IN VITRO IN VIVO METHODS AND PHARMACOKINETIC MODELS FOR SUBCUTANEOUSLY ADMINISTERED PEPTIDE DRUG PRODUCTS

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Virginia Commonwealth University

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IN VITRO IN VIVO METHODS AND PHARMACOKINETIC MODELS FOR SUBCUTANEously ADMINISTERED PEPTIDE DRUG PRODUCTS

A dissertation submitted in partial fulfillment of the requirements for the Doctor of Philosophy in Pharmaceutical Sciences at Virginia Commonwealth University.

By

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Advisors: Dr. William H. Barr, Pharm. D., Ph.D., Professor, Department of Pharmacotherapy and Outcomes Sciences, Director – Center for Drug Studies

&

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Virginia Commonwealth University, Richmond, Virginia, December 2012
Acknowledgement

First and foremost, I would like to sincerely thank my advisors, Dr. William H. Barr, and Dr. William R. Garnett for giving me the opportunity to pursue graduate studies under their guidance.

I started my journey as a graduate student in the department of Medicinal Chemistry, only to realize that my heart was not in the synthesis of new molecules, but in understanding and testing of the molecules in humans. Many thanks to the timely support from Dr. Patricia Slattum and Dr. Susanna Wu-Pong, I ended up working with the best mentor of my life Dr. William H. Barr.

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Dedication

This dissertation and the work that I do in the future are dedicated to the person who has been the biggest influence during my graduate program, Dr. William H. Barr.
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<td>ADR</td>
<td>Adverse Drug Reaction</td>
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<tr>
<td>ADME</td>
<td>Absorption Distribution Metabolism Excretion</td>
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<td>ALS</td>
<td>Acid Labile Subunit</td>
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<td>API</td>
<td>Active Pharmaceutical Ingredient</td>
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<td>AUC</td>
<td>Area Under Curve</td>
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<tr>
<td>BA</td>
<td>Bioavailability</td>
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<td>BCS</td>
<td>Biopharmaceutics Classification System</td>
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<td>BE</td>
<td>Bioequivalence</td>
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<tr>
<td>CDS</td>
<td>Center for Drug Studies</td>
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<tr>
<td>DEV</td>
<td>Deviation</td>
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<tr>
<td>DFN</td>
<td>Deviation Factor</td>
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<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>ER</td>
<td>Extended Release</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FD</td>
<td>FITC labeled dextran</td>
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<tr>
<td>USFDA</td>
<td>U S Food and Drug Administration</td>
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<tr>
<td>GE</td>
<td>General Electric</td>
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<tr>
<td>GH</td>
<td>Growth Hormone</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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HS               High Solubility
HP               High Permeability
RP-HPLC           Reversed-Phase High Performance Liquid Chromatography
rIFN             recombinant Interferon
HQC              Highest Quality Control
ICH              International Conference on Harmonization
ID               Intradermal
IGF              Insulin like Growth Factor
IGFBP            Insulin like Growth Factor Binding Protein
IGFD             Insulin Growth Factor Deficiency
IR               Immediate Release
IRB              Institutional Review Board
IM               Intramuscular
IV               Intravenous
IVIVC          *In Vitro - In Vivo Correlation*
kDa              kilo Daltons
kPa              kilo Pascals
LC               Liquid Chromatography
LLOQ             Lower Limit of Quantification
LRR              Leucine Rich Repeat
LQC              Lower Quality Control
LOD              Limit of Detection
MDT              Mean Dissolution Time
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>MQC</td>
<td>Middle Quality Control</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<td>MRT</td>
<td>Mean Residence Time</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
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<td>NFF</td>
<td>Normal Flow Filtration</td>
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<td>PDR</td>
<td>Product Development Report</td>
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<td>PES</td>
<td>Polyether Sulphone</td>
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<td>PK</td>
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<td>Pharmacodynamic</td>
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<td>Protein Data Bank</td>
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<td>PVDF</td>
<td>Poly Vinyl DiFlouro</td>
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<tr>
<td>RI</td>
<td>Ribonuclease Inhibitor</td>
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<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<td>SC</td>
<td>Subcutaneous</td>
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<td>TrifluoroAcetic Acid</td>
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<tr>
<td>TFF</td>
<td>Tangential Flow Filtration</td>
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<td>USP</td>
<td>United States Pharmacopoeia</td>
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Abstract

IN VITRO, IN VIVO METHODS AND PHARMACOKINETIC MODELS FOR
SUBCUTANEOUSLY ADMINISTERED PEPTIDE DRUG PRODUCTS

A dissertation submitted in partial fulfillment of the requirements for the
Doctor of Philosophy in Pharmaceutical Sciences at Virginia Commonwealth University.

By

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Sciences, Associate Director – Center for Drug Studies

Virginia Commonwealth University,
Richmond, Virginia, December 2012
Over the last several years, injectable drugs have been a growing area for the treatment of various therapeutic conditions and they are projected to comprise an even larger proportion among the drugs that will be available in the years to come. The injectable drugs are administered by various routes such as intramuscular (IM), intravenous (IV), subcutaneous (SC) and others, however, the majority of these drugs are administered subcutaneously.

Even though subcutaneous delivery has been utilized for a number of years, very little is known about the processes governing the absorption of macromolecules from the interstitial space; and the resulting impact of these processes on the bioavailability (BA) and pharmacokinetic (PK) profiles. Also, there is no established *In vitro - In vivo* correlation (IVIVC) for subcutaneously administered immediate release (IR) peptide based drugs in a biorelevant manner. The contribution of IVIVC in drug development of orally administered drugs is very well known. For oral drugs, the *in vivo* process of drug absorption is often rate limited by the rate at which drug dissolves in the gastrointestinal tract. This can be simulated by measuring the rate of dissolution in an *in vitro* apparatus, which can be correlated with the *in vivo* absorption rate to produce an IVIVC. This research program involved efforts to develop biorelevant IVIVC methods and model for subcutaneously administered peptide based drugs. The *in vivo* component of this Program involves the use of clinical data from a bioequivalence (BE) study of Iplex™ [(IGF-I (Insulin like growth factor-I)/IGFBP-3 (Insulin like growth factor binding protein-3)], administered subcutaneously, that was conducted at the Center for Drug Studies (CDS), VCU School of Pharmacy in the year 2005 (Barr et. al., 2005). The PK parameters for Increlex™ (IGF-I) are calculated from the clinical data obtained from another study (Rabkin et. al., 1996). Literature research and molecular modeling research formed the basis of our hypotheses that
unbound and bound IGF-I are absorbed from the blood capillaries and lymphatic capillaries respectively and that simulation of these physiologic variables is possible with the use of the modified Hanson Microette® device.

The Hanson Microette® device is a vertical diffusion cell system that has been modified to simulate the pores in the capillaries with the use of a synthetic membrane. The flow and composition of circulatory fluid was simulated by the use of modified Hanks balanced salts solution (HBSS). A validated RP-HPLC (reversed-phase high performance liquid chromatography) method has been used for the analysis of IGF-1/IGFBP-3 in the in vitro samples. The in vitro permeation/release results gave the in vitro component to conduct IVIVC analysis. The General Electric (GE) healthcare sourced polycarbonate nucleopore track etched membranes were the only set of membranes that resulted in significant permeation in the in vitro experiments.

IVIVC results demonstrated high inter and intra-membrane variability for the membranes (available from today’s technology) that were used to simulate the in vivo membrane characteristics.

Currently, there are no validated biorelevant IVIVC methods for SC formulations. The methods described here are the basis for future in vitro method development that will be of significant value for (a) predicting the in vivo performance of SC formulations based on the in vitro data, and (b) provide a reproducible in vitro method as the basis of developing an IVIVC
for other subcutaneously administered drugs. This will provide an important tool for both development and regulation of this growing class of drugs.
1. Background and rationale

The SC route is the route of administration for the delivery of many drugs, particularly drugs with low oral BA. These include drugs that have a high molecular weight, are very hydrophilic, or are degraded when administered orally. In the next 5 years, injectables will account for 40% of the drugs on the market (Data monitor Ltd., 2009). More than 95% of the new biotechnology derived drugs are injectable drugs and the majority of them are administered via SC route (Data monitor Ltd., 2009). The protein therapeutics market was estimated to be about one hundred billion dollars in 2010 (Data monitor Ltd., 2009).

However, even though SC delivery has been utilized for a number of years, very little is known about the processes that governs the absorption of macromolecules from the interstitial space; and the resulting impact of these processes on the BA and the PK profiles. Also, there is no established IVIVC for subcutaneously administered immediate release peptide based drugs in a biorelevant manner. The contribution of IVIVC to the drug development of orally administered drugs is very well known. For oral drugs, the in vivo process of drug absorption is often rate
limited by the rate at which drug dissolves in the gastrointestinal (GI) tract. This can be simulated by measuring the rate of dissolution in an in vitro apparatus, which can be correlated with the in vivo absorption rate to produce an IVIVC. These IVIVCs have been widely used for orally administered drug formulations at various stages of drug development, including proof-of-concept, formulation screening, dissolution specifications, and food and drug administration (FDA) biowaivers, which allows avoidance of additional expensive clinical trials (Emami, 2006). The regulatory acceptance of in vitro testing as a reliable surrogate for an in vivo BE study is commonly referred to as “biowaiver” (USFDA Guidance, 2000). The applications of biowaivers are discussed in chapter 2 of this dissertation. It will be of great value to develop similar in vitro methodologies and standards for subcutaneously injected drugs. This research program involves both an in vivo and an in vitro component.

1.1 In vivo component

The in vivo component of this Program involves the use of data from a BE study of Iplex™ administered subcutaneously, which was conducted at the Center for Drug Studies (CDS), VCU School of Pharmacy in 2005 (Barr et. al., 2005). The primary objective of this study was to determine if Iplex™, which was manufactured by Insmed, Inc. at two different facilities was bioequivalent to each other. The secondary objective was to describe the PK profile of subcutaneously administered 0.5 mg/kg Iplex™ in healthy, adult, male and female volunteers. The PK parameters for Increlex™ (IGF-I) are calculated from the clinical data obtained from another study (Rabkin et. al., 1996).
The PK analysis of the data from the BE study done at the CDS demonstrated that a single dose of 0.5 mg/kg of the larger complex, Iplex™ (IGF-1/IGFBP-3), when administered subcutaneously, was continuously absorbed for over 72 hours. The smaller compound,Increlex™ (IGF-1), was rapidly absorbed over only 1-2 hours. The mechanism for this remarkable 50-fold difference in absorption rates was not obvious. These plasma concentration data have been further analyzed by Wagner Nelson (model-dependent) method to obtain the amount of drug absorbed per unit time (input function).

1.1.1 Hypotheses

Based upon a thorough review of the relevant literature on drug absorption from SC tissue and estimates of relative molecular size from molecular modeling, we hypothesized that: (1) The smaller IGF-I (7.5 - 8 kDa) is absorbed mainly via the pores (50 – 100 nm) of venous blood capillaries (Flow Rate - 20 mL/min approximately) (2) The IGF-I/IGFBP-3 complex (36 – 50 kDa) is not absorbed through venous blood capillaries, but may be absorbed mainly via lymphatic pores (several microns to 15 – 20 nm) of lymphatic capillaries (Flow Rate - 2 mL/min approximately).

The Hanson Microette® device is a vertical diffusion cell system that was developed for quality control of topical preparations (FDA guideline SUPAC semi solid (SS), 1997). The device is currently used for testing the in vitro release rate of topical drug products such as creams, gels and ointments (Flynn et. al., 1999). We have modified the device to simulate the pores in the capillaries with the use of a synthetic membrane. The flow and composition of circulatory fluid is simulated by the use of biorelevant modified HBSS, which has been
established as a biorelevant medium for the study of implants (Iyer et. al., 2007). This medium has been used in the *in vitro* experiments as a biorelevant medium after testing the stability of Iplex™ drug in modified HBSS. The medium has been established a biorelevant medium for study of subcutaneously administered implants by Iyer and others. Since lymph is formed from blood, modified HBSS can be used to simulate both blood and lymph. Also, lymph contains most of the components of the plasma and the levels of electrolytes, nonelectrolytes such as creatinine and urea, and transferrin and iron are not significantly different in lymph than in plasma (Porter, 1997, Yoffey et. al., 1970, Werner, 1966, Friedman et. al., 1965).

This leads to our overall hypotheses that the absorption of Iplex™ and Increlex™ can be simulated in a biorelevant manner *in vitro* using the modified Hanson Microette®, device.

### 1.1.2 Objectives

The objectives of the research were:

1) To carry out the PK analysis of the data from the clinical study to compare the input functions of the two products.

2) To describe the explanation for the unusual findings from the clinical study results and to characterize the possible absorption pathways for the small and large molecular weight drugs using the examples of Increlex™ and Iplex™.

3) To describe and simulate the important variables affecting the absorption of the two drug products from SC tissue.

4) To develop a biorelevant *in vitro* method for a peptide based drug that incorporates pore size and flow rate as variables.
5) To perform *in vitro* permeability experiments with Increlex™ and Iplex™ and conduct IVIVC by using the *in vitro* and *in vivo* data.

1.1.3 Proposed Research Design and Methods

To evaluate this hypothesis, an *in vitro* method has been developed that will allow us to control both; (1) flow rate and (2) pore size, within the physiologic constraints described above, to determine if we can simulate the differences observed *in vivo*.

1.2 *In vitro* component

Based on a review of all commercially available *in vitro* devices, we selected and tested the applicability of the modified Hanson Microette® device to simulate absorption of IGF-I after subcutaneous administration in (a) an unbound form, Increlex™ or (b) a bound form with IGFBP-3, Iplex™, which will give us the *in vitro* component. Our hypothesis is that the modified Hanson Microette® device can be used to simulate drug absorption from subcutaneous tissue through the blood capillaries and lymphatic capillaries. The membranes of biorelevant pore sizes were used to study the permeability of IGF-I and IGF-I/IGFBP-3 through blood capillary pores or lymphatic capillary pores. Also, blood flow rates and lymphatic flow rates were simulated by modifying the Hanson Microette® device. A ‘biorelevant’ modified HBSS was used as the fluid for simulation of both blood and lymph after carrying out the stability studies of IGF-1/IGFBP-3 in the modified HBSS. The *in vitro* samples collected at various time points were analyzed using validated RP-HPLC method.
To our knowledge, there is no device that simulates drug absorption of a subcutaneously administered injection, which is the preferred route for most injectable drugs, in a biorelevant manner. Currently, there are no validated IVIVC methods for subcutaneous formulations. The methods described here are the basis for future \textit{in vitro} method development that will be of significant value for (a) predicting the \textit{in vivo} performance of SC formulations based on \textit{in vitro} data, and (b) provide a reproducible \textit{in vitro} method as the basis of developing an IVIVC for other subcutaneously administered drugs. This will provide an important tool for both development and regulation of this growing class of drugs.
2.1 The origin and current state for IVIVC methods and models

An IVIVC has been defined by the FDA as “a predictive mathematical model describing the relationship between an \textit{in vitro} property of a dosage form and an \textit{in vivo} response” (FDA guidance, 1995). An IVIVC describes a quantitative correlation between the \textit{in vitro} dissolution/release rate and the \textit{in vivo} absorption rate. The United States Pharmacopoeia (USP) defines IVIVC as “the establishment of a relationship between a biologic property, or a parameter derived from a biologic property produced in a dosage form, and a physicochemical property of the same dosage form” (Sirisuth et. al., 2004). The IVIVC methods are often used during pharmaceutical development in order to reduce drug development time and optimize the formulation (Emami, 2006, Cardot et. al., 2007). A successful IVIVC model can usually be
developed if *in vitro* dissolution/release is the rate-limiting step in the sequence of events leading to the appearance of the drug in the systemic circulation following oral or other routes of drug administration (Souliman et. al., 2006, Amidon et. al., 1995, Cardot et. al., 1993, Skelley et. al., 1990). IVIVC allows optimization of a dosage form with the fewest possible trials in man, establishes dissolution acceptance criteria, and can be used as a surrogate for some bioequivalence studies and assists in supporting biowaivers, if the developed IVIVC is predictive of *in vivo* performance of the product (Cardot et. al., 1993). IVIVC’s have been successfully used as a quality control tool during the manufacturing process (batch to batch variability and manufacturing site changes) and selecting appropriate formulations.

The first and most important role of establishing an IVIVC is to use the dissolution test as a surrogate for human studies, when possible. This helps minimize the expensive and time-consuming bioequivalence studies performed during the initial approval process and during the scaling-up and post approval changes (USFDA guidance, 1997).

In the case of orally administered drugs, the Biopharmaceutical Classification System (BCS) is a fundamental guideline for determining the conditions under which IVIVC’s are expected. BCS is also used as a tool for developing the *in vitro* dissolution specifications (Sirisuth et. al., 2004).

The classification is associated with dissolution of drug and an absorption model, which identifies the key parameters controlling drug absorption as a set of dimensionless numbers, which are defined below:
1. The Absorption number is the ratio of the mean residence time (MRT) to the absorption time.
2. The Dissolution number is a ratio of mean residence time to mean dissolution time.
3. The Dose number is the mass divided by an uptake volume of 250 mL and the drugs solubility.
4. The MRT in this context is the average of the residence time in the stomach, small intestine and the colon.

The fraction of dose absorbed then can be predicted based on these three parameters. For example, an Absorption number of 10 means that the permeation across the intestinal membrane is 10 times faster than the transit through the small intestine, indicating that 100% of the drug is absorbed (Sirisuth et. al., 2004).

In BCS, a drug is classified into one of four classes based solely on its solubility and intestinal permeability. In general, a drug is considered highly soluble if the largest dosage strength is soluble in 250 mL or less of water over a pH range of 1-8. A drug is considered highly permeable if the extent of drug absorption is greater than 90% given that the drug is stable in the gastrointestinal environment.

**Table 1** BCS classification of Drugs (Amidon et. al., 1995)

<table>
<thead>
<tr>
<th>Class I</th>
<th>Class II</th>
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<tr>
<td>HS/HP</td>
<td>LS/HP</td>
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<th>Class III</th>
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<tr>
<td>HS/LP</td>
<td>LS/LP</td>
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HS – High solubility, LS – Low solubility, HP – High permeability, LP – Low permeability
In the case of drugs that fall under BCS Class I (highly soluble/highly permeable), the rate-limiting step is drug dissolution or the rate of gastric emptying if dissolution is very rapid. The Class I drugs such as metoprolol exhibit a high absorption number and a high dissolution number. For BCS class II drugs, *in vivo* drug dissolution is a rate-limiting step for absorption (except at very high Dose number). BCS Class II drugs such as phenytoin have a very high Absorption number but a low Dissolution number. The rate of drug absorption is slower in case of Class II than Class I and occurs over a longer period of time. There is a high likelihood for Class I and Class II drugs to exhibit IVIVC. In the case of BCS Class III drugs, permeability is the rate-limiting step for drug absorption to occur. This class of drugs exhibits a high variability of rate and extent of drug absorbed. With dissolution being rapid for this class of drugs, the variation is due to alteration of GI physiological properties and membrane permeation rather than dosage form factors. Cimetidine is an example of a Class III drug. Lastly, Class IV drugs possess low solubility and low permeability characteristics. This class of drugs presents a significant challenge for effective oral administration. Chlorothiazide is an example of this class of drugs (Sirisuth et. al., 2004).

Similar classification for peptide based injectable drugs on the basis of size, which is based on the molecular weight characteristics of the drugs that affects the permeability through blood and lymphatic pathways into the blood circulation could prove to be extremely useful.
2.2 Conducting an IVIVC

2.2.1 In vitro data

The dissolution techniques from the FDA guidance are used to estimate the percent drug dissolved versus time. The selection of a suitable dissolution technique is done following the FDA guidelines. Selection of the dissolution apparatus selection depends on various parameters like drug characteristics and dosage form selection. The release/dissolution curve will be expressed as the percent of drug released/dissolved versus time and should reach 100 percent at infinity (Emami, 2006, Sirisuth et. al., 2004).

The selection of dissolution method should be the one that describes what happens in vivo. The main factors that govern dissolution selection are pH, surfactant, gastrointestinal movement, ionic strength, enzymes, and food, in vivo. All these factors are reproduced in vitro using suitable dissolution apparatus. Usually, USP Apparatus 1, 2 and 4 are preferred to correlate to the in vivo conditions (Sirisuth et. al., 2004).

2.2.2 In vivo data

In vivo data are obtained from the clinical trials on healthy human volunteers with collection of plasma samples at specified time intervals. These data provide an absorption profile or plasma concentration – time profile, which usually depends on the dosage form and properties of drug. As per the FDA guidance, the absorption input function can be calculated by estimating in vivo absorption using an appropriate deconvolution technique, such as the Wagner-Nelson method or numerical deconvolution (USFDA Guidance, 1997) After the calculations are performed, the absorption input curve can be presented as percentage of the fraction of dose
(%FD-0-100%) absorbed versus time. This can be calculated using the equation below (Wagner et. al., 1964):

\[
\% \text{ Absorbed} = \left\{ \frac{C_t}{K_e + \frac{\text{AUC}_{(0-t)}}{\text{AUC}_{(0-\infty)}}} \right\} \times 100
\]

Where, \( C_t \) is plasma concentration at time \( t \), \( K_e \) is the elimination rate constant for the drug administered; \( \text{AUC}_{(0-t)} \) and \( \text{AUC}_{(0-\infty)} \) represent the area under curve from zero to time \( t \) and infinity, respectively.

The trapezoidal rule is used for the calculation of the area under curve. The value of \( K_e \) can be estimated from the terminal slope of the semi-log plot of the plasma concentration–time profile. The information on percentage of fraction of dose absorbed versus time depicts not only percentage dose absorbed, but also gives us information on various phenomena such as dissolution and permeability throughout GI tract.

2.3 IVIVC Evaluation

The normalized in vivo absorption data is used for quantitative assessment of an in vitro – in vivo relationship. The percent absorbed in vivo versus time will be plotted versus the percent released in vitro versus time to yield the correlation. Linear regression analysis will be used to examine the relationship between the percent drug released in vitro and percent drug absorbed in vivo (USFDA Guidance, 1997).

Alternatively, non-linear functions (Weibull function) can be used to describe the relationship, if necessary; this can be done using Jump®, or WinNonLin® 5.1.
2.3.1 Three main levels of correlation

IVIVC is more of an *in vitro* – *in vivo* relationship than strict correlation. The three main levels of correlation, as defined by the FDA are described below (USFDA Guidance, 1997).

2.3.1.1 Level A

A level A is a correlation that is generally linear and represents a point-to-point relationship between the *in vitro* and the *in vivo* input rate. The percent drug absorbed (*in vivo* input rate) can be calculated by employing the use of model-dependent techniques (Wagner-Nelson or Loo-Riegelman approach) or by model-independent approach (numerical deconvolution). The *in vitro* dissolution and the *in vivo* input curves may be superimposable or may be made to superimpose by the use of a scaling factor. The purpose of level A correlation is to define a direct relationship between the *in vivo* data such that, *in vitro* dissolution rate alone is sufficient in determining the biopharmaceutical rate of the dosage form. The biggest advantage offered by a level A correlation is that an *in vitro* dissolution curve can serve as a surrogate for *in vivo* performance. This property enables a formulation to be considered for a biowaiver in case of a change in manufacturing site, manufacturing method, raw material supplies, minor modification in formulation and even product strength. Non-linear correlations, while uncommon, may also be acceptable. The model should be predictive of the entire *in vivo* time course from the *in vitro* dissolution data.
2.3.1.2 Level B

The statistical moment analysis principle forms the basis of level B IVIVC. The mean *in vitro* dissolution time (MDT *vitro*) is compared to either the mean *in vivo* dissolution time (MDT *vivo*) or the mean *in vivo* residence time (MRT).

The limitation of level B IVIVC is the fact that, a number of different *in vivo* plasma concentration time profiles will produce similar mean residence time (MRT) values, thus, resulting in not being considered to be a point-to-point correlation like the level A correlation. Another limitation is that, a level B correlation alone is not enough to justify formulation modification, manufacturing site change, excipient source change, etc.

2.3.1.3 Level C

A level C IVIVC establishes a single point relationship between a dissolution parameter (e.g. t₅₀, percent dissolved in 2 hours) and a PK parameter (e.g. AUC, C_max, T_max).

The level C correlation can be useful in the early stages of formulation development during the selection of pilot formulations. Biowaivers are unlikely with the presence of a level C correlation alone.

The limitation of a level C correlation is that it does not reflect the complete shape of the plasma concentration-time curve, which is a very critical and defining factor of the performance of extended release (ER) products and products requiring rapid or prolonged activity.
2.3.1.4 Multiple-level C correlation

A multiple level C correlation relates one or several PK parameters (AUC, $C_{\text{max}}$ or other suitable parameters) to the amount of drug dissolved at several time points of the dissolution profile curve.

A biowaiver is a possibility with this type of correlation, provided that the correlation has been established over the entire dissolution profile with one or more PK parameters of interest. A demonstration of the relationship should be made at each time point such that the effect of any change in dissolution profile can be assessed.

At least three dissolution time points covering the early, middle and late stages of dissolution profile form the basis of multiple level C correlation.

The level A correlation uses all the information from the dissolution and absorption profiles, as compared to level B and C correlation. The level B and C correlation are considered less powerful, due to their inability to utilize all the information from the in vitro dissolution data.

The FDA has ranked the levels as follows.

A level A IVIVC is considered to be the most informative and is recommended, if possible. Multiple level C correlations can be as informative and useful as level A correlations. However, when multiple level C correlations are possible, it suggests that a level A correlation is also likely and preferred. During the early stages of formulation development, when pilot
formulations are being selected, level C correlation can prove to be extremely useful. The level B correlations are least useful for regulatory purposes.

2.4 Demonstrated use and value of IVIVC for orally administered drugs.

Use of IVIVC’s has been recommended by several regulatory authorities (USFDA Guidance, 1997, ICH Q8, 2004). Investigations of IVIVC between in vitro dissolution and in vivo absorption are an integral part of most oral extended release (ER) drug development programs. Because of the scientific interest and associated regulatory utility of IVIVC as a valuable tool, the FDA published a guidance in September 1997, for “Extended Release Oral Dosage Forms: Development, Evaluation, and Application of IVIVC. Also, in the same year, another guidance, Non Sterile Semisolid Dosage Forms Scale-Up and Post Approval Changes: Chemistry, Manufacturing, and Controls; In Vitro Release Testing and In Vivo Bioequivalence documentation was published and IVIVC was discussed in a series of other guidances (FDA Guidance, 1995, FDA Guidance, 2000, FDA Guidance, 2003). IVIVC’s can be used in place of biostudies that may otherwise be required to demonstrate bioequivalence, when certain preapproval and post-approval changes are made in the formulation, equipment, manufacturing process, or in the manufacturing site. IVIVC development also helps improve product quality and decrease regulatory burden (Emami, 2006).

Over the years, several experimental methods have been developed to investigate drug release from vehicles and the percutaneous absorption of topically applied chemicals where the objective is often to find correlation between laboratory results (in vitro) and the transdermal
absorption experienced in living subjects (*in vivo*), so that *in vivo* experimentation may be curtailed.

An ideal *in vitro* method is one, where the *in vivo* variables affecting the absorption of drug can be simulated in a simple manner and the method is as biorelevant as possible. The term biorelevance has been used for dissolution media that simulate Gastrointestinal (GI) conditions. Prof. Jennifer Dressman used the term “biorelevant” for the first time to describe an artificial (*in vitro*) fluid that simulates and is biologically relevant to an *in vivo* fluid physically and chemically (Biorelevant.com, 01/10/2012).

The biorelevant media are designed specifically to represent conditions in the GI tract, unlike plain water or simple buffer solutions. Parameters such as the surface tension, the pH, the ionic strengths are tailored to match the average values in humans. Also, the bile salts, the natural surfactants in the GI tract and phospholipids, are used in physiologically relevant concentrations. All these factors are critical to the wetting and the solubilization of the drug and hence their solubility and dissolution in the GI tract. Time and time again it has been shown that solubilities and release (dissolution) rates measured in the biorelevant media are much more predictive of *in vivo* performance than those measured in plain water or simple buffer solutions (Biorelevant.com, 01/10/2012).
In the case of SC absorption of drugs, it is difficult to completely simulate the events that occur \textit{in vivo}. The absorption of drug via the blood and lymphatic capillary pores and the enzymatic degradation due to proteases are difficult to simulate \textit{in vitro}, as are the pharmacodynamic (PD) events such as corticosteroid-induced vasoconstriction, which in turn may affect the absorption rate of the drug.

So far, to our knowledge, no efforts have been made towards \textit{in vitro} simulation of absorption of a peptide-based drug from subcutaneous tissue in a biorelevant manner.

The \textit{in vivo} BE assessment of solid oral dosage forms is a commonly accepted surrogate for judging therapeutic equivalence of pharmaceutically equivalent drug products, thus offering the advantage of obviating the need for additional clinical evaluation. But, over the last few years, research on drug absorption in relation to the biopharmaceutical properties of the active pharmaceutical ingredient (API) has led to the acceptance of \textit{in vitro} testing as a sufficiently reliable surrogate for an \textit{in vivo} BE study for certain drug products.

The following categories of drug products can get a biowaiver:

A) Formulation development for a new drug product.

B) Line extensions, e.g. new strengths or new formulations for a new patient population, for instance, a pediatric formulation.

C) Formulation development of a generic drug product.

D) Post-approval changes both for innovator and generic drug products.
The present USA guidance that consider biowaivers are:

1. Immediate release solid oral dosage forms: Scale-up and post-approval changes (USFDA Guidance, 1995).

2. Extended release oral dosage forms: development, evaluation and application of IVIVC (USFDA Guidance, 1997).

3. Waiver of *in vivo* BA and BE studies for immediate release solid oral dosage forms based on a BCS System (USFDA Guidance, 2000).

4. BA and BE studies for orally administered drug products-general considerations (USFDA Guidance, 2003).

2.5 Advantages and limitations of drug administration by the oral route

2.5.1 Advantages of oral route

a. Patient convenience.

b. Safety.

c. Cost effectiveness.

2.5.2 Limitations of oral route

a. There may be limited absorption of some drugs because of their physical characteristics (hydrophilic or lipophilic characteristics), irritation to the gastrointestinal mucosa leading to nausea and vomiting, degradation of protein-derived drugs by digestive enzymes or acidic conditions (low pH) in the stomach.

b. The inter and intra-patient variability due to the inherent differences in Absorption, Distribution, Metabolism and Excretion (ADME) characteristics.
c. The effect of concomitant administration of the drug with another drug.

d. The effects of fasted or fed state on bioavailability, and compliance is necessary on part of the patient throughout the drug therapy.

e. The drugs in the GI tract may be metabolized by the enzymes of the intestinal flora, mucosa and the liver before they gain access to the systemic circulation, which will affect the bioavailability of the drug.

f. Also, the presence of transporters in the GI tract may influence the Absorption, Distribution, Metabolism and Elimination (ADME) characteristics of the drug in patients.

### 2.6 Rationale and need for additional research on injectable drugs

Injectables will account for 40% of the drugs on the market in the coming years (Data monitor Ltd. 2005, 2009). In the recent years, the market size of injectable products has grown exponentially and the proportion of subcutaneous injections have accounted for the largest route of protein delivery, followed by IV infusion with a distant margin (Data monitor Ltd., 2005). The share of the SC route as the preferred route for protein delivery is only expected to increase in the future (Data monitor Ltd., 2005).

Interestingly, more than 95% of biotechnologically derived drugs are injectable drugs (Biotechnology industry survey, 2005). The majority of peptide based injectable drugs (over 50%) are administered by the SC route and the choice of this route for delivery of protein based therapeutic drugs will only increase in the future. The protein therapeutics market was estimated to be over 100 billion dollars in 2010.
The protein therapeutic market is largely immediate release, but there is a trend towards an increase in sustained release formulations (Data monitor Ltd., 2005). A majority of the sustained release formulations are administered subcutaneously (Data monitor Ltd., 2005). Sustained release formulations offer the possibility of reducing the dosing frequency, which can further lead to improved patient compliance (Data monitor Ltd., 2005). Most of the information above points to the importance of the subcutaneous route as the preferred route for the protein based therapeutic class of drugs.

IVIVC’s have been widely used for orally administered drug formulations at various stages of drug development including proof-of-concept, formulation screening, dissolution specifications, and biowaivers. The avoidance of expensive clinical trials with the use of IVIVC is perhaps the most compelling feature of IVIVC. Principles of IVIVC have been mostly applied to oral products. There exists a need to develop methodologies and standards for subcutaneously injected drugs.

Currently, there is no FDA approved in vitro method available for subcutaneously injected drugs. In vitro permeation methods that simulate the drug absorption from tissue would serve as an important quality control tool during the development of subcutaneously administered drugs. IVIVC methods for injectable drugs could minimize the number of biostudies required and decrease the cost of drug product development.
2.7 Advantages and limitations of drug administration by parenteral route

2.7.1 Advantage of the parenteral route

a. Drug absorption is usually much more rapid, extensive, and predictable when a drug is injected in tissue compared to oral administration of the same drug.

b. Peptide-based drugs have to be administered as an injection to render their biologic activity, which may degrade at gastric pH, if administered orally.

c. When the patient is unconscious, uncooperative, or unable to retain anything given by mouth, the parenteral route is the only option to administer drug.

2.7.2 Limitations of the parenteral route

a. Asepsis must be maintained at all times.

b. Pain associated with injection.

c. When self-medication is desired, it is difficult for patients to self-inject the medication.

d. Inadvertent administration of drug, when it is not called for.

e. Injectable drugs are more expensive compared to orally administered drugs.

2.8 Major routes of parenteral administration

The major routes of parenteral administration are intravenous, subcutaneous and intramuscular, with the subcutaneous route being the most popular route. The reason of subcutaneous route being the preferred route over other routes of drug administration could be due to ability of SC tissue to act as a reservoir for the drug, due to slower absorption and longer duration of action of drug, when desired. The various widely used routes of drug administration into skin can be seen in Figure 1 below.
Absorption is slower when drug is administered via transdermal, implantation and SC route as compare to the IM and IV routes. This could be due to the difference in the presence of blood and lymphatic capillaries in the vicinity.

2.8.1 Intravenous route

An IV medication administration refers to the process of administering medication directly into a patient's vein. The IV medications are mainly administered when a rapid systemic response to medication is necessary. The IV route is one of the fastest ways to deliver medication. The drug is instantaneously and highly bioavailable to the body, when administered by this route. It is easier to control the actual amount of drug delivered to the body by using the
IV infusion and it is also easier to maintain drug levels in the blood for desired therapeutic response. The IV route for medication administration may be used to prevent degradation by digestive enzymes, when the drug is poorly absorbed by the tissue, or is painful or irritating when given by IM or SC injection. The IV route is preferably used to treat conditions that require immediate therapeutic response (e.g., epileptic seizures, acute asthma, or cardiac arrhythmias) (Elkin et. al., 1996, Kozier et. al., 1993).

The drugs administered by IV route are usually expensive compared to other dosage forms. Sterility, pyrogen testing and larger volumes of solvent add up leading to a greater cost for preparation, transport and storage.

2.8.2 Intramuscular route

An IM injection is an injection given directly into the central area of a specific muscle. In this way, the blood vessels supplying that muscle distribute the injected medication via the cardiovascular system. IM injection is used for the delivery of certain drugs not recommended for other routes of administration, for instance IV, oral, or SC (Elkin et. al., 1996, Kozier et. al., 1993).

Drugs that lead to irritation, when administered subcutaneously are good candidates for IM administration. The drug administration by the IM route compare to the SC route offers a faster rate of absorption, and muscle tissue can often hold a larger volume of fluid without discomfort. In contrast, medication injected IM is absorbed less rapidly and exhibits effect more
slowly than medication that is injected IV, which is favorable for some medications (Elkin et. al., 1996, Kozier et. al., 1993).

Careful consideration in deciding which injectable route is to be used for the prescribed medication is essential. In cases where muscle size and condition is not adequate to support sufficient uptake of the drug, the IM route should not be used. IM injection should be avoided if other routes of administration, especially oral, can be used to provide a comparable level of absorption and effect in any given individual's situation and condition. IM injections should not be given at a site where there is any indication of pain (Elkin et. al., 1996, Kozier et. al., 1993).

IM injections are given directly into the central area of selected muscles. There are a number of sites on the human body that are suitable for IM injections; however, the three sites that are most commonly used in this procedure are deltoid muscle, vastus lateralis and gluteus medius muscle.

Several variables affect absorption of drugs when administered intramuscularly: Blood flow to the nearby vicinity, where the drug is injected. Local heating, massage or exercise can increase the rate and extent of absorption. A slow and constant absorption rate from the IM site results if drug is injected in oil or various other repository vehicles.
2.8.3 Subcutaneous route of drug administration

Although usually liquid medications are injected, occasionally solid materials such as steroid hormones may be injected in small, slowly absorbed pellets to prolong their effect. SC injections may be given wherever there is SC tissue, usually in the upper outer arm, abdomen or thigh.

The SC method of injection is commonly ordered for medication that requires a slower absorption rate than IM injections provide. The needle must pass through the epidermis and dermis to reach the SC fatty (adipose) tissue. Small volumes of medication that are voluble and non-irritating to body tissues are administered by the subcutaneous route. A variety of medications, such as insulin and some immunizations, are given subcutaneously.

The SC route is the route of administration for the delivery of many drugs, particularly drugs with low oral bioavailability. These include drugs that have a high molecular weight, are very hydrophilic or are degraded when administered orally. SC administration represents the primary route of delivery for peptide-based drugs despite significant efforts to develop non-parenteral delivery systems for these drugs. The majority of the injectables are subcutaneously administered (Data monitor Ltd., 2005). In general, the injection of drugs into the SC tissue allows a deposit or ‘depot’ of drug to become established that will be released gradually into the systemic circulation over a period of time. By altering the formulation of the drug, the period over which it is released can be influenced.
Even though SC delivery has been utilized for a number of years, very little is known about the processes that govern the absorption of macromolecules from the interstitial space and the resulting impact of these processes on BA and PK profiles (Porter et. al., 2001, Porter et. al., 2000, Porter et. al., 1997). Large molecules in the form of injections e.g. Human growth hormone, IGF-I (Increlex™), IGF-1/IGFBP-3 (Iplex™) that act for a relatively short duration of time are administered subcutaneously.

There is limited literature regarding the mechanisms involved in drug absorption from SC tissue. In order to understand the mechanism involved in absorption of drug from SC tissue, understanding skin, its components and function become very important. The components (as seen in Figure 2) of skin are discussed below. On average, an adult has from 18-20 square feet (about 2 square meters) of skin, which weighs about 6 pounds (2.7 kg). Skin is usually divided into three layers: 1) Epidermis, 2) Dermis and 3) Subcutaneous tissue (www.cancer.gov, accessed on 01/20/2012).

2.8.3.1 Epidermis

The uppermost layer of skin consists of epidermis. Located at the base of epidermis are melanocytes that produce a dark pigment called melatonin that contributes to skin color and protects from ultraviolet (UV) light. The other types of cells in epidermis are dendritic cells, which functions to engulf the foreign material invading the epidermis and thereby stimulating an immune response. The very bottom of the epidermis consists of basal cells whose function is to continuously divide and send the newly formed cells towards upper surface of the skin pushing the older cell out. They are also known as the stratum germinativum.
2.8.3.2 Dermis

The dermis layer is much thicker than the epidermis and is made up of connective tissue. It makes up 90% of the thickness of the skin. It contributes to pliability of the skin, mechanical resistance and maintenance of the body temperature. Epidermis doesn’t have its own vascular supply and hence this layer supplies the nutrients. The sense organs for pressure, pain and temperature, touch, nerve fibres, blood vessels, sebaceous and sweat glands and hair follicles are located in this layer. Meissner’s corpuscles are touch receptors, which detects light touch and soft, fleeting movements. The function of pacinian corpuscles is to act as receptors for vibration and deep pressure. The free nerve endings are sensitive to temperature changes, itchiness and pain. The function of nerve fibres is to transfer information. Sebaceous glands are located all over the body. They are sacculated organs responsible for secreting sebum, which functions to moisturize hair and skin. Sweat glands function as thermoregulators for the body. Hair follicles are the downward growth into dermis and produce hair that helps maintain homeostasis. At the base of the follicle is the Arrector pilli muscle that stands upright when stimulated by cold or fright. Blood vessels help in supplying nutrients and oxygen to the skin and carry with them cell waste and cell products. Lymph vessels function to keep the skin supplied with lymph, which contains the cells responsible for destroying any infection or invading organisms as it circulates to lymph nodes.
2.8.3.3 Subcutaneous tissue

The subcutaneous tissue situated below the dermis consists of loose connective tissue and adipose tissue. It acts as a protective cushion and helps to insulate the body by monitoring heat gain and heat loss. Blood vessels and lymphatic vessels poorly supply this tissue.

The role of lymphatics is extremely important in the absorption of large molecular weight peptides, which is discussed in the later part of this chapter. From the extensive research findings in the recent years, it is evident that lymphatic transport plays a very important role in absorption of peptide-based drugs (Porter et. al., 2001, Porter et. al., 2000, Ballard et. al., 1968, Cuong et. al., 2011, Kota et. al., 2007, Oussoren et. al., 2001, Pessina et. al., 1993, Supersaxo et. al., 1990, Trevaskis et. al., 2008, Wang et. al., 2012, Wasan et. al., 2002, Wilting et. al., 2004, Yanez et. al., 2011). Currently, there are no biorelevant in vitro permeability methods for proteins and other high molecular weight injectables.

Figure 2 Cross section of skin (with permission from Terese Winslow, NCI at NIH)
2.9 Major variables affecting absorption of drugs from subcutaneous tissue

The SC administration of a drug results in delivery to the interstitial area underlying the dermis of the skin. The interstitium consists of a fibrous collagen network supporting a gel-phase comprised of negatively charged glycosaminoglycan (largely hyaluronan), salts and plasma-derived proteins (Porter et. al., 2001, Porter et. al., 2000). The proteins present within the interstitial space are essentially the same as those in plasma although they are thought to be present at approximately 50% lower concentration (Porter et. al., 2001).

A barrier to drug absorption after SC administration may also be presented in the passage through the interstitium to the blood or lymphatic capillaries. Interstitial diffusion of macromolecular drugs can be greatly influenced by their physico-chemical characteristics including size, charge, hydrophobicity, hydrophilicity, and their interactions with the endogenous components present within the interstitium. The interactions with interstitial proteins, the degree of interstitial hydration and the interactions with negatively charged glycosaminoglycans can all significantly affect the absorption and diffusion of the macromolecules into the systemic circulation (McLennan et. al., 2005). Various formulation characteristics such as pH, ionic strength, drug concentration, viscosity, injection volume along with the presence of formulation excipients can also influence the diffusion from SC site (Zuidema, 1994). Several other factors that may affect the drug absorption from the interstitial space are the presence of proteases in the near by vicinity, susceptibility to enzymatic degradation at the injection site, uptake by the phagocytic and endocytic mechanisms and aggregation or poor solubilization and precipitation may affect the drug absorption of drugs from the interstitium (Hawley, 1995).
Many variables may influence the drug absorption from SC site, but none are as critical as the molecular weight of the drug administered, which in turn may influence the rate and pathway of drug absorption by blood capillary or lymphatic capillary, where the pore size and flow rate in the two pathways could play a crucial role in determining the rate and extent of drug absorption into the systemic circulation following SC administration. The role of lymphatic capillary pathway in absorption of drug from SC tissue has been found to be much more significant than other variables that could cause loss of drug at injection site after administration of human growth hormone in sheep (Charman et. al., 1999).

After a drug is subcutaneously administered, it can be transported to the general cardiovascular pool either by blood capillaries or by the lymphatics. For small molecules, up to 1 kDa, the blood capillary wall diffusivity is very high and represents a small barrier to drug transport (Weinstein et. al., 1984). However, permeability of macromolecules through the blood capillary is low, and therefore, movement directly into the blood circulation is restricted. Hence, soluble macromolecules generally enter the bloodstream indirectly by way of lymphatic vessels. Supersaxo and coworkers found that absorption mainly occurs by the lymphatics following Intradermal (ID) or SC administration of human recombinant interferon (rIFN) alpha-2a (MW 19 kDa) (Supersaxo et. al., 1990).

The lymphatic absorption studies by Supersaxo and coworkers were performed with the use of lymph cannulated sheep model (Supersaxo et. al., 1988). This system allowed the collection of peripheral lymph draining directly from the injection site. The experiments were carried out 2-3 days post surgery. After collection of the blank lymph samples, SC administration
of test solutions rIFN alpha-2a, cytochrome c, inulin and 5-flouro-2’-deoxyuridine (FUDR) along with labeled substances were carried out into the lower part of lymph cannulated leg using a special injection device. Continuous collection of lymph was carried out in heparinized tubes (5 U heparin/mL), which were then centrifuged (200 g, 10 min, 4 °C) and stored at 4 °C until assay was performed. The effect of molecular weight on the cumulative recovery of test solutions in lymph was determined. The recovery in lymph expressed as the percentage of administered dose was calculated as the product of concentration in lymph and the volume of lymph collected for each time interval (Supersaxo et. al., 1990).

Supersaxo and coworkers demonstrated a linear relationship between the molecular weight of a drug and the proportion of the dose absorbed by the lymphatics, which drain the site of SC application in the investigated MW range (Figure 3). Molecules with MW > 16 kDa are absorbed mainly by the lymphatics, since more than 50% of the administered dose was recovered in lymph. Lymphatic vessels were not the primary mode of drug absorption for compounds with MW < 1 kDa (Supersaxo et. al., 1990). These results concur with the results from a study by Muranishi and co-workers, where they evaluated the absorption route of FITC-labeled dextrans (FD) of various molecular weights following rectal administration in rats (Muranishi et. al., 1997). Small amounts of the administered FD dose passed the epithelium of the rectum with help of an absorption enhancer. After this passage FDs with MW < 10,000 transferred into both blood and lymph, whereas FDs with MW > 20,000 preferentially entered the lymphatic capillaries (Supersaxo et. al., 1990).
Figure 3 Correlation between the Molecular Weight (kDa) and the cumulative recovery of IGFBP3, IFN-alpha-2a, Cytochrome C, IGF-1 & FUDR in the efferent lymph following SC administration into the lower part of the right hind leg. Data are represented as mean. (for n=3). The line was determined by linear regression and $r = 0.9960$ (Adapted with permission from Springer Link, from Supersaxo et. al., 1990). Prediction of cumulative lymph recovery of IGF-1 and IGFBP-3 were made using this graph.

Access of macromolecules from the SC space to blood and lymphatic capillaries may be related to the structural differences between blood capillary and lymphatic endothelial cell lining. The endothelial lining of the blood capillaries consists of continuous and uninterrupted sub endothelial basement membrane, whereas the lymphatic capillaries do not possess this membrane (Supersaxo et. al., 1990). The lymphatic capillaries may play an important role in facilitating access of interstitial macromolecules to the lymphatic system (Supersaxo et. al., 1990). Two
circulatory systems of the human body, blood circulation and lymphatic circulation are depicted in Figure 4.

Figure 4 Interlinking of the lymphatic, blood and pulmonary circulation (with permission from Razvanski B. from http://encyclopedia.lubopitko-bg.com/The_Lymphatic_System.html)
2.9.1 Physiological aspects of blood capillaries and lymphatic capillaries

Exchange of many small lipophilic molecules across blood capillary walls occurs by transendothelial diffusion. Also, there is a transfer of small and large hydrophilic molecules like water and some proteins. There are two types of blood capillary pores. Small pores (approx. 10 nm) are responsible for the transport of small hydrophilic molecules across the capillary endothelium and large pores (approximately 50 nm – 70 nm) that allow passage of relatively large molecules. These pores are a combination of plasmalemmal vesicles and transendothelial channels (large pores) and endothelial junctions (small pores). There are three types of blood capillaries within the vasculature depending on the basis of endothelia (continuous, fenestrated, and discontinuous). Capillaries with continuous endothelia have an uninterrupted basement membrane and are poorly permeable to large molecules and small particulates. They are widely distributed in mammalian tissues and are present in capillary beds supplying both subcutaneous tissue and skeletal muscle. They are of the greatest importance when evaluating lymphatic drainage after IM or SC administration. Capillaries with fenestrated endothelia (60 nm - 80 nm) are usually found in organs with high rates of fluid exchange (small intestine, kidney and salivary glands). Capillaries with discontinuous endothelia have large intercellular gaps (100 nm to 1 µm) and no basement membrane. They allow for unrestricted access between the blood capillaries and interstitium. However, they are present only in specialized organs such as liver, spleen and bone marrow (Porter et. al., 1997). The rate of filtration and reabsorption of fluid across the blood capillaries is high (approximately 20 mL/min).
Lymphatic capillaries are closed at their peripheral ends and consist of a single layer of non-fenestrated cells with an incomplete basal lamina. They have more ‘open’ intercellular junctions than those in blood capillaries. Intercellular junctions range from several microns to 15 nm – 20 nm, which are very high compared to the available pore sizes in blood capillaries and thus facilitate the clearance of macromolecules (proteins) from interstitial spaces into the lymphatics (Porter et. al., 1997). The structure of lymphatic capillaries is maintained by anchoring filaments that attach to the wall of the lymphatic capillary and to the collagen fibres of the interstitium. These filaments help prevent collapse of the lymphatic capillaries, particularly in the event of raised interstitial pressure (Casley-Smith et. al., 1980). A small quantity of interstitial fluid is cleared by lymphatic capillaries (approximately 2 mL/min) compare to blood capillaries. This results in the absorption of small molecules that may have similar affinity to blood and lymph predominantly via the blood capillaries with a much higher flow rate. Molecules with increasing size will more likely drain through the relatively more open capillaries in the lymphatic circulation.

2.10 Clinical Study Review

2.10.1 Study description of drug Increlex™ (IGF-1) and Iplex™ (IGF-1/IGFBP-3)

Increlex™ (mecasermin [rDNA origin] injection) is produced by recombinant DNA technology and is an aqueous solution for injection containing human insulin-like growth factor-1 (IGF-1). IGF-1, which has a molecular weight of 7649 daltons, consisting of 70 amino acids in a single chain with three intramolecular disulfide bridges. The amino acid sequence of the product is identical to that of endogenous human IGF-1. The IGF-1 protein is synthesized in bacteria (E. coli) that have been modified by the addition of the gene for human IGF-1.
Increlex™ is an aqueous, sterile, clear and colorless solution intended for SC injection. Each multi-dose vial of Increlex™ contains 10 mg/mL mecasermin, 9 mg/mL benzyl alcohol, 2 mg/mL polysorbate 20, 5.84 mg/mL sodium chloride, and 0.05 M acetate at a pH of approximately 5.4 (Tercica I., 2005). The structure of Increlex™ is depicted in Figure 5.

Figure 5 Primary amino acid sequence for IGF-1 (From Package insert of Increlex™, with permission from Tercica Inc, 2005)
Iplex™ (mecasermin rinfabate [rDNA origin] injection), produced by recombinant DNA technology, is an aqueous solution for injection containing a binary protein complex of IGF-1 and IGFBP-3.

IGF-1 and IGFBP-3 are produced by two separate *E. coli* strains: one containing the human gene for insulin-like growth factor-1 (IGF-1), the other containing the human gene for insulin-like growth factor-binding protein-3 (IGFBP-3). IGFBP-3 has a molecular weight of 28,732 daltons and consists of 264 amino acid residues. The amino acid sequence of the IGFBP-3 protein is identical to that of endogenous human IGFBP-3. All the 18-cysteine residues of endogenous IGFBP-3 are all paired in disulfide bonds to form the biologically active molecule, but the pairings have not been fully elucidated. The IGF-1 and IGFBP-3 proteins are complexed in a 1:1 molar ratio for formation of mecasermin rinfabate with a molecular weight of 36,381 daltons. IGFBP-3 obtained from human plasma is glycosylated, whereas IGFBP-3 produced in *E. coli* is non-glycosylated. Glycosylated and non-glycosylated IGFBP-3 bind IGF-1 with similar affinity. This is important as similar affinity suggests that the binary complex produced from *E. coli* may exhibit a pharmacological activity parallel to that of the endogenously present binary complex. Iplex™ consists of 105 mM sodium chloride and 36 mg/0.6 mL in 50 mM sodium acetate with a final pH of 5.5. Iplex™ is injected only subcutaneously, and is a sterile, clear, preservative-free, colorless-to-slightly-yellow liquid (Iplex™ package insert, 2010). The structure of Iplex™ is shown in the Figure 6.
Figure 6 Primary amino acid sequence for IGFBP-3 (From Package insert for Iplex™, with permission from Insmed™)
2.10.1.1 Chemistry and Pharmacology of IGF-I, IGFBP-3 and ALS

2.10.1.1.1 IGF-I

IGF-I, originally known as the sulfation factor and/or somatomedin-C, is the actual mediator of somatic growth. IGF-1 is a 70 amino acid peptide with a molecular weight of 7.5 kDa, a structure 70% homologous to IGF-2 structure and is 50 % homologous to proinsulin (Daughaday et. al., 1989).

IGF-1 is a mediator of statural growth (somatomedin) and it is formed after GH stimulates the GH receptors in liver, which results in stimulating bone and body growth. The type 1 IGF-1 receptor in the target tissues, which is homologous to the insulin receptor, is activated by the binding of IGF-1 leading to intracellular signalling which then stimulates multiple processes leading to statural growth, as well as mitogenic and “insulin-like” metabolic activities (Tercica Inc, 2005).

The administration of IGF-1 in animals and humans yields several effects. Some of the effects include promotion of linear growth (Laron et. al., 1992), anabolic effects (Froesch et. al., 1985), enhanced wound healing (Sommer et. al., 1991), promotion of bone formation (Bagi et. al., 1995), stimulation of lymphopoiesis (Clark et. al., 1993), motor neuron survival (Ishii et al., 1993, Lewis et. al., 1993), modified glucose metabolism (Guler et. al., 1987, Thraikill et. al., 1999), increased creatinine clearance (Guler et. al., 1987), and recovery of renal function following ischemic injury (Ding et. al., 1993).
IGF-1 has been manufactured on a large scale using both yeast & *E.coli* Several companies have evaluated IGF-1 in clinical trials for a variety of indications, including growth failure, type 1 diabetes, type 2 diabetes, Amyotrophic lateral sclerosis, burn injuries and myotonic muscular injuries.

### 2.10.1.1.2 IGFBP

Insulin like growth factor binding proteins (IGFBPs) are multifunctional proteins that modulate the biological effects of IGFs. Modulation is performed directly by sequestering IGFs, and by several mechanisms, including specific binding to a number of plasma proteins, extracellular matrices and cell surface molecules by conditional proteolysis, rapid internalization into target cells, and translocation into the nucleus (Firth et. al., 2002, Clemmons, 2001, Jones et. al., 1995, Bach et. al., 1995). In addition to their IGF-modulating functions, IGF and IGF receptor-independent actions of IGFBPs are characterized and well explained (Firth et. al., 2002, Baxter et. al., 2000, Butt et. al., 2000, Schendlich et. al., 2000).

In circulation and interstitial fluids, IGFBPs are the major carrier proteins for IGFs. IGFs are largely unavailable in these environments, as they are tightly associated with IGFBPs and are believed to be released – only after IGFBP proteolysis - for binding to the cell surface IGF receptors (Bunn et. al., 2003). Thus by sequestering IGFs away from IGF receptors, IGFBPs may inhibit mitogenesis, differentiation, survival, and other IGF-stimulated events. Alternatively, the interaction of IGFBPs with cell or matrix components may concentrate IGFs near their receptor, enhancing IGF activity (Clemmons et. al., 2001).
2.10.1.3 IGFBP-3 & ALS

IGFBP-3 is a 53 kDa binding protein that is 291 amino acids long with three potential N-linked glycosylation sites (Wood et. al., 1998). The majority of serum Insulin-like growth factors (IGFs) circulate within 130-150 kDa ternary complexes containing, IGF-1 or IGF-2, IGFBP-3, and the acid-labile subunit (ALS), an 85 kDa glycoprotein. The ternary complex does not cross the capillary barrier, and ALS is restricted to the intravascular space (Figure 7).

![Figure 7: Genetics, chemistry, and function of the IGF/IGFBP system (Adapted with permission from Springer Link, from Collett-Solberg et. al., 1996)](image-url)
The formation of ternary complex protects and prolongs the half-life of both IGFBP-3 and IGFs (Collett – Solberg et al., 1996). The half-life of unbound IGFBP-3 is between 20 and 90 min and the half-life of unbound IGF-1 is less than 10 min, while that of the 150 kDa ternary complex is approx. 12 h. The ternary complex maintains IGFs in the intravascular space for steady delivery of IGF-1 in contrast to the pulsatile levels of growth hormone (GH). ALS due to its size prevents IGF access to target cells, while free IGFs can easily cross the capillary endothelial barrier. The majority of IGFs are carried as a binary complex, and less than 1% circulates in unbound form. The ALS containing complex significantly increases the serum half-lives of both the IGFs and IGFBP-3 and in this way maintains the circulating store of these molecules (Rabkin et. al., 1996, Lewitt et. al., 1993).

Figure 8 Structural and size comparison of IGF-1, IGF-1/IGFBP-3, ALS, IGF-1/IGFBP-3/ALS (Zeslawski et. al., 2001, Sato et. al., 1993, Kobe et. al., 1996, Janosi et. al., 1999, Siwanowicz et. al., 2005. The crystal structures were obtained from http://www.rcsb.org/pdb/)
2.11 Study introduction, objective and design

IGF-1/IGFBP-3 (Iplex™, mecasermin rinfabate, Insmed Inc.) is a 1:1 recombinant complex consisting of IGF-1 and its most abundant binding protein, IGFBP-3. IGF-1/IGFBP-3 displays metabolic activities similar to those found with unbound IGF-1 alone in animal studies. An important advantage of administering binary complex, IGF-1/IGFBP-3 over unbound IGF-1 administration is a longer plasma half-life after administration of IGF-1 in bound form (IGF-1/IGFBP-3) and IGF-1/IGFBP-3 complex administration may have an improved safety profile compared to IGF-1.

IGF-1 is the most important hormonal growth regulator in humans (Backeljauw et. al., 2001). IGF-1 possesses structural and functional homology to proinsulin. IGF-1 also induces insulin-like effects, however its main effect is to promote cell division, growth, differentiation and proliferation in most tissues. According to the somatomedin hypothesis (somatomedin hypothesis originated in early efforts to understand how somatic growth was regulated by factors secreted by the pituitary), IGF-1 mediates many of the growth-promoting and metabolic effects of growth hormone (Clark et. al., 2004, Mauras et. al., 1995, 2000), although recently it has been suggested that the relationship between IGF-1 and GH is more complex, with IGF-1 augmenting the anabolic actions of GH to its receptor and counteracting some of its potentially deleterious effects (Kaplan et. al., 2007). The GH binds to IGF-1 receptors in the liver and other tissues and stimulates the production of IGF-1.
The objectives of the study were as follows:

1. To determine if two lots of Iplex™, which were manufactured by Insmed, Inc at two different facilities, were bioequivalent to each other.

2. To describe the pharmacokinetic profile of subcutaneously administered 0.5 mg/kg Iplex™ in healthy, adult, male and female volunteers.

### 2.12 CDS Clinical study results

Before the clinical study was initiated, IRB (Institutional Review Board) approval and informed consent from study volunteers was obtained. The clinical study done at CDS was a single-dose, randomized, open-label, two-period crossover study of the test drug product (IGF-1/IGFBP-3 (Iplex™, mecasermin rinfabate, Insmed Inc.)) and reference drug product (IGF-I Increlex™, Mecasermin, Terecica Inc.) in healthy adult volunteers. 28 healthy volunteers were enrolled in the study. Subjects who had qualified for the study at the screening visit checked into the clinic the evening prior to the first dose, which was given the following morning. Subjects were confined to the inpatient clinic through the 72-hour post dose procedures. There was a 7-day washout period between the doses. Blood samples were collected pre-dose (0 hour) and at the following time points post-dose: 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 48, 60 and 72 hours for determination of IGF-1, IGF-2, IGFBP-3, ALS and glucose concentrations. At the subsequent inpatient-dosing period, volunteers returned to the clinic the evening prior to administration of the second dose. The following morning, subjects received the alternate drug product at approximately the same time as the first dose such that, by the end of the study, each volunteers would have received both the drug products as treatments.
The evaluation of blood chemistry, adverse events, hematology, urinalysis, physical examination, vital signs and ECG assessed the safety of drug administered.

Pharmacokinetic parameters included:

- **AUC\(_{0-\text{Last}}\)**: Area under the concentration-time curve (AUC) from time 0 to last measurement using the linear trapezoidal rule.
- **AUC\(_{0-\infty}\)**: AUC from time 0 to infinity
- **C\(_{\text{max}}\)**: Maximum serum concentration
- **T\(_{\text{max}}\)**: Time to C\(_{\text{max}}\)
- **K\(_{\text{el}}\)**: Apparent elimination rate constant
- **T\(_{1/2}\)**: Apparent elimination half-life

Baseline-corrected values were calculated by subtracting the time 0 concentration from each post-injection concentration.

Thirty subjects were enrolled in the study and had at least one post-baseline safety assessment. The pharmacokinetic profile of total IGF-1, IGFBP-3, and free IGF-1 following administration of IGF-1/IGFBP-3 (Iplex™) was studied in 28 healthy adult volunteers. The demographics and baseline characteristics for evaluable subjects that were included in the PK analysis for the study done at CDS are listed in Table 2. The calculated PK parameters are listed in Table 3. Extensive PK analysis results will be discussed in detail in results section of this dissertation.
Table 2 Demographics and baseline characteristics for evaluable subject who were included in the PK analysis for the study done at CDS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Evaluation population (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (n,%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24 (86%)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (14%)</td>
</tr>
<tr>
<td>Race (n,%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>7 (25%)</td>
</tr>
<tr>
<td>Black</td>
<td>17 (61%)</td>
</tr>
<tr>
<td>Asian</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>36 ± 8.1</td>
</tr>
<tr>
<td>Range</td>
<td>21.0 – 48.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>76.4 ± 12.6</td>
</tr>
<tr>
<td>Range</td>
<td>55.0 – 98.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>173.5 ± 8.1</td>
</tr>
<tr>
<td>Range</td>
<td>160.0 – 185.0</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>368 ± 102</td>
</tr>
<tr>
<td>Range</td>
<td>151 – 700</td>
</tr>
<tr>
<td>IGF-2 (ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>448 ± 119</td>
</tr>
<tr>
<td>Range</td>
<td>238 – 775</td>
</tr>
<tr>
<td>IGFBP-3 (ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2925 ± 854</td>
</tr>
<tr>
<td>Range</td>
<td>1520 – 6267</td>
</tr>
<tr>
<td>ALS (µg/mL)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>14.3 ± 3.6</td>
</tr>
<tr>
<td>Range</td>
<td>4.8 – 20.9</td>
</tr>
</tbody>
</table>
Table 3 IGF-1 pharmacokinetic parameters after administration of a single dose of Iplex™ (Baseline-corrected, for n = 28)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$AUC_{0-T_{last}}$</td>
<td>ng/mL*hr</td>
<td>29,684</td>
<td>6850</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$</td>
<td>ng/mL*hr</td>
<td>38049</td>
<td>8951</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>ng/mL</td>
<td>721</td>
<td>209</td>
</tr>
<tr>
<td>$T_{max}$</td>
<td>hr</td>
<td>21.6</td>
<td>9.4</td>
</tr>
<tr>
<td>$K_e$</td>
<td>1/hr</td>
<td>0.027</td>
<td>0.0068</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>hr</td>
<td>27.6</td>
<td>6.95</td>
</tr>
</tbody>
</table>

The mean serum concentrations of IGF-1 and IGFBP-3, expressed as molar quantities, increased in parallel from baseline values, with similar profiles (Figures 9 and 10), suggesting contemporaneous absorption and circulation of the proteins, most likely as stable complexes (Barr et. al., 2005).

The administration of 0.5 mg/kg IGF-1/IGFBP-3 (Iplex™) resulted in a small increase in mean free IGF-1 to approximately the upper limit of the published normal range. The mean percent free IGF-1 remained below 1% throughout the 72-hour sampling period, consistent with normal physiological states. Only one subject had maximal percent free IGF-1 greater than 2% (Barr et. al., 2005).
2.13 Conclusions

IGF-1 therapy administered as IGF-1/IGFBP-3 (Iplex™), a stable protein complex, resulted in a small increase in free IGF-1 levels, mostly within the normal physiological range. The percent free IGF-1 remained < 1% in the vast majority of subjects.

It appears that supraphysiologic bursts of free IGF-1, as reported with isolated IGF-1 therapy (Bach et al., 1995, Baxter, 2000), can be avoided by administration of the IGF-1/IGFBP-3 complex. When IGF-1 is administered as an injection, it is absorbed for 1-2 hours, whereas absorption continues to occur for more than 72 hours from IGF-1/IGFBP-3 (Figure 9). Therefore, further development could possibly result in an once/week or twice/week administered product compared to the once/day or twice/day products that currently exist, thus greatly improving patient convenience and adherence.

![Mean free IGF-1(ng/mL) concentration with 0.1mg/0.4mg/kg dose of IGF-1/IGFBP-3 (Iplex™ ) in healthy volunteers](image)

**Figure 9** Mean free concentration of IGF-1 (ng/mL) over time after administration of 0.1 mg IGF-1/0.4 mg IGFBP-3/kg dose of Iplex™ in healthy volunteers
Figure 10 Mean free concentration of IGF-1 (ng/mL) over time after administration of 0.1 mg IGF-1/kg dose of Increlex™ in healthy volunteers

The two drugs, both containing IGF-1 have very different absorption profile. This is possibly due to the different absorption pathways that IGF-1 and IGF-1 in bound form with IGFBP-3 take to reach systemic circulation. It is proposed that, the unbound form will mostly be absorbed via blood circulation, whereas, the binary complex of IGF-1 and IGFBP-3 be absorbed via the lymphatic circulation.

The clinical study, which was carried out at the CDS, presented us with an opportunity to develop a biorelevant in vitro method for subcutaneously administered peptide based therapeutic agent. The two most important variables that would be necessary for simulating SC environment in a biorelevant manner are pores size in the blood capillaries and lymphatic capillaries and flow rate in the blood capillaries and lymphatic capillaries.
CHAPTER 3

METHOD DEVELOPMENT AND PARTIAL VALIDATION OF A REVERSE PHASE-HPLC METHOD FOR ANALYSIS OF IGF-1/IGFBP-3

In order to analyse the *in vitro* samples containing IGF-1/IGFBP-3 in our permeation/release experiments, a selective and sensitive method was needed. A reverse phase HPLC method for the analysis of IGF-1/IGFBP-3 was previously developed and validated by Insmed Inc. The method was found to be specific, linear, precise and accurate as per the ICH Q2B guidelines on validation of analytical procedures. Partial validation of this method was carried out in order to analyse the *in vitro* samples. It was performed, as there was a change in site and processing condition of the *in vitro* samples to be analysed. The only change in the prior validated method by Insmed was the introduction of a buffer - modified HBSS for *in vitro* permeation/release studies. According to the guidelines on bioanalytical method validation (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996), partial validation can range anywhere from within run precision and accuracy to almost full validation. For this research purpose
linearity, accuracy, precision and stability studies were carried out. The method employed UV detection and involved minimal sample preparation time. Although the limitation associated with the use of this method was high run time (65 minutes), there was no need to develop an alternative method such as ELISA or Mass Spectrometry, as the analytes to be measured in our in vitro studies were the same as the analytes for which, the method was previously developed by Insmed. So alternative methods were not explored which saved the time and resources required for this.

3.1 Preparation of modified Hanks Balanced Salt Solution (HBSS)

The modified HBSS was used as a biorelevant permeation medium for the present study, and it is widely used in cell culture studies for maintaining viability of the cells. The commercially available modified HBSS mixture consisted of anhydrous calcium chloride (0.1396 g/L), anhydrous magnesium sulfate (0.09767 g/L), potassium chloride (0.4 g/L), anhydrous potassium phosphate monobasic (0.06 g/L), sodium chloride (8 g/L), anhydrous sodium phosphate dibasic (0.04788 g/L), and D-glucose (1.0 g/L). The values in parenthesis represent the quantity of the respective salt required for preparing 1 L of the Hank’s Balanced Salt Solution. The Hanks’ medium was prepared by dissolving 9.8 g of the above salt mixture in 975 mL of deionized water followed by adjusting pH to 7.4 ± 0.1 with a 1 M of sodium hydroxide or a 1 M of hydrochloric acid solution in water. The HEPES solution (25 mL of 1 M) was added into the above medium, mixed well, and vacuum filtered through a 0.45 µm nylon filter.
3.2 Preparation of Stock solution

2 mL (1.5mg/mL of IGF-1) of Iplex™ was pipetted using a calibrated micropipette of 1 mL twice and transferred into 20 mL of volumetric flask. It was dissolved in modified HBSS to yield a stock solution of 150 µg/mL.

3.2.1 Preparation of Standards and Quality Control samples

From the stock solution, serial dilutions were made with modified HBSS to yield a calibration curve ranging from 7.5 to 150 µg/mL (7.5, 15, 30, 60, 120 µg/mL). Similarly, from other freshly prepared stock solution, quality control samples were prepared at three concentrations, 15 µg/mL, 60 µg/mL and 150 µg/mL, representing Low (LQC), Middle (MQC), and High (HQC), respectively. Aliquots were stored in polypropylene tubes, below -20 °C, until analysis.

3.3 Sample preparation

No sample filtration or extraction was required. The aliquots were thawed at room temperature and transferred into 2 mL HPLC vials for analysis.

3.4 Chromatography

A modification to the method, developed by Insmed, Inc was carried out. The Waters LC system consisted of a system controller, Alliance 2695, separation module, a high-pressure pump, an auto-sampler (part of Alliance 2695), and a Waters 2487 detector. The chromatographic separation was achieved using a Vydac 218TP54 protein and peptide C18 column (dimensions: 250 * 4.6 mm, 5 µm) maintained at 4 °C during the run time. The mobile
phase components consist of acetonitrile, water, and TFA (Trifluoro Acetic Acid). Acetonitrile was purchased from Sigma-Aldrich with purity greater than 99.93%. Water was procured from the lab using a Nanopure Diamond Barnstead D3750 irradiated hollow fiber filter system (Nanopure system), pore size of 0.2 µm, maximum operating pressure of 50 psi. TFA was purchased from Sigma-Aldrich. The preparation of the mobile phase was as below.

The mobile phase was used within a week of its preparation. The organic content in the mobile phase may be subjected to loss upon storage, affecting the relative composition of the mobile phase and performance of the method, so mobile phase were not used post one week of preparation.

3.4.1 Mobile phase A (0.1% TFA in water)

900 mL of water from the Nanopure system was added into a 1000 mL volumetric flask. 1 mL of TFA was added into the volumetric flask containing 900 mL water. This mixture was further diluted up to 1000 mL using water from Nanopure system.

3.4.2 Mobile phase B (0.1% TFA in 90% acetonitrile)

100 mL of water from the Nanopure system was added into a 1000 mL volumetric flask. 1 mL of TFA was added into the volumetric flask containing 100 mL water. This mixture was further diluted up to 1000 mL using water from Nanopure system.
3.4.3 Mobile phase C (0.1% TFA in 50% acetonitrile)

500 mL of water from the Nanopure system was added into a 1000 mL volumetric flask. 1 mL of TFA was added into the volumetric flask containing 500 mL water. This mixture was further diluted up to 1000 mL using water from Nanopure system. The instrument conditions and the gradient flow for mobile phase composition are listed in the table 4 and table 5 as follows.

**Table 4 Instrument conditions**

<table>
<thead>
<tr>
<th>Column</th>
<th>VYDAC 218 TP54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent A</td>
<td>Reverse phase C$_{18}$ 4.6 * 250 mm id</td>
</tr>
<tr>
<td>Eluent B</td>
<td>0.1% TFA in water</td>
</tr>
<tr>
<td>Eluent C</td>
<td>0.1% TFA in 50% acetonitrile</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1 mL/min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>100 µL</td>
</tr>
<tr>
<td>Column temperature</td>
<td>Ambient room temperature</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>210 nm</td>
</tr>
<tr>
<td>Run time</td>
<td>65 minutes</td>
</tr>
</tbody>
</table>

**Table 5 Gradient table for mobile phase**

<table>
<thead>
<tr>
<th>Time min</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>5.0</td>
<td>1.0</td>
<td>48</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>45.0</td>
<td>1.0</td>
<td>32</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>50.0</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>55.0</td>
<td>1.0</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>65.0</td>
<td>1.0</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>
At the end of the sequence of analysis, the column was flushed with eluent A for 10 minutes, then eluent B for 60 minutes. Following 60 minutes of eluent B, the flow rate was reduced to 0.0 mL/min and the lamp turned off. The column was stored in eluent B.

3.5 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. From the stock solution different standard solutions ranging from 7.5 to 150 µg/mL were prepared and were run with above chromatographic conditions. The table below derived from peak area vs. concentration plot represents the equation of the regression line, correlation coefficient, RSD values of slope and intercept. According to ICH Q2b guidelines (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996) a high correlation is often considered to be a criterion for linearity. The calibration curves were found to be linear in these ranges with $R^2 > 0.99$. The Figures 13, 14 and 15 shows representative chromatogram of blank medium, the lowest standard and a QC sample.

Table 6 Linearity along with regression line, correlation coefficient, slope and intercept.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength</th>
<th>Run</th>
<th>Equation</th>
<th>$R^2$</th>
<th>Slope</th>
<th>Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>210 nm</td>
<td>1</td>
<td>$y = 11859x - 78778$</td>
<td>0.9989</td>
<td>0.3710</td>
<td>2.8576</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>$y = 11870x - 77505$</td>
<td>0.9991</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>$y = 11789x - 74497$</td>
<td>0.9993</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.6 Accuracy and Precision

The accuracy of an analytical procedure describes the closeness of mean test results obtained by the method to the true value (concentration) of an analyte. Three working standard solutions with three determinations per concentration were prepared and analyzed to calculate the % deviation. From the respective peak area, concentration was back calculated using the linear calibration curve. For accuracy, % recovery was within 15% of the actual value for every concentration and hence was found to be acceptable (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996). Also the % deviation of mean from true serves as a measure of accuracy. % Deviation was calculated as below.

\[
\% \text{ Deviation} = \frac{\text{Spiked concentration} - \text{Mean measured concentration} \times 100}{\text{Spiked concentration}}
\]

Precision of an analytical method is the closeness of replicate measures under the same method and conditions. It was calculated by injecting the three working standards, with three determinations per concentration using the same operating conditions and the same column on a different day. For a method to be precise results were found to be not exceeding than 15% of CV (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996). Small differences in peak areas and good constancy in retention times were observed even after 3 days. RSD of less than 3.0 for peak areas were obtained. The comparable detector responses were obtained on different days, which indicated that the method is capable of producing results with high precision on different days.
Table 7 Intra-run & Inter-run accuracy and precision for three standard concentrations

| Intra-day and inter-day accuracy & precision for three standard concentrations for IGF1 (µg/mL) |
|---------------------------------|-----------------|---------------------|
| | 15 µg/mL | 30 µg/mL | 120 µg/mL |
| N | 6 | 6 | 6 |
| Mean | 16.06 | 28.04 | 120.02 |
| SD | 0.086 | 0.644 | 2.342 |
| %RSD | 0.536 | 2.297 | 1.952 |
| %DFN | 6.62 | -6.96 | 0.021 |
| Deviation range | 8.06 to 6.53 | -8.55 to 0.613 | -1.812 to 3.531 |

3.7 Limits of Detection (LOD) and Lower Limits of Quantification (LLOQ)

The LOD of method is defined as the smallest concentration that can be distinguished from the blank. It is also the concentration that has a signal of three times the noise in blank plasma samples across the run time. It was determined by calculating the Signal/Noise (S/N) ratio for a low concentration of 3.75 µg/mL. Signal was calculated by measuring the distance from the signal peak maxima to the peak minima while noise was calculated by measuring the
distance between the maximum and minimum baseline response, off peak (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996).

The LLOQ of method is the concentration for which the signal-to-noise ratio is at least 10:1. It was determined by spiking the samples in replicates at the standard concentration of 7.5 \( \mu \text{g/mL} \) and was found to be accurate with % RSD of 2.04 %. This confirmed that the analytical method was able to quantify the LLOQ in an accurate and precise manner (USFDA Guidelines, 2001, ICH Q2b Guidelines, 1996).

3.8 Stability studies

3.8.1 Freeze thaw stability study

The freeze thaw stability was evaluated because the analytical samples were stored in between chromatographic run during the experiments. It was done by comparing the mean peak areas of standard samples injected after 1, 2 and 3 freeze thaw cycles to that of the mean peak area of a freshly prepared sample. The storage for duration of 24 hours for cycle 1, 48 hours for cycle 2, and 7 days for cycle 3 at a temperature below -20 °C was done. As seen in Table 8, the mean value for these 2 cycles ranged from 98.0% to 100.0% of initial concentration at 0\(^{th}\) hour (freshly prepared). These results assured that repeated freeze and thaw of samples did not affect the integrity of an analyte for up to 48 hours. The mean observed concentrations after the 3\(^{rd}\) such cycle deviated by less than 96.0% of the each QC standard.
Table 8 Freeze thaw stability for IGF-1 at two standard concentrations

<table>
<thead>
<tr>
<th>IGF1 concentration (µg/mL)</th>
<th>Back predicted mean concentration (%RSD)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle 0 (Freshly prepared)</td>
<td>Cycle 1 (Post 24 hours)</td>
<td>Cycle 2 (Post 48 hours)</td>
<td>Cycle 3 (Post 1 week)</td>
</tr>
<tr>
<td>7.5 µg/mL</td>
<td>7.49</td>
<td>7.35 (1.35%)</td>
<td>7.20 (2.84%)</td>
<td>6.65 (8.34%)</td>
</tr>
<tr>
<td>30 µg/mL</td>
<td>30.12</td>
<td>29.94 (0.43%)</td>
<td>29.68 (3.03%)</td>
<td>28.00 (5.05%)</td>
</tr>
</tbody>
</table>

3.8.2 Auto sampler stability studies

These studies were done to check the stability of an analyte by keeping the standard solution in modified Hanks salt solution and in water at 2-8 °C for three different conditions as follows in Table 9. The graphs for stability studies (Figures 11 and 12) on the stability of analyte in the standard working solution showed that there was no decomposition products in the chromatogram as difference in areas was not observed during analytical procedures even after storage at 2-8 °C until 48 hours.

Table 9 Auto sampler stability studies for IGF-1

<table>
<thead>
<tr>
<th>Condition (2-8°C)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post 24 hours stability</td>
<td>Solution found to be stable</td>
</tr>
<tr>
<td>Post 48 hours stability</td>
<td>Solution found to be stable</td>
</tr>
<tr>
<td>Post 1 week stability</td>
<td>Slight decrease in concentration was found in both water and in Hanks media</td>
</tr>
</tbody>
</table>

3.9 Conclusion

Partial validation was carried out to ensure that the changes in the site and processing conditions did not affect the ability to detect the analytes of interest. The use of modified HBSS as a matrix did not interfere with the detection of IGF-1/IGFBP-3 and hence the method was
found to be suitable for identification and quantification of IGF-1/IGFBP-3. Also, the method requires only a small volume (100 µl) of injection with a run time of 65 minutes. The run time was considered to be an optimal one, as per the discussion with Insmed. This was to ensure that the column is completely flushed out of any analytes to prevent any interference with the analytes of subsequent samples. The calibration curve developed was found to be appropriate and the results for accuracy, precision and stability were within the acceptable limits. We concluded that the method was simple, sensitive and specific.
Figure 11 Auto sampler stability studies profile in Hanks medium over different time period

Figure 12 Auto sampler stability studies profile in water over different time period
Figure 13 Chromatogram with blank run

Figure 14 Chromatogram with QC standard
Figure 15 Chromatogram with lowest standard
CHAPTER 4

IN VITRO METHOD DEVELOPMENT FOR SUBCUTANEOUSLY INJECTED
PEPTIDE BASED DRUGS

Our research evolved from the PK results of a bioequivalence study done with Iplex™. The purpose of the study was to assess whether the drug Iplex™ manufactured at two different sites was bioequivalent or not. The mean PK plots reported by Insmed from the clinical study results were used to obtain concentration versus time values. For our analysis the concentrations were visually taken from the plots and the data were then used to calculate the PK parameters, using the Wagner Nelson method (Nelson, 1964). The clinical study was carried out as described in Chapter 2 of this dissertation (section 2.12).

This research work is an effort towards developing a biorelevant IVIVC method for peptide based injectable drugs. From the clinical study, it was found that the drug product Iplex™, manufactured at two different sites was bioequivalent relevant to the amount absorbed. However, the absorption rates from the PK analysis of Iplex™ were found to be remarkably different from that of the unbound IGF-1 (Increlex™) (Rabkin et. al., 1996). The concentrations were visually taken from the concentration vs. time plots for the clinical study of Increlex™.
carried out by Rabkin et. al and PK parameteres were then calculated using the Wagner Nelson method. The concentration versus time profile for both of the drug products are presented in Chapter 2 of this dissertation (section 2.13).

The graphs suggest that when IGF-1 (Increlex™) is administered as an injection, it is absorbed for 1-2 hours, whereas absorption continues to occur for more than 72 hours from IGF-1/IGFBP-3 (Iplex™). The two drugs, both containing IGF-1 have very different absorption durations. One hypothesis that may explain this discrepancy is that IGF-1 and IGF-1 in bound form with IGFBP-3 follow different pathways to reach the systemic circulation. The unbound form of IGF-1 may be mainly absorbed via blood circulation, whereas the binary complex of IGF-1 and IGFBP-3 absorption into the systemic circulation may occur via the lymphatic circulation. A review of the literature suggests that the absorption pathways may be different because of the significant differences in (1) flow rate and (2) porosity in blood capillaries and lymph capillaries, the two main pathways of drug absorption from the tissue (Porter et. al., 2001, Ballard, 1968, Cuong et. al., 2011, Oussoren et. al., 2001, Porter et. al., 2001, Supersaxo et. al., 1990, Wang et. al., 2012, Wasan, 2002, Wilting et. al., 2004, Yanej et. al., 2011, Kota et. al., 2007, Trevaskis et. al., 2008). The effect of pore size and flow rate in blood capillaries and lymphatic capillaries has been discussed in Chapter 2 of this dissertation (section 2.9).

The working hypothesis for our research project is as below:

(1) The smaller IGF-1 (7.5 - 8 kDa) is absorbed mainly via the pores (50 – 100 nm) of venous blood capillaries (Flow Rate - 20 mL/min approximately) because of the much higher flow rate of blood than lymph and their relatively smaller size as compared to the binary
complex of IGF-1/IGFBP-3. (2) The IGF-1/IGFBP-3 complex (36 – 50 kDa) is not absorbed through venous blood capillaries, but may be absorbed mainly via lymphatic pores (several microns to 15 – 20 nm) of lymphatic capillaries (Flow Rate - 2 mL/min approximately) due to large pore size. The objectives of this project were to:

1) To carry out the PK analysis from the clinical study plots of concentration vs. time and to compare the input functions of the two products.
2) Explain the unusual findings from the clinical bioequivalence study results and to characterize the possible absorption pathways for small and large molecular weight drugs using the examples of Increlex™ and Iplex™.
3) Describe and simulate the important variables affecting the absorption of the two drug products from SC tissue.
4) Develop a biorelevant in vitro IVIVC method for a peptide based drug that incorporates pore size and flow rate as variables.
5) Perform in vitro permeability experiments with Increlex™ and Iplex™ and conduct IVIVC.

The results related to Objective 1 and 5 will be discussed in Chapter 5 and our in vitro method development work to carry out Objective 2, 3 and 4 are discussed below.

4.1 Selection of an appropriate device

The selection of the device was made with the intent of simulating the in vivo conditions of pore size and flow rate. In order to simulate the in vivo conditions, the major variables need to be considered and a device needs to be chosen, so that the in vivo variables (flow rate and pore
size) can be simulated in a biorelevant way as possible. A biorelevant system has been previously developed and tested for implants (Iyer SS et al., 2007). Similar biorelevant *in vitro* methods for peptide-based, injectable drugs are needed. The limitation of the biorelevant system previously developed is that it tends to be a more expensive approach as reusing the device is not possible. Also, the pore size in the device was 80-100 times higher than the *in vivo* pore size of blood capillaries. The type of capillary device used in previous studies (Iyer SS et al., 2007) did not provide a wide range of pore size selection desired for this study. The use of a synthetic membrane in the Hanson Microette® device was evaluated as a potential method to overcome the limitations associated with the use of the earlier developed biorelevant system for implants.

The Hanson Microette® device, described below, was selected as the *in vitro* model to develop an IVIVC for injectable drugs because of the capability it provided to simulate both flow rates and pore sizes, which are the two major rate limiting factors affecting the amount of drug reaching systemic circulation following subcutaneous injection of peptide based drugs. Also, the device was donated to us by Hanson Research to test the applicability of the modified Hanson device to carry out IVIVC studies on peptide based drugs.

**4.1.1 Description of the Hanson Microette® Apparatus**

The Hanson Microette® Apparatus or the vertical diffusion cell system (Figure 16) was originally developed for quality control of topical preparations (Flynn GL et al., 1999). The device is widely used for testing the *in vitro* release rate of topical drug products such as creams, gels and ointments. The cell is made from clear glass and uses a clamp to secure the donor side of the cell to the receptor side. The clamp also ensures that all the components and the
membrane remain in place during the study. The cell is temperature controlled via the water jackets ports using a bath circulator. In this study, the temperature was controlled at $37.5 \pm 0.5 ^\circ C$ (Physiologic body temperature) to simulate the temperature which drug will be exposed to when administered subcutaneously. The temperature was monitored with the use of a thermometer that was placed in the water bath. The alignment ring ensures that the positions between the donor and receptor orifices are accurately aligned. The sampling and replacement ports have a Luer connection that facilitates the collection of sample and media replacement. In addition, a bubble trap is incorporated into the replacement port to seize any bubbles that may inadvertently be introduced during the sampling process. A magnetic stirrer is used to turn the helix and magnet, mixing the receptor medium to maintain a homogeneous mixture (Flynn GL et al., 1999).

A three Dimensional (D) representation of the SC site is possible using this device. This was possible by the extension of the donor chamber with the use of a tube to provide a continuous flow rate from the donor chamber and receptor chamber to the reservoir. During the start of the pump, the medium after reaching the donor chamber further continued to move up the tube length (approx. 5-6 cm). At this point equilibrium was established and a desired continuous flow rate was possible too. This provided a 3 D representation of the SC site, as the modified HBSS was present across both sides of the membrane.
4.1.2 Modifying the device for the *in vitro* release study

To our knowledge, this is the first attempt to develop a biorelevant *in vitro* IVIVC method for immediate release peptide-based drugs. From the literature, pore size and flow rate seem to be the most important factors governing the distribution of drug after an injection is administered (Porter et. al., 2001, Supersaxo et. al., 1990). Several modifications were made to the device, which included the use of a synthetic membrane, with pore sizes that would simulate the capillary pores and lymphatic pores as close as possible. Also, the sampling ports were modified to allow the continuous flow of Hanks solution, which would simulate lymph or blood flow rate in the SC environment. The sampling ports were connected to a media reservoir.
containing 200 mL Hanks solution and a pump was used to simulate blood or lymphatic flow rate. The temperature in the cell was maintained at 37.5 ± 0.5 °C. The drugs used in the *in vitro* studies, Iplex™ (IGF-1/IGFBP-3) and Increlex™ (IGF-1) were obtained from Insmed, Inc.

4.2 Membrane Specifications

The synthetic membrane was chosen to simulate the physiologic conditions (pore size) in the subcutaneous tissue. The approximate diameter estimates of the IGF-1, IGF-1/IGFBP-3 (binary complex of IGF-1 and IGFBP-3) and IGF-1/IGFBP-3/ALS (ternary complex of IGF-1, IGFBP-3 and ALS) were not known prior to this work. This was necessary information in order to choose a membrane with the pore size that would most closely simulate pore size of blood and lymphatic capillaries in the subcutaneous tissue. The lower molecular weight peptide based drugs may be absorbed via blood capillaries, pore size of which is a few nanometers, whereas large molecules may be absorbed via lymphatic capillaries, pore size of which ranges from a few nanometers to various microns. The diameter estimates using molecular modeling help predict the pathways, a drug molecule takes to reach systemic circulation (blood or lymphatic), after it is administered subcutaneously.

4.2.1 Use of Molecular modeling approach to investigate passive drug diffusion and binding characteristics of IGF-1, IGFBP’s and ALS

Molecular modeling is a technique used in computational chemistry, computational biology and material science to simulate the behavior of molecules to further understand its systems and how they bind to each other. Molecular modeling involves the description of the atomic and molecular interactions, which regulate macroscopic and microscopic behaviors of
physical system. The essence of molecular modeling resides in the connection between the microscopic world and the macroscopic world provided by the theory of statistical mechanics. Statistical mechanics is a branch of physics that applies statistical principles to the mechanical behavior of large numbers of small particles (such as molecules, atoms, or subatomic particles) in order to explain the overall properties of the matter composed of such particles.

Molecular mechanics enables the calculation of the total steric energy of a molecule in terms of deviation from reference unstrained bond lengths, angles and torsion plus non-bonded interactions. A collection of these unstrained values put together is known as force field (Holtje, 2008).

The goal of the force field is to provide high quality geometries and relative energies for a large variety of organic molecules by energy minimizations. In the molecular mechanics method, atoms in molecules are treated as rubber balls of different sizes (atom types) joined together by springs of varying lengths (bonds). The potential energy of the atomic ensemble can be calculated using Hook's law (Holtje, 2008).

The Hook's law states that, within the elastic limit of a solid material, the deformation produced by a force of any kind is proportional to the force. If the elastic limit is not exceeded, the material returns to its original shape and size after the force is removed, else it remains deformed or stretched. The force at which the material exceeds its elastic limit is called ‘limit of proportionality’.
During calculation, the total energy is minimized with respect to atomic coordinates, where

$$E_{\text{tot}} = E_{\text{str}} + E_{\text{bend}} + E_{\text{tors}} + E_{\text{vdw}} + E_{\text{elec}}$$

Where,

- $E_{\text{tot}}$ = total energy of the molecule
- $E_{\text{str}}$ = bond stretching energy term
- $E_{\text{bend}}$ = angle bending energy term
- $E_{\text{tors}}$ = torsional energy term
- $E_{\text{vdw}}$ = Van der Waals energy term
- $E_{\text{elec}}$ = electrostatic energy term

There are various software programs that are based on using the above basic principle, and with some modifications. The softwares used to perform the objectives for our research were SYBYL 7.3 and HEX. HEX is an interactive molecular graphics program that is used for calculating and displaying feasible docking modes of pairs of protein and DNA molecules (Ritchie, 2010). SYBYL 7.3 is a state-of-the-art program in molecular modeling that uses computer analysis to assist in the description and prediction of the behavior of molecules (SYBYL 7.3, Tripos International).

A molecular modeling approach was used to investigate passive drug diffusion and the binding characteristics of IGF-1, IGFBPs & ALS. The molecular modeling was performed to carry out the following objectives:
1. To investigate the permeability characteristics of IGF-1, IGFBP-3 and ALS through the capillary pores using SYBYL 7.3 (SYBYL 7.3, Tripos International).

2. To investigate the binding characteristics between the binary complex (IGF-1/IGFBP-3) and ternary complex (IGF-1/IGFBP-3/ALS) using HEX (Ritchie, 2010).

The IGFBP (Insulin like growth factor binding protein) family is comprised of six proteins (IGFBP-1, IGFBP-2, to IGFBP-6). The mature proteins have 216 to 289 amino acids. The family of IGFBPs shares a common domain organization & high degree of similarity (30-40%). The highest conservation is found in the N and C terminal cysteine-rich regions. Specifically, the highest conservation is found in the N- (residues 1 to ca. 100) and C- (from residue 170) terminal cysteine-rich regions (Zeslawski et. al., 2001). Due to the unavailability of the crystal structure of IGFBP-3, the residues of IGFBP-5 have been used for molecular modeling of IGFBP-3. Figure 17 below is the crystal structure of IGF-1/IGFBP-5 as it appears on the SYBYL 7.3 screen.
**Figure 17** Crystal structure of IGF-1/IGFBP (Zeslawski et. al., 2001)
Figure 18 Crystal structure of IGF-1 (1BQT) (Sato et. al., 1993)

The crystal structure above has been used for modeling of IGF-1 (Sato et. al., 1993).
According to the literature, Ribonuclease Inhibitor (RI) can be used as a template for homology modeling in the Leucine Rich Repeat (LRR) super family of proteins. The inner face of RI is lined with a substantial region of electronegative surface potential that could interact...
with the positively charged region of IGFBP-3 (Janosi et. al., 1999). This crystal structure has been used to carry out calculations for diameter estimates of IGF-1 in bound form as ternary complex (IGF-1/IGFBP-3/ALS).

**Figure 20** Ternary complex used to calculate diameter estimates of IGF-1/IGFBP-3/ALS
4.2.1.1 Results for diameter estimates post molecular modeling of IGF-1, IGFBP-3 and ALS using SYBYL 7.3

In order to predict the permeability characteristics (passive drug diffusion) of the bound and unbound form of IGF-1 through the lymphatic capillary pores and blood capillary pores, one needs to know the diameter estimates of the same. To carry out this objective, the diameter estimates for unbound IGF-1, IGF-1 bound to IGFBP-3, and the ternary complex of IGF-1, IGFBP-3 and ALS were calculated using SYBYL 7.3 and are stated below.

The crystal structure of IGFBP-3 was unavailable in the modeling library. According to the literature, IGFBP-5 can be used for size estimation due to structural and regulatory similarities between the two compounds. IGFBP-3 and IGFBP-5 have a common highly basic 18-amino acid sequence in their carboxyl terminal region that in the case of IGFBP-3 has been implicated in the interaction with ALS (Siwanowicz et al., 2005).

Also, the ribonuclease inhibitor (RI) can be used to get an estimate for ALS and a diameter estimate of the ternary complex. The diameter estimates are the mean values obtained by doing five estimates each, for IGF-1, IGFBP-5 and RI. The compute option in SYBYL was used to measure the diameter of the peptide. During each measurement, the two farthest atoms were chosen in the peptide. The diameter estimates for IGF-1, complex of IGF-1 and IGFBP-3 and ALS can be seen in the tables 10, 11 and 12 as following:
Table 10 Unbound IGF-1 size estimate using SYBYL 7.3

<table>
<thead>
<tr>
<th></th>
<th>Diameter (Å)</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O371-N151</td>
<td>39.00</td>
<td>19.50</td>
</tr>
<tr>
<td>N378-N151</td>
<td>32.36</td>
<td>16.18</td>
</tr>
<tr>
<td>N417-O252</td>
<td>37.98</td>
<td>18.99</td>
</tr>
<tr>
<td>N521-O15</td>
<td>21.15</td>
<td>10.57</td>
</tr>
<tr>
<td>N532-O15</td>
<td>38.44</td>
<td>19.22</td>
</tr>
<tr>
<td>Mean</td>
<td>33.8 ± 7.6</td>
<td>16.89</td>
</tr>
<tr>
<td>Capillary pore</td>
<td>48.00</td>
<td>24.00</td>
</tr>
</tbody>
</table>

Table 11 IGFBP-5 (structural homology to IGFBP-3) estimate using SYBYL 7.3

<table>
<thead>
<tr>
<th></th>
<th>Diameter (Å)</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O224-O469</td>
<td>39.69</td>
<td>19.85</td>
</tr>
<tr>
<td>O229-O461</td>
<td>38.62</td>
<td>19.31</td>
</tr>
<tr>
<td>N193-O454</td>
<td>38.43</td>
<td>19.22</td>
</tr>
<tr>
<td>O192-O456</td>
<td>38.29</td>
<td>19.15</td>
</tr>
<tr>
<td>O232-O469</td>
<td>41.66</td>
<td>20.83</td>
</tr>
<tr>
<td>Mean</td>
<td>39.3 ± 1.4</td>
<td>19.67</td>
</tr>
<tr>
<td>Capillary pore</td>
<td>48.00</td>
<td>24.00</td>
</tr>
</tbody>
</table>
Table 12 RI - Template for acid labile subunit (ALS) estimate using SYBYL 7.3

<table>
<thead>
<tr>
<th></th>
<th>Diameter (Å)</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N337-N3301</td>
<td>61.59</td>
<td>30.80</td>
</tr>
<tr>
<td>N337-O2494</td>
<td>59.26</td>
<td>29.63</td>
</tr>
<tr>
<td>O3478-O1371</td>
<td>68.79</td>
<td>34.39</td>
</tr>
<tr>
<td>O96-O2431</td>
<td>66.33</td>
<td>33.17</td>
</tr>
<tr>
<td>O3257-N1470</td>
<td>61.49</td>
<td>30.75</td>
</tr>
<tr>
<td>Mean</td>
<td>63.53 ± 3.92</td>
<td>31.77</td>
</tr>
<tr>
<td>Capillary pore</td>
<td>48.00</td>
<td>24.00</td>
</tr>
</tbody>
</table>

Results and conclusion are summarized as follows:

The results for the diameter estimates using SYBYL 7.3 indicate that IGF-1 and IGFBP-3 in unbound form may permeate through blood capillary pores, whereas IGF-1 bound to IGFBP-3, 73.12 ± 8.4Å° and IGF-1 bound to IGFBP-3 and ALS, should not permeate through the blood capillary pores. The unbound IGF-1 and IGFBP-3 diameter estimates were found to be 33.78Å and 39.34 Å, which are smaller than the average diameter estimates of the pores in blood capillaries, which is 48 Å, suggesting that the unbound form will permeate via blood capillaries. Also, from these results, it can be said that the bound form of IGF-1/IGFBP-3 may permeate mainly via lymphatic pores. The diameter estimates of RI suggest that ALS will be restricted to the vascular endothelium. Also, the ternary complex of IGF-1/IGFBP-3/ALS, which is restricted
to the vascular endothelium, is explained by the diameter estimate (136.65 Å > 48Å), from molecular modeling work.

IGF-1 in the bound form with IGFBP-3 may permeate mainly via lymphatic pores (size is from nm to microns), much larger in pore size and range than blood capillary pores. This information has proven useful to support our hypothesis that the differences in molecular weight of the subcutaneously administered drug could determine the pathways that drugs may take to reach the systemic circulation (Porter, 1997, Supersaxo, 1990). The effects of molecular weight on absorption pathways of subcutaneously administered drugs have been discussed earlier in Chapter 2 of this dissertation (section 2.9).

4.2.1.2 Results for binding characteristics of IGF-1, IGFBP-3 and ALS using HEX

Docking is a type of molecular modeling. Molecular docking is a method that predicts the preferred orientation of a molecule when it binds to another molecule to form a stable complex. It uses computers to create a 3-D image of two molecules and shows how they fit together. The knowledge of the preferred orientation that a molecule has for another can help predict the binding affinity strength that a substrate will have for an enzyme.

HEX has been used to get an idea of shape complementarities and possible binding modes of protein molecules. It is an interactive molecular graphics program and can be used for calculating and displaying feasible docking modes of pairs of protein and DNA molecules (Ritchie, 2010). It reads protein and DNA molecular structure from Protein Data Bank (PDB) format files. PDB files can be downloaded from the main PDB repository at Rutgers University
The structure of IGF-1, N and C – fragments of IGFBP-5 and RI have been obtained from the link above and after opening on the HEX panel, is further displayed as a solid model by using HEX. The solid model helps give a better understanding of the electrostatic potential across the molecule.

**Figure 21** Crystal structure of IGF-1 as seen on the docking panel of HEX, crystal structure of IGF-1 obtained with permission from Sato et. al., 1993
**Figure 22** Crystal structure of N and C fragments (essential for binding) of IGFBP-5 as seen on the docking panel of HEX, crystal structure of N and C fragments of IGFBP-5 obtained with permission from Zeslawski et. al., 2001

**Figure 23** Crystal structure of Ribonuclease Inhibitor - Template for Acid labile subunit (ALS) as seen on the docking panel of HEX, crystal structure of Ribonuclease Inhibitor obtained with permission from Kobe et. al., 1996
Figure 24 Ternary complex as appears on HEX panel before Docking
Figure 25 Figure as seen on HEX panel during docking

Figure 26 Solid model of the ternary complex as seen on HEX panel during docking
The docking was not completed, as it was not required to carry out our objectives as well as due to its undetermined nature and time constraints. However understanding the size complementarity characteristics and binding modes of the IGF-1/IGFBP-3/ALS has proven useful. The result demonstrates that there is a strong electronegative potential on the inner side of ALS, which is a prime target for IGFBP-3 binding. Also, the doughnut like hole in the ALS provides the binding cavity for IGF-1/IGFBP-3, thus leading to the formation of a ternary complex (IGF-1/IGFBP-3/ALS), which is a stable complex and raises the half-life of IGF-1 from 5 minutes to 12-14 hours.

4.3 Selection of synthetic membranes for in vitro studies

There were several challenges in the development of a biorelevant in vitro method for subcutaneously injected drugs, including simulation of pore size in the blood capillaries and lymphatic capillaries. The biggest challenge was to find a synthetic membrane that would simulate the pore size characteristics of the blood and lymphatic capillaries.

4.3.1 Supor® PES membrane

The first set of membranes that were tested in the in vitro method for biorelevance were the hydrophilic modified polyethersulfone membranes (Supor® PES membrane) donated by Pall Corporation. The specifications of the membrane that were used are given in table 13 and were taken from their website (www.pall.com) and characterization of the membranes itself in the table were not carried out as part of our research program, as we were primarily interested in screening for the membranes that would yield reproducible permeation in our in vitro method.
Table 13 Specifications of the Supor® PES membrane disc filters
(Reproduced with permission from Pall Corporation, www.pall.com).

<table>
<thead>
<tr>
<th>Filter Media</th>
<th>Hydrophilic PolyEtherSulfone (PES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore Size</td>
<td>10, 20, 45, and 80 nm</td>
</tr>
<tr>
<td>Diameter</td>
<td>13 - 293 mm (the one used in our <em>in vitro</em> experiments is 25 mm)</td>
</tr>
<tr>
<td>Typical Thickness</td>
<td>0.1 µm: 132 µm (5.2 mils)</td>
</tr>
<tr>
<td></td>
<td>0.2 µm: 145 µm (5.7 mils)</td>
</tr>
<tr>
<td></td>
<td>0.45 and 0.8 µm: 140 µm (5.5 mils)</td>
</tr>
<tr>
<td>Typical Water Flow Rate</td>
<td>mL/min/cm² at 0.7 bar (70 kPa, 10 psi)</td>
</tr>
<tr>
<td></td>
<td>0.1 µm: 5</td>
</tr>
<tr>
<td></td>
<td>0.2 µm: 26</td>
</tr>
<tr>
<td></td>
<td>0.45 µm: 58</td>
</tr>
<tr>
<td></td>
<td>0.8 µm: 165</td>
</tr>
<tr>
<td>Maximum Operating Temperature - Water</td>
<td>100 °C (212 °F)</td>
</tr>
<tr>
<td>Extractables - Soxhlet Extraction</td>
<td>&lt;4%</td>
</tr>
<tr>
<td>Minimum Bubble Point - Water</td>
<td>0.2 µm: 3.5 bar (350 kPa, 51 psi)</td>
</tr>
<tr>
<td></td>
<td>0.45 µm: 2.5 bar (250 kPa, 36 psi)</td>
</tr>
<tr>
<td></td>
<td>0.8 µm: 1.0 bar (100 kPa, 15 psi)</td>
</tr>
<tr>
<td></td>
<td>60% IPA/40% H₂O (v:v)</td>
</tr>
<tr>
<td></td>
<td>0.1 µm: 2.4 bar (240 kPa, 35 psi)</td>
</tr>
<tr>
<td>Biological Safety</td>
<td>Passes United States Pharmacopeia (USP) Biological Reactivity Test, In Vivo &lt;88&gt;</td>
</tr>
<tr>
<td>Sterilization</td>
<td>Provided non-sterile. Can be sterilized by autoclaving at 121 - 123 °C (250 - 253 °F) for 30 min.</td>
</tr>
</tbody>
</table>

These membranes have been extensively used for pharmaceutical, biological, and sterilizing filtration needs. Some of the main advantages offered by these membranes are fast filtration, low protein binding and extensive compatibility with the drug being filtered,
4.3.1.2 Performance of the Supor® PES membrane disc filters

As per the information from the Pall website (www.pall.com), the membranes exhibit good performance (performance here means that the membranes exhibit minimal biomolecule binding). This indicated that there would be minimal or no chances of membrane-protein interaction in our in vitro studies. Supor® membrane is low in biomolecule binding (Pall corporation, 2011). Additional information was not available from resources available to us such as, technical experts at Pall corporation, their literature and website. This could be due to our application being completely different than what they were primarily designed for.

There was no permeation obtained with the use of Supor® PES membrane as no peak area detection was obtained for IGF-1 with the use of RP-HPLC. This could be due to the lower pore size of the membrane and the characteristics of the membrane, which led us to screen other membranes available from Pall Corporation in our in vitro method.

After discussion with Pall Corporation, Omega™ Ultrafiltration Membrane Disc Filters were the next to be tested for biorelevance in our in vitro method. The cut offs for the various membranes from Omega™ Ultrafiltration Membrane Disc Filters were 300 kDa, 100 kDa, 50 kDa, 30kDa and 1 kDa, with nominal size being 30 nm, 10 nm, 5 nm, 3 nm and 0.1 nm respectively. The criterion for cut off and nominal size was that the membrane would retain 90% of the spherical particles, the size of which was equal to, or greater than, the nominal pore size for that membrane.
As per the discussion with Pall Corporation, the selection of a membrane should be based on the molecular weight of the protein of interest and cut-off value of the membrane. In order to retain the protein of interest, the membrane of choice should have three to four times smaller kDa value (as per the kDa cut-off) than the protein of interest. As our interest was not retention but rather in vitro permeation over time, higher kDa value membrane were screened as the first set of membranes and then the membranes of increasingly higher kDa value were tested in our in vitro method for biorelevant in vitro permeation. Also, these membranes are not designed to primarily operate under non-pressure conditions, thus the use of membranes with higher kDa value were required in our in vitro experiments.

There was no permeation obtained with the use of the Omega™ membrane as no peak area detection was obtained for IGF-1 with the use of RP-HPLC. This could be due to the lower pore size of the membrane and the characteristics of the membrane. Also, during the manufacturing of the membranes the pore size distribution is not uniform due to stretching involved in manufacturing process of these membranes. In order to achieve permeation through these membranes, application of pressure may be necessary, which was not originally included as part of our in vitro method. Introducing pressure would have added another variable, which was not desirable as, we wanted to keep the in vitro method as simple as possible and mimic the most important variables (flow rate and pore size). Also during experiments there may be some pressure due to the medium in donor chamber and atmospheric pressure over the donor chamber.

After investigating the modified PES Supor® membrane and Omega™ membrane and experiencing a lot of difficulties with those to achieve any permeation of the peptide based drug
in our set up, we contacted the VCU School of Engineering to use their expertise in selection of the biorelevant membranes for IVIVC method development to obtain reproducible permeation of the drug. After several meetings with Drs. Garry Tepper and Hooman Tafreshi of the VCU School of Engineering and phone conferences with Dr. Benjamin Fuchs at Dupont, there was a unanimous agreement on using the polycarbonate nucleopore track etched membrane. The highly hydrophilic nature, very uniform pore size distribution and minimal or non-protein binding nature of the membrane led us to using them in our in vitro method.

4.3.2 Cellulose membrane

Cellulose membrane filters were investigated for biorelevance under the same in vitro conditions. This membrane was of 10 kDa cut-off. No permeation was obtained with the use of this membrane. This could be due to the smaller pore size of the membrane. Larger pore sizes among the cellulose membrane were not investigated due to the advantages and minimal binding offered by polycarbonate membranes.

4.3.3 The polycarbonate nucleopore track etched membranes

The polycarbonate nucleopore track etched membranes were obtained from GE Healthcare Corporation. These membranes have pores that are spherical and have no known interactions with proteins. The pores in the membranes were thought to be the most biorelevant to the capillary pores in vivo. The polycarbonate nucleopore track etched membranes are manufactured using track etch technology. The manufacturing process involves exposure of film to high-energy particles (created in a cyclotron or linear accelerator) to produce “tracks”.

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These tracks are then chemically etched to produce pores that are very uniform in diameter.

The membranes of pore sizes 100 nm, 1000 nm, 5000 nm, 8000 nm, 12,000 nm and 15,000 nm were tested for biorelevant in vitro permeation/release in our in vitro method. Significant permeation/release was obtained with the use of these membranes. The in vitro permeation data was then used to test the device for IVIVC. The results and the in vitro permeation data are discussed in the Chapter 5 of this dissertation. Although, the pore size of this set of membranes that resulted in significant permeation in our in vitro method was higher than the earlier set of membranes (PES and Omega ultrafiltration disc filters), which would have been more biorelevant, it was a significant progress made in the area of in vitro method development for peptide based drugs. Also, the electron microscopic study of the membrane for pore size distribution supports the choice of polycarbonate nucleopore track etched membranes.

**4.4 Selection of biorelevant medium for in vitro studies**

In order to carry out the in vitro studies, an in vitro medium was necessary. A medium that had previously been developed and tested for biorelevance with implants (Iyer et. al., 2007) that are administered subcutaneously has been investigated for use with peptide based injectable drugs.

A modified HBSS has been found to be an acceptable medium for in vitro permeation studies of formulations of IGF-1, based on the results from our analytical stability studies. The
stability studies were carried out for IGF-1 in Hanks medium and water and the results are discussed in Chapter 3 of this dissertation.

4.4.1 Use of modified HBSS medium to simulate blood or lymphatic circulation

The modified HBSS was used as the biorelevant in vitro permeation medium for the present study. The HBSS is widely used in cell culture studies for maintaining viability of the cells. The preparation procedure of modified HBSS is discussed in chapter 3 of this dissertation.

4.4.2 Selection of the flow rates

The flow rate used in most of the experiments was 4 mL/min. For the membranes, where permeation was obtained, one exploratory experiment was carried out with the higher and lower flow rate to investigate the effect of flow rate on the rate of drug permeation into the reservoir. The results of this experiment are discussed in Chapter 5.

4.5 Modified device for IVIVC testing of peptide-based drugs

The reservoir volume was selected as 200 mL. However, it is to be noted that the volume to which the drug is exposed to the in vitro medium is much less at any point of time over the duration of the study. The 200 mL of reservoir volume was used with the idea that it would ensure that the concentration of the drug will not saturate the medium during the in vitro study duration. It was an approximate volume chosen to simulate the blood or lymph circulating in vivo and presenting the sink conditions that exist at the SC site of drug administration. The medium was allowed to flow through the tubes, until a constant flow rate of 4 mL/min was established. It
was at this point that the 1.6 mL (2.4 mg IGF-1) dose of Increlex™ was introduced to the modified Hanson Microette device. The 2 mL of sample was then collected at the following time points and replaced with 2 mL of freshly prepared HBSS: 0, 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, and 360 minutes. The samples were then stored at -20 °C. After every experiment, samples were analyzed using a validated RP-HPLC method for IGF-1/IGFBP-3. An image of the \textit{in vitro} set up can be seen in Figures 27 and 28.
Figure 27 Design of the modified Hanson Microette® device for IVIVC method development for peptide based drugs
**Figure 28** Real time Image of the modified Hanson Microette® device for IVIVC method development
4.6 *In vitro – in vivo correlations testing*

The PK data from the analysis of *in vivo* clinical study results gave us the *in vivo* input parameter for IVIVC testing. The percent IGF-1 absorbed from Increlex™ and Iplex™ over time (Input Function) was calculated using the Wagner-Nelson method (Wagner et. al., 1964).

The *in vitro* data was obtained for Increlex™ using the modified Hanson Microette® device. The percent *in vitro* permeation/release was plotted against percent *in vivo* absorbed to test for correlation. The results, summary, conclusion and future directions are discussed in Chapter 5 of this dissertation.
CHAPTER 5

IVIVC RESULTS, OVERALL CONCLUSION AND FUTURE DIRECTIONS

5.1 In vivo study results

The absorption pattern from the PK analysis of Iplex™ was found to be remarkably different from that of the unbound IGF-1 (Increlex™) (Rabkin et. al., 1996). The comparison of the absorption profile (concentration versus time profile) for both the drugs can be seen in the Figures 29, 30 and 31.

The Wagner-Nelson method (compartmental method) was used to obtain the PK parameters for Iplex™ and Increlex™ (Wagner et. al., 1964, Kawsar et. al., 2011).

The in vivo absorption can be calculated using appropriate PK methods such as the Wagner-Nelson procedure or the Loo-Riegelman method. The Wagner-Nelson and Loo-Riegelman methods are both model dependent methods, in which the former is primarily used
for a one-compartment model and the latter is used in case of a multi-compartment system. The Wagner-Nelson method was used to calculate the percent drug absorbed due to the simplicity of the method and applicability to one compartment model (assumption is that Iplex\textsuperscript{TM} and Increlex\textsuperscript{TM} follow one compartment model). Also, the unavailability of concentration time profile after IV administration of unbound IGF-1 makes the Wagner-Nelson a preferred method over the Loo-Riegelman method, as there is no requirement for IV data with Wagner-Nelson method. According to Wagner-Nelson method, the cumulative fraction of drug absorbed at time $t$ is calculated from the equation below:

\[
\% \text{ Absorbed} = \left\{ \frac{C_t}{K_e} + \frac{\text{AUC}_{(0-t)}}{\text{AUC}_{(0-\infty)}} \right\} \times 100
\]

Where, $C_t$ is plasma concentration at time $t$, $K_e$ is the elimination rate constant for the drug administered; $\text{AUC}_{(0-t)}$ and $\text{AUC}_{(0-\infty)}$ represent the area under curve from zero to time $t$ and infinity, respectively.

The PK parameters: $\text{AUC}_{0-T\text{last}}$, $\text{AUC}_{0-\infty}$, $C_{\text{max}}$, $T_{\text{max}}$, $K_{\text{el}}$ and $T_{1/2}$ have been calculated and reported. The comparison of PK parameters for the clinical study of Iplex\textsuperscript{TM} and Increlex\textsuperscript{TM} are tabulated in table 14. Also, the plot of percent drug absorbed versus time was calculated for Increlex\textsuperscript{TM} (unbound IGF-1) using Wagner-Nelson method and can be seen in Figures 29-31.
Table 14 Calculated PK parameters for total IGF-1 after administration of Increlex™ & Iplex™ mean concentration values

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>PK parameters from CDS study using WinNonlin for Iplex™</th>
<th>Calculated PK parameters using Wagner Nelson for Iplex™</th>
<th>Calculated PK parameters using Wagner Nelson for Increlex™</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;0-Tlast&lt;/sub&gt;</td>
<td>ng/mL*hr</td>
<td>29,684</td>
<td>30595</td>
<td>349</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt;</td>
<td>ng/mL*hr</td>
<td>38049</td>
<td>38002</td>
<td>360</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>ng/mL</td>
<td>721</td>
<td>700</td>
<td>20</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>hr</td>
<td>21.60</td>
<td>21.00</td>
<td>0.30</td>
</tr>
<tr>
<td>K&lt;sub&gt;el&lt;/sub&gt;</td>
<td>1/hr</td>
<td>0.020</td>
<td>0.027</td>
<td>0.260</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>hr</td>
<td>27.60</td>
<td>27.60</td>
<td>2.63</td>
</tr>
</tbody>
</table>
Figure 29 Comparison of absorption profiles of Increlex™ and Iplex™ for the same time points

Figure 30 Percent IGF-1 absorbed from Increlex™ over time calculated using the Wagner-Nelson method
Figure 31 Percent IGF-1 absorbed from Iplex™ over time calculated using the Wagner-Nelson method

5.2. In vitro permeation/release study results

It has been a major challenge to identify biorelevant synthetic membranes for the in vitro permeability studies. The membranes that were thought to be most likely to simulate the physiologic variables in the subcutaneous tissue after the administration of peptide-based drug (IGF-1 and IGF-1/IGFBP-3 for the purpose of this work) have been investigated in our in vitro method.

The biggest challenge was to find membranes that would yield significant permeation in the in vitro permeation/release apparatus. The modified Polyethersulfone (PES) membranes were
the first to be investigated and it failed to yield any permeation/release in our in vitro apparatus. The small pore size of the evaluated membranes and no pressure other than that exerted by the modified HBSS in the donor chamber and from the atmosphere outside the tube extension to the donor chamber could be a possible reason for no in vitro permeation/release as seen by no detection of IGF-1 in the in vitro samples using RP-HPLC. The next set of membranes was the Omega® ultrafiltration disk filters. No permeation/release was obtained with this set of membranes either. The reason for no in vitro permeation/release with this set of membranes may be very similar to that of the possible reasons for no in vitro permeation/release with the polyethersulfone membranes. The polycarbonate nucleopore track etched were the next set of membranes to be investigated in our apparatus for in vitro permeation/release studies. The polycarbonate nucleopore track etched membranes were the only set of membranes that gave significant permeation in the in vitro permeation/release experiments. The possible reason for getting significant permeation with these membranes could be the relatively higher hydrophilic characteristics of the membranes and more uniform pore size characteristics of the membranes as compare to the earlier investigated membranes.

The experiments were carried out in triplicates when the first experiment with a membrane of a given pore size gave permeation/release in the in vitro experiments. The percent of drug release was found to be almost 80 - 100 percent in the case of the freshly used polycarbonate membrane with 5 µm pore size than all the other membranes (Figure 32, 33, 34 and 35). This could be due to inter-membrane variability among the membranes of different pore size. Repeated use of the same membrane in different in vitro experiments under the same conditions resulted in a significant decrease (almost 50 percent) in vitro permeation. The intra-
membrane variability for the 5 µm membrane remained high (Figure 32), in spite of repeated washing (3 times) with water and carefully storing them until their reuse. Reusing the 5 µm membrane resulted in a decrease of almost 15 percent in amount permeated in the in vitro studies and there was almost a 50 percent decrease when the same membrane was used in in vitro experiment for the third time (Figure 32). This suggests that repeated use of the membrane is not possible even after sufficient washings. Also, the variability in the in vitro permeability was almost 30 percent when a new membrane of 5 µm was used suggesting a high inter-membrane variability between the membranes. There were some experiments in which no permeation/release was obtained with the membranes (fresh) of the same pore size and under the same experimental conditions. It was not possible to find out the reason for this inter-membrane variability among the membranes.

The in vitro permeation was found to be linear for up to 2 hours in almost all the experiments, where significant in vitro permeation was observed (Figures 32-35, in vitro permeation vs. time plots) and there was a slow increase in the percent permeated in vitro for up to 4 hours, saturation starts to occur at the same time. This may be due to blocking of the pores of the membrane by IGF-1 as the first few layers of molecules have permeated through the membrane. The blocking of pores may be due to the limited surface area offered by the synthetic membranes used in our experiments. The formation of a gel layer on the membrane could be another reason contributing to the high inter and intra-membrane variability observed in the percent in vitro permeation/release experiments. This can be tested by staining the protein, which would then be visible in the form of gel layer on the membrane (Kawsar et. al., 2011). Due to time constraints, the gel layer studies were not performed in our research project, but it is a
possible reason for building of cake on the membrane surface during filtration and slowing the filtration rate over time. The variability observed in the in vitro permeation/release is not likely to be due to protein binding to the membrane or the tubing used in the apparatus design, as this was taken into account prior to selection of all the components for our study. The selection of all the components such as the apparatus, pump tubing, reservoir, synthetic membrane were made after discussion with the manufacturer about our study and going thoroughly through their literature and website with regard to this. All the components used in the study present negligible or no protein binding and have no known interaction with peptides. In some studies, the sampling points were extended from 8 hours to 12 hours to see if there was any increase in percent permeation after 4 hours. It was found that, drug permeation/release saturates at around 4 hours and there is minimal or no permeation/release after this time point.

In spite of the variability, the best in vitro permeation/release was found with the membrane of 5 µm pore size and with a flow rate of 4 mL/min and the permeation started to saturate after 4 hours. The reason for the best in vitro permeation/release for this combination could not be determined. When in vitro studies using membranes of varying pore sizes, under different flow rate conditions (2 mL/min and 8 mL/min) there was no significant difference in the in vitro permeation/release of IGF-1 by changing the flow rate of modified HBSS.
Figure 32 Comparison of 5 µm membrane when used freshly and repeatedly (second time and third time) from the same batch to check intra-membrane variability in the *in vitro* permeation/release.
Figure 33 Comparison of 5 µm membrane when used freshly from the same batch to check inter-membrane variability in the in vitro permeation/release between the membranes.

Figure 34 Comparison of the in vitro permeation/release through a 5 µm membrane under different flow rate conditions.
Figure 35 Intra-membrane variability in the \textit{in vitro} release/permeability for a 12 µm membrane used freshly and repeatedly (second time)

5.3. \textit{In vitro-in vivo} correlation results

The IVIVC analysis was performed for the studies where significant percent permeation/release was obtained. The 5 µm and 12 µm membranes were the ones where significant permeation/release was obtained in our \textit{in vitro} studies. There was no proportional increase in percent permeation/release of IGF-1 in \textit{in vitro} studies for 12 µm as compare to the 5 µm membrane. The plot of percentage absorbed \textit{in vivo} versus percentage permeated/release \textit{in vitro} was done using a Level A, point-to-point correlation (IVVIC Guidance, FDA) and $R^2$ value
calculated using linear regression was found to be 0.83 for 5 µm membrane with 4 mL/min flow rate.

**Figure 36** Comparison of % IGF-1 absorbed/permeated over time for membranes with different pore size and flow rates
Figure 37 IVIVC of % IGF-1 absorbed *in vivo* vs. % IGF-1 permeated *in vitro* for membranes with 5 µm pore size

Figure 38 IVIVC of % IGF-1 absorbed *in vivo* vs. % IGF-1 permeated *in vitro* for membranes with 12 µm pore size
The IVIVC plot (% absorbed in vivo vs. % permeated in vitro) suggests that the developed in vitro method is able to simulate the lag time observed for the initial 1 to 2 hours of drug absorption in vivo. There is a good correlation between the percent drug permeated/released for the first 1 to 2 hours post administration of unbound IGF-1 (Increlex™), followed by a slow permeation post 2 hours. The slow permeation post 2 hours may be due to the saturable pore characteristics of the membrane due to the limited surface area available for permeation of the protein and the variability if any, in the pore size distribution among the membranes.

In order to understand the pore characteristics and the reasons for inter and intra-membrane variability found in the in vitro permeation/release obtained with polycarbonate nucleopore track etched membranes, electron microscopic characterization was carried out. The images were generated using JEOL JSM-5610 LV Microscope.
5.4 Membrane characterization using electron microscopy

**Figure 39** 1 μm polycarbonate nucleopore track etched membrane (n = 20, where 20 is the different parts of one 1 μm membrane studied under the electron microscope)
Figure 40 5 µm polycarbonate nucleopore track etched membrane (n = 20, where 20 is the different parts of one 5 µm membrane studied under the electron microscope)
The electron microscopic examination of the 1 µm, 5 µm and 12 µm polycarbonate nucleopore track etched membranes was carried out. Of these, only the 5 µm and 12 µm membranes gave significant permeation/release in the *in vitro* studies. On visualization of the membranes, it is evident that the majority of the pores in the membrane are of the labeled pore size. Although, at times, there are two, three or more pores fused together, as seen in the electron microscopic image, leading to the formation of a bigger pore, contributing to the inconsistency in the pore size distribution between and within the membranes, which could be one of the factors...
contributing to the variability observed during the *in vitro* experiments. Other reason may be the limited surface area available for the permeation/release of the protein in the *in vitro* experiments possibly leading to the formation of variable gel layer contributing to the inter and intra variability in the *in vitro* permeation/release of IGF-1.

### 5.5 Overall Summary and conclusion

The unusual findings from a bioequivalence study done at the CDS in 2005 prompted us to develop an *in vitro* method to simulate the absorption variables of subcutaneously administered peptide based drugs.

When IGF-I (*Increlex™*) is administered as an injection, IGF-1 is absorbed over 1-2 hours, whereas absorption continues to occur for more than 72 hours from IGF-1/IGFBP-3 (*Iplex™*). The two drugs, both containing IGF-1 have very different durations of absorption. This may be due to the different pathways that unbound IGF-1 and IGF-1 in bound form with IGFBP-3 takes to reach systemic circulation. The unbound form of IGF-1 may be mainly absorbed via blood capillaries into the systemic circulation, whereas, the larger binary complex of IGF-1 and IGFBP-3 may be absorbed via the lymphatic capillaries into the systemic circulation.

The CDS clinical study results and the availability of IGF-1 based drug products presented us with an opportunity to explore the development of a biorelevant *in vitro* method for subcutaneously administered peptide based therapeutic agents. From our literature review, we found pore characteristics in the blood capillaries and lymphatic capillaries and flow rate in the
blood capillaries and lymphatic capillaries to be the two most important variables that would be necessary to simulate the SC environment in a biorelevant manner. The other variables that affect drug absorption from site of SC injection are discussed in chapter 2 of this dissertation.

In order to develop a biorelevant in vitro method, these two factors need to be taken into consideration. This work is a first reported effort aimed towards the development of a biorelevant IVIVC method for a peptide-based drug.

In order to simulate the in vivo physiologic conditions, the Hanson Microette® device was modified. The modifications to the device are explained in Chapter 4 of this dissertation. The use of the synthetic membrane is an approach made to mimic the pores of blood capillaries and lymphatic capillaries. In order to establish our hypothesis regarding the absorption of unbound and bound IGF-1 absorption into the systemic circulation, molecular modeling was carried out.

The molecular modeling findings using SYBYL 7.3 indicate that the unbound form of IGF-1 and IGFBP-3 may permeate through blood capillary pores, whereas, the binary complex of IGF-1 to IGFBP-3 and the ternary complex of IGF-1 bound to IGFBP-3 and ALS may not permeate through the blood capillary pores. The unbound IGF-1 and IGFBP-3 diameter estimates were found to be 33.78 Å and 39.34 Å, which are smaller than the average diameter estimates of the pores in blood capillaries, which are 48 Å, suggesting that the unbound form will permeate via blood capillaries. Also, from these results, it can be said that the bound form of IGF-1/IGFBP-3 may permeate mainly via lymphatic pores (a few microns to a few nanometer pores) and will not permeate via blood capillary pores (73.12 Å > 48 Å). The explanation to the
ternary complex of IGF-1/IGFBP-3/ALS being restricted to the vascular endothelium (136.65 Å > 48Å) is explained by results from SYBYL 7.3 from molecular modeling work.

The size complementarity characteristics were better understood from the results of the HEX study that demonstrated the presence of a strong electronegative potential on the inner side of ALS, which is a prime target for IGFBP-3 binding. Also, the doughnut like hole in the ALS presents the binding cavity for IGF-1/IGFBP-3, thus leading to the formation of a ternary complex (IGF-1/IGFBP-3/ALS), which is a stable complex and raises the half-life of IGF-1 from 5 minutes to 12-14 hours. The longer half-life results from a slower degradation of IGFBP-3 from the complex by proteases as compare to rapid degradation of unbound IGF-1 by proteases in the nearby vicinity.

This information has proven useful to validate our hypothesis that the differences in molecular weight of the subcutaneously administered drug could determine the pathways (blood capillary or lymphatic capillaries) drugs may take to reach the systemic circulation (Porter et. al., 2001, Porter et. al., 2000, Porter, 1997, Supersaxo et. al., 1990). The relationship between molecular weight of drugs and their pathways of drug absorption after SC administration into systemic circulation have been discussed in Chapter 2 of this dissertation.

After the basis for membrane selection was established, it was necessary to identify a biorelevant medium for in vitro permeation studies. To our knowledge, the modified HBSS, which has been previously established as a biorelevant medium for in vitro release studies of implants (Iyer SS), has been investigated for use in our in vitro studies. The modified HBSS has
not been used as a biorelevant medium for *in vitro* studies of peptide based drugs prior to this study.

A RP-HPLC method, which was developed by Insmed Inc. for the analysis of IGF-1/IGFBP-3 was partially validated for the analysis of *in-vitro* samples. Partial validation was considered adequate because, the method was developed, validated and extensively used prior to this study for the analysis of IGF-1/IGFBP-3. The method was demonstrated to be accurate, linear, and precise in support of release and stability testing. The only change in the method is the introduction of a buffer, modified HBSS for the *in vitro* permeation/release studies. The modified HBSS did not interfere with the analysis of an analyte (IGF-1/IGFBP-3) during the *in-vitro* sample analysis. The method employed UV detection and involved minimum sample preparation time.

From the stock solution, different standard solutions ranging from 7.5 to 150 µg/mL were prepared and were run as per the chromatographic conditions. The calibration curves were found to be linear in this range of concentration with $R^2 > 0.99$. The LLOQ was determined to be 7.5 µg/ml with %RSD of 2.04. This confirmed that the analytical method was able to quantify the LLOQ in an accurate and precise manner. The freeze-thaw study was conducted by comparing the mean peak areas of standard samples that were injected after 1, 2 and 3 cycles to that of the mean peak area of a freshly prepared sample. The storage duration of 24 hrs for cycle 1, 48 hours for cycle 2 and 7 days for cycle 3 at below -20 °C was evaluated and was found to be acceptable. The mean value for these 2 cycles ranged from 98.0% to 100.0% of the initial concentration at 0th hour. These results assured that the repeated freezing and thawing of a sample did not affect the integrity of an analyte for up to 48 hours. The mean observed concentrations after the 3rd
such cycle deviated by less than 96.0% of the each QC standard. The graphs (Figures 11 and 12) for stability studies of an analyte in standard working solution showed that there were no decomposition products observed in the chromatogram, as no differences in areas were observed during the analytical procedures, even after storage at 2-8 °C for 48 hours.

After the analytical method and the in vitro method were developed, the in vitro studies were carried out; the samples were collected and analyzed according to the time points specified in the methods section of this dissertation.

The in vitro permeation was found to be linear for up to 2 hours in almost all the experiments, where significant in vitro permeation was observed. There was a slow increase in the percent permeated in vitro for up to 4 hours and saturation starts to occur at this time. This may be due to blocking of the pores of membrane by IGF-1 as the first few layers of molecules have permeated the membrane. The formation of a gel layer on the membrane could be another reason contributing to the high inter and intra-membrane variability observed in the percent in vitro permeation/release experiments. This can be tested by staining the protein, which can then be visible in the form as a gel layer on the membrane. The variability observed in the in vitro permeation/release is not likely to be due to protein binding to the membrane or the tubing used in the apparatus design, as this was taken into account prior to selection of all the components for our study. All the components used in the study present negligible or no protein binding and have no known interaction with peptides. In some studies, the sampling points were extended from 8 hours to 12 hours to see if there is any increase in percent permeation after 4 hours. It was
found that drug permeation/release saturates at around 4 hours and there is minimal or no permeation/release after this time point.

In spite of the high inter and intra-membrane variability in *in vitro* permeation/release of IGF-1, the best *in vitro* permeation/release was found with the membrane of 5 μm pore size and with a flow rate of 4 mL/min. The *in vitro* studies that were with membranes of varying pore sizes, under different flow rate conditions (2 mL/min and 8 mL/min), it was found that there was no significant difference in the *in vitro* permeation/release of IGF-1 by varying the flow rate of modified HBSS.

By using the percent *in vitro* permeation/release data from the *in vitro* permeation/release studies and the percent *in vivo* data from the clinical study, an IVIVC correlation was performed. The findings indicate that the polycarbonate nucleopore track etched membrane of 5 μm with a flow rate of 5 mL/min gave a good correlation ($R^2 = 0.83$) with the percent absorbed *in vivo* versus time for the same time points.

Efforts were made to choose the synthetic membranes that would best simulate the physiologic conditions in the closest possible manner. The ideal membrane would be the one that has a pore size as close to the average pore size of blood capillary and lymphatic capillary pores and is very hydrophilic and yields reproducible *in vitro* release/permeation in the *in vitro* method. The selection of membranes in this study was done by careful consideration of variables that would affect the release/permeation of peptides through them. Helpful discussions with
experts at Pall Corporation, GE Healthcare and the VCU School of Engineering were carried out when needed during the course of this research work.

In our work, it was possible to perform the experiments only for Increlex™. Experiments with Iplex™ could be carried out as part of a separate project, once the, inter and intra-variability among the membranes used in the \textit{in vitro} method has been further optimized.

This research effort work has improved our understanding of the absorption pathways of different molecular weight peptide based drug products after carrying out the PK analysis on the two drugs products (Iplex™ and Increlex™). A novel \textit{in vitro} method design was developed that is able to simulate physiologic conditions (pore size and flow rate characteristics) and that the reasonable permeation/release has been obtained with the use of polycarbonate nucleopore track etched membranes. It is variable but it is the best we could do with the disc membranes available with today’s technology. We have been in continuous touch with GE healthcare, Pall Corporation to use their expertise in this area, which has helped us in selection of the synthetic membranes. We have discussed the problems and possible solutions with their technical experts on these membranes during the course of this research project and best efforts have been made to optimize the \textit{in vitro} method. There have been many challenges in choosing a biorelevant membrane. We have chosen the most appropriate one from all the options available to us.
5.6 Future directions

Further modification in the developed *in vitro* method like varying the volume of modified HBSS, orientation of the reservoir, and different types of membranes (if better membranes become available to reduce inter and intra-variability) to optimize the IVIVC correlation.

Alternative approaches could be investigated that involve the use of a tangential flow filtration (TFF) approach rather than normal flow filtration approach (used in our study). Tangential Flow Filtration (TFF) works on the principle that the fluid is pumped tangentially along the surface of the membrane as compare to perpendicular flow to the surface of the membrane in case of Normal Flow Filtration (NFF). Also, an applied pressure serves to force a portion of the fluid through the membrane to the filtrate side. This approach may solve the problem of saturation of the pores of membranes by blocking the pores after the first set of molecules have permeated and could also provide a larger surface area for permeation to occur.

The MicroKros® hollow fiber filter modules that are made of modified polyethersulfone (mPES) are available in varied pore ratings (3 kDa, 10 kDa, 30 kDa, 50 kDa, 70 kDa, 100 kDa and 500 kDa) and could be evaluated using the same in the *in vitro* permeation/release methods used here. Due to the time, reusability and cost constraints, experiments were not carried out with these filter modules, but these offer could be explored in the future for optimizing the IVIVC method for peptide based drugs.
An alternative approach would be the use of a more expensive CELLMAX® hollow fiber bioreactor systems. The hollow fiber bioreactors are two-compartment, three-dimensional cell culture systems used for *in vitro* pharmacokinetic modeling. These bioreactor systems have been used to determine effects of a wide range of synthetic compounds, conjugates and biomolecules on cancers, parasites, fungi and viruses. These systems have been used to accurately predict *in vivo* drug kinetics including parameters like transport phenomena, clearance and reaction mechanisms. The reason for not investigating this instead of our *in vitro* method first is the cost associated with the bioreactors (> $400/unit), the difficulty in reusing the bioreactors and the limited pore size available (30 kDa and 10 kDa) as compared to the various synthetic membrane evaluated in our research.
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Vita

Amit A. Somani is an Indian citizen born on October 27\textsuperscript{th}, 1983 in Rajasthan. He received his Bachelors of Pharmacy degree from Shri B.M.C.P.E.R., Modasa, Gujarat, India in 2004. Since his Bachelors, he was keenly interested to pursue a career in the area of Clinical Research and Pharmacokinetics/Pharmacodynamics (PK/PD). To get the exposure of being involved in a clinical trial setting, he joined Cadila Pharmaceuticals for a month as a Bioequivalence intern in 2003. Upon the completion of Bachelors in Pharmacy degree, he joined Claris Life Sciences as a Research and Development Officer in 2004 in their Research and Development department for 7 months. It was then that he decided to pursue a Ph.D. in the area of clinical research and PK/PD. He started in the graduate program in Pharmaceutical Sciences in the Department of Pharmacotherapy and Outcomes Sciences, School of Pharmacy, VCU in the year 2006 with Dr. William H. Barr and Dr. William R. Garnett as his advisors, where his major research area was to develop IVIVC method and model for subcutaneously administered peptide based drugs. For this work, he won the graduate school dissertation assistantship award. Amit was supported by the Center for Drug Studies and Department of Pharmacotherapy and Outcomes Sciences, School of Pharmacy throughout his doctoral program as a teaching assistant and research associate. Apart from that he was also involved in clinical studies at the Center for Drug Studies at VCU along with Dr. Barr. He was involved in the development of protocols, PK/PD data analysis and clinical study report preparation. He got the exposure to work with the IRB, primary investigator, clinician, statisticians, nurses, CRA’s and sponsors and also got exposure of dose preparation, dosing, on site monitoring, collection and storage of study samples in accordance with GCP/ICH guidelines.