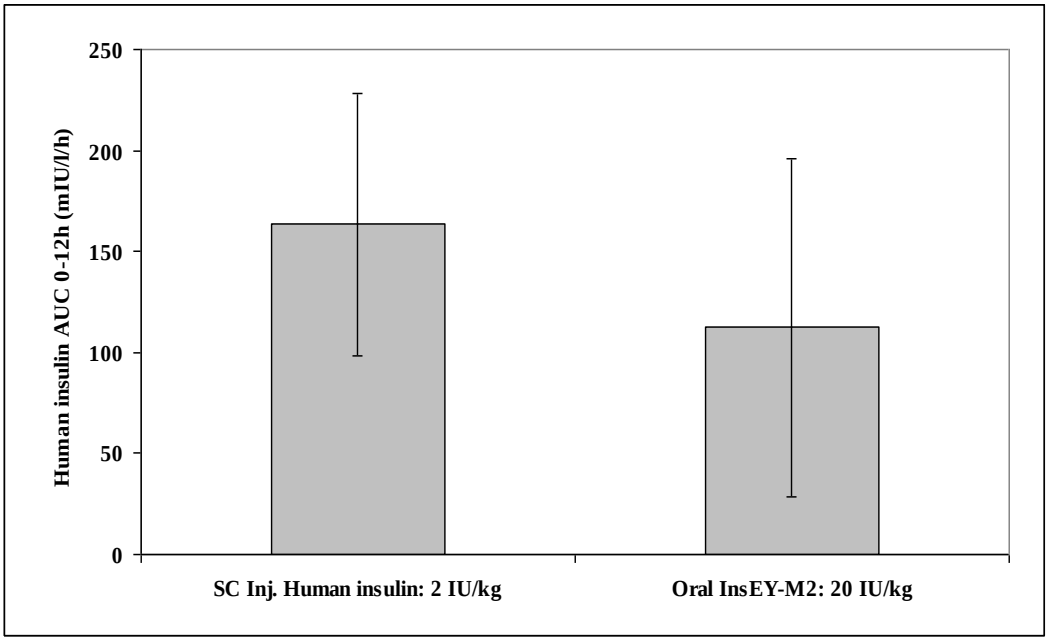


Figure 5.8: Comparison of AUC_{0-12h} for oral InsEY-SMEDDS (20 IU/kg) and subcutaneous injection of Human insulin solution (2 IU/kg)

5.5.2.2 Glucose estimation:

InsEY-SMEDDS dispersion (Batch: #InsEY-M2, Group-3) significantly reduced the blood glucose levels over the period of 4 to 12 hour compared to the placebo control (Group-1). Figure 5.9 shows the mean plasma % glucose fraction from the base line versus time profile for each group. Hypoglycemic effect of the 20 IU/kg dose was lower than 2 IU/kg subcutaneous injection of Human insulin injection with respect to the extent of reduction in glucose concentration from base line (~ 31% for 20 IU/kg – intragastrically administered Human insulin InsEY-SMEDDS and ~73.5 % of baseline for 2 IU/kg subcutaneously injected Human insulin solution). Area above glucose concentration curves, AAC_{0-12h} calculated from these curves (Figure 5.9). AAC_{0-12h} for both the formulations depicted in Figure 5.10 show that the effect of 20 IU/kg dose of InsEY-SMEDDS formulation was lower than subcutaneous injection of 2 IU/kg insulin (Figure 5.9), while the effect of the latter diminished 6 hour after injection, the oral InsEY-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity.

Table 5.21: Mean plasma glucose concentration for oral Placebo InsEY-SMEDDS dispersion, equivalent to 20 IU/kg (Group-1) N= 8

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	377.3	34.7	401.3	353.2
0.5	365.0	41.8	393.9	336.0
1	390.1	22.8	405.9	374.3
2	389.7	31.9	411.8	367.5
4	367.4	40.0	395.1	339.6
6	393.3	41.9	422.3	364.2
9	357.9	28.3	377.5	338.2
12	367.5	44.9	398.6	336.3

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

Table 5.22: Mean plasma glucose concentration for subcutaneous Human insulin injection, 2 IU/kg (Group-2) N= 7

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	352.6	38.5	379.2	325.9
0.5	199.4	98.3	267.5	131.2
1	90.7	56.2	129.6	51.7
2	142.6	67.7	189.5	95.6
4	226.7	64.9	271.6	181.7
6	304.2	44.5	335.0	273.3
9	379.3	21.4	394.1	364.4
12	385.3	13.6	394.7	375.8

***** Average of seven determination with standard deviation and, Upper and Lower 95% confidence limit

Table 5.23: Mean plasma glucose concentration for oral InsEY-SMEDDS dispersion, 20 IU/kg (Group-3) N= 8

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	370.3	37.3	396.1	344.4
0.5	359.5	40.69	387.6	331.3
1	307.6	46.4	339.7	275.4
2	273.1	76.9	326.3	219.8
4	252.1	97.9	319.9	184.2
6	314.1	63.9	358.3	269.8
9	340.8	22.5	356.3	325.2
12	363.1	39.2	390.2	335.9

***** Average of eight determination with standard deviation and, Upper and Lower 95% confidence limit

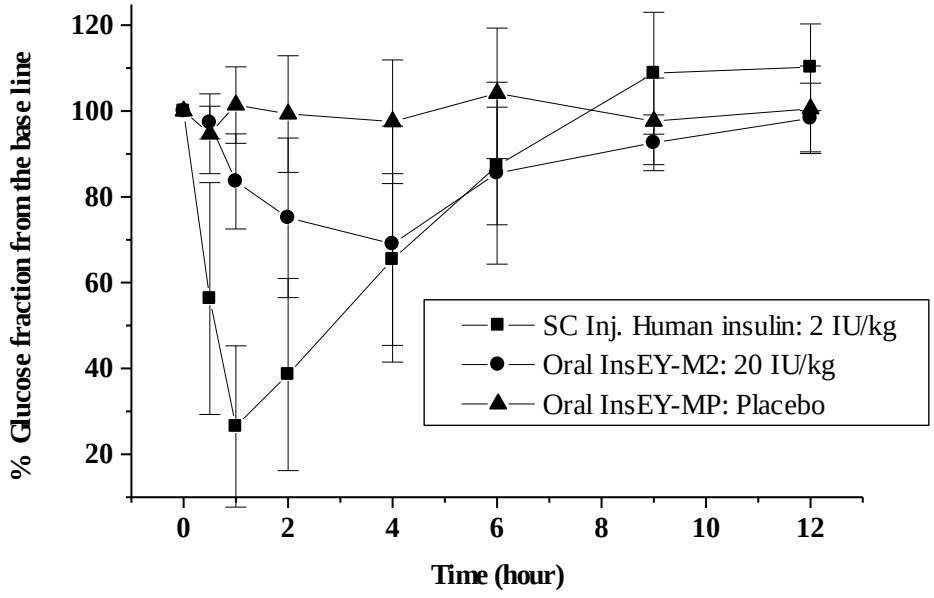


Figure 5.9: Mean plasma % glucose reduction versus time profile for Oral InsEY-SMEDDS dispersion

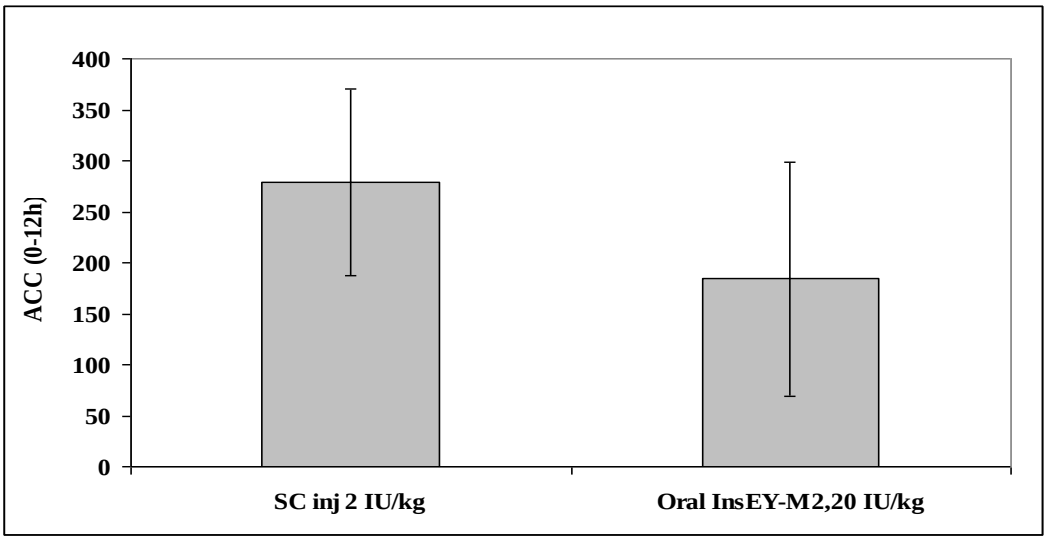


Figure 5.10: Comparison of area above the plasma glucose concentration versus time profile (% plasma glucose.h), AAC_{0-12h}, for subcutaneous injection and oral insEY-SMEDDS formulation (InsEY-M2).

5.6 CONCLUSION:

Self microemulsifying solutions (SMEDDS) have been reported to enhance the oral bioavailability of small molecules and semisynthetic dipeptide like Cyclosporine. However use of these hydrophobic systems possesses the problems of instability especially with high molecular weight proteins and peptides like insulin. In the present work combination of egg yolk and self microemulsifying solution has been evaluated to study the effect on insulin stability and oral bioavailability. The developed self microemulsifying formulation containing dispersion of Human insulin - egg yolk was found to possess good physical and chemical stability for insulin at long term storage of 2- 8°C. The formulations were also shown to retain biological activity of insulin.

The nanometer particle size of the formulation was achieved by unique process of making insulin-egg yolk dispersion by lyophilization and then dispersing it into SMEDDS solution. The particle size of SMEDDS solution was optimized by varying the surfactant and co-surfactant concentration. The particle size was found to increase after dispersion of Human insulin-egg yolk in SMEDDS. Reducing the surfactant and co-surfactant concentration showed increase in particle size from nanometer to micron range.

The duration of in-vitro insulin release was found to extend beyond 3 hour from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has been shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro.

In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0-\ 12h}$ for Human insulin serum versus time profile and $AAC_{0-\ 12h}$ for plasma glucose % glucose reduction of 20 IU/kg dose of InsEY-M2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished 12 hour after injection, the oral InsEY-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsEY-SMEDDS formulation was 6.87%.

5.7 REFERENCES:

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CHAPTER 6

HUMAN INSULIN-CASEIN DISPERSIONS IN SELF-MICROEMULSIFYING SYSTEM

6. HUMAN INSULIN - CASEIN DISPERSION IN SELF MICROEMULSIFYING SYSTEM:

6.1 INTRODUCTION:

The protection against enzymatic degradation and improvement in the permeability are the most preferred means to the success of oral delivery of therapeutic proteins and peptides like insulin. Variety of the stabilizers and permeability enhancers have been tried in past. The use of natural stabilizer would be of great value in reducing the potential side effect of the most studied proteolytic enzyme inhibitors and permeability enhancers.

Casein is a milk protein. The casein content of milk represents about 80% of milk proteins. The principal casein fractions are alpha(s1) and alpha(s2)-caseins, β-casein and kappa-casein. The distinguishing property of all caseins is their low solubility at pH 4.6 [1,2]. The common compositional factor is that caseins are conjugated proteins, most with phosphate group(s) esterified to serine residues. These phosphate groups are important to the structure of the casein micelle. Calcium binding by the individual caseins is proportional to the phosphate content. The conformation of caseins is much like that of denatured globular proteins [3,4].

Within the group of caseins, there are several distinguishing features based on their charge distribution and sensitivity to calcium precipitation:

alpha(s1)-casein: (molecular weight 23,000; 199 residues, 17 proline residues) Two hydrophobic regions, containing all the proline residues, separated by a polar region, which contains all but one of eight phosphate groups. It can be precipitated at very low levels of calcium [5].

alpha(s2)-casein: (molecular weight 25,000; 207 residues, 10 prolines) Concentrated negative charges near N-terminus and positive charges near C-terminus. It can also be precipitated at very low levels of calcium [5].

β-casein: (molecular weight 24,000; 209 residues, 35 prolines) Highly charged N-terminal region and a hydrophobic C-terminal region. Very amphiphilic protein acts like a detergent

molecule. Self association is temperature dependant; will form a large polymer at 20° C but not at 4° C. Less sensitive to calcium precipitation [5].

kappa-casein: (molecular weight 19,000; 169 residues, 20 pro lines) Very resistant to calcium precipitation, stabilizing other caseins. Rennet cleavage at the Phe 105-Met 106 bond eliminates the stabilizing ability, leaving a hydrophobic portion, para-kappa-casein and a hydrophilic portion called kappa-casein glycomacropeptide (GMP), or more accurately, caseinomacropeptide (CMP) [5].

Its biological function is to carry large amounts of highly insoluble calcium phosphate to mammalian young in liquid form and to form a clot in the stomach for more efficient nutrition. Besides casein protein, calcium and phosphate, the casein micelle also contains citrate, minor ions, lipase and plasmin enzymes and entrapped milk serum. These micelles are rather porous structures, occupying about 4 mg/g and 6-12% of the total volume fraction of milk. The casein micelle structure is presented in Figure 6.1 [6].

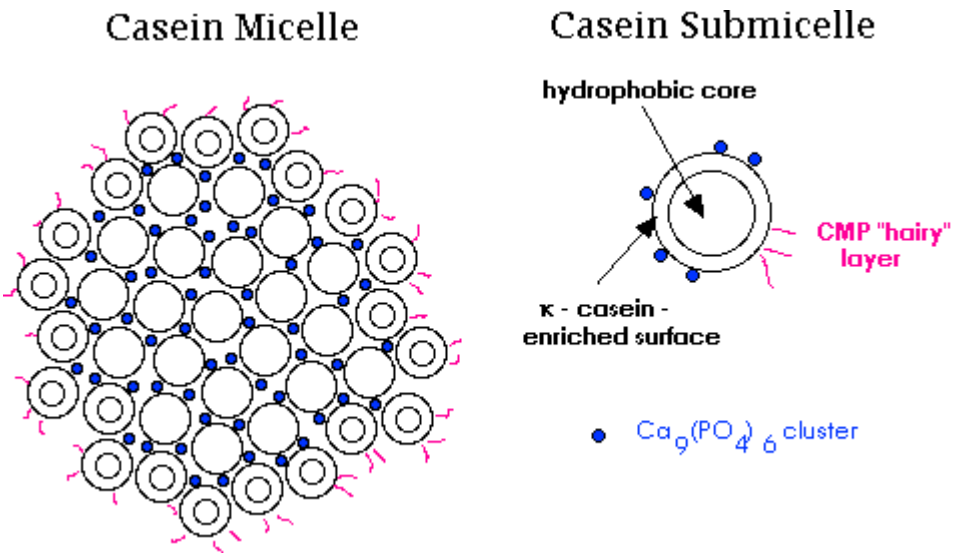


Figure 6.1: Casein micelle [6]

With respect to casein, some references have suggested the benefits of protective hydrolyzed casein (HC)-based diets to decrease diabetes frequency and the severity of insulitis [6]. See

Scot, et al. studied the effects of a protective hydrolyzed casein diet upon the metabolic and secretary responses of pancreatic Islets to IL-1 beta on Diabetes Prone BB Rats [7].

The inhibitory activity of casein on proteases has also been studied and documented, wherein the enzymatic activities of trypsin and chymotripsin were determined using a specific substrate for each protease (n-butoxycarbonyl-L-Gln-L-Ala-L-Arg-(4-methylcoumarinyl 7amine (MCA), a specific substrate for trypsin and N-succinyl-L-Ala-L-Ala-L-Pro-L PheMCA, a specific substrate for chymotripsin). It was suggested that not only the intact casein but also the products digested with trypsin or chymotripsin contribute to the inhibitory effect of casein on the proteases in the intestinal lumen [8,9].

In another study, insulin - calcium phosphate particles containing casein to improve the oral absorption of insulin has been investigated and improvement in oral absorption of insulin was seen [9,10].

In this study, dispersion of Human insulin with sodium caseinate in presence of combination of complexing agents such as zinc and calcium followed by its dispersion in self microemulsifying system (described in Chapter 5) was investigated to evaluate the improvement in in-vivo degradation and oral absorption of Human insulin.

Insulin in presence of zinc forms a hexamer which has been shown to improve the stability of insulin, while casein is known to interact with calcium to form agglomerated particles [11,12].

Lyophilization technique for the preparation of Insulin-sodium caseinate dispersion was designed to improve to the stability and encapsulation efficiency of insulin in the self mcroemulsifying system containing monoglycrides of linoleic acid.

6.2 MATERIALS AND METHODS:

Human insulin (Recombinant DNA origin, Akzo Nobel), Tris hydroxymethylaminomethane (Tris buffer, Merck), m-Cresol (Merck), Hydrochloric acid 36%, HCl (Merck), Sodium hydroxide pellets, (Merck), Hydroxypropyl β cyclodextrin (Roquette), Calcium chloride (SD Fine Chem), Zinc chloride (SD Fine Chem) Sodium caseinate (American Casein Company, New Jersy), Maisine® 35-1 (Monoglyceride of lineloic acid; Gattefosse), Cremophore® RH 40 (BASF), α-Tocopherol acetate (Sigma Aldrich), Polysorbate 80 (ICI India), Propylene glycol (Indian Glycol Ltd), Transcutol® (Gattefosse) , Glycerin (Indian Glycol Ltd).

6.2.1 Preparation of Tris buffer pH 7.4:

Tris buffer solution pH 7.4 (10 mM) was prepared by the procedure as described under Section 5.2.1.

6.2.2 Preparation of Human insulin-sodium caseinate solutions:

Human insulin and sodium caseinate solutions 75 IU/ml + 12.5 mg/ml, 75 IU/ml + 25 mg/ml and 75 IU/ml + 37.5 mg/ml respectively were prepared by suspending Human insulin crystals in 20 ml Tris buffer pH 7.4 (10mM) containing 0.781 mg/ml m-cresol and clear solution was prepared with drop by drop addition of 10 %v/v HCl until pH reached 2 ± 0.05. After formation of a clear solution, pH of the insulin solution was again raised to pH 7.4 ± 0.05 with drop wise addition of 1N NaOH and sodium caseinate (12.5 mg/ml, 25 mg/ml and 37.5 mg/ml) was added and dissolved under continuous stirring with the help of glass rod. Final volume of the solution was adjusted to 25 ml using Tris buffer pH 7.4 containing 0.781 mg/ml m-cresol. These neutral solutions of insulin were stored at 2-8°C temperature

6.2.3 Preparation of Hydroxypropyl beta cyclodextrin solutions:

HP-β CD solution of 25 mg/ml was prepared by dissolving HP-β CD in 20 ml Tris buffer pH 7.4 under stirring for 15 to 20 min in a beaker to get clear solution. The pH of the solution was adjusted to 7.4 ± 0.5 using 1N NaOH. Final volume of the solution was made up to 25 ml with Tris buffer pH 7.4 to obtain a solution of 25 mg/ml.

6.2.4 Preparation of Zinc chloride and calcium chloride solution:

Zinc chloride and calcium chloride solutions at concentrations of 0.125 mg/ml + 0.2 mg/ml, 0.125 mg/ml + 0.4 mg/ml, 0.125 mg/ml + 0.6 mg/ml, respectively were prepared by dissolving in purified water followed by sonication in water bath at 25°C for 10 min to form clear solution.

6.2.5 Preparation of Human insulin - sodium caseinate dispersion (InsSC):

Human insulin – sodium caseinate dispersions (InsSC) and its placebo dispersion (not containing insulin) were prepared by mixing individual different concentrations of Human insulin-sodium caseinate solution, HP-β CD solution and zinc chloride-calcium chloride solution to get various formulations with varying amount of insulin content and sodium caseinate content. The detailed formulation compositions prepared are presented in Table 6.1. The details of the process utilized for the preparation of these formulations is summarized below.

Human insulin-sodium caseinate solution was mixed with HP- β CD solution at ratio of 4:1 respectively in a beaker and stirred for 10 to 15 min on magnetic stirrer without any vortex formation. Zinc chloride-calcium chloride solution was added in the form of micro-droplets to the above insulin-sodium caseinate-HP- β CD solution at ratio of 1:2 respectively under continuous stirring. After complete addition of zinc chloride-calcium chloride solution, additional stirring was done for 10-15 min to obtain an opalescent solution of insulin.

The above opalescent solution was transferred to tubular glass vials and frozen at -40°C for 3 hour and lyophilized for 38 hour at condenser temperature of -40°C and pressure of 112×10^{-3} mbar. After complete drying vacuum was broken with nitrogen gas to purge the vials with nitrogen and capped with butyl stopper and aluminum seals. Vials were stored at 2-8°C.

Tablet 6.1: Formulations of InsSC with varying sodium caseinate content

Components	**Formulations (mg)**			
	InsSC-P*	**InsSC-1**	**InsSC-2**	**InsSC-3**
Human insulin	-	50	50	50
m-Cresol	12.5	12.5	12.5	12.5
Sodium caseinate	400	200	400	600
HP-β CD	100	100	100	100
Zinc chloride	1.25	1.25	1.25	1.25
Calcium chloride	4	2	4	6
Tris buffer pH 7.4	qs	qs	qs	qs

qs: quantity sufficient

***** Placebo formulation of InsSC-2

Theoretical potency of Human insulin : 25 IU/mg

6.2.6 Preparation of Self microemulsifying solution containing Insulin-sodium caseinate (InsSC-SMEDDS):

Lyophilized Human insulin-sodium caseinate powder was dispersed in the optimized self microemulsion solution (reference: SMEDDS-1 of Chapter 5) to get 40 IU/ml Human insulin content and mixed for 1 hour at room temperature (NMT 25°C) to get opaque dispersion. The solutions were stored at 2-8°C till use in a glass vial.

The compositions of self microemulsifying solution formulations prepared by this method is presented in Table 6.2 and Table 6.3

The process flow diagram for the preparation of Human insulin-sodium caseinate dispersions in SMEDDS (InsSC-SMEDDS) is depicted in Figure 6.2.

Table 6.2: Composition of Human insulin-sodium caseinate dispersions in SMEDDS (InsSC-SMEDDS)

Ingredient	**Formulation**			
	InsSC-MP*	**InsSC-M1**	**InsSC-M2**	**InsSC-M3**
Human insulin (equivalent to insulin-caseinate dispersion, #InsSC-2)	InsSC-P	40 IU/ml	80 IU/ml	120 IU/ml
Self microemulsifying solution (SMEDDS-1)	qs	qs	qs	qs

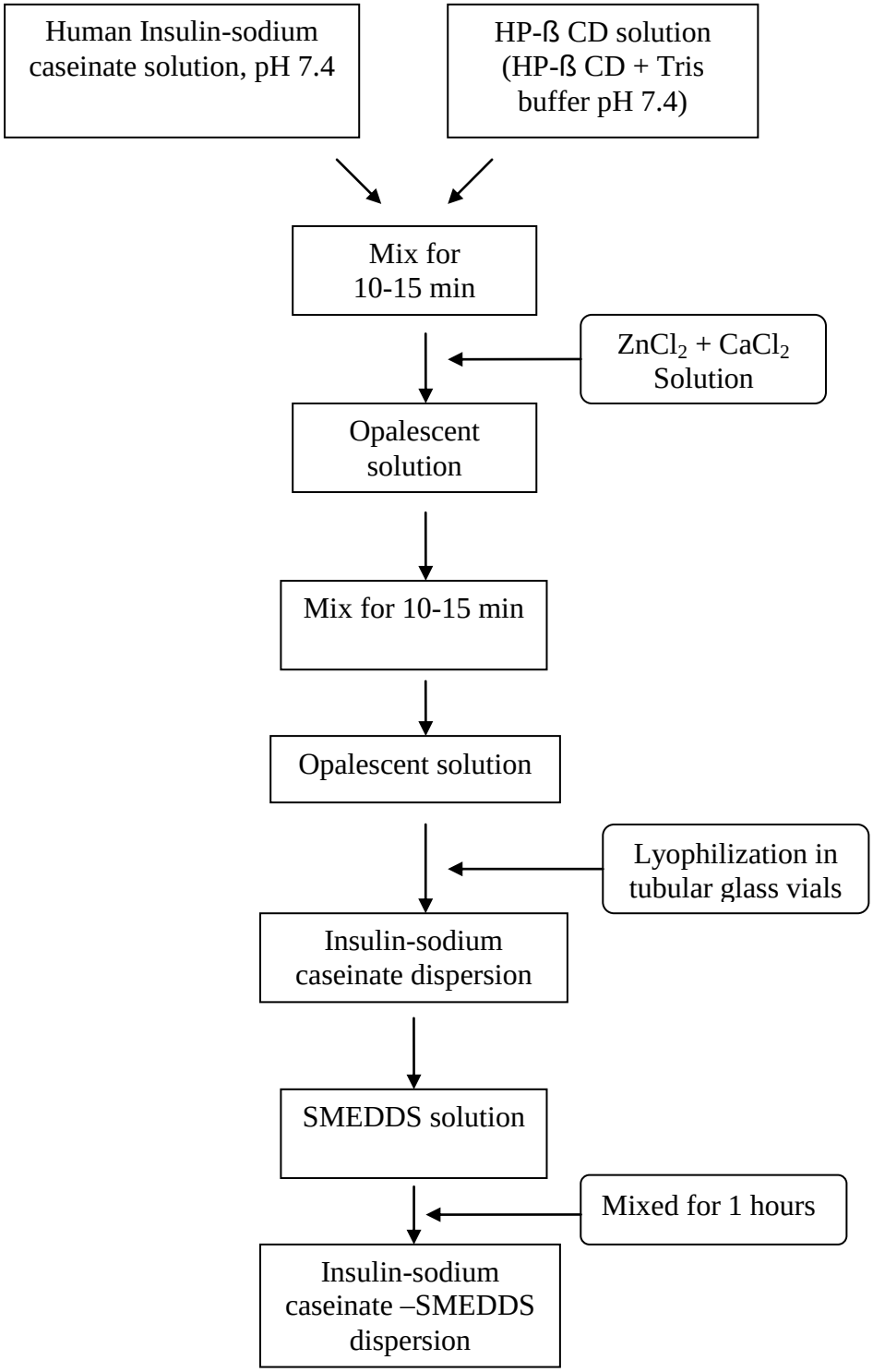
qs: quantity sufficient

***** Placebo formulation of InsSC-M2

Table 6.3: Composition of SMEDDS-1

Components	**Quantity (% w/v)** **SMEDDS-1**
Maisine® 35-1	27.2
α-Tocopherol acetate	1.0
Cremophore® RH 40	36.8
Transcutol®	11.4
Propylene glycol	23.6

Figure 6.2: Flow diagram for the preparation of Human insulin–sodium caseinate dispersions in SMEDDS (InsSC-SMEDDS)



6.3 FORMULATION CHARACTERIZATION:

Developed formulations were subjected to physicochemical characterization studies as described under Section 5.3. The procedure followed for each individual characterization study was similar to that presented under Section 5.3.

Following studies were performed to characterize insulin-sodium caseinate dispersions in SMEDDS (InsSC-SMEDDS) and the results obtained are presented and discussed under Section 6.5.

6.3.1 Appearance
6.3.2 Particle size
6.3.3 Scanning electron microscopy
6.3.4 Insulin entrapment efficiency
6.3.5 Residual water content
6.3.6 In-vitro insulin release study
6.3.7 In-vitro stability of Insulin from InsSC-SMEDDS in presence of proteolytic enzymes
6.3.8 Biological activity evaluation of entrapped Human insulin
6.3.9 Stability studies

6.4 IN-VIVO STUDIES:

The in-vivo studies on InSC-SMEDDS formulation was performed using streptozotocin induced diabetic male Wister rats (200-280 g). The studies were performed with approval from Animal Ethics Committee. The procedure followed for in-vivo studies was similar to that presented under Section 5.4.

The study was performed on day 4 following STZ injection after overnight fast of 8 hour. Water was allowed ad libitum. Diabetic rats were divided into three groups, each containing 6 rats. **Group-I:** Placebo of InsSC- SMEDDS (Batch: #InsSC-MP, equal to 20 IU/kg of insulin) was administered intragastrically to each diabetic rat as a control; **Group-2:** Human insulin injection (Huminsulin-R® 40 IU/ml Injection, Elli Lily) was administered subcutaneously to each diabetic rat at a dose level of 2 IU/kg. This group served as reference

standard; **Group 3:** InsSC-SMEDDS (Batch: #InsSC-M2 equal to 20 IU/kg of Human insulin) was administered intragastrically to each diabetic rat.

Serum Human insulin concentration and plasma glucose concentration were measured over 12 hour period to evaluate the effectiveness of oral InsSC-SMEDDS formulation versus subcutaneous injection of Human insulin solution. The analytical procedures followed for the estimation of serum Human insulin and plasma glucose concentration were similar to that presented under Section 5.4.

The pharmacokinetic parameters for serum Human insulin and plasma glucose were calculated by using the similar methodology as presented in Section 4.4.6.

The results obtained from the in-vivo studies are presented and discussed under Section 6.6.

6.5 RESULTS AND DISCUSSION:

6.5.1 Formulation characterization:

6.5.1.1 Appearance:

Visual appearance of the formulations developed was noted and the observations for InsSC are presented in Table 6.4.

Table 6.4: Physical appearance of the insulin-sodium caseinate (InsSC) dispersions

Formulation	Appearance (as such)	Appearance after dispersion in purified water
InsSC-1	White porous cake	Translucent solution
InsSC-2	White porous cake	Translucent solution
InsSC-3	White porous cake	Translucent solution

6.5.1.2 Human insulin - Sodium caseinate dispersion (InsSC):

A white, extremely porous and light weight cake formation was observed after lyophilization of a mixture of Human insulin solution and sodium caseinate. When it was dispersed in purified water a clear translucent solution observed.

6.5.1.3 Particle size:

Particle size of the SMEDDS solution and InsSC-SMEDDS dispersion in water was measured. For SMEDDS solution (SMEDDS-l) very fine particle size was observed with mean diameter of 0.387 ± 0.052 µm. The self microemulsifying solution after addition into water resulted into formation of nanoemulsion. When InsSC was dispersed into this SMEDDS system (InsSC-M2) and then added into water, the particle size increased to 0.904 ± 0.224 µm. It could be due to formation of dispersed particles of InsSC into SMEDDS system. When zinc chloride + calcium chloride solution was added to Human insulin + HP-β CD solution over 5 min with the help of fine needle syringe, slightly larger particles with little wider distribution range (1.212 ± 0.381 µm) was observed. When the addition time of zinc chloride solution was extended over 10, 15 and 30 min, slight reduction in mean diameter was observed with little narrow distribution range.

Table 6.5: Effect of addition time for zinc chloride + calcium chloride solution on particle size of InsSC-SMEDDS dispersions

Formulation	ZnCL2 + $CaCl_2$ addition time (min)	ZnCL2 + $CaCl_2$ addition rate (ml/min)	Mean diameter (µm)
InsSC-M2	5	~ 2.00	1.212 ± 0.381
	10	~ 1.00	1.086 ± 0.238
	15	~ 0.65	0.904 ± 0.224
	30	~ 0.35	0.946 ± 0.251

6.5.1.4 Scanning electron microscopy:

SEM images of the final InsSC -SMEDDS formulation (#InsSC -M2) are presented in Figure 6.3. The SEM images confirm that the solid spherical particles of about 0.9 µm, well dispersed and separated on the surface.

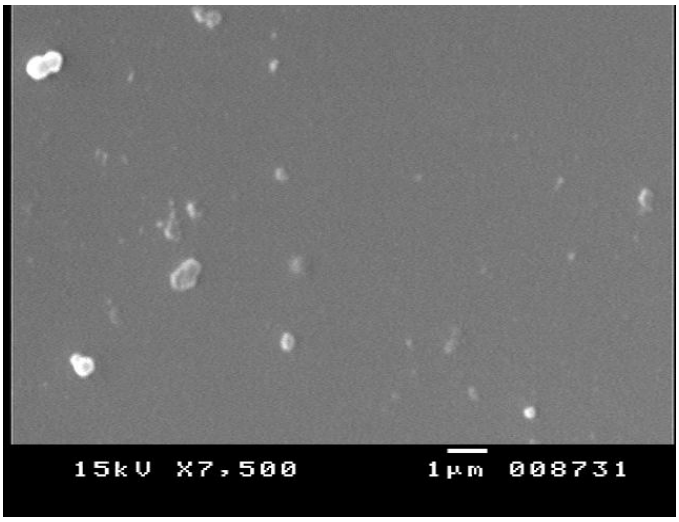


Figure 6.3: SEM images of InsSC-SMEDDS formulation

6.5.1.5 Insulin entrapment efficiency:

The entrapment efficiency of the InsSC and InsSC-SMEDDS were found to be satisfactorily high with an entrapment of ≥ 96.6 ± 0.9 %. This high entrapment efficiency could be attributed to the process of making Human insulin-sodium caseinate dispersion by lyophilization.

The entrapment efficiency of Human insulin in InsSC and InsSC-SMEDDS dispersion as a function of insulin content in the InsSC dispersions are tabulated in Table 6.6. Each formulation was tested in triplicate and average is reported.

Tablet 6.6: Insulin entrapment efficiency of insulin-sodium caseinate dispersions

Formulation	**Composition variable**	**Entrapment efficiency (%)***
InsSC-1	0.25 IU/mg, insulin content	98.4 ± 1.2
InsSC-2	0.5 IU/mg, insulin content	97.8 ± 1.4
InsSC-3	1.0 IU/mg, insulin content	98.2 ± 1.7
InsSC-M1	40 IU/mg, insulin content	97.1 ± 1.5
InsSC-M2	80 IU/mg, insulin content	96.6 ± 0.9
InsSC-M3	120 IU/mg, insulin content	98.7 ± 1.9

***** Mean and S.D. for three determinations

6.5.1.6 Residual water content:

Water content of the various InsSC dispersions was estimated after lyophilization by autotitration using Karl Fischer reagent. Each sample was analyzed in duplicate and the water content values obtained are reported in Table 6.7. Water content variation from 4.6 to 5.0 % w/w was observed when compared among various nanoparticle formulations.

Tablet 6.7: Residual water content of insulin-sodium caseinate (InsSC) dispersions

Formulation	Composition variable	Water content (%)
InsSC-1	0.25 IU/mg, insulin content	4.8
InsSC-2	0.5 IU/mg, insulin content	5.0
InsSC-3	1.0 IU/mg, insulin content	4.6

6.5.1.7 In-vitro release study:

Human insulin release profile from the dispersions is shown in Figure 6.4. Each is generally characterized by burst release followed by little slow release. The initial burst effect could be attributed to physical dispersion of insulin in sodium caseinate and releasing the insulin while forming spherical particles when InsSC-SMED is added to pH 7.4 phosphate buffer. There after little slower release of insulin was observed, could be due to the entrapment of insulin in the hydrophobic components of SMEDDS microemulsion. No effect of insulin content in the InsSC-SMEDDS on the release behavior was observed.

Table 6.8: In-vitro release of Human insulin from the InsSC-SMEDDS dispersions

Formulation	Composition variable	% release of Human insulin*			
		0 hour	0.5 hour	1 hour	2 hour
InsSC-M1	40 IU/mg insulin concentration	0	47 ± 2.0	62 ± 3.2	62 ± 2.5
InsSC-M2	80 IU/mg insulin concentration	0	52 ± 2.0	68 ± 3.0	65 ± 2.6
InsSC-M3	120 IU/mg insulin concentration	0	58 ± 2.5	65 ± 3.5	60 ± 2.5

***** Mean in % and S.D. for three determinations

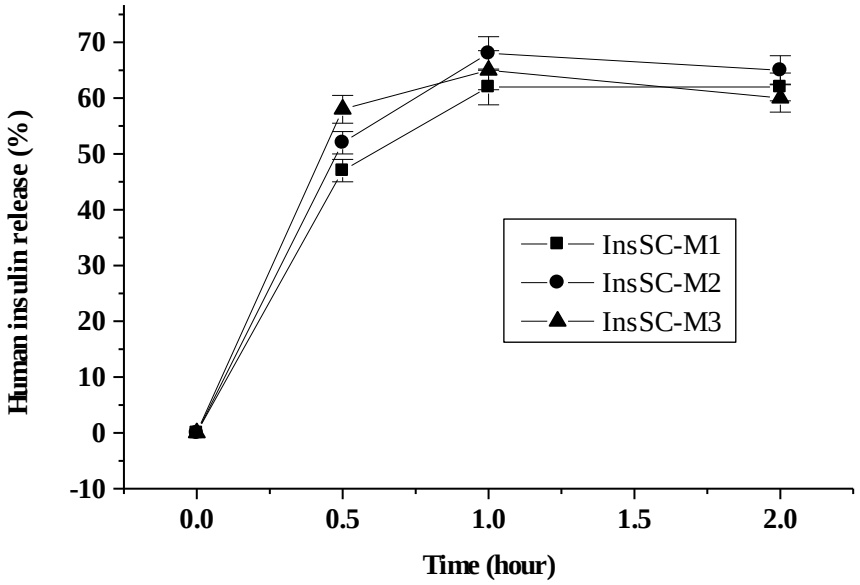


Figure 6.4: In-vitro release of Human insulin from InsSC-SMEDDS dispersions

6.5.1.8 In-vitro stability of InsSC-SMEDDS in presence of proteolytic enzymes:

The stability of Human insulin solution and lnsSC-SMEDDS formulations in presence of proteolytic enzyme is depicted in Table 6.9 and Figure 6.5. It can be seen that when insulin solution were incubated with α-chymotripsin, ≤ 18 % of Human insulin remained undegraded after 30 min. InsSC-SMEDDS incubated in the same medium, ≥ 70 % of entrapped insulin remained undegraded over the same period. Each sample was analyzed in triplicate.

Table 6.9: In-vitro stability of Human insulin – sodium caseinate (InsSC-SMEDDS) dispersions in presence of α-chymotripsin

Formulation	**Composition variable**	**% undegraded Human insulin* after 30 min**
Human insulin crystals	-	18 ± 3
InsSC-M1	40 IU/mg insulin concentration	70 ± 2
InsSC-M2	80 IU/mg insulin concentration	71 ± 4
InsSC-M3	120 IU/mg insulin concentration	72 ± 4

***** Mean in % and S.D. for three determinations.

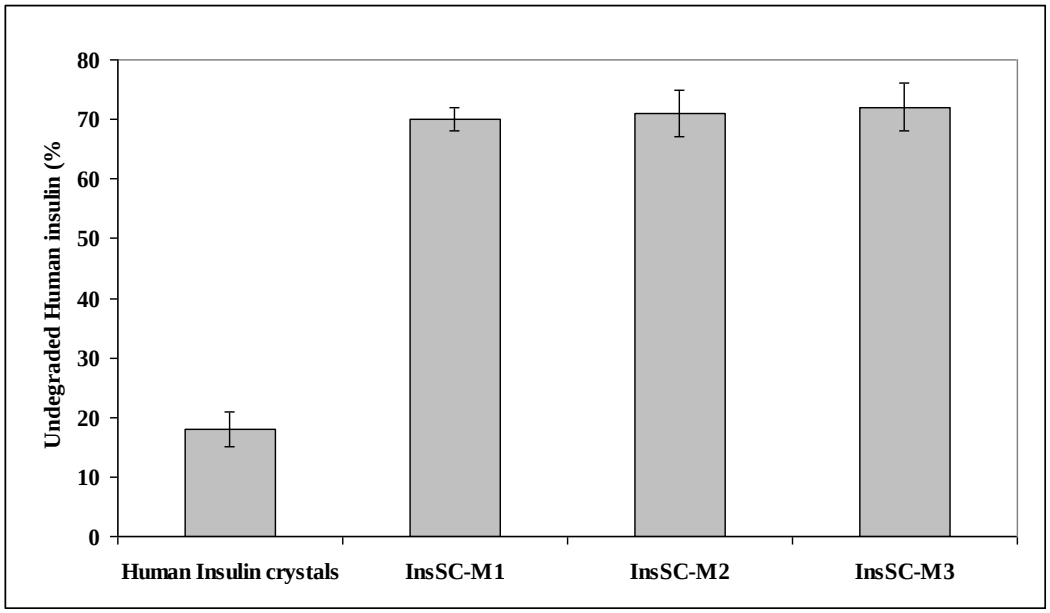


Figure 6.5: In-vitro stability of InsSC-SMEDDS dispersions in presence of α-chymotripsin after 30 min

6.5.1.9 Biological activity evaluation of entrapped Human insulin:

Biological activity of entrapped Human insulin in the InsSC-SMEDDS formulations was estimated using ELISA technique. It can be seen that biological activity of Human insulin from the InsSC-SMEDDS formulations was ≥97% (Table 6.10 and Figure 6.6). Samples were analyzed in triplicate. There was no considerable loss of biological activity observed indicating the InsSC-SMEDDS formulation components and the process of making the formulation preserved the integrity of the three dimensional structure of Human insulin. Proteins are fragile molecules with liable bonds and reactive side chains, disruption of this complex structure can lead to loss of biological activity. ELISA Human insulin assay measures biologically active insulin with high degree of specificity, using a pair of mouse monoclonal antibodies. The full biological activity of a protein is dependent on preserving the integrity of its three dimensional structure. ELISA results suggest that these nanoparticles are capable of preserving biological activity of entrapped insulin in presence of hydrophobic components of sodium caseinate dispersion and SMEDDS solution.

Tablet 6.10: Biologically active Human insulin content in InsSC-SMEDDS formulations

Formulation	Composition variable	% of biologically active Human insulin*
InsSC-M1	40 IU/mg insulin concentration	97.6 ± 0.5
InsSC-M2	80 IU/mg insulin concentration	97.0 ± 0.5
InsSC-M3	120 IU/mg insulin concentration	98.4 ± 0.3

***** Mean in % and S.D. for three determinations.

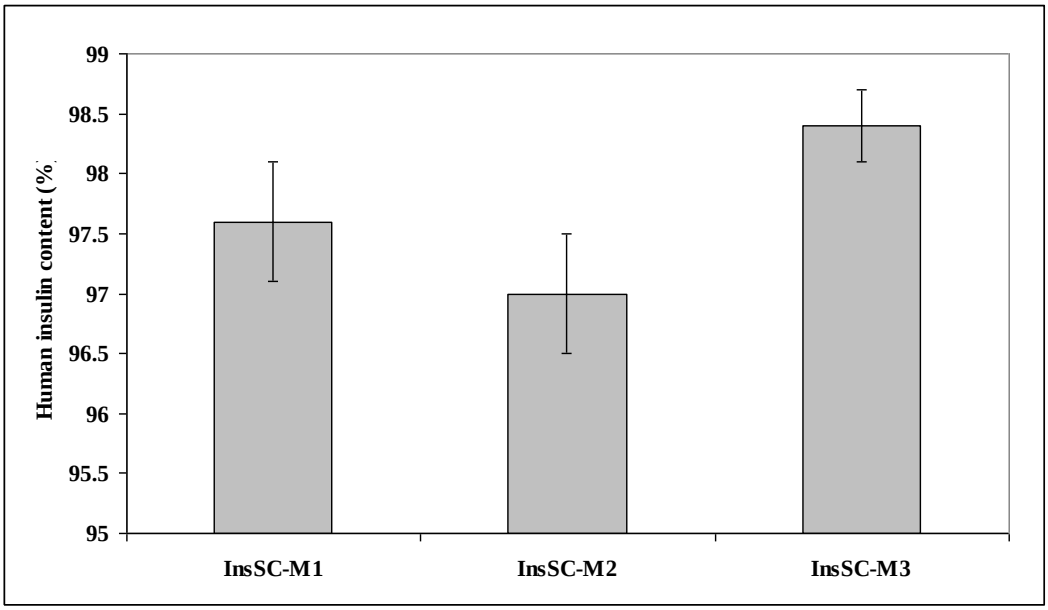


Figure 6.6: Biologically active Human insulin content in the InsEY-SMEDDS dispersions

6.6.2.10 Stability data:

InsSC and InsSC-SMEDDS dispersions were stored in nitrogen flushed vials at 2-8°C were tested for biologically active Human insulin content at 0, 1, 3 and 6 month time interval. The data obtained is presented in Table 6.11. There was no considerable loss of biological activity of Human insulin over the stipulated time interval, indicating the stabilization of Human insulin in sodium caseinate dispersion.

Tablet 6.11: Stability of InsSC and InsSC-SMEDDS dispersions at 2-8°C:

Formulation	**Human insulin content (%)***			
	Initial	1 Month	3 Month	6 Month
InsSC-2	98.5 ± 0.3	98.1 ± 0.4	97.4 ± 0.5	96.6 ± 0.7
InsSC-M2	97.2 ± 0.5	97.9 ± 0.4	96.7 ± 0.6	95.2 ± 0.8

***** Mean in % and S.D. for three determinations.

6.5.2 In-vivo studies:

6.5.2.1 Human insulin estimation:

InsSC-SMEDDS dispersion (Batch: #InsSC-M2, Group-3) showed significantly high serum Human insulin concentration levels over the period of 4 to 12 hour as compared to the placebo control (Group-1). Figure 6.7 and 6.8 shows the serum Human insulin concentration profile versus time for each group. The pharmacokinetic parameters such as C_{max}, T_{max}, AUC_{0-t}, $T_{1/2}$ were estimated for both group (Group-2 and Group-3) and compared. AUC_{0-12h} of both the formulations depicted in Figure 6.9 showed that the bioavailability of 20 IU/kg dose of insulin – sodium caseinate formulation was lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished 6 hour after injection, the oral InsSC-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsSC-SMEDDS formulation was 6.92%.

Table 6.12: Mean serum Human insulin concentration for Placebo of InsSC-SMEDDS, (Batch: #InsSC-MP) equal to 20IU/kg, (Group-1), N= 6

Time (hour)	Serum Human insulin concentration (mIU/l)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	3.070	1.036	4.951	3.121
0.5	4.036	1.143	4.835	2.899
1	3.867	1.210	4.936	2.850
2	3.893	1.304	5.259	3.309
4	4.284	1.219	4.274	2.556
6	3.415	1.073	4.625	3.229
9	3.927	0.872	5.149	3.469
12	4.309	1.050	3.657	2.347

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

Table 6.13: Mean serum Human insulin concentration for subcutaneous Human insulin injection, 2 IU/kg (Group-2) N= 6

Time (hour)	Serum Human insulin concentration (mIU/l)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	3.893	0.948	4.652	3.134
0.5	59.697	15.998	72.498	46.896
1	100.641	26.663	121.976	79.306
2	54.466	25.565	74.922	34.010
4	18.572	5.333	22.839	14.305
6	3.641	0.402	3.963	3.319
9	3.179	0.651	3.700	2.658
12	2.857	0.616	3.350	2.364

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

Table 6.14: Mean serum human insulin concentration for oral InsSC-SMEDDS dispersion, (Batch: #InsSC-M2), Dose: 10 IU/kg (Group-3); N=6

Time (hour)	Serum Human insulin concentration (mIU/l)*			
	Mean	SD	UL (95% CI)	LL (95% CI)
0	3.268	1.566	4.521	2.015
0.5	10.339	7.666	16.473	4.205
1	12.443	8.540	19.276	5.610
2	22.718	15.157	34.846	10.590
4	26.403	16.273	39.424	13.382
6	17.027	10.757	25.634	8.420
9	5.480	4.351	8.962	1.998
12	3.320	3.045	5.757	0.883

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

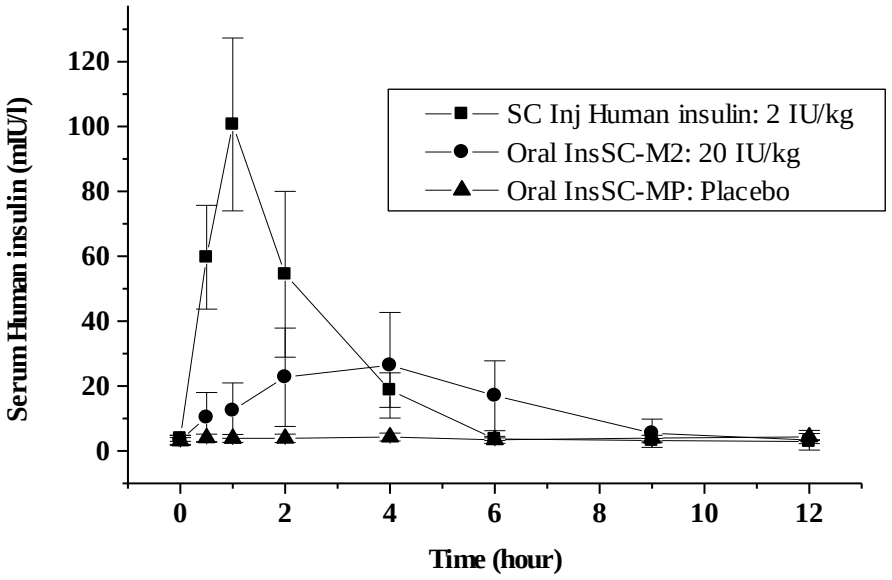


Figure 6.7: Serum Human insulin concentration versus time profile for Oral InsSC-SMEDDS dispersion

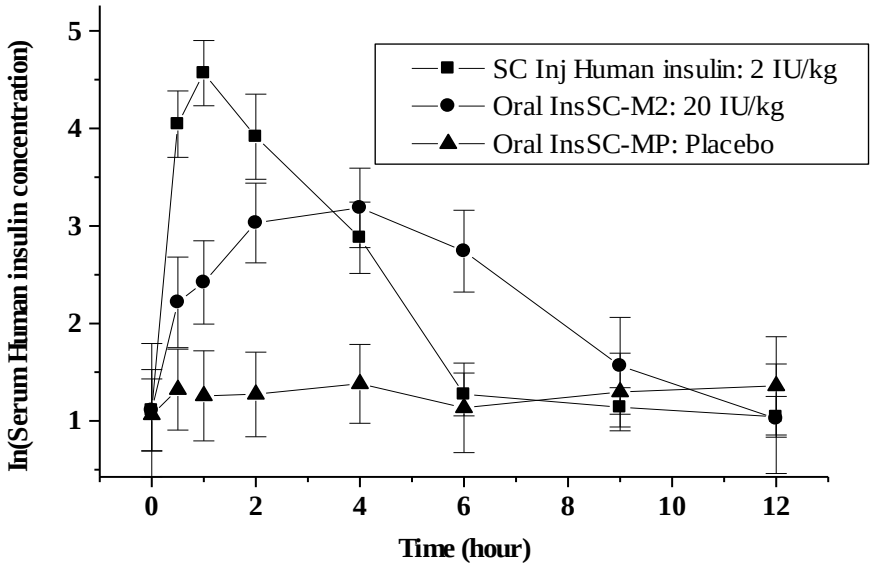


Figure 6.8: Semilogarithmic plot of Serum Human insulin concentration versus time for Oral InsSC-SMEDDS dispersion

Tablet 6.15: Pharmacokinetic parameters calculated from serum Human insulin concentration profile versus time profile for Oral InsSC-SMEDDS dispersion

Parameter	**Formulation**	
	Subcutaneous injection of Human insulin solution, 2 IU/kg*	**Oral, InsSC-M2 20 IU/kg***
C_{max} (mIU/l)	100.6 ± 29.0	26.4 ± 16.3
T_{max} (h)	1 ± 0.3	4 ± 0.4
$AUC_{0\text{-}12h}$ (mIU/l . h-1)	180.4 ± 96.8	124.7 ± 85.0
K_e	0.718 ± 0.082	0.762 ± 0.058
$T_{1/2}$ (h)	0.964 ± 0.110	0.910 ± 0.073
Relative oral bioavailability (%)	-	6.92

***** Mean and S.D. for six determinations

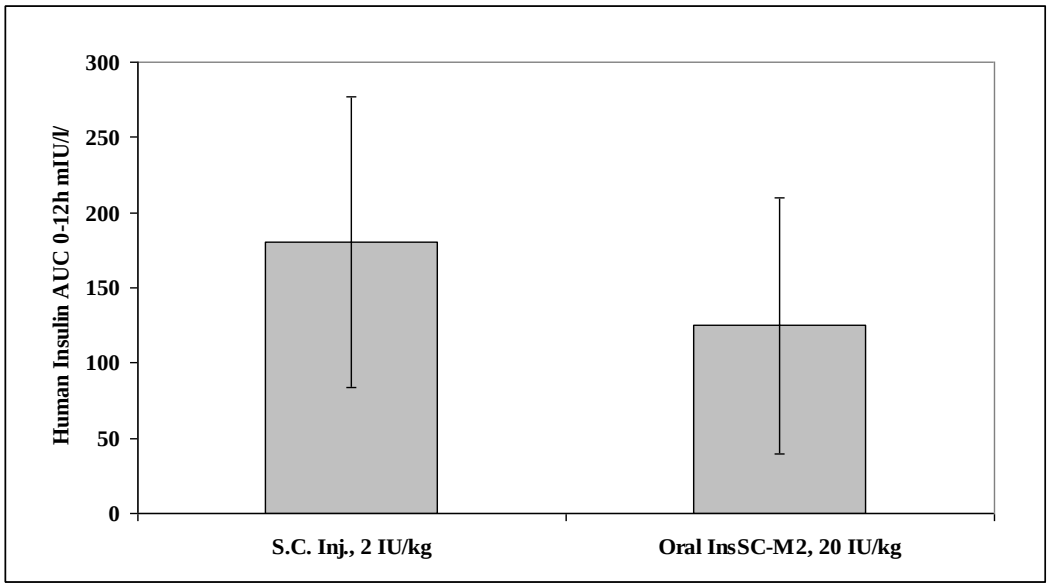


Figure 6.9: Comparison of $AUC_{0\text{-}12h}$ for oral InsSC-SMEDDS (20 IU/kg) and subcutaneous injection of Human insulin solution (2 IU/kg)

6.5.2.2 Glucose estimation:

InsSC-SMEDDS dispersion (Batch: InsSC-M2, Group-3) significantly reduced the blood glucose levels over the period of 4 to 12 hour compared to the placebo control (Group-1). Figure 6.10 shows the blood glucose profile for each group. Hypoglycemic effect of the 20 IU/kg dose was lower than 2 IU/kg subcutaneous injection of Human insulin injection with respect to the extent of reduction in glucose concentration from base line (~ 31% for 20 IU/kg – intragastrically administered Human insulin InsSC-SMEDDS and ~73.5 % of baseline for 2 IU/kg subcutaneously injected Human insulin solution). Area above glucose concentration curves, $AAC_{0\text{-}12h}$ calculated from these curves (Figure 6.10). $AAC_{0\text{-}12h}$ of both the formulations depicted in Figure 6.11 show that the effect of 20 IU/kg dose of InsSC-SMEDDS formulation was lower than subcutaneous injection of 2 IU/kg insulin, while the effect of the latter diminished 6 hour after injection, the oral InsSC-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity.

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Table 6.16: Mean plasma glucose concentration for oral Placebo of InsSC-SMEDDS, (Batch: #InsSC-MP) equal to 20IU/kg, (Group-1), N= 6

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	385.9	32.9	412.2	359.7
0.5	376.8	49.3	416.2	337.3
1	399.6	39.3	431.0	368.1
2	408.8	24.5	428.4	389.1
4	383.9	66.2	436.8	330.9
6	393.3	41.9	426.8	359.7
9	286.6	46.5	323.8	249.3
12	399.2	43.5	434.0	364.3

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

Table 6.17: Mean plasma glucose concentration for subcutaneous Human insulin injection, 2 IU/kg (Group-2) N= 6

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	381.0	39.4	412.5	349.4
0.5	199.4	98.3	278.0	120.7
1	75.7	31.4	100.8	50.5
2	122.1	42.6	156.1	88.0
4	224.0	68.7	278.9	169.0
6	304.3	44.6	339.9	268.6
9	379.3	21.4	396.4	362.1
12	385.3	13.6	396.1	374.4

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

Table 6.18: Mean plasma glucose concentration for oral InsSC-SMEDDS dispersion, (Batch: #InsSC-M2), Dose: 20 IU/kg (Group-3); N=6

Time (hour)	Mean plasma glucose concentration mg/dl)*			
	Mean	**SD**	**UL (95% CI)**	**LL (95% CI)**
0	378.4	50.3	418.6	338.1
0.5	359.5	40.6	391.9	327.0
1	307.6	46.3	344.6	270.5
2	263.6	80.4	327.9	199.2
4	228.9	102.6	310.9	146.8
6	302.7	52.2	344.4	260.9
9	340.8	22.4	358.7	322.8
12	363.1	39.2	394.4	331.7

***** Average of six determination with standard deviation and, Upper and Lower 95% confidence limit

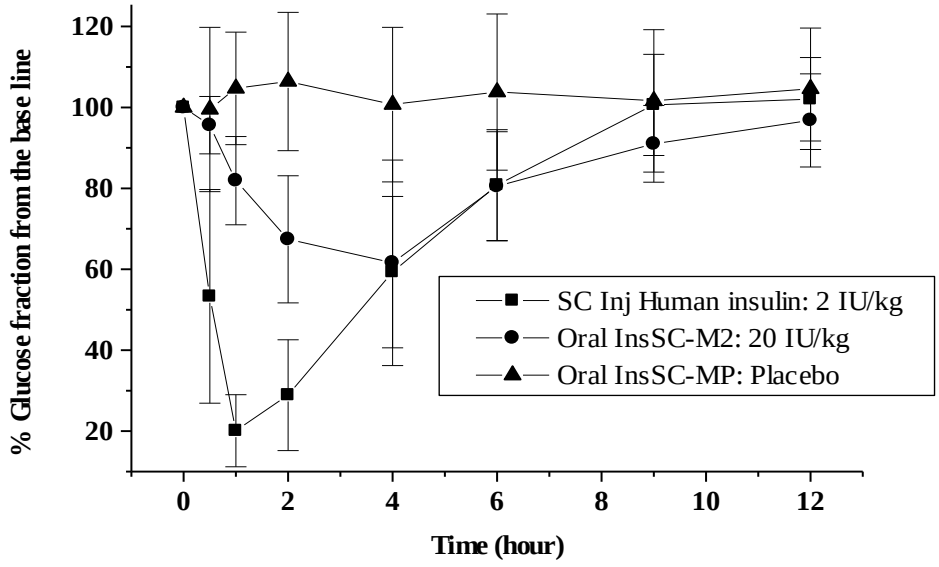

Figure 6.10: Mean plasma % glucose reduction versus time profile for Oral InsSC-SMEDDS dispersion

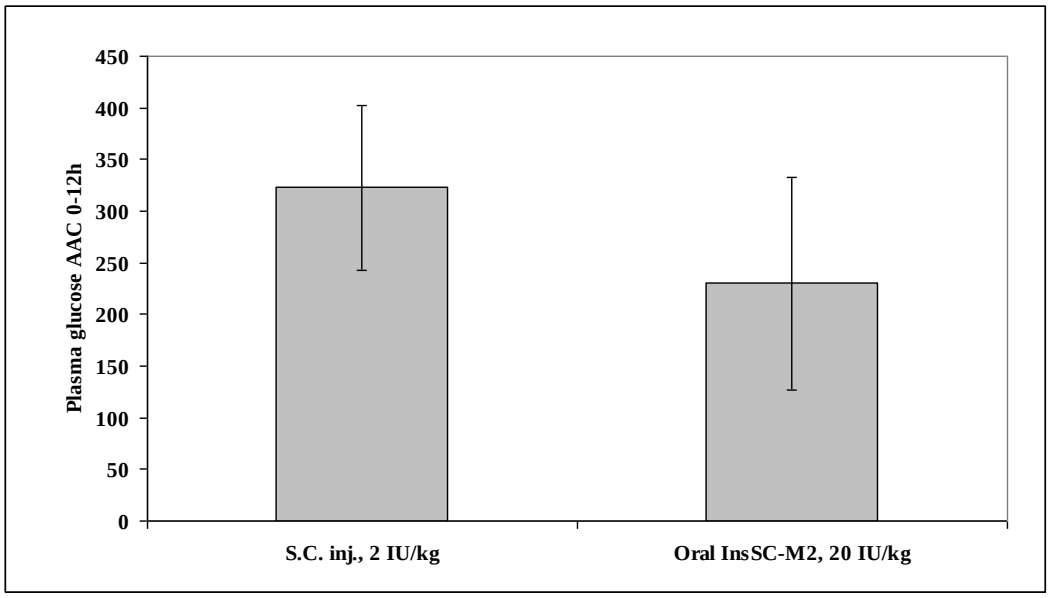

Figure 6.11: Comparison of area above the plasma glucose concentration versus time profile (% plasma glucose.h), $AAC_{0\text{-}12h}$, for subcutaneous injection and oral InsSC-SMEDDS formulation (InsSC-M2).

6.6 CONCLUSION:

In the present work, combination strategy for the use of sodium caseinate and complexing agents (zinc and calcium) was evaluated to stabilize and improve oral bioavailability of insulin in the hydrophobic environment of self microemulsifying (SMEDDS) solution. The developed oral SMEDDS formulation containing dispersion of Human insulin – sodium caseinate was found to possess good physical and chemical stability at long term storage at 2-8°C with retention of biological activity of Human insulin. The use of sodium caseinate in presence of zinc has been shown to improve the stability of Human insulin in the formulation.

The particle size of the formulation was achieved by unique process of dispersion formation by addition of zinc chloride and calcium chloride as complexing agent to Human insulin solution in presence of hydroxypropyl β cyclodextrin followed by lyophilization and then dispersing in SMEDDS solution.

The particle size of SMEDDS solution was optimized by varying the surfactant and cosurfactant concentration. The particle size was found to increase after dispersion of Human insulin-sodium caseinate in SMEDDS.

The duration of in-vitro insulin release was found to extend beyond 3 hour from the dispersions, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has been shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma glucose % glucose reduction of 20 IU/kg dose of InsSC-M2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished 6 hour after injection, the oral InsSC-SMEDDS formulation demonstrated longer duration of absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsSC-SMEDDS formulation was 6.92 %.

6.7 REFERENCES:

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CHAPTER 7

CONCLUSION

7. **CONCLUSION:**

An oral delivery of insulin is the most preferred form of delivery system. However delivering proteins and peptides by oral route is extremely challenging due to instability of insulin in the formulations and GI tract with poor permeability through GI tract.

The aim of the present study was to design and study better oral delivery system for Human insulin. Variuos hydrophobic delivery systems were envisaged to evaluate improvement in oral absorption of insulin. This has been achieved by approaches like (1) hydrophobic nanoparticles containing stearic acid, α-tocopherol acetate and soya phosphatidyl choline, (2) self microemulsifying delivery system containing dispersion of Human insulin with egg yolk, (3) self microemulsifying delivery system containing dispersion of Human insulin with sodium caseinate.

Insulin being unstable in the presence of hydrophobic excipients, different approaches like use of zinc chloride, calcium chloride, hydroxypropyl β cyclodextrin, egg yolk, sodium caseinate was studied to stabilize insulin in these hydrophobic delivery systems. Some of these excipients were also targeted to evaluate their effect on oral absorption of insulin and protection against enzymatic degradation of insulin.

The process for the preparation of nanoparticles greatly influence the encapsulation efficiency and stability of the protein drug, especially when protein molecule comes in contact with hydrophobic surfaces, air-water interfaces, shear stress, temperature and organic solvents. In addition, size of the nanoparticles also influences the oral absorption. In this work an innovative process utilizing ionic interactions and lyophilization was designed and optimized to produce well controlled particle size and high encapsulation efficiency.

The reported current official USP method for assay of insulin could not be used as such with the present formulations and excipients. Hence, as necessary to support the whole work, analytical method for estimation of Human insulin was developed by modification of current official USP method for assay of Human insulin. The method was validated as per ICH guidelines and by recovery studies. Additionally, biologically active Human insulin content from the formulations was estimated by pre-validated diagnostic kits (ELISA) for Human insulin.

These formulations developed were evaluated for physical appearance, particle size, insulin entrapment efficiency, in-vitro release of insulin, in-vitro stability of insulin in presence of proteolytic enzyme (α-chymotripsin) and long term formulation stability study at 2-8°C.

In-vivo studies were performed to evaluate oral bioavailability of insulin from the formulations developed. Pre-validated diagnostic kits for estimation of plasma glucose and serum Human insulin were used to measure the same from the base line. All these animal studies were performed with approval from Animal Ethics Committee.

The developed stearic acid nanoparticles formulation was found to possess good physical and chemical stability at long term storage of 2-8°C. The use of zinc and hydroxypropyl β cyclodextrin has been shown to improve the stability of Human insulin in the formulation. The nanometer particle size of the formulation was achieved by unique process of ionic interactions, solvent diffusion followed by lyophilization with excellent entrapment efficiency. The particle size of the nanoparticles was found to increase with increasing stearic acid content. The duration of in-vitro insulin release was found to extend beyond 3 hour from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The nanoparticle formulation has also been shown to protect Human insulin from proteolytic enzyme degradation in-vitro. Reducing concentration of stearic acid content has been shown to reduce the enzymatic protection due to higher in-vitro release of insulin. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The oral dose of 20IU/kg of nanoparticle formulation was found to be comparable with 2 IU/kg subcutaneous injection of Human insulin injection in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma % glucose reduction. C_{max} for Human insulin and extent of plasma glucose reduction was lower with oral formulation as compared to subcutaneous injection of Human insulin solution, but the serum Human insulin concentration of the latter diminished after 6 hour of injection, the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose and 10 IU/kg dose of nanoparticle formulation was 9.48% and 4.60% respectively.

The developed self microemulsifying formulation containing dispersion of Human insulin-egg yolk was found to possess good physical and chemical stability at long term storage of

2–8°C. The use of egg yolk has been shown to improve the stability of Human insulin in the formulation. The nanometer particle size of the formulation was achieved by unique process of dispersion formation with lyophilization and then dispersing in SMEDDS solution. The particle size of SMEDDS solution was optimized by varying the surfactant and co-surfactant concentration. The particle size was found to increase after dispersion of Human insulin-egg yolk in SMEDDS solution. The duration of in-vitro insulin release was found to extend beyond 3 hour from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has been shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma % glucose reduction of 20 IU/kg dose of InsEYM2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished after 12 hour of injection the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsEY-M2 formulation was 6.87 %.

Self microemulsifying formulation containing dispersion of Human insulin – sodium caseinate was also found to possess good physical and chemical stability at long term storage of 2-8°C. The use of sodium caseinate in presence of zinc has been shown to improve the stability of Human insulin in the formulation The nanometer particle size of the formulation was achieved by unique process of dispersion formation by addition of zinc chloride and calcium chloride as complexing agent to Human insulin solution in presence of hydroxypropyl β cyclodextrin followed by lyophilization and then dispersing in SMEDDS solution. The particle size of SMEDDS solution was optimized by varying the surfactant and cosurfactant concentration. The particle size was found to increase after dispersion of Human insulin – sodium caseinate in SMEDDS solution. The duration of in-vitro insulin release was found to extend beyond 3 hour from the nanoparticles, however estimation could not be performed for extended period due to degradation of insulin in the release medium at 37°C. The formulation has been shown to satisfactorily protect Human insulin from proteolytic enzyme degradation in-vitro. In-vivo study in the streptozotocin induced diabetic rats showed improvement in the oral absorption of insulin. The bioavailability in terms of $AUC_{0\text{-}12h}$ for Human insulin serum versus time profile and $AAC_{0\text{-}12h}$ for plasma % glucose reduction of 20

IU/kg dose of InsSC-M2 formulation was found to be slightly lower than subcutaneous injection of 2 IU/kg insulin, while the serum Human insulin concentration of the latter diminished after 6 hour of injection the former seemed to be continuing its absorption and hypoglycemic activity. The relative oral bioavailability for 20 IU/kg dose of InsSC-M2 formulation formulation was 6.92 %.

The developed formulations of Human insulin need to be further studied on higher animals and further refined to have successful oral delivery of proteins. These techniques developed serves as a broad base with reasonable amount of scientific data to demonstrate further success of oral delivery of not only insulin but other therapeutic peptides and proteins such as eptifibatide, human growth hormone, calcitonin, desmopressin, etc. in higher animals and Human beings. Further work could not be taken up due to limited time available, complexities of the project and unavailability of higher animals. However the present work done serves a sound scientific base with data to suggest a proof of concept on lower animals and feasibility of formulations having sufficient stability of insulin.

List of Publications/Presentations

1. Singnukar, P.S., Gidwani, S.K. (2008). Evaluation of hydrophobic nanoparticulate delivery system for insulin, Indian J. Pharm. Sci., Vol. 70(6), pp. 721-726.

2. Singnukar, P.S., Gidwani, S.K. (2008). Insulin-egg yolk dispersions in self microemulsifying system, Indian J. Pharm. Sci., Vol. 70(6), pp. 727-732.

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Purushottam S. Singnurkar did his B.Pharm and M.Pharm from Poona University, Maharashtra. He has been working as a Principal Scientist, Drug Delivery Research Laboratory of USV Limited, Mumbai for about ten years. He has a diverse experience in area of formulation development for conventional as well as New Drug Delivery Systems and clinical studies. He is involved in managing a team for formulation development for regulated markets and has eight international patents in the area of pharmaceutical research to his credit. Presently, he is Group Leader, Formulation development, Sandoz India, Mumbai.

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Dr. S. K. Gidwani did his B.Pharm form Delhi University with Gold Medal, M.S. and Ph.D in Pharmaceutics from the Birla Institute of Technology and Science, Pilani. He served in the field of Pharmaceutical research and development field in India in various capacities for the past three decades. He served as a leader for CSIR project from Government of India on the Development of new platform technologies with an initiative of Industry and Academia collaboration. He has more than 33 years of distinguished professional, administrative and academic experience. He has worked with leading Multinational and Indian companies and has 25 patents to his credit in the area of formulation development and technologies. He is actively engaged in research and development, resource development and training activities in the field of Pharmaceutical Research. His areas of research is in the filed of Pharmaceutical development and New Drug Delivery Systems. He also has substantial experience in analytical development and Clinical studies. He travelled extensively abroad and attended international conferences, symposiums and workshops in the area of drug delivery and analytical sciences. He has been acting as an examiner for research projects form University of Mumbai and B.I.T.S., Pilani. Presently, he is Senior Vice-President, Research and Development, Indoco Remedies, Mumbai.