DEVELOPMENT AND EVALUATION OF A NOVEL INSULIN ANALOGUE

By

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M.Tech

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>SGLT-2</td>
<td>sodium glucose transport protein – 2</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>dipeptidyl peptidase – IV</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon like peptide – 1</td>
</tr>
<tr>
<td>INGAP</td>
<td>islet neogenesis associated protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>g/kg/day</td>
<td>gram per kilogram per day</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>ZOT</td>
<td>zonula occludens toxin</td>
</tr>
<tr>
<td>PKCα</td>
<td>protein kinase C-alpha</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin like growth factor -1</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated haemoglobin</td>
</tr>
<tr>
<td>g/kg</td>
<td>gram per kilogram</td>
</tr>
<tr>
<td>°C</td>
<td>degree centigrade</td>
</tr>
<tr>
<td>NDA</td>
<td>New drug application</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxy succinimide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>g/cm³</td>
<td>gram per centimetre cube</td>
</tr>
<tr>
<td>kg/cm²</td>
<td>kilogram per centimetre square</td>
</tr>
<tr>
<td>MPa</td>
<td>mega pascal</td>
</tr>
<tr>
<td>kBar</td>
<td>kilo bar</td>
</tr>
<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris(hydroxymethyl) aminomethane hydrochloride</td>
</tr>
<tr>
<td>LC-ESI-MS</td>
<td>Liquid chromatography-electrospray ionisation-mass spectrometry</td>
</tr>
<tr>
<td>HCT</td>
<td>high capacity trap</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>ΔSu</td>
<td>change in entropy</td>
</tr>
<tr>
<td>ΔHu</td>
<td>change in enthalpy</td>
</tr>
<tr>
<td>HT[V]</td>
<td>high tension voltage</td>
</tr>
<tr>
<td>Cal.mol(^{-1}).K(^{-1})</td>
<td>calories per mole per degree kelvin</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>μg/mL</td>
<td>microgram per milliliter</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>mL/min</td>
<td>millilitre per min</td>
</tr>
<tr>
<td>mV</td>
<td>millivolts</td>
</tr>
<tr>
<td>m/z</td>
<td>mass per charge</td>
</tr>
<tr>
<td>L/h</td>
<td>liter per hour</td>
</tr>
<tr>
<td>ISTD</td>
<td>internal standard</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
</tbody>
</table>
Abstract

Oral delivery of proteins and peptides, particularly insulin, has been a major focus of research in recent times. While the earliest attempts to develop an oral insulin dates back to 1920s. Technology advances over the past few years have made it possible for several research groups to refocus on this area again. Research in this area has been primarily formulation centric with the exception of a few attempts to build in the desired properties at the molecular level itself.

Biocon, a leading biotechnology company has embarked upon developing oral insulin using a combination approach of modification at the molecular level and formulation development. Modification at the molecular level is achieved by conjugation of recombinant human insulin and a short chain alkyl-polyethylene glycol. The modified molecule is then converted into a tablet dosage form using standard unit processes. The modification provides resistance towards enzymatic degradation in gut. However, to ensure absorption of the molecule, the conjugated molecule is formulated with a permeation enhancer, the sodium salt of capric acid. Capric acid is a short chain fatty acid that is present in palm oil in large quantities and is known to increase gut permeability in a reversible manner.

The current thesis is focused on evaluation of various aspects of this oral insulin product. The first part focuses on evaluating the effects of formulation process on the structure and biological activity of pegylated insulin. The process of manufacturing involves high shear processes such as blending and compression of this blend. During blending the molecule experiences shear force due to friction against excipient particles. The tablet compression is a more complex process where the blend experiences friction while flowing through the hopper and on the rotating turret. Also, during compression of the table heat is released.
locally at the point of contact in each of the tablet. Since proteins are fragile molecules it was important to understand the effects of these process stresses and strains on the structural stability of the molecule as well as effect on biological activity.

The other two chapters of this thesis explore the in-vitro and in-vivo metabolism of the pegylated insulin analogue and also focus on determining the fate of the oligomer in a rat model. The pharmacokinetic profile mimics an ultra-rapid acting insulin profile, with peak maxima between 15 to 30 min from the time of administration, and the circulating levels of pegylated insulin coming back to baseline within 120 min. Since insulin therapy is a chronic potential life-long therapy, determining the fate of oligomer is important. The metabolism of pegylated insulin using a HepG2 cell line, which is a human hepatocyte cell line, was investigated and metabolites were identified over a period of 10 days using mass spectrometry. Subsequently, Pegylated insulin metabolism was studied in Wistar rats to trace the metabolites in Urine and faeces.

Pegylated Insulin is a first generation oral insulin and so in the last chapter the thought process and preliminary in-silico towards future potential development of improved oral insulin derivatives has been described. If oral insulin has to be truly successful and benefit patients in the long run, considerably more research is required. Making an orally bioavailable Insulin analogue also establishes technology that can be applied to developing orally bioavailable forms of other protein and peptide drugs.
Chapter 1

Introduction and Literature Review
1.1 Introduction

Diabetes is broadly categorized into type 1 and type 2. Type 1 diabetes is caused by loss of an ability to produce insulin due to the destruction of beta cells. Type 2 diabetes is a result of increased insulin resistance followed by loss of beta cell mass over a period of time. Type 2 diabetes is a progressive disorder. All type 1 diabetes patients require exogenous insulin throughout their lives to control hyperglycemia, while type 2 diabetes can be managed with oral anti diabetic agents (OAD) initially, depending on the stage of disease (Figure 1.1). Eventually, many type 2 diabetes patients require insulin treatment, which is shown to reverse insulin resistance (Scarlett et al. 1982). Early insulinization has been shown to have beneficial effects on preservation of residual beta cell function (Alvarsson et al. 2003). Even a transient insulin therapy has been shown to have beneficial effects in newly diagnosed patients with type 2 diabetes (Ryan et al. 2004, Ilkova et al. 1997). Irrespective of the aetiology of the diabetes, insulin remains the final treatment option.

![Diabetes continuum](image)

Figure 1.1: Diabetes treatment continuum and the duration of disease for type 2 diabetes
In type 2 diabetic patients the earliest defect observed is beta cell death, with the loss of first phase response (Weyer et al. 1999, Butler et al. 2003). The loss of first phase response results in inadequate suppression of endogenous glucose production resulting in postprandial hyperglycemia (Luzi et al. 1989). At the time of diagnosis of diabetes, it is believed that the beta cell mass is already reduced by 50% (Figure 1.2) (Lebovitz 1999).

Figure 1.2: Beta cell function and progression of type 2 diabetes (adapted from Lebovitz 1999)

Several years of research has not yielded any effective treatment for type 1 diabetes and insulin remains the only treatment for these patients. However, current insulin therapy is not physiological and has its own issues. Recently some new potential therapies are being developed for type 1 diabetes. Pramlintide, an analogue of amylin, enhances insulin action (Fineman et al. 2002) while others such as SGLT2 inhibitors increase the elimination of glucose via renal filtration and are not dependent on insulin action (Idris et al. 2009). Alternatively, alpha-glucosidase inhibitors prevent breakdown of starch to glucose (Chiasson et al. 1994) and thereby reduce glucose spikes. Some of these newer medicines
have the potential to be used in both Type 1 and Type 2 diabetes due to their non-specific insulin independent action. However, their long term safety and effectiveness is still being evaluated, which would be known over the next few years.

Over the years several drugs have been developed for the treatment of type 2 diabetes. The mechanisms of some of these drugs, biguanides, phenformin and metformin, are not fully understood even now. Although phenformin is no longer used in clinical practice due to increased death caused by lactic acid acidosis (Conlay et al. 1976), metformin is still considered a drug of choice as a first line therapy for type 2 diabetes (Bailey et al. 1996). Once metformin starts failing to control glycemic excursions, there are several other drugs like sulfonylureas, glitazones, DPP IV inhibitors, and SGLT2s inhibitors that can be used in conjunction with metformin. Sulfonylureas, insulin secretagogues, result in hypoglycemic events, and coax the pancreas to secrete more insulin, thereby causing increased rates of beta cell death (Matthews et al. 1998), and so these drugs have fallen out of favour. Clinical conclusions for these established drugs have been arrived at from meta-analysis of data available across multiple clinical trials and post marketing survey analysis and lack controlled experimental evidence. In developing countries such as India, sulfonylureas are still widely used as a second line of therapy after metformin therapy failure. Similarly, glitazones have been implicated in increased cardiovascular risk after long term treatment (Nissen et al. 2007).

Recently, there has been a spate of drugs which work on inhibition of DPP-IV enzyme, indirectly increasing circulating concentration of GLP-1 (Holst et al. 2005), which in turn helps in controlling glycemia. GLP-1 is a very promising therapy in the management of diabetes. The advantages of GLP-1 are that it increases insulin sensitivity, causes weight loss and has been shown to reduce insulin requirement over time. There is some animal
data which also indicates that GLP-1 may have a role in increasing beta cell mass. On the other hand, there has been a growing concern that GLP-1 treatments could be somehow connected to higher incidence of pancreatitis, as there are some sporadic reports of pancreatitis as well as thyroid cancer, although confirmatory evidence is still required.

Exenatide, a naturally occurring GLP-1 analogue is used for the treatment of diabetes. It works by increasing insulin secretion in response to increased glucose levels in blood and also reduces gastric motility, thereby reducing glycemic excursions (Cervero et al. 2008). Exenatide also increases satiety, resulting in weight loss in patients with type 2 diabetes (Kolterman et al. 2003, Dupre et al. 2004). Another synthetic GLP-1 analogue Liraglutide, was recently approved for the treatment of type 2 diabetes (Vilsboll et al. 2007). Despite so many different drugs being available for the treatment of type 2 diabetes, with time many patients stop responding to any of these therapies and finally have to resort to using insulin.

Despite close to eight decades of work on Insulin and diabetes, both remain incompletely understood. While there is considerable research which shows that diabetes affects the entire metabolism and eventually impact multiple different organs, the aetiology or the trigger to the disease is not very well established. We still do not know why insulin resistance increases in the first place in Type 2 diabetics. There are genetic and environmental factors that are known to increase the risk but there is no clear evidence of one event or series of events which conclusively leads to diabetes. There are other categories of latent onset diabetes in the young and in adults that have been identified over the last 2 decades or so. This occurs in a small percentage of the population having insulin deficiency similar to Type 1 diabetes, although it develops more slowly and can be misdiagnosed and mistreated as Type 2 diabetes (Groop et al. 1986).
For all forms of diabetes the treatments have been very glucose centric and symptomatic. The emergence of GLP-1 treatment has made the research community think beyond glucose and insulin and also emphasized that diabetes involves several other gut hormones in addition to insulin. Considerable focus has been given to understanding the biology of gut hormones, which affect gastric motility, gastric emptying satiety and play a role in glucose absorption and utilization by the body.

Besides these therapies, there have been attempts to regenerate beta cell mass and efforts are being taken to study beta cell biology, which may lead to the development of regenerative therapies. GLP-1s have been shown to increase beta cell mass in rodents but there is no evidence of increase in beta cell mass in humans (Wang et al. 1997). There are several proteins that have been reported to increase beta cell mass by either differentiation of ductal cells to beta cells or by increasing beta cell proliferations. One such protein which has been studied, Islet Neogenesis associated Protein (INGAP), belongs to the regulatory III protein family. It was shown to differentiate ductal cells into beta cells which produce insulin. In an experimental setting Rosenberg et al. wrapped a cellophane around the head of the pancreas in Syrian golden hamsters. After 5 days, ductal epithelia hyperplasia was observed, by 2 weeks lining of duct were formed by Pseudostratified epithelial cells with prominent goblet cells and new islet cells were observed (Rosenberg et al. 1983). The fact that the hyperplasia and new islet cells were observed without any external stimuli, led to the identification of INGAP protein (Rafaeloff et al. 1997).

A recent publication in Pancreas confirmed positive effects of chronological appearance of INGAP on beta cell mass, pdx-1 containing cells and insulin secretion in foetuses and neonatal wistar rats (Madrid et al. 2013). Researchers have tested a 15-mer peptide derived from INGAP protein in the clinic (Rosenberg et al. 2004). The molecule being tested in the
clinic is less potent than the native INGAP protein hence it would be interesting to see the clinical outcome. There are other peptides like betatrophin (22kDa) and osteocalcin that have been shown to have an impact on beta cell mass, however, the mechanism and role of these peptides is not yet fully understood. While regenerative therapies are some time away, there is enough evidence to show that they are likely to be a reality one day, given adequate research focus. Such a therapy may make insulin therapy redundant. However, until then there is likely to be no alternative to Insulin therapy especially in Type 2 diabetes.

Natural secretion of Insulin is glucose dependent whereas current therapies are not which puts the patient at a higher risk of hypoglycemia. There have been few attempts to develop glucose dependent insulin formulation. Hydrogels with concanavalin A have been shown to release insulin in glucose dependent manner by sensing glucose levels (Obaidat et al. 1996).

1.2 Glucose metabolism:

In normal healthy individuals, glucose once ingested is absorbed rapidly. There are receptors in the gut which trigger gastric hormone secretion and insulin. The gastric hormones help in slowing down the gut mobility, slowing down the absorption, while insulin helps in glucose removal from the circulation and uptake into various tissues. Liver is the primary organ where a large portion of ingested glucose is disposed off either by breakdown through glycolysis or storage into glycogen. In addition to glucose uptake, endogenous glucose production shuts down with glucose and insulin signalling, thereby reducing the total amount of glucose that is needed to be disposed of.

In diabetes patients, the glucose metabolism is significantly impaired when absorbed glucose is incompletely taken up by the liver, spilling out large quantities of glucose to be
taken care of by other peripheral tissues. In addition, gluconeogenesis in liver is incompletely suppressed, resulting in larger amount of circulating glucose. Earlier the belief was that gluconeogenesis is a major function of liver and incomplete suppression of gluconeogenesis contributes to hyperglycemia in diabetes. However, recently a better understanding of this process has evolved. It is now well established that inefficient glucose uptake by the liver contributes to hyperglycemia and is greater than the contribution of gluconeogenesis. A treatment improving glucose uptake by liver is likely to benefit patients more than the current injectable insulin therapy.

1.3 Development in Insulin treatment

Over eight decades of research has resulted in improved quality of the Insulin available and also in understanding the best insulin treatment regimen. In the first few decades after insulin discovery, animal derived insulin was being used, which had a limitation of supply as well as quality. The focus then was to solve these two problems. During 1960s and 70s the arrival of recombinant therapy enabled us to move from animal derived insulin to recombinant insulin as well as achieving higher purity of Insulin. In the past three decades or so, the focus has moved towards making insulin therapy more physiological or the development of alternate routes of delivery. Several new Insulin analogues have been developed in an effort to minimize variability, improve patient convenience/compliance and mimic insulin’s natural secretory pattern.

The natural insulin secretory pattern involves a rapid sharp burst of Insulin followed by a sustained release of insulin. The short burst of insulin, also called first phase response of insulin, is believed to play a critical role in switching the liver from anabolic state to catabolic state. The switch may be important since during anabolic states the liver produces glucose via gluconeogenesis and glycogen breakdown. When external glucose from food
starts getting absorbed, the switch helps in reducing the amount of glucose that needs to be disposed of.

During the progression of Type 2 diabetes especially, it is reported that in the pre-diabetic stage, the first phase insulin response starts diminishing, which is compensated by increased secretion of Insulin during second phase. Over time, with an increase in insulin resistance, the pancreatic cells get exhausted and produce less and less insulin due to progressive beta cell death. By the time a person is detected as diabetic, the beta cell mass is already down to about 50% of its original mass (Lebovitz 1999). A true replacement for Insulin therapy not only needs to restore the first phase, but also the second phase of insulin release in a physiologic manner. That is, insulin should primarily act on liver, which is the target organ. Ideally it should also mimic the glucose dependent secretory pattern of natural insulin.

In a normal individual, Insulin is secreted by beta cells of the pancreas in to the portal vein and is then carried to the liver first, where a large portion of insulin is extracted by the liver. The effect of variable insulin clearance on post prandial glucose was studied by Caumo et al. 2007. Caumo et al. in this study describe a mathematical model to predict the effect of variable hepatic clearance. The finding suggested that a small reduction to the tune of 20% reduction in hepatic extraction of insulin increased the plasma insulin concentration dramatically. Edgerton et al. 2006 showed a clear dominant effect of portal insulin on liver. The importance of first phase release of insulin has been extensively discussed in the literature (Mitrakou et al. 1992). Luzi and Defronzo 1989, in their hyperglycemic clamp study, where in phasic insulin release was artificially mimicked, concluded that the loss of first phase response may play a role in better glucose control. Pfifer et al. 1981, in a short review published in American Journal of medicine, captured
the difference between normal subjects. The study showed that while first phase response was lacking in non-insulin dependent diabetes, second phase response was more or less preserved. In the case of insulin dependent diabetes subjects, however, clearly both the phases were missing. The first phase insulin response has been suspected to play a role in signalling the liver to change from a catabolic state to an anabolic state. Insulin helps transfer glucose from the blood stream to adipocytes through translocation of glucose transporter 4 (Pessin et al. 1999) and gets stored as glycogen. It also increases body fat storage and increases protein synthesis (Zhang et al. 1999, Pilkis et al. 1974). On the other hand, it is possible that the first phase insulin response is an artefact of experimental settings where a sudden large bolus dose of sugar is administered, resulting in a sudden burst of insulin being released in response, which is unlike a meal situation. The research done by Caumo et al. 2004 concludes that while the first phase response against a meal might not exist in real life, the pulsatile release of insulin in portal vein serves the purpose.

Physiological insulin is secreted by the Pancreas under influence of glucose signal in the portal vein, which is then carried to the liver via the portal vein. In the liver, a significant amount of insulin is extracted by the liver and only a fraction of secreted insulin reached the peripheral circulation. The injectable insulins do not mimic this physiological gradient. In fact, the portal peripheral axis is reversed in the case of injectables with higher peripheral levels of insulin. This reversal in the physiological axis leads to weight gain, higher incidences of hypoglycemic events and other long term complications (Carlson et al. 1993). Weight gain is due to the fact that the higher exposure to insulin in the periphery results in storage of glucose in the muscle tissue instead of liver. This weight gain is associated with increases in peripheral insulin resistance, leading to high insulin dose requirements, increasing weight gain further, essentially resulting in long term complications, which may be attributed to the higher insulin exposure. It would be ideal to
develop an insulin therapy which does not cause weight gain and slows down progression of insulin resistance if not improving it.

1.4 Insulin Analogues:

Lispro, aspart and glulisine are faster acting analogues with rapid onset of action, as compared to soluble insulin formulation (Braak et al. 1996, Mudaliar et al. 1999, Becker et al. 2005). The rapid onset of action means patients now can take their insulin closer to the start of the meal and get a better control on the post prandial excursions. Also there has been a considerable reduction in hypoglycemic events as the late effect of insulin is minimized due to a shorter tail of the Insulin pharmacokinetic profile. The attempt to develop fast acting analogues is to provide a replacement of first phase insulin release, which is critical for reducing the post prandial glucose spike.

Insulin absorbed from the gut is the only insulin which may maintain physiologic portal peripheral axis. The rationale and potential advantages of oral insulin have been captured in few review articles (Hoffman et al. 1997, Arbit 2004). An effective, rapid acting oral insulin is likely to benefit not only from the physiology point of view but is likely to relieve patients by reducing the number of insulin injections that need to be administered on a chronic basis.

Insulin therapy for control of diabetes is suggested to generally involve a two step approach. Firstly the fasting glucose levels need to be brought down, and then the residual post-prandial glycemic excursions need to be controlled. Fasting plasma glucose indicates worsening insulin resistance and leads to a rapid increase in glycation of proteins and is linked to microvascular and macro vascular complications in the late stages of diabetes, while post-prandial glucose excursions have been shown to correlate strongly with cardiovascular risks.
From the patient point of view, multiple injections in a day is a major factor for poor compliance, and also increases the risk of hypoglycemia. Replacing these injections with alternate delivery could prove to be more beneficial than a traditional approach of development of long acting formulation. There are several potential advantages of alternate delivery. Since current therapy is an injectable therapy, by the time insulin therapy is initiated very little residual beta cell activity is remaining. It is hypothesized, that if insulin therapy is initiated early during disease progression, there may be a possibility of preserving beta cell mass, thereby slowing down disease progression. Alternate routes of delivery would potentially enable early initiation of insulin therapy.

1.5 Alternate routes of insulin delivery

1.5.1 Inhalable Insulin

Several alternate routes have been reported in the literature and a few have reached clinical development or market. Exubera was the first alternate insulin product to get approval for marketing authorisation and this was in the US in 2006. It was developed as an alternative to injectable rapid acting insulin, based on technology developed by Nektar Pharma. During the review, however, regulators in the UK and EU did not find Exubera to be any better than injectable insulin, reducing its utility only to that of enabling an alternative to injections. The product was withdrawn from the market in late 2009 due to poor sales and safety risks. The failure of this product in the market can also be attributed to high cost and difficulty in handling the device.

Recently, another inhalable product Afrezza was approved for marketing. The product is a rapid acting meal time dry powder inhalation insulin product. The second generation device is sleek, making it convenient for handling and usage. Afrezza was approved primarily on the basis of 2 clinical trials. The first trial was conducted to show superiority
over placebo in type 2 diabetes patients. The drug managed to show non-inferiority over Insulin aspart injection. The margin of non-inferiority was 0.4% in HbA1c change for the difference in the treatment efficacy. However, the study proved that over a background therapy of basal insulin Afrezza was non-inferior to Insulin aspart, but the treatment was statistically worse compared to Injectable insulin. This could be due to the fact that Afrezza in a way is an ultra-rapid acting insulin and controls glucose excursion over a period of 2 to 3 hours while Insulin aspart injection has a longer pharmacokinetic profile resulting in a post-prandial coverage for up to 4 to 5 hours (Rave et al. 2009). In addition, the Afrezza group needed to increase the dose of basal insulin to maintain a good glucose control. It is likely that if the basal insulin had not been adjusted, the difference between the two treatment groups would have been larger and not in favour of Afrezza. Considering these results, it would be interesting to see how Afrezza works in real life settings once larger patient pools are exposed.

1.5.2 Insulin transdermal patches

Transdermal drug delivery is an interesting option as a non-invasive delivery system. Several hydrophobic, low dose molecules are available in the form of transdermal patches. Scopolamine patches were the first ones to get approved by FDA followed by Nicotine patches (Paudel et al. 2010). Subsequently several patches containing analgesics, contraceptives and local anesthetic drug patches have been approved with increasing frequency. Transdermal delivery offers several advantages over oral or injectable delivery. It helps bypass the first pass metabolism in liver following oral delivery. Also, it is painless and easy to apply and prevents generation of unwanted medical waste (Praustnitz and Langer 2008).
Despite these advantages, the challenge of low skin permeability needs to be overcome, which has driven advancement of the patch technology. Lately, to improve the drug delivery efficiency, patches have been combined with microneedles (Zhu et al. 2013), sometimes made from dissolvable polymer. This has also been combined with a pump through which more sustained delivery has been achieved (Zisser et al. 2006).

Several attempts have been made to develop insulin transdermal patches. A group of Japanese researchers reported delivery of insulin using CaCO₃ nanoparticles suspended in petroleum jelly. The jelly was applied on the skin of ddY mice and samples were collected to study the pharmacokinetics.

ddY mice strain was introduced between 1910 and 1920s from Germany and established as a strain for hyperdyslipidemia at the National Institute of Infectious Diseases, Yoken. The ddY strain was derived from a mouse colony at the Institute of Infectious Diseases of Tokyo University (Denken) presently known as the Institute of Medical Sciences of Tokyo University. The strain name ddY stands for Deutschland, Denken, and Yoken (Yamakazi et al. 2012).

From figure 1.3 one can clearly see the difference in the profile of nanoparticle delivered insulin versus subcutaneous insulin monomer. While the overall bioavailability reported was between 2 and 3%, the nanoparticle delivery was found to be more sustained as against a rapid bolus profile for subcutaneous injection.
Figure 1.3: The closed black circle (○) represent insulin levels in serum from nanoparticle application. The open circle (●) represents subcutaneous administration of monomeric insulin. The error bars represent standard deviation (adapted from Higaki M et al., Diabetes Technology and therapeutics).

The nanoparticle Insulin reduced blood glucose levels in a dose dependent manner as seen from the figure 1.4 below.

Figure 1.4: Time course profiles of blood glucose in ddY mice. Blood glucose levels with transdermal nano insulin [200 (---), 100 (-- -- ), 50 (-----), and 0 (-----) μg] and subcutaneous monomer insulin (3 μg (---)) were measured. The results are shown as mean ± SD. (adapted from Higaki M et al., Diabetes Technology and therapeutics, 2006)
The decrease in blood glucose was dose dependent for insulin delivered transdermally using nanoparticles. The difference in the blood glucose at 4 hour and 6 hours was statistically significant compared to subcutaneous insulin. The pharmacodynamics profile of nanoparticles matched closely with the pharmacokinetic profile (Higaki et al. 2006).

Similarly, King et al. demonstrated absorption of insulin using a biphasic lipid system (King et al. 2002) delivered using a patch in a Sprague-Dawley rat model. The pharmacokinetic bioavailability of insulin was reported to be between 20 to 30% and the glucodynamic effect was reported to be around 39%. Subsequently, ultrasound along with sodium lauryl sulphate has been reported to have synergistic effect on mannitol absorption (Mitragotri et al. 2000). Similarly, a microneedle patch combined with Iontophoresis was reported as a delivery system for Insulin (Chen et al. 2009). In this system, the preparation of insulin solution in PBS encapsulated in Nano-vesicles prepared from soy lecithin and polyethylene glycol was shown to have a considerable impact on the rate of insulin uptake. Various charges on nano-vesicles were imparted by incorporating 0.4% cetyltrimethyl ammonium bromide (CTAB), 0.4% sodium dodecyl sulfate (SDS) and 0.03% SDS into the nanovesicles. Almost all the preparations tried in this system showed considerable reduction in blood glucose levels in male Sprague-Dawley rats.

In another study, the development of transdermal patches with dissolving microneedles has been described. The microneedles were made up of peptidoglycan chondroitin sulphate, which is naturally present in the skin and could be safe for long term use. The study proved the feasibility of transdermal delivery of insulin (Ito et al. 2012, Morawski et al. 2009). Overall, the transdermal delivery system offers a lot of promise in terms of non-invasive delivery of macromolecules. The challenges specifically for insulin that need to be overcome are an ability to mimic either a short acting profile, which would replace a first
phase insulin response, or a basal profile, which would provide cover for the basal insulin requirement.

1.5.3 Oral Insulin

1.5.3.1 Potential benefits of oral insulin

The earliest attempts reported towards the development of oral insulin date back to 1936 where Lasch et al. tried to inject insulin directly in the intestinal loop. Later, Draggs et al. in 1937 and Young et al. in 1939 used a formulation containing hexyl resorcinol to deliver insulin orally and showed reduction in blood glucose levels in dogs and later in healthy volunteers. Early research started off targeting improvements in patient acceptance since the injections were not readily acceptable by patients. Until a couple of decades ago, oral insulin was looked upon as an alternative delivery system from the patient’s point of view. However, several recent publications suggested that there is more to oral insulin than just patient convenience. From 1980 onwards, several research groups have tried to differentiate intraportal effects of insulin from peripheral insulin.

Stevenson et al. 1982 conducted a study using a dog model, wherein they tried to differentiate metabolic effects of intraportal insulin from peripheral insulin. The group monitored the effect on glycerol, 3-hydroxy butyrate, alanine and lactate. With peripheral insulin they noted a considerable rise in insulin levels in periphery. Correspondingly, there was a drop in glucose levels. It was found that peripheral insulin reduced glycerol and 3-hydroxybutyrate levels at all infusion rates but did not impact levels of alanine and lactate significantly. The portal delivery of insulin on the other hand resulted in very little rise in peripheral insulin levels and showed a drop in glucose, but the magnitude of this drop was less than that from peripheral insulin. However, there was a large drop in the levels of all four metabolic precursors at all infusion rates. The experiment clearly demonstrated that
portal insulin clearly affected gluconeogenic as well as protein synthesis precursors, and had a wider metabolic impact as compared to peripheral insulin.

Later Shishko et al. 1992 compared the effects of continuous peripheral infusion with intraportal insulin infusion for four months in insulin dependent diabetes patients and monitored the effects on glucose, HbA1c and metabolic precursors. They found that insulin requirement for intraportal infusion was lower compared to peripheral infusion, despite the difference, the fasting plasma glucose levels in intraportal group were near normal, whereas the group which received peripheral infusion had very high and abnormal fasting plasma glucose. By the end of four months, in the group which received intraportal insulin, HbA1c had dropped from a mean of 13.8% to 5.5%, whereas in the peripheral insulin infusion group, HbA1c had dropped from 14.8% to 10.0%, clearly indicating superior effect of intraportal insulin delivery. It was also noted that intraportal insulin caused a significantly larger decrease in the gluconeogenic and protein synthesis precursors compared to peripheral infusion.

Later the same group published that the intraportal infusion caused a greater decrease in growth hormone levels and IGFBP-1 protein levels, compared to continuous peripheral infusion, as well as conventional insulin therapy. Corresponding to the lowering of IGFBP-1, there was an increase in IGF-1 levels. Only intraportal insulin infusion restored the IGF-1 levels to normal levels, indicating that the route of administration of insulin played a major role in IGF-1 (Shishko et al. 1994).

Intraportal insulin achieves better glycemic control through suppression of endogenous glucose production and improved glucose uptake (Sindelar et al. 1998). Besides the positive effects on metabolic precursors, glycemic control and insulin resistance, portal insulin also has been reported to have a smaller fall in energy expenditure compared to
Injectable insulin and has better respiratory quotient (Freyse et al. 2006). Overall, portal delivery of insulin seems to have considerable physiological benefits, however, due to lack of effective delivery technologies these potential benefits have not been realised. Upon oral delivery, insulin once absorbed from the gut is expected to move into the portal vein and will get carried to the liver first, thus mimicking natural physiological secretion of insulin. Hence oral insulin has drawn considerable interest lately.

1.5.3.2 Challenges in development of oral Insulin:

While there are potential benefits of oral insulin, for a successful oral delivery of insulin, a variety of challenges must be overcome. First and foremost is what happens to insulin upon oral delivery? Insulin being a protein is degraded extensively by the stomach enzymes like pepsin, trypsin and chymotrypsin in different parts of the gut. Besides the digestive enzymes, there are brush border enzymes which hydrolyze proteins. These enzymes have been well characterized and are shown in Figure 1.5.

![Figure 1.5: Important brush border membrane peptidases and their substrate requirements (Image taken from Langguth et al. 1997).](image-url)
If anything survives the luminal enzymes, it gets filtered by the mucin layer, which essentially acts as a gel permeation layer and prevents large molecules from getting closer to the intestinal walls. Beyond this, the next challenge is to cross the tight junctions, where the non-specific proteases and insulin degrading enzymes are lurking to digest any remaining insulin. As the name suggests, tight junctions are the junctions which control the transport of ion, water and other molecules. Tight junctions were first identified under electron microscopy by Farquhar and Palade, 1963. It was only in late 1980’s and early 90’s when the proteins associated with tight junctions were identified (Citi et al. 1988, Gumbiner et al. 1991, Furuse et al. 1993). It was the same group of researchers who later established the interaction of Occludin proteins and Zonula Occludin-1(Furuse et al. 1994). In addition to that, the domains responsible for the association were identified by protein sequencing. This interaction has considerable importance for improving the absorption of macromolecules as will be evident in the later part of this chapter. All these barriers result in poor bioavailability. To overcome these challenges, considerable effort has been put into the development of formulations that will result in oral bioavailability of insulin, but with little success.

1.5.3.3 Approaches for Oral Insulin Delivery

Several methods have been explored and reported towards attempts to deliver insulin orally. Out of many approaches a few have focused on addressing the degradation in gut and the transportation barrier in the gastrointestinal tract (GIT). The enzymatic degradation has been commonly addressed using inhibitors (Langguth et al. 1997, Shah et al. 2004, Bernkop-Schnurch 1998), pro-drug strategies (Sood et al. 2001, Steffansen et al. 2004, Han et al. 2000), and encapsulation (Damage et al. 2007, Delie et al. 2005, Leobandung et al. 2002). Uptake through the gastrointestinal membranes is typically addressed by use of carriers (Thwaites et al. 2007, Pang et al. 2003, Foster et al. 2005, Ronnestad et al. 2007),
enhancing receptor-mediated transport (Liu et al. 2003, Tsuji et al. 1996, Shah et al. 1996), and the use of specific or non-specific permeation enhancers (Salama et al. 2006, Anderson et al. 1995, Salamat-Miller et al. 2005). Each of these approaches have had limited success and the proof of concept has been shown in animal models; but none of them have really moved into clinical development. The different approaches are discussed in more detail below.

1.5.3.4 Enzyme Inhibitors

The extent of absorption is likely to be a function of the residual concentration of intact protein available for absorption post enzymatic degradation. If protein can be made to survive longer, one can expect an improvement in the overall bioavailability. Hence to reduce the loss of protein, enzyme inhibitors have been tried. Multiple factors need to be considered for choosing the right inhibitor, for example, site of absorption. In the stomach, pepsin is present, in the duodenum and intestines trypsin and chymotrypsin are the main enzymes. In the lumen, brush-border and intracellular environments, non-specific or enzymes with broad specificity are present, which makes it challenging to find a single enzyme inhibitor. While making a choice of inhibitor, one needs to ensure that the inhibitory activity is reversible to prevent any potential safety concern that may arise due to long term usage of proteolytic inhibitors. Several enzyme inhibitors have been identified and reported in literature. Interesting approaches have been used to co-administer the inhibitors with the protein of interest. There are some reports of covalent binding the inhibitors with the protein of interest (Marschutz et al. 2000, Bernkop-Schurch et al. 1998, Bernkop-Schurch et al. 1998), which essentially is building up dual functionality in one molecule.
The enzymes of the Gastrointestinal tract are well studied, so a systematic assessment of the digestion of each protein therapeutic can be performed in vitro and in vivo (Bernkop-Schnurch 1998). Identification of the individual enzymatic boundary for any protein helps identify or develop specific enzyme inhibitors which are expected to just block the action of the specific enzyme. These enzyme inhibitors include but are not limited to pepsinostreptin, aprotinin, chymastatin, elastatinal, EDTA, chitosan-EDTA, soybean or essential pancreatic trypsin inhibitor, amastatin, puromycin, bestatin, phosphinic corrosive dipeptide analogues, and enterostatin (Sood et al. 2001, Bernkop-Schnurch 1998, Larionova et al. 1999, Hussain et al. 2004). Often enzyme inhibitors are concomitantly administered with the protein of interest. Plate et. al. 2002, in their study, developed a delivery system which included immobilized pancreatic and soybean trypsin inhibitor demonstrating low degradation of insulin in the presence of trypsin, α-chymotrypsin, and elastase. Similarly, citric acid and aminopeptidase inhibitors have been shown to prevent degradation of model peptide when released along with the drug (Langguth et al. 1997). In a similar study aprotinin was used as the enzyme inhibitor (Larionova et al. 1999).

Despite many attempts using various technologies, an oral insulin continues to be a distant reality. It may require a problem solving at multiple levels before oral insulin becomes a real option in the treatment of diabetes. For the past several decades, oral insulin development has been attempted across various groups. Various approaches have evolved and can be categorized as follows: Encapsulation, use of permeation enhancers and modification of Insulin.

1.5.3.5 Encapsulation

Encapsulation with chitosan, polylactide-co-glycolide co-polymer, cyanoacrylates, beta-cyclodextrin and liposomes are some technologies that have been reported extensively
(Co´zar-Bernal et al. 2011, Sullivan et al. 2004, Chao et al. 1994). However, these approaches solve only part of the problem. Encapsulation in micro- or nano-particles prevents enzymatic degradation, however, the absorption of particles is dependent on pinocytosis process or uptake via peyer’s patches, which could be slow and variable (Zhang et al. 2009). In addition, variability of the release of the payload, and the timing of such a release, have not been very well understood. Most of these studies are done in animals, primarily rodents, where under overnight fasting the data looks impressive. However, more studies are required to understand the usefulness of these systems in a real life setting.

1.5.3.6 Use of permeation enhancers

Proteins are generally absorbed by the active transport system, receptor mediated transport, or a paracellular pathway. Smaller peptides like octreotide, desmopressin, and thyrotropin releasing hormone are believed to be absorbed via paracellular transport (Pauletti et al. 1996). Large proteins cannot get absorbed through the tight junctions of epithelial cells. However, efficiency of paracellular transport can be increased in the intestine through the use of permeation or absorption enhancing materials to allow increased absorption of larger protein like insulin.

Often paracellular absorption enhancers are used in addition to enzyme inhibitors. Studies by Takeyama et al. 1991 and Komada et al. 1985 exhibited that nafamostat when used as an absorption enhancer inhibits enzymatic degradation of insulin. In the studies performed by Radwan et al. 2001, different permeation enhancers were tried and their effect on degradation with chymotrypsin was evaluated. The published data showed that glycerrhizic acid and deoxycholic acid were the most effective in preventing insulin degradation by chymotrypsin.
Several other chemicals have been tried as permeation enhancers, including short chain fatty acids, their salts and esters, zolnula occludens toxin (ZOT), bile salts, beta-cyclodextrin and its derivatives. ZOT in particular was found to have a profound effect on Insulin uptake. ZOT is derived from a toxin produced by *Vibrio cholera* that is known to reversibly change or modulate the tight junction permeability in intestine (Fassano et al. 1991). It is an octapeptide derived from the binding site of a large 399 amino acid native protein (Boudry et al. 1992). It increases permeability reversibly of the tight junction via PKCa mediated polymerisation of actin fibres in a dose dependent manner. While there are several publications which show promising results from the use of ZOT, it being a toxin means that concerns exist about its safety (Fassano et al. 1997). However, it is still a very promising permeation enhancer showing the desired safety profile and good effectiveness as a permeation enhancer.

Another interesting permeation enhancer is the sodium salt of capric acid. Lindmar et al. in their studies compared caproate (C6), caprylate (C8), caprate (C10) and laurate (C12) short chain fatty acids for the transport of mannitol across CaCo2 cell line in a dose dependent manner and found that concentrations above critical micellar concentrations were the ones which were effective (Lindmark et al. 1995). In an intresting experiment by Tomita et al. it was concluded that caprate improves colonic absorption more than the jejunal absorption of cefmetazole (Tomita et al. 1992). It is shown to be effective in increasing permeability of a variety of molecules (Maher et al. 2009) in a concentration range of 10 to 13 mM. It is shown to cause reversible permeation enhancement without causing damage to the epithelial layer. Sodium caprate has been shown to enhance uptake of several molecules. Kim et al. showed that in Caco-2 cells it can enhance transport of insulin by as much as 70 fold (Kim et al. 2003).
There have been some publications on the use of bile salts as permeation enhancers. Bile salts are secreted by the gall bladder to facilitate emulsification of fat and transport of fat across intestinal walls (Hofmann 1963). Several other permeation enhancers have been reported in the literature. Most of them have been shown to be effective in animal models but very few have been tried in humans, and those that were tried showed limited success. Considerable research is required to identify efficient and safe permeation enhancers that will take the field of oral delivery of peptides forward.

1.6 Protein modification

1.6.1 Pegylation

Pegylation as a protein/peptide modification is a technique of choice to improve the physicochemical properties of these drugs. Pegylation has been studied for several decades now with a considerable understanding of its toxicity in vivo across several rodent and non-rodent species. PEG of various different molecular sizes are used to either improve pharmaceutical properties like increasing solubility, or improving stability at room temperature. Several examples of modified proteins for sustained systemic exposure or for targeted delivery to the disease sites have been reported. Although several years of research has gone in the study on pegylation, the choice of PEG is still largely trial and error with limited ability to forecast the pharmacokinetic behaviour. The rate of absorption and extent of absorption of modified or pegylated proteins are believed to be governed by physiological factors, physicochemical properties and the interaction of the two in-vivo. Pegylation primarily results in increase in molecular size, which results in reduced renal clearance. This slower rate of clearance combined with amphilicity of the PEG results in peripheral distribution and possibly passive diffusion into several tissues. On the one hand, the large polymer size helps in prolonging the residence time in the body, while on the other hand, it might also result in tissue accumulation. There is some evidence of tissue
accumulation correlating with the molecular size of PEG. Lower molecular weight PEGs are readily cleared by the kidneys while for PEGs of molecular weights greater than 50 kDa the clearance was primarily hepatic (Yamaoka et al. 1994). Do the changes in the physicochemical properties of protein result in different clearance pathway? A very good example is a study published by Chen et al. 2000. In this study the authors reported that pegylated interleukin-2 showed 130 times lower partitioning into the lymph node from the peripheral compartment and also had a significantly reduced rate of clearance.

1.6.2 PEGs for reducing Immunogenicity

In another study adenosine deaminase (ADA) was modified with polyethylene glycol using cyanuric chloride chemistry. The modified adenosine deaminase (PEG-ADA) showed lesser immunogenicity in mice following multiple dosing via intravenous administration. PEG-ADA showed lesser reactivity to the antibodies raised against native ADA, suggesting a change in the binding epitope. Pegylation also resulted in increased circulating half-life of PEG-ADA. The reduced immunogenicity and longer circulating half-life made PEG-ADA suitable for the treatment of ADA deficiency (Davis et al. 1981).

On the other hand there are reports suggesting reduced pharmacokinetic exposure of pegylated liposomes. Ishida et al. have published multiple research articles clearly showing enhanced clearance of liposomes after repeated injections. The response across species is similar except for difference in time of preconditioning (Ishida et al. 2003, Ma et al. 2012). The same group reported the role of the spleen in enhancing the rate of clearance (Ishida et al. 2006). Koide et al. reported a connection between particle size of liposomes and the accelerated clearance of pegylated liposomes from circulation. The particles above 50 nm appeared to elicit an immune reaction resulting in faster clearance (Koide et al. 2008).
1.6.3 Pegylated insulins

Considerable work has been undertaken to improve various aspects of insulin therapy. Several approaches have been reported in the literature pertaining to insulin conjugation. The focus has been towards either making faster acting insulin, for example lispro, aspart and glulisine, or towards insulin analogues having longer duration of action, for example glargine and detemir. Along the lines of detemir, there have been several other attempts of modifying insulin by conjugating insulin with fatty acids, PEGs or mono or polysaccharides, sialic acid etc. (Gregory et al. 2005).

Work presented by Uchio et al. demonstrated that diglycosylated Insulin at B1 and Lys B29 position exhibit lower propensity for fibrillation while retaining biological activity. Conjugation at Gly A1 appears to result in decreased potency. On the other hand modification with PEG resulted in longer half-life and reduced immunogenicity as compared to glycosylated Insulins (Caliceti et al. 2003, Uchio et al. 1999).

1.6.4 Pegylation for increasing half-life

There are reports of pegylated insulin with increased half-life (Hinds et al. 2002). Hinds et al. studied the effect of site specific attachment of 750 Da and 2000 Da polyethylene glycol moiety. They tried conjugation at B1 and B29 sites and found that largely the secondary and tertiary structures remained unaffected. They reported a change in aggregation propensity with pegylation, pegylated insulin having lower propensity. B1 conjugated insulin showed significantly higher stability upon shaking. In addition, they also reported considerably lower antibody formation when injected in mice compared to unconjugated insulin. The conjugated insulins also showed a relatively high bioavailability when injected in dogs. The half-life increased depending on the size of PEG. Larger PEG showed longer half-life. Similar increased half-life due to pegylation has also been
reported for other proteins. Walsh et al. (2003) reported increase in half life and reduced antibody reactivity for a 27 kDa endoprotease lysostaphin by attaching it to a PEG. The use of branched PEG for conjugation increased the half-life to 24 h as compared to less than the 1 h for unconjugated lysostaphin. However, no information could be found in literature regarding metabolism and clearance of pegylated lysostaphin. Glucagon was pegylated with PEG 5000 for achieving longer circulating half-life. Structural analysis showed monopegylated glucagon to have better physical and chemical stability during purification and freeze drying (Stigsnaes et al. 2007). No information was found in the literature regarding the metabolism and clearance of pegylated glucagon.

Granulocyte colony stimulating factor (GCSF) and interferon are other two proteins which have been pegylated to increase circulating half-life of the molecule. The pegylated GCSF is marketed as Neulasta while Interferon alpha 2b is marketed as Interon and interferon alpha 2a is marketed as Pegasys. There are several reports stating the changes in the physicochemical properties as well as improved pharmacokinetic properties of proteins upon pegylation (Pasut et al. 2009, Bansal et al. 2011). Modi et al. reported that Peg interferon alpha 2a was primarily cleared by liver (Modi et al. 2000). There is very little information available on metabolism and clearance of pegylated proteins, although individually PEGs and proteins have been studied for many years.

Currently, the modification of PEG is done empirically and it is difficult to predict the changes in the biopharmaceutical properties. Similarly, it is difficult to predict the metabolism and clearance pathway for pegylated proteins.

1.7 Metabolism and elimination of PEG

Abuchowski et al., 1977 reported modification of bovine serum albumin by covalently linking it to polyethylene glycol methyl ethers of 1900 and 5000 Da. The pegylated bovine
serum albumin did not elicit any immunogenic response in rabbits on intramuscular or intravenous administration. Pegylated bovine serum albumin was double labelled with $^{125}$I and $^{14}$C for the study. Both the labels were recovered completely in urine after 30 days, indicating complete clearance from the body. Additionally, researchers also reported significantly different solubility, sedimentation, electrophoretic and chromatographic behaviour of modified bovine serum albumin (Abuchowski et al. 1977).

There are no definitive metabolism studies conducted on the PEG molecule. However, Webster et al. (2000) have published an extensive review of the available information in the literature on PEG metabolism. The metabolism of PEG appears to occur through the oxidation of the hydroxyl group of the PEG giving rise to dicarboxylic acid. For example, PEG metabolites like diacid and hydroxy acid were recovered in plasma and urine of burn patients, rabbits and in the biliary secretions of cats (Harold et al. 1989, Friman et al. 1993, Friman et al. 1990). Since PEG oxidation requires free alcohol groups, a similar degradation is not expected when the alcohols are blocked by methyl groups via ether linkages. PEG is reportedly metabolized via sulfation in the excised liver from guinea pig and in in vitro liver preparations of rat/guinea pig, by Roy et al., 1987, 1988. It can be concluded that ethylene glycol monomer is not recovered as a metabolite of PEG400 in humans (Schaffer et al. 1950).

1.8 PEG toxicity

PEG is metabolized by alcohol dehydrogenase in a phase 1 metabolism reaction in mammalian systems (Harold et al. 1989). P450s were suspected to play a role in PEG oxidation (Veronese and Pasut 2005). The literature evidence shows that PEG metabolites, generated by a series of first and second phase metabolism reactions, show very little serious toxic effect in animals. However, overdosing of PEG is reported to give rise to
carboxylic metabolites and has been correlated with increases in calcium concentration in serum (Bruns et al. 1982). The rise in serum calcium and reduction in ionized calcium was attributed to calcium chelation due to dicarboxylate metabolites of absorbed polyethylene glycol.

Upon chronic oral administration of PEG1500 at a dose of 0.06 g/kg/day and PEG4000 at 0.02 g/kg/day, for a period of 2 years, no serious adverse effects were seen in a rat model. When PEG1500 and 4000 were mixed with food at 4% concentration and given to rats, no adverse effects were observed. No effects were seen with PEG400 at 2% concentration in food, with higher doses not showing any specific effects on the liver. These data indicate that chronic administration of PEG in rats did not result in any serious toxicity issue. In a year long chronic toxicology study with PEG 400 to PEG 4000 in dogs no adverse effects were seen (Smyth et al. 1955).

In a study conducted in monkeys, lesions on kidney were observed post oral administration of PEG200 at a dose of 2.2 to 4.4 g/kg. The lesions were associated with renal deposition of oxalate crystals but no clear association with adverse effects was reported (Prentice et al. 1978). PEG400 also has been reported to show adverse events in Monkeys upon intravenous administration. The side effects involved loss of appetite, a greasy texture of their lower extremities, swelling on the genitals and legs, and deteriorating infusion sites (Lockard et al. 1979). PEGs are neither associated with any teratogenicity or reproductive toxicity, nor do they cause mutagenesis or cancer (Frujitier-Polloth 2005).

Yamaoka et al. 1994 conducted a very systematic clearance study of PEG. They established a clear relationship of molecular weight of PEG with the rate of clearance from the body as well as distribution.
Figure 1.6: Effect of molecular weight on distribution of PEG and relationship of PEG molecular weight and its circulating half-life. (Reproduced from the publication of Yamaoka et al., 1994)

The study covers the molecular weight range from 6 KDa to 170 KDa. The data presented clearly showed distribution of PEG to extravascular tissues as the PEG molecular weight decreased. Beyond molecular weights of 50 KDa, the PEG uptake by liver increased again, and the mechanism reported is pinocytosis. Does this mean that choosing the appropriate size of PEG for modification of a protein could be used for liver targeted delivery? And if it is indeed true, then it is all the more surprising that a liver targeted pegylated Insulin has not been reported yet.

1.9 Absorption, Distribution, Metabolism and Elimination (ADME)

Absorption, Distribution, Metabolism and Elimination (ADME) studies are conducted during development to assess metabolic/degradation pathway, rate of distribution, rate and route of elimination of the drug compounds, and to identify any potential toxic metabolites ahead of in-vivo testing. Typically, ADME studies are carried out by radiolabelling the molecule which enables the tracking of molecules even after transformation within the body. Several in-vitro systems have been developed to study the metabolites and have been reviewed by Plant 2004. No formal ADME studies are generally carried out for proteins as
the proteins are expected to get processed by proteases and the amino acid subsequently recycled.

1.9.1 Insulin degradation:

Insulin degradation has been extensively studied and reported in the literature. There are several reports on the mechanism of insulin metabolism in various in vitro and in vivo systems. Insulin degradation activity was reported in isolated liver cells by Varandani et al., 1976. The data presented in the publication tracked the sequential degradation of insulin, where two chains of insulins are separated first by glutathione insulin transhydrogenase followed by proteolytic digestion. It was also shown that the A-chain accumulated when the samples were treated with EDTA. At higher concentration of insulin EDTA was not effective in inhibiting the proteolysis. Duckworth et al. (1997) reported two separate pathways of insulin degradation, one pathway which could be inhibited by insulin degrading enzyme inhibitors and another pathway that could be blocked with inhibitors of cellular processing. It is generally recognized that the rate of insulin degradation is governed by the rate of binding of insulin to its receptor. The insulin bound to its receptor is metabolized by proteases associated with cell surface and the internalized insulin and insulin fragments are metabolized by lysosomes.

Monoiodinated and tritiated Insulin were studied for metabolism by rat hepatocytes. Upon incubation at 15°C and lower, hepatocyte bound insulin showed no degradation for a period of 2 h. While at 37°C, fragments with higher mobility in gel electrophoresis as compared to Insulin were detected. The fragments showed binding to anti-insulin antibodies, indicating that the binding epitope was unaltered. On the other hand, affinity towards the insulin receptor was greatly compromised. The fragments were identified to be Insulin cleaved at Arginine B22. Accumulation of Insulin upon incubation with lysozyme
in the presence of Chloroquine, an inhibitor of lysozyme, confirmed that one of the pathways involved in Insulin degradation involves lysozyme. The group reported the presence of two separate degradation pathways for Insulin (Misbin et al. 1983).

Yu et al. 1996 proved that the proteins tyrosine phosphatase inhibitors, Peroxovanadate and vanadate, inhibit insulin degrading enzymes in isolated rat liver endosomes. While studying the impact of peroxovanadium on receptor phosphorylation, Bevan et al. further confirmed modest inhibition of insulin degradation in rat liver using intra portal injection of vanadate. Insulin degradation within isolated rat liver endosomes was inhibited by 1,10-phenanthroline. In the presence of excess Cobalt the degradation got reactivated, confirming that the degradation of insulin involves metalloproteases. Based on kinetics data and subsequent recovery of intermediates with intact A-chain, it was further concluded that endosomal degradation occurs simultaneous to degradation of B-chain with the disulphide bond still intact. The two chains are later separated by disulphide bond cleavage. Simultaneous appearance of various intermediates led to the conclusion that degradation of Insulin does not happen through sequential reactions, but through more complex parallel processes (Bevan et al. 2000, Seabright et al. 1996). Despite these studies there is considerable ambiguity in the sequence of degradation of insulin.

Insulin processing has been fairly well studied. A significant proportion of secreted insulin is processed in liver during the first pass. The first step in the degradation involves separation of chain A and chain B. This reaction is reported to be mediated by an enzyme called glutathione insulin transhydrogenase. Subsequently each of the polypeptide chain undergoes proteolytic cleavage.
A conjugated insulin analogue Levemir® has been developed and marketed by Novo Nordisk. In their NDA submission package the comparison of metabolic pathway of Insulin Detemir (Des B30 analogue with myristic acid modification at lysine B29) and insulin in the presence of liver and kidney cytosol was presented to the FDA. The results indicated that Detemir and insulin degraded in a similar manner in the liver or kidney cytosol in in-vitro experiment and the first step in degradation was separation of two chains by cleavage of disulphide bonds (Medical review, US FDA document number 21-536).

1.10 Pegylated Insulin: A novel Insulin analogue:

Oral delivery of peptides and proteins is an attractive alternative to parenteral delivery and has remained as a formidable challenge despite several research groups attempting to improve the bioavailability of proteins upon oral delivery. Technologies to improve the translocation of proteins across membranes are needed to achieve high oral bioavailability of protein or peptides. The following approaches have been explored for achieving high bioavailability: modification or conjugation of proteins to improve solubility, improved resistance to digestive enzymes, increased transport across membrane, or novel formulation technologies which will increase survival of the molecule, as well as transport of the molecule through gut wall (Sakuma et al. 2001). So far the individual approaches have not yielded any approved new drug products. It is very likely that a hybrid approach of modification combined with a formulation technology may give better success.

1.10.1 Modification of Insulin:

Pegylated insulin, a novel insulin analogue, is being developed by Biocon Limited, Bangalore (India), for the treatment of diabetes. It has identical polypeptide sequence to that of native insulin, and has a modification with alkyl peg at ε-amino group lys-B29 (figure 1.7). Pegylated insulin is unique in that no new molecules with small molecular
weight PEG modification have made it to the clinical development stage. Preparation and purification process of pegylated insulin is disclosed by Dave et al. (2008). The human recombinant insulin conjugated using NHS ester of an oligomer via site specific reaction conditions. The oligomer was then purified using a series of preparative HPLCs to reach desired purity.

![Figure 1.7: Structure of pegylated insulin](image)

The pegylated insulin purified as described above is then converted into a tablet dosage form. The tableting process involves unit processes which can result in high shear during blending and high compression forces during the tablet punching. As part of our research for this thesis, we have tried to determine the impact of these high shear unit processes on the structure of pegylated insulin. Insulin having three disulphide bridges is a fairly stable protein. However, the tableting process exposes the molecule to high pressure at the time of compression in a dry powder form. All the literature that is available today is around understanding the effect of hydrostatic pressure in the liquid state. Little or no information is available regarding the effect of high pressure on protein crystals in a dry state.
1.10.2 Effect of pegylation and tableting on structural integrity of the molecule

Upon oral delivery of pegylated insulin, the peak plasma levels are reached in about 20 min and the molecule is cleared from circulation in about 60 to 80 min. This rapid pharmacokinetic profile helps in reducing the post prandial glycemia up to about 2 h. The rapid onset and peaking makes the molecule a fast acting insulin in development (Iyer et al. 2010, Khedkar et al. 2010).

1.11 Aim of research

In the work presented in this thesis, we have made an attempt to understand in vitro and in vivo metabolism for pegylated insulin, so as to understand the fate of oligomer. Once the pegylated insulin is absorbed, it is important to understand if the PEG moiety remains attached to the polypeptide chain or is likely to get cleaved off. In the case where it remains linked to the amino acid, there is a possibility that some fraction of the conjugated amino acid would get incorporated into protein synthesis and lead to accumulation, as this is expected to be a chronic therapy. The accumulation if found, could raise safety concerns which would need to be studied further. In addition, we were also interested in understanding if the pegylation alters or interferes with the degradation pathway of insulin. To our knowledge, no publications could be found that investigate the impact of pegylation on the insulin or any other pegylated protein degradation pathway. We also made attempts to identify the metabolites of pegylated insulin in the hepatoblastoma cell line using mass spectrometry. Besides studying degradation using a cell line we have also attempted to study in-vivo degradation in a rat model. The objective here was to understand how the pegylated insulin is likely to get processed and to understand the fate of the oligomer in a rat model.
Our hypothesis was that pegylated insulin is likely to be processed similarly to native Insulin in both in-vitro and in-vivo systems and that pegylation does not influence the processing of Polypeptide. Based on the literature survey on PEG metabolism and clearance, the alkyl PEG used in conjugation of Insulin is likely to get cleared from the system without any adverse effect on the body. The experimentation involved testing of this hypothesis in rat hepatocyte cell line and the in-vivo studies were carried out in a normal wistar rat model and the route of elimination of these metabolites was identified.

The aim of our research was to understand the impact of formulation on structural changes of pegylated insulin toward oral insulin development.

In addition, we also studied the metabolism of pegylated insulin in an in-vitro setting and identify the metabolites.

We also aimed to study metabolism in an in-vivo setting, and traced the route of elimination for these metabolites.

Lastly, we studied the enzymatic degradation of pegylated insulin, as well as degradation in blood, in an attempt to devise a strategy for future development.
Chapter 2

Effect of tableting on the structure of pegylated insulin
2.1 Background and Introduction

For a commercially viable product, development of a dosage form is critical. Formulation development of pegylated insulin is a key component of developing a bioavailable delivery form. Even though pegylation improves the in vivo survival of insulin, due to its large size a permeation enhancer is needed to take it across membranes. Since Insulin therapy is a chronic therapy, safety of the permeation enhancer is an important concern. Several permeation enhancers are reported in the literature, out of which salts of short chain fatty acids are considered safest. Amongst the short chain fatty acids, lauric acid and capric acid were found to be better than other fatty acids. Based on PK/PD studies carried out in a pancreatomized dog model at Vanderbilt University, capric acid was chosen as the permeation enhancer as use of capric acid showed higher systemic exposure to insulin.

Capric acid is a liquid in its acid form, hence a sodium salt was used in the formulation. Sodium caprate is an unconventional excipient and there is practically no experience of using it as a pharmaceutical excipient. Sodium caprate is a waxy, fibrous material (figure 2.3) and is difficult to handle during the manufacturing process. Much of the challenge faced during process development was due to sodium caprate. Sodium caprate can cause local irritation of nasal mucosal layers if inhaled, which makes handling it difficult. Additionally, the powder has poor and inconsistent flow properties, compression index and low bulk density, which makes it difficult to compress tablets of required hardness. Hardness of the tablet is important for physical stability during manufacturing and transportation. In addition, the bulk density of sodium caprate varied considerably. During development, the bulk density was found to vary from 0.04 g/cm$^3$ to 0.27 g/cm$^3$. This variation causes significant variation in capacity utilization and also affects mixing homogeneity, which poses content uniformity challenges. To overcome inconsistency in the flow properties, the powder was converted to granules by using a wet granulation
technique with isopropyl alcohol as the granulating agent, which helped fusing the fibres together resulting in considerable change in surface properties of the caprate particles. The granulated sodium caprate had higher bulk density (~ 0.3 g/cm³ vs 0.04 g/cm³) and better flow properties and compressibility index than the initial powder. Increase in the density had an impact on the batch size and capacity utilization of high shear granulator. Improved flow property resulted in better flow through the hopper, less segregation on the turret and more consistent filling of tablet die, thereby improving consistency on tablet weight. Better compressibility index enabled compression of tablets with high hardness, which gave the tablet better physical stability without affecting dissolution or other parameters.

Mannitol was selected as a diluent. Mannitol being a non-reducing sugar was chosen over lactose, starch or maltodextrin. The concern with lactose, starch and maltodextrin are that they contain glucose, which is a reducing sugar. Proteins are known to undergo the maillard reaction with reducing sugars. This concern was eliminated by the choice of Mannitol. The other components of formulations are disintegrating agent, glidant and lubricant.
A flowchart on the manufacturing process of pegylated insulin tablets is presented below;

**Dispensing and Sieving**
Spray dried mannitol, crosspovidone and fumed silica Pegylated insulin and Magnesium stearate were dispensed and passed through a sieve.

**Granulation of Sodium caprate**
Sodium caprate is granulated using alcohol as the granulating agent. The granules are dried and sized as per requirement using Quadro Co mill.

Checked for residual IPA content
Checked for moisture content

**Blending**
Pegylated insulin Drug substance is blended with other excipients using geometric mixing for 30 min using an octagonal blender.

**Lubrication**
The blend is lubricated with Magnesium stearate prior to compression for 3 min.

- Tested for Moisture Content
- Bulk density (tapped & untapped)
- Compressibility index, Hausner ratio
- Repose angle and Blend Uniformity

**Tablet Compression**

*In-process testing (Weight, Hardness, Thickness, Friability and Disintegration), Complete Analysis of Tablets*

2.1.1 Solid solid mixing:
The tablet manufacturing process involves mixing of all the excipients in the solid state. For any solid-solid mixing to happen homogeneously, the physical compatibility of all the material is important. There are no mathematical models currently available which can predict multi component solid-solid mixing accurately. Hence it is a good practice to ensure that the size and shape of all the components are closely matched as much as possible to prevent segregation of individual components from the mixture. Typically,
smaller particles tend to segregate from the larger particles in the hopper due to the vibrations of the machine, leading to variation in content uniformity or weight variation. In addition, segregation is also possible in the turret due to the continuous motion. The difference in density, and differential amounts of each material, adds to the complexity of solid-solid mix and are leading causes of segregation of material (Ottino et al. 2000). The surface morphology of the various excipients was studied under scanning electron microscope to get an idea of size and shape of the particles.

2.1.2 Proteins under pressure

Behaviour of proteins has been a subject of intrigue for several years. For example, life in the ocean can exist at very high pressure and studies in this area have led to some understanding of protein requirements at high pressure. Several publications subsequently have shown that high pressure prevents fibrillation. Dzwolak et al. 2003, reported that when bovine insulin is subjected to pressure it prevents aggregation. Later Jansen et al. 2004 reported that under pressure insulin tended to grow fibrils elongating in one direction and formed circular loops of fibrils which was contradictory to the previous reports. In some cases, preformed aggregates dissociate back to non-aggregated state (Gorowits et al. 1998) and in some cases pressure leads to formation of aggregates (Ferro-Gonzales et al. 2000).

Since, there are no tablet dosage forms for therapeutic proteins available in the market, hence virtually no studies have been reported towards understanding the behaviour of proteins in solid dosage forms, nor is the impact of the manufacturing process well understood. The compression of pegylated insulin tablets involves a pressure of 25 kg/cm² to ensure that the tablets have sufficient hardness, so that they can survive the packaging process and later do not lose integrity during shipping. In addition, an increase in
temperature during tablet compression is a well-documented phenomenon. Pressure is one of the factors known to affect the secondary and tertiary structure of proteins and has been well studied. At pressures below 200 MPa, the structural changes are typically reversible, however, at higher pressure (pressures above 200 MPa) the compaction brings the molecules close enough to result in changes that are irreversible in nature. When the molecules are close to each other and pressure is applied, one of the major changes that was expected was aggregation of pegylated insulin.

The functionality of a protein is closely associated with its structure, which is sensitive to high temperature and pressure (Meersman et al. 2006). Application of pressure to proteins may lead to reversible or irreversible denaturation (Mozhaev et al. 1996). Hummer et al. 1998 reported that protein destabilization under pressure results from insertion of water into the molecule, which leads to disturbance in hydrophobic bonds within the molecular structure, leading to destabilization. Hence dry proteins do not undergo denaturation due to temperature or pressure (Goossens et al. 1996).

Pressure induced changes in the three dimensional structure, dynamics and function of proteins has been reviewed extensively in the literature (Mozhaev et al. 1996). A pressure greater than 200 MPa or 2 kBar has been shown to cause irreversible denaturation of biological membranes and proteins (Jonas et al. 1994). Studies suggest that pressure induced denatured proteins have a high percentage of residual structure conserved and the changes are of a reversible nature (Li et al. 1976). Research by another group suggested that the pressure induced changes are irreversible in nature (Wong et al. 1988). The difference between these two conclusions could have been due to the concentration of the protein. At lower concentrations the changes were found to be reversible while at 10 fold higher concentration, the same protein exhibited irreversible pressure induced changes.
Thus once the pressure influence is removed there is a high probability that a protein regains its native structure if denaturation is not complete. Most of the literature is focused on studying pressure effects in the liquid state, primarily because traditionally proteins are used in solution form. Attempts to understand the pressure effects on protein in a solid state are important for the potential future development of solid dosage forms of proteins. Existing literature was not directly applicable to our investigation due to the difference in the state of the samples.

While the data from animal experiments (not published) and human experiments (Khedkar et al. 2010) showed that pegylated insulin gets absorbed intact, bioavailability of it is still low. There is considerable scope for improvement in bioavailability. As mentioned earlier, proteolytic degradation could play a big role in bioavailability. It is also important to understand if there are any structural changes to the molecule during the process of tablet manufacturing. The potential structural changes were studied primarily using circular dichroism in the experimentation study described in this thesis chapter. Pullen et al., 1976 have studied biological properties of chemically modified insulins (modification at A1) and they concluded that the modification at A1 leads to change in conformation and loss of biological activity. Molecular conformation is critical for activity. Hence studying the impact of the tableting process on structure is important.

The ensemble of therapeutic proteins has expanded exponentially in recent years due to rapid biotechnological advances (Dimitrov 2012). Although the common route of administration of therapeutic proteins has been through injections, a lot of research and development is being done towards achieving oral delivery of such proteins (Singh et al. 2008). Pegylated orally bioavailable Insulin is one such anti diabetic therapeutic protein that is expected to be amenable to oral delivery (Khedkar et al. 2010) in the form of tablets.
The unit processes involved in tablet manufacturing involves high shear and high compression forces, which may affect structural stability of proteins. The heat release during the tablet compression has been well documented (Wurster et al. 1986, Zavaliangos et al. 2008).

Figure 2.1: Infrared temperature map of the tablets. Below the tablet, image of the lower die is visible. (Image reproduced from Zavaliangos et al. 2008)

From figure 2.1 it can be seen that the temperature at the top surface and the sides, which comes in contact with upper punch and the walls of the die, had higher temperature compared to the edges and the lower surface. The magnitude of the temperature rise was reported to be approximately 8 to 12°C. If a similar magnitude of temperature rise occurred during tablet compression for pegylated insulin, it could potentially be enough to affect protein structure and function.

The tableting process involves mixing of pegylated insulin API with the excipients sodium caprate, mannitol, polyvinyl pyrrolidone, colloidal silica, and magnesium stearate, which is referred to as ‘blend.’ The tablet is formed by compression (under pressure of 25 kg/cm²) of the pegylated insulin blend, and this is referred to as ‘tablet’ throughout this chapter. In this study, it was assumed that any structural attribute that is present in the solid state of the blend and the tablet will be carried over into the solution state and would thereby influence the solution results obtained. It was also assumed that the results obtained were likely to represent irreversible changes, as during transition of the tablet to a solution any reversible changes would be lost.
With the above background, as part of this work, the pressure induced effects on pegylated insulin were studied using CD spectroscopy, mass spectrometry, and size exclusion chromatography (SEC).

### 2.1.3 Selection of pH for the experiment

The physicochemical attributes with respect to structure of pegylated insulin in blend and tablet in physiological solution conditions (pH 7.4) were studied to decipher the effect of the tableting procedure on pegylated insulin’s structural integrity. Besides the physiological condition, the studies were also performed in alkaline solution conditions (pH 9.6). It is known that at pH>9 the oligomer formation tendency of the Insulin molecule increases (Brange 1994) owing to differential disulphide interactions, arising probably due to changes in the side chain packing of Insulin at high pH. Considering that pegylated insulin is analogous to Insulin, any change in the side-chain packing should be reflected, more so under high pH condition, hence the selection of this pH condition.

One of the ways to study the effect of pressure on the protein structure is through unfolding studies by temperature and chemical melting. Unfolding by temperature melt enables determination of the melting temperature and the enthalpy change associated with melting, thus giving both quantitative and qualitative insight into the stability of and structural attributes of the molecule (Benjwal et al. 2006). Chemical denaturation using Urea or Guanidine hydrochloride helps to determine the free energy change associated with unfolding, as well as aids in understanding the protein unfolding process, since depending on the denaturant concentration used, one can trap and study, besides the unfolded and native states, the partially folded intermediate states and low energy excited states (Pace 1986). Thus using both thermal and chemical denaturation studies one can experimentally access the widely accepted ‘funnel view’ model of protein folding, which
is also known as the ‘energy landscape model’ (Plotkins et al. 1997). With this additional background, we also studied the thermal denaturation of pegylated insulin present in tablet and blend monitored at 222 nm by CD spectroscopy. It is expected that this study will form the basis to analyze the conformational stability of pegylated insulin in tablet and thereby provide insights into its circulatory time in the body.

2.2 Materials and Methods

2.2.1 Pegylated insulin blend and tablet preparation and dissolution in solvents for solution studies

The pegylated insulin was prepared as described previously by Partha et al. 2012. Blend was prepared by the addition of all the excipients in a geometric ratio of 1:1.6. The tablets, containing 5 mg of pegylated insulin, were prepared by direct compression of pegylated insulin API blended with sodium caprate, mannitol, polyvinyl pyrrolidone, colloidal silica, magnesium stearate as excipients.

For preparation of the stock solutions used in this study, 337 mg of blend containing 5 mg of pegylated insulin and one 5 mg tablet were dissolved in 15 mL of buffers to give a final concentration of 50 μM of pegylated insulin. The basic solution was prepared by dissolving one tablet in 20 mM Tris- HCl buffer (pH=9.6). Similarly for the preparation of neutral solution, dissolution was done in 7 mM Phosphate buffer (pH=7.4). Working stocks were appropriately prepared for each experiment.

2.2.2 Scanning electron microscopy:

SEM scans of all the excipients were done using a JSM 6701 F field emission scanning electron microscope manufactured by JEOL Company limited, Japan. The samples were fixed on a stub of metal with carbon paste adhesive, coated with 40-60nm gold and then scanned under the microscope.
2.2.3 CD spectroscopy

Samples were prepared in both 20 mM Tris- HCl buffer (pH=9.6) and 7 mM Phosphate buffer (pH=7.4) to give final concentration of 50 μM of pegylated insulin, for which Far-UV (260-200 nm) scans were acquired using a JASCO J-815 CD Spectrometer was obtained using 1 mm cuvette, with data pitch as 0.1nm, Data Integration Time (D.I.T) of 1 second and scanning speed of 200 nm/min.

2.2.4 Thermal denaturation and melting curve data fitting

Thermal denaturation was studied using CD technique by temperature melting experiments. The temperature melting was carried out from 15°C to 95°C with the help of the Peltier Temperature Controller (PTC-423S/15) with ramp rate and the temperature interval optimised at 3°C/min and 2°C, respectively. The data were fitted into the equation,

\[
y_{obs} = \frac{(y_n + m_nT) + (y_u + m_uT)e^{\frac{\Delta H_u}{RT} + \frac{\Delta S_u}{R}}}{1 + e^{\frac{\Delta H_u}{RT} + \frac{\Delta S_u}{R}}}
\]

……………….. (Dignam et al. 2001)

Where,

\[ y_{obs} = \text{Observed ellipticity, } y_n = \text{y intercept of native baseline, } m_n = \text{slope of native baseline,} \]

\[ y_u = \text{y intercept of unfolded baseline, } m_u = \text{slope of unfolded baseline, } T = \text{absolute temperature (°K),} \]

\[ R = \text{Gas constant (1.98 cal/mol °), } \Delta H_u = \text{Enthalpy change of unfolding,} \]

\[ \Delta S_u = \text{Entropy change of unfolding, using Microsoft Solver which is a very powerful routine based on the Marquardt–Levenberg algorithm and is used for the nonlinear least-squares curve-fitting. The structural content were calculated using data fitting into Chen’s equation.} \]
2.2.5 LC-ESI-MS data acquisition and analysis:
The LC-ESI-MS analysis was carried out using ESI HCT Ultra PTM discovery system (Bruker Daltonics, Germany) using 10 mM Ammonium acetate buffer (pH=7.4 and pH=9.6) and 100% Acetonitrile (ACN) as mobile phases and ACE® C-18, 250 mm x 4.6 mm, 5 μm particle size, as column. The column was initially equilibrated with 25% acetonitrile and the percentage of acetonitrile increased gradually to 40 in 15 min, during which the compound eluted at around 12 min. This was further followed by a wash step of 5 min with 60% acetonitrile and column re-equilibration. Mass Spectrometric analysis was carried out for the before and after melt samples in the respective buffers and data acquired in positive ionization mode with nebulizer gas pressure of 65 psi, dry gas flow of 11.0 L min⁻¹, drying temperature of 365°C and a target mass of 1400 m/z. 15 μL of sample was injected with a flow rate of 1.0 mL/min.

2.2.6 Size Exclusion Chromatography:
The buffers employed were 7 mM phosphate (pH 7.4), 20 mM Tris (pH 9.6) and an Arginine buffer (L-Arginine (1 g/L)/acetic acid/acetonitrile 65/15/20 v/v/v). Size exclusion column manufactured by Waters (Insulin HMWP SEC Column, 125Å, 10 μm, 7.8 mm X 300 mm, 5K - 150K, [WAT201549]), was employed for an isocratic method of 30 min duration having a flow rate of 0.5 ml/min with the injection volume of 100 μl sample (1 mg dissolved in 1ml of the respective buffer). 1mg/ml lysozyme served as a marker.

2.2.7 Hydrogen Deuterium exchange:
The pegylated insulin was reconstituted in 5 mM phosphate buffer for structural studies through D₂O exchange. The hydrogen atoms in pegylated insulin were replaced with deuterium by maintaining a ratio of 1:15 (hydrogen: deuterium) for 10 min. The experiment is purely temperature dependent and hence the temperature was maintained at
21°C. The exchange was stopped by the addition of ice cold 25 mM citric acid. In order to maintain low levels of back exchange, the samples were spotted immediately using alpha-cyano-4-hydroxy-cinnamic acid, on a chilled Matrix assisted laser desorption ionisation (MALDI) target plate and the spectrum was acquired within a time frame of 3-5 min.

### 2.3 Results and Discussion

As part of an exercise to understand the nature, size and shape of the particles, all the components of the formulation were studied under scanning electron microscope. The SEM pictures below reveal the detailed information on the particles for each of the excipient.

The particles were observed under different magnification wherein the magnification was chosen based on the particle size distribution found during the initial scan. For SEM to work, the samples need to be dry and should not be deliquescent. Pegylated insulin being a freeze dried material has tendency to absorb moisture when exposed to atmosphere but was not found to be deliquescent in nature which allowed us to scan it under SEM. None of the other excipients posed any challenge in handling during sample preparation for SEM.
Figure 2.2: SEM image of crystals of pegylated insulin. First image of the particles (top left, Panel A) was acquired at 1000X magnification, the top right image (panel B) was acquired was under 10000X magnification and the image at the bottom left (panel C) was acquired under 25000X. All the images were acquired from nearby fields.

From the SEM images (figure 2.2) of pegylated insulin, it appears that the crystals are fractured and contains a wide size distribution. The particles range from 100 nm to 20 micron size. Smaller particles appear to be present in clusters and the larger particles have non-homogeneous shape and size. Some crystals are cuboidal with well-defined edges where as several particles appear to be fractured. The impact of this wide distribution in particles size and shape is not clear but it was conceivable that it could be playing a role during blending.

Figure 2.3: Above SEM scans show that sodium caprate before granulation is fibrous in nature (panel A). The diameter of the fibre is around 60 nm to 300 nm (panel B).
Figure 2.4: SEM scan of sodium caprate granules (panel A), after granulation the fibres appear to be fused with one another (panel B).

The SEM scans, figure 2.3, of sodium caprate before granulation shows that the caprate in its native state is fibrous with fibres ranging from 60 nm to 300 nm in thickness. Caprate is also waxy in nature and develops static charge easily. These properties of sodium caprate make it a very challenging excipient to work with during tableting. Figure 2.4 left image (panel A) shows SEM scans of sodium caprate acquired after granulation of sodium caprate. The image on the left shows sodium caprate granules which look completely different from the sodium caprate before granulation. The granulation was done using isopropyl alcohol as a solvent at very low volume. The amount of solvent used was just enough to wet the sodium caprate. Granulation results in the fibres fusing together and forming slightly more compact particles. The use of isopropyl alcohol perhaps results in dissolution of the surface of caprate (figure 2.4, right image, panel B), which helps during the granulation stage. Granulation of caprate improves the flow properties of the caprate significantly and also helps during handling.

SEM scans of all the other excipients were taken (not shown here), which clearly showed that particle size and shape of each of the excipient varied considerably, which could potentially create issues during mixing as well as could result in segregation during off loading the blend, or in the hopper of the tableting machine, or on the turret during
compression. Hence the blending was done in a geometric fashion to attain a homogenous blend.

2.3.1 Pegylation does not alter structure of insulin.

The CD spectra in these experiments were recorded from 200 nm to 260 nm. There was considerable noise observed when we attempted to record the spectra below 200 nm. Due to this, there was a limitation in getting a definitive estimate of random coil and to some extent alpha helical content. The minima for random coil typically appears at 204 nm and one of the minima for helix is expected at 207 nm. During the melting experiments it was observed that the RMS values obtained from curve fitting using Chen’s equation (Chen et al 1974), increased beyond 20% consistently at higher temperature, hence the calculations of structural contents were less reliable. But from the graphs, it was possible to understand the changes in the structure.

The first set of experiments was performed to compare recombinant human insulin and pegylated human insulin. When the purified lyophilized crystals of either product were dissolved in 0.1N hydrochloric acid (figure 2.5 and figure 2.6) at room temperature, the CD profile showed clear minima at 208 nm and 222 nm, indicating well-formed structure with helical content. Beta sheet content was also seen, with a CD minima around 214 nm. During the melting experiment, upon heating to 95°C, the structure was stable up to temperatures of around 50°C, after which there was a visible sign of unfolding. The helical content decreased from 36% to about 16% with increase in temperature in both the products.
At pH 7.4 in phosphate buffer, upon melting (figure 2.7 and figure 2.8), the minima shifted towards 204 nm and we also saw a lowering of ellipticity at 200 nm, indicating unfolding of
the structure. The unfolding resulted in increases in coil content for both the products. The extent of retention of residual structure appeared to be higher for pegylated insulin.

Figure 2.7: CD spectra overlay at various different temperatures for pegylated insulin dissolved in Phosphate buffer at pH 7.4

Figure 2.8: CD spectra overlay at various different temperatures for insulin dissolved in Phosphate buffer at pH 7.4
In tris buffer at pH 9.6 (figure 2.9 and figure 2.10), there was no change in the minima from 208 nm. However, the minima at 208 nm became more pronounced. There was no or minimal change in the minima with rise in temperature but the magnitude was less pronounced at higher temperature. The profile appeared to be different from what was seen at pH 7.4 in phosphate buffer, although the CD data doesn’t enable determination of the exact structural differences.

Figure 2.9 CD spectra overlay at various different temperatures for pegylated insulin dissolved in Tris buffer at pH 9.0
The helical content of the pegylated insulin molecule in the blend and tablet was found to be similar (~30%) by Far-UV Circular Dichroism (CD) spectra showing that the compaction pressure did not affect the global secondary structure of pegylated insulin. This was substantiated by similar electro-spray-ionization (ESI) spectra, Hydrogen-Deuterium (HD) exchange values, and profiles for blend and tablet which are discussed later in the chapter.

2.3.2 Formulation excipients may have a protective effect on pegylated insulin

For facilitating tablet dosage for manufacturing, the active drug was mixed with excipients prior to compression. These excipients play a key role in ensuring that the tablets are aesthetic, and release the drug at the site of absorption in the desired manner. To ensure that all the tablets behave consistently, homogenous mixing is critical. At large scale, mixing is done using blenders; such as an octagonal blender, ribbon blender, cone or double cone blender. Blending is typically achieved with a tumbling motion inside the blenders. Due to this tumbling motion, the large bulk of particles move inside the blender.
resulting in friction between the particles. The particle surface properties, density and particles size all play an important role. The blend of pegylated insulin and its excipients was dissolved in HCl buffer at pH 2.0, and separately in phosphate buffer 7.4, and the blend was subjected to CD, mass spectrometric analysis and hydrogen-deuterium exchange.

A hydrostatic pressure greater than 300 MPa is known to irreversibly damage the structure of proteins in solution state, whereas lower pressure results in reversible changes in proteins (Heremans K 1993). No literature is available regarding the effect of pressure on completely powdered forms of proteins. When pressure is applied onto pegylated insulin during tableting process, irreversible changes to the pegylated insulin structure were anticipated. However, the tableting pressure was not detrimental to the pegylated insulin structure. The overall global structural attributes were intact even after high pressure applied during tablet manufacturing process and the local effects were small.

Figure 2.11: CD spectra overlay at various different temperatures for pegylated insulin blended with all the formulation excipients, dissolved in HCl at pH 2.0
Figure 2.11 shows CD spectra from melting experiment performed with pegylated insulin blended with excipient dissolved in HCl. The excipients fumed silica, magnesium stearate and crosspovidone were insoluble in water and were removed by centrifugation. The supernatant was used for the melting experiment. The spectra of blend and the spectra of purified pegylated insulin were similar and showed similar temperature profiles. When the Tm values were compared, an increase of approximately 9 °K was observed for the blend over Tm value of the drug itself.

When pegylated insulin was dissolved in phosphate buffer at pH 7.4, a slight increase of about 5 °Kelvin in the Tm value was observed, compared to that for a solution in HCl. This increase was expected, as at pH 2.0 human insulin is known to be in a monomeric state whereas at neutral pH, it is known to be present in a hexameric state. When the pegylated insulin blend was dissolved in phosphate buffer (figure 2.12) and CD spectrum was compared with purified pegylated insulin solution in phosphate buffer. It was noted that the
degree of unfolding was less in presence of excipients. Only at temperature above 85 °C was a slightly higher amount of random coil observed in the blend, as seen from a slight shift in the minima from around 208 nm to 204 nm.

An explanation of the intriguing fact that the protein pegylated insulin was not affected by a 25 kg/cm² (2.45 MPa) pressure may lie in the shielding that the excipients provide to the protein molecules. To explore this aspect we compared the size exclusion chromatograms of pegylated insulin API and blend (Fig. 2.13A). It was appealing to see that the blend exhibited a higher oligomeric state when compared with that of insulin, giving direct evidence that the excipients play a role in the oligomerization of pegylated insulin. A closer look at excipients shows that two of them, namely polyvinyl pyrrolidone and colloidal silica, can act as pressure absorbers. The colloidal silica may be visualized as cushioning pegylated insulin from high pressure and polyvinyl pyrrolidone might be triggering higher oligomer formation for pegylated insulin, considering its ability to bind rather strongly with polar moieties.

To obtain insight into the type of oligomerization (covalent or non-covalent), we acquired the SEC data for the samples in buffers of different pH. Thus size exclusion chromatography for blend and tablet solutions prepared in basic buffer was performed using neutral buffer and vice versa. In the chromatograms (Figs. 2.13B & C) corresponding to these acquisitions it is interesting to observe that the oligomers present in the neutral buffer are highly stable (Fig. 2.13B) and hence are most likely covalent in nature. However, the same is not true for the basic conditions, since the higher molecular size oligomers break into lower molecular size oligomers (Fig. 2.13C) when the SEC data is acquired in neutral solution for the blend and tablet dissolved in basic solution. This may be attributed to pegylated insulin accessing a different conformation in basic buffer.
This is corroborated by the thermal unfolding data for the blend and tablet at basic pH (Fig. 2.13D), which shows a $T_m$ shift of 10 °C when compared with the $T_m$ in neutral condition (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>Average $\Delta$Hu (cal/mol)</th>
<th>Average $\Delta$Su (cal.mol-1K-1)</th>
<th>$T_m$ (°Kelvin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegylated insulin Blend</td>
<td>7mM Phosphate buffer</td>
<td>5769.65</td>
<td>17.54</td>
<td>328.57</td>
</tr>
<tr>
<td>Pegylated insulin Tablet</td>
<td>7mM Phosphate buffer</td>
<td>8266.395</td>
<td>24.42</td>
<td>338.41</td>
</tr>
<tr>
<td>Pegylated insulin API</td>
<td>0.01N HCl</td>
<td>11213.04</td>
<td>34.69</td>
<td>323.65</td>
</tr>
<tr>
<td>Pegylated insulin Blend</td>
<td>20 mM Tris – HCl buffer</td>
<td>7154.8</td>
<td>21.47</td>
<td>337.58</td>
</tr>
<tr>
<td>Pegylated insulin Tablet</td>
<td>20 mM Tris – HCl buffer</td>
<td>9498.79</td>
<td>27.244</td>
<td>348.96</td>
</tr>
</tbody>
</table>

Table 2.1 Average $\Delta$H, $\Delta$S and $T_m$ values for three repeats of thermal denaturing acquisitions in neutral (7mM Phosphate buffer; pH 7.4) and basic (20 mM Tris –HCl buffer; pH 9.6) buffers

The enthalpy and melting temperature values obtained from fitting the CD melting curve at 222 nm, showed that the compaction pressure did influence the three dimensional structure of pegylated insulin. The higher melting temperature obtained for the tablet seemed to indicate that the application of compaction pressure led to formation of a stabilized pegylated insulin molecule. This was corroborated by the differences observed in the SEC chromatograms after melt. However these structural changes must be subtle and local since any global change should have reflected in the CD spectra and SEC chromatograms.

The assumption that tablet formation stabilizes the pegylated insulin structure, however, held true since the tablet shows an increase of 10°K when compared with the blend.
From Figure 2.13A, it can be observed that the pegylated insulin in blend elutes earlier than the pegylated insulin API, indicating higher state of association in the blend. However, the chromatograms of blend and tablets overlap completely (fig 2.13B), suggesting the association state of pegylated insulin in blend and tablet does not show any difference. The increased $T_m$ at basic pH may be attributed to non-covalent oligomer formation that is not very different from that formed in neutral pH and hence does not lead to a substantial change in retention time in the SEC (figs. 2.13A and B). These non-covalent oligomers may be thought of as arising due to a different conformation of pegylated insulin that is driven by electrostatic forces. Due to this it appears stable in terms of $T_m$ but is vulnerable to pH changes and shows higher association state inferred from earlier retention time, when the pH is changed from basic to neutral (Fig. 2.13C).
Figure 2.13: Overlay of Size Exclusion Chromatogram of (A) Pegylated insulin Blend (---) and Pegylated insulin API (-) prepared in basic buffer run in basic buffer, (B) Pegylated insulin Blend (- - ) and Pegylated insulin Tablet (---) prepared in basic buffer run in neutral buffer, (C) Pegylated insulin Blend (-.-) and Pegylated insulin Tablet (...) prepared in neutral buffer and run in basic buffer. The curves in (D) represent the overlay of the normalised fraction folded values of Pegylated insulin Tablet (solid line) and Pegylated insulin Blend (broken line) at 222 nm in basic pH obtained from the melting curve.
2.3.3 Tablet formation stabilizes pegylated insulin structure:

As described previously, pegylated insulin tablet formation involves the application of about 2.45 MPa pressure (25 kg/cm²) on the blend. Hence it may be expected that pegylated insulin present in the tablet will be irreversibly denatured. Surprisingly however, a Far UV CD spectrum, which can be regarded as a selective probe for changes in the secondary structure of proteins (Greenfield 2006), showed no difference between purified drug, blend and tablets with helical content of more than 30% in the tablet. The melting temperature \(T_m\) of a protein is indicative of its overall stability in a particular solution condition (Mergny et al. 2003). Furthermore, the enthalpy change \(\Delta H\) associated with thermal unfolding provides insight into the thermodynamic stability of the protein (Ross et al. 1981). Differential scanning calorimetry (DSC) and CD spectroscopy are the two techniques that are used to monitor and decipher the corresponding thermodynamic parameters for thermal unfolding of proteins. CD based analysis of the temperature dependent unfolding gives a direct insight into the secondary structure melts. Thus, observing the changes in the CD signal at 222 nm as a function of temperature helps in elucidating the behaviour of the helices present in a protein when exposed to temperature ramps. This not only aids in knowing if the thermal unfolding is multi-step or not, but also gives quantitative outputs like \(T_m\) and \(\Delta H\).

CD based Thermal unfolding data on blend and tablet in neutral pH was acquired at 222 nm and was fitted to equation 1. The average \(T_m\) and \(\Delta H\) values obtained from three repetitions of this experiment are summarized in Table 2.1. The single sigmoidal curves for blend and tablet show a single step unfolding of pegylated insulin in both blend and tablet, again indicating that the tableting pressure did not dramatically alter the structure of pegylated insulin. However, a shift in the \(T_m\) value by 10 °C indicates that the pegylated insulin structure has been stabilized after tablet formation. This stabilization may be
attributed to the increased van-der-waals and/or electrostatic interactions amongst the pegylated insulin molecules that come close to each other due to the pressure. One may argue that once the tablet is dissolved such interactions must also disappear, but the fact that an increased $T_m$ is seen for the tablet indicates that these interactions are stable but perhaps localized within the structural folds and hence are not picked up by the experiments described in the previous sections, probably due to the plasticity of the molecule that does not let the local interactions affect the overall globular fold of pegylated insulin. The Far-UV CD scan before and after temperature melt further corroborates this stabilization, since the spectra for blend after melt seems to show almost complete loss of structure for pegylated insulin, but this is not so for the tablet. The tablet seems to retain most of the secondary structural characteristics even after the melt as seen from the overlay of normalized fraction folded (2.13D).

2.3.4 CD spectra of melting experiments on pegylated insulin tablets

The tablets of pegylated insulin are compressed directly after the blending step. During tableting the blend or mixture with excipients pass through the hopper onto the turret. During the compression, there is release of heat, as noted earlier. Temperature mapping shows that local temperatures at the surface and the sides can rise as high as 10 to 15 °C, which is a large change for protein molecules. The effects of changes in temperature are well documented to be of more severe and irreversible in nature as compared to effect of pressure. These changes were studied and results have been captured in the figures below.
Figure 2.14: CD spectra overlay at various different temperatures for pegylated insulin tablet dissolved in HCl at pH 2.0.

Figure 2.15: CD spectra overlay at various different temperatures for pegylated insulin tablet dissolved in Phosphate buffer at pH 7.4.

Figure 2.14 is the CD spectra of tablets dissolved in HCl. The data showed that at lower temperatures the structure of insulin is largely intact but with an increase in beta sheet content, which may be due to the existence of higher oligomeric states. At higher temperature, the unfolding was more pronounced compared to purified crystals and tablet.
blend. When the tablets were dissolved at neutral pH the data again showed more beta sheet and turn at lower temperature, as seen from the shift in the minima to around 215 nm. As the temperature increased there was a significant loss of structure with an increase in random coil content.

Size Exclusion Chromatography is a time tested technique to deduce the amount of oligomers present in a protein solution (Gabrielson et al. 2007). One can also infer the tendency of oligomerization of a protein in different solution conditions based on the size exclusion chromatograms, where the higher oligomers elute before the lower oligomers and the monomer. Brange et.al. 1994 showed that Insulin at high pH has an increased tendency for oligomerization, probably due to disulphide scrambling. Insulins show a tendency for oligomer formation and the hexameric state is known to be the most stable oligomer that is physiologically active (Dunn MF 2005). Since pegylated insulin is an Insulin analogue, one may expect similar behaviour for pegylated insulin in SEC to that of Insulin.

The Size Exclusion Chromatograms in figures 2.16A and 2.16 B do not show any differences between the association states pegylated insulin in the blend and the tablet in neutral or basic pH conditions, which contradicts the finding from CD data. This could be due to reversible nature of oligomeric state or the oligomeric state could be insoluble and hence could not be detected in the chromatography.

2.3.5 Globular fold of pegylated insulin is not affected by the tableting compression:
Charge state distribution in ESI spectrum and HD exchange are elegant techniques to probe the folding and dynamics of proteins (Nettleton et al. 2000, Katta et al. 1993). Any change in the globular fold of a protein due to unfolding or conformational change will
result in changes in the charge states in ESI mass spectrum when compared with charge states observed for the completely folded native conformation of the protein.

![Graph A](image)

![Graph B](image)

Figure 2.16 Overlay of Size Exclusion Chromatogram of (A) Pegylated insulin Blend (-) and Pegylated insulin Tablet (---) sample prepared in basic buffer run in basic buffer. (B) Pegylated insulin Blend (-) and Pegylated insulin Tablet (---) sample prepared in neutral buffer run in neutral buffer.

When the blend samples and the tablets were dissolved in Tris buffer, pH 9.6 and were chromatographed at basic pH, the profiles were perfectly overlapping for blend and tablet. There was no visible peak of aggregate observed. In size exclusion chromatography, higher molecular weight peaks elute earlier and lower molecular weight peaks appear later. The monomer peak of pegylated insulin eluted at around 12 min. No peak was seen before the main peak. After the main peak a small peak was seen at the base on the main peak around 12.5 min which was a smaller molecular weight species. It could be due to some of the excipients or small quantities of separated A-chain and B-chain, due to high pH where
disulphide scrambling is known to occur. The peak at 15 min is probably due to the buffer itself, as it was also seen in blank injections (not shown here).

Figure 2.17 ESI-MS spectrum of Pegylated insulin (A) Blend, (B) Tablet and (C) Deconvoluted mass. The left panel corresponds to acquisitions in neutral pH while the right panel corresponds to acquisitions in basic buffer conditions.

Positive ion ESI mass spectrum of pegylated insulin tablet and blend acquired in physiological solution pH of 7.4 shows a similar profile with respect to the charge state distribution, with a relatively intense 4+ peak (Fig. 2.17). This similarity is also seen for the ESI-MS spectra acquired in solution with basic pH of 9.6 for tablet and blend. These similarities suggest that the overall globular fold of pegylated insulin is not altered due to the pressure applied for tablet formation. The ESI spectrum acquired in the basic solution shows a relative increase in the 5+ charge state when compared to the spectrum acquired at pH 7.4. This change may be attributed to opening up of the three dimensional structure, resulting in exposure of more protonating sites through the structure leading to populating higher charge states. Another reason for this change may come from the buffer effect of the charge state distributions. This stems from the fact that the ammonium acetate buffer results in increased ionization in a molecule due to its higher ion-pairing effect. Whatever the reason, it is reasonable to conclude from this data that the overall structural attributes of pegylated insulin is similar in both blend and tablet. Furthermore, the ESI spectrum also serves the purpose for identifying pegylated insulin, from the deconvoluted mass (Fig. 2.17)
C) that matches with the theoretical average mass of pegylated insulin (6025.6 Da). Complete characterization of this molecule has been described previously (Partha et. al. 2012). The deconvoluted mass \((M+H^+)\) was 6026.3, which corresponded to the mass of intact pegylated insulin. The charge envelop consisted of \(3^+\), \(4^+\) and \(5^+\) charge states.

![Figure 2.18 MALDI-TOF spectra of Hydrogen - Deuterium exchanged Pegylated insulin blend (A) and Tablet (B). The exchange times are mentioned in the individual panels. In all cases the deuteration results in addition of about 40 Daltons.](image)

In the hydrogen-deuterium exchange experiment (fig 2.18), when the samples were subjected to MALDI-MS at different time points, the blend samples showed an increase in mass by 38 Da over mass of intact pegylated insulin within 2 min and the mass increase stabilized across all the samples up to 30 min. For tablet samples, the 2 min samples showed an increase of 34 Da, which increased further to 6066.5 Da at 5 min, a 38 daltons
increase. The 30 min sample showed a slightly lower mass. No additional masses were observed in the samples.

These observation corroborate results of ESI/MS experiment. If a protein is partially unfolded there exists a high probability that a higher number of exchangeable protons, especially the backbone and side chain amides, will be exposed to the solvent and hence such a moiety will take up higher number of deuterons. Using this argument one can say that the pegylated insulin does not show any degree of unfolding in the tablet since the extent of deuteration is similar in blend and tablet.

2.4 Conclusion

At higher temperatures the insulin protein structure partially unfolded. At higher pH the amount of helix was about 10 to 12% lower than at acidic or neutral pH. There was no difference found in the structures of pegylated insulin and non-pegylated insulin, indicating that pegylation did not affect the structure of recombinant human insulin.

When the crystals were dissolved in Phosphate buffer or Tris buffer, upon melting pegylated insulin retained its structure better than recombinant human insulin did at higher temperatures. Pegylated insulin continued to show higher helical content even at higher temperature, although unfolding of the structure was clearly observed. Recombinant human insulin unfolded more readily with temperature than pegylated insulin. At pH 9.6 there were visible differences in the behaviour of the two proteins. Pegylated insulin retained a higher helical content even at higher temperature, whereas human insulin showed significantly larger amounts of beta sheet structure. Overall this shows that conjugation with small alkyl PEG improves the structural stability of human insulin.
In the next set of experiments, purified crystals of pegylated insulin were compared with excipient blend before and after compression of the tablet. Based on retention of the structure and observed increase in the Tm value, it was concluded that the excipient had a protective effect on pegylated insulin and also the blending process did not have any destabilization effect in acidic pH.

However, overall behaviour of the pegylated insulin was not altered in the presence of excipients. In HCl solution or phosphate buffer solution there was a protective effect of the excipients on the structural stability of pegylated insulin.

The CD spectra of pegylated insulin tablets (figure 2.14) dissolved in HCl buffer at pH 2 showed that the structure of pegylated insulin at lower temperature was largely unaffected, with helical minima observed at 208 nm and 222 nm. However, from the melting profile, at 67°C and 87°C it appeared that there was some aggregation, with a distinct increase in beta sheet and turn content, and corresponding decreases in both alpha helical content and random coil. When the tablet was dissolved in Phosphate buffer (figure 2.15) at pH 7.4, again it was evident that there was a difference in the melting behaviour of pegylated insulin. At higher temperature there was more beta sheet and random coil, with less helical structure. The spectra in phosphate buffer was noticeably different from that in HCl buffer. At high temperature, a minima was observed at around 215 nm, which indicated increased beta sheet content. Near 200 nm ellipticity shifted downwards compared to that at lower temperature, indicating there was more random coil present. The reason for the different CD behaviour was further explored using size exclusion chromatography with phosphate buffer, where higher molecular aggregates would be seen as separate peaks in SEC chromatogram. However, no peaks were observed for high molecular weight species (figure 2.16). The changes observed by CD may have been of a temporary nature and
weren’t due to any irreversible aggregation. Hence, when the samples were solubilized in HCl buffer where the solubility is very high, any temporary changes that occurred due to compression were reversed and the structure in the monomeric state matched closely between pegylated insulin from tablet with that from purified crystals dissolved in HCl. However, basic pH is known to be detrimental to the insulin structure due to disulphide scrambling (Yang et al. 1993) and so for that reason a significant structural change is observed at basic pH. The behaviour of pegylated insulin was found to be significantly different from that of insulin during melting experiments.

The high Tm values seen in Tris buffer of pH 9.6 could be due to a possible high oligomeric state. This may be due to a combination effect of compression related changes, followed by exposure to not so favourable pH conditions and exposure to high temperature.

When the samples were analysed using electrospray ionisation mass spectrometer (figure 2.17), no changes in the charge envelope were seen, further confirming that there was no real structural change at neutral or basic pH. The results from basic pH were surprising, as we had speculated that the higher Tm values and the changes seen for the CD data could be due to a higher oligomerization state, perhaps as a result of disulphide scrambling. Higher oligomerization states would change the charge envelop, as the larger molecules are likely to hold more charges. In this case, since neither the charge state was different nor the charge envelop, it can be concluded there were structural changes, but they were not due to aggregation or a higher association state, but could be simply due to the opening of the structure, and were of temporary nature. If oligomerization did take place it was reversible in nature.
The hydrogen deuterium exchange experiment (figure 2.18) also showed that the time taken to incorporate deuterium was probably slightly longer with the tablets than with the blend, but within about 20 min the final equilibrium state of the two samples was the same. Lastly, when the tablets were extracted in phosphate buffer at pH 7.4 and tested for glucose uptake in an in vitro assay, the activity of the pegylated insulin extracted from the tablet was identical to that of the purified pegylated insulin crystals.

From the results in this chapter it can be concluded that the effect of tableting has minimal or no impact on the structure and function of pegylated insulin. Any changes that were observed were only detected at higher temperature in the CD experiments and were probably reversible in nature. Pegylated insulin was found to be more stable than human recombinant insulin. However, it is not clear if the differences were significant. The pressure effects were also reversible in nature, which is consistent with the literature available on pressure effects on protein structure. Melting experiments show that the effect of temperature is pronounced, and in combination with higher pH may be detrimental to the protein stability and function. Pegylation has the potential to provide some protection against temperature and pH. Insulin is an interesting molecule where there are three disulphide linkages within a small peptide, making it comparatively more resistant to structural changes. However, the research described in this chapter indicates that there is potential to develop conventional oral dosage forms of proteins without the process of manufacturing the dosage form being detrimental to the structure-function of proteins, particularly for insulin. As more and more proteins are developed for oral delivery, a more complete understanding of the effects of tableting and formulation technologies will be required for successful oral delivery.
Chapter 3

In-vitro metabolism and identification of metabolites of a pegylated insulin analogue in a HepG2 cell line
3.1 Introduction

The development of pharmaceuticals and biopharmaceuticals in recent years has seen an increased use of polymer conjugated drugs that lead to higher efficacy (Jevesevar et al. 2010). Pegylated insulin (Iyer et al. 2010), a novel insulin analogue, is one such conjugate drug that is being developed for the treatment of diabetes. It has a polypeptide sequence which is identical to that of native insulin, and has a modification with an alkyl peg at ε-amino group lys-B29. The modification allows absorption of Insulin from the GUT with peak maxima seen between 10-30 min, with the levels rapidly coming to baseline within 2h (Khedkar et al. 2010). Pegylated insulin is unique in that no other molecules with a small molecular weight PEG modification have so far made it to clinical development.

3.1.1 Metabolism of Polyethylene glycols

Pegylation, as a protein/peptide modification, is a technique of choice to improve physicochemical properties, and has been studied for several decades, with considerable understanding of its safety (Webster et al. 2007). However despite several years of research on pegylation, the ability to forecast the balance of pharmacokinetics and pharmacodynamics behaviour of a pegylated protein/peptide is still limited (Fishburn 2008). The rate and extent of absorption of modified or pegylated proteins are governed by physiological factors, physicochemical properties and the interaction of the two in-vivo. Pegylation primarily results in increased molecular size and thereby reduce renal clearance. This slower rate of clearance combined with amphiphilicity of PEG results in peripheral distribution, and possibly passive diffusion in to several tissues, leading to PEG-mass related tissue accumulation as observed by Yamaoka et al.. It is interesting to note that lower molecular weight PEGs are readily cleared by the kidney’s, while for PEGs of molecular weights greater than 50 KDa, the clearance is primarily hepatic (Yamaoka et al. 1994). The size and solubility perhaps play a role in the processing of PEGs.
Although literature on definitive metabolism studies of Pegylated molecules are unavailable, it appears that the metabolism of PEG happens through oxidation of the hydroxyl group giving rise to di-carboxylic acid. For example, PEG metabolites di-acid and hydroxyl-acid were recovered in plasma and urine of burn patients, rabbits and in the biliary secretions of cats (Harold et al. 1989, Friman et al. 1993, Friman et al. 1990). Roy et al. reported that PEG metabolized via sulphate ion in the excised liver from guinea pig and in an in-vitro liver preparations of rat/guinea pig (Roy et al. 1987, Roy et al. 1988). The literature evidence shows that PEG metabolites, generated by a series of first and second phase metabolism reactions, do not lead to serious toxic effects in animals (Harold et al. 1989, Roy et al. 1987). However, overdosing of PEG is reported to give rise to carboxylic metabolites and has been correlated with increased calcium concentration in serum (Bruns et al. 1982). Nevertheless, no serious adverse effects were seen upon chronic oral administration of PEG in rats over a period of 2 years. Similarly, in a year-long chronic toxicology studies with PEG 400 to PEG 4000 in dogs, no adverse effects were seen (Smyth et al. 1955). In this context it is important to understand how the processing of pegylated protein occurs by the cells, to identify the major metabolites associated with the use of pegylated proteins as biopharmaceutics as well as to understand the fate of the PEG moiety. With this background we present here the identification of the major metabolites associated with the protein pegylated insulin in an in-vitro system.

### 3.1.2 Insulin processing

Processing of insulin has been extensively studied both in-vitro and in-vivo (Krupp et al. 1982, Varandani et al. 1976). In a study of Insulin metabolism by rat adipocytes, it was shown that Insulin was prone to fragmentation at Arginine B22 (Misbin et al. 1983). In the same study, it was also reported that the protein tyrosine phosphatase, Per-oxo-vanadate and vanadate, inhibit insulin degrading enzymes in the isolated rat liver endosomes. Bevan
et al. 2000 showed that the endosomal degradation of Insulin occurred simultaneous to degradation of B-chain with disulphide bonds still intact. The two chains were later separated by cleavage of both the inter-chain disulphide bonds. Though these studies seem to indicate that the Insulin metabolism occurs via specific pathways, simultaneous appearance of various intermediates in a few studies (Seabright et al. 1996) led to the conclusion that degradation of Insulin does not happen through sequential reactions, but must be happening through more complex reactions or through the simultaneous action of multiple enzymes.

Nonetheless, these studies showed that cultured cells with specific functionalities can form a significant in-vitro system to study Insulin metabolism. The major target tissues for Insulin are fat, muscle and liver. Hence HepG2, a liver cell line derived from a human hepatoblastoma and expressing a wide variety of liver-specific metabolic functions (Javitt et al. 1990, Bouma et al. 1989, Thrift et al. 1986), serves as an acceptable system for in-vitro metabolite studies of Insulin. HepG2 maintains both the gross liver cell morphology and liver cell function as determined by the synthesis and secretion of several liver specific proteins including albumin, alpha-feto protein, transferrin (Knowles et al. 1980).

3.1.3 Choice of cell line:

Since HepG2 cells express high-affinity insulin receptors meeting all the expected criteria for classic insulin receptors, we chose them for the study of pegylated insulin metabolism. We report here studies of the metabolism of a pegylated insulin analogue using HepG2 cells. Our studies demonstrate that pegylated insulin metabolism occurs via B-chain degradation. Although typical ADME studies, as well as all the earlier reports on insulin degradation, were carried out using labelled compounds, in the case of pegylated insulin the conjugated alkyl PEG molecule acted as an intrinsic label for these studies. Therefore,
an LC/MS/MS method was used for tracking the chronological appearance of metabolites formed during HepG2 mediated pegylated insulin metabolism.

3.2 Materials and Methods

3.2.1 Chemicals and reagents:
Lysine.HCl was purchased from S.D fine chemicals (Mumbai, India, N-Hydroxy Succinamide activated Oligomer was prepared in-house, HPLC grade Acetonitrile was purchased from JT Baker (Center Valley, PA, US). All other chemicals were analytical-reagent grade and were used without further purification. The water used for mobile phase preparation was purified with a Milli Q system (Millipore, Billerica, MA USA).

3.2.2 Preparation of standards
Lysine-ε-Oligomer was synthesized by conjugating Lysine and Activated NHS-Ester of oligomer at pH 8.5 in borate buffer. Three products were observed. Lysine-N-oligomer and lysine-ε-oligomer were found to have different fragmentation; Lysine-ε-Oligomer (mass and fragmentation matches with metabolite observed in the HepG2 cell supernatants). For further analysis the Lysine-ε-Oligomer was purified to greater than 90% purity using RPHPLC followed by lyophilisation.

Free oligomer was obtained by hydrolysis of NHS ester of Oligomer at pH 8.5 in borate buffer. The free oligomer was purified using RPHPLC and Lyophilized. Samples were analyzed with LCMS/MS for comparison of intact mass and fragments in MSMS mode.

3.2.3 Experimental Procedure
Human Hepatoblastoma cells (HepG2) were obtained from ATCC and were grown in T25 flasks at 3x10⁵ cells /ml in RPMI 1640 media with 10% FBS, 1mM HEPES and 1% sodium pyruvate. Flasks were incubated at 37°C incubator which was maintained
constantly at 5% CO₂. At the time of plating 25 μg/ml of pegylated insulin was added to the various time point assigned flasks. Samples were collected at 16, 40, 48, 64, 72, 96, 168, 192, 207, 216, 232 and 240 h. All the samples were stored at -20°C until the time of analysis. Media alone, cells alone and Media with pegylated insulin were kept as controls to avoid any confusion.

Confirmation of role of glutathione insulin transhydrogenase:
Experimental procedure described above was used. Five sets were prepared. To set 1, 25 μg/mL of pegylated insulin was added while plating. To set 2, only 0.5 mg/mL of bacitracin was added, to set 3, 1 mg/mL of bacitracin was added, to set 4, 25 μg/mL pegylated insulin along with 0.5 mg/mL of bacitracin were added, and to set 5, 25 μg/mL pegylated insulin with 1 mg/mL of bacitracin were added. Samples were harvested every 24 h up to 168 h. Chromatographic separation was achieved using a Vydac C18 column (250 mm x 4.6 mm ID, 5 μm particle size) at a column temperature of 40°C, with the flow rate kept at 1 mL/min, and UV detection at 215 nm. The analysis was carried out using gradient conditions (Mobile phase A: 90% Acetonitrile 0.1% TFA; Mobile phase B: water with 0.1% TFA)

In this experiment, since the cell supernatant did not undergo any specific sample preparation, hence an internal standard was not required. Additionally, considering the objective of the experiment of identifying the metabolites, a highly quantitative method was not developed.

3.2.4 MS Instrument parameters
MS was performed using an ESI-Ion Trap HCT Ultra PTM Discovery system (Bruker Daltonics, Bremen, Germany) as well as LTQ orbitrap XL (Thermo fisher, San Jose, CA, USA) with Nexera LC (Schimadzu, Kyoto, Japan) as a front end. The analysis was carried
out using electrospray ionization in positive mode. The scan range was from 50 m/z to 2200 m/z. Capillary exit voltage was kept at 181 mV, skimmer voltage 40 mV, trap drive 104.6%, accumulation time 100 ms. For orbitrap, ESI parameters were maintained as given here: capillary temperature 340 °C, sheet gas flow 5 L/h and sweep gas flow 2 L/h.

3.3 Results and discussion

Pegylated insulin analogue was seen as multiply charged species when injected in ESI-MS figure 3.3. The m/z of 1507 had four charges and the deconvoluted mass was seen as 6025 Da, which matched with the theoretical mass of pegylated insulin. The standard lysine-ε-oligomer peak showed a value of 365.2. In addition to the protonated mass, a sodium adduct of mass 387 was also seen. The fragmentation pattern of 365.2 m/z shows loss of water molecule (347 m/z), a combined loss of NH$_2$ and CO (302 m/z), an additional loss of CH$_2$ resulting in 288 m/z, a loss of 44 m/z gave 244. This loss of 44 m/z could be arising out of a loss of O-CH$_2$CH$_2$. Further loss of O-CH$_2$CH$_2$ gave 200 m/z. A mass of 154.8 m/z appears to have a mass error of 1.1 Da and could be due to additional loss of O-CH$_2$CH$_2$ from the 200 m/z fragment. A mass of 181.9 m/z could not be assigned to any particular fragment. It appears to be arising out of a water loss from the 200 m/z fragment. A loss of O-CH$_2$CH$_2$ from 181.9 m/z gave 137.8 m/z. Since the MS/MS was performed in an ion trap, fragment below 30% cut off of 365 m/z could not be seen.

Figure 3.1 shows overlay of total ion chromatograms (TIC) obtained from 240 h sample of pegylated insulin incubated with HepG2 cells overlaid along with the control of only HepG2 cells incubated for 240 h. From this overlay, clearly an additional peak at 8.3 min which is circled could be seen above the control profiles. The mass of this peak was found to be 365 m/z and the fragmentation of 365 m/z matches with that of MS/MS of lysine-ε-oligomer. All the other peaks were essentially the cell components.
The samples being highly complex, besides the lysine-ε-oligomer, no other metabolite could be identified from TIC, hence extracted ion chromatogram (EIC) was used for further analysis. Peak 2 depicted in figure 3.2 corresponds to lysine-ε-oligomer at RT ~8.0, Peak 2 in EIC clearly showed presence of some residual pegylated insulin, peak 3 was identified as deamidated pegylated insulin and peak 4 was identified as reduced B-chain.
Figure 3.1: TIC of pegylated insulin incubated with HepG2 Cells at 240 h (blue line) overlaid with supernatant of HepG2 Cells grown as control. The samples from control were drawn at different timepoints from (16-240 h) and overlaid with the test sample. Only the test sample (blue line) showed presence of a unique peak at approximately 8.0 min.
Figure 3.2: EIC chromatogram showing lysine-ε-oligomer, Chain-B of pegylated insulin, pegylated insulin in HepG2 Cells. The levels of lysine-ε-oligomer were found to be increasing with time of incubation.
Figure 3.3: Mass Spectra for observed Metabolites after start of incubation (A) Full-Scan Mass spectrum of the peak eluting at 41.3mins pegylated insulin, (B) Full-Scan Mass spectrum of the peak eluting at 42.2mins. Chain-B of pegylated insulin, (C) Full-Scan Mass spectrum of the peak eluting at 8.8 mins (D) MS/MS Mass spectrum of the peak eluting at 8.8 mins.
Figure 3.3 represents the experiment, where pegylated insulin was incubated with HepG2 cells in presence of bacitracin. The data shows that the mass spectra of different peaks seen in the samples. Panel A shows a mass profile of intact pegylated insulin. Along with that a mass of 1359.8 was seen in all the samples. The mass appeared to have a charge state of 3 and was found to be present only in the sample set containing bacitracin. Panel B shows m/z which corresponded with deconvoluted mass of B-chain of pegylated insulin (3646 Da). The mass of B-chain of human insulin is 3429. Pegylation increases the mass of B-chain by 217 Da which works out to a total mass of 3646 Da. Panel C shows mass of lysine-ε-oligomer which corresponds to 365 Da and panel D shows MS/MS fragmentation of 365 Da.

Figure 3.4: Fragments of lysine-ε-oligomer observed after performing MS/MS.

Figure 3.4 shows potential structures that were assigned to the fragments. However, some of the masses could not be assigned to any structure, which indicates that during the MS/MS experiment, it is likely that the lysine-ε-oligomer undergoes some sort of
rearrangement and the unassigned fragments could be the result of this rearrangement. The fragments appeared similar to the ones which have been reported for polyethylene glycol fragments post low energy collision induced dissociation (Chen et al. 2002) where PEG ethers showed repeating loss of 44 Da.

Figure 3.5: A. TIC chromatogram showing Lysine-ε-Oligomer and B29 Oligomer in pegylated insulin HepG2 Cells. B. EIC chromatogram showing the Lysine-ε-Oligomer and lysineB29-Oligomer in pegylated insulin HepG2 Cells.
Figure 3.6: Panel A. Mass Spectra showing Lysine-ε-Oligomer m/z 365 and B29 Oligomer in pegylated insulin HepG2 Cells m/z 365. In addition 387.1 which is a sodium adduct is seen as well. Panel B. MS/MS Spectra showing the fragmentation of Lysine-ε-Oligomer (top figure) and B29 Oligomer in pegylated insulin HepG2 Cells (bottom figure).

Further confirmation that the peak showing molecular mass of 365 m/z indeed corresponded to lysine-ε-oligomer, the TIC was overlaid with the TIC obtained after injecting standard lysine-ε-oligomer (figure 3.5). Both peaks overlaid perfectly and showed identical mass and fragmentation pattern (figure 3.6).
Figure 3.7: Extracted Ion Chromatogram extracted for free oligomer for all the timepoints.

Figure 3.8: EIC chromatogram showing Oligomer standard and free Oligomer in pegylated insulin in HepG2 Cells 

Free oligomer peak seen in sample containing Peg-Insulin with Hep G2 Cells

Free Oligomer observed in pegylated insulin + HepG2 Cells after 168 h of
In addition to lysine-ε-oligomer, another small peak was identified from the total ion chromatogram. The molecular mass was found to be 237 m/z (figure 3.7), which was similar to the molecular mass obtained after injecting a standard of free oligomer in acid form. The peaks seen in extracted ion chromatograms from both the sample overlaid well (figure 3.8) and the molecular mass for both peaks was identical (Figure 3.9).

The Insulin-receptor complex is known to degrade via a pathway that includes simultaneous occurrence of chain separation and B-chain degradation. It appears that the degradation pathway of modified insulin follows a similar pattern to that reported for insulin, where the two polypeptide chains were separated first and subsequently broken down into smaller peptides.
3.3.1 Pegylated Insulin degradation occurs via chain separation

From figure 3.10, it can be seen that when a control sample using pegylated insulin incubated with media alone showed no decline in TIC intensity for intact pegylated insulin, although a small increase in deamidated pegylated insulin was observed over time in the control set. The MS and MS/MS data obtained clearly showed the presence of various peaks, which could be traced back to pegylated insulin. In a sample where pegylated insulin was incubated with HepG2 cells, a steady loss of intensity was seen with a corresponding increase in the intensity of the separated B-chain. Pegylated insulin as well as the B-chain intensity dropped sharply after 96 hrs of incubation, which corresponds to the time of cells reaching 100% confluence. Post 72 hrs, a peak at 365 m/z in the MS spectrum increased steadily until 168 hrs. No free A-chain was seen during the duration of the experiment, which could be due to rapid degradation of the A-chain. Free insulin B-chain was observed from the beginning of incubation, indicating that the first major step in degradation is probably separation of the two chains by reduction of disulphide linkage.
3.3.2 B-chain degradation is a prominent feature of the metabolism of Pegylated Insulin

![Graph A](image1)

**Figure 3.10:** Panel A shows time course of pegylated insulin and its metabolites by Hep G2 cells in absence of Bacitracin. Control refers to pegylated insulin in the media incubated without any cells, no metabolites were seen. Panel B shows time course of metabolite formation in presence of bacitracin. 4075 Da (1359.3+3) metabolite is observed in presence of bacitracin due to suppression of glutathione insulin transhydrogenase.
Soon after the observation of separated B-chain, a consistent increase in lysine-ε-oligomer was seen from around 40 hrs until the end of the experiment. The increase in signal of lys-ε-oligomer correlated with reduction in B-chain signal, indicating that perhaps proteolytic cleavage of the B-chain gives rise to lys-ε-oligomer in a step subsequent to disulphide reduction. As mentioned in the previous section, from 168 hrs onwards a small but steadily increasing signal was seen for free oligomer, indicating further hydrolysis of ε-amide bond to give rise to free oligomer (figure 3.10, upper panel). No further metabolites of oligomer were found in the supernatant.

3.3.3 The chain separation is mediated by Glutathione Transhydrogenase

Tomizawa isolated Glutathione insulin transhydrogenase from human liver and showed that the enzyme was responsible for degradation of insulin (Tomizawa et al. 1965) and later Varandani et al. 1972 characterized the role of glutathione insulin transhydrogenase extensively and also reported that bacitracin inhibits glutathione insulin transhydrogenase activity and prevents separation of two chains (Duckworth 1981). Recently, a mechanism of how bacitracin inhibits Protein disulphide isomerase, an enzyme having functional similarity to glutathione insulin transhydrogenase, has been reported (Dickerhof et al. 2011). The insulin degradation is thought to be important in the signal transduction process of insulin inside the cell as well as helps in keeping check on the effect of insulin on various cellular processes (Caro et al. 1980).

To confirm the role of glutathione transhydrogenase or a similar enzyme in the observed degradation, bacitracin, reportedly a non-specific inhibitor of glutathione transhydrogenase, was added to the media along with pegylated insulin in the next experiment (Roth et al. 1981). Upon analysis, samples containing bacitracin did not show any presence of B-chain, instead they showed a new metabolite wherein partially clipped
A-chain and B-chain were still bound together via the disulphide linkage. The molecular mass of this new metabolite was deconvoluted to 4075 da.

![Figure 3.11: Structures of all major metabolites observed. Metabolite A and B were observed in absence of bacitracin along with B-chain. While the 4075 Da fragment was observed in presence of bacitracin.](image)

It is likely that the separation of the two chains could be mediated by the glutathione insulin transhydrogenase, which was indirectly confirmed by bacitracin induced inhibition of reduction of disulphides (figure 3.10, lower panel). All the metabolites of pegylated insulin, identified under these experimental conditions with and without bacitracin, are listed in Table 3.1 and their structures are given in Figure 3.11. Most of the metabolites that have been identified as fragments of the B-chain are around the B29 lysine. The presence of oligomer on B-29 lysine perhaps makes it easier to identify the fragments around that site.
Table 3.1: List of metabolites

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Pegylated insulin with Hep G2 cells</th>
<th>Pegylated insulin and 0.5 mg/ml of bacitracin with Hep G2 cells</th>
<th>Pegylated insulin and 1 mg/ml of bacitracin with Hep G2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-chain</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>B-chain</td>
<td>√</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Free-oligomer</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>K(oligo)</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>K(oligo)T</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>PK(oligo)T</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>TPK(oligo)T</td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>4075 da fragment</td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

3.3.4 Metabolite characterization and identification in the Supernatant

The major metabolite observed with m/z 365 was found to match in retention times with an in-house standard Lysine-ε-Oligomer solution (t_R = 8.3 mins), with further confirmation obtained by matching the fragmentation pattern of 365 m/z with the standard. Similarly, other metabolites were identified using MS and MS/MS patterns. This metabolite, as mentioned previously, was stabilized for 168 hours of incubation after which there was a slight decline in its intensity. This loss appeared to correspond to subsequent rise in a peak of 259 m/z, which was later confirmed as free oligomer by comparing the retention times and fragmentation with an Oligomer standard.
3.4 Conclusion

Abundant literature is available on the topic of insulin degradation with several research groups having investigated insulin degradation. Many cleavage sites for insulin in the A-chain and B-chain have been identified and reported in the literature. The B-chain has been proven to undergo extensive proteolytic degradation. Whereas only couple of cleavage sites have been identified in the A-chain, namely A13-A14 and A14-A15 (Duckworth et al. 1987). It is possible that the A-chain undergoes cleavage at other sites, which have not been identified yet or reported. For insulin, several cleavage sites have been reported in the B-chain between B7 to B19 residues and between B22 to B26 residues.

There are reportedly two pathways for insulin degradation. Insulin in bound form to its receptor and the free circulating form of insulin are processed differently. Insulin–receptor complex internalizes into endosomes and undergoes proteolytic cleavage of the B-chain and separation of the chains from each other (Carpentier et al. 1979, Gliemann et al. 1978). It was speculated that both these processes occur simultaneously. With the current experiment, we have proven indirectly that the two processes could be simultaneous and not sequential. It was observed that the proteolytic cleavage happens from N- and C-terminal of both the chains (figure 3.11). In the presence of bacitracin, no cleavage was observed between A7 to A20 and B7 to B19, which could be due to the sites becoming inaccessible to proteases due to intact disulphide bonds.

When pegylated insulin was incubated with HepG2 cells, a significant amount of B-chain was seen in the supernatant in the initial stages, indicating that reduction of the disulphide bond plays a big role in degradation of insulin. On the other hand, when pegylated insulin was incubated with HepG2 cells in the presence of bacitracin, the separation of two chains was inhibited. 4075 m/z could be a metabolite with the A-chain cleaved between A5-A6
and A20-A21, all the disulphides still intact, B-chain cleaved between B1-B2 and another cleavage between B22-B23. A chain cleavage positions have not been previously reported for insulin. This could be because no metabolite identification studies have been reported in the presence of bacitracin. While we could not find any reports for these cleavage sites, we do not anticipate it to be different from insulin, since the A-chain is unmodified in our analogue. It is interesting to note that both these cleavage sites were found between charged amino acids and cysteine, however, the data is not sufficient to form a hypothesis on the specificity of the protease involved. We have captured the cleavage sites reported for Insulin as well as cleavage sites identified for Pegylated insulin in figure 3.12 below.

Figure 3.12: Proteolytic degradation sites in the insulin molecule (top) and degradation sites in Pegylated insulin (bottom). \text{K} is modified lysine with alkyl PEG.

Most of the previously reported work on Insulin processing involves $^{125}$I labelling, chromatographic separation and Edman degradation for identification of cleavage products. As far as we can deduce, this is the first report where insulin metabolites have been identified using LC/MS without using a radiolabelling. The presence of oligomer served as a tag and allowed us to identify metabolites which had the oligomer tag still attached. This also indicates that small alkyl-PEG in conjunction with LC/MS could prove
to be useful in identifying potential metabolites of proteins without the use of radiolabelling. In case of traditional methods of radio-labelling, the scintigraphy makes it easier to identify metabolites but there is a possibility of labelling falling off the molecule and the presence of radioactivity in certain tissue would not necessarily mean that the metabolites are accumulating in those organs. Hence the conclusions are not confirmatory in certain cases. On the other hand if the molecule is tagged covalently with alkyl PEG, the presence of a metabolite can be conclusive, especially when used in conjunction with a sensitive and accurate technique like mass spectrometry.

Although degradation of the pegylated analogue followed a largely similar pattern to that of insulin processing, some differences were observed in the cleavage sites in the presence of bacitracin. It is possible that additional cleavage occurs similarly to what has been reported for insulin, but were just not observed under our experimental conditions. Any actual differences in the degradation steps are unlikely to affect the safety or efficacy of pegylated insulin, since eventually the molecule, being a protein, will be completely digested. Therefore, in conclusion, modification of insulin using small molecular weight alkyl-PEG does not appear to affect protein processing significantly, while providing a useful tool for altering the pharmaceutical properties.

Insulin degradation via Insulin degrading enzyme also seems to play an important role in reducing protein degradation. Duckworth et al. 1997 hypothesized that insulin interacts with insulin degrading enzyme in the cytosol. As a direct result of this interaction proteosomal catalytic activity is inhibited, hence they went on to propose that insulin degrading enzyme had a regulatory role as well.

Further, the lack of metabolism of free PEG suggests that there is no induction of alcohol dehydrogenase or the cytochrome system. It can be hypothesized that a free terminal
alcohol group on PEG molecule is likely to induce liver enzymes to a greater extent compared to the methoxy ether terminal.

In addition, an understanding of cellular processing may be useful for designing new analogues that are resistance to proteolytic enzymes, leading to desired pharmacological profiles. It would also be important to understand if a proteolytic resistant insulin derivative would similar cellular actions of the human insulin molecule, like inhibition of protein degradation, improved protein synthesis and inhibition of β-oxidation of lipids. A molecule which is more resistant to cellular processing could have a longer half-life in circulation and could prove to be useful in developing Insulin. With longer duration of pharmacokinetic profile.
Chapter 4

Fate of oligomer, a modifier used for pegylated insulin analogue, in Wistar rats
4.1 Introduction

Pegylated insulin analogues are modified human insulin compounds currently being tested in non-clinical studies to support eventual clinical development. The pegylated insulin used in this study is derived from human Insulin and contains an oligomer (methoxy triethylene glycol propionate) attached to the B29-Lys amino group of human Insulin. The outcome of *in-vitro* experiments conducted in hepatic carcinoma cell lines revealed that B29-Lysine-ε-Oligomer was the distinct major metabolite of pegylated insulin. In addition, free oligomer was also found along with other cellular metabolites (Dave et al., 2008).

Pegylated insulin is reported to be available systemically upon oral administration in patients with type 2 diabetes (Khedkar et al. 2010). From a safety perspective, it is important to understand the metabolites and fate of the metabolites.

4.1.1 Absorption, Distribution, Metabolism, Elimination (ADME) studies:

ADME studies are conducted primarily for synthetic molecules in an effort to establish tissue distribution, metabolic pathways, identification of metabolites and routes of elimination and elimination kinetics. Typically, metabolic profiling involves studying the molecules in *in-vitro* experiments followed by *in-vivo* models. Liver is a central organ where most drugs undergo metabolism due to the presence of several metabolizing enzymes. If the metabolites turn out to be reactive then they can irreversibly bind to cellular molecules and may cause hepatotoxicity or adverse reactions. The metabolism occurs in two phases. The first phase refers to hydrolysis, oxidation/reduction and dehydrogenation reactions (Meyer 1996) and the second phase typically involves glucoronidation or sulfation. The first phase is designed to break down the molecule or transform it in an attempt to detoxify, while the second phase increases molecular polarity to enhance the elimination process (Zimmerman et al. 1999). The reactive
metabolites are hard to detect as their half-life is short (Antoine et al. 2008). To determine the presence of reactive metabolites in early stages of development, metabolism is studied in in vitro systems like microsomal extracts, hepatocytes, cytosol, transgenic cell lines, liver sections or perfused liver (Brandon et al. 2003). The effort is to mimic the conditions as close to in vivo conditions as possible.

Intact liver cells provide relevant information about the metabolism of drugs. However, isolation of liver cells is tedious and any damage to the cells would affect the quality of information. In an artificial environment, some enzymes may be over expressed or under expressed and hence the information may not exactly match the real situation (Pelkonen et al. 2005). Liver slices may give a good representation of actual metabolism but preparation of liver slices is tedious and the preparations do not last long. An isolated liver closely mimics the in vivo condition, but has to be harvested from a live animal, which means that for every assay one animal needs to be sacrificed. Liver cells do not express all the metabolic enzymes, on the other hand transgenic cell lines overexpress only one of the isoforms of enzyme. The cytosolic fraction of the liver homogenate contains soluble phase II enzymes, whereas S9 fractions have all the enzymes, albeit at very low levels. Hence to generate near complete information regarding metabolism combining more techniques might be prudent (Antoine et al. 2008).

4.1.2 Analytical techniques in ADME:

Traditionally, radiolabelled compounds are used in ADME studies, which makes detection of the compounds in various tissues and body fluid easier by a scintillation counter. With the previous generation scintillation counters, the dose requirement was high. With the
development of high sensitivity liquid scintillation beta counter, the dose requirement has been greatly reduced (Deroubaix et al. 2004). Recently, another technique has been developed that uses stable isotope labelling and measurement by flow isotope ratio mass spectrometry. This technique is not yet widely used as it required further validation (Browne et al. 1998).

Mass spectrometry is becoming widely used as a technique of choice for ADME. Accelerated mass spectrometry is a high sensitivity detection method for $^{14}$C. It may be possible to reduce the dose of radioactivity by 1000 fold to 50 nCi from 50 μCi that is required in standard ADME studies. The mass spectrometric methods have some disadvantages, such as the requirement to transformation (graphitization) of the sample in to solid graphite (Chung and Kim 2013). In addition it requires a high energy Van der Graaf accelerator as a special requirement, which makes the technique expensive to use. Also, the required sophisticated equipment is not widely accessible (Gamer 2000).

Besides accelerated mass spectrometry, a new mass spectrometer called the Orbitrap, which is a high resolution instrument and with a good sensitivity, is becoming popular for qualitative and quantitative work.

Figure 4.1: Spindle shaped ion trap of orbitrap instrument
The orbitrap mass analyser consists of a spindle shaped electrode surrounded by a coaxial electrode. The ions which enter the orbitrap oscillate around this spindle at a frequency proportional to their m/z. The frequency of these ions is detected and Fourier transformed to convert into mass spectrum by the following equation:

$$\omega = \sqrt{\frac{k}{m/z}}$$  
(Hu et al. 2005)

where $\omega$ is the z-axis frequency, $k$ is the force constant, $m$ is mass of the ion and $z$ is charge of the ion. The frequency based mass measurement results in high resolving power to the tune of 100,000. Ion trap has one limitation, which is, it cannot hold large numbers of ions at the same time. Large ion injection in the trap leads to broad distribution of kinetic energy that reduces the resolving power and accuracy of the mass spectrometer. This problem has been solved by injecting the ions into the trap radially via an Rf only quadrupole called a C-trap (Makarov 2000). The accuracy of mass measurement can be high with an error of up to 3 ppm.

Peterman et al. showed that the orbitrap can be a very useful tool for examination of metabolic pathways of the anti-depressant compound nefazodone. In this study, high accuracy of the mass data helped them assign the structures of known and new metabolites of nefazodone. A
mass accuracy below 3 ppm was reported for all the metabolites detected (Peterman et al. 2006).

Prior to orbitrap mass spectrometer, triple quadrupole mass spectrometer (QqQ), and linear trap quadrupole-time of flight (Q-TOF) mass spectrometers were available of which QqQ mass spectrometer and linear trap instruments have been reportedly used for metabolite identification work (Bateman et al. 2009). Multiple reaction monitoring using the QqQ instrument is very useful in identifying multiple metabolites of the given drug (Baillie 1992). QqQ also has the capability to generate neutral loss and precursor ion scans, which help identify the larger group of metabolites. The full scan mode of QqQ however is not sensitive enough, hence ion trap instruments and Time of flight instruments have become popular as well. The advent of higher sensitivity and high accuracy machines has made mass spectrometry a technique of choice especially in the field of metabolite identification (Kostiaenen et al. 2003).

ADME studies are conducted using a single dose in rodent or non-rodent small animals, usually rats, rabbits, dogs or monkeys. At times human studies may also be performed but there are ethical challenges in performing human ADME studies, especially when radiolabelled drug needs to be administered. Besides understanding how the pharmacological agent is cleared by the body and identify, the metabolites ADME studies help evaluate potential toxicological issues. Radiolabelling helps in identifying major metabolites in circulation, understand clearance pathways better and help understand tissue accumulation. While ADME studies performed in animals may not be directly applicable to humans due to differences in physiological aspect due to species differences, they provide enough indication
on the safety aspects of the pharmacological agent. ADME conducted in normal animals may not always reflect the actual picture and might require ADME to be studied in disease models. If the disease condition for which the drug is being developed affect kidney or liver, there is imminent possibility that the metabolism of the drug would be different from a normal subject. To precisely rule out these differences, disease models are usually preferred for ADME studies.

In designing the ADME studies, the choice of labelling, specific activity and position of labelling are critical to the success of the trial. The choice of labelling is from tritium, $^{14}$C, $^{32}$P, $^{35}$S and $^{131}$I (Heggie et al. 1987, Chando et al. 1998, Boado et al. 1995, Lu et al. 1982, Wafelman et al. 1997), of which $^{14}$C and $^{3}$H are the most popular ones. In the case of proteins/peptides $^{131}$I can be preferable over $^{14}$C or $^{3}$H. If $^{3}$H is used, then higher doses are required as $^{3}$H emits weak $\beta$-radiation (Beumer et al. 2006). $^{14}$C has a long half-life and gives a good amount of radio-activity. If full body autoradiography is to be used during the study, the amount of radioactivity becomes critical for getting enough signal to enable a clear understanding of tissue distribution.

The animal strains used for the ADME studies should be well characterized and the source should be reliable. Normally, ADME studies are conducted only in male animals but if differences between genders are expected or known then studies should involve both male and female animals. Choice of gender is also governed by the country of registration and the regulations followed by different countries.

In the past few years several molecules have been modified using Pegylation technology in an effort to slow down the elimination, thereby increasing the circulating half-life. Walsh et al.
(2003) reported increased half-life and reduction of antibody reactivity of lysostaphin conjugated to polyethylene glycol. GCSF, interferon and organophosphorus hydrolase have been pegylated to increase their circulating half-life (Zhai et al. 2009, Bansal et al. 2011, Novikov et al. 2010, Soares et al. 2002). Abuchowski et al. 1977 reported alteration of immunological properties of bovine serum albumin by conjugating with PEG. The same group later reported changes in circulating half-life as well as changes in immunological properties of bovine adenosine deaminase in mice (Davis et al. 1981).

A considerable amount of literature has been generated over the years in an attempt to understand the ADME of polyethylene glycols of different molecular weight ranges. Small molecular weight PEGs are used as solvents or co-solvents in formulations and have been tested for toxicity in animal models. Lockard et al. studied the efficacy and toxicity of PEG 400 in rhesus monkeys, for its use in the formulation of sodium phenobarbital. They reported that animals receiving PEG400 at 60% concentration, as well as animals receiving PEG400 and drug both, exhibited greasy lower extremities and oedema of genitals and legs along with loss of appetite (Lockhard 1979). Furuichi et al. (1984) examined absorbability of radiolabelled PEG4000 from gastrointestinal tract and compared with Intravenous administration. They observed that i.v PEG400 was almost completely recovered in urine and no hepatic or renal uptake was observed. Similarly, PEG administered in the gastric region directly was eliminated in urine as well with only 43% of the dose over 24 hrs.

PEGs are largely unchanged in the body and are reported to get eliminated primarily in urine. Yamaoka et al. have studied the rate of elimination as a function of molecular weights and demonstrated that the rate of elimination slows down with the increase in molecular weight of
PEG. Hepatic elimination has also been reported for large molecular weight PEG (Yamaoka et al. 1994).

In this chapter we have summarized our findings of a follow on study conducted with pegylated insulin to identify and confirm the possible metabolites of the compound in vivo in a rat model. The primary objective of this study was to identify and confirm the presence of Lys-Oligomer or free oligomer, the possible metabolites of pegylated insulin; as determined by in-vitro experiments, in rat urine and faeces. The study was an attempt to understand ADME of the oligomer in a qualitative manner.

4.2 Regulatory Compliance:

This study was conducted in an investigative manner to study the metabolic profile of Pegylated insulin. The in-life phase of this study was conducted at Syngene International Ltd, Bangalore, India. The study was approved by the animal ethics committee of Syngene International Ltd.

4.3 Materials and methods:

A total 8 male Wistar Rats of 8 - 12 weeks age and weighing approx 250 g were included in the study and were assigned to two groups with 4 rats each. The test item administration and blood collection was staggered for 3 - 5 days. The study design is provided in Table 4.1.
Table 4.1: Study design

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Group &amp; Group Size</th>
<th>Dose</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegylated insulin</td>
<td>Group -1 (4 Males)</td>
<td>200 mg/kg</td>
<td>3% w/v Sodium Caprate in 50 mM Sodium Phosphate buffer (pH ~7.8)</td>
</tr>
<tr>
<td>Lysine-ε-Oligomer (Positive Control)</td>
<td>Group -2 (4 Males)</td>
<td>20 mg/kg</td>
<td>3% w/v Sodium Caprate in 50 mM Sodium Phosphate buffer (pH ~7.8)</td>
</tr>
</tbody>
</table>

Pegylated Insulin, batch# B-0860021A, was provided by Biocon Limited. The average molecular mass of Pegylated insulin was 6025 and protein content was 93.5%. Lysine-ε-Oligomer standard used as a control was prepared and supplied by Biocon Limited, and was a viscous yellow liquid having 98.2% purity. A 3 % solution of Sodium caprate in 150mM sodium phosphate buffer was used as vehicle for administration of test and control substances.

4.3.1 Metabolic cages:

Rats were accommodated in metabolic cages from Lab products Inc. The design of these cages allows up to 99% separation efficiency of urine and faeces, which assures minimum contamination. Components below the cage floor are removable without disturbing animals allowing tests to be conducted over a prolonged period of time without interruptions. These
cages are easy to assemble, maintain, disassemble and clean and are made up of autoclavable polycarbonate and stainless steel components.

Animal waste is directed through a collection funnel and onto a linear diffuser. The diffuser allows solid matter to travel down the top ridges of a 50% incline, passing over a urine port, and into a faecal collection vessel. Liquid waste flows along grooves down the inclined diffuser, passing through the urine port, and into a urine collection vessel. A urine deflector prevents excess urine from splashing and contaminating faecal specimens. Separate urine and faeces samples are collected in two standard 50 ml centrifuge vessels.

Figure 4.3: Photo of a metabolic cage.
4.3.2 Dose Administration

The animals were fasted at least 10 h prior to administration. The overnight fasting condition was selected as it is known that the presence of food will interfere with the absorption of pegylated insulin and also probably impact absorption of oligomer and would have impacted the study outcomes. A single dosage of 200 mg/kg and 20 mg/kg of the pegylated insulin and Lysine-ε-Oligomer were administered to Groups 1 and 2, respectively by oral route, on day 1, day 2 and day 3, at a dose volume of 5 mL/kg. The dose of pegylated insulin was chosen from prior unpublished toxicology studies. The dose of Lysine-ε-Oligomer was chosen arbitrarily. Food was provided to all the animals at approximately 0.5 hours post-dosing and was available ad-libitum. Animal weight was recorded prior to dosing on each day and the dose was calculated as mentioned.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat_No.</th>
<th>Dose (mg/Animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dose 1</td>
</tr>
<tr>
<td>G1</td>
<td>1</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>53.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>57.7</td>
</tr>
<tr>
<td></td>
<td><strong>Average Dose</strong></td>
<td><strong>55.38</strong></td>
</tr>
<tr>
<td>G2</td>
<td>1</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td><strong>Average Dose</strong></td>
<td><strong>5.75</strong></td>
</tr>
</tbody>
</table>

4.3.3 Sample Collection & Handling

Individual urine and faeces samples were collected from all animals at the intervals listed in the Table 3 below.
Table 4.3: Sample time and interval plan

<table>
<thead>
<tr>
<th>Dose no./ Day</th>
<th>Time intervals (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 1/ Day 1</td>
<td>Pre-dose, 0 - 6, 6 - 12 &amp; 12 - 18, 18 - 24 hours post dose</td>
</tr>
<tr>
<td>Dose 2/ Day 2</td>
<td>0 - 6, 6 - 12 &amp; 12 - 18, 18 - 24 hours post dose</td>
</tr>
<tr>
<td>Dose 3/ Day 3 to 5</td>
<td>0 - 6, 6 - 12, 12 - 18, 18 - 24, 24- 30, 48 &amp; 72 hours post dose</td>
</tr>
</tbody>
</table>

All the urine and faeces samples were stored frozen at -80 °C before taking up for analysis.

4.3.4 Analytical Procedures

The rat urine and faeces samples were processed and analysed for Lysine-ε-oligomer and free oligomer in rat urine and faeces using a suitable in-house analytical LC/MS/MS method. The term ‘metabolite recovery’ indicates the cumulative amount of a metabolite eliminated into urine and faeces which was calculated using the formula “∑(Concentration*Volume)”. Percent recovery was calculated using the formula “100xAmount Recovered/Dose”.

All animals were observed before and after dosing for clinically relevant abnormalities. Blood glucose concentrations were measured from all the animals using calibrated glucometer at pre-dose and 15 minutes post-dose.
4.3.5 Equipment

HPLC System – SHIMADZU Prominence (Tokyo, Japan), Mass Spectrometer – TSQ QUANTUM ULTRA AM, Thermo Electron corporation (San Jose, CA, US), Waters Solid Phase Extraction Vacuum Manifold (Waters corporation, Milford, MA, US), Ultra Low Temperature Freezer (Thermo Fisher scientific, Ashville, NC, US), Weighing Balance – Sartorius AG (Gottingen, Germany), Water purification system – Milli-Q Synthesis, (Millipore, Billerica, MA)

4.3.6 Preparation of reagents

**Mobile phase A:**
Solution A was prepared by adding 10mL of Acetic acid into 990mL of Milli-Q water, and sonicated for 10min and degassed.

**Mobile phase B:**
Solution B was HPLC grade Acetonitrile.

**Diluent:**
Diluent was prepared by mixing 90 parts of water and 10 parts of acetonitrile to make 100 mL of diluent.

**Elution solvent:**
Elution solvent was prepared by mixing 90 parts of 1mM NaCl and 10 parts of acetonitrile to make 100mL of elution solvent.
4.3.7 Preparation of Stock Solutions

4.3.7.1 Oligomer Stock Solution:

10 mg of Oligomer standard was accurately weighed and transferred into a 10 mL volumetric flask, dissolved and made up to 10 mL with 90:10 water and acetonitrile. Then the Stock solution was stored in the cold room (4°C).

4.3.7.1.1 Preparation of Stock dilutions for Oligomer in Urine:

The stock dilutions were prepared as per Table given below from the Oligomer stock solution, using 90:10 water and acetonitrile, in the concentration range from 500.0 ng/mL to 5000.0 ng/mL.

4.3.7.1.2 Lysine-Oligomer stock solution:

About 1.0 mg of L-Oligomer standard was accurately weighed into a 1 mL volumetric flask, dissolved and made up to 1 mL with 90:10 water and acetonitrile. The Stock solution was stored in a cold room (4°C).

4.3.7.1.3 Oligomer Stock solution:

About 10 mg of Oligomer standard was accurately weighted and transferred into a 10 mL volumetric flask, dissolved and made up to 10 mL with 90:10 water and acetonitrile. The Stock solution was stored in a cold room (4°C).

Table 4.4: Illustration of the dilution scheme followed for calibration standards

<table>
<thead>
<tr>
<th>Stock.ID (ng/mL)</th>
<th>Stock Conc. (ng/mL)</th>
<th>Stock.vol. (mL)</th>
<th>Make upto (mL)</th>
<th>Final Conc. (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000000.0</td>
<td>1000000.0</td>
<td>0.1</td>
<td>10.0</td>
<td>10000.0</td>
</tr>
</tbody>
</table>
4.3.8 Preparation of standards

4.3.8.1 Preparation of Calibration Curve Standards

4.3.8.1.1 Preparation of Spiked Urine Standards:

The blank rat urine was spiked with the above-prepared stock dilutions (spiking solutions) to prepare the urine standards ranging from 0.1\(\mu\)g/mL to 75\(\mu\)g/mL as per the Table given below:

Table 4.5: Illustration of the dilution scheme followed for calibration standards

<table>
<thead>
<tr>
<th>Stock.ID (ng/mL)</th>
<th>Stock Conc. (ng/mL)</th>
<th>Stock.vol. (mL)</th>
<th>Making up the volume with Urine(mL)</th>
<th>Final Conc. in urine (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50000.0</td>
<td>50000.0</td>
<td>0.1</td>
<td>1.0</td>
<td>5000.0</td>
</tr>
<tr>
<td>20000.0</td>
<td>20000.0</td>
<td>0.1</td>
<td>1.0</td>
<td>2000.0</td>
</tr>
<tr>
<td>10000.0</td>
<td>10000.0</td>
<td>0.1</td>
<td>1.0</td>
<td>1000.0</td>
</tr>
<tr>
<td>5000.0</td>
<td>5000.0</td>
<td>0.1</td>
<td>1.0</td>
<td>500.0</td>
</tr>
</tbody>
</table>
4.3.8.1.2 Preparation of L-Oligomer spiked fecal standards

To nine separate 10 mg faecal aliquot, 10 μL of L-Oligomer from each of the stock solution with concentrations of 1, 2, 4, 10.0, 20.0, 40.0, 100.0, 200 and 400 μg/mL was added to get final standards of 1, 2, 4, 10, 20, 40.0, 100, 200 and 400 ng/mg standards.

4.3.8.1.3 Preparation of Oligomer spiked fecal standards

To nine separate 10 mg faecal aliquot, 10 μL of Oligomer from each of the stock solution with concentrations of 1, 2, 4, 10, 20, 40, 100, 200, 300 μg/mL was added to get final standards of 1, 2, 4, 10, 20, 40, 100, 200 and 300 ng/mg standards.

4.3.9 Sample Preparation

4.3.9.1 Preparation of samples

4.3.9.1.1 Extraction of urine samples

200 μL of milli-Q water was added to 200 μL of urine sample and vortexed for 1 min. Samples were treated with solid phase extraction technique for the removal of the matrix. Oasis HLB cartridges with 30mg, 1CC were used for the extraction. Cartridges were equilibrated with methanol followed by water and loaded with the sample. Washing was done with 1mL of water and analytes were eluted with 300 μL of 90:10 1mM NaCl and acetonitrile. Samples were transferred to HPLC vials and 1 μL was injected for the analysis.
4.3.9.1.2 Extraction of Fecal samples

Dried faecal sample, 10 mg was added to an eppendorf tube to which 300μL of 1mM NaCl solution was added. This was vortexed for 15 minutes and centrifuged at 10,000rpm for 10 min. The aqueous layer was transferred into HPLC vials and 1μL of this sample was injected into the HPLC for analysis.

4.3.9.1.3 Solid phase extraction procedure:

Cartridge conditioning was done using 1 mL methanol.

Equilibration was done using 1 mL water.

A 400 μl sample was loaded on each of the cartridge and washed with 1 ml of water.

Elution was performed with 300 μL of 90:10, 1mM NaCl: Acetonitrile.

4.3.9.1.4 Chromatographic conditions used during sample analysis

Column: TSKgel amide column, 4.6 x 150mm

Flow rate: 0.700mL/min.

Volume of injection: 1μL

Run Time: 11.00 min

Column oven temperature: 40°C

Autosampler temperature: 4 ± 2°C

Gradient:
<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90.0</td>
</tr>
<tr>
<td>6.0</td>
<td>10.0</td>
</tr>
<tr>
<td>6.1</td>
<td>90.0</td>
</tr>
<tr>
<td>11.0</td>
<td>90.0</td>
</tr>
</tbody>
</table>

**MS Conditions:**

L-Oligomer : Transitions that were monitored in MRM

\[ 387.110 \rightarrow 258.900 \text{ m/z} \]

\[ 387.110 \rightarrow 342.360 \text{ m/z} \]

Oligomer : \[ 258.960 \rightarrow 187.100 \text{ m/z} \]

Spray Voltage : 400 V

Vaporizer Temperature : 300 °C

Sheath Gas Pressure : 45 psi

Ion sweep Gas Pressure : 2.0 psi

Aux Gas Pressure : 60 psi

Capillary Temperature : 275 °C

Tube Lens offset : 150 V
Skimmer Offset : 0 V

Collision Pressure : 1.5 psi

Collision Energy : 27/22 V(L-Oligomer/Oligomer)

Probe Position : C

Lateral Position of needle : 0

Micro meter : 1.5 μm

4.3.10 Data Processing:
Calibration curves were tested for linear fit. Where ever linear fit was not possible, data was linearized using a log transformation and then fitted. A standard linear equation was used for calculating the concentrations of unknowns. The equation is given below:

Y = mx + C, where

x = Analyte conc.

Y = Analyte Area / ISTD Area

m = Slope of the calibration curve

C = y-axis intercept value.

Mass spectrometer used was a TSQ Quantum ultra AM, triple quadrupole mass spectrometer manufactured by Thermo Electron corp (San Jose, CA, US)
Internal standards were not used in the analytical method. The concentrations were expected to be on the higher side hence lack of internal standard was not expected to affect the recovery of the metabolites. Typically an internal standard is used to help tighten the variability of the assay. In this case, the variability was not expected to affect the outcome of the experiment. In addition, no appropriate internal standard, which would show similar behaviour to the metabolites, could be identified at the time of planning the experiment.

For HPLC, a generic small molecular weight compound method was chosen. The MS conditions were optimized for maximum signal intensity in multiple reaction monitoring mode. The transition followed for quantitation are provided above.

The recovery of oligomer and lysine-oligomer was calculated by calculating expected molar equivalents of metabolites to pegylated insulin. Each mole of pegylated insulin (6025 gm) was expected to give rise to either 365 gm (1 mole) of lysine oligomer or 233 gm (1 mole) of free oligomer.

If dose given was 50 mg per animal then

\[
\text{oligomer dose} = \frac{\text{molecular wt of oligomer} \times \text{Dose of pegylated insulin}}{\text{Molecular weight of pegylated insulin}}
\]

Similarly, dose of oligomer was calculated using equation given below:

\[
Lys - \text{oligomer dose} = \frac{\text{molecular weight of lys - oligomer} \times \text{Dose of pegylated insulin}}{\text{Molecular weight of pegylated insulin}}
\]
4.4 Results and discussions

The response of oligomer in urine and feces was found to be non-linear when all the concentrations were included as part of calibration curve. The linearization of the response was done by log-log plot (Figure 4.4 and Figure 4.5), which was found to fit well and gave an R squared value of 0.9988. The back calculated recovery was found to be erroneous at the lower concentrations using this calibration curve. The calibration curve of lysine-ε-oligomer showed a linear response but yet again the back calculated recovery at the lower concentration showed over estimation by a significant margin, implying that the quantification below 1 μg/mL would not be reliable in the case of urine standard and in the case of oligomer measurement in feces the limit would be 1 ng/mL. The recovery was found to be acceptable between 80-120% over the rest of the range. In the real samples while calculating absolute recovery, it was thought the lower concentrations would not make significant difference to the total recovery as lower concentrations would contribute only a small fraction of total recovery and the overall error percentage would not affect the outcome of the study and conclusion. Hence it was decided to complete analysis of all samples with the said method.

The following graphs are the calibration curves obtained for oligomer and lysine-ε-oligomer after spiking in blank urine and faeces.
Figure 4.4: Calibration curve for free oligomer standard in blank urine.

Table 4.6: Calibration curve for oligomer spiked in Blank Urine.

<table>
<thead>
<tr>
<th>Conc. (ng/mL)</th>
<th>Peak Area</th>
<th>log (conc)</th>
<th>log (PA)</th>
<th>Back calculated conc</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>136741</td>
<td>-0.3010</td>
<td>5.1359</td>
<td>-0.4023</td>
<td>134%</td>
</tr>
<tr>
<td>1000</td>
<td>271690</td>
<td>0.0000</td>
<td>5.4341</td>
<td>-0.0142</td>
<td>-</td>
</tr>
<tr>
<td>2000</td>
<td>476072</td>
<td>0.3010</td>
<td>5.6777</td>
<td>0.3028</td>
<td>101%</td>
</tr>
<tr>
<td>5000</td>
<td>1145459</td>
<td>0.6990</td>
<td>6.0590</td>
<td>0.7991</td>
<td>114%</td>
</tr>
<tr>
<td>10000</td>
<td>2061755</td>
<td>1.0000</td>
<td>6.3142</td>
<td>1.1314</td>
<td>113%</td>
</tr>
<tr>
<td>20000</td>
<td>2992292</td>
<td>1.3010</td>
<td>6.4760</td>
<td>1.3419</td>
<td>103%</td>
</tr>
</tbody>
</table>
### Table 4.7: Calibration curve for lysine-oligomer standard in blank urine

<table>
<thead>
<tr>
<th>Conc. (ng/mL)</th>
<th>Peak area</th>
<th>Back calculated conc</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>233180</td>
<td>-2.8756</td>
<td>-288%</td>
</tr>
<tr>
<td>2</td>
<td>277679</td>
<td>1.0430</td>
<td>52%</td>
</tr>
<tr>
<td>5</td>
<td>320396</td>
<td>4.8046</td>
<td>96%</td>
</tr>
<tr>
<td>10</td>
<td>357325</td>
<td>8.0565</td>
<td>81%</td>
</tr>
<tr>
<td>20</td>
<td>489629</td>
<td>19.7071</td>
<td>99%</td>
</tr>
</tbody>
</table>
Figure 4.6: Calibration curve for lysine-oligomer standard in blank feces

Table 4.8: Calibration curve for lysine-oligomer standard in blank feces

<table>
<thead>
<tr>
<th>Conc. (ng/mg)</th>
<th>Peak area</th>
<th>Back calculated conc</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1080668</td>
<td>0.9474</td>
<td>95%</td>
</tr>
<tr>
<td>2</td>
<td>2111863</td>
<td>1.9932</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>4494484</td>
<td>4.4095</td>
<td>110%</td>
</tr>
<tr>
<td>10</td>
<td>10547300</td>
<td>10.5480</td>
<td>105%</td>
</tr>
</tbody>
</table>
Figure 4.7: Calibration curve for oligomer standard in blank feces.

Table 4.9: Calibration curve for oligomer standard in blank feces.

<table>
<thead>
<tr>
<th>Conc. (ng/mg)</th>
<th>Peak Area</th>
<th>Log (conc)</th>
<th>Log (PA)</th>
<th>Back calculated conc</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20785918</td>
<td>20.9316</td>
<td>105%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>41559901</td>
<td>41.9997</td>
<td>105%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>97561747</td>
<td>98.7945</td>
<td>99%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>187969915</td>
<td>190.4827</td>
<td>95%</td>
<td></td>
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### Table 4.10: Individual Animal Data of Metabolite Recovery in Urine Following Oral Administration of IN-105 to Wistar Rats

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Table 4.12: Individual Animal Data of Metabolite Recovery in Faeces Following Oral Administration of pegylated insulin to Wistar Rats

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NA: Sample Missing; ‘-’: Sample Not Collected
Table 4.13: Individual Animal Data of Metabolite Recovery in Faeces Following Oral Administration of Lys-e-Oligomer to Wistar Rats

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Time Intervals (h)</th>
<th>Dose 1</th>
<th></th>
<th></th>
<th></th>
<th>Dose 2</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Dose 3</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Free Oligomer (ng/mg)</td>
<td>Lys-Oligomer (ng/mg)</td>
<td>Weight (g)</td>
<td>Free Oligomer (ng/mg)</td>
<td>Lys-Oligomer (ng/mg)</td>
<td>Weight (g)</td>
<td>Free Oligomer (ng/mg)</td>
<td>Lys-Oligomer (ng/mg)</td>
<td>Weight (g)</td>
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<td>0.00</td>
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<td>-</td>
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<td>-</td>
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<td>0.06</td>
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<td>597.53</td>
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<td>141.60</td>
<td>280.69</td>
<td>0.95</td>
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<td>24 - 48</td>
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<td>-</td>
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<td>0.18</td>
<td>12.72</td>
<td>2.44</td>
<td></td>
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</tr>
</tbody>
</table>

NA: Sample Missing; ‘-’: Sample Not Collected
Tables 4.10 to 4.13 also exhibited the few time points no urine or feces samples were obtained. In one case a few of the samples, which were kept separately, were accidently mixed up with samples from another study and hence were labeled as missing.

All the animals showed a drop in blood glucose to the extent of 30 to 70% from baseline, indicating Insulin was being absorbed after the oral gavage (Table 4.14). Since the trial was over a period of 3 days, and volume requirement for pharmacokinetic analysis was high, it was decided not to draw blood for PK measurements. For antidiabetic drugs a reduction in blood glucose can be used as a surrogate marker. No drop in blood glucose levels was seen in control animal’s treated with Lys-e-Oligomer, which ruled out any impact of overnight fasting on the blood glucose levels and the reduction in glucose in test group was clearly attributed to the absorption of pegylated insulin. There was no adverse impact of low blood glucose noted in the study. There was a small increase in blood glucose level seen in group 2, which may be due to the stress animals experienced during handling.

Table 4.14: Mean blood glucose reduction (N=4) in positive control and pegylated insulin treated animals

<table>
<thead>
<tr>
<th>Study Day</th>
<th>Group</th>
<th>Test Compound</th>
<th>^Change in Mean % Blood Glucose (N=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1</td>
<td>Pegylated insulin</td>
<td>↓ 71.39</td>
</tr>
<tr>
<td>Day 2</td>
<td>1</td>
<td>Pegylated insulin</td>
<td>↓ 63.72</td>
</tr>
<tr>
<td>Day 3</td>
<td>1</td>
<td>Pegylated insulin</td>
<td>↓ 29.15/60.99*</td>
</tr>
<tr>
<td>Day 1</td>
<td>2</td>
<td>Lys-e-Oligomer</td>
<td>↑ 20.50</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>Lys-e-Oligomer</td>
<td>↑ 10.61</td>
</tr>
<tr>
<td>Day 3</td>
<td>2</td>
<td>Lys-e-Oligomer</td>
<td>↑ 10.05</td>
</tr>
</tbody>
</table>

^: % Change as compared to pre-dose blood glucose values; ↓ Decrease from baseline; ↑ Increase from baseline
*: 2 Non-responders (data presented with & without outliers)
4.4.1 Metabolite Recovery:

Both free oligomer and Lysine-ε-oligomer were quantified in rat urine and faeces samples collected over the 5 day observation period. The quantity of metabolite recovered in urine/faeces is more after dose 3 (Study days 3 to 5) as compared to dose 1 and 2. This could be due to very high doses being used in the study leading to incomplete clearance of metabolites within 24 hours. The accumulation is perhaps an artefact of very large doses and is not likely to be the case in practice at the pharmacologically effective doses in patients.

Table 4.15: Mean concentration of metabolites recovered in rat urine following oral administration of 200 mg/kg of pegylated insulin.

<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* (μg)</th>
<th>Volume (mL)</th>
<th>Amount Recovered (μg)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (*Pegylated insulin 200 mg/kg)</td>
<td>Free Oligomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 / Day 1</td>
<td>1976</td>
<td>13.00</td>
<td>259.00</td>
<td>13.11</td>
<td></td>
</tr>
<tr>
<td>Dose 2 / Day 2</td>
<td>2125</td>
<td>13.98</td>
<td>282.37</td>
<td>13.29</td>
<td></td>
</tr>
<tr>
<td>Dose 3 / Day 3–5</td>
<td>2112</td>
<td>51.53</td>
<td>476.38</td>
<td>22.56</td>
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</tr>
<tr>
<td>Lysine Oligomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 / Day 1</td>
<td>3308</td>
<td>13.00</td>
<td>0.34</td>
<td>0.010</td>
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</tr>
<tr>
<td>Dose 2 / Day 2</td>
<td>3558</td>
<td>13.98</td>
<td>0.53</td>
<td>0.015</td>
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<tr>
<td>Dose 3 / Day 3–5</td>
<td>3536</td>
<td>51.53</td>
<td>1.11</td>
<td>0.031</td>
<td></td>
</tr>
</tbody>
</table>

*: Dose is a theoretical maximum recovery possible for each of the metabolite after administration of 200 mg/kg dose of pegylated insulin.

Following administration of pegylated insulin to Wistar Rats, the major metabolite quantified in urine was free oligomer. The free oligomer formed 99% of the fraction of the total amount recovered in urine. The percent recovery of free oligomer in urine was approximately 13 –23% with highest amount recovered after the day 3 dosing as the samples were collected up to day 5.
Table 4.16: Mean concentration of metabolites recovered in rat urine following oral administration of 20 mg/kg of Lysine-ε-oligomer.

<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* (μg)</th>
<th>Volume (mL)</th>
<th>Amount Recovered (μg)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free Oligomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2 Lysine-ε-Oligomer</td>
<td>Dose 1 / Day 1</td>
<td>3434</td>
<td>14.03</td>
<td>239.68</td>
<td>6.98</td>
</tr>
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<td>Dose 2 / Day 2</td>
<td>3507</td>
<td>14.18</td>
<td>283.41</td>
<td>8.08</td>
</tr>
<tr>
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<td>Dose 3 / Day 3 – 5</td>
<td>3557</td>
<td>57.95</td>
<td>458.58</td>
<td>12.89</td>
</tr>
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<td>Lysine Oligomer</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dose 1 / Day 1</td>
<td>5750</td>
<td>14.03</td>
<td>0.85</td>
<td>0.015</td>
</tr>
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<td>Dose 2 / Day 2</td>
<td>5873</td>
<td>14.18</td>
<td>1.20</td>
<td>0.020</td>
</tr>
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<td></td>
<td>Dose 3 / Day 3 – 5</td>
<td>5955</td>
<td>57.95</td>
<td>1.96</td>
<td>0.033</td>
</tr>
</tbody>
</table>

*: Molar equivalent to free oligomer & Lysine oligomer

Following administration of Lysine-ε-oligomer to Wistar Rats, the major metabolite quantified in urine was again free oligomer. The amount of free oligomer recovered was higher than that of lysine oligomer. The percent recovery of free oligomer in urine was about 7 to 13%. The highest recovery was found in the day 3 samples. Interestingly, very little lysine-ε-oligomer was recovered in Urine.

Table 4.17: Mean concentration of metabolites recovered in rat faeces following oral administration of 200 mg/kg of pegylated insulin.

<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* (μg)</th>
<th>Weight (g)</th>
<th>Amount Recovered (μg)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free Oligomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 Pegylated insulin</td>
<td>Dose 1 / Day 1</td>
<td>2004</td>
<td>2.63</td>
<td>407.17</td>
<td>20.32</td>
</tr>
<tr>
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<td>Dose 2 / Day 2</td>
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<td>1.60</td>
<td>330.31</td>
<td>15.55</td>
</tr>
<tr>
<td></td>
<td>Lysine Oligomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dose 1 / Day 1</td>
<td>3355</td>
<td>2.63</td>
<td>341.16</td>
<td>10.17</td>
</tr>
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<td>Dose 2 / Day 2</td>
<td>3558</td>
<td>1.60</td>
<td>79.60</td>
<td>2.24</td>
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<td>Dose 3 / Day 3 – 5</td>
<td>3536</td>
<td>7.39</td>
<td>99.09</td>
<td>2.80</td>
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</table>
*: Molar equivalent to free oligomer & Lysine oligomer

Following administration of pegylated insulin to Wistar Rats, the major metabolite quantified in faeces was free oligomer. The concentration of free oligomer recovered was about 3 folds higher than that of lysine oligomer (Table 8). The percent recovery of free oligomer in faeces was about 15 to 21% and recovery of unchanged lysine oligomer was between 2 and 10% of the expected theoretical recovery. In terms of absolute quantity, lysine-oligomer was present in significantly higher quantity in faeces compared to that which was recovered in Urine. It is difficult to say if this was due to the presence of amino acid, due to lower solubility of lysine-oligomer, or some other as yet undetermined reason.

Table 4.18: Mean concentration of metabolites recovered in rat urine following oral administration of 20 mg/kg of Lysine-\(\varepsilon\)-oligomer.

<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* ((\mu)g)</th>
<th>Weight (g)</th>
<th>Amount Recovered ((\mu)g)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
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<td>G2 Lysine-(\varepsilon)-Oligomer (20 mg/kg)</td>
<td>Dose 1 / Day 1</td>
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<td>16.06</td>
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<td>91.10</td>
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<td>6.59</td>
<td>99.65</td>
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</table>

*: Molar equivalent to free oligomer & Lysine oligomer

A similar result was obtained following administration of Lysine-\(\varepsilon\)-oligomer to Wistar Rats. The major metabolite quantified in faeces was free oligomer. The concentration of free oligomer recovered was about 6 to 12 fold higher than that of lysine oligomer (Table 9). The percent recovery of free oligomer in faeces was about 5 to 13% and unchanged lysine oligomer was 0.2 to 1.7%.

Table 4.19: Total recovery of free oligomer and lysine oligomer metabolites in rat urine and faeces following oral administration of pegylated insulin
<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* (μg)</th>
<th>Recovery in Urine (μg)</th>
<th>Recovery in Faeces (μg)</th>
<th>Total Metabolite Recovered (μg)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free Oligomer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1 (Pegylated insulin 200 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 / Day 1</td>
<td>2004</td>
<td>259.00</td>
<td>407.17</td>
<td></td>
<td>666.17</td>
<td>33.25</td>
</tr>
<tr>
<td>Dose 2 / Day 2</td>
<td>2125</td>
<td>282.37</td>
<td>330.31</td>
<td></td>
<td>612.68</td>
<td>28.83</td>
</tr>
<tr>
<td>Dose 3 / Day 3 – 5</td>
<td>2112</td>
<td>476.38</td>
<td>448.37</td>
<td></td>
<td>924.75</td>
<td>43.79</td>
</tr>
<tr>
<td><strong>Lysine Oligomer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 / Day 1</td>
<td>3355</td>
<td>0.34</td>
<td>341.16</td>
<td></td>
<td>341.50</td>
<td>10.18</td>
</tr>
<tr>
<td>Dose 2 / Day 2</td>
<td>3558</td>
<td>0.53</td>
<td>79.60</td>
<td></td>
<td>80.13</td>
<td>2.25</td>
</tr>
<tr>
<td>Dose 3 / Day 3 – 5</td>
<td>3536</td>
<td>1.11</td>
<td>99.09</td>
<td></td>
<td>100.20</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* Molar equivalent of free oligomer and Lysine oligomer

The additive results of total recovery of the metabolites from both urine and faeces are summarized in Table 4.19. The total amount of metabolite recovered showed a mass balance between 30 to 45% of the total expected after administration of pegylated insulin. After each of the dosing, amount of free oligomer was larger as compared to lysine oligomer.

Table 4.20: Total recovery of free oligomer and lysine oligomer metabolites in rat urine and faeces following oral administration of Lysine-g-oligomer

<table>
<thead>
<tr>
<th>Trt Group, Test item</th>
<th>Dose No. / Study Day</th>
<th>Dose* (μg)</th>
<th>Recovery in Urine (μg)</th>
<th>Recovery in Faeces (μg)</th>
<th>Total Metabolites Recovered (μg)</th>
<th>Percent Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free Oligomer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2 Lysine-g-Oligomer (20 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 / Day 1</td>
<td>3434</td>
<td>239.68</td>
<td>183.85</td>
<td></td>
<td>423.53</td>
<td>12.33</td>
</tr>
<tr>
<td>Dose 2 / Day 2</td>
<td>3507</td>
<td>283.41</td>
<td>284.25</td>
<td></td>
<td>567.66</td>
<td>16.18</td>
</tr>
<tr>
<td>Dose 3 / Day 3 - 5</td>
<td>3557</td>
<td>458.58</td>
<td>472.99</td>
<td></td>
<td>931.56</td>
<td>26.19</td>
</tr>
<tr>
<td><strong>Lysine Oligomer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1 /</td>
<td>5750</td>
<td>0.85</td>
<td>16.06</td>
<td></td>
<td>16.91</td>
<td>0.29</td>
</tr>
<tr>
<td>Day 1</td>
<td>Dose 2 / Day 2</td>
<td>5873</td>
<td>1.20</td>
<td>91.10</td>
<td>92.30</td>
<td>1.57</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>Dose 3 / Day 3 – 5</td>
<td>5955</td>
<td>1.96</td>
<td>99.65</td>
<td>101.61</td>
<td>1.71</td>
<td></td>
</tr>
</tbody>
</table>

* Molar equivalent to free oligomer & Lysine oligomer

Similarly, upon oral gavage of lysine–oligomer, significantly higher amounts of metabolites were recovered in urine than in faeces, indicating lysine–oligomer gets absorbed systemically and is cleared by the kidneys. Total percentage of free oligomer and lysine -oligomer recovered following lysine-oligomer gavage was 12 to 26% and 0.3 to 1.7%, respectively. The low recovery of the metabolites in both groups could be due to the fact that at the time of developing it was not possible to predict the expected concentrations of the metabolites. Hence the linear range was later found to be inadequate to cover the actual concentrations found in the sample, especially for the urine samples. Typically, since the response of the calibration curve saturates beyond a certain concentration, the estimation of actual samples falling in the non-linear range of the calibration curve is expected to be low. It was not possible to identify the extent of dilution that would have been required for some of the samples containing high amounts of metabolites. The lower recovery was not attributed to the absence of an internal standard for mass spectrometric analysis. In addition, the missing or unavailable samples may have resulted in lack of mass balance.

When these results were obtained, it was decided to repeat the analysis for the urine samples only, as close to 99% metabolites was in the form of oligomer and present in urine. The samples were stored at -80°C until the time of the repeat analysis. The gap between the two analyses was close to 1 year. When the samples were reanalysed, the overall mass balance was found to be greater than 100% which obviously is incorrect. The
reason for this high recovery was not determined and has been attributed to long term storage problems. Hence only the results from first analysis are presented here and the repeat analyses have been ignored. Triethyleneglycol, which is structurally similar to the oligomer, was used as internal standard and a calibration curve was prepared along with quality control (QC) samples. There was no difference found in terms of the recovery of the QC samples, indicating that the recovery of the samples was not affected by the presence or absence of internal standard.

4.5 Conclusions

Based on the reduction in blood glucose levels (table 4.14) it can be concluded that pegylated insulin was systemically available following oral administration. A large proportion of administered pegylated insulin was broken down in gut and is excreted via faeces. The metabolite seen in the faeces was primarily lysine-oligomer while a significantly smaller fraction was seen as intact oligomer. Apart from lysine-oligomer and free oligomer no other metabolites could be detected in any of the samples. This could be due to the fact that the polypeptide of insulin is likely digested completely into dipeptides and amino acids and then recycled by the body for protein synthesis. PEGs are known to induce liver enzymes, but as described in previous chapter, the published data suggests that the liver enzyme induction happens with PEG alcohol. It is not clear if the PEG ether would induce the enzymes in a similar fashion. It is highly likely that the presence of methoxy ether groups either prevents the metabolism of PEG altogether or slows it down sufficiently that the excretion via kidney results in excretion of the metabolite unchanged.

The total recovery of free oligomer was found to be between 30 to 46% of the theoretically possible recovery post pegylated insulin dosing. Similarly, lower recoveries of free oligomer were observed in the control arm post lysine–oligomer dosing. It appears that
pegylated insulin is broken down in the gut and during circulation to lysine-oligomer, followed by further hydrolysis of lysine-oligomer to free oligomer. Lysine-oligomer, upon oral administration, appears to get absorbed rapidly resulting in > 90% recovery in urine, when administered either in its intact form or as a free acid. Lysine-oligomer was also metabolized to the free oligomer form. The linkage between lysine and oligomer is an amide linkage. It is possible that a peptidase or an amidase is involved in the conversion of lysine-oligomer to free oligomer and it would be interesting to identify the specific enzyme responsible. However, this is beyond the scope of the present research.

The reason for incomplete mass balance could not be determined. However, it can probably be attributed to the metabolite concentrations exceeding the linear range resulting in gross underestimation of metabolites. This study was not designed along the lines of classical ADME studies, which determine the rate of elimination or metabolites as well as tissue accumulation if any. There is a possibility that the incomplete mass balance could be due to the limitation of the study scope and/or an artefact of the study design. It could also be possible that given the use of very high doses, the complete elimination would have taken longer than anticipated, and had sampling been done over longer period of time, better mass balance could have been achieved.

The results confirmed the findings of the earlier in-vitro metabolite identification study that pegylated insulin is broken down to lysine-oligomer and free oligomer. However, in the in vivo experiment, the ratio of free oligomer to lysine-oligomer was reversed, which may be due to more rapid metabolism in a living organism as opposed to laboratory grown cells. A more controlled study would help establish the mass balance. Also radiolabelling of oligomer would help determine the exact fate of oligomer and give an idea on tissue accumulation status. If the complete mass balance of oligomer is not achieved, then it
raises questions on the fate of remaining oligomer. A classical ADME study with more extensive tissue samples would give better clarity on oligomer fate. However, it is a good sign that most of the oligomer is recovered in its free acid form in urine, which alleviates the concern of accumulation to some extent.

In a small study in human subjects with varying degree of renal impairment, it was determined that for Liraglutide, a novel GLP-1 analogue, there was no change in pharmacokinetics or renal clearance (Linnebjerg et al. 2007). On the other hand, for Exenatide, a natural analogue of GLP-1 with 57% homology to GLP-1, the half-life increased with increasing severity of renal impairment. Also, exposure to Exenatide appeared to be higher with increasing severity of the disease. Because of this higher exposure, there were more incidences of nausea and vomiting in subjects with renal impairment and Exenatide was poorly tolerated in such subjects (Jacobsen et al. 2009).

Another ADME study was reported by a group of scientists from Japan. They studied the ADME of ε-polylysine, which is used as a preservative in food. While the end use of the product is non-therapeutic, ADME is important as food is consumed in large quantities making safety of preservative a major concern. In this study, ε-polylysine was radiolabelled using $^{14}$C and ADME was studied in a rat model. The group reported that there was no toxicity observed and close to 100% mass balance was achieved with greater than 90% radio activity being recovered in faeces. Metabolite profiling by HPLC showed that the primary metabolite was L-lysine and the group could conclude safe usage of ε-polylysine as a preservative (Hiraki et al. 2003).

The use of mass spectrometry facilitated by the presence of an oligomer tag worked well as a non-radioactivity method in identifying at least some of the metabolites. Lim et al. 2015 recently published a non-radioactivity method for investigating metabolism of
adrenocorticotropic hormone (ACTH). In this method, the researchers tagged the ACTH fragment with $^{127}$I. The measurement of 127 m/z was done using Ion coupled plasma mass spectrometry (ICP-MS). $^{127}$I is a non-radioactive isotope of iodine and does not affect the metabolic profile of the peptide. The group was successful in measuring the ACTH fragment in various different human tissue preparation in an in-vitro setting.

In the case of proteins, modification with a small oligomer is not likely to affect the ADME profile of the drug. However, more studies are required to validate and fully determine the utility of the use of oligomer tagging and mass spectrometry for ADME profiling. This method appears to be a useful method for ADME profiling, particularly for proteins and peptides drugs.

Traditionally ADME studies are not carried out for peptide drugs as it is believed that the peptides are broken down into amino acids are recirculated in the body. The studies that are performed are mostly to understand qualitative aspects of ADME. With more and more peptide and modified peptide drugs coming to market there is a need for improved analytics to enable the study of metabolism and elimination of these drugs.
Chapter 5

Summary and Future Directions
5.1 Summary

Based on the work described in the thesis following conclusions were made:

A novel pegylated insulin analogue is currently under clinical development. The therapeutically active compound is available in tablet dosage form. Sodium caprate as a novel permeation enhancer was used as an excipient. Upon granulation, we showed that sodium caprate particle properties are completely changed. Before granulation sodium caprate was present as fibrous material which led to high variation in the bulk density and flow properties. After granulation, the fibres fused, forming denser particles with a significant improvement in flow properties.

The structural differences between recombinant human insulin and pegylated recombinant human insulin were studied using circular dichroism. These studies revealed that pegylation does not affect overall global structure of the molecule. Melting experiments using CD showed that the extent of unfolding of both the molecules were similar, however, pegylated insulin showed higher elevated Tm values, as compared to those for human insulin, indicating a more stable structure for the pegylated protein. The stabilization effect was more apparent at pH 7.4 and pH 9.6 where the extent of unfolding was significantly lower for pegylated insulin. At basic pH, the unfolding due to temperature was greater for human insulin than for pegylated insulin. The excipients appeared to stabilize the insulin in a higher order association state, which was observed in the higher Tm value. The higher association state could not be observed using size exclusion chromatography, which indicates that the higher association state was reversible in nature.

Tableting affected the structure of pegylated insulin, resulting in an intact structure with higher levels of beta sheet. Unfolding of the molecular structure was greater at higher temperatures for pegylated insulin after tableting, than for pure pegylated insulin or its
blend with the excipients. The higher oligomeric state could not be observed using size exclusion chromatography. The overall globular structure was shown to be intact, based on hydrogen-deuterium exchange and ESI-MS data, suggesting that the changes during tableting are reversible.

This shows that modification of protein with a short chain alkyl PEG was not detrimental to insulin and the formulation excipients offered some protection against unfolding due to pressure and temperature during tableting. This indicates that there is a considerable scope for using protein modification for making designer molecules for oral delivery, and also that a tablet dosage form is a feasible option for protein drug delivery. To our knowledge, this is first ever study exploring the impact of compression on protein structure in solid state.

As part of this dissertation, the fate of the oligomer was studied in vitro using a hep G2 cell line. Several pegylated metabolite fragments of the polypeptides were identified using mass spectrometry combined with liquid chromatography. A potential sequence of chronological events for degradation of pegylated insulin was identified, wherein the separation of two chains was identified as a potential first step breakdown of pegylated insulin possibly involving glutathione transhydrogenase or a similar enzyme.

This was further confirmed by using bacitracin, a known inhibitor of glutathione transhydrogenase. In presence of bacitracin, a new potential metabolite was identified where the individual chains were partially cleaved, yet the disulphides were found to be intact. It was also shown that the amide linkage between the epsilon amine and alkyl-PEG was hydrolysed to a small extent. Although the A-chain of the two molecules are identical, we identified two new sites of hydrolysis in the A-chain that have not been previously reported for insulin.
During the in vivo study of metabolism in a rat model, free oligomer was identified as a primary metabolite. A large portion of this metabolite was found in urine, suggesting that the small alkyl PEG, being highly water soluble, is excreted via kidney into urine. This is a significant finding as it re-enforces the safety of using small alkyl PEG for modification of proteins.

5.2 Future Direction:

Based on the work done in this thesis several areas have been identified where future work would be valuable.

ADME studies for proteins and peptides are not very commonly performed. However, as more and more therapeutic proteins and their modified versions come to the market, understanding the ADME will become critical. ADME studies need to be tailored for specific structures and their functions. In the case of pegylated or modified molecules, an important issue is determining the fate of the pegylation unit or the modification.

For any oral insulin it is important to show that the insulin when given orally gets absorbed and goes straight to the liver via the portal vein. In addition, it is important to understand the distribution in terms of what percentage of absorbed dose goes to the liver and what percentage of absorbed dose spills over into the periphery. This would help establish that oral insulin indeed restores the portal-peripheral axes of insulin secretion, which is the primary benefit expected of oral insulin. It is also important to study different dosage forms and the impact of the unit processes involved on protein structure and function, as the forces involved in making dosage forms could play a significant role in developing therapeutically active and commercially viable alternate delivery systems for proteins.
**Future of oral insulin**

There is a need to develop a more physiological form of insulin delivery, and pegylated insulin is just a beginning. The pegylated insulin studied as part of this work, is a prandial insulin which provides glucose control for up to 2 to 3 hours after a meal. There may be scope for developing a better product with a longer duration of action. A basal oral insulin may have a larger impact on control of diabetes, hence there is scope for developing a basal oral insulin. In addition to improving the duration of action, there is scope to improve bioavailability of insulin.

To achieve longer duration of action, without compromising onset of action, the molecule may have to be modified for slower clearance. Following are the options that can be explored:

a) Increasing the molecular size by modifying insulin by conjugation with a large molecule like a high molecular weight polyethylene glycol or a large protein that will reduce renal clearance.

b) Increase the protein binding which will prolong the circulating half-life of insulin.

c) Modifying the insulin sequence to make the molecule more resistant to degrading enzymes

To ensure adequate bioavailability, it will be important to make the molecule resistant to different proteases, so that a sufficient amount of pharmaceutically active form survives long enough to be available for absorption. In this study, we focused on comparing the degradation of pegylated insulin versus human insulin by pepsin. If any protein is delivered in the intestinal region, then the onset of action would be governed by the drainage of the dosage form from stomach to intestinal region hence gastric motility would play a critical role. The time to passage to intestine varies greatly from day to day and person to person.
which would make the availability of insulin less predictable. For insulin, having a tight control over onset and peak of action is important, as it can result in serious consequences if the timing does not match with presence of food or high glucose levels. Considering this, for a successful oral delivery, the best place for insulin delivery is the upper Gastrointestinal tract (stomach or upper duodenum), which would provide a reasonable control over insulin action. Thus, ensuring resistance to pepsin degradation is of the key property to ensuring good bioavailability.

As an initial proof of concept we did some basic in-silico work to show that by replacing amino acids, it is possible to make the molecule resistant to digestive enzymes. The work is presented in the following section.

5.3 Protein modeling toward future insulin analog design

The following modelling work was done using freely available proteomics resources from the website www.expasy.org.

It is valuable to evolve or model the insulin molecule so as to devise strategies to make it more resistant to proteases, increase the solubility, increase permeability and perhaps increase circulating half-life to be able to mimic different desirable profiles.

Identification of Proteolytic degradation sites in Insulin:

A-chain primary sequence: GIVEQ CCTSI CSLYQ LENYC N

pI 3.79, Calculated Molecular weight: 2383.7

Pepsin at pH 1.3

Total cleavage sites 8 cleavage positions 12, 13, 14, 15, 16, 18, 19
Pepsin at pH > 2

Total cleavage sites 4  cleavage positions 12, 13, 15, 16

B-chain primary sequence: FVNQH LCGSH LVEAL YLVCG ERGFF YTPKT

pI 6.9, Calculated Molecular weight: 3429

Pepsin at pH 1.3  Total cleavage sites 11  cleavage positions 1, 5, 6, 10, 11, 14, 15, 16, 17, 24, 25

Pepsin at pH > 2  Total cleavage sites 11  cleavage positions 1, 5, 6, 10, 11, 14, 15, 16, 17, 23, 25

Amino acids at these cleavage sites were replaced in order to test for pepsin resistance without affecting the secondary and tertiary structure of insulin, insulin receptor binding, and IGF receptor binding, so that the structure function relationship remains intact. In the A-chain cleavage occurred between Ser-Leu, Leu-Tyr, Tyr-Asp, Asp-Leu, Asn-Tyr and Tyr-Cys. In an in-silico peptide cutter, when Leu was replaced with iso-leucine or valine at 13 and 16 position of the peptide, and tyrosine at 14th position were replaced with histidine or tryptophan, the peptide cutter did not show any possible cleavages at those positions.

A-chain primary sequence: GIVEQ CCTSI CSIHQ IENYC N

Pepsin at pH 1.3 , Total cleavage sites 2  cleavage positions 18, 19

Pepsin at pH > 2  Total cleavage sites 0

Name of the sequence is A-chain.

Sequence consists of 21 amino acids.
Target Sequence:

GIVEQCCTSICSLYQLENYC N

Secondary Structure was predicted using http://www.biogem.org/tool/chou-fasman/ server. (Chou and Fasman 1974)

• * Query 1 GIVEQCCTSICSLYQLENYC N 21
• Helix 1 <-------------> 21
• Sheet 1 EEEEEEEEEEEEEEEEE 21
• Turns 1 T 21
• Total Residues: H: 15 E: 17 T: 1 Percent: H: 71.4 E: 81.0 T: 4.8

Name of the sequence is A-chain.

Sequence consists of 21 amino acids.

Target Sequence:

GIVEQCCTSICSIYQLENYC N

Secondary Structure:

• * Query 1 GIVEQCCTSICSIYQLENYC N 21
• Helix 1 <-------------> 21
• Sheet 1 EEEEEEEEEEEEEEEEE 21
• Turns 1 T 21
• Total Residues: H: 15 E: 17 T: 1 Percent: H: 71.4 E: 81.0 T: 4.8
Name of the sequence is A-chain.
Sequence consists of 21 amino acids.

**Target Sequence:**
GIVEQCCTSICSIHQIENYC N

**Secondary Structure:**
- * Query 1 GIVEQCCTSICSIHQIENYC 21
- Helix 1  <-------------------> 21
- Sheet 1  EEEEEEEEEEEEEEEE 21
- Turns 1  T 21
- Total Residues: H: 15 E: 17 T: 1 Percent: H: 71.4 E: 81.0 T: 4.8

The exercise shown here is only an illustration of what is possible and how replacing amino acids may or may not change the secondary structure dramatically. The secondary prediction software showed that there may not be any change in structure with these replacements of amino acids, yet these changes are predicted to make the chain more resistant to pepsin. Since the prediction is only for standalone A-chain, once it is associated with B-chain, depending on the accessibility of these sites, the number of changes required might be different for improving resistance.

A similar exercise was performed for B-chain.

B-chain primary sequence: FVNQHLCGSHYLVCGERGFFYTPKT
Pepsin at pH 1.3  
Total cleavage sites 14  
cleavage positions 1, 5, 6, 10, 11, 14, 15, 16, 17, 24, 25

Pepsin at pH > 2  
Total cleavage sites 11  
cleavage positions 1, 5, 6, 10, 11, 14, 15, 16, 17, 24, 25

Name of the sequence is B-chain.
Sequence consists of 30 amino acids.

Target Sequence:
FVNQHLCGSH LVEALYLVCG ERGFFYTPKT

Secondary Structure:

- * * Query 1 FVNQHLCGSHLVEALYLVCGERGFFYTPKT 30
- Helix 1  <>  <--------------->  30
- Sheet 1  EEEEE  EEEEEEEE  EEE  30
- Turns 1  T  T  TT  T  30
- Total Residues: H: 10  E: 16  T: 5  Percent: H: 33.3  E: 53.3  T: 16.7

Name of the sequence is B-chain mutant.
Sequence consists of 30 amino acids.

Target Sequence:
FVNQHTCGSH IVEATYIVCG ERGFWYTPKT

Secondary Structure:

- * * Query 1 FVNQHTCGSHIVEATYIVCGERGFWYTPKT 30
- Helix 1  <--------------->  30
B-chain primary sequence: FVNQH TCGSH IVEAT YIVCG ERGFW YTPKT

Pepsin at pH 1.3  Total cleavage sites 6  cleavage positions 1, 15, 16, 23, 25, 25

Pepsin at pH > 2  Total cleavage sites 2  cleavage positions 1, 23

Similar to the A-chain, in the B-chain when Leu at the 6th position was replaced with threonine, leu at the 11th position was replaced with isoleucine, Val at the 12th position was replaced with iso-leucine, Leu at the 15th position was replaced with threo, Leu at the 17th position with Iso-leu and phenyl alanine at the 25th position with tryptophan. Despite so many changes the resultant peptide was predicted to have only 2 possible cleavage sites, hence is likely to be more resistant to pepsin compared to the natural analogue.

The secondary structure prediction of these individual modified chains showed that there was minimal or no change in the secondary structure. These pepsin resistant sequences of A-chain and B-chain were used in combination for predicting a 3D structure of protein. The structure prediction was done using Phyre server (Kelly and Sternberg 2009). For the purpose of modelling, a hypothetical 6 amino acid long connecting sequence was used to create a single chain molecule.
The predicted structures (Figure 5.1) showed a high degree of similarity for the mutated sequence despite a change in 8 amino acids. A similar exercise needs to be performed to test more combinations of amino acids to select a mutant sequence with least structural difference. The confidence in the presented modelling work is low, as the accuracy of prediction has not been tested. In addition, the freely available resources are capable of predicting the structures of single chain proteins and also are based on the structures of known proteins. Insulin consists of two chains which makes the prediction unreliable to some extent. For more accurate predictions, more exhaustive work needs to be done using energy minimisation models.

Once a more proteolytic resistant active molecule is designed, any further modification may be done to achieve the desired pharmacological profile. Modification may be done using appropriate pegylation. It is necessary to check for immunogenicity, since proteolytic resistant proteins are more likely to be immunogenic (Delamarre et al. 2006). Besides immunogenicity, the molecule might need evaluation for protease resistance (in vitro), protein binding, glucose uptake, lipogenesis activity, insulin receptor binding, IGF receptor binding, aggregation behavior etc. There is a considerable work that would be needed to be
done to take the development of oral delivery toward clinical use. A good target product profile can help focus the development of such molecules with tailored properties.

Figure 5.2 shows a potential development path that can be followed for developing a next generation of insulin. There is a significant scope for further development of a next generation of molecules, which would provide the desired glycemic control at lower doses of insulin. The above modeling work was done toward proof of concept for future insulin analog design.

In addition to tinkering with insulin itself at the molecular level, finding more efficient permeation enhancers is another area of interest. There is a need to improve the bioavailability of proteins when given orally without compromising the safety of the patients. Thus designing insulin analogs, finding safer and efficient permeation enhancers, and the development of better formulation technologies, are key to developing successful oral insulin products.
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