

Peroral and Nasal Delivery of Insulin with PheroidTM Technology

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Peroral and Nasal Delivery of Insulin with Pheroid™ Technology

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Trust in the Lord with all your heart
And lean not on your own understanding;
In all your ways acknowledge Him,
And He shall direct your paths.

King Solomon, Proverbs 3:5-6 NKJV

TABLE OF CONTENTS

Abstract.....	viii
Uttreksel.....	x
Introduction and Aim of Study.....	xii

Part I: Insulin Delivery

CHAPTER 1

Peroral Insulin Delivery: A Review of Barriers and Recent Developments

1.1	Introduction.....	1
1.2	Insulin.....	3
1.2.1	The discovery of insulin.....	3
1.2.2	Pharmacology and function of insulin.....	6
1.2.2.1	The endocrine pancreas.....	6
1.2.2.2	Insulin chemistry.....	7
1.2.2.3	Insulin synthesis, secretion and degradation.....	8
1.2.2.4	Different types and duration of action of insulin preparations.....	9
1.2.2.5	Oral hypoglycaemic agents.....	11
1.2.2.5.1	Sulfonylureas.....	11
1.2.2.5.2	Biguanides.....	13
1.2.2.5.3	Thiazolidinedione derivatives.....	14
1.2.2.5.4	α -Glucosidase inhibitors (aldose reductase inhibitors).....	14
1.3	Oral delivery of insulin.....	15
1.3.1	Strategies for oral insulin delivery.....	15
1.3.2	Barriers limiting peptide bioavailability and ways to overcome it.....	16
1.3.2.1	The metabolic barrier.....	17
1.3.2.2	The physical barrier.....	20
1.3.3	Recent developments in oral insulin delivery.....	23
1.3.3.1	Hydrogel polymers.....	23
1.3.3.2	Transferrin mediated insulin delivery.....	26
1.3.3.3	Insulin-transferrin (ins-Tf) conjugates in complexation hydrogels.....	27

1.3.3.4	Nano- and microparticles.....	28
1.3.3.5	Alginate microspheres.....	30
1.3.3.6	W/O/W emulsions.....	32
1.3.3.7	Co-administration with specific enzyme inhibitors.....	33
1.3.3.8	Mucoadhesive intestinal patches.....	35
1.3.3.9	Chitosan and its derivatives as absorption enhancers.....	35
1.3.3.10	Insulin derivatives.....	37
1.3.3.11	Insulin receptor activators.....	38
1.4	Conclusion.....	39

CHAPTER 2

Nasal Insulin Delivery

2.1	Introduction.....	40
2.2	Biological and pharmaceutical aspects for nasal drug delivery.....	41
2.2.1	Anatomy and physiology of the nasal cavity.....	41
2.2.2	The nasal epithelium and mucosa.....	43
2.2.3	Nasal mucus secretion and mucociliary clearance.....	45
2.2.4	Enzymatic degradation.....	47
2.2.5	Limitations of intranasal drug delivery.....	48
2.2.6	Overcoming the barriers to nasal insulin absorption.....	49
2.2.6.1	Surfactants.....	51
2.2.6.2	Fatty acids and fatty acid derivatives (phospholipids).....	52
2.2.6.3	Bile salts and bile salt derivatives.....	53
2.2.6.4	Enzyme inhibitors.....	54
2.2.6.5	Cyclodextrins.....	55
2.2.6.6	Bioadhesive polymer delivery systems.....	56
2.2.6.6.1	Chitosan.....	56
2.2.6.6.2	Carbopol.....	58
2.2.6.6.3	Microspheres.....	59
2.2.6.7	Resin microparticles.....	60
2.2.6.8	Lipid emulsions.....	61
2.3	Conclusion.....	61

Part II: Peptide Drug Delivery Systems

CHAPTER 3

Pheroid Technology as an Insulin Delivery System

3.1	Introduction.....	62
3.2	Pheroid technology.....	63
3.3	Pheroid types, characteristics and function.....	63
3.4	Pheroid and other lipid-based delivery systems.....	66
3.5	Pharmaceutical applicable features of the Pheroid delivery system.....	68
3.5.1	Increased delivery of active compounds.....	68
3.5.2	Decreased time to onset of action.....	68
3.5.3	Reduction in minimum inhibitory concentration.....	68
3.5.4	Increased therapeutic efficacy.....	69
3.5.5	Reduction in cytotoxicity.....	69
3.5.6	Immunological responses.....	69
3.5.7	Transdermal delivery.....	70
3.5.8	Entrapment and transference of genes to nuclei and expression of proteins.....	70
3.5.9	Reduction and elimination of drug resistance.....	70
3.5.10	Pro-Pheroid.....	71
3.6	Therapeutic and preventative uses of Pheroid.....	71
3.6.1	Therapy of tuberculosis.....	71
3.6.2	Preventative therapies: Vaccines.....	72
3.6.2.1	A virus based vaccine: Rabies.....	73
3.6.2.2	A peptide based vaccine: Hepatitis B.....	73
3.7	Conclusion.....	74

CHAPTER 4

Chitosan and *N*-trimethyl Chitosan Chloride (TMC) as Drug Delivery Systems

4.1	Introduction.....	75
4.2	Chitosan.....	76
4.2.1	History.....	76
4.2.2	Synthesis and physicochemical properties.....	77
4.2.3	Applications of chitosan in pharmaceutics and medicine.....	79
4.2.4	Mechanism of action of chitosan.....	82
4.2.4.1	Why the need for chitosan derivatives?.....	83
4.3	<i>N</i> -trimethyl chitosan chloride.....	85
4.3.1	Synthesis of TMC.....	85
4.3.2	Mucoadhesive properties of TMC.....	86
4.3.3	Mechanism of action of TMC.....	87
4.3.4	The effect of TMC on the TEER of human intestinal epithelial cells (Caco-2).....	87
4.3.5	TMC as absorption enhancer of peptide drugs and hydrophilic model compounds.....	89
4.3.6	The effect of the degree of quaternisation of TMC on its absorption enhancing capabilities.....	89
4.3.7	Cytotoxic evaluation of TMC.....	91
4.4	Conclusion.....	93

Part III: Experimental

CHAPTER 5

Entrapment of FITC-Insulin in Pheroid Vesicles and Analysis with CLSM

5.1	Introduction.....	94
5.2	Pheroid preparation and synthesis.....	94
5.2.1	Materials.....	94
5.2.2	Method.....	94

5.3	Characterisation of Pheroid vesicles.....	95
5.3.1	Particle size analysis.....	95
5.3.1.1	Materials.....	95
5.3.1.2	Method.....	95
5.3.2	Confocal Laser Scanning Microscopy.....	96
5.4	Loading of Pheroid vesicles with FITC-insulin.....	96
5.4.1	Materials.....	96
5.4.2	Method.....	96
5.5	CLSM analysis of Pheroid vesicles containing FITC-insulin.....	97
5.5.1	Materials.....	97
5.5.2	Method.....	97
5.6	Results and discussion.....	97
5.7	Conclusion.....	100

CHAPTER 6

In vivo Evaluation of Peroral and Nasal Absorption of Insulin with Pheroid Technology and N-trimethyl Chitosan Chloride

6.1	Introduction.....	101
6.2	Experimental design and <i>in vivo</i> procedures.....	101
6.2.1	Experimental animals.....	101
6.2.2	Breeding conditions.....	102
6.2.3	Experimental design.....	103
6.2.4	Preparation of experimental formulations.....	104
6.2.4.1	Materials.....	104
6.2.4.2	Method.....	104
6.2.4.2.1	Pheroid formulations.....	104
6.2.4.2.2	TMC formulations.....	105
6.2.5	Laboratory animal preparation and administration of TMC and insulin formulations to rats.....	107
6.2.5.1	Materials.....	107
6.2.5.2	Induction and maintenance of anaesthesia.....	107

6.2.5.3	Surgical procedures.....	108
6.2.5.3.1	Cannulation of the artery <i>carotis communis</i>	108
6.2.5.3.2	Abdominal surgical procedures.....	111
6.2.5.4	Administration of insulin formulations.....	112
6.2.5.4.1	Oral administrations.....	112
6.2.5.4.2	Nasal administrations.....	114
6.2.5.4.3	Intravenous and subcutaneous administrations.....	116
6.2.5.5	Collection of blood samples.....	116
6.2.6	Determination of blood glucose levels.....	117
6.2.7	Quantitive analysis of plasma insulin concentrations.....	117
6.2.7.1	Principles of the procedure.....	118
6.2.7.2	Reagents supplied.....	118
6.2.7.3	Storage and stability.....	119
6.2.7.4	Assay procedure	119
6.3	Results and discussion.....	122
6.3.1	Intravenous administration	122
6.3.1.1	Intravenous administration of insulin 0.5 IU/kg.....	122
6.3.1.2	Intravenous administration of normal saline.....	124
6.3.1.3	Comparison between the reference (intravenous administration of 0.5 IU/kg insulin) and control (intravenous administration of normal saline).....	126
6.3.2	Intragastric administration.....	127
6.3.2.1	Intragastric administration of insulin (50.0 IU/kg) in saline (control)....	127
6.3.2.2	Intragastric administration of insulin (50.0 IU/kg) in Pheroid vesicles.....	129
6.3.2.3	A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after intragastric administration.....	131
6.3.3	Intracolonic administration.....	133
6.3.3.1	Intracolonic administration of insulin (50.0 IU/kg) in saline (control).....	133
6.3.3.2	Intracolonic administration of insulin (50.0 IU/kg) in Pheroid vesicles.....	134
6.3.3.3	A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after intracolonic administration.....	136

6.3.4	Intraileal administration.....	138
6.3.4.1	Intraileal administration of insulin (50.0 IU/kg) in saline (control).....	138
6.3.4.2	Intraileal administration of insulin (50.0 IU/kg) in Pheroid vesicles.....	140
6.3.4.3	A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after interileal administration.....	142
6.3.4.4	Intraileal administration of insulin (50.0 IU/kg) in Pheroid microsponges.....	148
6.3.4.5	A comparison between the intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles and Pheroid microsponges.....	150
6.3.4.6	The effect of time on the entrapment and efficiency of insulin in Pheroid vesicles.....	152
6.3.4.7	Intraileal administration of insulin and TMC (0.5% w/v).....	154
6.3.5	Subcutaneous administration.....	158
6.3.6	Intranasal administration.....	160
6.3.6.1	Intranasal administration of insulin (8.0 IU/kg) in saline (control).....	160
6.3.6.2	Intranasal administration of insulin (8.0 IU/kg) in Pheroid vesicles.....	162
6.3.6.3	Intranasal administration of insulin (12.0 IU/kg) in Pheroid vesicles.....	164
6.3.6.4	Intranasal administration of insulin (8.0 IU/kg) in Pheroid microsponges.....	166
6.3.6.5	Intranasal administration of insulin (12.0 IU/kg) in Pheroid microsponges.....	167
6.3.6.6	Comparison between insulin at 8.0 and 12.0 IU/kg in Pheroid vesicles and Pheroid microsponges.....	169
6.3.7	Absolute availability.....	161
6.4	Conclusion.....	173
Part IV:	<u>Summary and Future Prospects</u>	176
ANNEXURE 1.....	179	
ANNEXURE 2.....	180	
ANNEXURE 3.....	181	
ANNEXURE 4.....	192	
REFERENCES.....	193	
ACKNOWLEDGEMENTS.....	211	

ABSTRACT

Since its initial discovery in 1922 by Banting and Best, the formulation of an oral insulin delivery system has ever been so troublesome. Unfortunately, insulin is indispensable in the treatment of diabetes mellitus, which affects approximately 350 million people worldwide. Various factors contribute to the peptide being such a persistently difficult hormone to be used in an oral formulation. The gastrointestinal tract is home to various protein digestive enzymes such as pepsins in the stomach and trypsin, chymotrypsin and carboxypeptidases in the small intestine, which digests insulin. Also the physical barrier of the gastrointestinal tract, i.e. the columnar epithelial layer which lines the tract, is a tightly bound collection of cells with minimal leakage and is thus a sound barrier for the absorption of peptides and hormones. The aim of this study is to determine whether a dosage form for insulin, entrapped in Pheroid™ vesicles and -microsponges, can overcome these barriers and successfully deliver insulin at the site of action resulting in a significant therapeutic response.

Initial phases of the study consisted of the manufacturing of Pheroid™ vesicles and -microsponges, entrapment of fluorescein-isothiocyanate labelled insulin (FITC-insulin) into the Pheroid™. The Pheroid™-insulin complex was analysed with confocal laser scanning microscopy (CLSC) to determine drug loading. *In vivo* experiment in Sprague - Dawley rats were done where blood glucose levels as well as insulin blood levels were monitored after administration of different Pheroid™-insulin formulations. Firstly a standard reference was set by subcutaneous injection of insulin (0.5 IU/kg) in rats followed by a comparative study where administration to the stomach, colon and ileum (50.0 IU/kg insulin) were compared by means of blood insulin levels and therapeutic effect between the control and Pheroid™ complexes (Pheroid™ vesicles and micro-sponges). Each study was done by means of direct injection into the stomach, ileum or colon through which the insulin in saline (control) or insulin-Pheroid™ complex was administered. Nasal administration of 8.0 and 12.0 IU/kg insulin in saline (control) or insulin-Pheroid™ complex was done in the right nostril of Sprague - Dawley rats. Blood samples were taken from the artery *carotis communis* by means of an inserted cannula.

Blood samples were taken just before administration and then at 5, 10, 15, 30, 60, 120 and 180 minutes after administration. Blood glucose levels were measured just after every blood sample was taken and plasma insulin levels were determined with a human insulin specific radioimmunoassay. The results were compared to the reference as well as the control to determine relative bioavailability.

Through the results obtained it was discovered that in comparison with the various parts of the GI tract, the ileum showed undoubtedly to be the best area of absorption where Pheroid™ vesicles revealed a peak 42.0 % lowering in blood glucose levels after 60 minutes and a peak plasma concentration of 244.0 μ IU/ml after 5 minutes together with an 18.7 % lowering in blood glucose levels after just 5 minutes. After nasal administration of Pheroid™ microsponges (8.0 IU/kg insulin) a remarkable lowered blood glucose level of 19.2 % after 10 minutes and 36.5 % after 30 minutes as well as a peak plasma insulin level of 220.2 μ IU/ml after 3 hours was observed. Insulin entrapped in Pheroid™ microsponges administered at 12.0 IU/kg showed a maximum blood glucose lowering effect of 72.4 % after 3 hours with a peak plasma level of 154.8 μ IU/ml also after 3 hours, thus showing a long acting effect.

In conclusion, the delivery system based on Pheroid™ technology shows a sufficient therapeutic effect for insulin and is therefore promising for further *in vivo* evaluation and ultimately for medicinal use to patients suffering from diabetes mellitus.

Key words: Insulin; nasal delivery; Pheroid™; oral delivery; microsponges; diabetes mellitus.

UITTREKSEL

Sedert die ontdekking van insulien deur Banting en Best in 1922, was daar herhaaldelik bewys dat die formulering van ‘n orale insulien doseervorm heelwat problematies is. Ongelukkig is insulien noodsaaklik in die behandeling van diabetes mellitus, ‘n siekte wat ongeveer 350 miljoen mense in die wêreld affekteer. Hierdie peptied-hormoon toon verskeie faktore wat daartoe bydrae dat die formulering van ‘n orale doseervorm so problematies is. Verteringsensieme soos tripsien, chemotripsien, karboksiepeptidase en pepsiene in die maag- en dermkanaal se vertering is almal geteiken op insulien. Ook die fisiese skans van die spysverteringskanaal, naamlik die diggepakte eenlagige silindriese epiteel sellaag wat die spysverteringskanaal aan die binnekant uitvoer. Hierdie diggepakte selle vorm ‘n digte skans wat die absorpsie van peptiede en hormone soos insulien bemoeilik. Die doel van hierdie studie is om vas te stel of ‘n formulering vir insulin, geenkapsuleer in Pheroid™ druppeltjies en -mikrosponsies, hierdie skans kan oorkruis en ook die suksesvolle aflewering van insulien by die plek van werking kan bewerkstellig om so ‘n genoegsame terapeutiese effek te gee.

Initiële fases van die studie het bestaan uit die vervaardiging van Pheroid™-druppeltjies en -mikrosponsies, die vasvanging van florisien-isotiosianied gemerkde insulien (FITS-insulien) in die onderskeie Pheroid™ formulerings. Hierdie Pheroid™-insulien formulerings is daarna geanaliseer met konfoliale laser skanderingsmikroskopie (KLSM) om die geneesmiddellading te bepaal. *In vivo* eksperimente is op Sprague – Dawley rotte gedoen waar bloedglukosevlakke en plasma insulien waardes gemonitor was na die toediening van Pheroid™-insulien komplekse. ‘n Standaardverwysing was eerstens opgestel deur insulien (0.5 IU/kg) subkutaneus toe te dien waarna ‘n vergelykingsstudie gedoen was tussen toedienings in die maag, dunderm en dikderm (50.0 IU/kg) van insulien-Pheroid™ komplekse en die kontroles in terme van terapeutiese effekte en plasma insulien waardes. Toediening was in elke geval gedoen deur direkte inspuiting van die insulien-Pheroid™ komplekse en insulien in soutwateroplossing (kontrole) in onderskeidelik die maag, dunderm en dikderm. Nasale toediening van insulien-

Pheroid™ komplekse en kontroles was gedoen in konsentrasies van 8.0 en 12.0 IU/kg en in die regter nasale openinge van die Sprague – Dawley rotte.

Bloedmonsters was geneem deur middel van ‘n kannule in die arterie *carotis communis* net voor toediening en op tye 5, 10, 15, 30, 60, 120 en 180 minute na toediening. Bloedglukosewaardes was gemeet net na monsterneming en plasma insulienwaardes was bepaal deur middel van ‘n radio-immuno-essai. Resultate was vergelyk met die verwysing en die kontroles om relatiewe biobeskikbaarheid te bepaal.

In vergelyking tussen die verskillende gebiede in die spysverteringstelsel toon die ileum die beste area vir absorpsie van insulien waardeur bloedglukosewaardes ‘n daling van 18.7% getoon het na toediening van insulien in Pheroid™ druppeltjies na net 5 minute en ‘n maksimum verlaging van 42.0% na drie ure. Hierdie formulering het ook ‘n piek plasmakonsentrasie van 244.0 µIU/ml gelewer na net vyf minute na toediening. Na nasale toediening van inulien (8.0 IU/kg) in Pheroid™ mikrosponsies was daar ‘n merkwaardige verlaging in bloedglukosewaardes van 19.2% na 10 minute en 36.5% na 30 minute met ‘n piek plasmakonsentrasie van 220.2 µIU/ml insulien na 3 ure. Insulien (12.0 IU/kg) in Pheroid™ mikrosponsies het ‘n maksimum verlaging van 72.4% in bloedglukosewaardes getoon en ‘n piek plasmakonsentrasie van 154.8 µIU/ml getoon, drie ure na toediening en het dus gedui op ‘n langdurige effek.

Daar is tot die gevolg trekking gekom dat die afleweringsisteem wat op Pheroid™ tegnologie gebasseer is ‘n merkwaardige terapeutiese effek teweegbring vir insulien en toon belowend te wees in verdere *in vivo* studies. Gevolglik mag dit lei tot die ontwikkeling van ‘n medisinale doseervorm wat verligting kan bring vir pasiënte wat ly aan diabetes mellitus.

Sleutelsterme: Insulien; nasale aflewing; Pheroid™; orale aflewing; mikrosponsies; diabetes mellitus / suikersiekte.

Introduction and Aim of Study

An excess of 350 million human beings on earth are suffering from diabetes mellitus. Insulin, which is indispensable in the treatment of this disease is and has, since its discovery in 1922 by Banting and Best, been ever so troublesome in the formulation of an oral delivery system. Several factors contribute to this protein being such a persistently difficult hormone to be used in an oral formulation. One main factor is the various protein digestive enzymes such as pepsins in the stomach and trypsin, chymotrypsin and carboxypeptidases in the small intestine, which digests this peptide molecule. The physical barrier of the gastrointestinal tract i.e. the columnar epithelial layer which lines the GI tract is a tightly bound collection of cells with minimal leakage and is therefore a sound barrier for the absorption of peptides and hormones.

Numerous strategies have been developed to improve the oral bioavailability of insulin in a unique oral formulation. These strategies mainly focused on overcoming or bypassing the enzymatic barrier or the physical barrier in the GI tract. Some of these strategies made use of permeation enhancers, enteric coatings, protease inhibitors, combination strategies and microsphere encapsulation. Despite intensive research an effective peroral formulation of insulin seems to be an ever elusive goal and is, even after years of research, still in the beginning phase of development and requires much research and initiative.

It is clear that the peroral administration of insulin is deemed necessary and essential, however the success of formulating such a unique and effective dosage form has still to be discovered.

MeyerZall Laboratories (Pty) Ltd (South Africa) has developed a unique delivery system comprising of a submicron emulsion type formulation called Pheroid™ (further referred to in text only as Pheroid). The patent protected Pheroid consists mainly of plant and essential fatty acids. The system contains stable vesicular or sponge-like structures that

can be manipulated in terms of morphology, structure, size and function. Pheroid can entrap, transport and deliver active compounds and other useful substances. Pheroid is not liposome-based technology. Enabled by its essential fatty acid components, which are manipulated in a very specific manner, Pheroid has high entrapment capabilities, a very fast rate of transport and delivery and is very stable. Furthermore, the essential fatty acid component of Pheroid, while necessary for several cell functions in the human body, cannot be manufactured by human cells and must be ingested. Some of the inherent therapeutic attributes of Pheroid are the maintenance of the membrane integrity of mammalian cells, energy homeostasis, the modulation of the immune system through amongst others, the prostaglandins/leukotriens and some regulatory aspects of programmed cell death (apoptosis).

The objectives of this study were to-

- a) conduct a literature study on insulin and advancements on insulin delivery.
- b) entrap Flourescein-isothiocyanate labeled insulin (FITC-insulin) into the Pheroid and analyse the Pheroid-insulin complex with confocal microscopy.
- c) do an *in vitro* experiment on Sprague - Dawley rats where insulin in Pheroid and TMC formulations were administered orally and blood glucose levels as well as insulin blood levels were monitored and analysed; and
- d) evaluate the results in terms of efficiency and relevancy.

This thesis is divided into four main parts. The first part is a literature study, which concentrates on insulin and insulin delivery in terms of oral as well as nasal delivery. Part two, also a literature study, mainly focuses on the drug delivery systems tested for insulin delivery, namely Pheroid technology en *N*-trimethyl chitosan chloride or TMC. Experiments performed and results obtained are discussed in part three and an overall summary and future prospects are concluding the thesis in part four.

Part I: Insulin Delivery

CHAPTER ONE

**Peroral Insulin Delivery:
A Review of Barriers and Recent Developments**

1.1 Introduction

Diabetes mellitus is classified as a syndrome with disordered metabolism and inappropriate hyperglycaemia, which is due to either a deficiency of insulin secretion or to a combination of inadequate insulin secretion and insulin resistance. It is almost certainly caused by the autoimmune destruction of insulin secreting β cells of the pancreas. An estimated 16 million people in the United States of America suffer from diabetes of which 1.4 million have insulin-dependent diabetes mellitus (IDDM) or Type 1 diabetes, a more severe form of diabetes associated with ketosis if untreated. Type 1 diabetes is a catabolic disorder in which circulating insulin is virtually absent, plasma glucagon is elevated and pancreatic β cells fail to respond to any insulinogenic stimuli. Diabetics cannot properly utilize glucose and have remarkably elevated glucose levels (hyperglycaemia) while intercellular glucose levels are generally low. Exogenous insulin administration is therefore required to normalise blood glucose, reverse the catabolic state, prevent ketosis and reduce hyperglucagonemia. It occurs most commonly in juveniles and occasionally in adults and varies in prevalence in different parts of the world.

Approximately 350 million people are affected by diabetes mellitus worldwide. Scandinavia has the highest occurrence of Type 1 diabetes with as many as 20 % of diabetes sufferers being classified as Type 1. This tendency decreases from southern Europe (15 %) and the United States of America (10 %) to China and Japan (less than 1%) (Masharani, 2004:1146-1149; Karam, 1999:1118-1121; Carino & Mathiowitz, 1999:250).

In northern Europe the yearly incidence per 100 000 juveniles (14 years of age or younger) is found to be 37 in Finland, 27 in Sweden, 22 in Norway and 19 in the United Kingdom. This decreases to the rest of Europe to 10 in Greece and 8 in France. The island of Sardinia has a surprisingly high incidence of 37 although the rest of Italy has an incidence of 10 per 100 000 per year. The United States presents with 15 per 100 000 juveniles per year (Masharani, 2004:1146).

Diabetes is considered by many as an epidemic and has acquired a lot of attention in the field of research as this disease is life threatening and deteriorates the quality of life. Maintaining near normal glycaemic levels is complex and requires multiple daily subcutaneous insulin injections. Failing to maintain such a normal physiological profile results in hypoglycaemia, peripheral hyperinsulinemia and weight gain (Takei & Kasatani, 2004:578). Chronic complications of consistently high glucose levels are very serious and include retinopathy (diabetes is the most common cause of blindness), neuropathy, nephropathy (diabetes is the leading cause of chronic renal failure), cardiovascular disease, peripheral vascular disease (diabetes is the leading cause of limb amputation) and causes the patient to be more susceptible to infection (Carino & Mathiowitz, 1999:250).

Various drugs have been developed in aiding the maintenance of glucose levels. These include sulfonylureas, biguanides, thiazolidinediones, drugs modifying the absorption of glucose and several insulin formulations. Diabetes has been treated with much success with proper drug regimens but however showed several shortcomings. Insulin remains the cornerstone in treating Type 1 and 2 diabetes but is unfortunately only available as a subcutaneous injection. This in itself has several disadvantages such as time lag between peak insulin levels and postprandial glucose levels, hypoglycaemia, weight gain, peripheral hyperinsulinemia and poor patient compliance due to frequent painful and uncomfortable injections (Takei & Kasatani, 2004:578). An overdose of insulin may evoke severe hypoglycaemia which, in turn, leads to a series of secondary effects such as

the release of growth hormone, catecholamines, glucagon and corticosteroids to mention a few (Hasselblatt & Bruchhausen, 1975:V).

In summary, the ideal for treating and managing diabetes mellitus with insulin therapy will be to, through research and development, find a way to administer a stable insulin by means of a more patient friendly dosage form and still maintain stable and acceptable blood glucose levels. A common goal will be to, in other words, develop a dosage form provoking better patient compliance and thus a better management of diabetes, prolonging the life of the diabetes sufferer.

1.2 Insulin

1.2.1 The discovery of insulin

Many similarities still exist between the approach and performing of research and development of the past and present. The main idea was, and still is, to present data together with ideas and notions underlying the experimental approach. Discussing these ideas and sharing information, together with the aid of new concepts, will benefit future research. The history of the discovery of insulin is a classic (and dramatic) example of how new perspectives are opened up by pursuing the way dictated by experimental results.

It mostly began when a German medical student named Paul Langerhans noted in 1869 that the pancreas contains two distinct groups of cells, the acinar cells and cells that are clustered in islands or islets. He further concluded that the acinar cells secrete digestive enzymes and that the islet cells must perform a second function (Davis & Granner, 2001:1679). In 1889 Joseph von Mering and Oskar Minkowski discovered that the extraction of the pancreas in dogs lead to the induction of diabetes. Minkowski continued his research and in 1893 discovered that diabetes could be prevented by the implanting of tissue from the pancreas under the skin of the abdomen. In 1900 a scientist

named Schulze ligated the pancreatic duct and classified the surviving islets as blood vessels of the same type as the pituitary. Ssobolew suggested in 1902 that pancreatic glands from newborn animals should be used for the organotherapy of diabetes and concluded that this will bring relief for people suffering from diabetes (Hasselblatt & Bruchhausen, 1975:V).

This was proven to be correct when, in the early 1900's, Gurg Ludwig Zuelzer attempted to treat a dying diabetes patient with extracts from the pancreas. The patient improved temporarily but went into a coma and died (Davis & Granner, 2001:1679). It was also substantiated by Minkowski, Sandmeyer, Pfluger and several others that the feeding of pancreas extracts presented with several negative and harmful effects to dogs and humans (Banting *et al.*, 1922:141). In 1911 a student from the University of Chicago named E. L. Scott made another attempt to isolate the mysterious active principle. He used alcoholic extracts of the pancreas on diabetic dogs with very positive results, but lacked the clear measures of blood glucose concentrations and thus his professor considered his findings inconclusive. Between 1916 and 1920 Nicolas Paulesco also found that injections of pancreatic extracts reduced urinary sugar and ketones in diabetic dogs.

All of this research proved to be very inconclusive, but it wasn't until 1921 when a young Canadian surgeon named Frederick G. Banting set out to search for the antidiabetic principle of the pancreas. He assumed that the islets secreted insulin but that the insulin was destroyed by proteolytic digestion prior or during extraction. He then, together with Charles H. Best, attempted to overcome this problem by tying the pancreatic duct, causing the acinar tissue to degenerate, leaving the islets intact. The remaining tissue was then extracted using ethanol and acid, and they obtained an extract that effectively reduced blood glucose levels in diabetic dogs (Davis & Granner, 2001:1679; Banting *et al.*, 1922:141-141). Banting *et al.* concluded in their preliminary report that this extract or "concentrated internal secretion", as they referred to it, was clinically significant in the treatment of diabetes mellitus. They also concluded that after administration this extract-
a) markedly reduced blood glucose levels, even to normal values; b) abolished glycosuria; c) eliminated acetone bodies from the urine (ketoacidosis); d) lead to an

increased utilization of carbohydrates; and e) showed a definite improvement in the general condition of patients and the patients themselves reported to “a subjective sense of well being and vigour” (Banting *et al.*, 1992:146). This was in fact the discovery of the peptide hormone insulin.

Banting, Best and MacLeod had an arrangement with the Eli Lilly Company to manufacture and distribute insulin in North America and there were also possibilities that the British Medical Research Council might have had a comparable role in Europe. The large-scale production had a problematic start, as the insulin appeared to be inadequate in both quantities and potency. Also the lack of an adequate simple test to measure the characteristics of insulin made the situation more complicated and troublesome. Another complication was that the protection afforded by the patent on insulin was inadequate and easily circumvented. Before the end of 1922 numerous rival patents on insulin were filed in America, leaving the Toronto inventors without any control over the price and quality of insulin production. The original patent filed so poorly defined insulin that the United States government’s Hygienic Laboratory had difficulty in legally adding insulin to its list of regulated substances (Liebenau, 1990:95).

Within a few years of its discovery, insulin was purified and crystallized. Abel Sanger established the amino acid sequence of insulin in 1960 and this lead to the complete synthesis of the protein in 1963 and the elucidation of its three dimensional structure by Hodgkin and co-workers in 1972. Insulin was also the first hormone for which a radioimmunoassay was developed (Davis & Granner, 2001:1680), but this was only one of the first instances of insulin.

Insulin was the first protein to be sequenced completely, one of the first proteins to be crystallised in pure form, one of the first proteins of which the structure was investigated with X-ray crystallography and the first protein to be chemically synthesised. It was the first Biotech drug (Bhatnagar *et al.*, 2005:199).

1.2.2 Pharmacology and function of insulin

1.2.2.1 The endocrine pancreas

As mentioned earlier, the pancreas contains islands of cells or islets. An adult human has approximately one million islets interspersed throughout the pancreatic gland. Thus far at least four hormone-producing cells have been identified and is summarised in Table 1.1.

Insulin is one of the hormones found in these hormone-producing cells. Other hormones include islet amyloid polypeptide (IAPP or amylin), of which the metabolic function is still uncertain, glucagon, the hyperglycaemic factor that facilitates glycogen secretion, somatostatin, an inhibitor of secretory cells, and pancreatic peptide, a small protein that modulates digestive processes by a mechanism not clarified as yet (Karam, 1998:684).

Table 1.1: Types of islet cells and their secretory products (Karam, 1998:685).

Cell types	Approximate percentage of islet mass	Secretory products
A cell (alpha)	20	Glucagon, pro-glucagon
B cell (beta)	75	Insulin, C-peptide, pro-insulin, islet amyloid polypeptide (IAPP)
D cell (delta)	3 to 5	Somatostatin
F cell (PP cell)	< 2	Pancreatic polypeptide (PP)

1.1.1.1 Insulin chemistry

Human insulin is a small protein with a molecular weight of 5807.7 Da. It contains 51 amino acids arranged in two chains, A and B, which are linked by disulfide bridges. There are species differences in the amino acids of both chains (Karam, 1998:685).

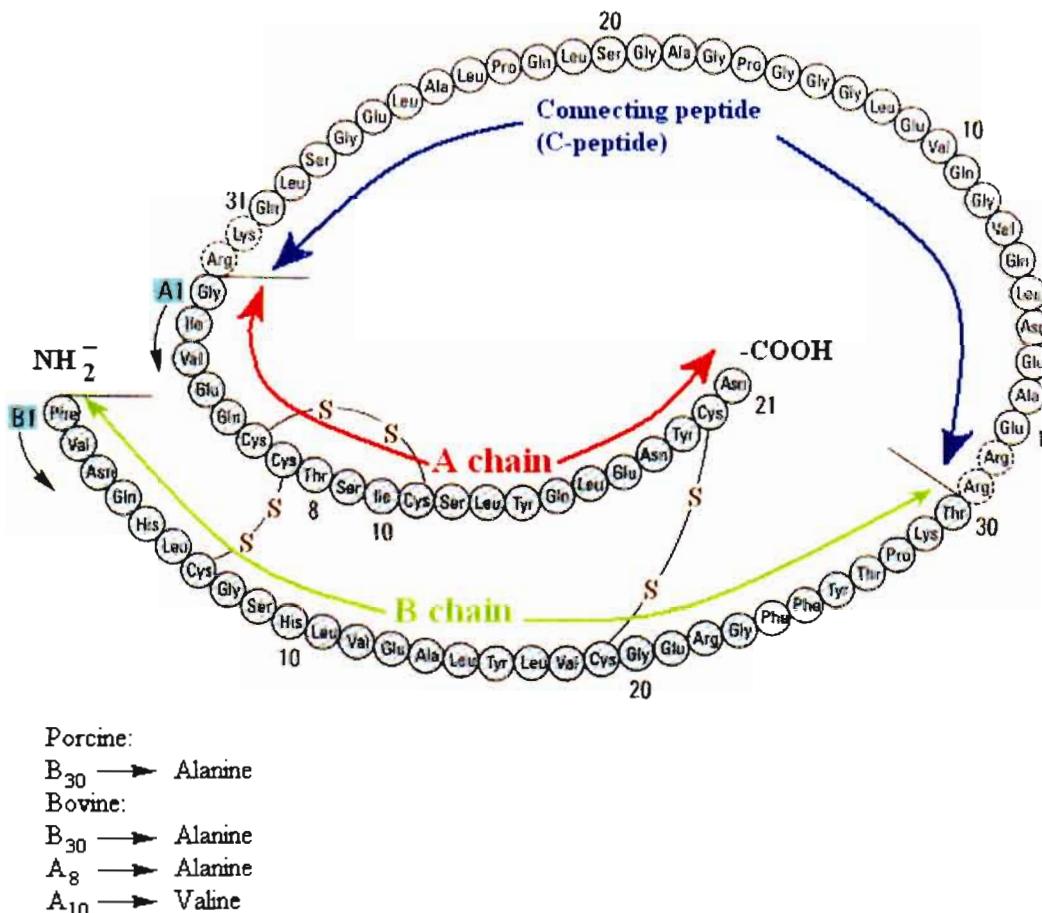


Figure 1.1: Human pro-insulin. Chains A and B (shaded peptide chains) constitute insulin. Species differences in chains A and B are noted in the inset (Derived from Davis & Granner, 2001:1680; Karam, 1998:686).

Insulin is synthesised by the β cells of the pancreatic islets from a single chain precursor consisting of 110 amino acids called *prepro-insulin*. The 24-amino acid N-terminal signal peptide of pro-insulin is cleaved off after translocation through the membrane of the rough endoplasmic reticulum to produce pro-insulin. At this stage the molecule folds and the disulphide bonds are formed. In the conversion of pro-insulin to insulin in the Golgi complex the remaining connector or C-peptide and four amino acids are removed by proteolysis, which then produces the two chains (A and B) of the insulin molecule, which contains one intrasubunit and two intersubunit disulphide bonds. The A chain constitutes 21 and the B chain 30 amino acids which result in a total molecular weight of 5734 Da (Davis & Granner, 2001:1680).

1.2.2.3 Insulin synthesis, secretion and degradation

Insulin production, storage and secretion by the β cell and ultimate degradation by its target tissues have been studied to a great extent and have served as a model for the study of other cell types in the pancreatic islet. The β cells constitute between 60.0 and 80.0% of islets of Langerhans and are responsible for the synthesis of insulin. As mentioned in the previous section, insulin is synthesised as a single-chain precursor in which the A and B chains are connected by the C peptide and is known as pro-insulin. The unique sequence of the amino acids in pro-insulin gives it the ability to penetrate into the lumen of the rough endoplasmic reticulum from where it is transported to the Golgi complex and packed into small secretory granules along with anabolic insulin enzymes. In the secretory granules, pro-insulin is converted to insulin and the synthesis is therefore almost complete at the time of secretion whereby equimolar amounts of C peptide and insulin are released into the blood stream. Small amounts of pro-insulin and des-31,32 pro-insulin are also released from the β cells. As only a part of the insulin which is secreted is not completely synthesised, it means that up to 20.0% of the immunoreactive insulin in plasma is in fact pro-insulin (Davis & Granner, 2001:1682).

The β cells of the islets also facilitate insulin secretion. Insulin is released at a low basal rate but this increases to a much higher rate in response to a variety of stimuli, in

particular glucose. Other stimulants include sugars (i.e., mannose) and certain amino acids (i.e., leucine, arginine). Vagal activity is also recognised. When a stimulus such as glucose is recognised, potassium diffuses down its concentration gradient by means of ATP-gated potassium channels (ATP closes the channels), maintaining the intracellular potential at a fully polarised, negative level. Insulin secretion at this time is minimal but as the glucose levels become elevated, ATP production increases and potassium channels close and the cell becomes depolarised and calcium channels open and calcium enters the cell. As the influx of calcium increases, more insulin is secreted (Karam, 1998:687).

Under normal fasting conditions the pancreas secretes about 40.0 µg (1.0 international unit [IU]) of insulin per hour into the hepatic portal vein resulting in a portal blood concentration of 2.0 to 4.0 ng/ml (50.0 to 100.0 µIU/ml) and a peripheral blood concentration of 0.5 ng/ml (12.0 µIU/ml). The half-life of insulin in plasma is about 5 to 6 minutes in healthy subjects and patients without complicated diabetes, and the half-life of pro-insulin is about 17 minutes. The degradation of insulin occurs mainly in the liver, kidneys and muscle whereby 50.0 % of the insulin that reaches the liver via the hepatic portal vein is totally degraded and never reaches the general circulation. In the kidneys insulin is filtered by the renal glomeruli and is reabsorbed by the tubules where it is degraded and as a result removes about 35.0 to 40.0 % of the circulating insulin. Degradation is presumably by the hydrolysis of the disulfide connections between the A and B chains by means of glutathione insulin transhydrogenase (insulinase) and is followed by proteolysis. Insulin is also degraded by peripheral tissues such as fat, but to a much lesser extent (Davis & Granner, 2001:1683; Karam, 1998:685).

1.2.2.4 Different types and duration of action of insulin preparations

Commercially available insulin preparations present with a number of differences, including purity, concentration, solubility, time of onset, duration of therapeutic activity and species of origin. Over the last decade human insulin has replaced many types of animal insulin and in 1997 over 22 different human insulin formulations were available on the market in the USA. Depending on the constitution, there are mainly four types of

insulin available- a) ultra short acting, with very fast onset and short duration of action; b) short-acting, with very fast onset of action; c) intermediate-acting; and d) long-acting, with slow onset and a longer duration of action (Karam, 1998:689). A summary of currently available insulin preparations on the market is given in Table 1.2.

Ultra short acting and short acting insulins are available as clear solutions at neutral pH and contain small amounts of zinc to improve their stability and prolong shelf life. All the other preparations have been modified to give a prolonged therapeutic effect and are turbid suspensions at a neutral pH containing either protamine in a phosphate buffer (NPH insulin) or varying concentrations of zinc in acetate buffer (ultralente and lente insulins). At present, conventional subcutaneous insulin therapy mainly consists of split-dose injections consisting of mixtures of short-acting and intermediate-acting insulin (NPH or lente), or multiple preprandial doses of short-acting insulin together with any of the three insulin suspensions (NPH, lente or ultralente) for prolonged duration of action for overnight basal insulin levels (Karam, 1998:689).

Table 1.2: Several insulin preparations currently available in the USA (Karam, 1998:690; Masharani, 2004:1165)

Preparation	Species source	Concentration
Ultra-short-acting insulins		
Insulin lispro (Humalog, Lilly) Insulin aspart (Novolog, Novo Nordisk)	Human analog (recombinant) Human analog (recombinant)	U100 U100
Short-acting insulins "Purified"		
Regular Novolin (Novo Nordisk) Regular Humulin (Lilly) Regular Iletin II (Lilly) Velosulin (Novo Nordisk)	Human Human Pork Human	U100 U100, U500 20.0ml U100 U100
Intermediate-acting insulins "Purified"		

Lente Humulin (Lilly)	Human	U100
Lente Iletin II (Lilly)	Pork	U100
Lente Novolin (Novo Nordisk)	Human	U100
NPH Humulin (Lilly)	Human	U100
NPH Iletin II (Lilly)	Pork	U100
NPH Novolin (Novo Nordisk)	Human	U100
Premixed insulins		
%NPH/%regular		
Novolin 70/30 (Novo Nordisk)	Human	U100
Humulin 70/30 and 50/50 (Lilly)	Human	U100
%NPH/%insulin lispro		
Humalog mix 75/25 (Lilly) 70 % insulin aspart protamine/ 30 % insulin aspart (Novolog Mix 70/30)	Human analog (recombinant)	U100 (insulin pen, prefilled syringes, 5x3.0ml cartridges)
Long-acting insulins		
"Purified"		
Ultra-lente Humulin (Lilly)	Human	U100
Insulin glargine (Lantus, Aventis)	Human analog (recombinant)	U100

1.2.2.5 Oral hypoglycaemic agents

Being a successful treatment for diabetes, insulin always had and still has several shortcomings. Because of its extensive proteolytic degradation it deems to be unsuitable for oral administration and because of its socially undesirable subcutaneous administration the (re)search for oral hypoglycaemic agents presented an exciting new field of study.

1.2.2.5.1 Sulfonylureas

This group of hypoglycaemic agents was accidentally discovered in 1942 when scientists noted that some sulphonamides caused hypoglycaemia in experimental animals. After extended research the first clinically useful sulfonylurea, 1-butyl-3-sulfonylurea was used in the treatment of diabetes. Even though the drug was later recalled because of its

adverse effect on bone marrow, it lead to the development of the whole range of sulfonylureas (Davis & Granner, 2001:1701).

Sulfonylureas used today are divided into two main groups or generations of agents. These compounds are arylsulfonylureas with substitution at the *para*-position of the benzene ring and of one nitrogen residue of the urea groups. These agents causes hypoglycaemia by stimulating the secretion of insulin from the pancreatic β cells but their effects on diabetes are more complex. Sulfonylureas also decrease the hepatic clearance of insulin and cause a reduction in serum glucagon levels. The mechanism by which glucagon concentrations are reduced is unclear but may however, involve indirect inhibition due to the enhanced release of both insulin and somatostatin, which inhibits A cells secretion and hence glucagon secretion (Davis & Granner, 2001:1702; Karam, 1998:697).

Another proposed mechanism by which sulfonylureas reduce blood glucose is with insulin that tends to bind to tissue receptors with sulfonylurea treatment in patients with type II diabetes. This causes an increase in the receptor number of insulin and such an action of the sulfonylureas would then potentiate the effect of the patients' low insulin levels as well as the exogenous insulin levels. These observations strongly suggest an indirect action of sulfonylureas on insulin action. Sulfonylureas include tolbutamide, chlorpropamide, tolazamide and acetohexamide and second-generation sulfonylureas being glipizide, glyburide and glimepiride (Karam, 1998:697).

Although not related to sulfonylureas, repaglinide and nateglinide are also used in the treatment of diabetes and poses the same mechanism of action as sulfonylureas. Repaglinide is a derivative of benzoic acid and stimulates insulin release by closing ATP-dependent potassium channels in the pancreatic β cells. Nateglinide is derived from D-phenylalanine and works on the same mechanism as repaglinide but promotes a more rapid but less sustained release of insulin than other oral antidiabetic agents (Davis & Granner, 2001:1705).

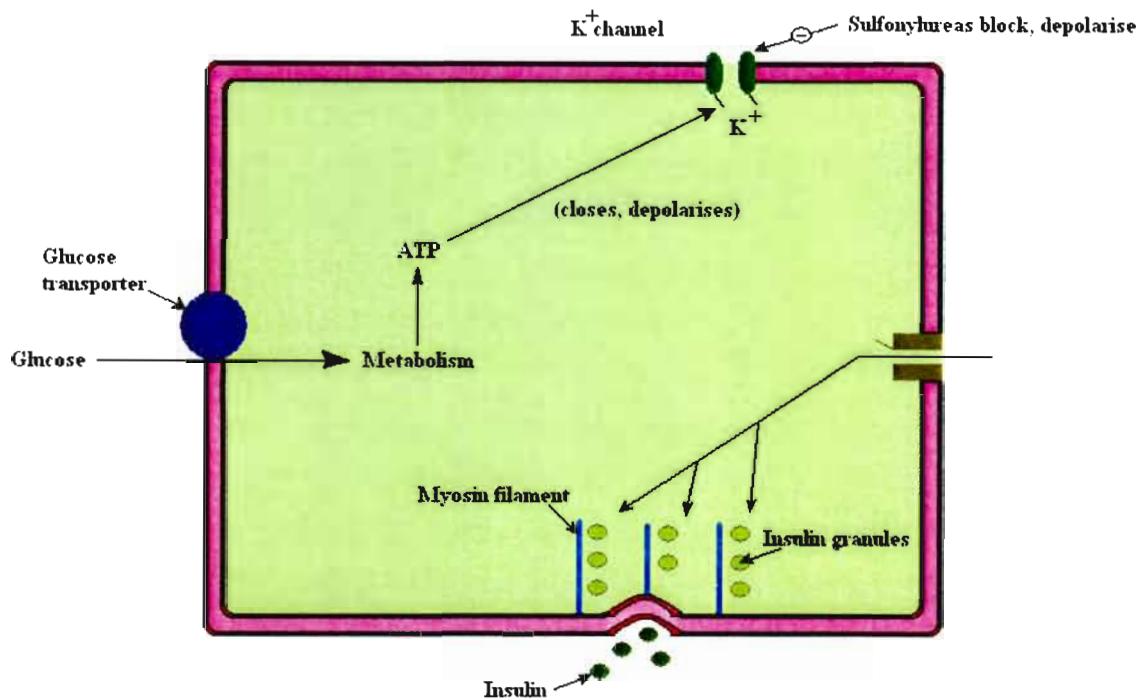


Figure 1.2: A model for the release of insulin from the pancreatic β cell by glucose and by sulfonylurea drugs (Adapted from Karam, 1998: 687).

1.1.1.1.1 Biguanides

Another group of oral hypoglycaemic agents called the biguanides also made their debut in the late 1950's. One of the vastest utilised biguanides is metformin. This antihyperglycaemic drug does not cause insulin secretion nor does it cause hypoglycaemia or have any effect on the secretion of glucagon, cortisol, growth hormone or somatostatin. Metformin mainly reduces blood glucose levels by decreasing hepatic glucose production and by increasing insulin action in muscle and fat tissue. A full and specific explanation for metformin's lowering effect on hepatic glucose production remains unattained but the preponderance of data suggests that it reduces gluconeogenesis (Davis & Granner, 2001:1705).

Other proposed mechanisms of action of biguanides are that they inhibit the absorption of glucose from the gastrointestinal tract, reduce plasma glucagon levels and directly stimulate glycolysis in tissues. It is therefore that biguanides are indicated for patients suffering from “insulin resistance syndrome” and also therefore tend to suffer from obesity (Karam, 1998:700).

1.2.2.5.3 Thiazolidinedione derivatives

This group of agents are selective agonists for nuclear peroxisome proliferator-activated receptor-gamma (PPAR γ). When these drugs bind to PPAR γ , insulin-responsive genes that regulate carbohydrate and lipid metabolism are activated and insulin resistance in the peripheral tissue is alleviated. Thiazolidinediones decrease circulatory glucose by transporting it into muscle and adipose tissue by enhancing the synthesis and translocation of specific forms of the glucose transporter proteins. Rosiglitazone and pioglitazone are both given once daily and are metabolised by cytochrome-P450 enzymes in the liver and up to date no clinically significant drug interactions between them and other drugs have been reported (Davis & Granner, 2001:1706).

1.2.2.5.4 α -Glucosidase inhibitors (aldose reductase inhibitors)

Acarbose is an oligosaccharide analogue of microbial origin that binds 1000.0 times more avidly to the intestinal disaccharides, such as α -glucosidase, than natural carbohydrates. This hampers the absorption of most carbohydrates including starches, dextrans, maltose and sucrose but not lactose and hence limits the postprandial rise in blood glucose levels and results in an insulin-sparing action. Unfortunately, the unabsorbed carbohydrates reach the lower bowel and, together with the bacteria, produces gas, which in turn causes flatulence, abdominal bloating and diarrhoea in many patients (Karam, 1998:701).

1.3 Oral delivery of insulin

1.3.1 Strategies for oral insulin delivery

What makes the oral route so attractive for drug administration is the mere simplicity and comfort associated with it. Insulin injections are both painful and uncomfortable, not to mention the risk of infection because of the constant re-use of needles. Moreover, oral preparations are usually cheaper to manufacture, as they don't need to be sterile (Fasano, 1998:152). Another great advantage of this route of administration is that peroral insulin mimics the endogenous secretion of insulin much more closely. Insulin is absorbed from the intestine and reaches the liver via the hepatic portal vein and can thus have a direct effect on the hepatic glucose production. This is very desirable since the liver plays a crucial role in the maintenance of blood glucose levels by taking up and storing energy from carbohydrates in the form of glycogen. Parenteral administered insulin primarily targets the peripheral tissue rather than the liver and the pharmacokinetics does not replicate the normal dynamics of endogenous insulin secretion (Fasano, 1998:152). Oral insulin administration therefore avoids peripheral hyperinsulinemic effects, which are also considered to be a very important contributor in the development of arteriosclerosis (Marschütz *et al.*, 2000). Unfortunately, in the case of insulin, less than 0.5 % of the orally administered dose is absorbed from the GI tract (Allémann *et al.*, 1998:178) and less than 0.1 % reaches the central bloodstream intact (Kumar *et al.*, 2006:117; Lowman *et al.*, 1999:933), which presents a number of problems that need to be overcome.

There has been an explosion in the field of creating and researching new oral delivery systems over the past few years. More recently more attention has been given to the delivery of larger more complex drug molecules such as proteins and hormones, which has become available through recombinant DNA technology. This also attracted a lot of attention towards increasing the intestinal permeability for these larger molecules and other molecules already in use that has low bioavailability due to poor absorption (Fasano, 1998:152). Despite this fact, many large molecule drugs are still generally

administered through injection, and insulin, the most widely utilized protein drug, being amongst those (Carino & Mathiowitz, 1999:250).

There exist three transepithelial pathways for drug molecules to cross from the intestinal lumen to the bloodstream- a) transcellular (i.e. through the cell) carrier mediated active or facilitated transport; b) transcellular passive transport; and c) paracellular (i.e. between adjacent cells) transport (Fasano, 1998:152). Insulin is mainly absorbed via transepithelial absorption, but is limited due to enzymatic degradation (Bai & Chang, 1995:1171). Many strategies have been investigated to improve the bioavailability of peroral administered proteins, insulin included. These strategies focused mainly in counter acting or hurdling one or more of the barriers to intestinal absorption, which will be discussed in the following paragraphs.

1.3.2 Barriers limiting peptide bioavailability and ways to overcome it

Notwithstanding being the favourite route for drug administration, the oral route presents a number of difficulties especially in the delivery of peptide and protein drugs such as insulin. Unfavourable gastric pH ranges and mucosal barrier conditions prevent or hamper both drug stability and absorption. This is mainly due to the epithelium lining in the gastrointestinal tract that acts as a strategic interface between the external and internal environment of the human body. It presents both a physical barrier that restricts peptide flux to paracellular and transcellular pathways only, and a biochemical barrier, that includes mechanisms of metabolism, and apically polarised efflux systems. Oral active peptide drugs are also restricted by their unfavourable physicochemical properties, which render them vulnerable to poor intestinal mucosal permeation and extensive enzyme degradation (Pauletti *et al.*, 1996:4).

Intestinal peptide and protein delivery presents with several challenges and involves systematic case-by-case investigations in proteolytic degradation mechanisms and kinetics, physiological factors and biochemical considerations. These barriers have been

researched and several methods of overcoming them have been discovered and investigated.

1.3.2.1. The metabolic barrier

Digestion of dietary macromolecules such as proteins is one of the main functions of the GI tract. It is therefore designed to have several digestive processes in place to ensure that peptides and proteins are catalysed by means of various enzymes, specialized in the hydrolysis of peptide bonds (Gangwar *et al.*, 1997:155; Pauletti *et al.*, 1996:4). Proteolytic enzymes, or proteases, break down peptide drugs in the GI tract and there are three different groups of proteases, divided according to their location in the GI tract- a) luminal enzymes, b) membrane bound and c) cytosolic enzymes (Lee, 1986:87). Luminal enzymes often initiate the degradation of orally administered peptides and include the endopeptidases trypsin and α -chymotrypsin. These endopeptidases degraded peptides are further digested by a variety of exopeptidases such as aminopeptidases, carboxypeptidases, di- and oligopeptidases that are embedded in the brush border membrane of the intestinal epithelium and also the lumen of the gut (Bai, 1994:898; Lee *et al.*, 1991:304).

Amino-oligopeptidase (AP-N), a zinc containing protein is the most abundant peptidase in the intestinal and renal microvilli. It has a very broad substrate specificity when a free α -amino group is available and it is in the L-configuration. Dipeptides, in contrast, containing D-amino acids or Pro at the carboxy- or amino-terminus are relatively stable. Intracellular peptide and protein metabolism may also occur following endocytosis and uptake into lysosomes where proteolytic degradation is mainly catalysed by the cathepsins and may involve exo- and endopeptidases activity (Langguth *et al.*, 1997:41). A cytosolic enzyme called insulin-degrading enzyme is mostly responsible for the metabolism of insulin in the intestine (Bai & Chang, 1995:1173). A number of ways to improve oral peptide bioavailability and to overcome enzyme degradation have been investigated and researched. Chemical modifications of several peptide drugs to improve oral bioavailability have proven to be successful. Various tools and methods available

for limiting peptide bond hydrolysis, *in vivo*, ranges from simple additions that chemically protect the peptide bond from catabolism, or by replacing it or by changing it in such a way that it can't be recognised by the protease enzyme applicable. Such chemical modifications include N-methylation, C_α-methylation, replacement with a D-amino acid or replacement of the peptide bond altogether.

Such modifications have proven to be very efficient and are not restricted to protease inhibitors. Practically every class of biologically active peptide known has been modified in such a way (Pauletti *et al.*, 1997:239). The modification of drugs lead to the formulation of prodrugs. Prodrugs are molecules that have to undergo several chemical or biochemical conversions (to the active form thereof) in order for it to exert a pharmacological effect. Prodrugs are designed to overcome some of the limitations that exist with parent drugs such as poor solubility, poor chemical and/or enzymatic stability, poor membrane permeability, rapid metabolic elimination by the liver or kidneys and poor or lack of target delivery. Examples of prodrugs that have yielded very good results are those synthesised for an orally active platelet fibrinogen receptor (GP IIb/IIIa) antagonist. The ACE inhibitor benazepril and desmopressin (DDAVP). These prodrugs have shown to be more lipophilic than their parent peptides and significantly less reluctant towards enzymatic degradation (Gangwar *et al.*, 1997:152).

Enhanced peptide drug absorption may also be facilitated by the co-administration of penetration enhancers to alter the membrane permeability and protein inhibitors to restrain the activity of proteolytic enzymes (Lee, 1986:87). Penetration enhancers such as detergents, fatty acids and bile salts are meant to permeabilise the mucus and epithelial layers and open the intercellular tight junctions. Disadvantages of penetration enhancers are high toxicity and local cell damage. One penetration enhancer that does not compromise the overall intestinal integrity is a protein named Zonula occludens toxin or ZOT. This protein is produced by the bacteria *Vibrio cholera*, which infects the intestine and causes serious disease. ZOT acts specifically on the actin filaments of the zona occludens and is only effective in areas in the jejunum and ileum but not the colon. The use of ZOT is known to be safe, reversible, time- and dose dependent and limited to the

relevant tissue. ZOT does not affect the viability of the intestinal epithelium *ex vivo* and does not completely abolish the intestinal transepithelial resistance. *In vivo* studies have shown ZOT to increase the absorption of insulin by 10 fold in rabbit ileum and jejunum and had no effect in the colon (Carino & Mathiowitz, 1999:252; Fasano, 1998:155).

In recent studies it was also proven that buffered polyacrylic acid polymer dispersions at pH 6.7 inhibits the activity of trypsin, α -chymotrypsin and carboxypeptidase A and the cytosolic leucine aminopeptidase (Lueßen *et al.*, 1996:126). The use of enteric coatings to protect peptides from protease enzymes are widely utilized in the pharmaceutical industry with much success and have been researched extensively in attempts to deliver insulin orally. This will be discussed later on in this chapter. One major factor in overcoming the enzymatic barrier is the co-administration of enzyme inhibitors, or more specifically, protease inhibitors (Carino & Mathiowitz, 1999:253). An enzyme inhibitor can be defined as any compound that slows down or blocks enzyme catalysis. Many of the drugs used today function on the mechanism of enzyme inhibition or inactivation and therefore work on major metabolic pathways in the body. It is important that an enzyme inhibitor should be totally specific for the one target enzyme. Since this is rare, if attained at all, highly selective inhibition is a more realistic objective (Silverman, 1992:147). Inhibiting enzymes that break down or metabolise peptide drugs in the gastrointestinal tract may lead to an increase in peptide drug absorption (Lee *et al.*, 1986:87).

Unfortunately some of the major problems of enzyme inhibitors are their high toxicity, especially in chronic drug therapy, and their limited activity, which is mainly for luminal enzymes with preference to endopeptidases. Since it is difficult to achieve a direct interaction between the enzyme and inhibitor, protease enzymes imbedded in the mucus layer or located in the apical membrane of the epithelial cells are not easily affected. This holds true particularly for high molecular weight structures for which diffusion is hampered by the mucus layer such as soybean trypsin inhibitor, aprotinin and Bowman-Birk inhibitor (Lueßen *et al.*, 1996:118).

Delivering insulin orally by means of enteric coatings has been researched extensively. pH Sensitive materials have been used to encapsulate insulin in order to protect it from degradation and facilitate site-specific release in the colon. A number of different polyacrylic (Eudragit)-coated gelatin capsules loaded with insulin have been studied in rats and showed statistically significant reductions in blood glucose levels compared to normal fasted animals (Touitou & Rubinstein, 1986:95). These results encouraged further investigation and other studies followed using different polyacrylic coatings (Eudragit L100 and S100) where the S100 showed slow release of insulin in the ileum. However, these formulations only showed a 10% decrease in blood glucose levels but in combination with the protease inhibitor, aprotinin, the effects were enhanced significantly (Morishita *et al.*, 1993:68).

1.3.2.2 The physical barrier

Apart from digestion, the GI tract is also designed to impair the entry of pathogens, toxins and undigested macromolecules. Compared to keratin, which provides a very sufficient physical barrier to the skin against bacteria, the intestinal mucosa uses biochemical and physiological mechanisms to compliment this physical barrier (Daugherty & Mrsny, 1999:144).

The stomach wall (Figure 1.3) is composed of the same four basic layers as the rest of the GI tract but with slight differences. In the mucosa there is a layer of simple columnar epithelium that contains many narrow openings that extend down into the lamina propria called gastric pits. At the bottom of the pits are the gastric glands (Tortora & Anagnostakos, 1987:740). The gastric glands contain five different types of cells that have different secretory functions. Goblet cells secrete protective mucus, parietal cells secrete hydrochloric acid (HCl), principal or chief cells secrete pepsinogen (inactivated pepsin), argentaffin cells secrete serotonin, histamine and autocrine regulators, and endocrine cells secrete the hormone gastrin into the blood stream (Van de Graaf, 2000:529). Three additional layers are located deeper than the mucosa. The submucosa is composed of areolar connective tissue. The muscularis has three layers of smooth

muscle: an outer longitudinal layer, a middle circular layer and an inner oblique layer that is distinctive of the stomach. The serosa is made up of simple squamous epithelium (mesothelium) and areolar connective tissue, which is part of the visceral peritoneum that extends upward to the liver as the lesser omentum. At the greater curvature of the stomach the visceral peritoneum extends downward as the greater omentum and hangs over the intestines (Tortora & Derrickson, 2006:913).

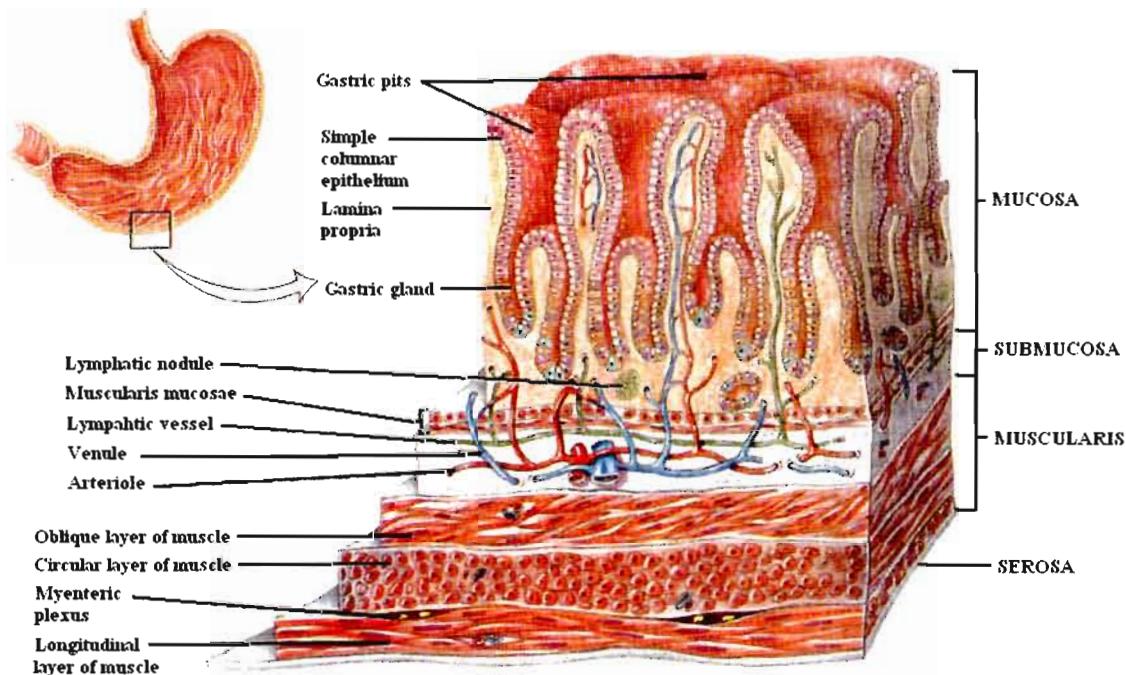


Figure 1.3: The mucosa of the stomach: Peptide drugs, absorbed in the gut, must pass through the mucosa and submucosa to be absorbed into the bloodstream (Tortora & Derrickson 2006:913).

Intestinal epithelial cells provide this physical barrier by means of tight intercellular junctions or zona occludens and a lipid matrix. Peptide permeation across the cell barrier to the paracellular and/or transcellular routes is restricted due to the unique organization

and architecture of the intestinal mucosa (Figure 1.3). The paracellular pathway is an aqueous extracellular pathway across the epithelium and has gained a substantial amount of attention for the delivery of peptide drugs because it is believed that it is deficient in proteolytic activity (Gangwar *et al.*, 1997:149). Epithelial folds, or villi, possess microvilli in the brush border (Figure 1.4). Microvilli are uniform 1.0 μm finger-like projections that increase the absorptive area of the intestine by approximately two orders of magnitude but in this also hinder the absorption of proteins as it also contains digestive enzymes. In addition to this the top of the epithelial layer consists of the glycocalix, which consists of a layer of sulphated mucopolysaccharides, and a mucus layer consisting mainly of glycoproteins, enzymes, electrolytes and water. The glycocalix and mucus layer greatly contributes to the physical barrier to oral protein and peptide delivery (Carino & Mathiowitz, 1999:251).

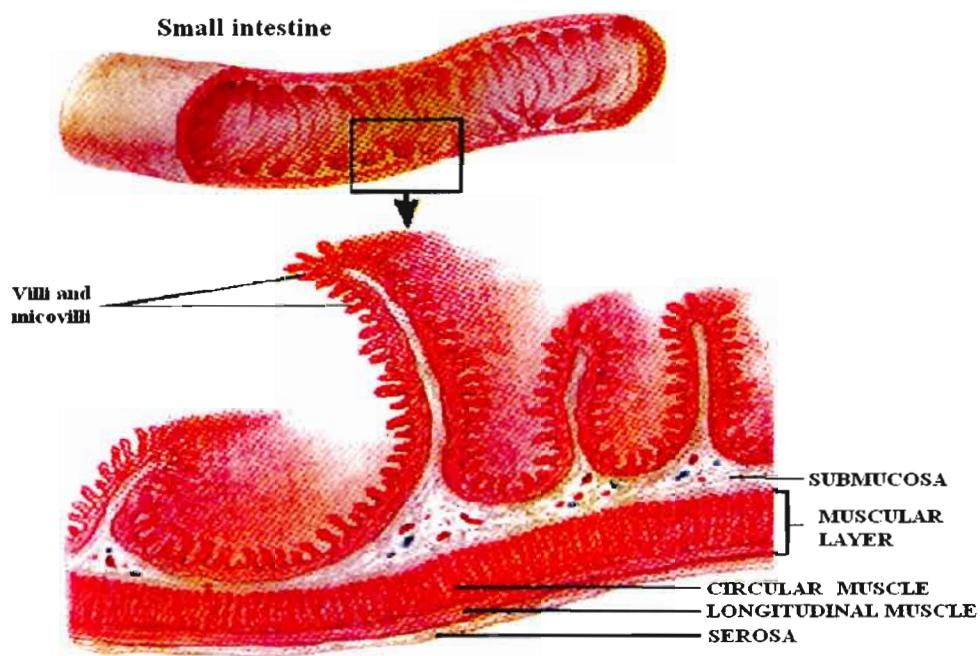


Figure 1.4: The interior lining of the small intestine containing epithelial folds named villi, which also contains microvilli (Shier *et al.*, 1999:688).

It is of absolute importance to overcome mucosal membrane penetration as a barrier in order to achieve oral absorption and systemic availability. Permeation can be passive or carrier-mediated and paracellular or transcellular, the latter being more common for peptide drugs (Lipka *et al.*, 1995:122).

1.3.3 Recent developments in oral insulin delivery

Oral insulin delivery has come a long way since Banting processed the first administration of insulin to human patients in the 1920's. Almost all barriers limiting insulin bioavailability have been addressed and many of them have been overcome with much success. Combined efforts in research have opened various doors for oral insulin delivery making the once thought to be a dream a part of reality. The main goal of insulin delivery devices is to protect the sensitive insulin from proteolytic degradation in the stomach and upper intestine. Subsequent is an overview of recent developments in the effort to produce a practical and useful oral delivery system for insulin.

1.3.3.1 Hydrogel polymers

pH-responsive poly(methacrylic-g-ethylene glycol) (P(MAA-g-EG)) hydrogels have been synthesised and loaded with insulin at different doses. These hydrogels are cross-linked co-polymers of poly(methacrylic acid) that is grafted by ethylene. These co-polymers exhibit pH-sensitive swelling behaviour due to the formation/dissociation of interpolymer complexes stabilized by hydrogen bonding between the carboxylic acid protons and the etheric groups on the grafted chains. The pK_a of PMAA is 4.8 and thus at neutral pH the MAA groups are almost completely deprotonated. Hydrogen bonds that are present at a low pH dissociate at a near-neutral pH value resulting in swelling of the network structure.

The acidic environment of the stomach causes the gels to remain in its complex state and insulin cannot diffuse through the membrane because of the small mesh size. When the particles reaches higher, more favourable pH ranges, the gel transforms and the

complexes dissociate immediately and the network pore size increase rapidly and consequently releases the insulin. The results of these experiments showed clearly that the insulin was protected by the hydrogels from enzyme degradation. Blood glucose levels were decreased significantly for up to 8 hours and showed that biologically active insulin was delivered via the oral route (Lowman *et al.*, 1999:933-936).

Another pH-sensitive hydrogel was synthesized by means of dispersion polymerisation of methacrylic acid and methoxy-terminated poly(ethylene glycol) monomethacrylate. Tetra(ethylene glycol) dimethacrylate is added to provide crosslinks in the network structure. This hydrogel showed similar characteristics to those of P(MAA-g-EG) and showed to inhibit the activity of Ca^{2+} - dependent proteolytic enzymes thus furthering the decrease in insulin degradation (Peppas & Kavimandan, 2006:191).

Dorkoosh *et al.* (2002:433) studied the release of insulin from superporous hydrogel (SPH) and superporous hydrogel composite (SPHC) delivery systems. Factors such as peptide stability in SPH and SPHC and the integrity of insulin in the polymeric matrix of SPHC was also studied and determined. The studies revealed that the insulin was almost completely released from the polymers and that the rate of release was faster from SPH than SPHC due to the more porous structure of the SPH polymer. Insulin showed to be stable under different environmental conditions (ambient temperature, 37.0 °C, light and darkness and at pH 3.2 and 7.2). It was also found that there was no covalent binding of insulin with the polymeric SPHC matrix and was determined by Fourier transform infrared spectroscopy (FTIR). Drug release profiles showed an insulin release of at least 80.0% within 40 minutes and a 90.0 % release after 90 minutes. Overall the SPH and SPHC delivery system showed appropriate and promising *in vitro* results and is awaiting further *in vivo* studies (Dorkoosh *et al.*, 2002:433).

Another insulin release profile was studied when hydrogels with a phospholipid polar group, namely 2-methacryloyloxyethyl phosphorylcholine (MPC), which was prepared from two aqueous solutions of polymers, namely water-soluble poly[MPC-*co*-methacrylic acid-(MA)] (PMA) and poly[MPC-*co*-*n*-butyl methacrylate (BMA)] (PMB).

The hydrogel was formed by spontaneous physical cross-linking without any chemical reactions or physical stimuli and showed a controllable insulin release even through a pH change in the medium. Several aspects were examined during the study including mechanical strength, erosion of the hydrogel caused by polymer dissociation, and the release of insulin. This was all done at a neutral pH (6.8). Three parameters of the MPC polymer was investigated namely the molecular weight, the PMA/PMB ratio and the polymer concentration inside the hydrogel. Changing these parameters brought a change in the dissolution rate and the dissolution could therefore be controlled and modified.

It was found that the hydrogel with a PMA/PMB ratio of 3/7 was mechanically the strongest and the hydrogel with a ratio of 5/5 presented with the slowest dissolution. Polymers of different weight percentage (wt.%) were also prepared to see what effect it will have on dissolution. An increase in wt.% showed an increase in complex modulus E and viscosity over a range of 5.0 to 70.0 wt.%. Hydrogels with a wt.% of 5.0 and 10.0 showed fast continual dissolution rates, because the carboxyl groups that formed hydrogen bonds became ionised and dissociated, thus releasing the insulin. However, hydrogels over 20.0 wt.% showed a high water uptake from the media, because of the higher polymer concentration, which causes the polymer network in the hydrogel to be rearranged and resulting, in part, the hydrogel to dissolve. Only after that the gel showed continual dissolution. Insulin release compared to the hydrogel dissolution was also investigated. The dissolution percentage of the 5.0 and 10.0 wt.% hydrogels with the respective PMA/PMB ratio showed a 30.0 and 70.0 % insulin release over 4 hours. It was observed that with the 5.0 wt.% the insulin release was almost the same but slightly lower than the erosion of the hydrogel, when the PMA/PMB ratio was higher than 5/5. In the case of ratios 8/2 up to 6/4 the release was faster than erosion, indicating that the release was much more dependent on the diffusion of the insulin. Hydrogels with ratios of 8/2 and 6/4 have a slower dissolution and a lower mechanical strength. For the 10.0 wt.% hydrogels the release of the hydrogel was always slightly slower than the erosion thereof and it was obvious that the release was regulated by erosion rather than diffusion of the hydrogel (Nam *et al.*, 2004:261 – 269).

Even more recently poly(ethylene glycol) dimethacrylates (PEGMA) were synthesized by etherification reactions of different molecular weight poly(ethylene glycol) (PEG) with methacrylic acid (MAA) in the presence of an acid catalyst. The PEG molecular weights ranged from 400.0 to 4000.0 Da and the MAA was co-polymerised by suspension polymerisation to obtain pH-sensitive hydrogel microparticles. The hydrogels were loaded with mono-component insulin (80.0 IU/kg) and the *in vivo* experiment was done on diabetes-induced male Sprague-Dawley rats. It was found that poly(PEGDMAA4000:MAA) co-polymeric microparticles had the highest efficiency to reduce blood glucose levels in diabetic rats. The dose was also sufficient to control blood glucose levels between 100.0 and 300.0 mg/dl (Kumar *et al.*, 2006:123).

1.3.3.2 Transferrin mediated insulin delivery

Transferrin (Tf) is a large single protein with a molecular weight of ~ 80.0 kDa and Tf-receptors (TfRs) have been widely investigated and researched for the receptor mediated delivery of anti-cancer agents by enhancing the transport of drugs across the blood brain barrier and even more recently, across the intestinal epithelium. TfR is a homodimer that is expressed by many cell types including human intestinal cells. TfR binds to ironbound Tf (holo-Tf) on the cell surface and stimulates the cell that results in the uptake of the Tf-TfR complex. A change in the endosomal pH causes the ligand-bound Tf to loose its bond to iron and the complex is recycled to the cell surface where the iron-free Tf (apo-Tf) is released. TfRs are present in vast amounts in human gastro-intestinal epithelium and Tf is resistant to tryptic and chymotryptic degradation (Peppas & Kavimandan, 2006:193).

Insulin has been conjugated to Tf via disulphide linkages and resulted in 5- to 15-fold increase transport across Caco-2 cell monolayers. This enhancement is specific for the conjugated insulin and occurs without the opening of tight junctions and is thus desirable in terms of toxicity and damage to the epithelium (Shah & Shen, 1996:1306). Insulin-Tf (ins-Tf) conjugates were consequently administered orally to diabetic rats and the results showed a slow but prolonged hypoglycaemic effect. Ins-Tf administered subcutaneously

prolonged the maintenance of low blood sugar by at least four times compared to unconjugated insulin, thus showing to be beneficial in terms of frequency of the dosage. The mechanism of this action is yet to be clarified. It can be concluded that Tf conjugated with insulin is able to resist proteolytic attack and reach the systemic circulation intact and thus increases the bioavailability of insulin (Peppas & Kavimandan, 2006:193; Xia *et al.*, 2000:597).

A comparison between monomeric and oligomeric Tf as a carrier for oral insulin delivery has also been investigated. Tf-oligomer induced cross-linking of TfR was studied in polarized Caco-2 cells in order to determine whether or not an alteration in the intracellular trafficking of the receptor-ligand complex do occur, which will increase the transcellular drug delivery. An *in vivo* study, done on stretozotocin induced diabetic rats, showed that conjugated insulin and Tf-oligomer was more effective in reducing blood glucose levels than the conventional monomeric insulin-Tf complex (Lim & Shen, 2005:274).

1.3.3.3 Insulin-transferrin conjugates in complexation hydrogels

Kavimandan *et al.* (2006:3854) went yet one step further and developed an oral formulation that incorporates insulin bioconjugates together with complexation hydrogels as a delivery vehicle. These hydrogels were classified as environment-sensitive hydrogels and specifically showed pH-dependent swelling behaviour. These hydrogels were synthesized by the use of UV-initiated free radical polymerisation of MAA and methoxy-terminated poly(ethylene glycol) monomethacrylate (PEGMA), which consisted of cross-linked PMAA grafted with PEG chains and was thus designed as P(MAA-g-EG). Insulin was conjugated with Tf mainly for reasons discussed in subsection 1.3.2.2 by means of a modified procedure of Shah and Shen (1996:1310).

There was one concern in these experiments and that was the physical size of the ins-Tf conjugate. The hydrodynamic radius of the Tf molecule alone is ~ 40.0 Å and that of insulin is ~ 20.0 Å. This may have caused the diffusion coefficient of the conjugate

molecules to be lower than for non-conjugated insulin molecules for this *in vitro* method using Caco-2 cells. The primary mechanism of transport was identified as being Tf-mediated transcytosis and the hydrogel polymers increased the permeation of the insulin conjugate and the combination of the hydrogel and the Tf was reported to have a 22-fold net increase of insulin permeability (Kavimandan *et al.*, 2006:3853). These results prove to be very significant and are definitely a stepping-stone on the road to increasing the efficacy of formulations used in peroral insulin administration.

1.3.3.4 Nano- and microparticles

As mentioned previously, insulin is degraded by intestinal enzymes and poorly absorbed because of its hydrophilic properties. Another way of overcoming these problems is to physically protect or “shield” insulin from catabolic enzymes and at the same time improve absorption or membrane permeability. A substantial amount of research has been done on the investigation in the use of different particles with certain significant characteristics in the aid of insulin absorption in the gastrointestinal tract.

Currently, particles used in drug delivery fall into two classes, namely nanoparticles, ranging in size from 10.0 to 1000.0 nm, and microparticles ranging in size between 1.0 to 1000.0 μm . For oral delivery it seems that smaller particles, such as nano- and microparticles, are absorbed more efficiently because the absorption of particles in the intestine increase with a decrease in size and an increase in hydrophobicity (Fasano, 1998:152).

Insulin’s physicochemical properties are not ideal for the preparation of nanoparticles as it is slightly soluble in water and practically insoluble in alcohol, chloroform and ether. It is also amphoteric and thus forms salts with weak acids and bases. Novel insulin nanoparticles have been manufactured from a neutral insulin solution cross-linked with glutaraldehyde to form solid nanospheres of about 200.0 nm in diameter. In 1982 Oppenheim and co-workers administered these nanoparticles to rats and mice and it was reported to be partially absorbed in the GI tract of mice as well as in diabetic and normal

rats. Lowering blood glucose levels by between 15.0 and 20.0% in some animals indicated that the absorbed insulin had to remain biologically active. It was also mentioned that these insulin nanoparticles appeared to exert a slower but more pronounced response compared to Actrapid®, a similarly administered commercial formulation, but the amount of nanoparticles administered did not make it viable as a commercial product (Allémann *et al.*, 1998:179).

Poly-alkylcyanoacrylate nanocapsules (<300.0 nm) surrounded by a biodegradable isobutylcyanoacrylate polymeric wall has been used as a colloidal insulin delivery vehicle in a subsequent study. This was done because of previous findings by Oppenheim that nanocapsules of less than 200.0 nm in diameter passes through the intestinal mucosal, most probably by means of paracellular transport. It was found that insulin entrapped in nanocapsules remain stable and biologically active as it decreased glycemia in streptozotocin-induced diabetic rats (Michel *et al.*, 1991:1).

It was also found that the duration, and not the intensity, of the therapeutic response was dependent on the amount of capsules administered, which lasted from 6 up to 20 days after a single oral administration. Using an insulin/polymer ratio of 0.25 units (mg of polymer)⁻¹ it was shown that glycemia was normalized from day 2 up to day 6, 9 and 20 with doses of 12.5 IU/50.0mg of polymer/kg, 25.0 IU/100mg of polymer/kg and 50 IU/200mg of polymer/kg respectively (Michel *et al.*, 1991:3). Up to now the biological activity of insulin absorbed after oral nanocapsule administration has not been evaluated nor characterised. In order to do this researchers administered Humalog® loaded nanocapsules (50IU/kg) to streptozotocin-induced diabetic rats. Results obtained showed that orally administered nanoparticles showed very heterogeneous absorption but it was however concluded that oral administered nanocapsules deliver noticeable amounts of insulin to the central circulation of rats and that insulin was absorbed very quickly (peak plasma values after 1 hour). The reason for the fast absorption was that Humalog® is a hexameric form of cross-linked insulin and quickly dissociates into insulin dimers and then into monomers, the active form of insulin (Cournarie *et al.*, 2002:327).

Another way of synthesising poly(isobutylcyanoacrylate) nanoparticles is by *in situ* polymerisation using an aqueous pluronic acid solution. It was discovered that a decrease in particle size was found in accordance with an increase in pluronic acid concentration. A 2.5% pluronic acid solution produced nanoparticles of 85.0 nm in average diameter and a 59.0% intra-particular insulin load. A prolonged blood glucose-lowering effect was achieved on streptozotocin-induced diabetic rats from 6 to 72 hours (Mesihha *et al.*, 2004:289).

Insulin microspheres have been synthesized using Eudragit L100, a pH-dependent co-polymer that is soluble at pH 6.0 and above. The formulation was also loaded with several specific enzyme inhibitors such as aprotinin, Bowman-Birk inhibitor, soybean trypsin inhibitor, protease inhibitor and chymostatin. *In vitro* insulin release and enzyme degradation was studied. It was found that about 80.0% of the insulin was entrapped in the microspheres and the insulin release was found to be 94.3 ± 4.2 and $94.0 \pm 5.9\%$ at 120 and 180 minutes respectively. The amount of non-degraded insulin was also measured and was found to be much higher with the co-administration of the relevant enzyme inhibitors (Morishita *et al.*, 1992b:12; Morishita *et al.*, 1991:4).

Several of these formulations were also compared in the aspect of enteral insulin delivery. Three types of insulin microspheres were synthesised as previously mentioned but with the addition of Eudragit S100. Insulin microspheres made from Eudragit S100 seemed to have insulin absorption in a lower part of the ileum whereas Eudragit L100 microspheres released the insulin more distal in the duodenum. In conclusion it was found that insulin microspheres made from Eudragit L100 and containing enzyme inhibitors showed a larger blood glucose lowering effect than Eudragit S100 microspheres containing enzyme inhibitors (Morishita *et al.*, 1992a:36).

1.3.3.5 Alginate microspheres

Silva and co-workers (2006:1) have recently developed another form of microparticles called alginate microspheres to be utilised in oral insulin delivery. These microspheres

were prepared through emulsification/internal gelation. An Actrapid® formulation in water (1:2 v/v) was mixed with a polymer (giving a 0.1 % insulin solution) which was then dissolved in a 2.0 % (w/v) sodium alginate solution. A 5.0 % (m/v) CaCO₃ suspension was then added to the alginate giving a 7.3 % Ca/alginate mass ratio. After homogenisation the mixture was dispersed into paraffin oil (30.0 % internal phase ration, v/v) containing 1.0 % Span® 80 and stirred at 400.0 rpm. After 15 minutes of emulsification 20.0ml of paraffin oil containing glacial acetic acid (acid/Ca molar ratio of 3.5) was added to the w/o emulsion and stirring continued to prevent solubilisation of the CaCO₃. Microspheres with sizes ranging from 21.0 to 288.0μm were obtained. Encapsulation of insulin varied from 65.0 to 79.0 % and insulin release was found to be as high as 75.0 % after just 5.0 minutes and a slower cumulative release of up to 80.0 % after 2.0 hours (Silva *et al.*, 2006a:6).

In a further investigation Silva and co-workers (2006:153) used different anionic polymers as additives to the formulation such as cellulose acetate phthalate (CAP), Eudragit® L100, sodium carboxymethylcellulose (CMC) polyphosphate (PP), dextran sulphate (DS) and cellulose sulphate (CS). CAP, PP and DS showed no significant increase in mean diameter sizes of the microspheres but Eudragit® L100, CMC and CS showed a size increase of mean diameter nearing 100.0μm. A majority of the anionic polymer additives showed an increase in insulin encapsulation with the highest efficiency being with DS and CS. It was also found that DS and CS causes insulin to be encapsulated in the centre of the microspheres which further enhances the protection and shielding of insulin from degrading enzymes. PP, DS and CS showed to be the most effective additive for insulin release (Silva *et al.*, 2006b:151).

A chitosan coating was also added in this study and it was found that chitosan only slightly increased particle size and SPAN factor but no changes were found in the morphology of the microspheres. Insulin encapsulation however, showed to have an increase of greater than 100.0 % with the chitosan coating when DS (1.0% w/w) was used. This could be explained by the partial loss of additive polymer which decreased the theoretical polymer mass and changed the theoretical insulin content to higher values.

The chitosan coating however, had no effect on the insulin release when DS was used but showed an increase when PP was used as an additive at pH 1.2 (Silva *et al.*, 2006b:155).

Another alginate-chitosan formulation has recently been developed but this time as nanoparticles. This was done by adding 200.0 IU of insulin to the alginate solution to provide a pre-gel and then 7.5ml of a 18.0mM calcium chloride solution was dropped for 60.0 minutes into 117.5ml of the alginate solution (0.063% w/w) stirring at 800.0 rpm. A chitosan solution (0.05 to 0.09% w/w) was then added, drop wise, to the pre-gel for 120.0 minutes and nanoparticles were held with a 30-minute additional stirring to improve curing.

The nanoparticles were then separated with centrifugation and analysed. Particle size increased with a decrease in chitosan to alginate ratio. From ratios 6:1 to 3.3:1 showed an increase of 764.0 to 2209.0 nm in diameter. Taken the ratio being 4.3:1 the optimum pH range for the particle size was 4.7, giving an average particle size of 797.0 ± 48.0 nm. Using DSC and FTIR analysis it was determined that new chemical entities were indeed formed and that significant insulin entrapment did occur (Sarmento *et al.*, 2006:3).

1.3.3.6 Water-in-oil-in-water emulsions

A theory that emulsions could be utilised in the enhancement of peptide bioavailability has been proposed as long ago as the 1960's. Since then it was discovered that, being the nature of emulsions, it promotes the absorption of water-soluble substances normally not absorbed in the intestine and also protects peptide drugs from proteolysis (Morishita *et al.*, 1998:190)

Water-in-oil-in-water (W/O/W) emulsions consisting of an inner aqueous phase, oily phase and outer aqueous phase was prepared using a basic emulsification process. The inner aqueous phase comprised of different gelatin concentrations to ensure that entrapped insulin does not escape the inner phase. The oily phase consisted of 5.0 % lecithin, 20.0 % Span 80 and 75.0 % soybean oil and the outer aqueous phase also

contained 3.0 % Tween 80. Smaller emulsion particles were made by filtration through a 0.45 μ m filter. Enteral loops were used to administer the emulsions to the stomach, ileum and colon of male Wistar rats. Results showed that significant blood glucose lowering effects were attained in the ileum and colon and thus it was concluded that W/O/W emulsions containing 5.0 % gelatin in the inner aqueous phase improved insulin absorption in the ileum and colon (Matsuzawa *et al.*, 1995:1723).

Further study incorporated and evaluated the effect of lipoidal absorption enhancers such as fatty acids. Different insulin emulsions containing soybean oil, triolein or trilinolein were prepared. When 3.0 % limonene or 3.0 % menthol was added to the triolein emulsion it was shown that the blood glucose lowering effect was enhanced in the ileum but not in the colon. Emulsions containing 2.0 % fatty acids such as oleic acid, linoleic acid and linolenic acid exerted a strong blood glucose lowering effect and the efficacy thereof was more pronounced in the colon than in the ileum and no tissue damage was observed after close light microscopic examination (Morishita *et al.*, 1998:196).

1.3.3.7 Co-administration with specific enzyme inhibitors

Considering the nature of insulin and being such a sensitive peptide towards physical and biochemical barriers it was elementary that certain aids should be implemented to counteract these effects in order to elevate the effects of insulin. Two major areas that have to be addressed are those of enzyme inhibition and methods to enhance absorption. In previous sections enzyme inhibitors and absorption enhancers have been mentioned in aiding existing oral insulin delivery formulations. But more specifically, insulin formulations consisting mainly of enzyme inhibitors have also been developed in order to elevate insulin bioavailability. The complete mechanism and types of enzyme inhibition has been described by Oberholzer (2003:13).

Polymer-inhibitor conjugates have proven to exert a strong protective effect on insulin. Carboxymethylcellulose (CMC) was conjugated with Bowman-Birk inhibitor by direct linkage through means of a condensation reaction mediated by 1-ethyl-3,3-

dimethylaminopropyl carbodiimide hydrochloride (EDAC). A second polymer-inhibitor complex consisted of CMC conjugated with elastatinal, whereby the elastatinal was firstly coupled with the spacer putrescine dihydrochloride (tetramethylenediamine .2HCl) that provided the NH₂ groups for the covalent bond to the polymer. The polymer to inhibitor ratio was 20:1 (w/w) in both cases. These conjugates were then homogenised with a polycarbophil-cysteine conjugate, insulin and mannitol and then compressed into 2.0 mg microtablets enteric coated with polymethacrylate. These tablets were evaluated *in vitro* for the degree of insulin protection against enzyme degradation as well as for its insulin release profile and also *in vivo* for its blood lowering effect on diabetic mice. An artificial intestinal fluid containing trypsin, chymotrypsin and elastase was used to evaluate the insulin formulation and showed that microtablets only containing insulin showed a 91.6 ± 7.4% degradation of insulin whereas the formulation containing the enzyme inhibitors showed 49.7 ± 5.5% of the insulin to remain intact over a period of 3.0 hours. The *in vivo* study also showed a significant 20.0 to 40.0% decrease in basal blood glucose levels in the diabetic mice (Marschütz *et al.*, 2000:1471).

Co-administration of several protease inhibitors with insulin into the small and large intestine has also proven to be of significance. Insulin (80.0 IU/kg) in phosphate buffer (pH 7.4) was co-administered with Na-glycocholate (20.0 mM), aprotinin (10.0 mg/ml), bacitracin (10.0 mM and 20.0 mM) soybean trypsin inhibitor (1.5 and 10.0 mg/ml) and camostat mesilate (20.0 mM). Data was analytically compared to those found with an intravenous insulin (0.1 IU/kg) injection. Although no significant increase in pharmacological availability was noted in the small intestine it was found however that in the large intestine Na-glycocholate and camostat mesilate showed a pharmacological availability of 5.1% and that of bacitracin was 3.5%. These results correlated well with the blood glucose lowering effect observed in these experiments as Na-glycocholate, camostat mesilate and bacitracin showed a significant lowering in blood glucose levels. It was concluded that co-administration of enzyme inhibitors with insulin is useful in aiding the absorption of insulin in the large intestine (Yamamoto *et al.*, 1994:1496).

1.3.3.8 Muco-adhesive intestinal patches

Another way of shielding insulin from enzymes and enhancing its absorption is by keeping the peptide close to the absorption surface away from the lumen of the intestine by means of intestinal patches. Patches such as these have been manufactured using a mixture of Carbopol 934, pectin and sodium CMC with a weight ratio of 1:1:2, respectively. The patches were compressed under approximately 0.5 to 4.0 tons of pressure by means of a hydraulic press. These patches were approximately 400.0 μ m thin and had a radius of between 1.0 to 4.0mm. Bovine insulin was added to the patches and administered at a dose range of 1.0- 10.0 IU/kg. A significant adhesion force of 1.0 to 2.7 N/cm² was observed between the patch and the mucosa for over 4.0 hours. More than 90.0 % of the insulin was released over a period of 4.0 hours from the muco-adhesive side of the patches. Patches co-administered with sodium glycocholate showed a significant dose dependent blood glucose lowering effect. Patches containing doses of 5.0 and 10.0 IU/kg decreased blood glucose levels by 60.0 and 75.0 % respectively (Whitehead *et al.*, 2004:41).

1.3.3.9 Chitosan and its derivatives as absorption enhancers

Muco-adhesive polymers, similar to muco-adhesive patches, have been used especially in combination with enzyme inhibitors, to enhance the bioavailability of peptide drugs such as insulin. Bernkop-Schnürch and co-workers have developed a drug delivery system based on polymer-enzyme inhibitor complexes that provides specific enzyme inhibition from enzymes such as α -chymotrypsin (Bernkop-Schnürch & Apprich, 1997:247; Bernkop-Schnürch & Göckel, 1997:6), aminopeptidase (Bernkop-Schnürch & Marschütz, 1997:184; Bernkop-Schnürch *et al.*, 1997b:917), trypsin (Bernkop-Schnürch *et al.*, 1997a:17) and elastase (Bernkop-Schnürch *et al.*, 1997c:119). Muco-adhesive polymers deliver drugs and keep the drugs at the site of absorption thus decreasing the distance between the drug and the delivery system as well as the absorptive tissue and finally decrease degradation by luminal protease enzymes (Bernkop-Schnürch *et al.*, 1997a:18).

Some muco-adhesive polymers exert inhibition of luminal proteases to some extent but are not sufficient to be used singularly in dosage forms in order to protect embedded peptides from enzyme degradation. It is therefore necessary, especially with insulin in mind, to co-administer enzyme inhibitors for sufficient enzyme inhibition. One such study incorporated the use of chitosan as muco-adhesive polymer and antipain as a trypsin inhibitor in a unique formulation with insulin. Results showed that the inner matrix of the conjugated carrier completely inhibited proteolysis of insulin for up to 1.5 hours compared to 11.3 ± 9.5 % degradation in tablets not containing the conjugate and thus concluded that the chitosan-anti-pain conjugate sufficiently protects insulin in such a dosage form (Bernkop-Schnürch *et al.*, 1997a:22).

Chitosan has also been covalently linked to 2-iminothiolane to produce chitosan-4-thiobutylamidine (chitosan-TBA). In addition Bowman-Birk inhibitor and elastatinal were also covalently linked to chitosan representing 3.5 ± 0.1 % and 0.5 ± 0.03 % of the polymer conjugate respectively. The conjugate (5.0 mg), together with insulin (2.75 mg), were compressed to chitosan-TBA-insulin tablets which showed a 8.0 hour controlled release and a 60 fold increase in the muco-adhesive/cohesive properties of chitosan because of the immobilisation of the thiol groups on the polymer. *In vivo* studies on non-diabetic rats showed a significant decrease in blood glucose levels over a period of 24.0 hours and a pharmacological efficacy of 1.69 ± 0.42 % compared to subcutaneous injection (Krauland *et al.*, 2003:547).

Hydroxypropylmethyl cellulose phthalate enteric coated chitosan capsules containing insulin 20.0 IU and sodium glycocholate compared to capsules containing only lactose or only 20.0 IU insulin were also administered to non-diabetic rats. Transit times for these capsules were 1.0 to 10.0 hours into the small intestine and 10.0 to 12.0 hours for the colon. 5(6)-Carboxyflourescein was used to track the capsules and monitor the insulin release and it was found that most of the insulin was released after 8.0 hours with a high concentration in the cecal area indicating that most of the insulin was released in the area of the colon. There was a significant effect on blood glucose levels and the chitosan capsules containing the insulin showed a pharmacological availability (PA %) of 3.49 %

compared to capsules containing only insulin or lactose (1.62 %) thus suggesting that chitosan may assist in insulin delivery in the colon (Tozaki *et al.*, 1997:1019).

1.3.3.10 Insulin derivatives

As mentioned previously, it is possible to modify a drug (peptide mimetics) or create a prodrug from an existing drug in order to minimise metabolic degradation. Such a molecule will have several different chemical and physical characteristics and may therefore not be recognised as its true entity and will consequently not be metabolised to such an extent as the original molecule.

Efforts in designing peptide mimetics for insulin have focused on the C-terminal of the B-chain and it was reported in cell culture and animal studies that a decapeptide showed hypoglycaemic activity. It was revealed that the activity of insulin was increased when amino acid residues 6 and 7 in the decapeptide, which correlates to positions 24 and 25 on the B-chain, were converted to their D-isomers. Substitution of D-Phe, D-Ala and D-Tyr at position 24 on the B-chain also increased the receptor binding affinity for the molecule where, in contrast to D-Tyr at the 25 position of native insulin, resulted in lower receptor binding affinity (Bhatnagar *et al.*, 2005:216).

Insulin was modified in such a way that numbers 1 to 8 of the amino acid residues of the decapeptide corresponded to numbers 19 to 26 of the B-chain residues while residues 9 and 10 of the decapeptide corresponded to positions 20 and 21 on the A-chain (Maslov *et al.*, 2002a:261). Substitution by the D-isomers of the 6 and 7 residues of the decapeptide, corresponding to residues 24 and 25 on the B-chain, has been reported to increase and prolong the therapeutic response in streptozotocin-induced diabetic rats. By acylation of the N-terminal it was also found that the time of onset of action was shortened. The efficacy and clinical safety of these drugs has still to be researched and evaluated (Maslov *et al.*, 2002b:103).

Another insulin derivative comprises a single amphiphilic oligomer, covalently linked to the free amino group on the 29-Lys residue on the B-chain, resulting in a substance called hexyl-insulin monoconjugate 2 (HIM2). This research is conducted by the Nobex Corporation and is currently undergoing clinical trials. This modification confers resistance to proteolysis and increased absorption and therefore improves bioavailability. The drug can be encapsulated in hard gelatin capsules and will be administered orally in doses of 0.5 and 0.1 mg/kg corresponding to about 8.0 IU of regular subcutaneous administered insulin. HIM2 is a rapid-acting insulin (therapeutic effect within 30.0 minutes) but also exerts a longer lasting effect for up to 2.0 to 3.0 hours after administration. It can therefore be used to control early post-prandial hyperglycemia without the cause of peripheral hyperinsulinemia, a common occurrence with subcutaneous insulin administration (Bhatnagar *et al.*, 2005:216).

1.3.3.11 Insulin receptor activators

For many decades, Chinese traditional medicine has successfully treated type 2 diabetes, which is associated with insulin resistance. Insulin resistance is possibly due to attenuated signalling from the insulin receptor and thus inhibiting insulin signal transduction and hence insulin release. Termination of insulin signal transduction requires the dephosphorylation of the β insulin receptor and its downstream effector molecules and also increased activity of a substance called protein tyrosine phosphatase 1B (PTP1B) responsible for the negative regulation of the insulin-signalling pathway ultimately resulting in insulin resistance. It is believed that traditional Chinese medicine contains potential PTP1B inhibitors that may be used to enhance insulin sensitivity and treat type 2 diabetes and obesity. After screening the fruit of *Cornus officinalis*, used as traditional medicine, by a high-throughput assay it was found that ursolic acid was the main medicinal compound. Ursolic acid has many important medicinal functions, as it has anti-inflammatory, anti-cancer, hepatoprotective, hypolipedemic, as well as anti-atherosclerotic activity. After several tests it was discovered that ursolic acid actively inhibits PTP1B, TCPTP and SHP2 and several other protein tyrosine phosphatase enzymes (Zhang *et al.*, 2006:1511).

Currently there are no clinical studies done on small molecule inhibitors of PTP1B concerning diabetes and obesity as their efficacy is insufficient and side effects indicate it to be non-viable. However, the pharmaceutical company Merck Co. discovered a dihydroxyquinone derivative named asterriquinone (4-hydroxy-2-furoic acid), which is a potent and selective insulin receptor activator. Further bioconversions of 4-hydroxy-2-furoic acid yielded several products that are active insulin receptor activators and showed a significant blood glucose lowering effect. Currently further studies are being conducted to improve the selectivity of 4-hydroxy-2-furoic acids by regio-selective synthesis of several analogs (Chou *et al.*, 2006:7579).

1.4 Conclusion

Insulin, after its classic and dramatic discovery in 1922 by Banting and Best, was known as a wonder drug but ever elusive in the attempt to formulate it as an oral preparation being more aesthetic and socially acceptable. Research has come a long way in understanding the way in which insulin functions, the manner in which it behaves naturally and when it is being manipulated or physically altered. Even though the reality of a feasible oral delivery system for insulin is not entirely certain, science has come a lot closer in achieving such a marvel. Pharmaceutical technologies such as hydrogel polymers, micro- and nanoparticles, conjugation with absorption enhancers and enzyme inhibitors and several formulations such as intestinal patches and W/O/W emulsions, together with peptide mimetics, have brought us much closer in realising that insulin administered orally can and will be achieved. It is only a matter of time and enduring keen scientific approaches before such a preparation will be used by millions of diabetes sufferers all around the world.

Part I: Insulin Delivery

CHAPTER TWO

Nasal Insulin Delivery

2.1 Introduction

In pursuit of finding a “more tolerable” route of administration for insulin other than subcutaneous (SC) injection, scientists had to look at different approaches, new and existing, to formulate such an alternative insulin delivery system. Several other methods of insulin delivery have been explored and researched with much success, where some of them have reached the consumer market and is still in use today. Some of these delivery systems include- a) continuous subcutaneous insulin infusion by wearing an infusion pump; b) a total or segmental pancreas transplant; c) isolated islet cell transplant; d) implantation of a programmable insulin pump; e) implantation of polymeric capsules that gives continuous or time-pulsed release of insulin; f) implantation of a biohybrid artificial pancreas and g) oral, nasal, rectal and transdermal mechanisms of insulin delivery (Kennedy, 1991:213).

Researching and ultimately formulating such an alternative insulin delivery system was and is not done with ease. Insulin is well known for its low non-parental bioavailability due to poor absorption and vast proteolytic degradation. Thus one must bear in mind with much discernment all possibilities, feasibilities and implications when considering formulating such a delivery system. One attractive route of administration of insulin that is quite viable is the nasal route as it shows rapid absorption of peptide molecules across the nasal membrane with much less enzymatic degradation than in the gastrointestinal tract and also the mere simplicity and comfort associated with it (Dondeti *et al.*, 1995:92).

Several other advantages of nasal drug delivery are:

- a) Rapid absorption which gives higher bioavailability and therefore needs lower doses;
- b) fast onset of therapeutic action;

- c) exclusion of first-pass hepatic metabolism;
- d) exclusion of gastric metabolism and degradation;
- e) no irritation of the gastrointestinal membrane;
- f) risk of overdose is reduced;
- g) non-invasive, therefore, there is a reduced risk of infection and transmission of infectious diseases;
- h) transport of drugs into the central circulation and central nervous system is possible;
- i) formulation requirements are simple;
- j) convenient and simple;
- k) self medicated;
- l) improved patient compliance; and
- m) can be used as an adjunct to an existing product (*Arora et al.*, 2002:968; *Behl et al.*, 1998:96).

Nasal administration of peptide drugs also has its limitations and much research has been done on improving the efficacy of this delivery system. The use of excipients such as viscosity enhancing agents, muco-adhesives, considering solid versus liquid preparations and co-administration of absorption enhancers have all improved the absorption of peptide drugs to a viable standard (*Merkus et al.*, 1996:70). The nasal route is, however, not a viable route for all drugs (*Behl et al.*, 1998:96).

With so many peptide drugs in use and even more in demand it is inevitable that, with their poor bioavailability, methods other than oral and parental administration will be developed and researched. The following is an overview and discussion of nasal insulin delivery, it's barriers and recent developments.

2.2 Biological and pharmaceutical aspects in nasal drug delivery

2.2.1 Anatomy and physiology of the nasal cavity

The physiological function of the nasal cavity, as it is hollow and the main route by which ambient air enters the body, is to condition inspired air by cleaning,

humidifying and warming the air prior to reaching the sensitive alveolar tissue in the lungs. The nasal cavity is divided into approximately equal left and right halves divided by the nasal septum and extend posteriorly to the nasopharynx, while the most anterior part, called the nasal vestibule, opens to the face through the nares or nostrils. Each half consists of a medial wall, formed by the septum, a roof, which is parallel to the hard palate, and an outside wall which is formed by the bones and cartilage of the skeleton of the skull (Hinchcliffe & Illum, 1999:202).

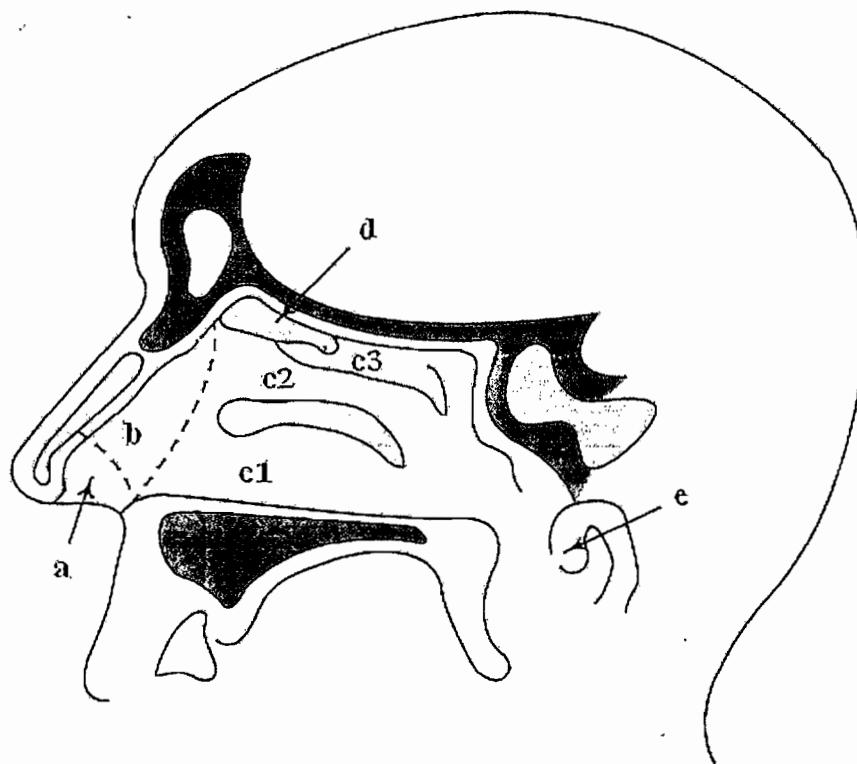


Figure 2.1 A schematic representation of a sagittal section of the nasal cavity showing the a) nasal vestibule, b) atrium, c) respiratory area: inferior turbinate (c1), middle turbinate (c2) and the superior turbinate (c3), d) olfactory region and e) nasopharynx (Arora *et al.*, 2002:969; Ugwoke *et al.*, 2005:1642).

The atrium (Figure 2.1) is an intermediate area in between the vestibule and the respiratory region. This respiratory region, the nasal conchae (turbinate bone), occupies the major part of the nasal cavity and comprises lateral walls dividing it into three sections: the superior, middle and inferior nasal meatuses. The nasal conchae curl out and forms folds, which provide the nasal cavity with a large surface area

compared to its small volume. They also provide support to the mucus membrane lining the cavity and further help to increase the surface area (Ugwoke *et al.*, 2005:1643; Shier *et al.*, 1999:740). The volume of the nasal cavity is between 15.0 and 20.0 ml but its total surface area is about 160.0 cm² of which the vestibular region occupies about 10.0 to 20.0 cm², the respiratory region about 130.0 cm² and the olfactory region about 10.0 to 20.0 cm². Of these the respiratory region is mainly responsible for the absorption of drugs into the circulatory blood stream (Dahlin, 2000:8; Hinchcliffe & Illum, 1999:202).

2.2.2 The nasal epithelium and mucosa

Pseudostratified ciliated epithelium containing mucus-secreting goblet cells covers most of the nasal mucosa. The composition or content of the nasal mucosa, however, differs in the various regions in the nasal cavity (Shier *et al.*, 1999:740). The nasal epithelium cells (Figure 2.2) in the vestibule are stratified, squamous and keratinised with sebaceous glands making the vestibule very resistant against dehydration and noxious environmental substances, and makes permeation of substances difficult. The area of the atrium poses as a transitional epithelium region with stratified squamous cells anteriorly and pseudostratified columnar cells, containing microvilli posteriorly (Ugwoke *et al.* 2005:1643; Hinchcliffe & Illum, 1999:202).

The respiratory region is covered by pseudostratified columnar epithelial cells interspersed with goblet cells and seromucus ducts (openings of subepithelial seromucus glands). Many of these cells possess actively beating cilia with microvilli whereas each ciliated cell contains about 100.0 cilia, that constantly moves the thick mucus layer towards the posterior apertures of the nasal cavity. Both ciliated and non-ciliated cells contain about 300.0 microvilli each (Shier *et al.*, 1999:740). The four main types of epithelium cells in the respiratory region are ciliated columnar-, non-ciliated columnar-, goblet-, and basal cells (Dahlin, 2000:8).

Columnar cells are covered by about 300 microvilli, uniformly distributed over the entire apical surface. These cells are short, slender finger-like cytoplasmic expansions that increase the surface area of the epithelium cells and thus promote exchange processes over and across the epithelium. The microvilli also prevents

dehydration of the surface by retaining moisture, which is essential for ciliary function. One-third of the anterior nasal cavity is non-ciliated as cilia starts occurring just behind the front edge of the inferior turbinate and the posterior part of the nasal cavity as well as the paranasal sinuses, which are also densely covered with cilia. The distribution of cilia corresponds well with the formation of nasal airflow where it is found that the density of ciliated epithelial cells in the nasal cavity is inversely proportional to the linear velocity of inspired air. Consequently there are less cilia in the upper nasal cavity than on its floor (Mygind & Dahl, 1998:5).

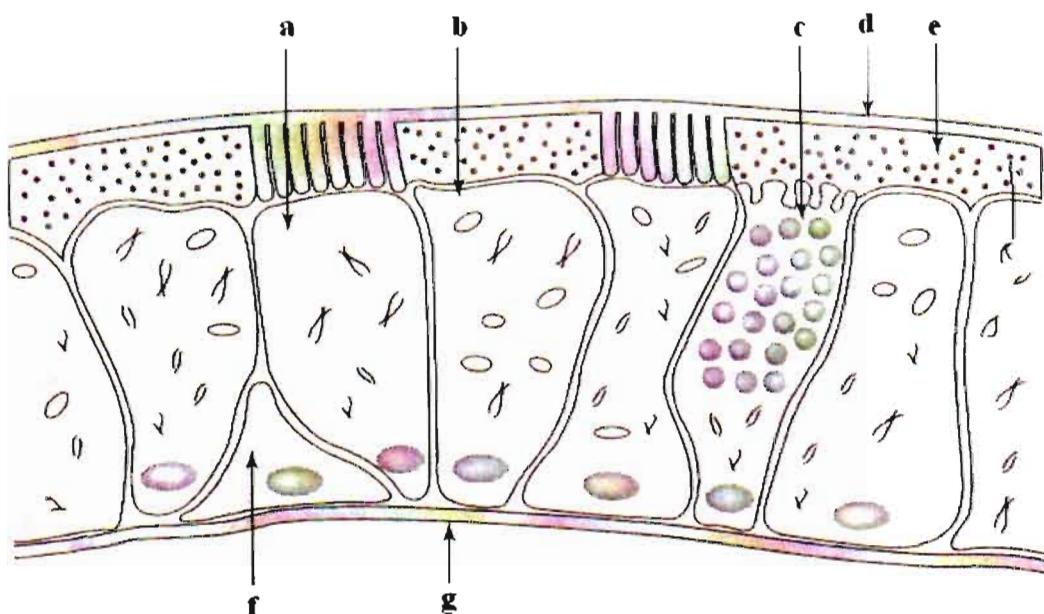


Figure 2.2: The nasal epithelium consists of a) ciliated cells, b) non-ciliated cells, c) goblet cells, d) gel mucus layer, e) sol layer, f) basal cells and g) a basement membrane (Ugwoke *et al.*, 2005:1643; Arora *et al.*, 2002:970).

Goblet cells are characteristic to nasal epithelium cells. There are slight topographical differences where they are more in abundance in the posterior than anterior part of the nasal cavity. The mean concentration of goblet cells in the nasal cavity (4000.0 to 7000.0 per mm²) is similar to that of the trachea and bronchi. Compared to the submucosal cells, the goblet cells contribute little to nasal secretion and little is known about the secretory mechanism of the goblet cells. Surface epithelial cells are joined together by tight junctions and it has been found that fragmentation and discontinuity

occurs where filled goblet cells are located. This may contribute to the absorption of certain aerosol drugs administered intranasally (Mygind & Dahl, 1998:5-6).

The mucus lining itself contains an extensive network of blood vessels thus giving it its pinkish appearance. The mucus also heats up the inspired air passing over it and moisturises the inspired air by evaporation from the mucus lining, adjusting it to the temperature of the body. Serving as a filter, the mucus membrane also entraps dust, pathogens and small particles in the air. These particles are pushed towards the pharynx by the movement of the cilia on the epithelial cells and as it reaches the pharynx it is swallowed. Once the mucus containing the particles reaches the stomach the microorganisms and pathogens are destroyed by gastric juices (enzymes and acids) thus the mucus membrane also protects the body from respiratory and systemic infections (Shier *et al.*, 1999:740).

2.2.3 Nasal mucus secretion and mucociliary clearance

Nasal mucus is secreted by submucosal glands and comprises both mucus cells, which secrete the mucus gels, and serous cells, producing a watery fluid. It has been estimated that the human nose contains about 100 000.0 seromucus glands. The goblet cells also secrete mucus in the form of mucus granules that swell in the nasal fluids. Nasal mucus is a complex mixture of several substances and comprises of about 95.0 % water, 2.0 % mucin, 1.0 % salts, and 1.0 % protein such as albumin, immunoglobins, lysosomes and lactoferrin, and less than 1.0 % are lipids (Ugwoke *et al.* 2005:1643).

Mucin is a glycoprotein and gives mucus its characteristic rheological properties, which makes it ideal in providing a protective coating to the nasal epithelium as well as for its function of mucociliary clearance. Mucus further consists of two fluid layers; a viscous gel layer (mucus or epiphase) floats on a less viscous periciliary fluid layer, or hypophase, which is immediately adjacent to the epithelial surface. Cilia and mucus facilitate the removal of deposited materials from the nasal cavity and this is known as mucociliary clearance (Hinchcliffe & Illum, 1999:203).

The bioavailability of nasal administrated drugs is dependent on and influenced by the contact time between the drug and epithelial tissue. Intranasally administered drugs show a rapid peak in blood plasma concentrations and studies have shown absorption within 5.0 to 15.0 minutes after administration of protein and peptide drugs. Fast nasal absorption of drugs is dependent on two factors namely the permeability of the nasal mucosa and the rate of mucociliary clearance. The permeability of the nasal epithelium is relatively high for large molecules and the path of diffusion is short as it consists only of two layers. Nasal mucociliary clearance, on the other hand, limits the resistance time of nasally administered drugs, therefore decreasing the time for the drug to be in contact with the site of absorption. Mucociliary clearance is also responsible for the rapid clearance of drugs from the nasal cavity to the nasopharynx. The normal clearance half-life of nasal deposited particles in humans is about 20.0 to 30.0 minutes (Hichcliffe & Illium, 1999:205; Marttin *et al.*, 1998:28; Schipper *et al.*, 1991:808).

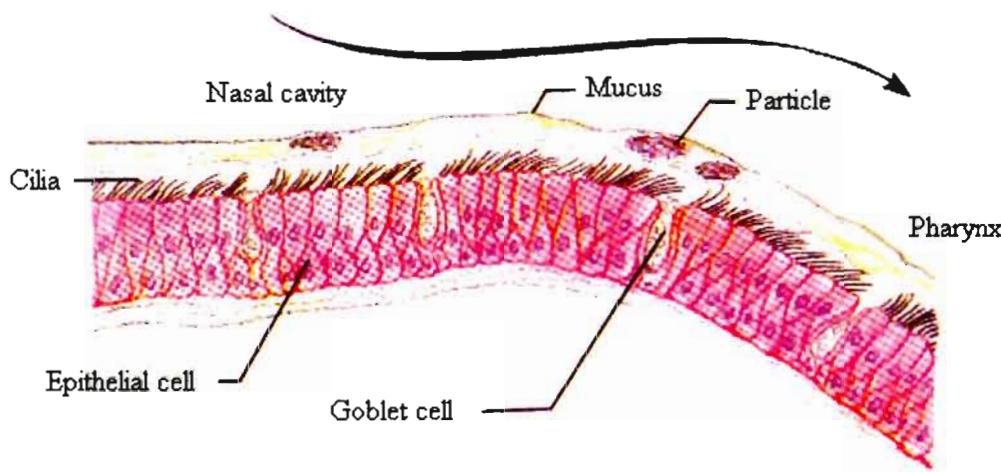


Figure 2.3: Mucociliary clearance is facilitated by the movement of cilia “pushing” trapped particles to the pharynx (Shier *et al.*, 1999:741).

There exist various factors that influence the rate of mucociliary clearance and ultimately influence drug absorption. Several drugs, hormonal changes of the body, pathological conditions and formulation factors alter the rate of mucociliary clearance and have a significant influence on drug permeability (Arora *et al.*, 2002:971).

2.2.4 Enzymatic degradation

Though not as problematic as the enzyme activity of the gastro-intestinal tract, nasal enzymes present a significant barrier to the systemic absorption of protein and peptide drugs (Duchêne & Ponchel, 1993:111). There can be said, however, that most of the work published on nasal enzyme degradation is overestimated as studies that have been done used nasal tissue homogenates, which incorporates enzymes from all mucosal cells and subcellular fractions including lysosomes. Both the amount and type of enzymes exposed in the nasal cavity, as well as the extent of degradation, is therefore mostly exaggerated (Illum, 1999:517).

None the less, the nasal mucosa contains a variety of enzymes that are present in nasal secretions, in the cytosol of cells where they can occur free or membrane bound, and in the lamina propria where they are more specifically associated with glandular tissue. It was found that despite inter-species variations, the profile of nasal drug degrading enzymes in humans seem to be similar (Hinchcliffe & Illum, 1999:203). This is very fortunate as this may reduce inter-patient variance with the use of nasal drug preparations and promote constant bioavailability and makes proper treatment more feasible.

Enzymes that have been identified in the nasal mucosa are monooxygenase, reductase, transferase, esterase and several proteolytic enzymes (Irwin *et al.*, 1995:25). It is thought that mono-oxygenase enzymes, such as cytochrome P450-dependent mono-oxygenases plays an important role in the first line defence mechanism against inhaled xenobiotics, especially environmental pollutants, and volatile and odorous chemicals which are then transformed into harmless products (Hinchcliffe & Illum, 1999:204). Nasal P-450 dependent mono-oxygenase is also known to metabolise other substances such as nasal decongestants, essences, anaesthetics, alcohols, nicotine and cocaine (Behl *et al.*, 1998:109). Aminopeptidases are the main proteolytic enzymes in the nasal mucosa and nasal secretions where membrane bound aminopeptidases account for almost half of nasal enzyme activity, and this most probably serves as a protective mechanism against the incursion of foreign petidagenous and proteinaceous substances (Hinchcliffe & Illum, 1999:204).

Apart from cytochrome P-450 and aminopeptidase, there exist several other enzymes in nasal secretions such as lactate dehydrogenase, oxidoreductases, hydrolases, acid phosphatase and esterases. There has been found that in addition to cytochrome P-450 enzymes, that some oxidative Phase 1 enzymes and Phase 2 conjugative enzyme activity are also present in nasal epithelium (Behl *et al.*, 1998:109).

Insulin undergoes degradation by means of hydrolysis by leucine aminopeptidase in the nasal cavity. It has been reported that insulin is degraded to about 9.0 % of the original dose after being in contact with rat nasal tissue homogenate for just 60.0 minutes without the addition of absorption enhancers or enzyme inhibitors (Behl *et al.*, 1998:109; Hirai, 1981:178b). With the addition of 1.0 % of the surfactant sodium glycocholate there was almost no degradation in the rat nasal mucosa for up to 60 minutes. Other surfactants such as sodium laurylsulfate, -oleate, -taurocholate and sodium cholate also showed extensive inhibition of leucine aminopeptidase in the nasal mucosa (Hirai, 1981b:178). It was later reported that the aminopeptidase inhibitor bestatin also increases the nasal absorption of insulin (Illum, 1999:518).

2.2.5 Limitations of intranasal drug delivery

As with almost any route of administration the nasal route presents certain restrictions or limitations and is not suited for every type of drug. Regardless of the high permeability of the nasal mucosa, many drugs are still not adequately absorbed. There are a few reasons for this such as a lack of adequate aqueous solubility as well as the doses, which are given nasally, are also very small (25.0 to 200.0 µl). Other drugs may also undergo extensive enzymatic degradation or may cause nasal irritation. Drugs that require sustained blood levels are also not suitable for nasal administration. There is currently no conventional way of formulating a sustained release nasal dosage form (Behl *et al.*, 1998:97).

Other limitations are that high molecular weight compounds cannot be delivered through the nasal route; the route is adversely affected by pathological conditions; large interspecies variability is observed; normal defence mechanisms such as mucociliary clearance and ciliary beating affects the permeability of drugs and; there

is a limited understanding of the mechanisms and there are only less developed models at present (Arora *et al.*, 2002:968).

2.2.6 Overcoming the barriers to nasal insulin absorption

It goes without saying that it is of absolute importance to overcome the various absorption barriers in order to achieve significant nasal insulin absorption and hence, bioavailability.

The use of several absorption enhancers has become popular in enhancing the bioavailability of several nasally administered peptide drugs. Using the term “absorption enhancer” refers to any compound that modulates nasal absorption. An ideal absorption enhancer should have several principle characteristics such as fast onset of action, result in transient and reversible modulation of the absorptive properties or physiology of the nasal mucosa and must not be absorbed systemically. The degree of absorption enhancement should also be significant, predictable and reproducible. In the case of diabetes, the substance should also be safe for chronic nasal administration and exert no significant damage to the nasal mucosa (Hinchcliffe & Illum, 1999:207). A list of compounds used in promoting nasal insulin absorption is given in Table 2.1.

An enormous amount of research has gone into enhancing nasal insulin absorption by finding ways to overcome the barriers that prohibits insulin absorption. A discussion of several entities that has been used recently to promote nasal insulin absorption will follow.

Table 2.1 A summary of compounds used in promoting nasal insulin absorption
 (partly adapted from Hinchcliffe & Illum, 1999:209).

Type of compound	Examples	Possible mechanism of action
<i>Absorption enhancers</i>		
Surfactants	Sodium lauryl sulphate, saponin, polyoxyethylene-9-lauryl ether	Disrupt membranes
Fatty acids and derivatives	Sodium caprylate, sodium laurate phospholipids (e.g., didecanoyl-phosphatidylcholine, lysophosphatidylcholine)	Disrupt membranes
Bile salts	Sodium deoxycholate, sodium glycocholate, sodium taurodihydrofusidate	Disrupt membranes, open tight junctions, enzyme inhibition, mucolytic activity
Enzyme inhibitors	Bestatin, amastatin	Enzyme inhibition
Cyclodextrins	Dimethyl- β -cyclodextrin	Disrupt membranes, open tight junctions
<i>Bioadhesive polymers</i>		
Powders	Carbopol, microspheres Chitosan	Reduce nasal clearance, open tight junctions
Liquids	Chitosan, carbopol	Reduce nasal clearance, open tight junctions
<i>Other</i>		
Resins	Sodium polystyrene sulfonate Styrene-divinylbenzene copolymer	Ionic interactions with nasal mucosa
Lipid emulsions	Oil-in-water	Interactions between oil droplets and nasal mucosa

2.2.6.1 Surfactants

A surfactant or surface-active agent is a compound that contains two separate regions namely hydrophilic and hydrophobic. These compounds, because of their dual structure, tend to absorb at interfaces, orientating themselves in such a manner that the regions are associated with the proper solvent or air (Fell, 2002:65)

Surfactants are often used in pharmaceutics in dosage forms as solubilising agents, emulsifying agents, suspension stabilisers or wetting agents. Surfactants are, however, not “inert” excipients as they are able to either increase or decrease the effect on drug absorption. Surfactant monomers are known to potentially disrupt the function and integrity of biological membranes, such as those of the gastrointestinal tract, which may lead to enhanced absorption. Poorly soluble drugs orally administered in the form of tablets or hard gelatine capsules may also benefit from the inclusion of a surfactant/s in the formulation. The surfactants’ ability to reduce the solid/liquid interfacial tension will allow the gastrointestinal fluids to wet the solid phase more effectively, resulting in better disintegration and dissolution of the dosage form (Ashford, 2002:250).

It has previously been mentioned that insulin is degraded to about 9.0 % of the original dose after being in contact with rat nasal tissue homogenate for only 60 minutes without the addition of absorption enhancers or enzyme inhibitors (Behl *et al.*, 1998:109; Hirai *et al.*, 1981b:178). However, with the addition of 1.0 % of the surfactant sodium glycocholate (also a bile salt) there was found that there was almost no significant degradation in the rat nasal mucosa for up to 60 minutes. Other surfactants such as sodium laurylsulfate, -oleate, -taurocholate and sodium cholate also showed extensive inhibition of leucine aminopeptidase in the nasal mucosa (Hirai *et al.*, 1981b:179). Earlier studies by Hirai *et al.* (1978:296) already showed that non-ionic surfactants such as polyoxyethylene-9-lauryl ether (Laureth-9) and saponin at concentrations of 1.0 % both showed an increase in intranasal absorption of insulin in dogs. Anionic surfactants such as sodium- and potassium laurylsulphate, saponin and surfactin (a peptidelipid) promotes nasal insulin absorption but is not as potent as Laureth-9 which showed maximal absorption at a concentration of 0.3 % compared to 0.5 % of saponin (Hirai *et al.*, 1981a:179).

The use of Laureth-9 as nasal absorption enhancer for insulin has been extensively researched. It has also been investigated in aerosolised insulin with patients with insulin dependent diabetes in fasting conditions and non-diabetic controls. Insulin given at 1.0 IU/kg and 1.0 % Laureth-9 was rapidly absorbed (within 15.0 minutes) and lowered blood glucose levels to 50.0 % of basal values after 45.0 minutes in normal subjects compared to 50.0 % in 120 minutes in diabetics. (Hinchcliffe & Illum, 1999:215 – 216; Merkus *et al.*, 1996:71). Unfortunately, in view of the extent of epithelial damage caused by surfactants such as Laureth-9 and sodium laurylsulphate at their effective concentrations, it is doubtful that these compounds will be successfully used in nasal insulin delivery (Hinchcliffe & Illum, 1999:215 - 216).

2.2.6.2 Fatty acids and fatty acid derivatives (phospholipids)

Being the main component of lipids, fatty acids consist mainly of straight chains of even numbers of carbon atoms. Medium chain fatty acids such as sodium caprylate (C8), sodium caprate (C10) and sodium laurate (C12) have been investigated and optimum absorption was observed with 1.0 % sodium caprate, which produced a 10-fold increase in insulin absorption compared to insulin control. Bioavailability of insulin was elevated to 15.0, 30.0, and 50.0 % with caprylate, laurate and caprate salts respectively. Fatty acid derivatives such as phospholipids are potent absorption enhancers for insulin. Phospholipids are insoluble swelling amphiphiles that contain a large polar head group and two long chain acyl groups each joined by a glycerol backbone. Novo Nordisk A/S, Denmark, has screened various phospholipids in order to develop effective insulin absorption enhancers without any local or systemic toxic or immunological effects. It was found that the medium chain phospholipids, didecanoyl-L- α -phosphatidylcholine or DDPC, administered in a microemulsion formulation with vegetable oil, showed to be non-irritant whilst retaining its absorption-enhancing efficacy (Illum, 2002:1188; Hinchcliffe & Illum, 1999: 217).

DDPC has proven to give a repeatable, consistent increase in the bioavailability of insulin of between 8.3 and 13.2 %, when compared to insulin IV, which has an inter-subject variability of 40.0 %. Insulin pharmacodynamics also closely simulated

normal physiological insulin secretion and action post-prandial than with that of SC injection. The blood glucose lowering effect was slower than with IV insulin but faster than SC administered insulin. Acute administration of intranasal insulin formulations containing DDPC showed to be more effective than insulin administered intravenously (IV) in reducing post-prandial blood glucose elevations in non-insulin dependent diabetes sufferers. Unfortunately however, in a two-month trial done comparing intranasal and SC administration, the same formulation showed poor efficacy and metabolic deregulation in insulin dependent diabetes patients. Serum insulin concentrations increased more rapidly but also decreased more rapidly with nasal administration compared to SC administration. Also because of the higher dose (20.0 times that of SC administration), the bioavailability of the DDPC formulation was also lower (Pantiroli, 1998:84; Merkus et al., 1996:72).

2.2.6.3 Bile salts and bile salt derivatives

Bile salts are amphiphilic molecules consisting of distinct non-polar and polar regions. In earlier studies of bile salts to improve the bioavailability of insulin it was found that the most hydrophilic bile salt, ursodeoxycholate hardly affected insulin absorption whilst sodium deoxycholate, the most lipophilic compound, showed to be very potent. It was also found that conjugation with glycine or taurine made the compound less lipophilic but did not affect its potency. The results indicated that the lipophilicity of the steroid nucleus is the main determinant of the absorption enhancing activity of bile salts. Nasal application of 0.5IU/kg insulin and 1.0 % sodium deoxycholate showed a blood glucose lowering effect of 50.0 % within 10.0 to 20 minutes in normal subjects and bioavailability was about 10.0 % (Merkus et al., 1996:70).

Hirai *et al.* (1978:296) demonstrated that a formulation containing 1.0 % sodium glycocholate enhanced the nasal absorption of insulin in dogs. In man, intranasal drops containing insulin and 1.0 % sodium glycocholate showed to possess approximately 10.0 % of the activity of intravenously administered insulin and even better efficacy was shown with a spray formulation containing 4.0 % sodium glycocholate (Merkus et al., 1996:70). Later on a variety of bile salts (taurocholate, cholate, deoxycholate, glycocholate and chenodeoxycholate) was reported to be

equally effective in enhancing insulin absorption in rats (Hirai *et al.*, 1981b:179). After investigating the reproducibility of nasal insulin administration with sodium glycocholate (1.0 % m/v) in man, a large intra-subject coefficient of variance was found of between 40.0 and 75.0 %. Overall, minimal nasal irritation and morphological damage was observed with 1.0% sodium glycocholate (Merkus *et al.*, 1996:73).

A bioavailability of 16.0 % of insulin, co-administered with 1.0 % m/v sodium taurodihydrofusidate (STDHF), a bile salt derivative, was obtained when administered to sheep. Administration of a similar formulation showed an increase in bioavailability from 0.9 to 5.2 % in rabbits and from 0.3 to 18.0 % in rats (Hinchcliffe & Illum, 1999:210). When tested in humans, a formulation containing insulin (0.35 IU/kg) and STDHF was administered nasally to eight healthy volunteers. It was reported that the inter-subject variation coefficient was lower than that of SC administration. Maximum insulin blood plasma concentration was observed after 8.7 minutes and a maximum blood glucose lowering effect after 20.0 minutes and absolute bioavailability was calculated to be 11.4 % (Hinchcliffe & Illum, 1999:213; Merkus *et al.*, 1996:71).

2.2.6.4 Enzyme inhibitors

As mentioned earlier, insulin undergoes degradation through means of hydrolysis by leucine aminopeptidase in the nasal cavity (Behl *et al.*, 1998:109). The full nature of the nasal enzymatic barrier and the extent to which it affects nasal drug delivery has to date not yet been fully elucidated although it has been concluded that, especially for smaller peptides, the barrier prove to be significant. Several of the compounds mentioned earlier such as bile salts, surfactants and fatty acids are known to have intrinsic enzyme inhibiting properties which may overall contribute to their effectiveness as absorption enhancers (Hinchcliffe & Illum, 1999:220).

As with oral insulin delivery, the use of enzyme inhibitors in nasal insulin delivery also enhances the bioavailability of insulin by decreasing enzymatic degradation. It has been reported that the aminopeptidase inhibitor, bestatin, increases the nasal absorption of insulin by inhibiting aminopeptidase activity (Illum, 1999:518). The

protease inhibitor aprotinin has also been reported to increase the activity of insulin after intranasal co-administration. Efficacy, compared to the intramuscular dose, increased from 0.4 % for the insulin control solution to 9.6 % in the presence of aprotinin. Enzyme inhibition at this stage is not as efficient as other absorption enhancing systems when it comes to increasing the bioavailability of nasally administered peptide drugs and is not competent enough to be used as an absorption enhancer without the aid of other promoting compounds (Hinchcliffe & Illum, 1999:220).

2.2.6.5 Cyclodextrins

Cyclodextrins are cyclic oligosaccharides, which through the formation of inclusion complexes, have been used to improve the solubility and stability of drug molecules in solution. The most common types of cyclodextrins are α -cyclodextrin (α -CD), β -cyclodextrin (β -CD) and γ -cyclodextrin (γ -CD) containing six, seven or eight glucopyranose units respectively (Hinchcliffe & Illum, 1999:220). All three of these cyclodextrins have proved to increase nasal insulin absorption in rats. The addition of 5.0 % α -CD to a nasal preparation resulted in a total bioavailability of about 30.0 %. Dimethyl- β -cyclodextrin (DM β CD), 5.0 % (w/v) co-administered with a 2.0 IU/kg insulin solution resulted in an astounding 100.0% bioavailability with a coherent strong hypoglycemic response in rats. The lowest concentration of DM β CD to exert an increase in insulin absorption was 2.0 % (w/v). Increased concentrations of 3.0, 4.0 and 5.0 % (w/v) resulted in higher insulin absorption but the area under curve of the plasma concentration-time curve showed little increase from 2.0 % (w/v) measured up to 1.0 hour after administration. The results were, unfortunately, insignificant in studies done on humans and rabbits and showed a decrease in ciliary beat frequency in both chicken embryo trachea and human adenoid tissue *in vitro*, but at 2.0 % DM β CD the ciliary beat frequency was still at 40.0 % (Illum, 2002:1186; Schipper *et al.*, 2002:173; Merkus *et al.*, 1996:72).

A further attempt to increase the bioavailability of insulin in rabbits made use of a lyophilised insulin/DM β CD powder formulation which resulted in 13.0 ± 4.0 % bioavailability compared to an insulin/DM β CD liquid and insulin/lactose powder formulation which both resulted in a 1.0 ± 1.0 % bioavailability (Schipper *et al.*,

1993:682). This powder formulation also showed to be effective in humans at a dose of 1.2 IU/kg insulin. Peak insulin plasma levels were reached after 5 to 10 minutes followed by a slow decrease from 10 to 120 minutes and it was concluded that the intranasal absorption closely mimics the physiological meal response profile. Bioavailability was measured at 3.4% in healthy volunteers ($n = 5$) and 5.1% in diabetic patients ($n = 5$) (Merkus *et al.*, 1996:73).

2.2.6.6 Bioadhesive polymer delivery systems

One of the major factors contributing to the low bioavailability of nasally administered drugs is the rapid mucociliary clearance by which drugs are being removed from the nasal cavity. Prolonging the resistance time, and hence, the period of time that the drug is in contact with the nasal mucosa will improve drug absorption (Schipper *et al.*, 1991:808). Administering drugs in the form of a spray intranasally has shown to decrease the mucociliary clearance as the solution viscosity is increased and this has been suggested as a way to improve the therapeutic effect of nasal spray preparations. Several approaches have been made to prolong the resistance time of drugs in the nasal cavity of which many of these approaches involve polymeric materials. These polymeric compounds increase the viscosity and/or have bio- or muco-adhesive properties in solutions or powder formulations thus prolonging contact time between the drug and the mucosal layer. Many of these polymeric compounds exhibit both bio- and muco-adhesive properties. Bioadhesion involves interaction between the compound and the nasal mucosa and will be affected by the nature of the compound and surrounding media (Hinchcliffe & Illum, 1999:222). The polymeric compounds that have been investigated in nasal insulin administration are chitosan, carbopol and polymer microspheres.

2.2.6.6.1 Chitosan

Well known for its pharmaceutical application as an absorption enhancer for protein and peptide drugs (Illum, 2003:190; Hinchcliffe & Illum, 1999:224; Dodane & Vilivalam, 1998:247; Li *et al.*, 1997:9; Aspden *et al.*, 1995:23;), chitosan has also been studied and trialed as an aid in nasal drug delivery. Chitosan, a cationic polysaccharide, is a linear copolymer of $\beta(1\rightarrow4)$ linked 2-acetamido-2-deoxy- β -D-

glucopyranose and 2-amino-2-deoxy- β -D-glycopyranose (Yu *et al.*, 2004:11; Dodane & Vilivalam, 1998:246). The history, synthesis, characterisation and application of chitosan will be discussed in detail in Chapter 4.

Illum and co-workers were the first to report the application of chitosan as absorption enhancer in nasal insulin administration and after preliminary studies in sheep they found an increase of peak plasma insulin levels from 34.0 to 191.0 mIU/l and a seven-fold increase in the area under curve with the use of formulations containing 0.5 % chitosan (Illum *et al.*, 1994:1186). Further studies in humans confirmed the effectiveness of chitosan, which increased insulin bioavailability to between 9.0 and 15.0 % compared to SC injection. These results showed to be repeatable and it was believed that the mechanism of action of chitosan improving the transport of polar drugs across the epithelial membrane is a combination of bioadhesion and the transient opening of tight junctions between the cells to enable the movement of polar drugs (Illum, 2002:1187; Aspden *et al.*, 1995:24).

It was first thought that chitosan nanoparticles are better absorption enhancers for nasally administered insulin than insulin-chitosan solutions alone (Fernandez-Urrusuno *et al.*, 1999:1576) but contrary results were found with a more comprehensive study. Chitosan nanoparticles (loaded and post-loaded) has been investigated and compared to various chitosan solution formulations for nasal insulin administration in both rats and sheep. Insulin was administered nasally at 100.0 IU/kg in the various formulations and injected SC at a concentration of 10.0 IU/kg serving as a reference. The chitosan nanoparticles were found to be less effective, lowering blood glucose levels to 59.7 % (in rats) and 72.6 % (in sheep) than the insulin-chitosan solution formulation, which lowered blood glucose to 40.1 % (in rats) and 53.0 % (in sheep) of the basal blood glucose levels. An insulin-chitosan powder delivery system (128.0 ± 13.0 IU/kg) administered to sheep in the same range of experiments showed the highest bioavailability of 17.0 % for insulin compared to the 1.3 % of the chitosan nanoparticles and 3.6 % of the chitosan solution formulations (Dyer *et al.*, 2002:998).

Extensive toxicological and tolerance studies in animals and humans have shown chitosan to be non-toxic and non-irritant to the nasal membrane (Illum, 1999:530;

Dodane & Vilivalam 1998:249) and can be formulated as powders, liquids or liquid gelling systems. Powders can be administered as freeze-dried or spray-dried particles or microspheres (Illum, 2003:190). Chitosan has also been proven to be more efficient and safer than surfactants such as Laureth-9 (Aspden *et al.*, 1996:28) and cyclodextrins such as dimethyl- β -cyclodextrin (Dodane & Vilivalam, 1998:249) and hydroxypropyl- β -cyclodextrin (Yu *et al.*, 2004:21).

2.2.6.6.2 Carbopol

Carbopol, a synthetic polyacrylic acid derivative, has a high molecular weight and forms aqueous gels at low concentrations. Carbopol has muco-adhesive properties and has been used to enhance intranasal absorption of insulin in rats. Both a 0.1 and 1.0 % Carbopol solution promoted insulin absorption with the 1.0 % solution being slower because of its higher viscosity. Solutions of similar viscosity containing 1.0 % CMC, a bioadhesive polymer, did not enhance insulin absorption suggesting that bioadhesive properties and viscosity were not the determining factors in insulin absorption. It was suggested that the water influx from the gel would allow the movement of hydrophilic and macromolecular compounds through intercellular channels. Polyacrylic acid therefore promotes drug absorption through the paracellular route (Hinchcliffe & Illum, 1999:222). The absorption enhancing effect of Carbopol on insulin was not influenced by pH. It was also shown that insulin (5.0 IU/kg) in combination with polyacrylic acid administered nasally to rats showed a 18.0 % decrease in blood glucose levels compared to the 7.0 % of the control (Illum, 1999:520).

Further studies in rabbits investigated the effect of Carbopol 974P® mixed with cospray dried Amioca® starch and drum dried waxy maize starch in daily nasal administrations of insulin. Amioca® starch and Carbopol 974P® in a mixture of 1:3 and a mixture of waxy maize starch and Carbopol 974P® were administered nasally to rabbits for eight consecutive days and the bioavailability and maximal decrease in blood glucose were monitored. A significant increase in nasal absorption (bioavailability above 10.0 %) and a decrease in blood glucose levels had occurred when no administrations from day 2 to 7 were made, concluding that Carbopol 974P® showed to be a significant absorption enhancer for nasally administered insulin.

However, after multiple administrations of these polymer and starch mixtures absorption showed to be slower because of the higher viscosity inhibiting the movement of the peptide and hence its absorption (Callens *et al.*, 2002:421).

Another study noted that the administration of a Carbopol based nasal gel to rabbits increased the absorption and bioavailability of insulin compared to the control. A significant decrease in blood glucose levels was obtained and bioavailability was calculated at 20.6 % compared to IV injection. It was concluded that Carbopol significantly enhances insulin absorption and, because of its sprayability, can serve as a platform for nasal insulin administration (Najafabadi *et al.*, 2004:295).

The use of Carbopol 934[®] in bioadhesive powder delivery systems has also been investigated. Freeze-dried insulin inherently has good bioavailability but with the addition of Carbopol 934[®] showed a vast and prolonged decrease in blood glucose levels of 49.0 %. Addition of crystalline cellulose powder further enhanced bioavailability to such an extent that it can be compared to a third of the effect of IV administered insulin at the same dose. This insulin-Carbopol-crystalline cellulose powder formulation was reported to be readily acceptable in terms of tolerability after administration to human volunteers nonetheless, plasma glucose levels between patients showed to be quite variable (Illum, 1999:523).

2.2.6.6.3 Microspheres

In section 1.3.3 the characteristics and use of microparticles or microspheres in oral insulin delivery has been discussed in detail. In nasal insulin delivery, however, the application of microspheres has also been extensively researched. The *raison d'être* behind the use of microspheres (in powder form) with good bioadhesive properties would permit such microspheres to swell when in contact with the nasal mucosa and form a gel, and simultaneously control (decrease) the rate of clearance from the nasal cavity. Poorly absorbed drugs will then have a longer contact period with the nasal mucosa and hence have more time to be absorbed and will, theoretically, result in higher bioavailability of these drugs (Illum, 1999: 525).

Investigators have found that degradable starch microspheres administered to rats resulted in a bioavailability of approximately 30.0 % for insulin compared to IV administered insulin (Hinchcliffe & Illum, 1999:223). Another study done on sheep comprised of a bioadhesive microsphere system containing a biological absorption enhancer, lysophosphatidylcholine. Compared to IV and SC injection the bioavailability of insulin in the microsphere system alone was found to be 10.7 % and that of insulin administered with the microsphere and absorption enhancer system was reported to be 31.5 % (Farraj *et al.*, 1990:253). A study in rats using the same method showed a bioavailability of 30.0 % for insulin (Illum, 1999:527).

Pereswetoff-Morath and Edman (1995:37) experimented with dextran (Sephadex®) microspheres for nasal insulin delivery and found that microspheres with insulin in the core dextran matrix of the microspheres are prone to be less effective than microspheres with insulin on the surface of the spheres. Microspheres with insulin on the surface showed higher insulin absorption than microspheres with insulin trapped in the dextran matrix and it was concluded that the size of the particles also influences the kinetics of the insulin effects curve. It was found that these microspheres had no apparent toxic effects on human subjects. It was also stated that starch microspheres tend to be more efficient in enhancing insulin absorption than dextran microspheres

Hyaluronic acid ester microspheres for nasal insulin delivery in sheep has also been investigated and it was found that the increase in nasal insulin absorption was independent of the dose of microspheres in the range of 0.5 to 2.0 mg/kg and the mean bioavailability was calculated to be 10.0 % (Illum, 1999:527).

2.2.6.7 Resin microparticles

The success of bioadhesive microparticles in nasal insulin administration has lead to the investigation of other microparticle technologies. Fractionated sodium polystyrene sulfonate powder (an anionic resin) together with human insulin (28.0 IU) was administered intranasally to rabbits. A rapid increase of plasma insulin levels up to $413.0 \pm 71.7 \mu\text{U/ml}$ was observed after 15 minutes and blood glucose levels decreased from $118.8 \pm 18.5 \text{ mg/dl}$ to $65.8 \pm 13.8 \text{ mg/dl}$ after 45 minutes from

administration. Styrene-divinylbenzene copolymer (a non-ionic resin) showed similar increases in bioavailability (Takenaga *et al.*, 1998:81).

2.2.6.8 Lipid emulsions

Both oil-in-water (o/w) and water-in-oil (w/o) emulsions have been investigated in improving the bioavailability of nasally administered insulin. Insulin was incorporated in the aqueous phase of the emulsions that was then perfused *in situ* through the nasal cavity of rats. Enhancement of insulin absorption was observed only with o/w emulsions, whereas w/o emulsions showed no change in absorption through the nasal mucosa. These results were also confirmed by *in vivo* administration. It appears that the presence of a small fraction of oil droplets as well as insulin in the aqueous phase favoured the absorption of insulin through the mucosa. It is believed that there occurs a certain interaction between the oil phase and the nasal mucosa that promotes the absorption of insulin (Mitra *et al.*, 2000:133).

2.3 Conclusion

Nasal administration of peptide and protein drugs has become a popular and more acceptable subject as several poorly absorbed drugs have been successfully formulated to deliver adequate bioavailabilities when administered intranasally. In the case of insulin the agonising daily SC administration of insulin has long called for a more gentle and satisfactory route of administration. The enormous amount of research that has been done in making the sought-after dream of formulating a nasally administrated insulin formulation that is effective, non-toxic, consistent and reliable, is almost a reality.

With several minor correct steps in the field of research that addresses the unresolved problems in nasal insulin delivery, we may soon witness a viable product for most type 1 diabetes sufferers around the world.

Part II: Peptide Drug Delivery Systems

CHAPTER THREE

Pheroid Technology as a Drug Delivery System

3.1 Introduction

In 1992 a company named MeyerZall (Pty) Ltd (South Africa) was established. MeyerZall discovered a unique drug formulation that seemed to have drug delivery properties when it was used as a basic formulation for the remission of psoriasis. One of the basic ingredients of this first formulation was present in banana peel extract and was later identified as essential fatty acids (Schlebusch, 2002:7; MeyerZall Laboratories (Pty) Ltd, 2004:2). The company also discovered various other applications for the product but currently the most pertinent being drug delivery. Later on in 1999 MeyerZall expanded its research and development and defined a unique drug delivery system named Pheroid™ technology (further referred to as Pheroid and previously known as Emzaloid). Above suspected and far reaching findings emerged from the research done by MeyerZall and indicated that Pheroid was effective in the treatment of infectious diseases such as tuberculosis, malaria and AIDS. Other drug applications were gene delivery and the use of it in generic medication. Pheroid also has the potential to entrap the active compound in the vesicles and to deliver these agents to specific organelle and tissue types (MeyerZall Laboratories (Pty) Ltd, 2004:2)

In December of 2003, MeyerZall and the Northwest University (NWU) entered into an agreement where the NWU acquired the patented intellectual property of MeyerZall. The company however retained the right to market some new products developed by the NWU thus diverting all their resources to efforts in commercialising existing and selected new products (MeyerZall Laboratories (Pty) Ltd, 2004:2).

3.2 Pheroid technology

Pheroid is a patented system, based on a unique submicron or micron emulsion formulation. A Pheroid is a stable structure within a system that can be manipulated in terms of morphology, structure, size and function (Grobler, 2004:4).

Pheroid consist mainly of plant and essential fatty acids, namely, ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid, emulsified in water, which is saturated with nitrous oxide. Pheroid can entrap, transport and deliver pharmacologically active compounds and other useful molecules. Depending upon the clinical indication it can also act in synergism with such compounds or molecules, resulting in an enhanced therapeutic action of such compound (Saunders *et al.*, 1999:99).

3.3 Pheroid types, characteristics and function

There exist many potential barriers for effective drug delivery, as discussed in previous chapters. Pheroid can overcome these barriers as it entraps drugs and deliver them to their specific sites of action in the body. Pheroid can penetrate keratinised tissue, skin, the intestinal lining, nasal epithelium, the vascular system, fungi, bacteria and parasites.

There currently exist various delivery systems in research and the pharmaceutical industry but what makes Pheroid so unique is that its components are manipulated in such a specific manner that it ensures high entrapment capabilities, fast rate of transport, delivery and stability of the active compound or molecule (Grobler, 2004:4).

Essential fatty acids are required for many cell functions, but cannot be manufactured by human cells and therefore have to be ingested. It has been shown that western diets lack these basic lipid molecules. Some of the functions of the fatty acid components of the Pheroid system are the maintenance of membrane integrity of cells, energy homeostasis

and modulation of the immune system through leukotriins and prostaglandins and some regulatory aspects of programmed cell death. The Pheroid system therefore has inherent therapeutic qualities that present it with significant advantages compared to various other delivery systems (Grobler, 2004:4).

Various types of Pheroid exist as illustrated in Figure 3.1, which shows confocal laser scanning micrographs of the following formulations of the Pheroid delivery system:

- a) Lipid bilayer vesicles in both the nano- and micrometer size range.
- b) Microsponges.
- c) Depots/reservoirs that contain pro-Pheroid.

Each type of Pheroid consists of a specific formulation and size and shape of vesicles, which can be reproduced within the size ranges of 80.0 – 30.0 nm for vesicles and 0.5-5.0 μm for microsponges. The sizes of depots / reservoirs are determined by the amount of pro-Pheroid contained in the reservoir. The absorption capabilities and drug release characteristics of the Pheroid can thus be controlled (Schlebusch, 2002:8).

Pheroid is composed mainly of 3 phases namely an aqueous phase, an oil phase and nitrous oxide. The aqueous phase is mainly water while the oil phase is a unique combination of essential fatty acids. The entrapment of drugs, which can be either in the aqueous or oil phase, within the Pheroid, generally creates a safer, more effective formulation than a formulation containing the drug alone (Grobler, 2004:4).

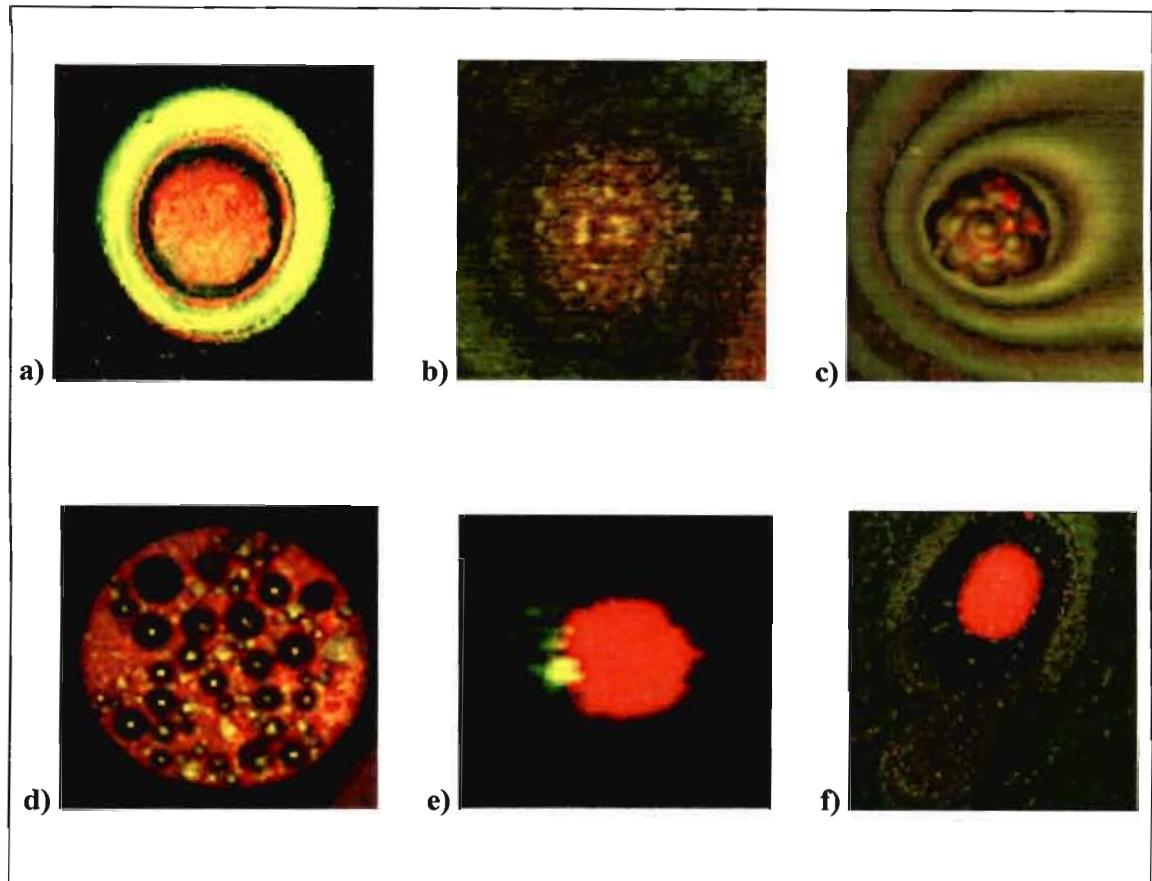


Figure 3.1: Confocal laser scanning micrographs of some of the basic Pheroid types: **a)** A bilayer membrane vesicle with a diameter of 100.0 nanometer containing rifampicin. **b)** A highly elastic or fluid bilayered vesicle with loose lipid packing, also containing rifampicin. **c)** Small pro-Pheroid that is used in oral drug delivery. **d)** A reservoir that contains multiple particles of coal tar. These reservoirs have large loading capacity to surface area ratios and are good entrappers of insoluble compounds with general sizes of 1.0 to 10.0 μm . **e)** A Pheroid in the process of entrapping fluorescently labelled water-soluble diclofenac. It has a very small diameter (about 30.0 nm) and the membrane packing is sponge-like. **f)** A depot with a hydrophobic core containing pro- Pheroid, a surrounding hydrophilic zone and an outer vesicle-containing zone. Selective addition of fluid results in the release of vesicles from a release zone (MeyerZall Laboratories (Pty) Ltd, 2004:3).

3.4 Pheroid and other lipid-based delivery systems

Although Pheroid systems are often confused with lipid based delivery systems, the fundamentals of the Pheroid system clearly shows that it differs substantially from conventional macromolecular carriers, such as the liposomal delivery systems.

Table 3.1 provides a comparison of the variation and key advantages of the Pheroid to other lipid-based delivery systems.

Table 3.1: Differences and advantages of Pheroid and other lipid-based delivery systems (Grobler, 2004:6).

Pheroid	Lipid- based delivery systems
Consists mainly of essential fatty acids, a natural and essential ingredient of the human body.	Delivery systems generally contain a proportion of substances foreign to the human body.
Cytokine studies demonstrated that Pheroid elicits no immune response in man.	Some liposomal formulations have been shown to elicit immune responses in man.
By using different combinations of fatty acids and/or other substances, the Pheroid can be targeted at subcellular level to some extent.	Since phospholipids are metabolised in the cell membrane, it is difficult to envisage how subcellular organelles can be targeted by this approach.
Since it is part of the natural biochemical pathways, Pheroid cause no cytotoxicity and assists with cell maintenance.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body. Liposomal systems may decrease the cytotoxicity of compounds or may cause cytotoxicity.
It is polyphonic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.

Due to its composition it is sterically stabilised without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be sterically stabilised. This generally leads to an increase in size and rigidity of the carrier.
It contains no cholesterol and the interior volume remains stable.	Most lipid-based delivery systems contain phospholipids and cholesterol to stabilise the interior of the vesicles.
Due to Pheroid composition, it is able to inhibit the drug efflux mechanism in the intestinal lumen and can thereby enhance the bioavailability of a compound.	Liposomal systems containing this feature have not been described. A separate compound i.e. Cremophor is co-administered to achieve the same effect.
The entrapment efficiency of compounds inside the Pheroid is high (between 85 and 100 %).	Due to the charge and steric limitations of liposomal delivery systems, entrapment efficiencies may be problematic.
The type of Pheroid formulated for a specific compound determines the loading capacity of the Pheroid.	The loading capacity of most lipid-based delivery systems is dependant on the interior of the intramembrane volume and is therefore limited.
Pheroid microsponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other drug in the sponge spaces. Geographical separation of active compounds into different interior spaces minimises drug interactions or interactions between drug compounds.	Combination therapies are problematic in most liposomal drug delivery systems.
Batch-to-batch reproducibility and stability has been proven with existing products containing Pheroid, such as the registered product Exorex®.	Large scale manufacturing of other liposomal delivery systems sometimes shows low batch-to-batch reproducibility as well as problems with size control.

<p>It is designed to show a high degree of elasticity and fluidity, with a relatively high phase transition temperature.</p>	<p>Because of the general use of stabilising compounds and cholesterol, liposomal systems lose fluidity and elasticity. The phase transition temperatures of phospholipid groups are higher than that of essential fatty acids, resulting in loss of elasticity.</p>
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3.5 Pharmaceutical applicable features of the Pheroid delivery system

3.5.1 Increased delivery of active compounds

Both *in vitro* and *in vivo* studies have revealed that by entrapment of active compounds such as acyclovir and miconazole nitrate in Pheroid, the percentage active compound delivered to the target site of action can be dramatically increased by using Pheroid (Grobler, 2004:8).

3.5.2 Decreased time to onset of action

Research has signified that the Pheroid delivery system rapidly transverses most physiological barriers and delivers the active compound intact. Rifampicin delivered in conjunction with Pheroid has been shown to act considerably faster than rifampicin alone delivered via a conventional method, therefore suggesting potentially faster onset of therapeutic action (Grobler, 2004:8).

3.5.3 Reduction in minimum inhibitory concentration

Research performed with the Pheroid system has shown that, for isoniazid, using as little as 2.5% of the active compound may result in an effective drug plasma concentration.

Ultimately this characteristic will result in a reduction of drug side effects as well as a major reduction in manufacturing and treatment cost (Grobler, 2004:9).

3.5.4 Increased therapeutic efficacy

It has been revealed, that by using Pheroid vesicles the efficacy of several antibiotics incorporated in the Pheroid system has shown a significant increase in inhibiting bacterial growth. Research done with Pheroid microsponges showed a further enhancement in the efficacy of ciprofloxacin and erythromycin (Grobler, 2004:9).

3.5.5 Reduction in cytotoxicity

Side effects of drugs are in most cases caused by cellular damage. The Pheroid system has the potential to minimise cellular damage that occurs as a result of membrane damage caused by active compounds. Given that the components of Pheroid are also part of the natural biochemical pathway, it appears not to be cytotoxic. A study was done by entrapping miconazole nitrate in Pheroid, which showed that damage to human cells were neutralised and simultaneously proved to be extremely effective as an antifungal formulation (Grobler, 2004:10).

3.5.6 Immunological responses

Some drugs, such as proteins and peptides, may induce an immunological response or adverse intolerance reactions. By masking these compounds using the Pheroid system, this may prohibit the human immune system from recognising these compounds. By doing this the dosage can be reduced without diminishing the potency, or the dosage can be increased to enhance therapeutic effects (Grobler, 2004:11).

3.5.7 Transdermal delivery

Whilst the Pheroid system is not limited to topical application, research has indicated that many medications can be administered topically instead of orally. In this way the digestive system, which is the origin of many side effects, is therefore bypassed and transdermal delivery will therefore eradicate many of the unwanted side effects caused by oral medication.

An independent bioequivalence study revealed that capsaicin entrapped in Pheroid showed an enormous increase in absorption of the compound compared to commercially available applications such as Novasome A and B and Zostrix (Grobler, 2004:11).

3.5.8 Entrapment and transference of genes to nuclei and expression of proteins

Experiments performed on the Pheroid delivery system demonstrate its applicability in DNA vaccines and gene therapy. *In vitro* studies have shown entrapment of human and viral DNA of various lengths into Pheroid. Reproducible expression of appropriate proteins was observed after transfection of cells by Pheroid-entrapped genes (Grobler, 2004:12).

3.5.9 Reduction and elimination of drug resistance

In vitro studies have demonstrated that by incorporating a drug into the Pheroid delivery system, drug resistance can be reduced or eliminated. Analysis of bacterial growth of multidrug-resistant tuberculosis has shown that formulations containing the standard antimicrobial rifampicin entrapped in Pheroid, obviated pre-existing drug resistance. The ability to enhance the effectiveness of antibiotics such as penicillin can have widespread implications in the healthcare industry (Grobler, 2004:12).

3.5.10 Pro-Pheroid

Pro-Pheroid is developed with the use of increased concentrations of polyethylene glycol (PEG) polymers in the Pheroid manufacturing process. It has been shown that PEG renders a protein therapeutically effective and contributes to the following aspects of drug administration:

- a) Increased bioavailability;
- b) increased drug stability and extended circulating life;
- c) lower toxicity; and
- d) enhanced solubility (Grobler, 2004:11).

The development of pro-Pheroid makes it possible for oral and systemic drugs to be orally administered in Pheroid (MeyerZall Laboratories (Pty) Ltd, 2004:2)

3.6 Therapeutic and preventative uses of Pheroid

3.6.1 Therapy of tuberculosis

According to the World Health Organisation (WHO) tuberculosis (TB) kills 2 million people each year. Overall, one-third of the world's population is currently infected with the TB bacillus. It was suggested by previous studies that the Pheroid delivery system might have significant benefits when combined with TB medication. Therefore, a bio-equivalence study was performed to determine whether the Pheroid delivery system would remain as effective in oral administration as it was in topical use (Grobler, 2004:14).

The purpose of the study was to prove that the pro-Pheroid formulation could:

- a) Increase the availability of the antimicrobials in human plasma;

- b) enhance the absorption of the formulated antimicrobials from the gastrointestinal tract without an increase in toxicity;
- c) increase the intracellular concentration of the antimicrobial in the target cells where the tuberculosis bacteria breed;
- d) extend the circulatory time of the active drugs;
- e) increase the bactericidal effect of the antimicrobials inside the target cells; and
- f) decrease the side effects caused by the antimicrobials (Grobler, 2004:14).

The results of the bio-equivalence study however, showed the following:

- a) The entrapment of the antimicrobials into Pheroid led to an increase in absorption of the antimicrobials after oral administration, with a resulting increase in plasma levels of these antimicrobials.
- b) The entrapment of the antimicrobials led to an increased rate of absorption and cellular response.
- c) The therapeutic concentrations of the drugs were maintained for longer and the circulatory time of the drugs was extended, indicating that the exposure of the bacteria to the antimicrobials was increased.
- d) The entrapment of the antimicrobials in the Pheroid delivery system increased the delivery of the antimicrobials to the target cells, thereby decreasing the minimum inhibitory concentration.
- e) A lower dosage can therefore be used to obtain similar effects.
- f) A decrease in side effects was observed, with an increase in patient compliance, therefore suggesting that it could prevent the development of multi-drug resistance (Grobler, 2004:17).

It could therefore be concluded that Pheroid can be seen as being successful in enhancing the efficacy and hence the bioavailability of oral peptide drugs and needs further investigation to determine to which boundaries this can be exploited.

3.6.2 Preventative therapies: Vaccines

Historically, vaccination is the only strategy that has led to the elimination of the viral disease, smallpox. An indirect relationship has been observed for vaccine

immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor; hence there is an urgent need for effective vaccine adjuvants to enhance the immunogenicity and immuno-stimulatory properties of vaccines (Grobler, 2004:17).

3.6.2.1 A virus based vaccine: Rabies

Each year approximately 50 000 people die from rabies and more than 10 million people receive post-exposure vaccination against rabies. Currently more than 2.5 billion people live in areas where rabies is seen as an endemic. The efficacy of a commercially available rabies vaccine and a rabies vaccine incorporated in a Pheroid delivery system was investigated and compared. An inactivated virus was used in the formulation of rabies vaccines. For the comparative animal studies, different formulations of the virus were used namely the inactivated virus, the inactivated virus with alum (aluminium hydroxide) as adjuvant and the inactivated virus incorporated in a Pheroid delivery system. The inactivated virus incorporated in a Pheroid delivery system showed a 9-fold increase in antibody response when compared to the other formulations (Grobler, 2004:15).

3.6.2.2 A peptide based vaccine: Hepatitis B

The efficacy of a commercially available hepatitis B vaccine and a hepatitis B vaccine incorporated in a Pheroid delivery system was investigated and compared. Non-recombinant hepatitis B vaccines are generally based on the use of the surface molecules of the virus as antigen. For the comparative animal studies, different formulations of this peptide-based vaccine were used namely the peptide, the peptide with alum as an adjuvant and the peptide incorporated in a Pheroid delivery system.

The use of Pheroid as a drug delivery system led to a more than 10-fold increase in the efficacy of the peptide-based hepatitis B vaccine as measured by antibody response. The Pheroid therefore has an obvious dual role in vaccinology, firstly as delivery system for disease specific antigens, and secondly as immuno-stimulatory adjuvant (Grobler, 2004:19).

3.7 Conclusion

It has been substantiated that drugs such as rifampicin, isoniazid, miconazole nitrate and capsaicin have been entrapped in Pheroid and with much success have contributed and enhanced the efficacy of these drugs in one or several ways. It can therefore be suggested that the absorption of peptide drugs could be greatly improved by using the Pheroid delivery system. Pheroid is an ideal delivery system as it is polyphilic and capable of encapsulating drugs that have different solubilities as well as insoluble drugs, many of which are highly toxic (miconazole nitrate) (Schlebusch, 2002:12). Pheroid is also able to actively transport these peptide drugs through various physiological membrane barriers and ultimately increase the bioavailability of certain drugs (Schlebusch, 2002:13). Pheroid has also been proven to be non-toxic which means that it will not contribute to the side effects of such a medicinal formulation. The full potential of Pheroid however, has still to be determined but seem to be plentiful and promising.

Part II: Peptide Drug Delivery Systems

CHAPTER FOUR

Chitosan and *N*-trimethyl Chitosan Chloride (TMC) as Drug Delivery Systems

4.1 Introduction

In recent years a vast amount of research has been done on the uses and applications of chitosan and its derivatives, as they have been proven to be valuable commodities in the enhancement of drug absorption. Today chitosan is readily commercially available in a range of different grades with different molecular weights and degrees of deacetylation, all with different applications (Aspden *et al.*, 1996:23) including a novel peptide carrier in drug delivery systems (Dodane & Vilivalam, 1998:246).

Even owning such ideal characteristics, chitosan has inert limitations such as insolubility in neutral physiological pH ranges. Further investigations have resulted in the synthesis of partially quaternised chitosan derivatives that are freely soluble over a wide pH range and still possess exceptional absorption enhancing capabilities. *N,N,N*-Trimethyl chitosan chloride, or TMC, is such a derivative and has proven to be a potent intestinal absorption enhancer for peptide drugs, even in neutral environments (Polnok *et al.*, 2004:77). Today the word chitosan refers to a large number of polymers that differ in their degree of *N*-deacetylation (40.0 – 98.0 %) and molecular weight (50 000.0 – 2 000 000.0 Da) as these properties differentiate their physicochemical and biological properties (Hejazi & Amiji, 2003:152). This chapter will focus on chitosan and TMC and their potential as delivery systems for protein and peptide drugs.

4.2 Chitosan

4.2.1 History

Chitin (Figure 4.1), a structural polysaccharide, is a major constituent of the exoskeleton of crustaceous water animals and of the hyphae or spores of lower plants and is the second most abundant polysaccharide in nature (Hejazi & Amiji, 2003:151). In 1811 chitin was described as a distinct substance identified in plants and that it occurs naturally in three polymorphic forms namely: a) α -chitin, b) β -chitin and c) γ -chitin.

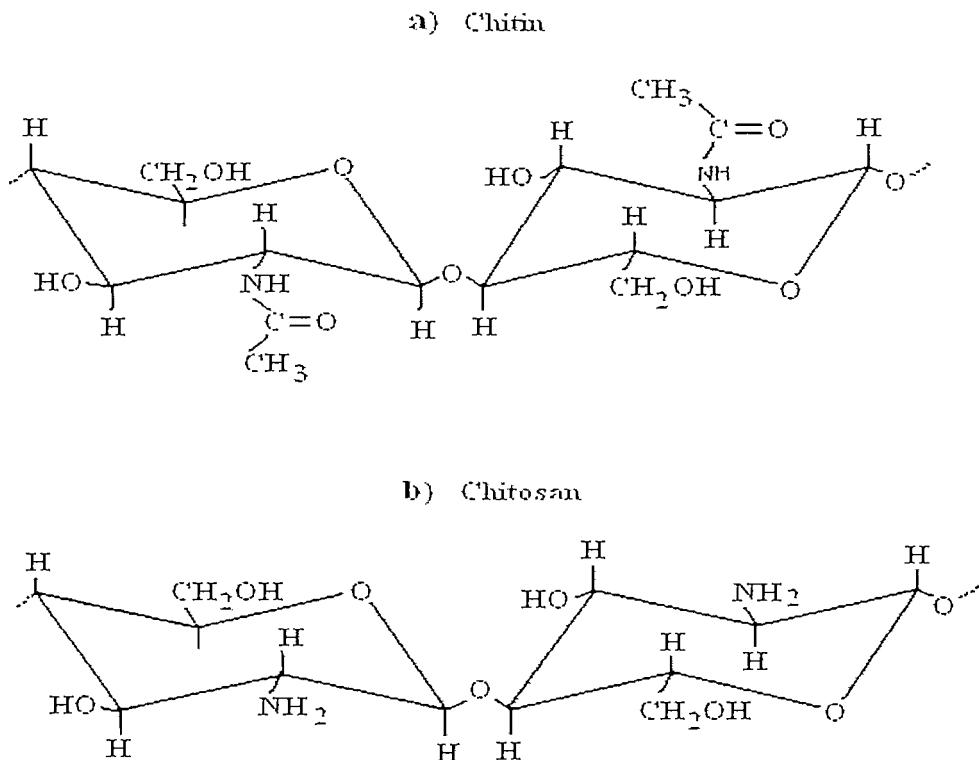


Figure 4.1: The structures of a) chitin and the chitin derived mucopolysaccharide b) chitosan (adapted from Hejazi & Amiji, 2003:152).

In 1859 chitin was boiled in a concentrated potassium hydroxide solution and found that the product dissolved in dilute iodine and acid solutions and in 1894 this substance was named chitosan. Chitosan, or [α -(1 \rightarrow 4)-2-amino, -2-deoxy, - β -D-

glucan] (Figure 4.1), was noted as the deacetylated form of chitin and as a mucopolysaccharide, which has similar structural characteristics than that of glycosaminoglycans (Paul & Sharma, 2000:5).

4.2.2 Synthesis and physicochemical properties

Synthesising chitosan incorporates the deacetylation of chitin and is illustrated in Figure 4.2. Crab or shrimp shells are decalcified in dilute (3.0 – 5.0 % w/v) hydrochloric acid and deproteinated in a dilute (3.0 – 5.0 % w/v) aqueous sodium hydroxide solution at 80.0 – 90.0 °C at room temperature overnight for a few hours. The beige or white product is chitin.

Chitin is then boiled in an aqueous 40.0 – 45.0 % (w/v) sodium hydroxide solution at 90.0 – 120.0 °C for 4.0 – 5.0 hours and forms *N*-deacetylated chitin (Hejazi & Amiji, 2003:152; Paul & Sharma. 2000:5). This process of deacetylation of chitin in an alkaline solution is never completed, even under harsh conditions, and therefore different degrees of deacetylation occurs which ranges between 70.0 and 95.0% (Li *et al.*, 1997:5).

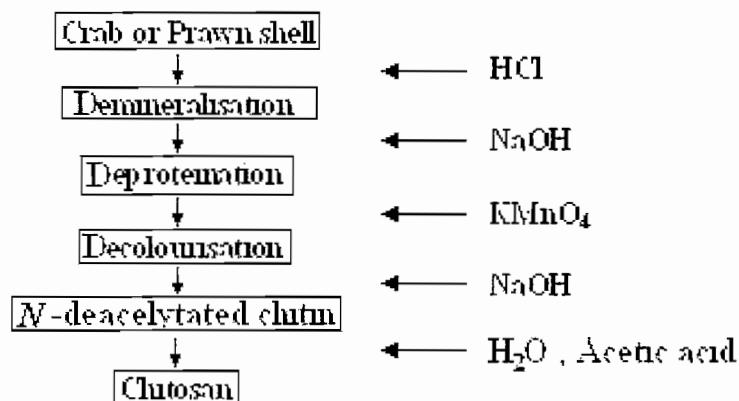


Figure 4.2: The basic steps in the synthesis of chitosan from chitin (adapted from Paul & Sharma, 2000:5).

The insoluble precipitate is then washed with water, which produces a crude form of chitosan. The conditions in which the deacetylation is done will determine the

molecular weight and the degree of deacetylation. The crude sample is then dissolved in aqueous acetic acid (2.0 % m/v) and the insoluble material is then removed that results in a clear supernatant solution that is neutralised with NaOH solution that produces a pure white chitosan product. Further purification is done to prepare medical and pharmaceutical grades of chitosan (Hejazi & Amiji, 2003:152)

In Japan the production of chitosan showed an increase of 37.0 % each year from 1978 up to 1983. Production reached a total annual amount of 311.0 tons by 1983 and 1270.0 tons by 1986 worldwide (Li *et al.*, 1997:5). Chitosan used as pharmaceutical grades has a degree of deacetylation between 90.0 and 95.0 % and the food grade is deacetylated between 75.0 and 80.0 % (Paul & Sharma, 2000:5).

The degree of deacetylation is determined through various analytical techniques such as UV spectrophotometry, dye adsorption, IR spectroscopy, metachromatic titration and gas chromatography. It is suggested that UV-spectrophotometry at 199.0 nm is probably the best non-destructive and accurate method when determining the degree of deacetylation of chitosan samples. Chromatography, light scattering or viscometry can be used to determine the molecular weight of chitosan. The latter is found to be the most simple and fastest method, but is influenced by the ionic strength of the solution. It has been found that the molecular weight of native chitosan is usually larger than 1 000 000.0 g/mol while commercially used chitosan products have molecular weights between 100 000.0 and 1 200 000.0 g/mol. This may be as a result of the degradation of the chitosan product during the manufacturing process (Li *et al.*, 1997:6).

Chitosan is a weak base with a pK_a value (of the D-glucosamine residue) of between 6.2 and 7.0. It is for this reason that chitosan is insoluble at neutral and alkaline pH values. Chitosan does, however, form salts with inorganic and organic acids such as hydrochloric-, acetic- and lactic acid. In an acidic medium the amine groups of the polymer are protonated producing a soluble, positively charged polysaccharide with a high charge density of about one charge per D-glucosamine unit (approximately between 1.35 and 1.4 g/cm³). Chitosan also forms gels when it interacts with different types of divalent and polyvalent anions and has a particle size of <30.0 µm (Hejazi & Amiji, 2003:153; Paul & Sharma. 2000:6).

Like many polymers, chitosan has a relatively high viscosity. Chitosan's viscosity, while in solution, is influenced by factors such as molecular weight, polymer deacetylation, concentration, temperature, pH and ionic strength. Chitosan is insoluble in water, alkali and organic solvents but it is, however, soluble in organic acids if the pH of the solution is less than 6.0. Acetic and formic acids are most widely used for dissolving chitosan (Li *et al.*, 1997:6).

Chitosan salts are soluble in water. The degree of deacetylation and the pH of the solution will determine the solubility of different salts; chitosans with a low degree of deacetylation ($\leq 40.0\%$) are soluble up to a pH of 9.0 and highly deacetylated chitosans ($\geq 85.0\%$) are soluble only up to a pH of 6.5. An increase in deacetylation also increases the viscosity. This is due to the differences in the conformation of high and low deacetylated chitosan in aqueous solution. When chitosan has a high percentage of deacetylation it has an extended conformation with a more flexible chain because of the charge repulsion in the molecule. However, if chitosan has a low degree of deacetylation the molecule has a rod-like shape or coil shape, resulting from a low charge density in the polymer chain (Hejazi & Amiji, 2003:153).

4.2.3 Applications of chitosan in pharmaceutics and medicine

Because of all its unique properties, chitosan has become a precious commodity in the world of medicine, pharmaceutics, cosmetics, the food industry, water purification, papermaking, biotechnology and agriculture. Properties such as low toxicity and good biocompatibility make it well suited for application in pharmaceutics and pharmaceutic related subjects and it has already proven to fulfil quite a unique and important role (Sinha *et al.*, 2004:2; Li *et al.*, 1997:5).

Chitosan in itself is haemostatic. Derivatives of chitosan such as sulphated chitosan have been used as anticoagulants in chitosan bandages, sponges, artificial blood vessels and membranes with quite an amount of success. It has also been found to lower cholesterol levels by 66.0 % in *in vivo* studies on mice. This hypocholesterolemic activity of chitosan was supposedly due to the inhibition of micelle formation that contains cholesterol, fatty acids and monoglycerides. Thus

these “building blocks” of cholesterol were radically reduced in quantities. Studies have also shown that chitosan inhibit tumour cell growth *in vitro* as well as *in vivo* when covalently linked to 5-flouroracil and tested on mice. The chitosan bound prodrug showed an enhanced inhibition effect against tumour cells without any apparent toxicity (Dodane & Vilivalam, 1998:246; Li *et al.*, 1997:8).

Furthermore it has been suggested that chitosan may be used to inhibit fibroplasia in wound healing and that it promotes tissue growth and differentiation in tissue culture (Kumar, 2000:9).

Additionally it was also found that chitosan is a fairly good coagulating agent and flocculant. This is due to the high density of amino groups which can interact with negatively charged substances such as proteins, dyes, solids and polymers. Chitosan also has the ability to adsorb metal ions and the chitosan-glucan complex is able to recover several metal ions such as Cr, Co, Ni, Cu, Cd and Pb from water (Li *et al.*, 1997:6).

Inherent properties of chitosan made it suitable for use in hypobilirubinaemi, as an antacid, and anti-ulcer effects and it has been used in formulation as a vehicle for directly compressed tablets, as a disintegrant, a binder, granulating agent, in ground mixtures, as well as a co-grinding diluent for the enhancement of the dissolution rate and bioavailability of water insoluble drugs. It has also been used in ocular bandage lenses for the treatment of acute or chronic trauma to the eyes (Sinha *et al.*, 2004:2). Furthermore chitosan has also been proven to have a bacteriostatic action towards skin bacteria such as *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Li *et al.*, 1997:9) and it has been proven to inhibit the growth of *Escherichia coli*, *Fusarium*, *Alternaria* and *Helminthosporium* (Kumar, 2000:21).

Other studies described the manufacturing and testing of artificial skin on rats as well as chitosan’s usefulness in treating bone disease and accelerating bone formation. Furthermore chitosan is used in cosmetics such as shampoos (conferring shine and strength to hair), coloured powders, nail polishes, moisturisers, hair and skin fixatives, hair conditioners, cleansers and also bath lotions. The use of chitosan as a carrier for

enzyme immobilisation has also been researched. Published reports have shown chitosan to be useful as a polyligand, flocculating agent and polymer support for the separation and recovery of proteins and cells (Li *et al.*, 1997:9). Additionally it has also been proven useful in eye preparations for surgery as is ideal for the manufacturing of soft contact lenses such as chitin *n*-butyrate lenses, chitosan lenses and blue chitosan lenses (Kumar, 2000: 11).

Essentially chitosan is considered a novel material in controlled release technology and drug delivery systems, which has been researched extensively. In parenteral drug delivery chitosan has proven to be successful in delivering bovine serum albumin, diphtheria toxoid and bisphosphonates by entrapment in chitosan microparticles. Chitosan-based colloidal suspensions have proven to be successful in *in vivo* studies aimed on improving ocular drug bioavailability. Chitosan-coated nanocapsules enhanced the ocular penetration of indomethacin compared to uncoated nanocapsules and appeared to have a good ocular tolerance as measured by ocular-lesion indices. Acyclovir loaded chitosan microparticles showed a significant sustained ocular release *in vivo* whereby it was suggested that apart from chitosan's muco-adhesive properties, it also has the ability to prolong drug release (Dodane & Vilivalam, 1998:248).

Formulations of chitosan and chitin in drug delivery systems include hydrogels such as chitosan interpenetrating polymer network (IPN) hydrogel, semi-IPN hydrogel networks, β -chitin and β -chitosan hydrogels, chitosan-amine oxide gel; chitin and chitosan tablets that include directly compressed tablets and controlled release tablets; microcapsules and microspheres that include cross-linked chitosan microspheres coated with polysaccharides or lipids, chitosan/gelatin network polymer microspheres, controlled release microspheres, chitosan-polyethylene oxide nanoparticles, chitosan/calcium alginate beads, multiporous chitosan beads; and chitosan membranes in transdermal drug delivery systems (Kumar, 2000:14).

The use of chitosan in oral and nasal insulin drug delivery has already been discussed in detail in Chapters 1 and 2.

4.2.4 Mechanism of action of chitosan

Muco-adhesive polymers have been used in formulations to increase the bioavailability of poorly absorbed peptide drugs. The rationale of this is to enhance the efficacy of certain aspects on drug delivery such as prolonged residence time of the drug, localisation of the drug molecule in specific regions and optimising contact with the absorbing epithelium (Junginger, 1990:111).

As mentioned, chitosan exhibit bioadhesion properties. Bioadhesion may be defined as the attachment of a synthetic or biological macromolecule to biological tissue. In the case of chitosan the term muco-adhesion is more commonly used and refers more specifically to epithelium that is covered with mucus. For a polymer to be muco-adhesive it must possess certain characteristics such as to- a) have strong hydrogen bonding groups (-OH, -COOH); b) have strong anionic charges; c) have a high molecular weight; d) have sufficient chain flexibility; and e) surface energy properties favouring adhesion to mucus (Peppas & Buri, 1985:258).

Even though chitosan, which has considerable muco-adhesion properties, does not have any anionic charges it does form a positively charged hydrogel in acidic environments. This could develop additional molecular attraction forces by electrostatic interactions with the negatively charged mucosal surface. Another factor effecting muco-adhesion is the molecular weight of the polymer molecule. It was established that high molecular weight chitosan exhibits better muco-adhesive properties than lower molecular weight chitosans (Lehr *et al.*, 1992:46).

Chitosan's muco-adhesion properties was suggested to contribute to a large part of the mechanism by which chitosan enhances drug bioavailability (Illum, 1994:1186). It has also been suggested, however, that the mechanism of action is a combination of muco-adhesion and the transient opening of tight junctions (Dodane & Vilivalam, 1998:250). Caco-2 cell monolayers and a vertically perfused intestinal loop model in rats have been used to evaluate the potential of chitosan glutamate (1.0 % w/v) to improve the intestinal transport of 9-des-glycinamide, 8-L-arginine vasopressin (DGAVP). Epithelial permeability was assessed by measuring transepithelial electrical resistance (TEER) and it was found that chitosan glutamate (pH 5.6)

decreased TEER to 45.0 ± 2.0 % of the control solution. A lower dose of chitosan glutamate (0.4 % w/v) showed to be even more effective in transporting DGAVP across the Caco-2 monolayers. The absorption of DGAVP across the intestinal mucosae was also significantly improved in the vertically perfused loop model (Lueßen *et al.*, 1997:19).

Another study revealed that chitosan hydrochloride reduced TEER even to a greater extent than chitosan glutamate. Again Caco-2 monolayers were used and after 1.0 hour of incubation with the solutions (1.5 % w/v and pH 6.2) chitosan hydrochloride reduced TEER with 71.0 ± 4.0 % compared to the 64.0 ± 6.0 % of chitosan glutamate (Kotzé *et al.*, 1997:248). Chitosan hydrochloride also increased the absorption of buserelin where, after 4.0 hours, 4.3 ± 0.3 % of the total dose applied was absorbed. The absorption of insulin across Caco-2 monolayers (pH 4.4) was also increased by chitosan hydrochloride (Kotzé *et al.*, 1997:249).

Lueßen *et al.* (1996:1668) also investigated the absorption enhancement of buserelin by chitosan hydrochloride compared to other absorption enhancers. Chitosan hydrochloride was compared with carbopol 934P (Carbomer or C934P) and its freeze-dried neutralised sodium salt form (FnaPC934P) in 1.5 % (w/v) gel formulations. Chitosan hydrochloride showed the highest bioavailability of buserelin of 5.1 ± 1.5 %.

4.2.3.1 Why the need for chitosan derivatives?

Chitosan presents ideal absorption enhanced properties but only when in an acidic medium where the pH is equal to or less than the pK_a of chitosan (5.5 to 6.5). As mentioned earlier, chitosan forms a positively charged water-soluble gel in acidic environments. In neutral and basic environments, however, chitosan loses its charge and precipitates from solution, making it insignificant as absorption enhancer as the molecule remains in a coiled configuration (Kotzé *et al.*, 1999a:351).

Kotzé *et al.* (1999:149) also found that, in dissimilarity to the reduction of TEER of Caco-2 cell monolayers after incubation with chitosan glutamate and chitosan hydrochloride at a pH pf 6.2, no decrease in TEER was found at pH 7.4. Investigation of the transport of [^{14}C]-mannitol also revealed negative results for these chitosan

salts, after incubation at pH 7.4, as no increase of transport was observed. It will seem that more basic environments, such as those found in the ileum, colon and nasal cavity, will render chitosan ineffective as absorption enhancer in these anatomic regions which are well suited for drug administration.

It is therefore evident that chitosan derivates with more suited physicochemical properties such as pK_a and water solubility at neutral and basic pH values are needed as they are in demand as absorption enhancers in these environments. Hence, chitosan polymers with different substituents, different碱碱ities or different charge densities may result in the same or even more pronounced opening of tight junctions (Kotzé *et al.*, 1999a:351).

One such a chitosan derivative, TMC, has been found to be just as significantly effective in neutral and basic environments concerning enhanced drug absorption. Studies revealed that TMC, at lower pH ranges (6.2) was not as effective as chitosan glutamate (reducing TEER by 60.0 %) or -hydrochloride (84.0 %) in reducing TEER in Caco-2 monolayers but however demonstrated satisfying results (reducing TEER by 24.0 %). Transport studies done showed that chitosan glutamate and chitosan hydrochloride increased the transport of [^{14}C]-mannitol 25-fold and 34-fold respectively and TMC increased it by 11-fold (Kotzé *et al.*, 1998:35).

At pH 7.4, however, TMC-H (highly quarternised TMC which is 19.9 % quarternised) reduced TEER by 53.0 ± 6.0 % and increased the transport of [^{14}C]-mannitol 97-fold. All polymers were incubated at a concentration of 2.5 % (w/v) except chitosan hydrochloride and -glutamate in the transport studies of [^{14}C]-mannitol which was 0.25 % (w/v) (Kotzé *et al.*, 1999b:269).

It is therefore evident that, even displaying exceptional drug absorption enhancing capabilities, chitosan has several critical restrictions that have to be overcome. One of the ways to overcome these restrictions or limitations is by deriving molecules from chitosan or by modifying chitosan in such a way that its efficacy is improved in such a way that it is rendered effective, even at higher pH levels.

4.3 N-trimethyl chitosan chloride

Confirmed by confocal scanning laser microscopy (CSLM), it is apparent that TMC has the same mechanism of action than chitosan, namely the opening of tight junctions between epithelial cells (Kotzé *et al.*, 1998:44). Of several chitosan derivatives synthesised, only TMC seems to be perfectly soluble in neutral and basic pH values, deeming it ideal as absorption enhancer for protein and peptide drugs (Van der Lubben *et al.*, 2001:204).

4.3.1 Synthesis of TMC

The method used to synthesise TMC is based on the method described by Domrad *et al.* (1986:105). Experimental conditions involve the reductive methylation of chitosan with iodomethane in a strong basic environment at 60.0 °C for 60.0 minutes. The quaternised polymer is then dissolved in a sodium chloride solution where the counterion (Γ) is changed to Cl^- (Kotzé *et al.*, 1997a:1197) During the synthesis the amino groups in the C-2 positions of the chitosan subunits are methylated to form quaternary amino groups with fixed positive charges as shown in Figure 4.3. The number of positive charges is therefore increased, causing the polymer to enlarge in the solution due to repelling forces between the functional groups. During the reaction amounts of acid are produced. By using a strong base to bind to the acid the methylation of the primary amine to the quaternary stage is accomplished and protonation of the unreactive primary amino groups is prevented. Although a high degree of substitution on the amino group is complicated due to the presence of the acetyl group, the hydroxyl group of chitosan can be substituted in the methylation process (Polnok *et al.*, 2004:78).

Repeating the reaction steps several times and under prolonged conditions will determine the degree of quaternisation (Hamman & Kotzé, 2001:374; Sieval *et al.*, 1998:157). Achieving absolute quaternisation will probably be troublesome due to the presence of the acetyl groups on adjacent quaternary amino groups. $^1\text{H-NMR}$ spectra also showed that a high percentage of the amino groups are mono- and

dimethylated and can thus still be protonated in an acidic environment. TMC, even at low degrees of quaternisation, is soluble at all pH ranges (Sieval *et al.*, 1998:158).

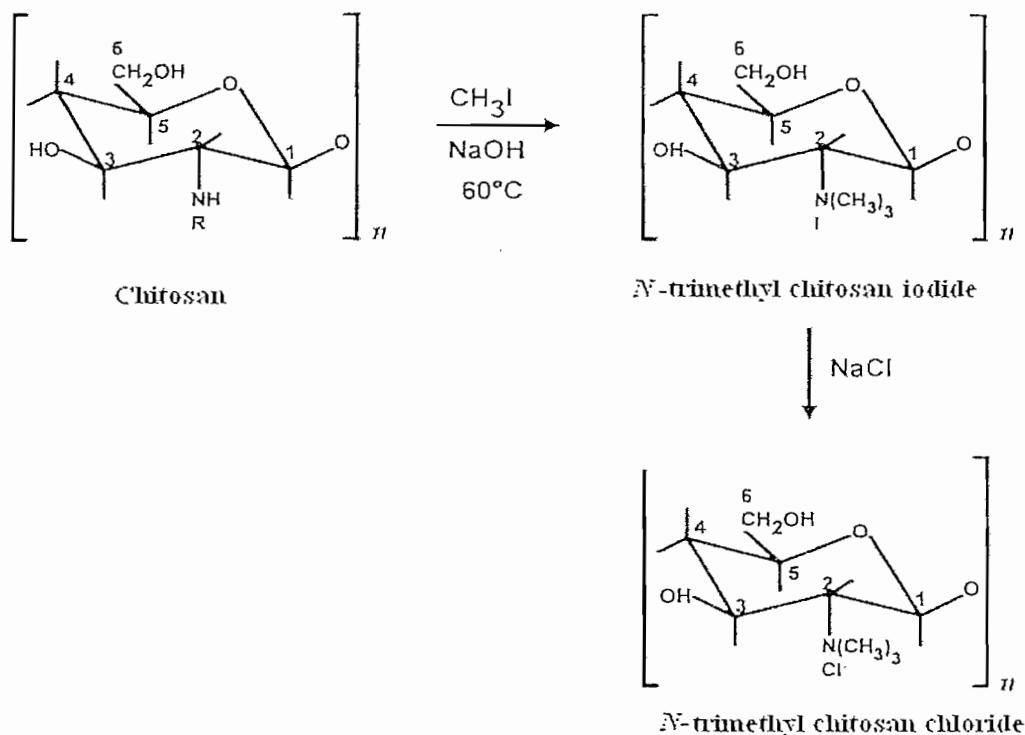


Figure 4.3 Synthesis of *N*-trimethyl chitosan chloride (TMC) from chitosan by reductive methylation.

4.3.2 Muco-adhesive properties of TMC

As mentioned earlier, it is essential for an absorption enhancer to have certain muco-adhesive properties. Snyman *et al.* (2003:61-68) have investigated the muco-adhesive properties of TMC with different degrees of quaternisation ranging from 22.1 to 48 % and the molecular mass was above 100 000.0 g/mole for all the polymers. An increase in quaternisation showed a decrease in muco-adhesive properties and it was also found that TMC had a lower intrinsic mucoadhesivity than the chitosan salts (chitosan glutamate and -hydrochloride) but superior muco-adhesive properties compared to the reference polymer, pectin. The observed decrease in mucoadhesivity

from chitosan to TMC is probably due to interactions between the fixed positive charges on the quaternary amino groups, which also possibly decreases the flexibility of the polymer. Quaternised TMC in the range tested was still considered to have sufficient muco-adhesive properties in order to be included into muco-adhesive drug delivery systems.

4.3.3 Mechanism of action of TMC

Chitosan's effect on paracellular permeability is initiated by its direct and specific binding to cell membranes. It has been shown that heparin inhibits this binding, indicating that the positive charge on the chitosan molecule is essential for chitosan's binding properties. It is most likely that TMC has the same or similar mechanism of action than chitosan and chitosan salts, as TMC at all degrees of quaternisation has positive charges (Schipper *et al.*, 1997:923).

After extensive research it was concluded that TMC reduces the TEER of epithelial cells, thereby opening tight junctions and therefore allows for the transport of hydrophilic molecules along the paracellular transport pathway (Thanou *et al.*, 2001:122; Kotzé *et al.*, 1999b:273; Kotzé *et al.*, 1998:44). The results were confirmed by visualisation using CSLM.

4.3.4 The effect of TMC on the TEER of human intestinal epithelial cells (Caco-2)

It has been suggested by Boulenc *et al.* (1995:14), after investigating tight junction permeability of biophosphates on human intestinal epithelium cells, that the measurement of TEER is a good indication of paracellular transport of hydrophilic drugs. Tight junctions between epithelial cells serve as a barrier to the paracellular diffusion of molecules and TEER is believed to be an accurate indication of the tightness of the junctions between these cells (Kotzé *et al.*, 1998:39).

Studies done revealed that incubation of intestinal epithelial cells (Caco-2) with TMC, with a degree of quaternisation of 12.0 %, in concentrations of 1.5, 2.0 and 2.5 % (w/v) immediately reduces TEER values. TEER was reduced by 9.0 ± 4.0 , 52.0 ± 3.0

and $79.0 \pm 0.3\%$ respectively after 20.0 minutes. It was evident from the results that the reduction in TEER was directly proportional to the TMC concentration and was therefore concentration dependant (Kotzé *et al.*, 1997a:1200).

Reduction in TEER by these polymers was also found to be reversible. By removing the polymer and repeatedly washing and substituting the apical medium with fresh Dulbecco's Modified Eagles Medium, showed a slow recovery of the monolayers and a slight increase in TEER was measured. The slow and partial recovery was most probably due to the damage done to the cells in the process of removing the polymers. Removing the polymers was found to be problematic because of their high viscosity. However, staining the monolayers with trypan blue after incubation with TMC assessed the viability of the polymers. There was no visible uptake of the marker and it was concluded that the viability of the monolayers was not compromised by incubation with TMC (Kotzé *et al.*, 1997a:1200).

A comparison made between chitosan hydrochloride, chitosan glutamate and TMC (12.28 % quaternised) on the effect of TEER across Caco-2 cell monolayers revealed that, at the same concentration, TMC was less effective in reducing TEER than the chitosan salts. The reduction in TEER at 0.25 % (w/v) polymer concentration was in the order of chitosan hydrochloride ($71.0 \pm 4.0\%$) > chitosan glutamate ($56.0 \pm 1.0\%$) > TMC ($28.0 \pm 1.0\%$) after 20.0 minutes (Kotzé *et al.*, 1998:39).

The difference in the effect of these polymers could probably be explained in terms of the equivalent weights of each repeating unit in the polymer backbone of the respective polymers, thus determining the amount of free chitosan base and therefore the density of the amino groups available for protonation at similar weight concentrations. It was suggested that the attached methyl groups on the C-2 position of TMC may cause steric effects and also partially hide the positive charge on the quaternary amino groups and thereby altering the time needed for interaction with the negatively charged cell membranes and tight junctions (Kotzé *et al.*, 1998:41). At higher concentrations of TMC (2.0 – 2.5% w/v) similar effects to those of chitosan hydrochloride, were observed. The exceptional solubility of TMC at higher pH values may therefore compensate for its lower effect on TEER at the same weight concentrations (Kotzé *et al.*, 1998:35).

4.3.5 TMC as absorption enhancer of peptide drugs and hydrophilic model compounds

As discussed in Chapter 1 and 2, the main contributing factors to the poor bioavailability of oral and nasal administered peptide and protein drugs are proteolytic and acidic degradation, poor solubility, cell- and first pass hepatic metabolism and the physical epithelium barrier. Furthermore, these drugs are mostly excluded from transcellular pathways as they are large, hydrophilic and do not partition into cell membranes a great deal. The absorption of these drugs is mostly limited to the alternative paracellular pathway, which is primarily restricted by tight junctions (Kotzé *et al.*, 1998:36).

As previously discussed, TMC (12.28 % quaternised) was able to improve the transport of the hydrophilic compound [¹⁴C]-mannitol at a pH of 6.2. The transport of other hydrophilic compounds such as [¹⁴C]-PEG 4000 and FD-4 across intestinal epithelial membranes (Caco-2) has also been improved by TMC (Kotzé *et al.*, 1998:39; Kotzé *et al.*, 1997a: 1197).

Similarly, TMC was also able to transport peptide drugs such as insulin, buserelin and DGAVP across Caco-2 monolayers. A 4-hour experiment at pH ranges between 4.4 and 6.2 was conducted and TMC (1.5 % (w/v)) showed a 1.4 ± 0.2 % increase (from the initial dose) in transport for buserelin, 0.3 ± 0.1 % increase for insulin and 0.96 ± 0.28 % increase for DGAVP. At a higher concentration of 2.5 % (w/v) TMC, the results showed a 2.7 ± 0.3 % increase (from the initial dose) in transport for buserelin, 0.8 ± 0.1 % increase for insulin and 1.09 ± 0.08 % for DGAVP across Caco-2 monolayers (Kotzé *et al.*, 1997:248).

4.3.6 The effect of the degree of quaternisation of TMC on its absorption enhancing capabilities

Compared to chitosan glutamate and chitosan hydrochloride, TMC with the same weight per volume, tends to be less effective in increasing the transport of hydrophilic compounds across Caco-2 monolayers. As mentioned, the proposed reason for this occurrence is the difference in the density of amino groups available for interaction,

and the partial hiding of the positive charge on the amino groups by attached methyl groups in the C-2 position. It was then proposed that TMC with higher degrees of quaternisation would be more effective as absorption enhancers of hydrophilic compounds in neutral environments (Kotzé *et al.*, 1999b:253).

Further studies by Kotzé *et al.* (1999c:253) investigated the different effects of TMC-H (61.2% quaternised TMC), TMC-L (12.28 % quaternised TMC) and chitosan hydrochloride on the TEER and permeability of Caco-2 cell monolayers at pH values of 6.2 and 7.4. At pH 6.2 and polymer concentrations of 0.5 % (w/v), all of the polymers showed a reduction in TEER of between 37.0 and 67.0 %. At pH 7.4 however, only TMC-H was able to decrease TEER in Caco-2 cells and even at a concentration of 0.05 % decreased TEER by 35.0 %. Similar results were found with transport studies done with [¹⁴C]-mannitol. At pH 6.2 large increases in the transport rate (increased by 18 – 23 fold) was found at concentrations of 0.5 %, while at pH 7.4 only TMC-H was able to increase the permeation of [¹⁴C]-mannitol 31.0 to 48.0 fold from concentrations 0.05 to 1.5 % (w/v) respectively.

Another study only used TMC with quaternisation between 22.1 and 48.8 % in an in vitro and in situ transport study with [¹⁴C]-mannitol as model compound. Everted rat intestinal sacs were used to test the polymers at different concentrations (0.0625 – 0.5 % (w/v)) and degrees of quaternisation at pH 7.4. A single pass intestinal perfusion method was also used to evaluate the permeation enhancing properties of the different TMC polymers under the same conditions. The results clearly showed that permeation was enhanced much more with TMC with higher degrees of quaternisation and in both models the best permeation enhancement was obtained with the highest degree of quaternised TMC (48.8 %) at a concentration of 0.5 % (w/v) (Jonker *et al.*, 2002:205). This clearly indicates that the absorption enhancing effect of TMC is dependent and directly proportional to the degree of quaternisation and polymer concentration.

A study done on the effect of chitosan salts and TMC on semisynthetic human insulin showed comparable results to those found with [¹⁴C]-mannitol. Insulin was administered nasally *in vivo* at 4.0 IU/kg body weight at a pH of 4.4 and 7.4 and again chitosan hydrochloride, TMC-H and TMC-L was investigated. At pH 4.4 all the

polymers were able to increase insulin plasma levels and reduce blood glucose levels. In this case, however, there was a minimal difference between the results of TMC-H and TMC-L. At pH 7.4 again only TMC-H showed a significant reduction in blood glucose levels. Neither chitosan hydrochloride nor TMC-L was able to reduce blood glucose levels to a significant level. TMC-H (0.5 % (w/v)) reduced blood glucose levels by about 34.0 % after 30 minutes of co-administration (Kotzé *et al.*, 1999a:253).

The co-administration of the somatostatin analogue, octreotide, with TMC and chitosan hydrochloride in rats showed matching results in contrast with what Kotzé *et al* (1999a:253) found relating to semisynthetic human insulin. Experiments performed at pH 7.4 showed that only 1.0 % (w/v) TMC (60.0 % quaternised) was able to increase the absorption of octreotide 5-fold compared to the control. Chitosan hydrochloride (1.0 % w/v) showed no enhancement of absorption whatsoever (Thanou *et al.*, 2000:951). This study was repeated but octreotide was intrajejunally administered to juvenile pigs through an in-dwelling fistula implanted one week prior to the administrations. Octreotide (10.0 mg) was co-administered with 5.0 and 10.0 % (w/v) TMC which resulted in a 7.7 and 14.5-fold increase in octreotide absorption, respectively. Absolute bioavailability was also calculated at $13.9 \pm 1.3\%$ and $24.8 \pm 1.8\%$ respectively (Thanou *et al.*, 2001:826).

Hamman *et al.* (2002:241) also concluded that TMC with a degree of quaternisation higher than 48.0 % showed no significant increase in absorption enhancing effects, which may indicate that TMC's optimum absorption enhancing effect is at a degree of quaternisation of 48.0 %. This may be attributed to steric effects caused by a high density of methyl groups and changes in the flexibility of TMC polymers found at degrees of quaternisation higher than 48.0 %.

4.3.7 Cytotoxic evaluation of TMC

Safety evaluations are required for novel drug absorption enhancers to ensure non-toxic effects and the absence of tissue damaging effects. Even though chitosan is known to be non-toxic and biodegradable, other cationic polymers, including

chitosan, can exhibit dose-dependant damaging effects to epithelial cells (Schipper *et al.*, 1996:1686).

In order to evaluate the damaging effect of TMC to epithelial cells, a viability test has been conducted. Caco-2 cell monolayers were incubated apically with a 0.1 % (w/v) trypan blue solution in 0.01 M phosphate buffer solution at pH 7.4 for 30.0 minutes. The basolateral side was incubated only with phosphate buffer solution. After the 30.0-minute incubation period the medium was removed from both sides and the cells were examined by light microscopy for the exclusion of the marker. Cells that did not contain trypan blue were considered to be viable. Prolonged incubation with TMC polymers showed no visible uptake of trypan blue into the cells, which suggested that the Caco-2 cell monolayers were undamaged and functionally intact. The integrity of the cells seemed to be uncompromised by TMC polymers (Kotzé *et al.*, 1997:246).

Another viability test made use of trypan blue and propidium iodide. Caco-2 cell monolayers were incubated with TMC polymers with degrees of quaternisation of 40.0 and 60.0 %, respectively. Similar to the previous experiment, the results revealed that, even at high concentrations of TMC (1.0 % w/v), there was no uptake of trypan blue into the cell monolayers and only a very small uptake of propidium iodide. This indicated that TMC has negligible cytotoxic effects (Thanou *et al.*, 2000:23).

Another viability test done by Thanou *et al.* (1999:77) used a fluorescent probe (YO-PRO-1) to test the possible membrane damaging effects of TMC on Caco-2 monolayers. The probe only emits fluorescence when it bonds with the nuclei of cells. Cells that do not absorb the fluorescent probe were considered to be viable. Caco-2 cell monolayers were incubated with 1.0 % (w/v) TMC with a degree of quaternisation of 60.0 % for 4.0 hours. CLSM revealed no staining of the horizontal cross sections of the Caco-2 monolayers. The ciliary beat frequency (CBF) of chicken embryo trachea also revealed a slight decrease when it was incubated with a 1.0 % (w/v) TMC (60.0 % quaternised) solution. This decrease in frequency was, however, less pronounced than that of incubation with physiological saline (0.9 % NaCl) and strongly suggested that TMC is a safe absorption enhancer for hydrophilic macromolecules such as peptide and protein drugs across nasal and other mucosal

tissues (Thanou *et al.*, 1999:81). Jordaan (2001:79) also concluded that TMC in concentrations as high as 1.0 % (w/v) has minimal influence on CBF after tests for local toxicity. It was also concluded that TMC with a high molecular weight exerted a more pronounced effect on CBF than low molecular weight TMC.

It has also been found that the degree of methylation has an influence on the toxicity of TMC, in that if the trimethylation of TMC increases so does its toxicity. TMC polymers trimethylated to 93.0 %, however, became less toxic. TMC was considered to be non-toxic when compared to the “gold standard” polyethylenimine (PEI) (Kean *et al.*, 2005:651).

4.4 Conclusion

It is evident from literature that both chitosan salts and TMC chloride are exceptional absorption enhancers for protein and peptide drugs. The absorption enhancing properties of chitosan and its salts are however, limited only to acidic and neutral environments, whereas TMC seems to be very efficient in neutral and basic environments. The reason for this seems to be that TMC is more soluble than chitosan and its salt forms in neutral and basic pH ranges. TMC has significant muco-adhesive properties, which renders it suitable as an absorption enhancing entity. TMC binds to cell membranes and lowers the TEER that in turn opens the tight junctions between epithelial cells and renders better permeability for protein and peptide drugs.

Several protein and peptide drugs, including insulin, have shown to have an improvement in absorption and bioavailability when co-administered with TMC. The higher the quaternisation of TMC, the more pronounced are its effects. Furthermore, TMC has been shown to be deacetylated, non-toxic and safe for medicinal use and seems therefore to be an ideal absorption enhancing agent.

Part III: Experimental Procedures

CHAPTER FIVE

Entrapment of FITC-Insulin in Pheroid Vesicles and Analysis with CLSM

5.1 Introduction

Pheroid comprises different exceptional characteristics that are described in Chapter 4. These characteristics are distinctive of Pheroid because of its unique formation which is a result of a sensitive formulation process. Apart from the formulation of Pheroid vesicles and microsponges, it is also important to determine whether or not and to what extent, insulin is entrapped in Pheroid.

This degree of entrapment will be a determining factor of the efficiency of these formulations as drug delivery systems. This chapter will discuss the preparation of the Pheroid delivery system used in experimental procedures and insulin entrapment in Pheroid vesicles will be quantified.

5.2 Pheroid preparation and synthesis

5.2.1 Materials

The following materials were used in the synthesis of Pheroid vesicles and microsponges: Vitamin F Ethyl Esther CLR (Chemisches Laboratorium, Germany), Cremaphor® RH 40 (BASF, Germany), DL-a-Tocopherol (Chempure (Pty) Ltd., South Africa) and medical grade Nitrous Oxide (Afrox, South Africa).

5.2.2 Method

Initial steps included the preparation of an oil-in-water (o/w) emulsion. Cremaphor® RH 40 was preheated on a hotplate until it was translucent and then added to an oil

phase consisting of Vitamin F Ethyl Esther CLR and DL- α -Tocopherol giving a total concentration of 4.0% (w/v). The mixture was then stirred and heated in a microwave (LG Electronics (Pty) Ltd., South Africa) at 900 watts for 2 minutes or until transparent.

A water phase was prepared by gassing deionised water with nitrous oxide for four days. The water phase was then heated to \pm 75.0 °C on a hotplate. As soon as the oil and water phases were at the same temperature, the oil phase was added to the water phase and the mixture was then homogenised using a Heidolph Diax 600 homogeniser at 13 500 rpm for 120 seconds. The emulsion was then poured into an amber glass container and sealed to protect it from sunlight and oxidation. A GFL shaker (GFL Gesellschaft für Labortechnic mbH, Germany) was then used to shake the emulsion until it reached room temperature, whereafter it was refrigerated at 6.0 °C.

5.3 Characterisation of Pheroid vesicles

5.3.1 Particle size analysis

5.3.1.1 Materials

Pheroid samples were analysed using a Malvern Mastersizer (Malvern Instruments Ltd., United Kingdom).

5.3.1.2 Method

Preparation for the procedure included switching on the Malvern Mastersizer half an hour prior to performing the analysis in order to let the laser beam warm up and stabilise. Pheroid samples were then mildly stirred to optimise homogenous particle distribution without causing air bubbles to form that may have an effect on readings.

A 2.0 ml Pheroid sample was then added to 800.0 ml distilled water and was pumped at a pump speed of 2500.0 rpm until an obscuration rate was obtained of 10.0–20.0 %. Measurement time for the background and samples was set at 12.0 seconds and the

delay between readings was 20.0 seconds. The refractive index was set at 1.4564 for emulsion droplets and at 1.33 for the dispersion medium (distilled water). The absorption value of the emulsion droplets was 0.100. These settings were used for every evaluation of Pheroid samples and every sample was measured in duplicate.

5.3.2 Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to visualise and analyse Pheroid samples in order to determine the size and formation of the vesicles. CLSM was also used to visualise and analyse fluorescent-labelled insulin in Pheroid samples. The mechanism by which it operates comprises a laser as energy source and an optical microscope. When a molecule is exposed to the laser beam it progresses into an excited state and emits photons at a specific wavelength. This molecule can then be “dissected” and fully visualised by the optic microscope.

It can also be used to determine the percentage of loading of drug molecules in Pheroid vesicles.

5.4 Loading of Pheroid vesicles with FITC-insulin

5.4.1 Materials

Pheroid vesicles (North-West University, Department of Pharmaceutics), Fluorescein-isothiocyanate (FITC) labelled insulin (Sigma-Aldrich, South Africa), GFL shaker (GFL Gesellschaft für Labortechnic mbH, Germany).

5.4.2 Method

FITC-insulin was carefully weighed and dissolved in 10.0 ml of freshly prepared Pheroid vesicles at a total concentration of 100.0 nmol/kg. The mixture was then slowly shaken on the GFL shaker for 10.0 minutes to ensure optimum entrapment.

5.5 CLSM analysis of Pheroid vesicles containing FITC-insulin

5.5.1 Materials

A Nikon PCM 2000 Confocal Laser Scanning Microscope with digital camera DMX 1200 Confocal Laser Scanning Microscope (Nikon, The Netherlands), Nile Red (Molecular Probes, Inc., USA) and Pheroid vesicles containing FITC-insulin.

5.5.2 Method

A Helium/Neon laser was used with an optical zoom of 60x and an emission of 568 nm. The Pheroid sample was prepared by which 100.0 µl of Pheroid vesicles loaded with FITC-insulin was stained with 2.0 µl of Nile Red for 10 minutes. 20.0 µl of the stained solution was than placed on a glass slide and covered with a glass cover slip. Any excessive Pheroid vesicles were removed with tissue paper.

Immersion oil was placed on the optic opening of the CLSM prior to placing the glass slide in the microscope. As the fluorescent wavelength band of FITC-insulin (488.0–494 nm) fell within the range of that of the detector system, it could easily be detected and analysed.

5.6 Results and discussion

The particle sizes as analysed were all according to specification. An example of such a measurement is given in Annexure 4.

Photographical images (micrographs) of the formulated Pheroid vesicles were captured and it was concluded that the Pheroid vesicles formed were within standard specifications as illustrated in Figure 5.1.

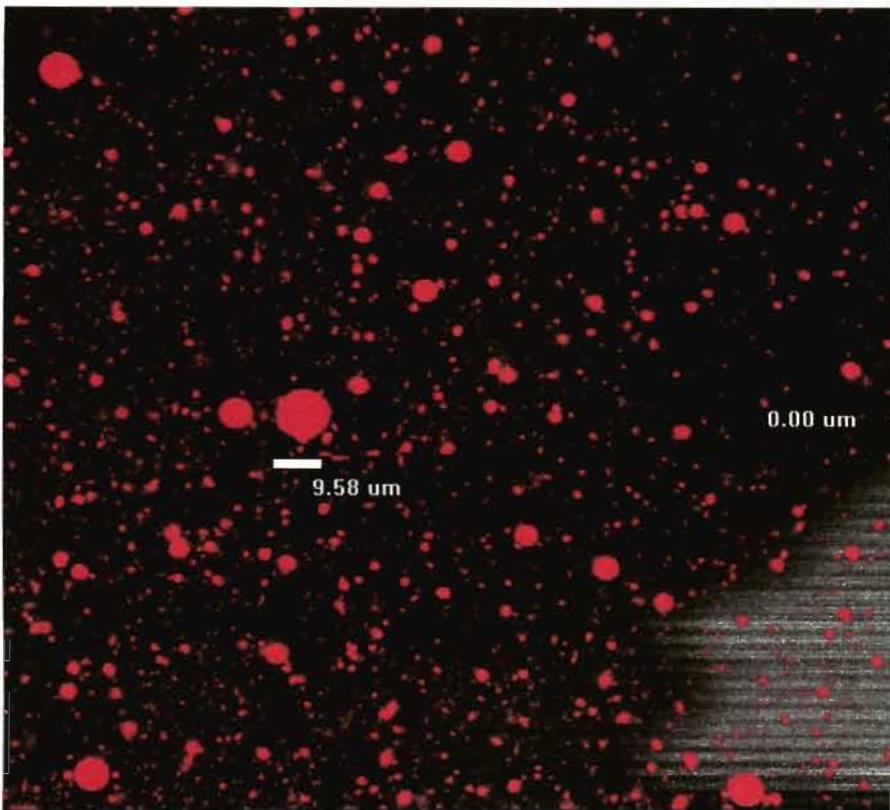


Figure 5.1: A captured CLSM micrograph of formed Pheroid vesicles coloured with Nile Red and which fall within required specifications.

Computer software concluded that the loading of FITC-insulin in Pheroid vesicles was >90.0 % and therefore totally sufficient. The yellow fluorescence of the FITC-insulin can clearly be seen in Figure 5.2, concentrated in the core of the Pheroid vesicles.

Figure 5.3 shows the fluorescence of the FITC-insulin without the fluorescence of the Nile Red. Only a very small amount of FITC-insulin was visible outside the core of the Pheroid vesicles as illustrated in Figure 5.3. The FITC-insulin that was not entrapped can be seen as small green fluorescent spots suspended in the medium.

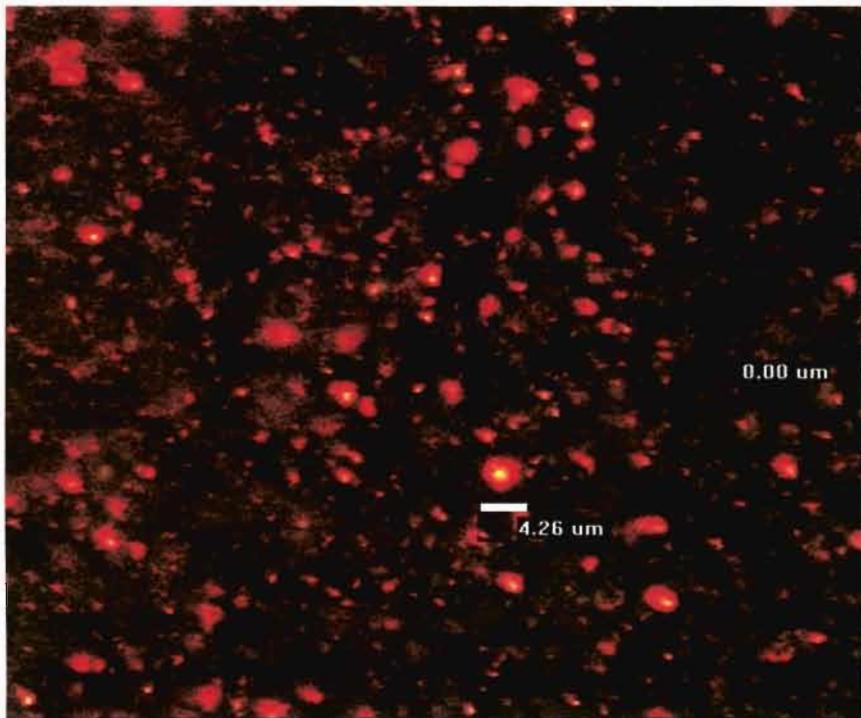


Figure 5.2: CLSM micrograph of Pheroid vesicles containing Nile Red loaded with FITC-insulin.

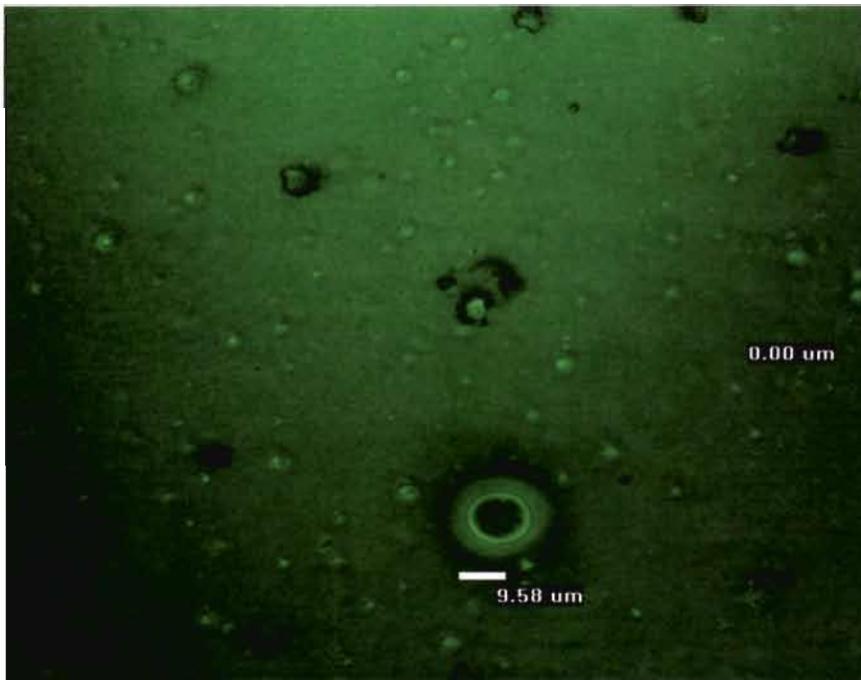


Figure 5.3: CLSM micrograph of Pheroid vesicles loaded with FITC-insulin in the absence of Nile Red.

5.7 Conclusion

After loading Pheroid vesicles with FITC-insulin and analysed with CLSM it can be concluded that the entrapment efficacy of FITC-insulin in Pheroid vesicles is totally sufficient at more than 90.0 %. Therefore Pheroid vesicles can be deemed ideal for the entrapment of insulin.

Part III: Experimental procedures

CHAPTER SIX

In vivo evaluation of per oral and nasal absorption of insulin with Pheroid technology and N-trimethyl chitosan chloride

6.1 Introduction

As discussed in previous chapters Pheroid technology and *N*-trimethyl chitosan chloride (TMC) have promising absorption enhancing and drug carrier qualities. Both have shown to be able to increase the bioavailability of degrading enzyme sensitive and poorly absorbed drugs, such as insulin, with minimal cytotoxic effects. It has been said that TMC increases the transport of insulin across Caco-2 cell monolayers (Kotzé *et al.*, 1997:249) and have proven to increase the absorption of insulin after nasal administration (Kotzé *et al.*, 1999a:253). This chapter describes the *in vivo* experimental procedures performed with male Sprague Dawley rats as model. Both nasal and oral co-administration of recombinant human insulin with Pheroid and TMC and their effect on blood glucose levels as well as blood plasma insulin levels will be evaluated.

6.2 Experimental design and *in vivo* procedures

6.2.1 Experimental animals

Male Sprague Dawley rats with a body mass of between 240.0 and 336.0g were used as experimental models to investigate the absorption enhancing capabilities of Pheroid and TMC. Sprague Dawley rats were chosen as they are readily available, breed successfully and quick in captivity and are easy to handle, as they are relatively small.

Their gastro intestinal and nasal physiology and biochemistry also show several similarities to that of humans in terms of anatomical sequence and morphology.

Direct administration into the stomach, ileum and duodenum was chosen to simulate the oral absorption studies. The experimental procedures for using rats in the selected *in vivo* method are well documented and illustrated in literature (Baudyš *et al.*, 1996:146; Matsuzawa *et al.*, 1995:1719; Michel *et al.*, 1991:2; Morishita *et al.*, 1998:192; Prego *et al.*, 2005:154; Thouitou *et al.*, 1980:108). Direct administration into the nasal cavity, and more precisely the left nostril, was chosen as the *in vivo* procedure for nasal administration and is also well documented in literature (Aspden *et al.*, 1996:25; Pereswetoff-Morath & Edman, 1995:39; Yu *et al.*, 2004:14).

An application for the experimentation on and use of Sprague Dawley rats in this study was compiled and was approved by the Ethical Committee of the North-West University (05D17) (Annexure 1). Six rats were used in each experiment to ensure that any significant statistical differences would be detected between the control and experimental groups (Statistical Consultation Services, North-West University).

6.2.2 Breeding conditions

Male Sprague-Dawley rats were bred and kept in a closed, controlled environment at the Animal Research Centre, North-West University, Potchefstroom. The animals were kept under controlled conditions to ensure an ideal growth environment with minimum exposure to pathogens, which was accomplished by a constant airflow. All the variables in the Animal Research Centre were kept constant. The conditions, to which the rats used in this study were exposed to, are shown in table 6.1. Rats had free access to water and were fed with Epol® mice cubes (Epol Pty (Ltd), Pretoria, RSA) provided by the Research Animal Centre of the North-West University.

Table 6.1 Conditions under which rats were kept at the Animal Research Centre (North-West University).

CONDITION	RECOMMENDED VALUE*	VALUE IN ANIMAL RESEARCH CENTRE
Temperature	$19 \pm 2 \text{ }^{\circ}\text{C}$	$21 \pm 2 \text{ }^{\circ}\text{C}$
Relative humidity	$55 \pm 15\%$	$55 \pm 10\%$
Rate of ventilation/air movement	15-20 changes per minute	18 changes per minute
Light intensity	350-400 lux one meter above floor level	350-400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

*Values recommended according to international standards.

6.2.3 Experimental design

Six Sprague Dawley rats were used in each experiment in accordance with literature (Apden *et al.*, 1996:25; Baudyš *et al.*, 1996:146; Lowman *et al.*, 1999:934; Pereswetoff-Morath & Edman, 1995:39; Yu *et al.*, 2004:18) to determine the effect on blood glucose and the extent of insulin absorption from the different formulations. The experimental design was a parallel design where the experimental animals were arranged in various test groups and a single treatment was administered to each individual rat in the different groups. Several control groups of six rats each also received a single dose of insulin in saline solution in order to determine absorption without the presence of any absorption enhancing agent.

One control group of six rats only received normal saline for the determination of a normal rat glucose and insulin profile during the experiment, which may serve as a reference. This was deemed necessary as physiological stresses such as injury, surgery, renal failure, burns and infections causes blood cortisol, glucagon,

catecholamines, and growth hormone levels to increase. The patient (rat) also tend to be resistant to insulin and the basal metabolic rate and blood glucose and free fatty acid levels are elevated (Harris & Crabb, 2002:892).

6.2.4 Preparation of experimental formulations

6.2.4.1 Materials

Recombinant human insulin expressed in yeast (28.7 International Units (IU) per mg dry base) was supplied by Sigma-Aldrich (South Africa) (Annexure 2). *N*-trimethyl chitosan chloride (TMC). Sodium chloride (Saarchem, South Africa). TMC and freshly prepared Pheroid vesicles and -micro-sponges were kindly provided by the Department of Pharmaceutics at the School of Pharmacy, North-West University, Potchefstroom.

6.2.4.2 Method

6.2.4.2.1 Pheroid formulations

Pheroid™ vesicles and -micro-sponges were freshly prepared before each experiment, as described in chapter 5, and supplied by the Department of Pharmaceutics at the School of Pharmacy, North-West University, Potchefstroom. Insulin was entrapped in Pheroid vesicles and -micro-sponges 24 hours prior to each experiment except where indicated. Insulin was weighed according to the concentration and volume of the formulation.

The Pheroid vesicle or -microsponge solution was then warmed to 37.0 °C in a waterbath where after the insulin was added and entrapped in the Pheroid solutions. These formulations were then slowly shaken for 15 minutes and then kept at ± 1.0 °C on ice until administration. The content of the different formulations used and the volumes of the doses administered are summarised in table 6.1.

Table 6.1. Examples of Pheroid and insulin formulations prepared for the *in vivo* experiments.

Type of Pheroid	Formulation used for	Dose ($\mu\text{l}/250\text{g}$)	Total concentration of insulin (IU/kg)
Pheroid vesicles	Intra-gastric administration	500	50
Pheroid micro-sponges	Intra-gastric administration	500	50
Pheroid vesicles	Intra-ileal administration	500	50
Pheroid micro-sponges	Intra-ileal administration	500	50
Pheroid vesicles	Intra-colonic administration	500	50
Pheroid micro-sponges	Intra-colonic administration	500	50
Pheroid vesicles	Subcutaneous administration	300	4
Pheroid micro-sponges	Subcutaneous administration	300	4
Pheroid vesicles	Nasal administration	30	8
Pheroid micro-sponges	Nasal administration	30	8
Pheroid vesicles	Nasal administration	30	12
Pheroid micro-sponges	Nasal administration	30	12

6.2.4.2.2 TMC formulations

TMC with a degree of quaternisation of 48.0% and a molecular weight of 166 000.0 g/mole was used. This TMC was previously synthesised from Primex® chitosan raw material with a degree of deacetylation of 97.0% and was a generous gift from the Department of Pharmaceutics at the School of Pharmacy, North-West University, Potchefstroom. A TMC solution was prepared by weighing and then dissolving TMC in distilled water accordingly to give a 1.0% (w/v) TMC solution.

Insulin was weighed accordingly (examples illustrated in table 6.2) and dissolved in freshly prepared 0.9% normal saline. Then 1.0 ml of the TMC solution and 1.0ml of the insulin in saline solution was mixed together to give insulin concentrations of 10.0, 25.0 and 50.0 IU/kg in a 0.5% TMC solution. Several times the insulin/TMC

solution appeared murky white, which indicated that TMC and insulin might have interacted to form a complex that will render insulin bound and ineffective. The solution was then treated with 0.1M NaOH until it became clear or transparent. A summary of the TMC and insulin formulations that were prepared is given in table 6.3.

Table 6.2. Examples of amounts of insulin weighed to solution volume and final solution concentrations after mixing with dissolved TMC.

Amount of Insulin weighed (mg)	Insulin Units/kg	Solution volume (ml)	Final TMC/Insulin concentration (%/[IU/kg])
3.488	100	2	0.5/50
1.744	50	2	0.5/25
0.698	20	2	0.5/10

Table 6.3. Examples of the TMC and insulin formulations prepared for the *in vivo* experiments.

Formulation used for	Strength of TMC solution (%)	Dose (μ L/250g)	Total insulin concentration (IU/kg)
Intra-ileal administration	0.5	500	10
Intra-ileal administration	0.5	500	25
Intra-ileal administration	0.5	500	50

6.2.5 Laboratory animal preparation and administration of TMC and insulin formulations to rats

6.2.5.1 Materials

Freshly prepared insulin in Pheroid (4.0, 8.0, 12.0, and 50.0 IU/kg) solutions Freshly prepared insulin and 0.5% TMC (10.0, 25.0 and 50.0 IU/kg) solutions. Sodium chloride supplied by Saarchem, South Africa. Heparin sodium 5000.0 IU/ml was supplied by Fresenius, South Africa. Halothane 4.0% (v/v) (Fluothane[®]) supplied by Zebeca South Africa (Pty) Ltd. Fine bore polythene tubing, 0.58 mm inner diameter and 0.96 mm outer diameter (Portex Ltd., Hythe, Kent, England).

6.2.5.2 Induction and maintenance of anaesthesia

All rats were fasted 18 hours prior to the commencement of each experiment but water was available *ad libitum*. Anaesthesia was induced by placing each rat, individually, in a closed glass container with a metal grid floor above a cotton wool layer. The cotton wool layer contained 4.0% liquid halothane of which each rat was allowed to inhale the vapours of. At no time was there any direct contact between the rat and the liquid halothane. Each rat was removed from the container after it has lost consciousness.

Directly after each rat was removed from the container, its' head was placed in a latex rubber sheath that was connected to two plastic bags by means of a three-way valve. The two plastic bags had each a volume of 5000.0 cm³ and contained a mixture of 2.0% and 4.0% (v/v) halothane in medical oxygen respectively. The latex rubber sheath fitted securely but comfortably over the head of the rat to prevent air from escaping.

By simply rotating the lever on the three-way valve the concentration of anaesthesia was easily altered. The 2.0% (v/v) halothane was used to maintain anaesthesia and the 4.0% (v/v) halothane was only used to deepen the anaesthesia when deemed necessary. Each bag also contained a small amount (\pm 5.0g) of soda lime granules,

located at the entrance to the valve to absorb the CO₂ expired by the rat. Figure 6.1 clearly depicts the apparatus used for the maintenance of anaesthesia.

Prior to the commencement of surgery the rat was placed on a thermal electric blanket to maintain a constant body temperature of 37.0 °C throughout the experiment. At the end of each experiment euthanasia was performed, without the rat gaining consciousness, by deepening the anaesthesia with 4.0% (v/v) halothane until breathing and heart rate ceased. Depth of anaesthesia was monitored continuously using the pedal reflex and by monitoring the depth and rate of breathing.

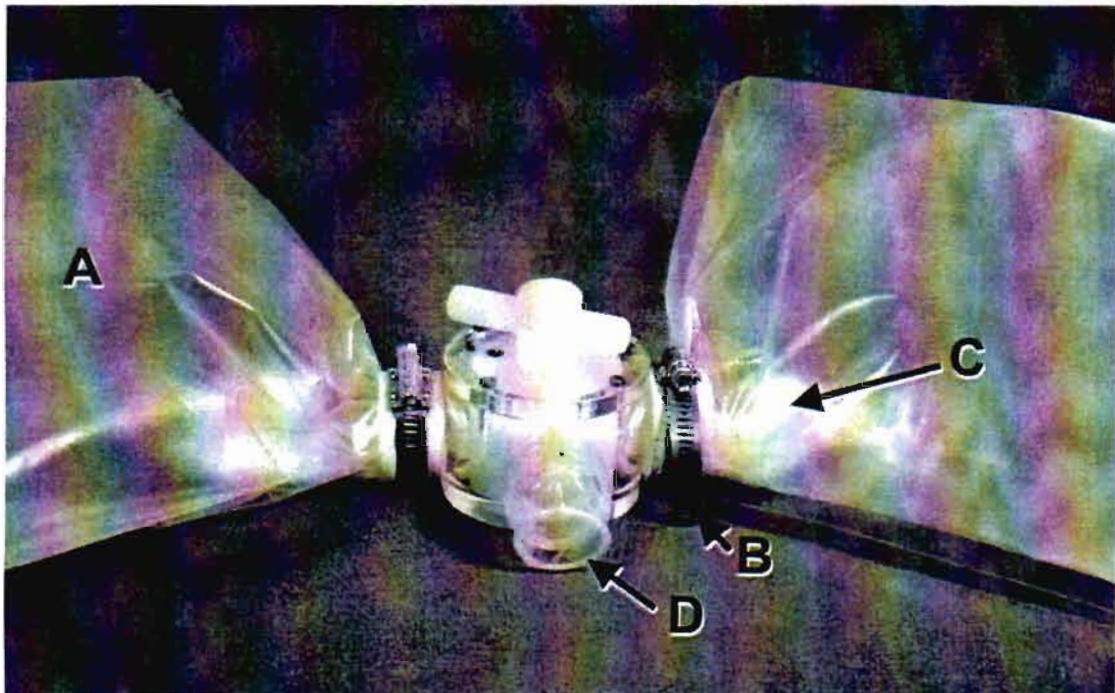


Figure 6.1. Apparatus needed to maintain anaesthesia: A) Plastic bag containing halothane and oxygen mixture; B) Clamp; C) Soda lime; D) Latex rubber sheath.

6.2.5.3 Surgical procedures

6.2.5.3.1 Cannulation of the *artery carotis communis*

All surgical procedures were performed on rats while under anaesthesia and were conducted by a veterinary technician of the Animal Research Centre, North-West University, Potchefstroom.

Firstly the hair was shaved in the area of the ventral neck with a sharp razor and the skin disinfected. The rat was then placed prostrate in a supine position with the head stretched in slight opistotonus. A mid ventral incision of about 2.0 cm was made in the neck skin. In-between the two bilateral *muscularis sternohyoideus* up to the area lateral to the trachea, a blunt dissection was made to expose the *artery carotis communis* beneath the *muscularis sternohyoideus*. The *a. carotis communis* was then loosened from surrounding membranes and lifted out of the surgical incision and was kept wet with physiological saline at body temperature. The rostral part of the artery was ligated with silk. The artery was then clamped shut with a mosquito artery clamp proximal to the ligature (figure 6.2).

Another ligature was then pre-placed loosely caudal to the first ligature. A "V" shaped incision was then made with a pair of scissors in the artery wall between the two ligatures. A thin polythene tube (about 30.0 cm long) was pre-filled with saline heparin (5.0 IU/ml) and connected via a needle to a 10.0 ml syringe containing the same content at 37.0 °C. The addition of the heparin was to prevent blood clotting in the tube and in the proximal artery. The other end of the tube was diagonally cut in order to sharpen it for easier insertion into the artery. The tube was also slightly lubricated with K-Y gel (Johnson & Johnson, South Africa) and then guided for approximately 2.0 cm into the artery via the incision. As soon as a strong and constant blood flow was established the clamp was released and the loose ligature was tied around the artery with the tube inside. Another ligature was also tied to ensure that the tube is fitted tightly and will not come loose (figure 6.3). The wound was then covered with sterile gauze and wetted with the saline-heparin solution to prevent dehydration of the wound.

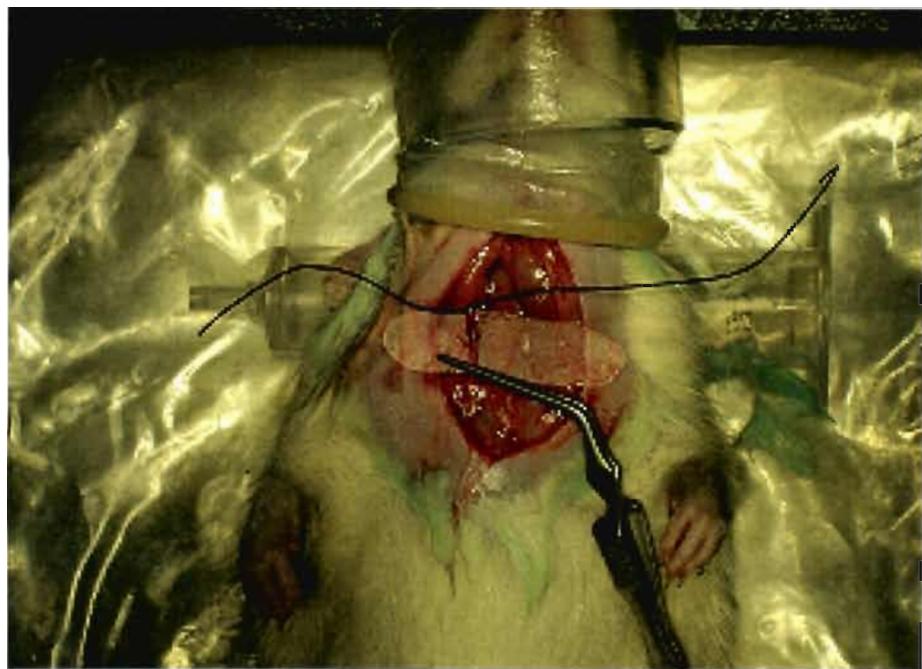


Figure 6.2. The *artery carotis communis* is ligated and lifted out of the incision.



Figure 6.3 The *artery carotis communis* after surgical cannulation.

6.2.5.3.2 Abdominal surgical procedures

Abdominal surgical procedures were performed under the same conditions than with the cannulation of the *artery carotis communis*. The rat was also placed prostrate in a supine position before surgery commenced.

First the skin of the ventral abdomen was shaved with a sharp razor and then disinfected. A midline abdominal incision (laparotomy) of approximately 2.0 cm was carefully made through the linea alba caudal to the sterno without cutting the intestines. Either the stomach, ileum or colon was then identified and carefully lifted out of the incision for administration of the insulin formulations where after it was then carefully replaced in the abdominal cavity in its anatomical position (figure 6.4). The incision was then covered with sterile gauze and kept moist with the saline-heparin solution to prevent dehydration



Figure 6.4. The shaven abdomen of the rat after laparotomy.



Figure 6.5. Conditions during experimental procedures

6.2.5.4 Administration of insulin formulations

6.2.5.4.1 Oral administrations

After the laparotomy the stomach, ileum or colon was identified and lifted out of the abdomen (figure 6.6). Oral insulin Pheroid and -TMC formulations were then injected with a needle and syringe directly into the specific area (figure 6.7).

Intra-gastric injections were made into the lumen of the stomach. The stomach was ligated at the start of the duodenum to ensure that the formulations did not move down the small intestine by peristaltic movement. Intra-ileal administrations were made directly into the lumen of the small intestine, 7.0 cm from where it exits from the stomach to ensure that the formulation is administered into the ileum. The small intestine was not ligated in order to simulate the normal peristalsis and hence absorption of insulin in a non-artificial environment at normal transit times.

Intra-colonic administrations were made directly into the colon. The colon was ligated at the proximal end to ensure that the formulation did not pass back into the ileum where additional absorption may occur. Volumes of 500.0 μ l per 250g body weight were administered via an insulin syringe. Insulin formulations were kept on ice before and during the experiment and administrations were done slowly to prevent spillage.

Initially a preliminary comparison was made by administering the insulin in Pheroid into the stomach, ileum and colon in order to determine which area of the gastro intestinal tract will be the optimum site for absorption. The insulin and TMC formulations would then be administered only to the site of maximal absorption to determine the efficacy thereof. A summary of the orally administered insulin formulations is given in table 6.4.

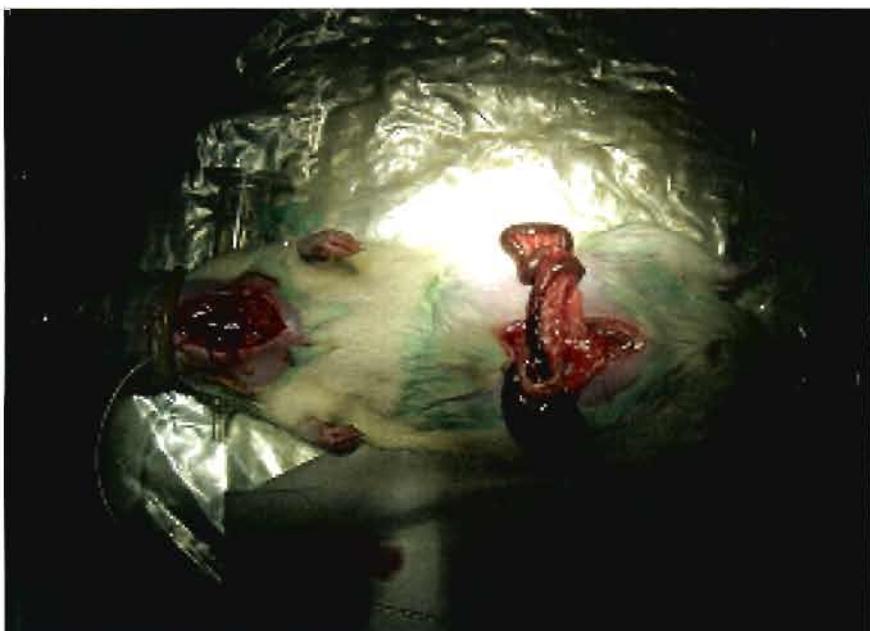


Figure 6.6. Intestines lifted out of the abdomen after laparotomy.

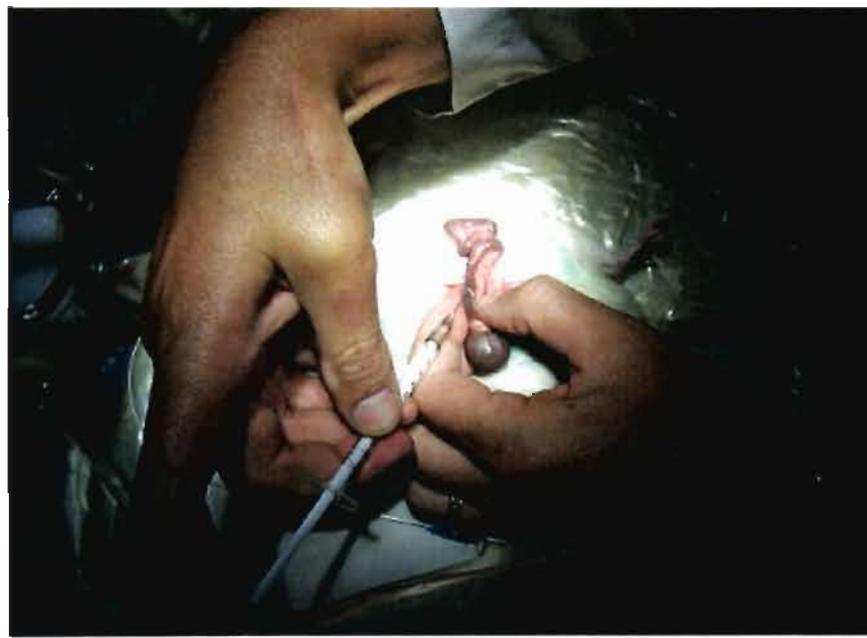


Figure 6.7. Direct intra-colonic administration of insulin in Pheroid formulation.

Table 6.4. Insulin formulations administered perorally.

Insulin formulation	Type of administration	Dose ($\mu\text{l}/250\text{g}$)	Total concentration
		of insulin (IU/kg)	
Insulin in saline (control)	Intra-gastric	500	50
Pheroid vesicles	Intra-gastric	500	50
Pheroid micro-sponges	Intra-gastric	500	50
Insulin in saline (control)	Intra-ileal	500	50
Pheroid vesicles	Intra-ileal	500	50
Pheroid™ micro-sponges	Intra-ileal	500	50
TMC 0.5%	Intra-ileal	500	10
TMC 0.5%	Intra-ileal	500	25
TMC 0.5%	Intra-ileal	500	50
Insulin in saline (control)	Intra-colonic	500	50
Pheroid vesicles	Intra-colonic	500	50
Pheroid micro-sponges	Intra-colonic	500	50

6.2.5.4.2 Nasal administrations

30.0 μ l / 250.0g body weight of each insulin in Pheroid formulation was administered to the left nostril of each rat (figure 6.8). Administrations were done while the rat was unconscious. The rat was briefly taken out of the rubber sheath and the formulation was administered with an Eppendorf® micropipette with a range of 10.0 to 100.0 μ l. The administration was done slowly and tactfully to prevent damage to the nasal epithelium and to minimise irritation which could have caused the rat to sneeze the formulation out of the nasal cavity. A summary of nasally administered insulin formulations is given in table 6.5.



Figure 6.8. Nasal administration of insulin in Pheroid formulation.

Table 6.5. Insulin in Pheroid formulations for nasal administration.

Type of insulin formulation	Dose (μ l/250g)	Total concentration of insulin (IU/kg)
Insulin in saline (control)	30	8
Pheroid vesicles	30	8
Pheroid micro-sponges	30	8
Insulin in saline (control)	30	12
Pheroid vesicles	30	12
Pheroid micro-sponges	30	12

6.2.5.4.3 Intravenous and subcutaneous administrations

A 200.0 μ l / 250.0g body weight intravenous insulin (0.5 IU/kg) in saline administration was made into the tail of each rat. This served as a reference to determine the efficacy of recombinant human insulin in rats and to determine relative bioavailability for the insulin-Pheroid and -TMC formulations.

Subcutaneous administrations were also made to determine whether if insulin-Pheroid and -TMC formulations would be suitable for subcutaneous use compared to present commercially available products. Subcutaneous injections were made just beneath the abdominal skin in volumes of 300.0 μ l / 250.0g of body weight. Insulin was administered at a concentration of 4.0 IU/kg, which seemed comparable to commercial products. Table 6.6 is a summary of the intravenously and subcutaneously administered insulin formulations.

Table 6.6. Intravenous and subcutaneous administration of insulin- Pheroid formulations.

Type of insulin formulation	Type of administration	Dose volume (μ l/250g)	Total concentration of insulin (IU/kg)
Insulin in saline (control)	Intravenous	200	0.5
Insulin in saline (control)	Subcutaneous	300	4
Pheroid vesicles	Subcutaneous	300	4
Pheroid micro-sponges	Subcutaneous	300	4

6.2.5.5 Collection of blood samples

Blood samples with a volume of 1.0 ml were collected from the cannula in 1.5 ml Eppendorf® micro test tubes (Merck, South Africa). Eight samples were collected from each rat at different time intervals. The first sample was collected 1 minute before administration and served as time 0. Thereafter samples were collected at 5, 10, 15, 30, 60, 120 and 180 minutes after administration. One drop of blood was

taken directly from the micro test tube for the determination of blood glucose values. All the samples were then stored at ± 1.0 °C in a cold storage box filled with ice. All the samples were then centrifuged (Eppendorf® Centrifuge 5415C) within 30.0 minutes of collection at 7000.0 rpm for 7 minutes. The blood plasma was then extracted using an Eppendorf® micropipette with a range of 10.0 to 100.0 μl after which the samples were stored at -65.0 °C until it was analysed.

6.2.6 Determination of blood glucose levels

Blood glucose levels of each rat were monitored to determine the therapeutic effect of each formulation. A drop of blood was taken immediately after each blood sample was taken. Blood glucose levels were measured with a Glucometer® II reflectance meter (Boehringer Ingelheim Pharmaceuticals, South Africa). A single drop of blood was used on a Glucostix® reagent strip (Bayer, South Africa). The drop was applied and had to be blotted after 30 seconds where after it was inserted into the slot of the Glucometer® II reflectance meter and a reading in mmol/l was given after 20 seconds.

6.2.7 Quantitive analysis of plasma insulin concentrations

Plasma insulin levels were determined *in vitro* by the quantitive measurement of human insulin in plasma using a human specific radioimmunoassay (RIA) kit. A LINCO (LINCO Research, USA) human insulin specific RIA kit (catalogue number HI-14K) was obtained from Diagnostic System Laboratories (Laboratory Specialities, South Africa). A human insulin specific radioimmunoassay was chosen to eliminate interference of rat insulin as much as possible, which would give a more accurate indication of the actual absorption of recombinant human insulin. The specificity of the RIA kit was 100.0% for human insulin and 0.1% for rat insulin and the kit does not cross-react with human proinsulin, which ensures that only true insulin levels are measured.

The lowest level of insulin that can be detected by the assay is 2.0 $\mu\text{IU}/\text{ml}$.

6.2.7.1 Principles of the procedure

In radioimmunoassay, a fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited. For example, the antibody may bind only 50% of the total tracer concentration. If unlabeled antigen is added to this system, there is competition between labelled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labelled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The LINCO Research Human Insulin assay used utilises ^{125}I -labeled human insulin and a human insulin antiserum to determine the level of insulin in serum, plasma or tissue culture media by the double antibody/PEG technique (Linco Research, 2006:3).

6.2.7.2 Reagents supplied

Each kit was sufficient to run 250.0 tubes and contained the following reagents:

A. Assay buffer

0.05M Phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, and 1.0% RIA grade BSA. Quantity: 40.0 ml/vial. Preparation: Ready to use.

B. Human insulin antibody

Guinea pig anti-human insulin specific antibody in assay buffer quantity: 26.0 ml / vial. Preparation: Ready to use.

C. ^{125}I -insulin

^{125}I -insulin Label, HPLC purified (specific activity 367.0 uCi / fig). Lyophilised for stability. Freshly iodinated label contains less than 5.0 uCi (185.0 kBq), calibrated to

the 1st Monday of each month.

Quantity: 27.0 ml / vial upon hydration.

Preparation: Contents is lyophilised. Hydrate with entire contents of label hydrating buffer. Allow setting at room temperature for 30 minutes, with occasional gentle mixing.

D. Label hydrating buffer

Assay buffer containing normal guinea pig serum as a carrier. It was used to hydrate the ¹²⁵I-insulin. Quantity: 27.0 ml / vial. Preparation: Ready to use.

E. Human insulin standards

Purified recombinant human insulin in assay buffer at the following concentrations: 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 µU/ml. Quantity: 2.0 ml / vial. Preparation: Ready to use.

F. Quality controls 1 and 2

Purified recombinant human insulin in assay buffer. Quantity: 1 ml/vial. Preparation: Ready to use.

G. Precipitating Reagent

Goat anti-guinea pig IgG serum, 3.0% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide.

Quantity: 260.0 ml / vial.

Preparation: Ready to use; chill to 4°C (Linco Research, 2006:5).

6.2.7.3 Storage and stability

All reagents were refrigerated between 2.0 and 8.0 °C after delivery until used. All reagents were kept cold during use in cold boxes containing crushed ice.

6.2.7.4 Assay procedure

Day one:

1. 300.0 µl of assay buffer was added to the non-specific binding (NSB) tubes (3-4), 200.0 µl to the reference (Bo) tubes (5-6), and 150.0 µl to tubes 7 through the end of the assay.
2. 100.0 µl of the standards and quality controls were added in duplicate to all the samples.

3. 50.0 μ l of each sample was added in duplicate. (NOTE:
4. Hydrated ^{125}I -insulin solution was prepared and 100.0 μ l was added to all tubes.
5. 100.0 μ l of the human insulin antibody was added to all tubes except total count tubes (1-2) and NSB tubes (3-4).
6. All samples were then vortexed, covered, and incubated overnight (20-24 hours) at room temperature (22.0- 25.0 °C).

Day two:

7. 1.0 ml of cold (4°C) precipitating reagent was added to all tubes (except total count tubes).
8. All samples were then vortexed and incubated for 20 minutes at 4.0 °C.
9. After incubation the samples were centrifuged at 4.0 °C, except total count tubes (1-2), for 20 minutes at 2300.0 Xg.
10. Immediately after centrifuging the supernatant was decanted from all tubes except total count tubes (1-2). Tubes were drained for at least 20 seconds and excess liquid was blotted from the lip of each tube. Tubes were inverted only once.
11. All tubes were counted for 60 seconds in a Cobra Gamma Counter (Packard, USA). The bound fraction was counted and insulin concentrations were determined in $\mu\text{U}/\text{ml}$ using Packard® software package (Linco Research, 2006:10).

A summary of the procedure is given as a flow chart on table 6.7.

Table 6.7. Assay procedure flow chart (Linco Research, 2006:11).

Day one						Day two		
Set-up	Step 1	Steps 2 & 3	Step 4	Step 5	Step 6	Step 7	Step 8	Steps 9- 11
Tube Number	Add assay buffer	Add standard / quality control sample	Add I- 125 human insulin tracer	Add human insulin antibody	Vortex, cover, and incubate 20-24 hrs at room temperature	Add precipitating reagent	Vortex, and incubate for 20 min. at 4.0 °C	Centrifuge at 4.0 °C for 20 min.. decant, and count
1,2	N/A	N/A	100.0 µl	N/A				
3,4	300.0 µl	N/A	100.0 µl	N/A		1.0 ml		
5,6	200.0 µl	N/A	100.0 µl	100.0 µl		1.0 ml		
7,8	100.0 µl	100.0 µl of 2.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
9,10	100.0 µl	100.0 µl of 5.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
11,12	100.0 µl	100.0 µl of 10.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
13,14	100.0 µl	100.0 µl of 20.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
15,16	100.0 µl	100.0 µl of 50.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
17,18	100.0 µl	100.0 µl of 100.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
19,20	100.0 µl	100.0 µl of 200.0 µU/ml	100.0 µl	100.0 µl		1.0 ml		
21,22	100.0 µl	100.0 µl of QC 1	100.0 µl	100.0 µl		1.0 ml		
23-24	100.0 µl	100.0 µl of QC 2	100.0 µl	100.0 µl		1.0 ml		
25,26	150.0 µl	50.0 µl of unknown	100.0 µl	100.0 µl		1.0 ml		
27-n	150.0 µl	50.0 µl of unknown	100.0 µl	100.0 µl		1.0 ml		

Results and discussion

Blood glucose levels (mmol/l) were calculated as a percentage (%) of the original value and represented against time in the form of a graph. Y error bars represent the standard error in that range of data. Even though blood glucose levels are a clear indication of the therapeutic effect of the formulations the exact extent and amount of insulin absorbed has to be measured and quantified. Subsequently is a series of data depicting blood plasma insulin levels in μ IU/ml after administration of the various formulations. All average blood glucose and plasma insulin values over time are given in the form of tables in Annexure 3.

Intravenous administration

6.3.1.1 Intravenous administration of insulin 0.5 IU/kg

Blood glucose levels of the intravenous administration of 0.5 IU/kg insulin in saline solution (reference) are represented in figure 6.9.

Intravenous injection of insulin 0.5 IU/kg in saline resulted in a rapid and pronounced blood glucose lowering effect. After just 5 minutes average blood glucose levels drops by 13.3% and a maximum affect is reached after 30 minutes where blood glucose levels falls to 34.9% of the origin value at time 0. A glucagon response may be the reason for elevated blood glucose levels back to normal after 150 minutes and is slightly elevated by 9.7% 3 hours after administration.

Blood plasma insulin levels after the intravenous administration of 0.5 IU/kg insulin in saline solution (reference) are represented in figure 6.10.

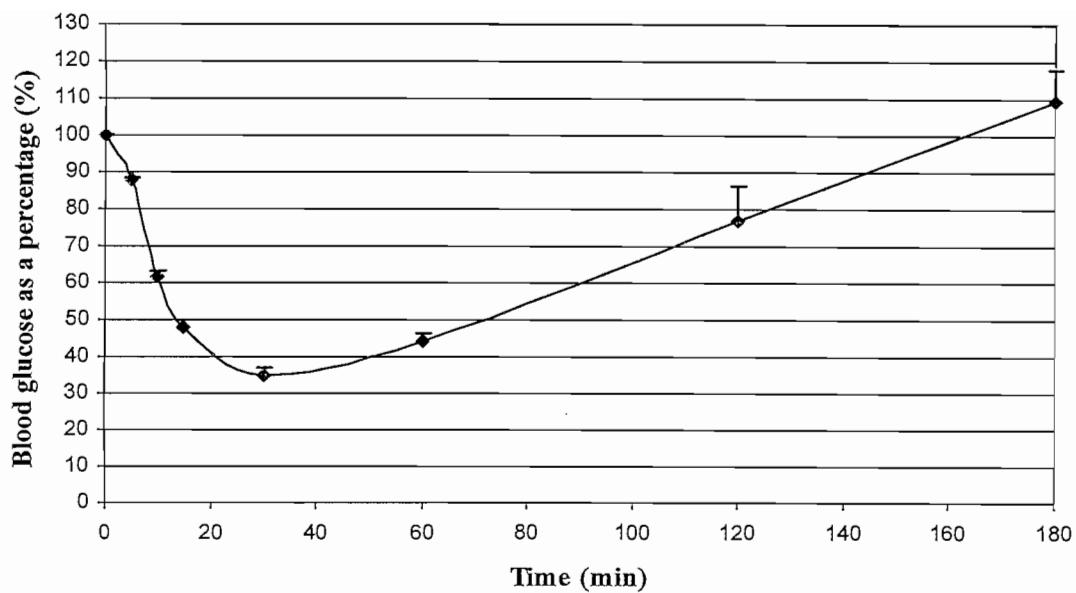


Figure 6.9. Average blood glucose as a percentage against time after intravenous administration of 0.5 IU/kg insulin in saline solution.

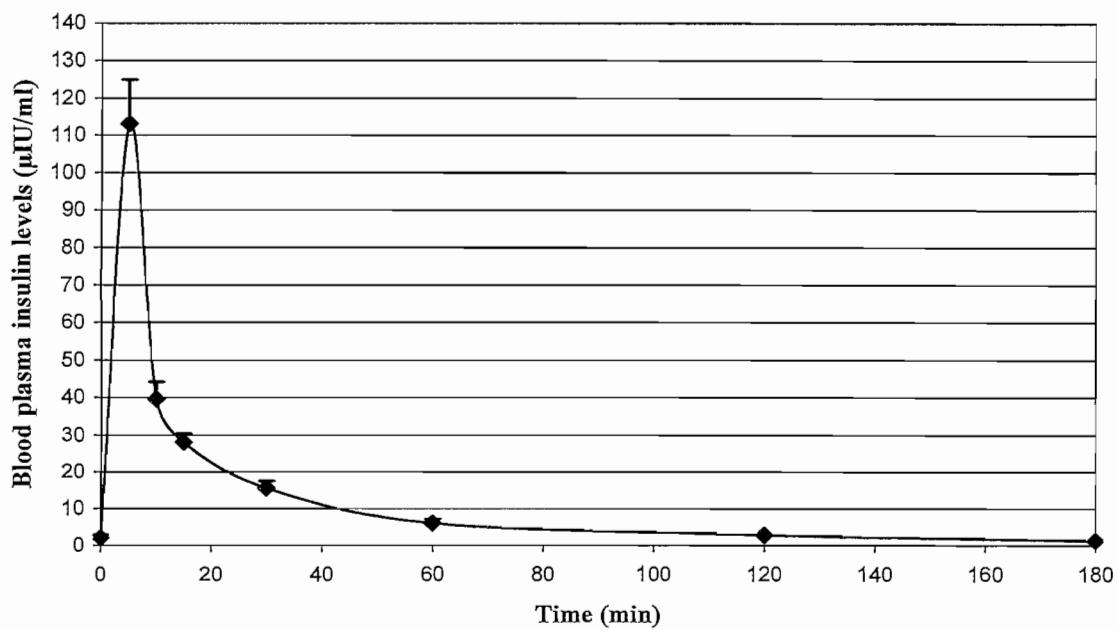


Figure 6.10 Blood plasma insulin levels ($\mu\text{IU}/\text{ml}$) after intravenous administration of insulin in saline.

The data depicted in figure 6.10 clearly shows the rapid distribution and elimination of insulin after intravenous administration. A maximum plasma level of 113.1 $\mu\text{IU}/\text{ml}$ is reached after only 5 minutes but slowly decreases to 39.6 $\mu\text{IU}/\text{ml}$ after 10 minutes. A normal elimination presents whereby only 1.3 $\mu\text{IU}/\text{ml}$ insulin remains in the plasma after 3 hours.

6.3.1.2 Intravenous administration of normal saline

As mentioned earlier, a control group of six rats received an intravenous administration of normal saline for the determination of a normal rat glucose and insulin profile during the conduction of the experiments. This was deemed necessary as physiological stresses such as injury, surgery, renal failure, burns and infections may cause blood cortisol, glucagon, catecholamines, and growth hormone levels to increase. The patient (rat) also tend to be resistant to insulin and the basal metabolic rate and blood glucose and free fatty acid levels are elevated (Harris & Crabb, 2002:892). Figure 6.11 clearly depicts the blood glucose profile during a 3-hour experiment after cannulation of the *artery carotis communis*.

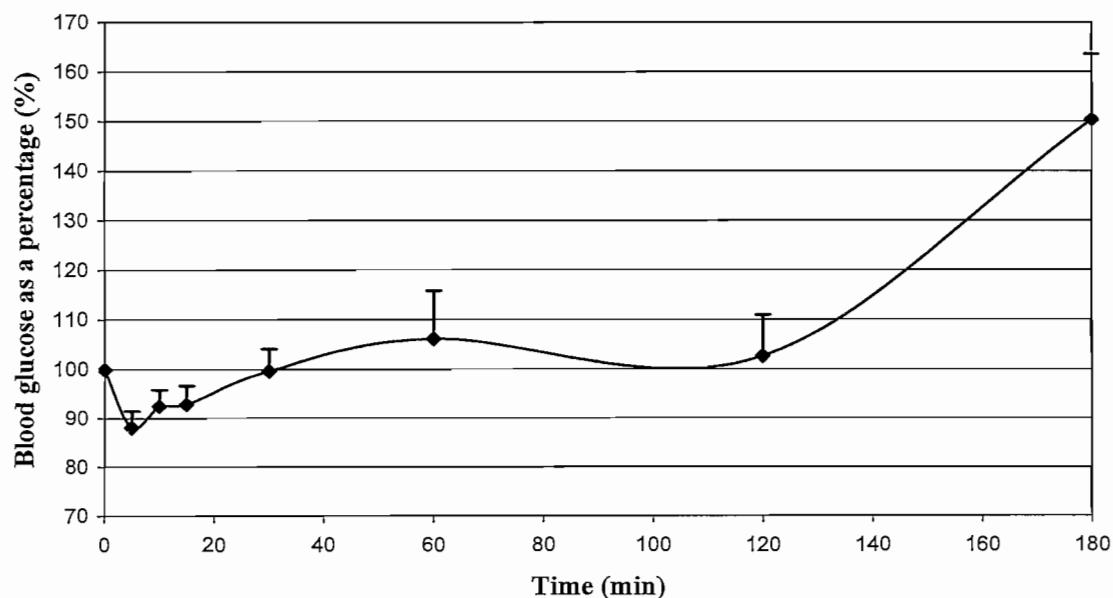


Figure 6.11 Average blood glucose as a percentage against time after intravenous administration of normal saline solution.

From the profile in figure 6.11 it can be clearly seen that, for up to 2 hours, the blood glucose levels of the rats seems to be fairly stable, varying with no more than 12.0%. Between 2 and 3 hours however, the blood glucose levels starts to rise because of elevated glucagon levels due to physiological stress and fatigue. Even though stress levels were kept to a minimum, it is difficult to determine exactly how much stress the rats experienced throughout the extent of the surgery and the experiments.

Figure 6.12 illustrates the effect of normal saline on plasma insulin levels in rats during the 3-hour experiment.

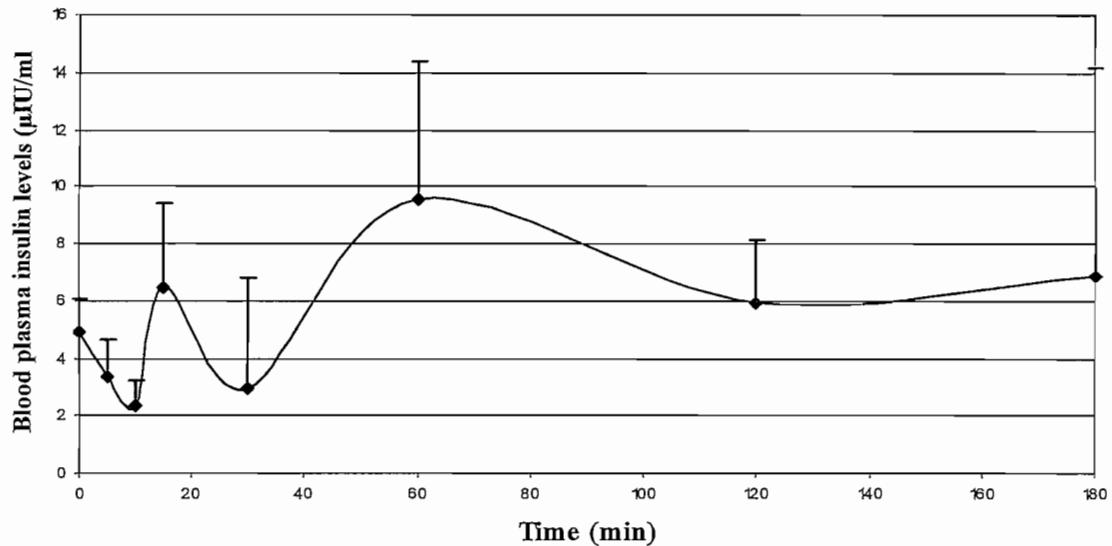


Figure 6.12 Blood plasma insulin levels ($\mu\text{IU}/\text{ml}$) after intravenous administration of normal saline (0.9%).

It can be clearly seen that there exists a distinct, but not so pronounced, fluctuation in plasma insulin levels during the experiment. There is a definite release of insulin at 15 minutes after an initial decrease directly after administration. Insulin levels are raised to a maximum of $9.6 \mu\text{IU}/\text{ml}$ at 60 minutes after which the levels decrease to

6.9 μ IU/ml after 3 hours. The elevated insulin levels at 60 minutes may be reflected as the glucagon response in the blood glucose levels seen in figure 6.11.

6.3.1.3 Comparison between the reference (intravenous administration of 0.5 IU/kg insulin) to control (intravenous administration of normal saline)

A comparison of the intravenous administration of 0.5 IU/kg insulin (reference) and the intravenous administration of normal saline (control) is given in figure 6.13.

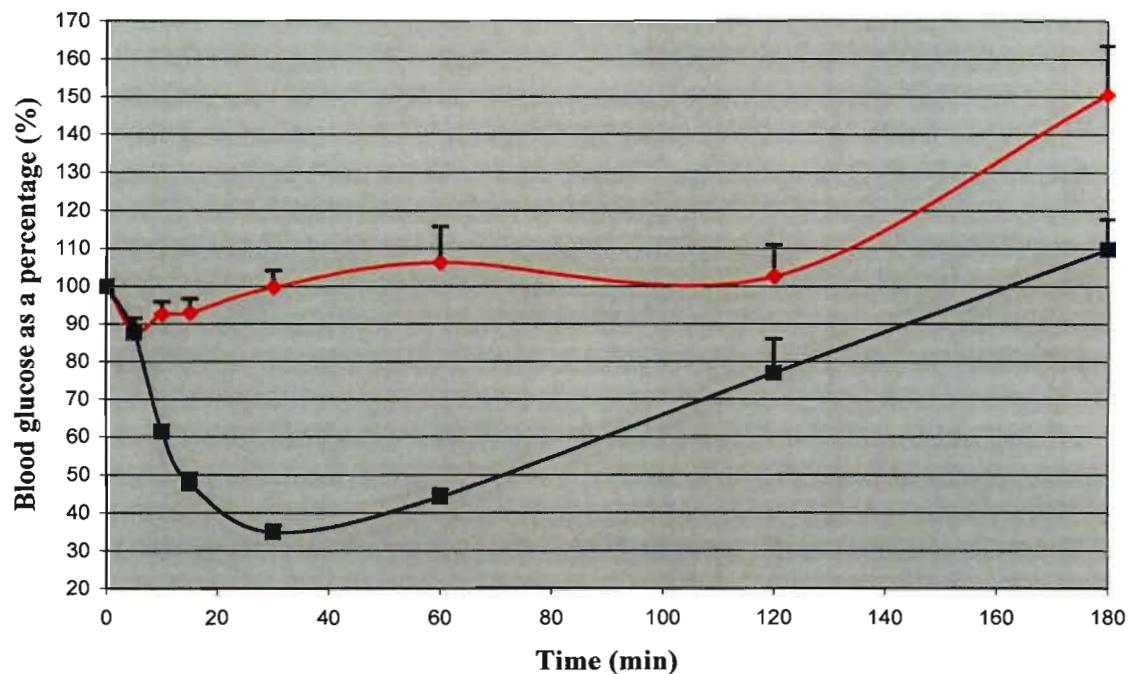


Figure 6.13 A comparison of the average blood glucose levels as a percentage of the reference (intravenous administration of 0.5 IU/kg insulin) (■) to the control (intravenous administration of normal saline) (◆).

As seen in figure 6.13 it is clear that exogenous administrated insulin has a major impact on normal blood glucose levels, even when the rat is under surgery.

A comparative graph of blood insulin levels is given in figure 6.14. A clear distinction can be seen between “normal” insulin levels and insulin levels after the addition of exogenous insulin.

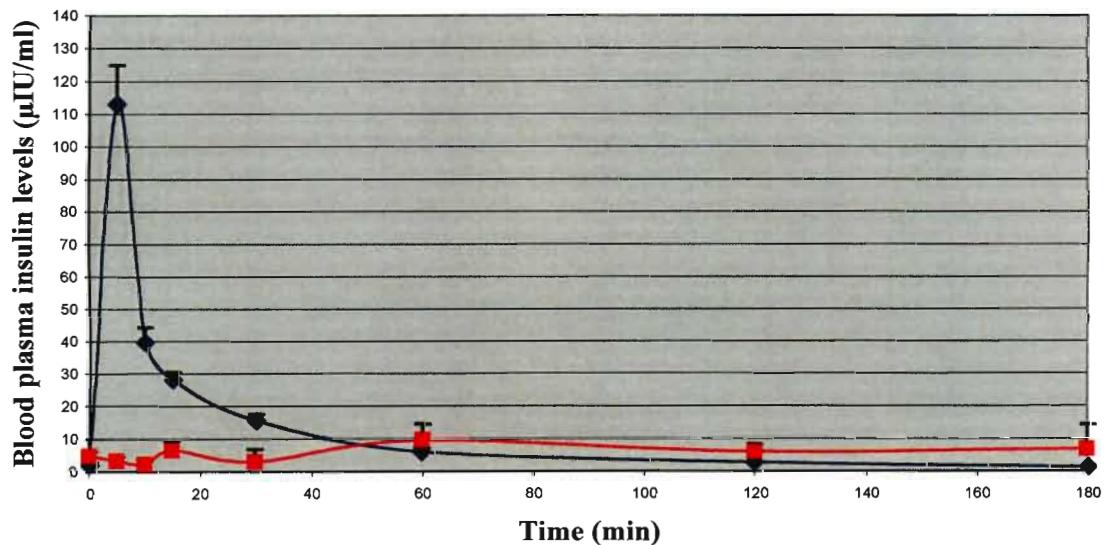


Figure 6.14 Blood plasma insulin levels ($\mu\text{IU}/\text{ml}$) after intravenous administration of insulin 0.5 IU/kg (◆) and normal saline (0.9%) (■).

6.3.2 Intra-gastric administration

To follow is a series of “peroral” administrations of insulin whereby insulin was administered directly into the gastrointestinal tract as part of a liquid formulation.

6.3.2.1 Intra-gastric administration of insulin (50.0 IU/kg) in saline (control)

A graph of blood glucose levels after intra-gastric administration of insulin (50.0 IU/kg) in saline, which served as a control, is given in figure 6.15.

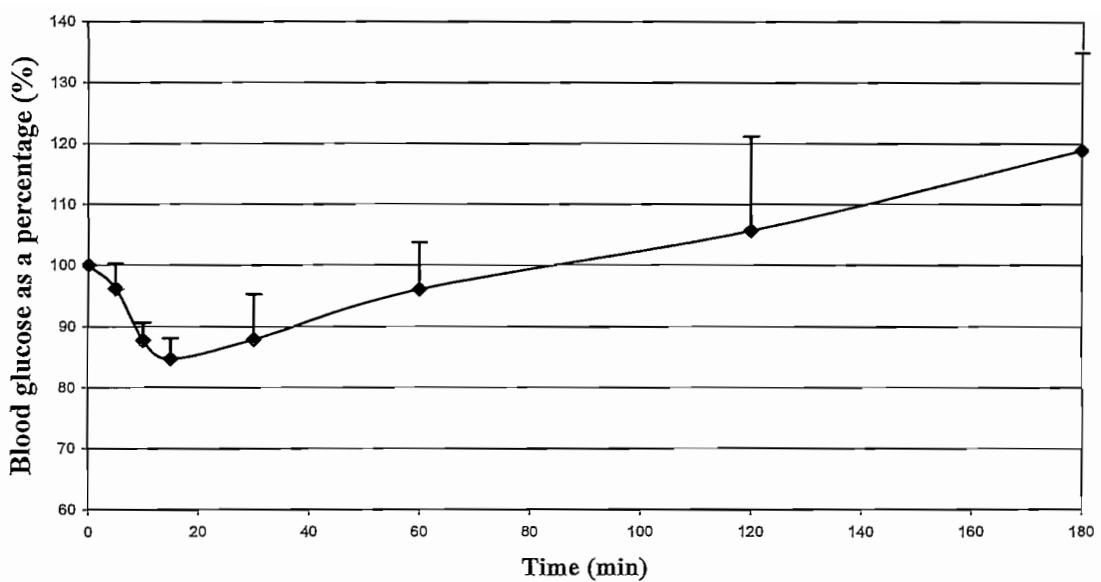


Figure 6.15 Average blood glucose as a percentage against time after intra-gastric administration of insulin (50.0 IU/kg) in saline solution.

After 5 minutes it seems that there is a slow response where blood glucose levels decreases by only 3.8%. A maximum decrease in blood glucose levels of 15.3% is observed at 15 minutes. At approximately 90 minutes the average blood glucose levels returns to normal but continue to increase to 118.9% at 3 hours due to a glucagon response. The duration of action seemed to be relatively short (less than one and a half hour) with a pronounced unwanted glucagon response.

As a control insulin (50.0 IU/kg) was dissolved in normal saline and administered directly into the stomach and the results are given in figure 6.16.

Initial values seem to be high, starting at 13.0 μ IU/ml and then increase to a maximum of 19.1 μ IU/ml insulin in plasma. Insulin levels then rapidly declines for the next 10 minutes where after it shows a slight increase at 30 minutes. Plasma insulin levels then gradually decrease towards the 2-hour measurement and then slightly increase to give a reading of 13.5 μ IU/ml after 3 hours.

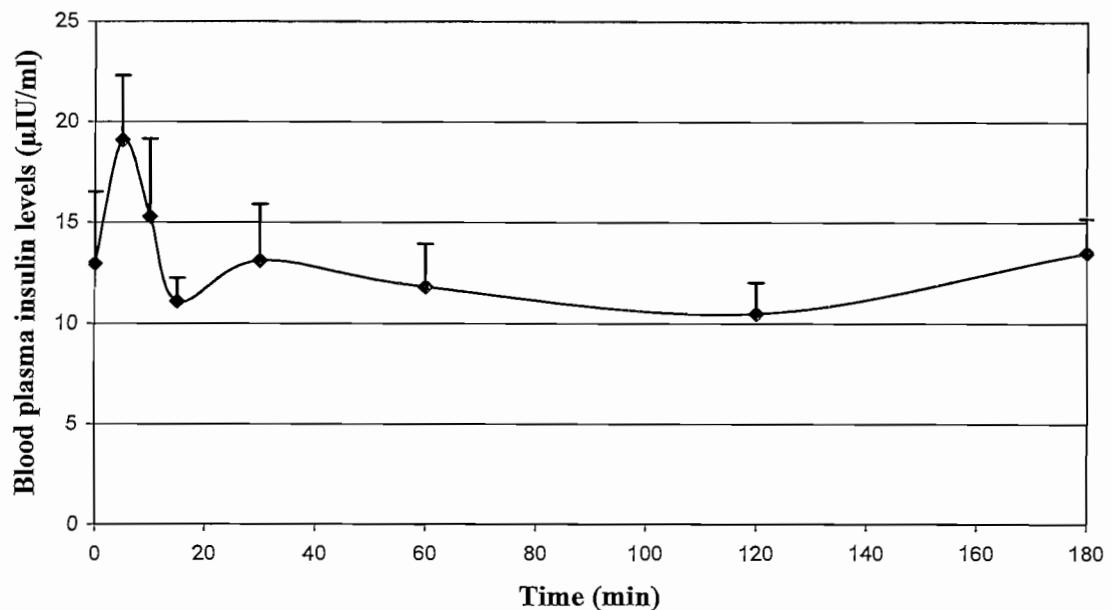


Figure 6.16 Blood plasma insulin levels after the intra-gastric administration of insulin (50.0 IU/kg) in saline (0.9%).

6.3.2.2 Intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles

Blood glucose levels after intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles is given in figure 6.17. No pronounced decrease in blood glucose levels is observed after administration of insulin (50.0 IU/kg) in Pheroid vesicles. A slight decrease of almost 4.0% is observed after 15 minutes and is mainly maintained for up to 90 minutes where after a glucagon response is observed which amounts to an increase of blood glucose levels for up to 131.9%.

Pheroid vesicles are therefore not able to lower blood glucose levels sufficiently when administrated into the stomach, nor does it have the ability to prevent the occurrence of a hyperglycaemic effect. Insulin was entrapped in Pheroid vesicles and administered intra-gastric to a group of six rats at a concentration of 50.0IU/kg. The data obtained from blood plasma insulin levels are represented in figure 6.18.

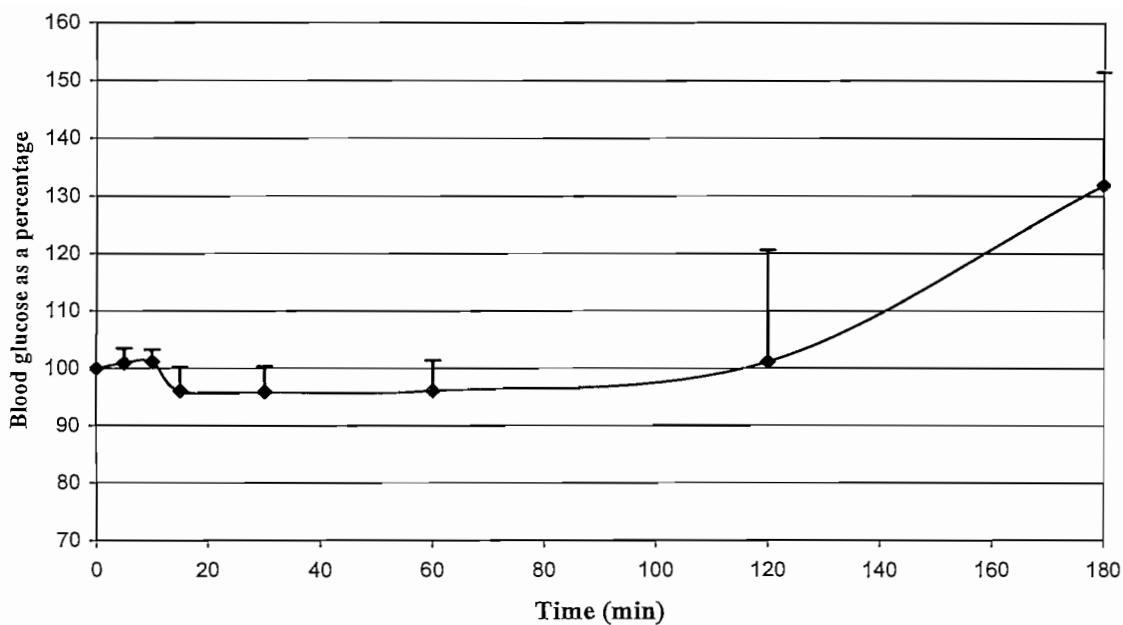


Figure 6.17 Average blood glucose as a percentage against time after intra-gastric administration of insulin (50.0 IU/kg) in PheroidTM vesicles.

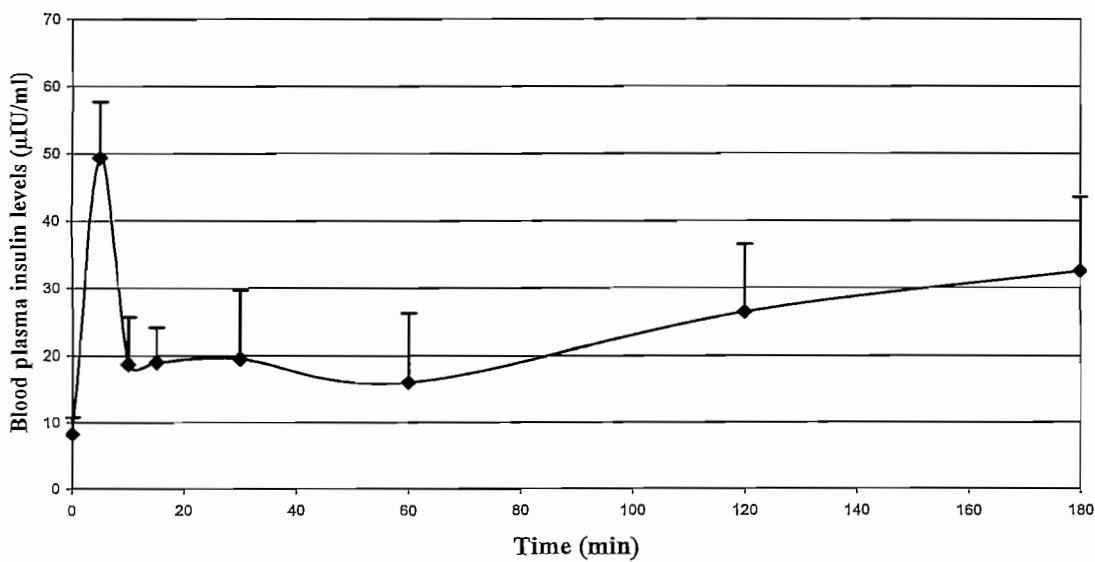


Figure 6.18 Blood plasma insulin levels after the intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles.

After the intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles a notable increase in plasma insulin levels is observed after just 5 minutes. Initial values at time 0 are 8.3 μ IU/ml and reach a maximum of 49.4 μ IU/ml at 5 minutes. Insulin levels then rapidly decline to 18.9 μ IU/ml where it stays stable for 50 minutes after which it declines again at 60 minutes. After 60 minutes insulin levels increase to reach 32.6 μ IU/ml at 3 hours.

6.3.2.3 A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after inter-gastric administration

A comparison of blood glucose levels after administration of insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline, as given in figure 6.19, clearly shows that Pheroid vesicles decreases the blood glucose levels to a lesser extent than the control, but however has the ability to maintain a longer blood glucose lowering effect than the control. The glucagon response and ultimately hyperglycaemic effect is however more pronounced in the case if insulin in Pheroid vesicles as seen after 3 hours.

Blood plasma insulin levels after the intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles are compared to those of insulin (50.0 IU/kg) in saline after inter-gastric administration in figure 6.20.

A vast increase in average plasma insulin levels can be observed after administration of insulin in Pheroid vesicles compared to that of the control. Pheroid vesicles clearly aid the absorption of insulin and maintain a higher concentration of insulin in the blood through the course of the 3-hour experiment. It also seems that insulin is released over time, as levels appear to still be rising after 3 hours.

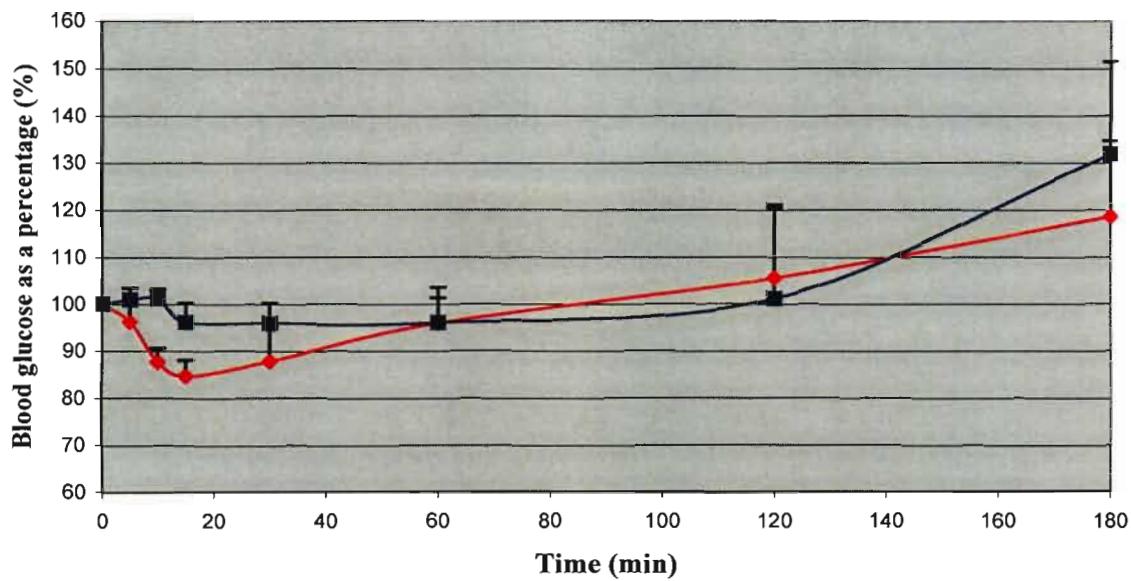


Figure 6.19 A comparison of the average blood glucose levels as a percentage after intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) and insulin (50.0 IU/kg) in saline (◆).

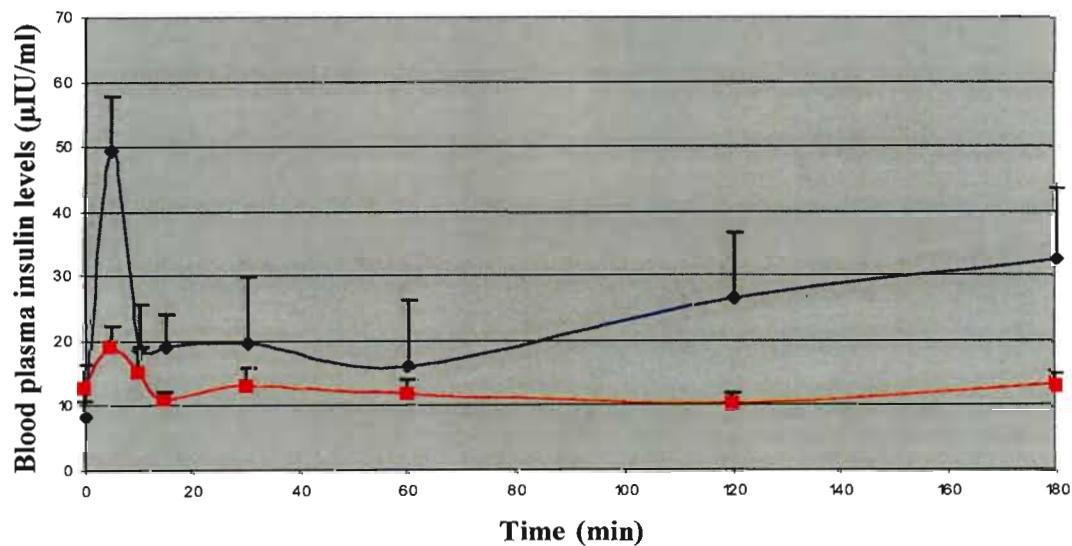


Figure 6.20 Blood plasma insulin levels after intra-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles (◆) and insulin (50.0 IU/kg) in saline (■).

6.3.3 Intra-colonic administration

6.3.3.1 Intra-colonic administration of insulin (50.0 IU/kg) in saline (control)

The blood glucose results obtained after intra-colonic administration of insulin (50.0 IU/kg) in saline to rats are illustrated in figure 6.21.

From the results obtained it is evident that insulin has no clear effect on lowering blood glucose after colonic administration. A large hyperglycaemic effect is observed after 60 minutes for the duration of the experiment.

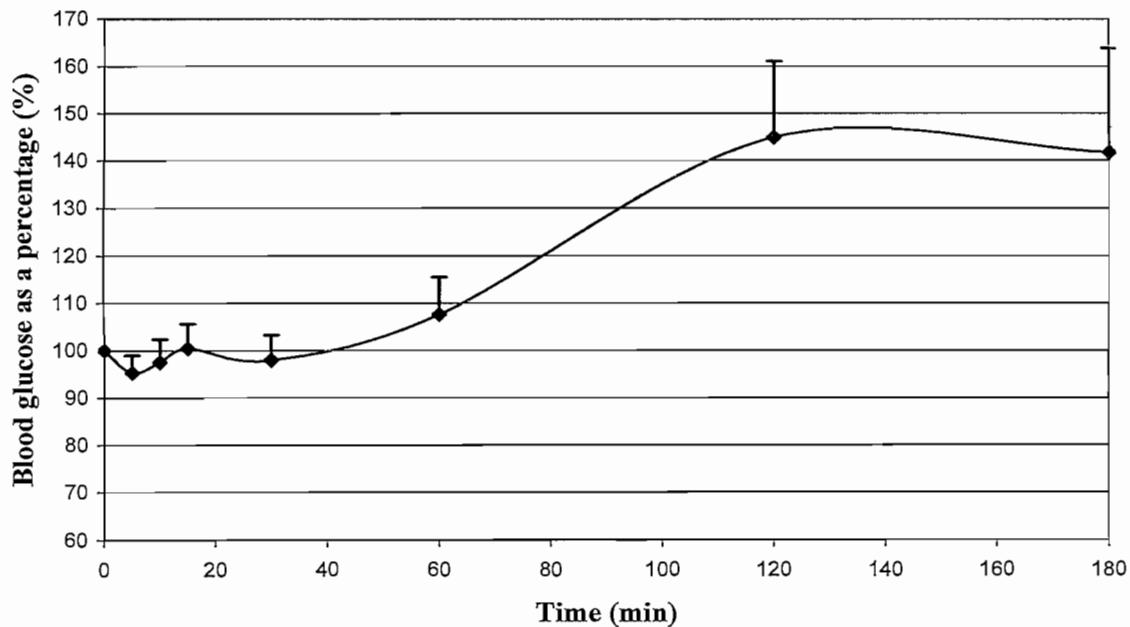


Figure 6.21 Average blood glucose levels after inter-colonic administration of insulin (50.0 IU/kg) in saline.

The plasma insulin level results obtained after intra-colonic administration of insulin (50.0 IU/kg) in saline to rats are illustrated in figure 6.22.

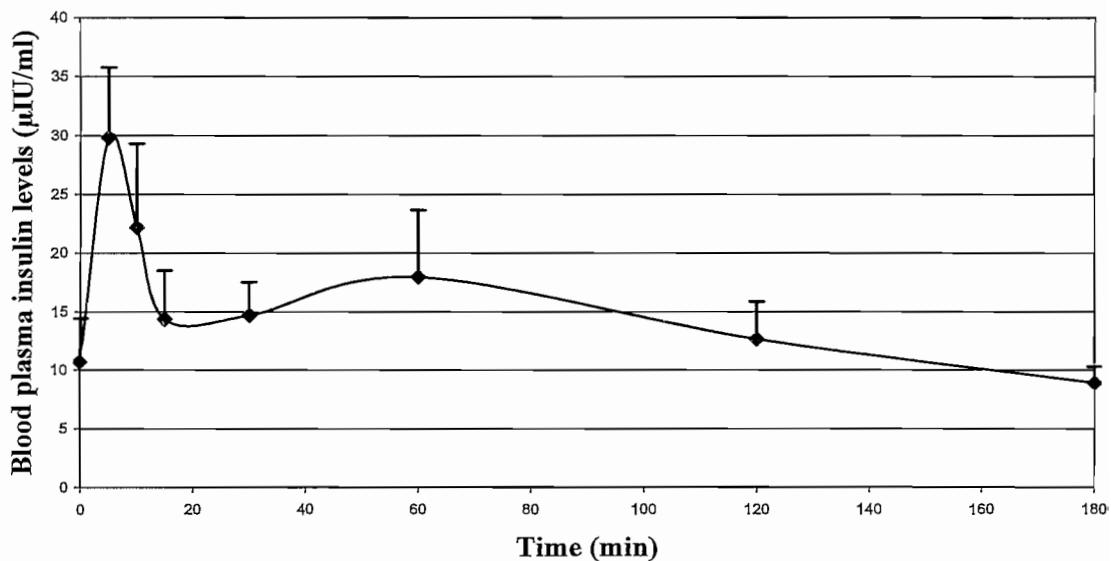


Figure 6.22 Blood plasma insulin levels after the intra-colonic administration of insulin (50.0 IU/kg) in saline (0.9%).

A rapid absorption of insulin is shown after 5 minutes (peak at 29.9 $\mu\text{IU}/\text{ml}$) followed by a steep decline for up to 15 minutes. Plasma insulin levels are then fairly steady for the next 15 minutes but then show a slight increase at 60 minutes where after levels drop gradually to 8.9 $\mu\text{IU}/\text{ml}$ at the 3-hour mark.

6.3.3.2 Intra-colonic administration of insulin (50.0 IU/kg) in Pheroid vesicles

Results of blood glucose levels of rats obtained after intra-colonic administration of insulin in Pheroid vesicles are given as a graph in figure 6.23. Blood glucose levels shows an immediate decrease of 8.6% after 5 minutes that further decreases by 5.6% to 14.2% after 15 minutes.

Blood glucose levels starts to rise however and reaches normal levels after approximately 100 minutes and a maximum of 132.1% after 3 hours. Blood glucose levels seem to be rising even after 3 hours.

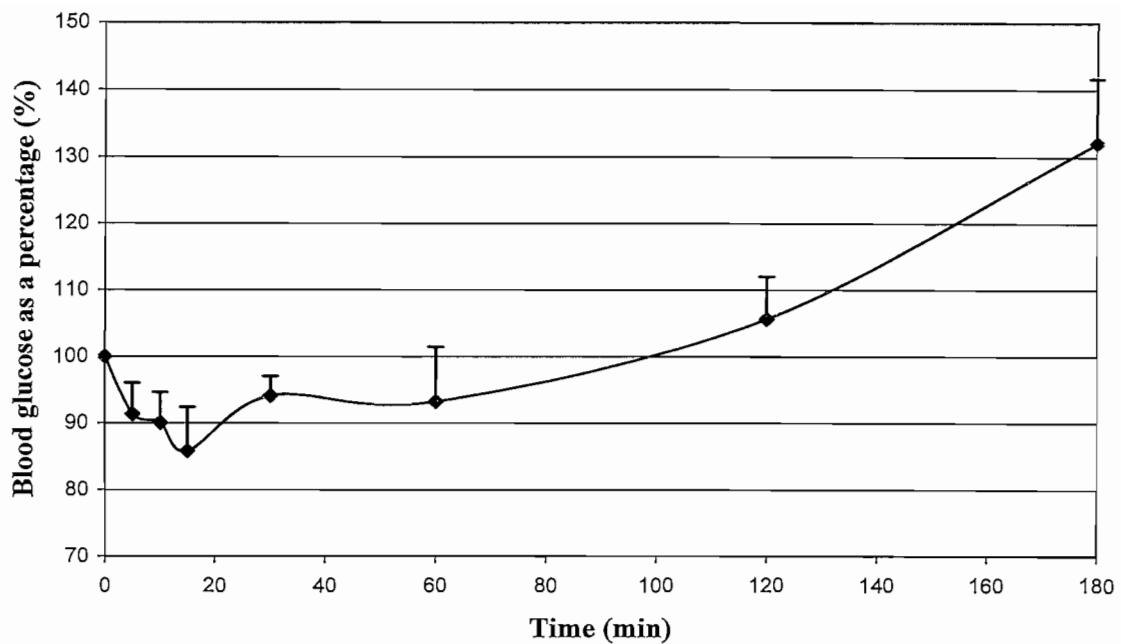


Figure 6.23 Average blood glucose levels after inter-colonic administration of insulin (50.0 IU/kg) in Pheroid vesicles.

Results of blood plasma insulin levels of rats obtained after intra-colonic administration of insulin in Pheroid vesicles are given as a graph in figure 6.24.

A sheer drop follows an initial rise in blood plasma levels to a minimum plasma insulin level of 21.8 $\mu\text{IU}/\text{ml}$, 15 minutes after administration. Insulin levels then gradually increase up to the 120-minute mark to reach a maximum of 40.9 $\mu\text{IU}/\text{ml}$ where after a slight decline is observed. At 3 hours the average reading is 36.5 $\mu\text{IU}/\text{ml}$, showing a slight decrease in the last hour.

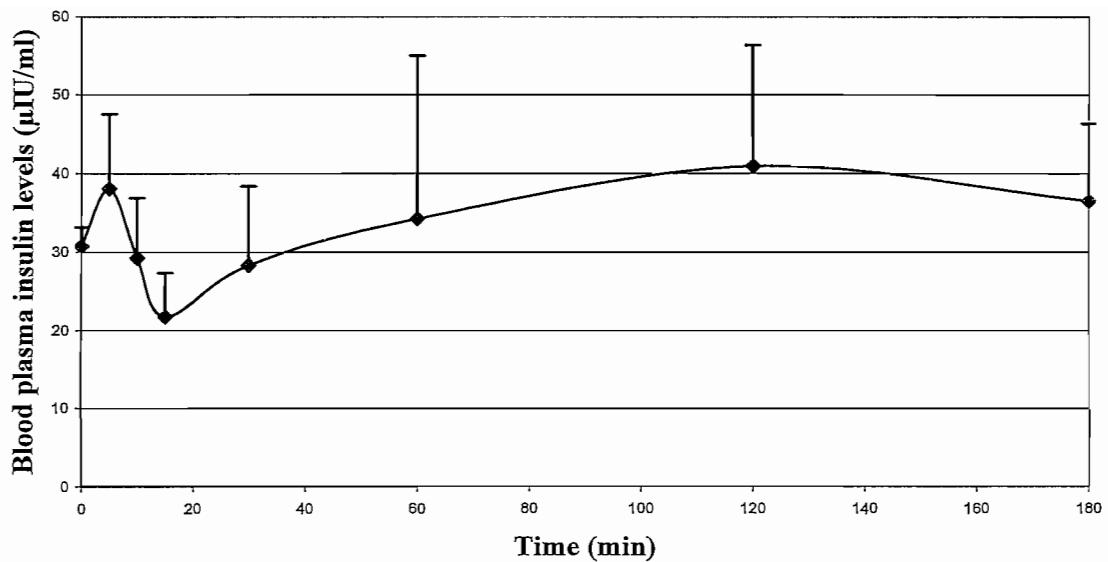


Figure 6.24 Blood plasma insulin levels after the intra-colonic administration of insulin (50.0 IU/kg) in Pheroid vesicles.

6.3.3.3 A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after inter-colonic administration

A graphic comparison between the results obtained after inter-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline is given in figure 6.25. From the graph it is evident that insulin in Pheroid vesicles shows to have a more pronounced and longer blood glucose lowering effect than the control and does not present with such a vast increase in blood glucose levels.

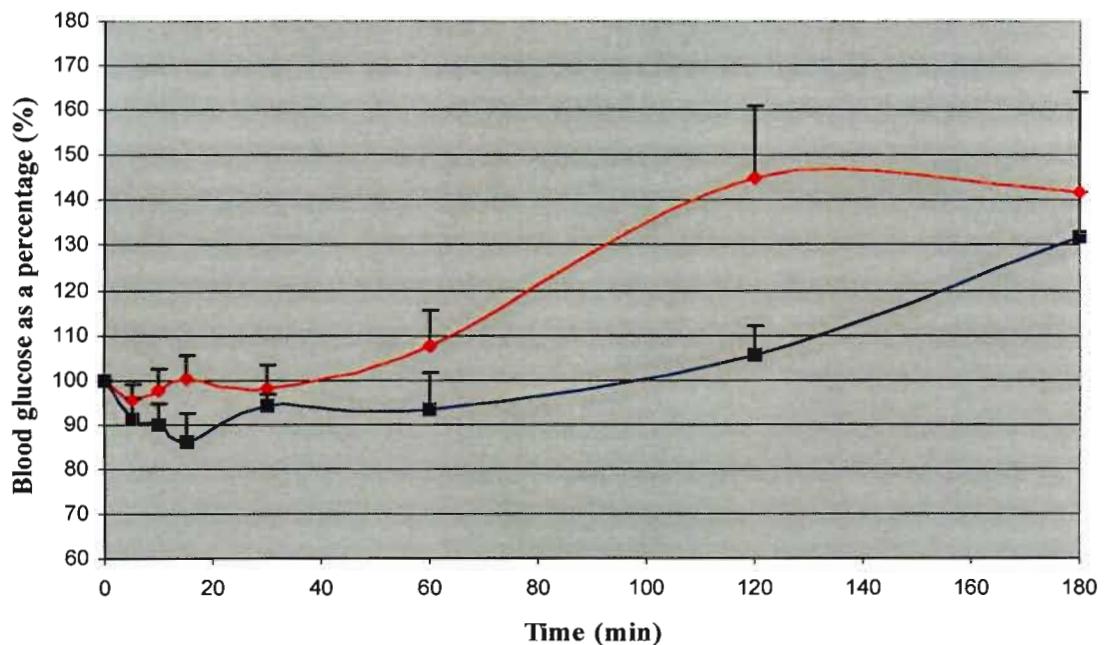


Figure 6.25 A comparison of the average blood glucose levels as a percentage after intra-colonic administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) and insulin (50.0 IU/kg) in saline (◆).

Even though insulin in Pheroid vesicles show a promising blood glucose profile it is still not ideal in that the effects thereof only appear to last for an hour and a half, followed by an unwanted hyperglycaemic effect.

A graphic comparison between the results obtained after inter-gastric administration of insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline is given in figure 6.26.

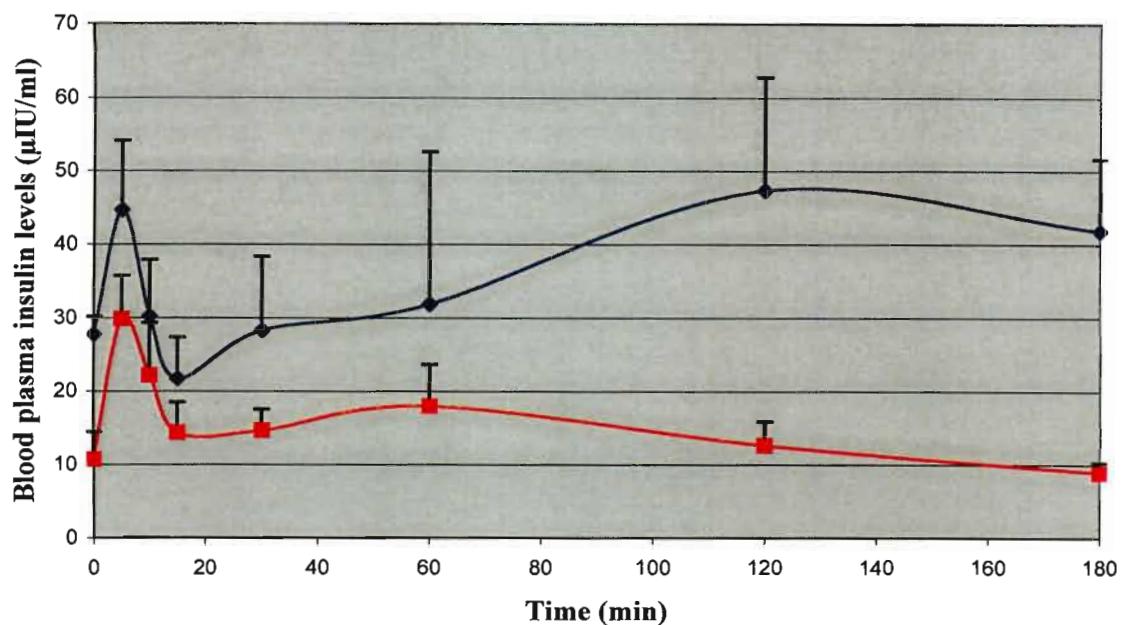


Figure 6.26 A comparison of the average blood plasma insulin levels after intra-colonic administration of insulin (50.0 IU/kg) in Pheroid vesicles (◆) and insulin (50.0 IU/kg) in saline (■).

Even though initial values of plasma insulin at time 0 is higher for insulin in Pheroid vesicles, there still exist a further increase and a higher overall average of plasma insulin levels than for the control group. Plasma insulin levels after administration with Pheroid vesicles indicate that insulin is continually being released and absorbed into the blood for up to 2 hours opposed to the control where insulin levels decline as from 60 minutes, signifying the elimination of insulin.

6.3.4 Intra-ileal administration

6.3.4.1 Intra-ileal administration of insulin (50.0 IU/kg) in saline (control)

Results obtained from blood glucose levels after intra-ileal administration of insulin in saline (0.9%) are given as a graph in figure 6.27.

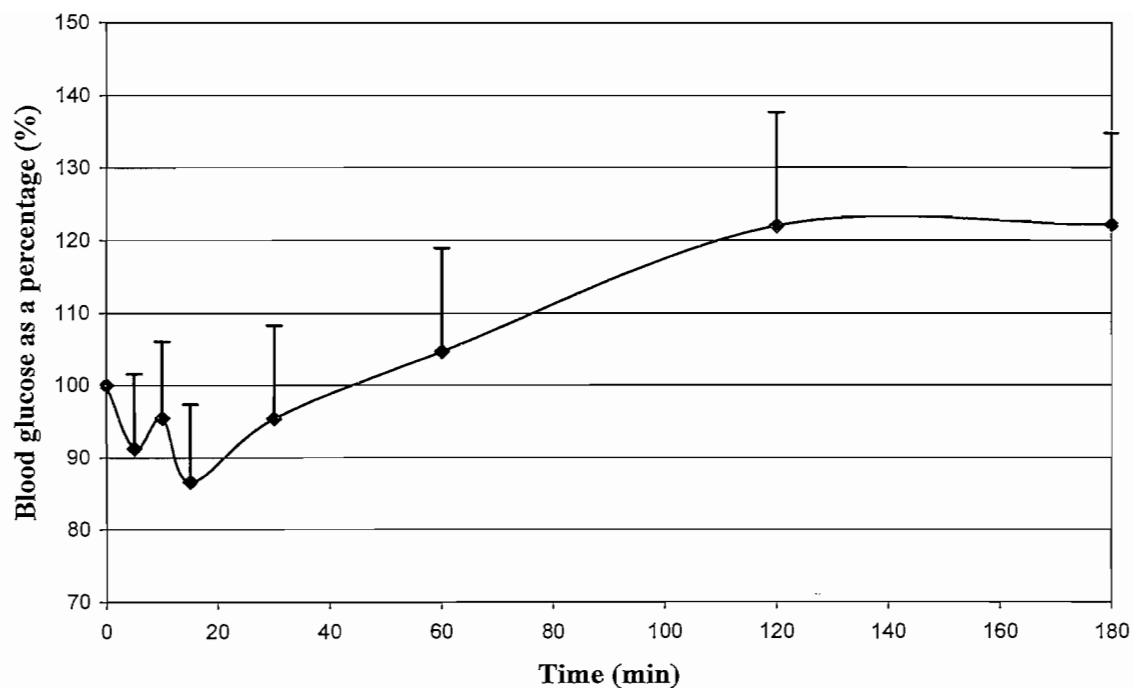


Figure 6.27 Average blood glucose levels after inter-ileal administration of insulin (50.0 IU/kg) in saline.

Figure 6.27 clearly shows that shortly after administration a clear decrease in blood glucose occurs, followed by a slight increase and then a further decrease up to 13.4% (very similar to intra-gastric administration) after 15 minutes. A steady increase follows and normal values are reached at approximately 45 minutes. Blood glucose levels continue to increase up to the 120-minute mark where it appears to have reached steady state at 122.0%.

Results obtained from blood plasma insulin levels after intra-ileal administration of insulin in saline (0.9%) are given as a graph in figure 6.28.

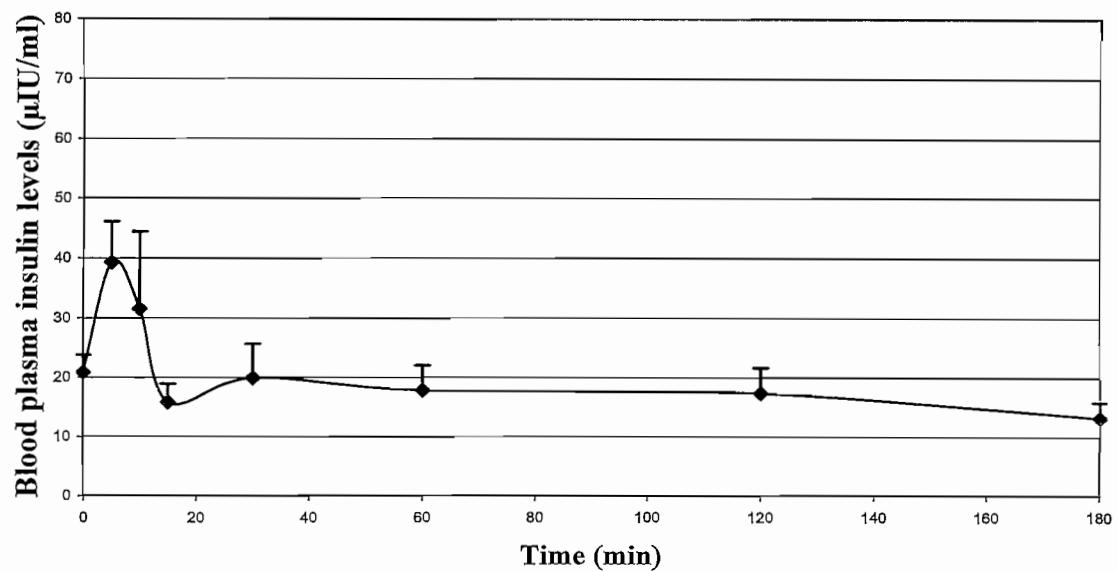


Figure 6.28 Blood plasma insulin levels after the intra-ileal administration of insulin (50.0 IU/kg) in saline (0.9%).

Much similar to the profile of the control groups of intra-colonic and intra-gastric administration an initial rise in plasma insulin levels is subsequently followed by a decline in blood plasma insulin levels. Plasma insulin levels peak with a maximum of $39.3 \mu\text{IU}/\text{ml}$ at 5 minutes. After 30 minutes a fairly steady state is maintained with a slow but sure decrease in plasma levels up until 3 hours where a minimum of $13.2 \mu\text{IU}/\text{ml}$ is observed.

6.3.4.2 Intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles

Average blood glucose levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles to rats are given in figure 6.29.

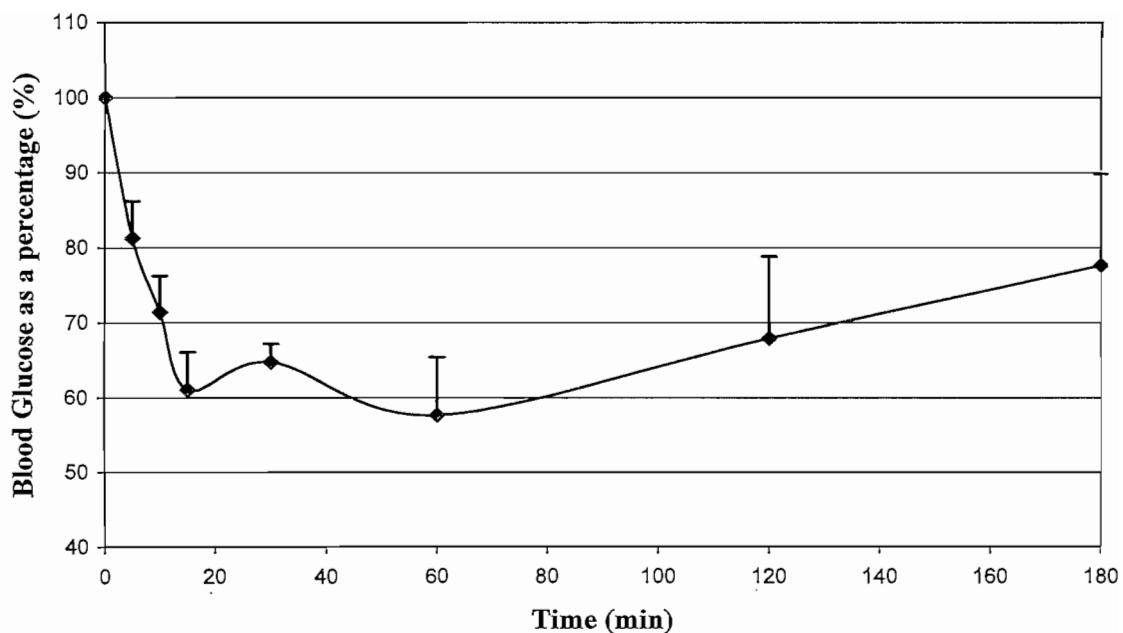


Figure 6.29 Average blood glucose levels after inter-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles.

From the results in figure 6.29 it can be clearly seen that there is a rapid and pronounced decrease in blood glucose levels directly after administration of insulin in Pheroid vesicles. Average blood glucose levels show a constant decrease for up to 15 minutes with a total decrease of 39.0%. Blood glucose levels show a slight increase in blood glucose levels of 3.7% after 30 minutes but then decreases again after 60 minutes. Blood glucose levels then starts to rise steadily but after 3 hours still shows a 22.4% decrease from initial values at time 0.

Average plasma insulin levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles to rats are given in figure 6.30.

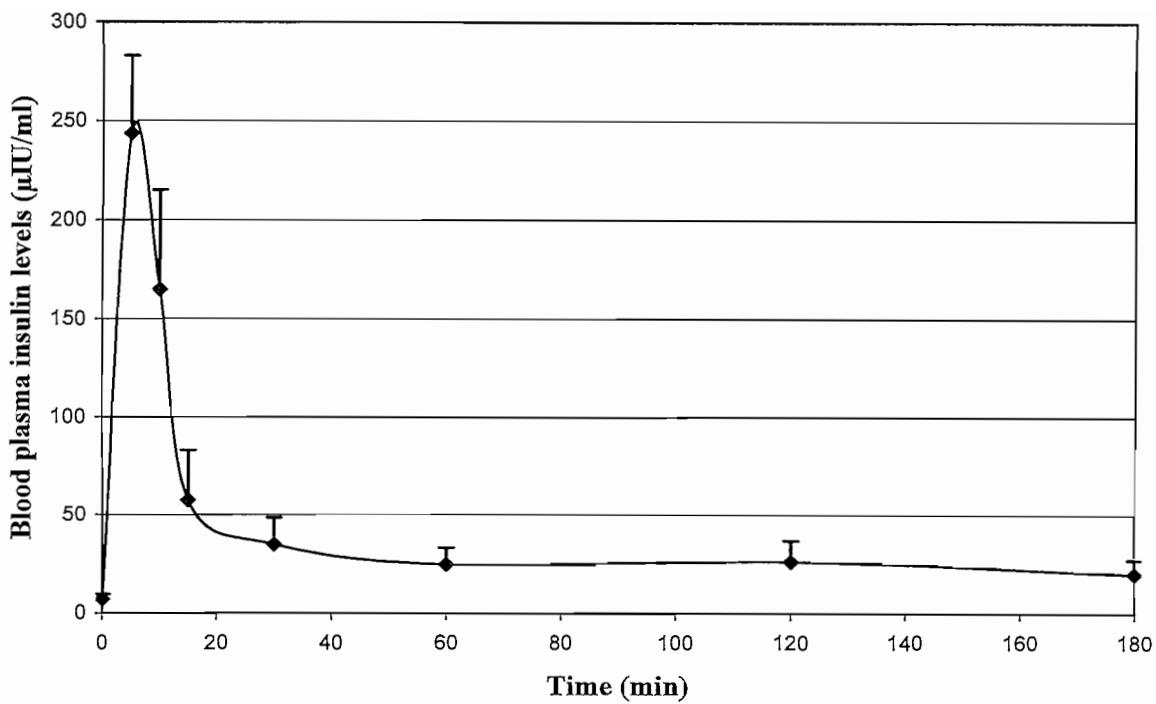


Figure 6.30 Blood plasma insulin levels after the intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles.

A vast increase of blood plasma insulin levels of up to $243.8 \mu\text{IU}/\text{ml}$ is reached after just 5 minutes followed by a sheer decline for up to 30 minutes. After 30 minutes there is a very steady decline in plasma insulin levels, which reaches a minimum of $20.0 \mu\text{IU}/\text{ml}$ at 3 hours. The profile of this administration seems to be much similar to that of intravenous administered insulin demonstrated in figure 6.10.

6.3.4.3 A comparison between insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline after inter-ileal administration

A graphic comparison between the results obtained after inter-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline is given in figure 6.31. From the graph it can be undoubtedly seen that insulin in Pheroid vesicles shows to have a more pronounced and longer blood glucose lowering effect than the control and does not present with such a vast increase in blood glucose levels.

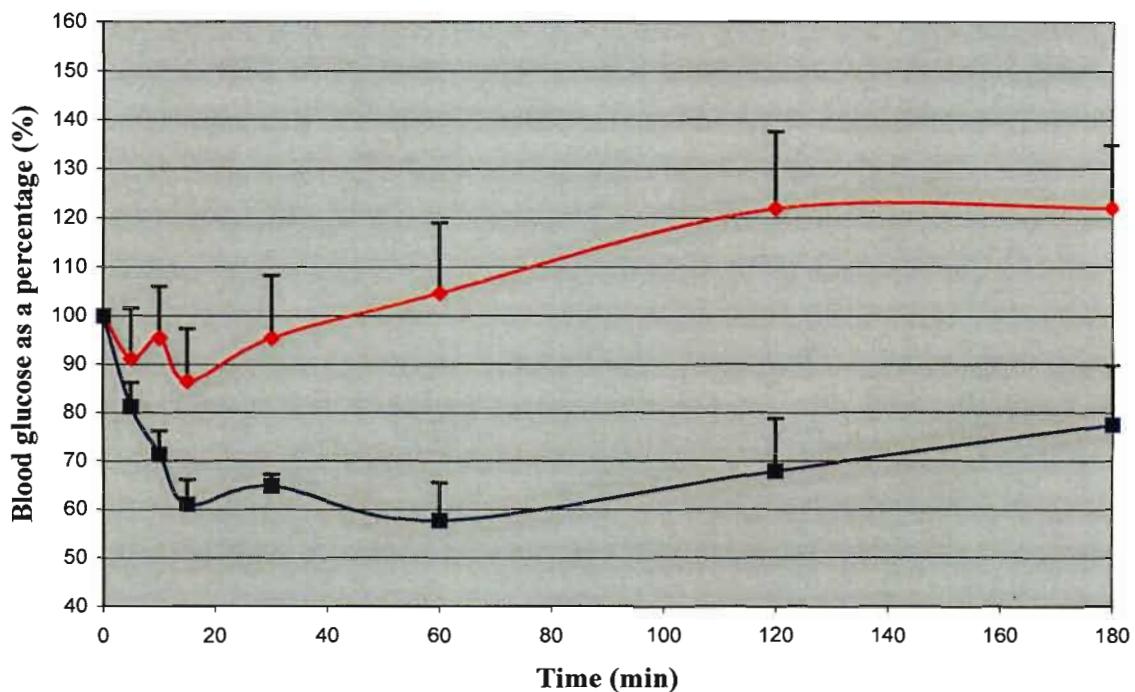


Figure 6.31 A comparison of the average blood glucose levels as a percentage after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) and insulin (50.0 IU/kg) in saline (◆).

Of all the peroral results observed so far, it is evident that after administration of insulin in saline (control groups) there is a slight decrease in blood glucose levels followed by a vast increase, which results in hyperglycaemic effects. Of all the peroral insulin in Pheroid formulations administered it is clear that the inter-ileal administration is by far the most promising as illustrated in figure 6.33 which compares the average blood glucose levels obtained after inter-gastric, -colonic and – ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles.

A graphic comparison between the results obtained after inter-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles and insulin (50.0 IU/kg) in saline is given in figure 6.32.

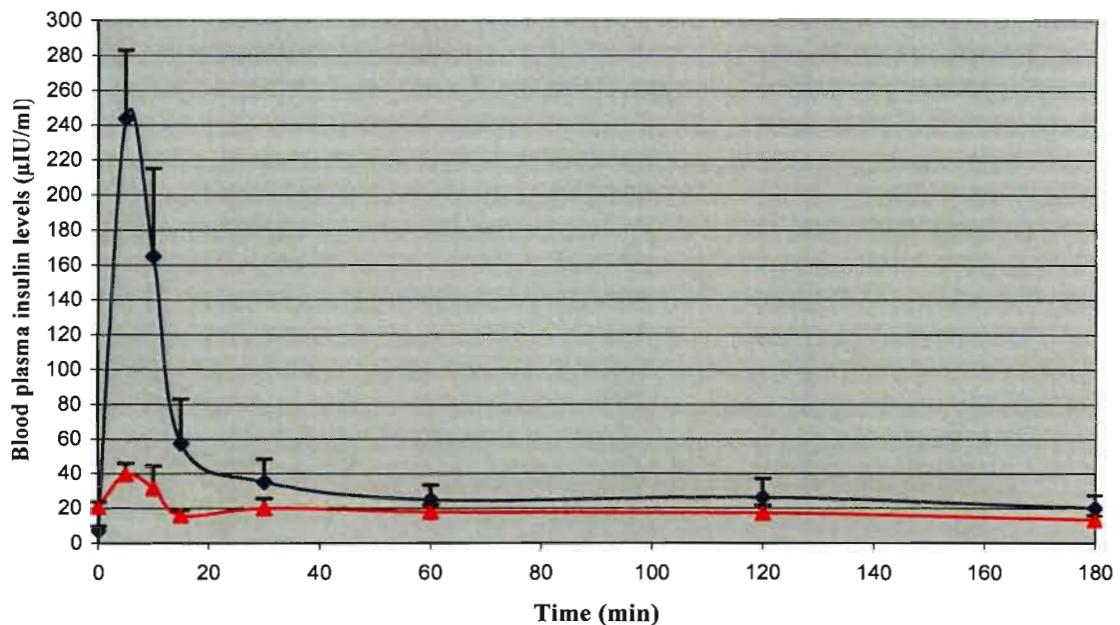


Figure 6.32 A comparison of the average plasma insulin levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid™ vesicles (◆) and insulin (50.0 IU/kg) in saline (▲).

From the graph depicted in figure 6.32 it can be clearly seen that Pheroid vesicles aid the absorption of insulin in the ileum. There is a distinct difference in plasma insulin levels between the control and insulin in Pheroid vesicles.

Again, corresponding to the therapeutic blood glucose values, it seems that the ileum presents with ideal characteristics for optimum insulin absorption. To better illustrate this assumption, figures 6.33, 6.34 and 6.35 compares the absorption of insulin in the various parts of the gastrointestinal tract after administration of insulin in Pheroid vesicles. Compared to the ileum, the stomach and colon did not nearly provide as much insulin absorption.

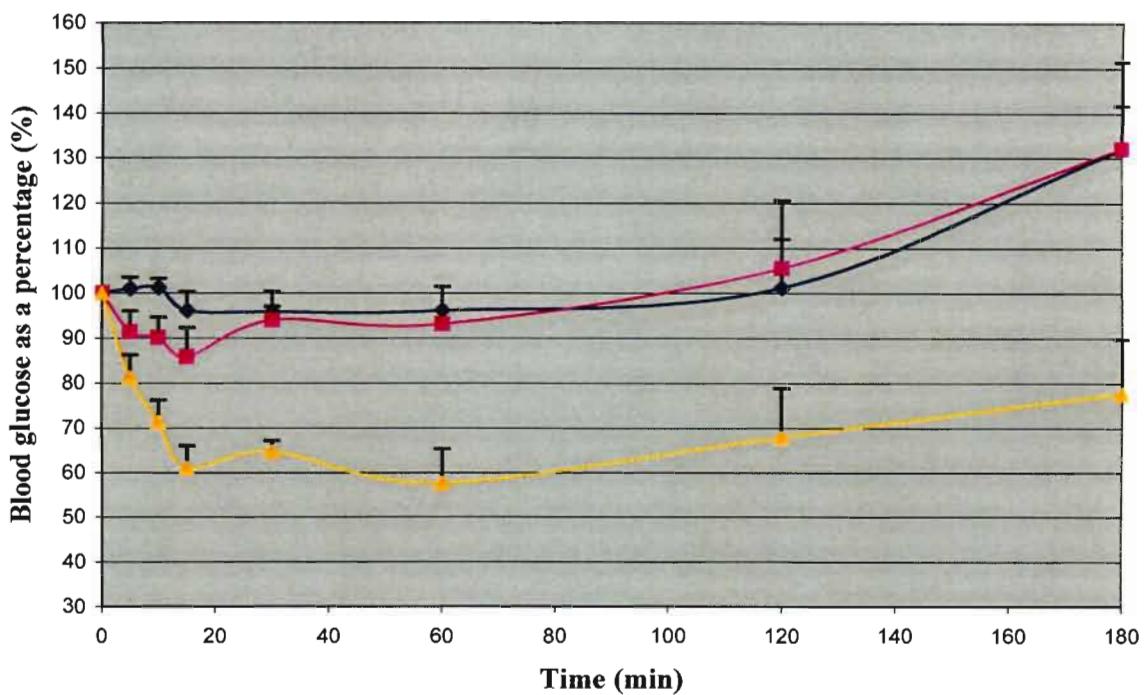


Figure 6.33 A comparison of the average blood glucose levels as a percentage after administration of insulin (50.0 IU/kg) in Pheroid™ vesicles intra-gastric (◆), intra-colonic (■) and intra-ileal (▲).

Intra-ileal administration presents with ideal properties such as rapid onset of action, prolonged action and the absence of hyperglycaemia after 3 hours. It is also for this reason that intra-ileal administration was selected as the sight of administration for the subsequent experiments.

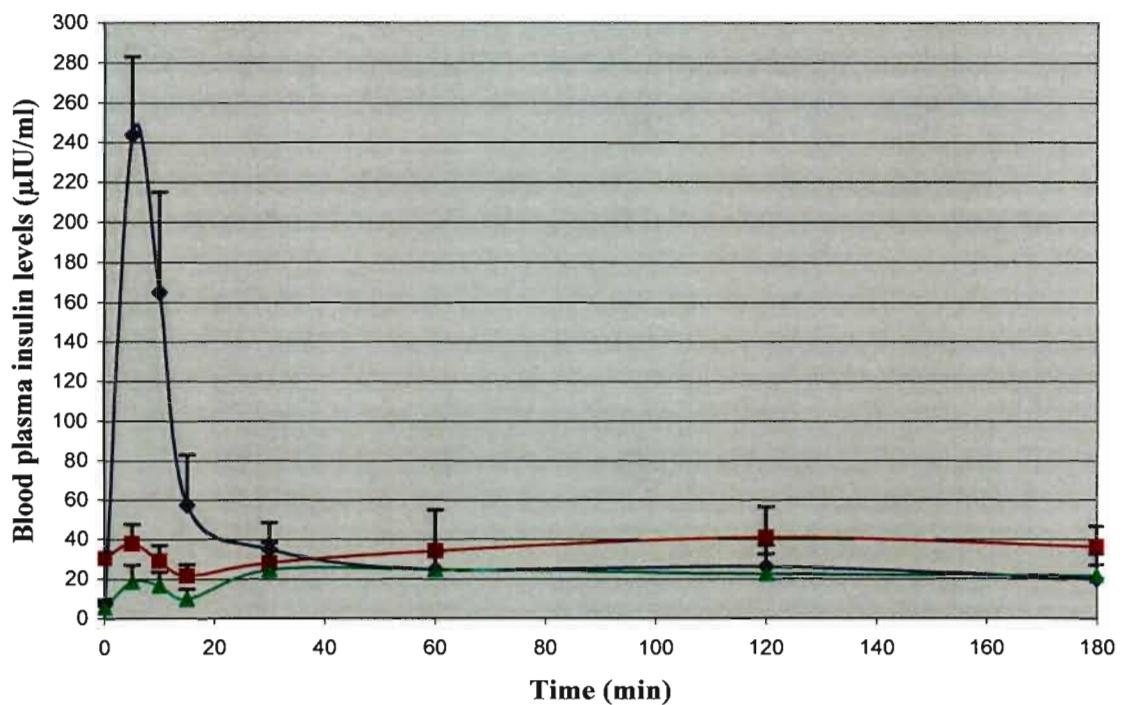


Figure 6.34 A comparison of the average blood plasma insulin levels after administration of insulin (50.0 IU/kg) in Pheroid vesicles intra-gastric (▲), intra-colonic (■) and intra-ileal (◆).

Concurring with the blood glucose levels again inter-ileal administration was chosen to determine the blood plasma insulin levels after co-administration of insulin and other Pheroid formulations as well as with TMC 0.5%. These formulations included insulin (50.0 IU/kg) in Pheroid micro-sponges and TMC 0.5% and insulin concentrations prepared at 10.0, 25.0 and 50.0 IU/kg.

Another comparison of the blood glucose lowering effect of the insulin in Pheroid vesicles administered in the different areas of the gastrointestinal tract is illustrated in figure 6.35.

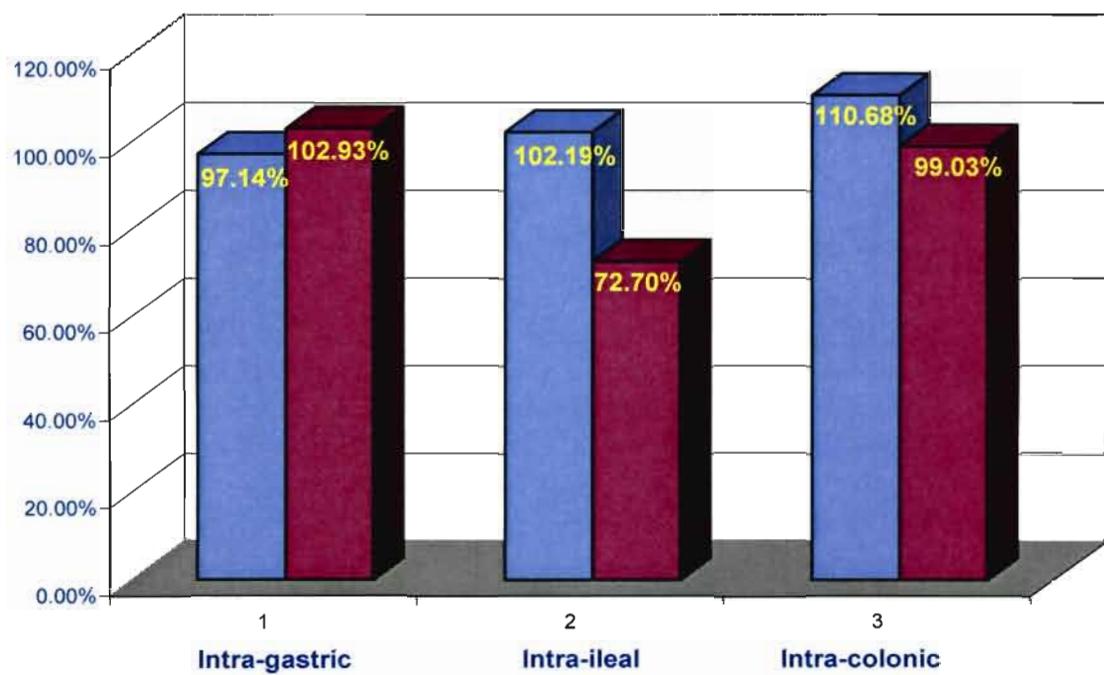


Figure 6.35 A comparison of the average blood glucose levels, expressed as a percentage, after intra-gastric, intra-colonic and intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) compared to the control (□).

Figure 6.36 illustrates the average blood plasma insulin levels during the 3-hour experiments after administration. In figure 6.36 the administration of insulin in Pheroid vesicles shows a more than 3-fold increase in plasma insulin levels after intra-ileal administration and a 2-fold increase after intra-colonic administration compared to the control.

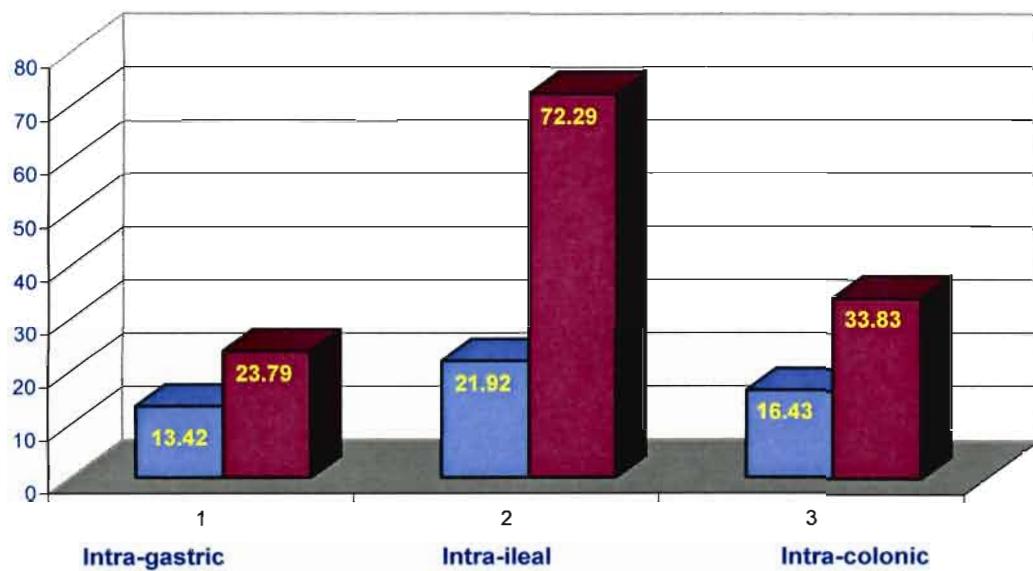


Figure 6.36 A comparison of the average blood plasma insulin levels after intra-gastric, intra-colonic and intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) compared to the control (□).

6.3.4.4 Intra-ileal administration of insulin (50.0 IU/kg) in Pheroid micro-sponges

A formulation containing another type of Pheroid, namely Pheroid micro-sponges, has also been tested via intra-ileal administration and the results that were obtained are depicted in figure 6.37.

It can be clearly seen that the response is prolonged and only after 10 minutes is there a significant decrease in blood glucose levels of 14.25%. Blood glucose levels remain low but steadily start to increase and then reach normal values (100.0%) after approximately 80 minutes. Blood glucose levels continue to increase and reaches 37.2% above normal after 3 hours.

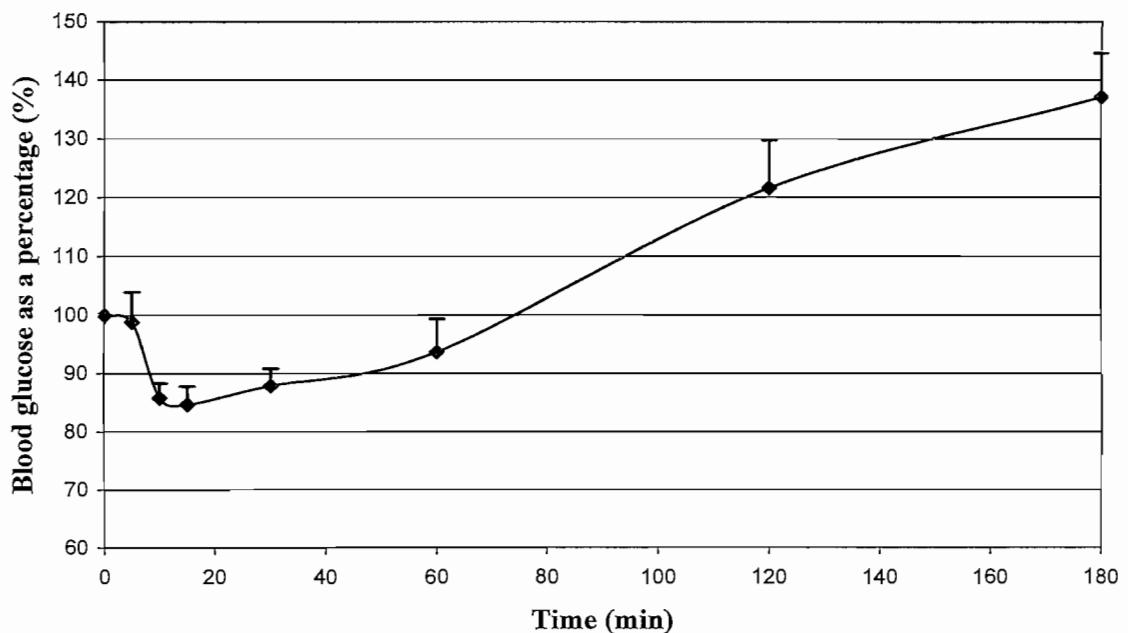


Figure 6.37 Average blood glucose levels after inter-ileal administration of insulin (50.0 IU/kg) in Pheroid micro-sponges.

A formulation containing another type of Pheroid, namely Pheroid micro-sponges, has also been tested via intra-ileal administration and the results from plasma insulin levels that were obtained are depicted in figure 6.38.

Pheroid micro-sponges show a much slower and less pronounced absorption of insulin. A maximum plasma insulin level of 43.4 μ IU/ml is reached after 10 minutes where after a slow and steady decline takes insulin levels down to 15.9 μ IU/ml at 2 hours. The 3-hour level has been slightly elevated to 17.6 μ IU/ml.

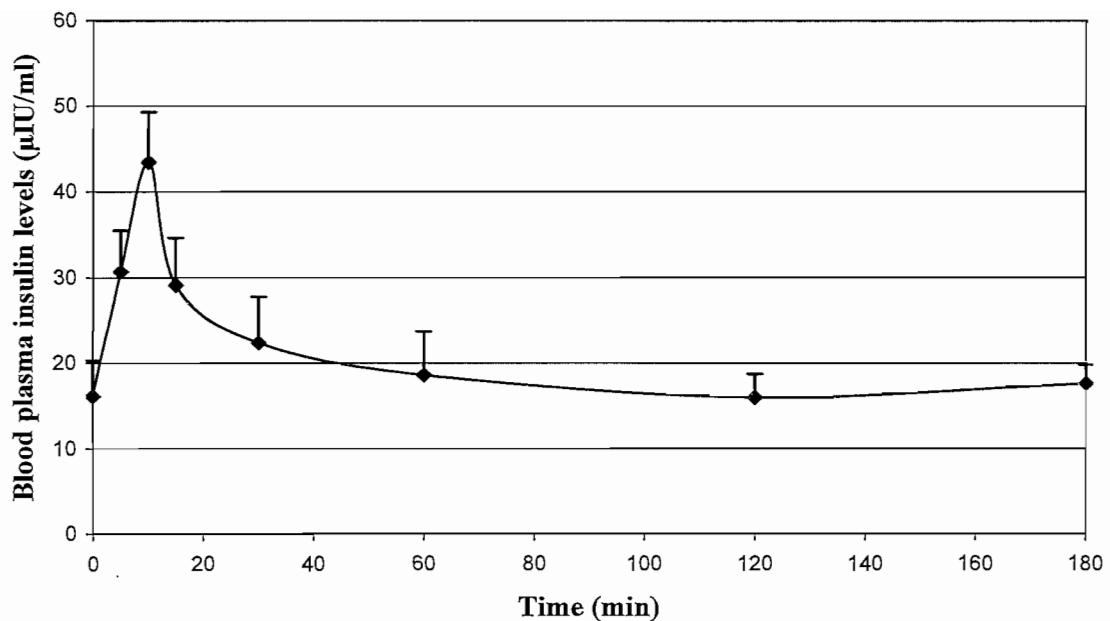


Figure 6.38 Average plasma insulin levels after inter-ileal administration of insulin (50.0 IU/kg) in Pheroid micro-sponges.

6.3.4.5 A comparison between the intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles and –micro-sponges

A comparison between the intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles and Pheroid micro-sponges together with the control group is illustrated in figure 6.39.

It is clear that only insulin (50.0 IU/kg) in Pheroid vesicles illustrates the idyllic profile needed for a successful insulin formulation. Pheroid micro-sponges initially presents with good blood glucose lowering capabilities but tends to result in a pronounced hyperglycaemic response.

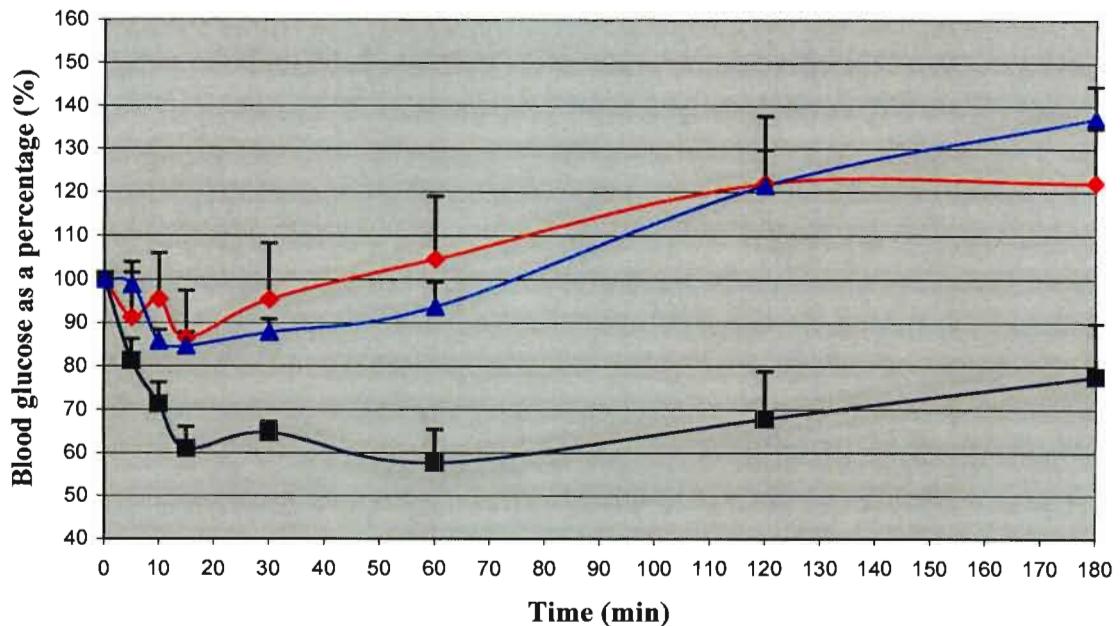


Figure 6.39 A comparison between the average blood glucose levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (■) and Pheroid micro-sponges (▲) together with the control group (♦).

A comparative graph of the effects of Pheroid vesicles and –micro-sponges on the absorption of insulin is given as plasma insulin levels against time in figure 6.40.

It can be clearly seen that Pheroid micro-sponges has almost no enhancing effect on the absorption of insulin and has a poor effect compared to Pheroid vesicles. Insulin in Pheroid micro-sponges shows much the same effect as insulin in saline (0.9%) and follows much the same profile of an initial increase and subsequent decrease in insulin levels followed by a steady state of plasma insulin levels.

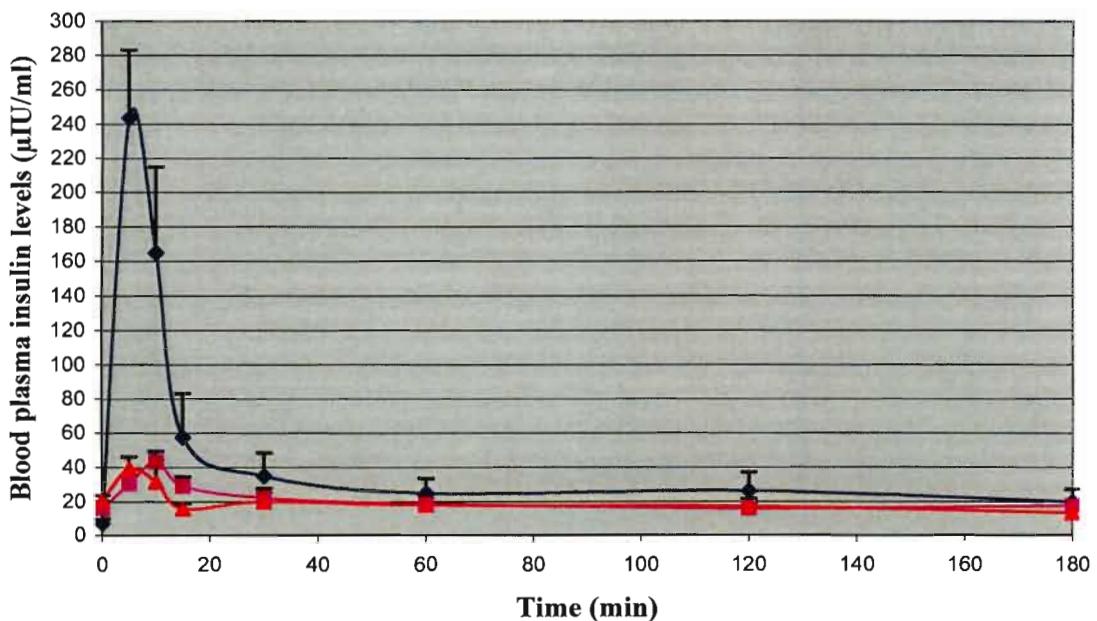


Figure 6.40 A comparison between the average blood glucose levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (◆) and Pheroid micro-sponges (■) together with the control group (▲).

6.3.4.6 The effect of time on the entrapment and efficiency of insulin in Pheroid vesicles

To illustrate why insulin must be entrapped in Pheroid formulations 24 hours prior to administration a comparison was sketched between two Pheroid vesicle formulations in which insulin (50.0 IU/kg) was entrapped 1 and 24 hours prior to administration. The results are given in figure 6.41.

It is clear, as seen in the graph, that the complete entrapment and efficiency of insulin in Pheroid is time dependent. If insulin is dissolved in Pheroid for only one hour it does not necessarily mean that entrapment had occurred nor has it been completed.

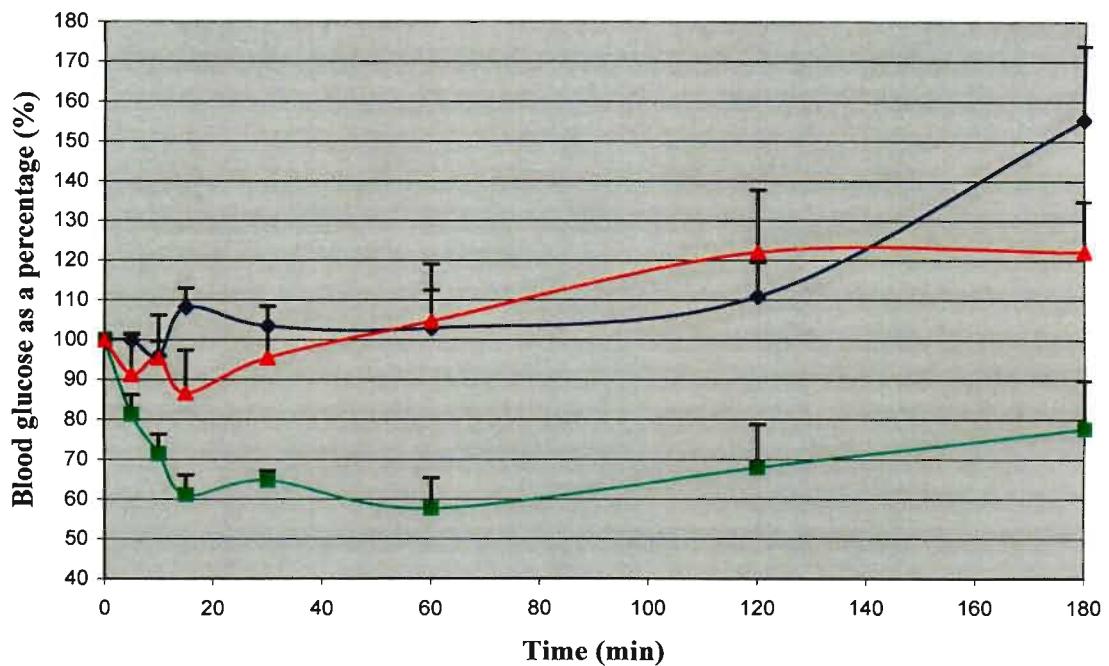


Figure 6.41 A comparison between the average blood glucose levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles entrapped for 1 hour (◆) and 24 hours (■) together with the control group (▲).

It is clear from the results obtained that insulin was either not entrapped or not absorbed as well after 1 hour as after 24 hours of entrapment. There appears to be only a brief lowering on blood glucose levels for up to 10 minutes where after blood glucose levels never decreases below normal. Between 2 and 3 hours there also seem to be a glucagon response with Pheroid containing insulin for just 1 hour.

The reason for this may be that Pheroid vesicles form over time subsequent to manufacturing. This may cause the insulin molecules to move into the vesicles as they form, ensuring better entrapment and ensure the entrapment of insulin that was not initially entrapped in Pheroid vesicles directly after dissolving insulin in the Pheroid vesicle formulation.

As mentioned earlier, the efficacies of the Pheroid formulations are determined by the duration of entrapment of insulin. To illustrate this in respect of blood plasma insulin levels a comparative graph of insulin entrapped for 1 hour against insulin entrapped for 24 hours in Pheroid vesicles is given in figure 6.42. A clear distinction can be seen between the formulations. It is as if the Pheroid vesicles containing insulin for only 1 hour is totally ineffective compared to the control.

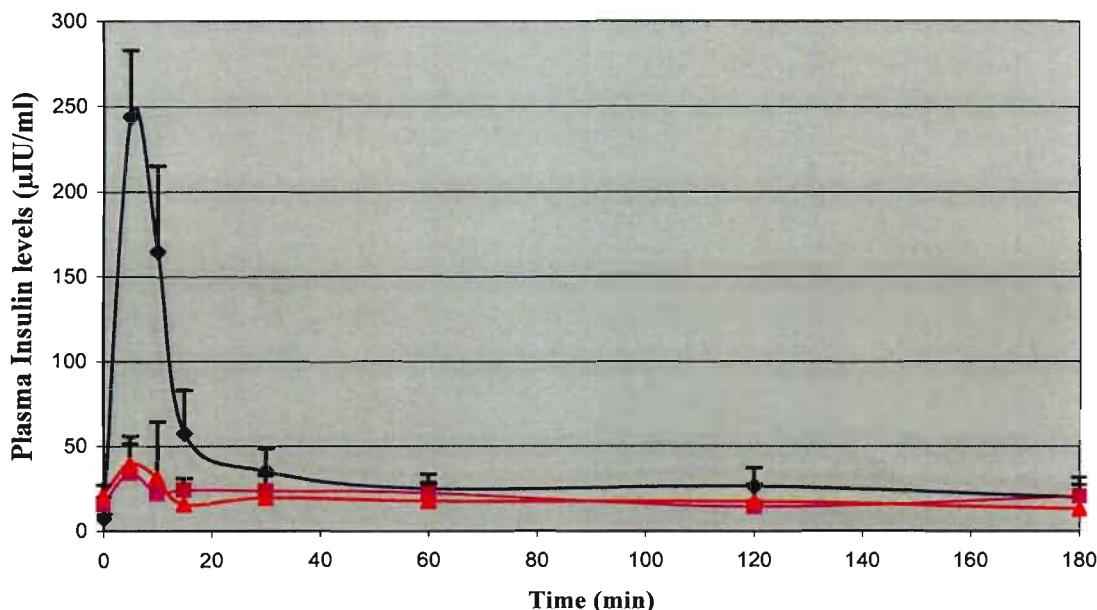


Figure 6.42 A comparison between the average blood plasma insulin levels after intra-ileal administration of insulin (50.0 IU/kg) in Pheroid vesicles entrapped for 1 hour (◆) and 24 hours (■) together with the control group (▲).

6.3.4.7 Intra-ileal administration of insulin and TMC (0.5% w/v)

N-trimethyl chitosan chloride solutions (0.5%) containing insulin at concentrations of 10.0, 25.0 and 50.0 IU/kg respectively, have been administered intra-ileal to different groups of rats. The results of the different administrations are given in figure 6.43.

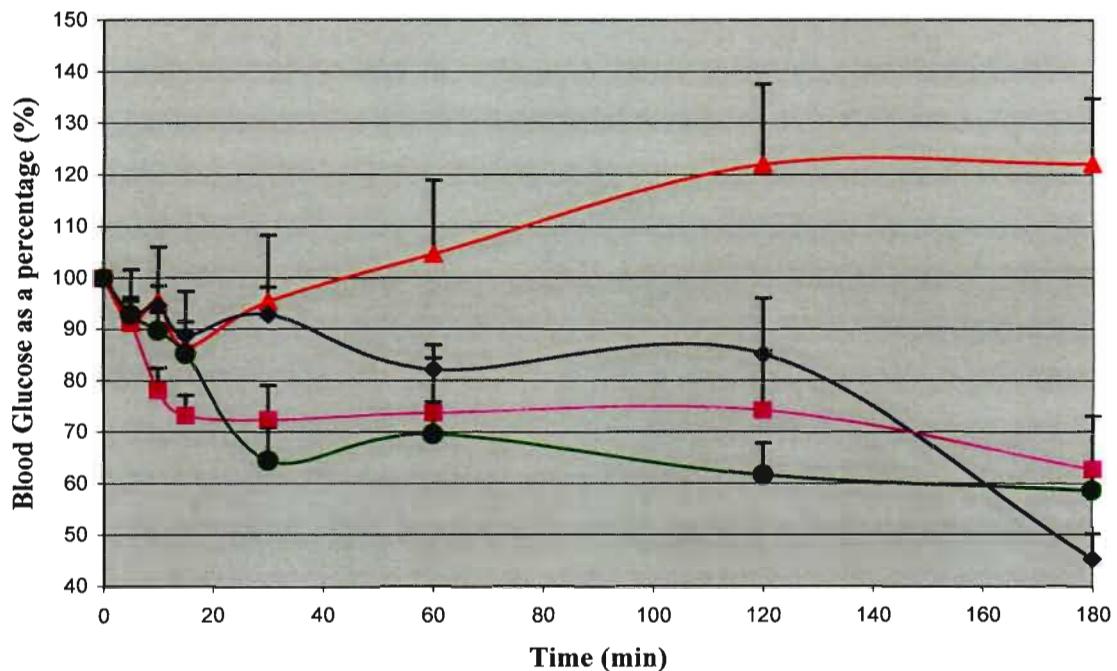


Figure 6.43 A comparison between the average blood glucose levels after intra-ileal administration of TMC 0.5% (w/v) containing insulin 10.0 IU/kg (♦), 25.0 IU/kg (■) and 50.0 IU/kg (●) together with the control group (▲).

From the results depicted in figure 6.43 it is evident that the blood glucose lowering effect of the formulations is insulin dose-dependent. TMC and insulin 10.0 IU/kg forms a pattern, initially similar to that of the control, for up to 30 minutes. It is only

after 30 minutes that a pronounced decrease in blood glucose is observed. A maximum lowering effect of almost 55.0 % is observed after 3 hours, after dropping from 85.2% two hours after administration.

TMC and insulin 25.0 IU/kg shows a rapid blood glucose lowering effect of 26.7% after just 15 minutes, which stays fairly constant for up to 2 hours. After 2 hours blood glucose levels decreases gradually to a minimum of 62.8%.

This formulation shows a faster and more pronounced initial effect than TMC and insulin 50.0 IU/kg, which only decreases blood glucose levels by 14.7% after 15 minutes, but shows a greater overall decrease in blood glucose levels between 30 and 180 minutes. A maximum blood glucose lowering effect of 41.3% is achieved after 3 hours. The average blood glucose lowering effect of TMC 0.5% and insulin 50.0 IU/kg is 22.2% over the 3-hour course of the experiment.

N-trimethyl chitosan chloride solutions (0.5%) containing insulin at concentrations of 10.0, 25.0 and 50.0 IU/kg respectively, have been administered intra-ileal to different groups of rats. The results of the plasma insulin levels over time after the different administrations are given in figure 6.44.

It appears that the effect of TMC on the absorption of insulin is dependent on the concentration of insulin. It appears that TMC and insulin 10.0 IU/kg had a poor effect, lower than that of the control group, but keeping in mind that the control group was insulin 50.0 IU/kg, then effect of TMC does not seem poor at all.

Insulin administered at 25.0 IU/kg however, has a marked effect. A peak and maximum plasma insulin level of 238.3 μ IU/ml is reached after just 5 minutes.

Subsequently a sharp decline to 86.2 μ IU/ml is reached at 15 minutes followed by a slight increase to 98.2 μ IU/ml, which is observed at 30 minutes. Insulin declines from thereon forward to reach a minimum level of 16.0 μ IU/ml at 3 hours.

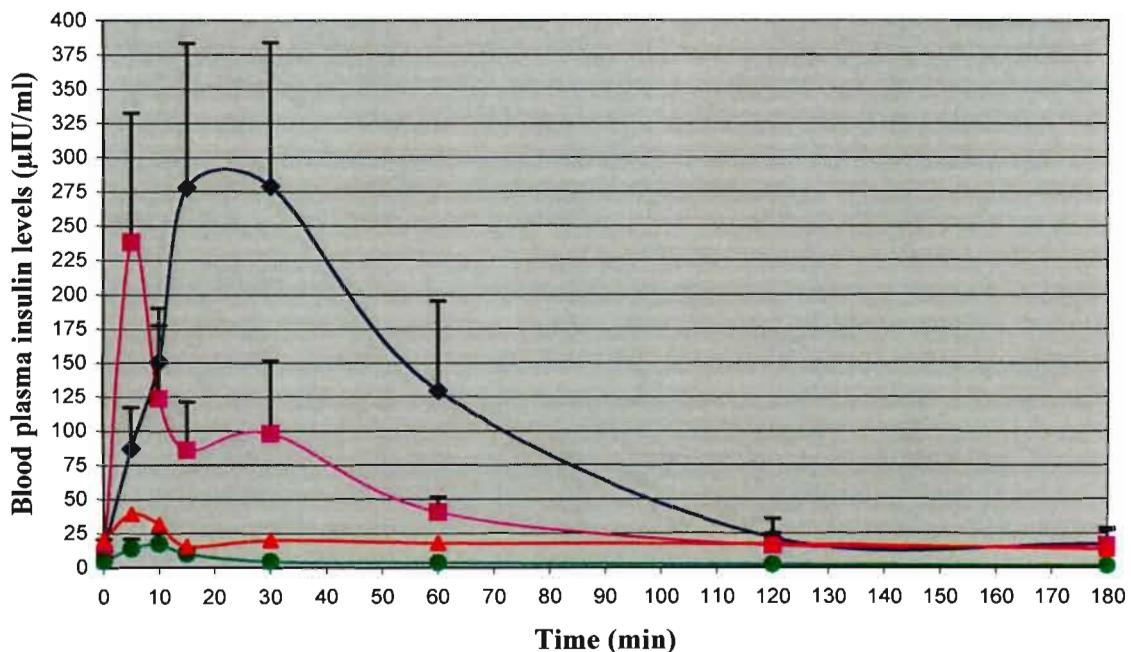


Figure 6.44 A comparison between the average blood glucose levels after intra-ileal administration of TMC 0.5% (w/v) containing insulin 10.0 IU/kg (●), 25.0 IU/kg (■) and 50.0 IU/kg (◆) together with the control group (insulin 50.0 IU/kg in saline) (▲).

Insulin administered at 50.0 IU/kg however, shows an enormous increase in insulin absorption, reaching a peak level between 15 and 30 minutes of 277.6 and 278.7 $\mu\text{IU}/\text{ml}$ respectively. This maximum peak is followed by a steady decline in plasma insulin levels that reach a minimum of 17.2 $\mu\text{IU}/\text{ml}$ at 3 hours.

Another illustration of the effect of TMC and insulin (50.0 IU/kg) on plasma insulin levels is given in figure 6.45. The figure illustrates the average plasma insulin values over the 3-hour course of the experiment compared to the control. This illustrates that insulin levels are increased more than 5 and a half times when administered intra-ileal together with TMC 0.5%, compared to the control.

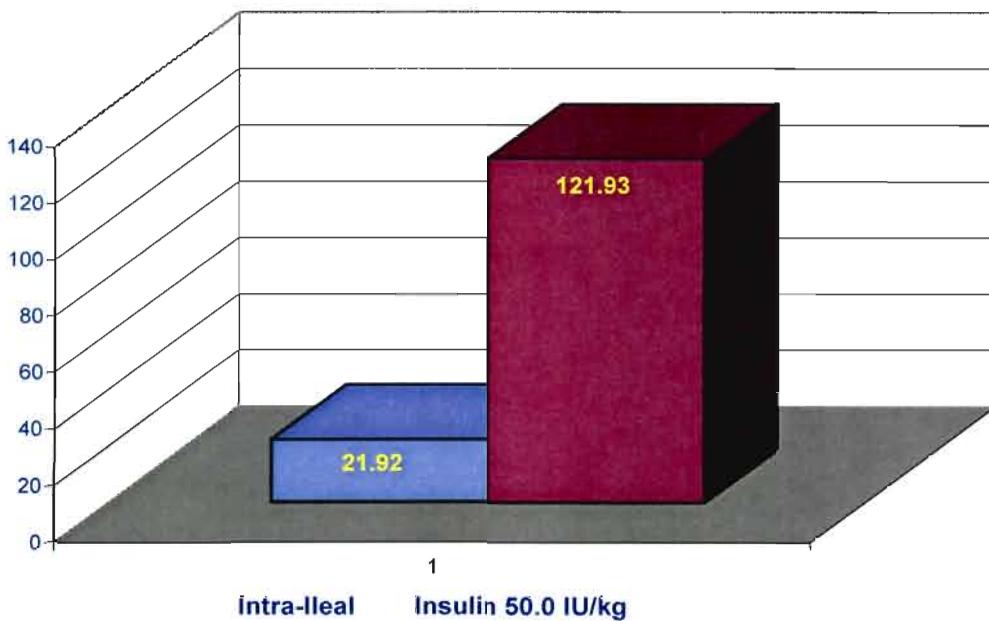


Figure 6.45 Average blood plasma insulin levels after the intra-ileal administration of TMC 0.5% and insulin 50.0 IU/kg (■) against the control (□).

6.3.5 Subcutaneous administration

Commercially available products of insulin used in the treatment of diabetes mellitus are in the form of subcutaneous injectables. A comparative study was done to determine if vesicles and -micro-sponges might be suitable for subcutaneous administration.

A comparative graph of the blood glucose levels after subcutaneous administrations is given in figure 6.46. It can clearly be seen that neither Pheroid vesicles nor -micro-sponges (both containing insulin 4.0 IU/kg) gives such an idyllic blood glucose lowering profile as normal subcutaneous administered insulin (4.0 IU/kg) (control). Pheroid micro-sponges show an initial lowering in blood glucose levels followed by a short-lived increase in blood glucose levels. There after it follows much the same profile as the control but with higher blood glucose levels, reaching a minimum of 35.6% after 2 hours.

Pheroid vesicles shows a fairly constant decrease in blood glucose levels for up to 60 minutes where after glucose levels steadily starts to rise until the 3 hour mark. A minimum blood glucose level of 67.5% is reached at 2 hours.

Insulin 4.0 IU/kg in saline decreased blood glucose by 74.9% after 3 hours.

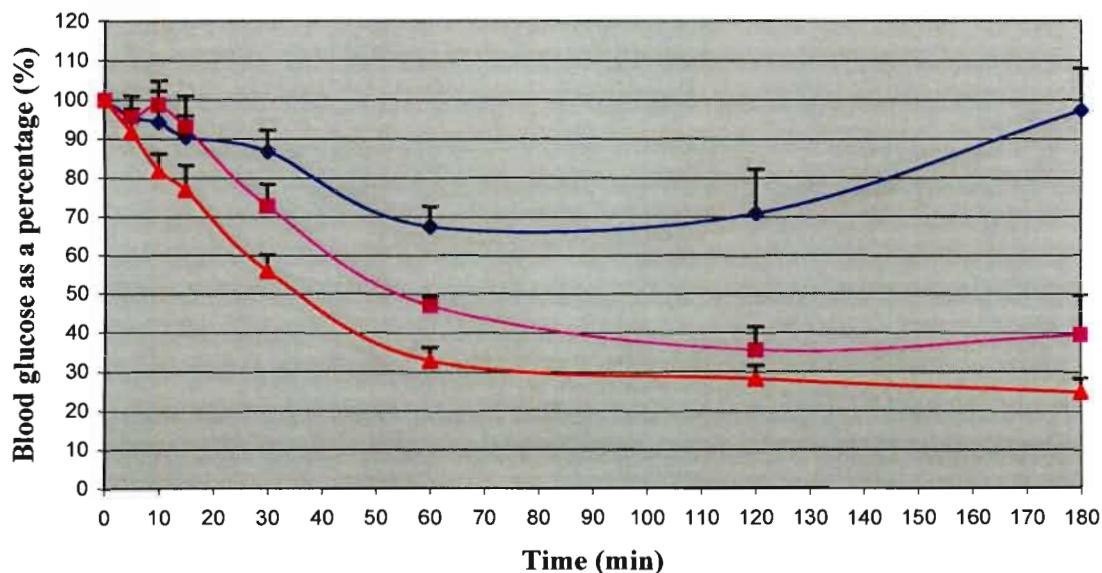


Figure 6.46 A comparison of the average blood glucose levels as a percentage after the subcutaneous administration of insulin (4.0 IU/kg) in Pheroid vesicles (◆), Pheroid micro-sponges (■) and saline (0.9%) (▲).

A comparison of the blood plasma insulin levels after the subcutaneous administration of the various insulin formulations is given in figure 6.47 in the form of a graph.

In correlation to the blood glucose values it can be clearly seen that the therapeutic response was a direct effect of the amount of insulin absorbed. Insulin in saline shows the best absorption with a maximum level of 499.8 µIU/ml of insulin after 30 minutes. From thereon insulin levels decline to reach 254.0 µIU/ml at 3 hours.

Insulin in Pheroid micro-sponges shows the same profile as the control, but with much lower plasma insulin levels. A maximum level of 250.9 μ IU/ml is reached after 30 minutes after which it steadily declines to 116.0 μ IU/ml at 3 hours. Pheroid vesicles presents with the lowest plasma insulin levels with a maximum of 109.6 μ IU/ml after 1 hour. Plasma insulin levels then decrease to 25.8 μ IU/ml at the 3-hour mark.

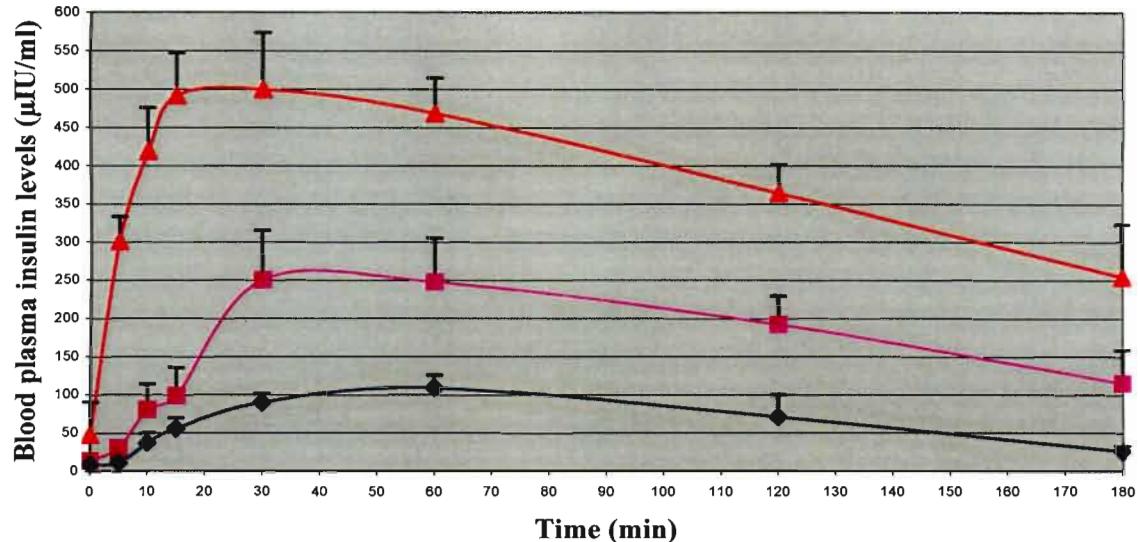


Figure 6.47 A comparison of the blood plasma insulin levels after the subcutaneous administration of insulin (4.0 IU/kg) in Pheroid vesicles (\blacklozenge), Pheroid micro-sponges (\blacksquare) and saline (0.9%) (\blacktriangle).

6.3.6 Intra-nasal administration

6.3.6.1 Intra-nasal administration of insulin (8.0 IU/kg) in saline (control)

Blood glucose levels obtained after intra-nasal administration of insulin in saline is represented as a graph in figure 6.48. Blood insulin levels after intra-nasal administration of insulin in saline is illustrated in figure 4.49.

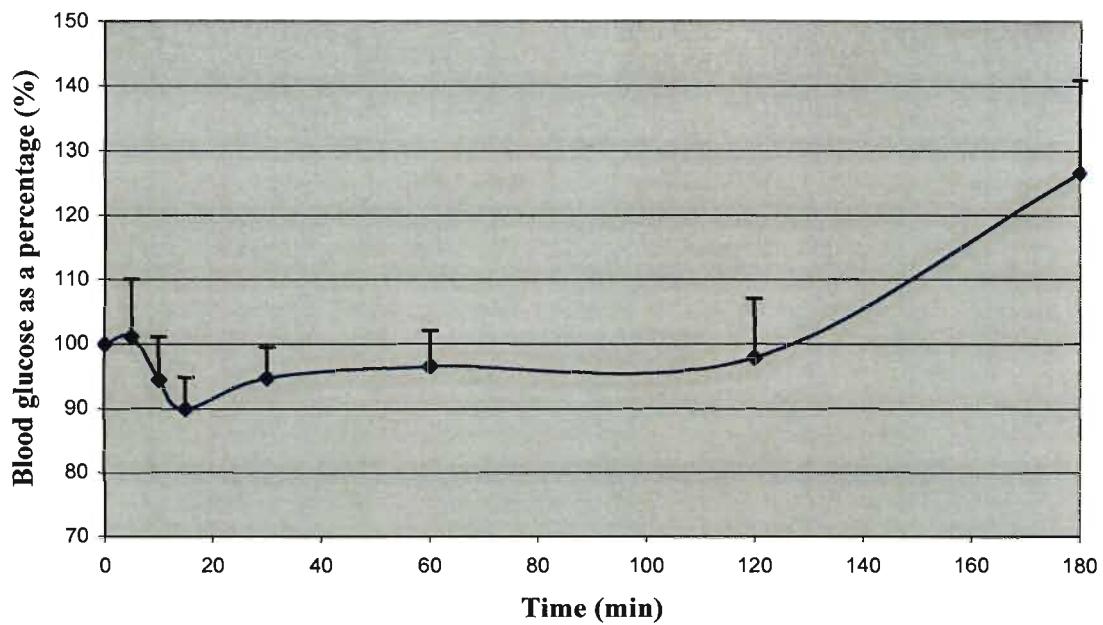


Figure 6.48 Blood glucose levels as a percentage after intra-nasal administration of insulin (8.0 IU/kg) in saline (control).

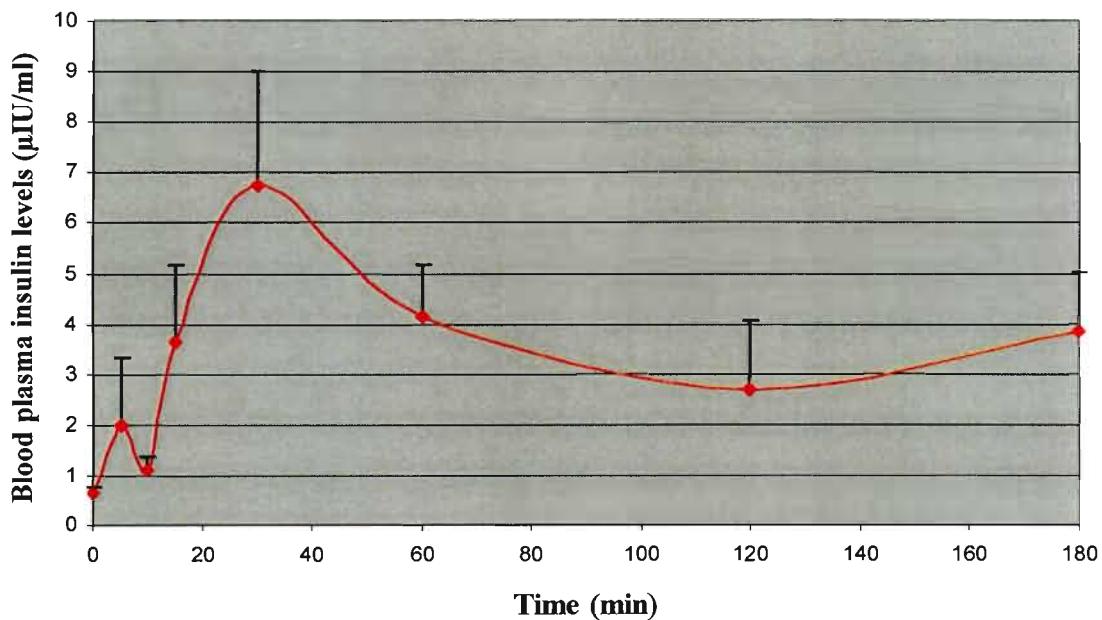


Figure 6.49 Blood plasma insulin levels after intra-nasal administration of insulin in saline (control).

After the intra-nasal administration of insulin in saline there seems to be a slight rise in blood glucose levels for the first five minutes where after the levels sharply decline to 90.0%. The blood glucose levels then show a steady rise but stays below the 100.0% level up until after the 120-minute mark. There after the blood glucose levels are rapidly elevated to 126.5% after 180 minutes, probably due to a glucagon-response.

Intra-nasal absorption of insulin seems to be very rapid as there is a sharp increase in plasma insulin levels up to 2.0 μ IU/ml in the first five minutes after administration. At 10 minutes it seems that insulin clearance has set in and blood plasma levels drops back to 0.7 μ IU/ml.

The next twenty minutes shows a substantial increase in blood insulin levels where it reaches a maximum of 6.7 μ IU/ml at 30 minutes. Blood plasma insulin levels show a steady decline to 2.7 μ IU/ml at the 2-hour mark from which it steadily increases to reach 3.8 μ IU/ml at three hours.

Insulin at a concentration of 8.0 IU/kg shows to have significant even though relatively small effects on blood glucose and blood plasma insulin levels. For this reason 8.0 IU/kg was considered as the lowest concentration for further studies on intra-nasal insulin administration.

6.3.6.2 Intra-nasal administration of insulin (8.0 IU/kg) in Pheroid vesicles

Insulin in Pheroid vesicles was administered nasally to the laboratory rats and the following blood glucose and blood plasma insulin levels are depicted in figures 6.50 and 6.51 respectively.

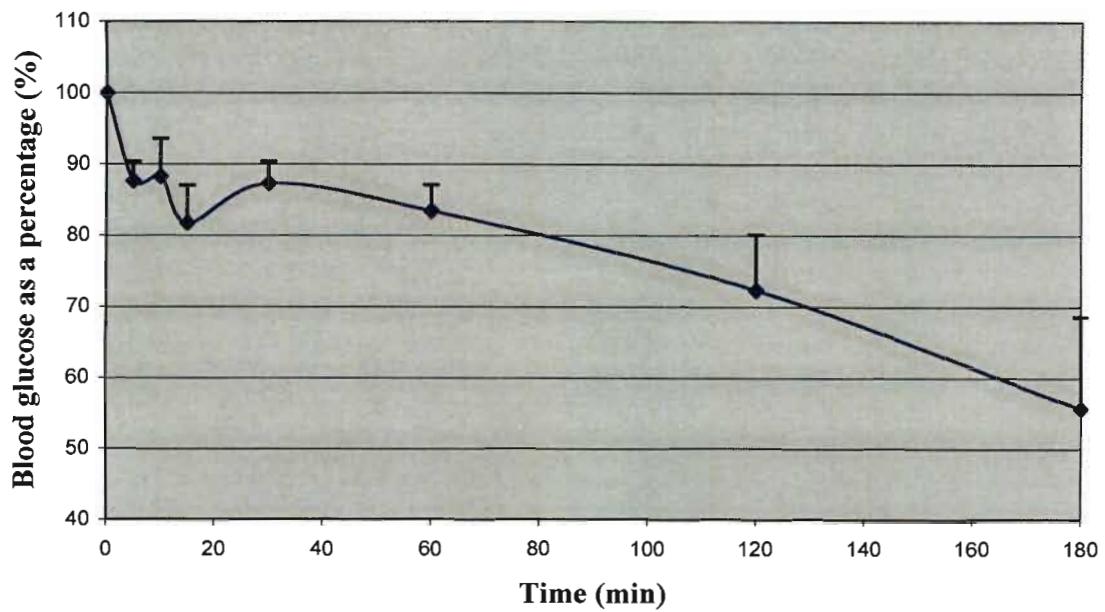


Figure 6.50 Blood glucose as a percentage after intra-nasal administration of insulin in Pheroid vesicles (8.0 IU/kg).

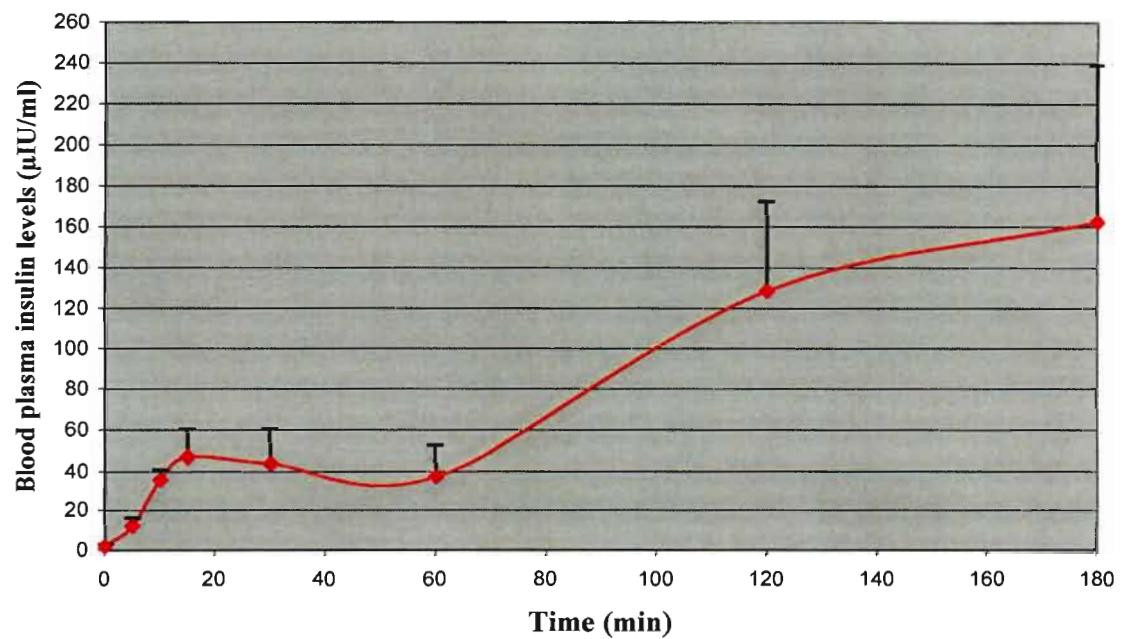


Figure 6.51 Blood plasma insulin levels after intra-nasal administration of insulin in Pheroid vesicles (8.0 IU/kg).

Insulin in Pheroid vesicles shows a dramatic decline in blood glucose levels and shows a decrease of 12.3% after just 5 minutes. At ten minutes levels are slightly elevated but at 15 minutes levels are down to 81.8%. Blood glucose levels then rise again up until 30 minutes, after which it shows a steady decline, which continues for at least until the 3-hour mark where it has already declined by 44.4% from time 0.

There is a clear correlation between blood glucose levels and blood plasma insulin levels. Initially there is a steady absorption of insulin in the first 30 minutes, which accounts for the drop in blood glucose levels. This rise in blood plasma insulin levels possibly triggered a more rapid insulin clearance but after 60 minutes plasma insulin levels showed a steady increase up until at least the 3-hour mark where levels were measured at 162.5 µIU/ml. This also correlates well with the constant decline in blood glucose levels after 60 minutes.

6.3.6.3 Intra-nasal administration of insulin (12.0 IU/kg) in Pheroid vesicles

After obtaining substantial results with insulin at 8.0 IU/kg in Pheroid vesicles it was necessary to investigate what the effect of a higher concentration of insulin in Pheroid vesicles on blood glucose- and plasma insulin levels will be. The results obtained are illustrated in figures 6.52 and 6.53 respectively.

Initially blood glucose levels do not show such a dramatic decrease (only 6.3%) but after 15 minutes blood glucose levels rapidly declines to 58.4%. Fifteen minutes after administration blood glucose levels show a sharp, linier decline from 93.7% to 58.4% at the 3-hour mark. Blood glucose levels are still declining at 3 hours after administration.

Blood insulin levels, as depicted in figure 6.53, sharply increases to 68.8 µIU/ml after just 15 minutes but reaches its maximum after approximately 20 minutes. After 30 minutes plasma levels decline to a lower level of 44.0 IU/ml after which it increases to 59.6 IU/ml at 3 hours.

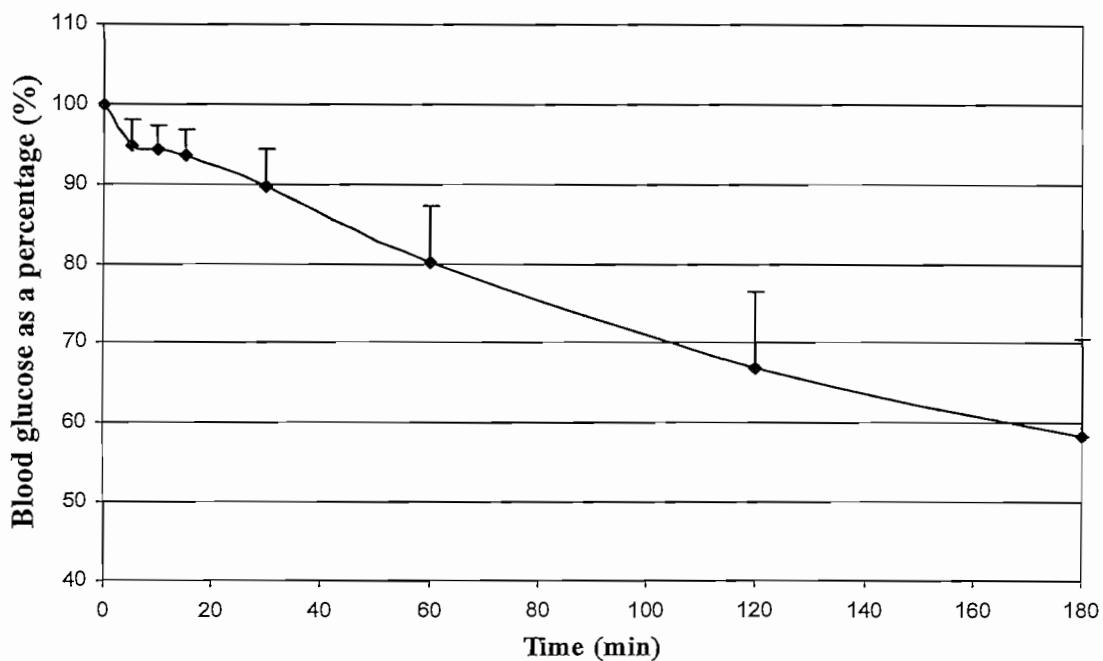


Figure 6.52 Blood glucose levels after intra-nasal administration of insulin (12.0IU/kg) in Pheroid vesicles.

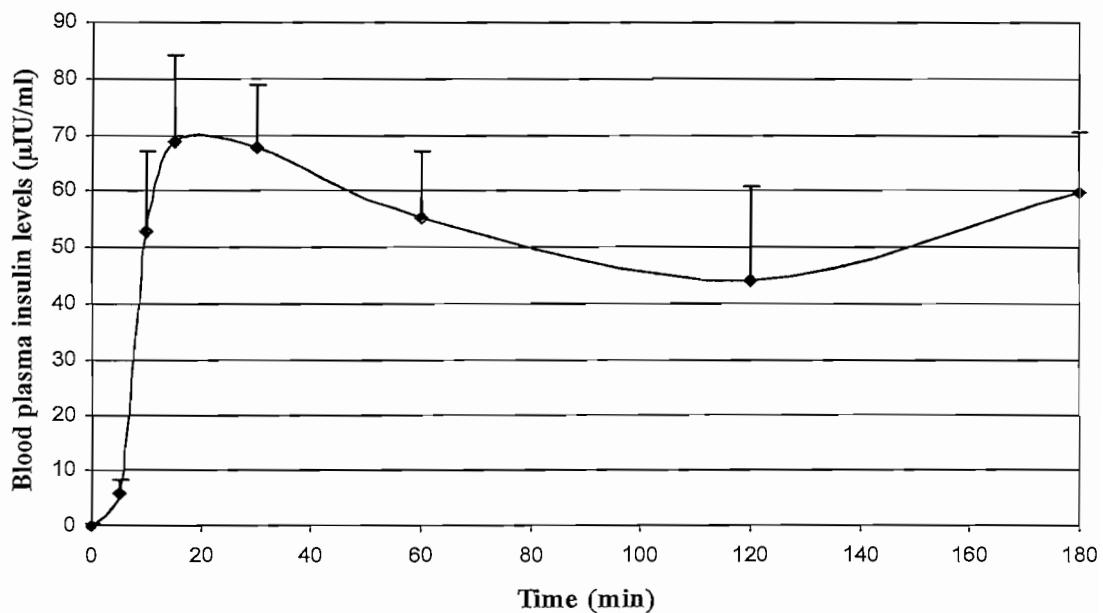


Figure 6.53 Blood plasma insulin levels after intra-nasal administration of insulin (12.0 IU/kg) in Pheroid vesicles.

6.3.6.4 Intra-nasal administration of insulin (8.0 IU/kg) in Pheroid micro-sponges

Another Pheroid formulation, namely Pheroid micro-sponges, were used wherein insulin was entrapped and administered at concentrations of 8.0 and 12.0 IU/kg. Figures 6.54 and 6.55 depict the results obtained from blood glucose- and blood plasma insulin levels respectively after intra-nasal administration of insulin (8.0 IU/kg) in Pheroid micro-sponges.

Blood glucose levels show a substantial decline after just 10 minutes of 19.2% where after it stays stagnant for five minutes. For the next 15 minutes glucose levels drop by another 17.3% to 63.5% after which it shows a slight elevation until the one-hour mark where it registers at 65.5%. Between 120 and 180 minutes blood glucose levels drop yet another 18.0% to reach a staggering 45.1%.

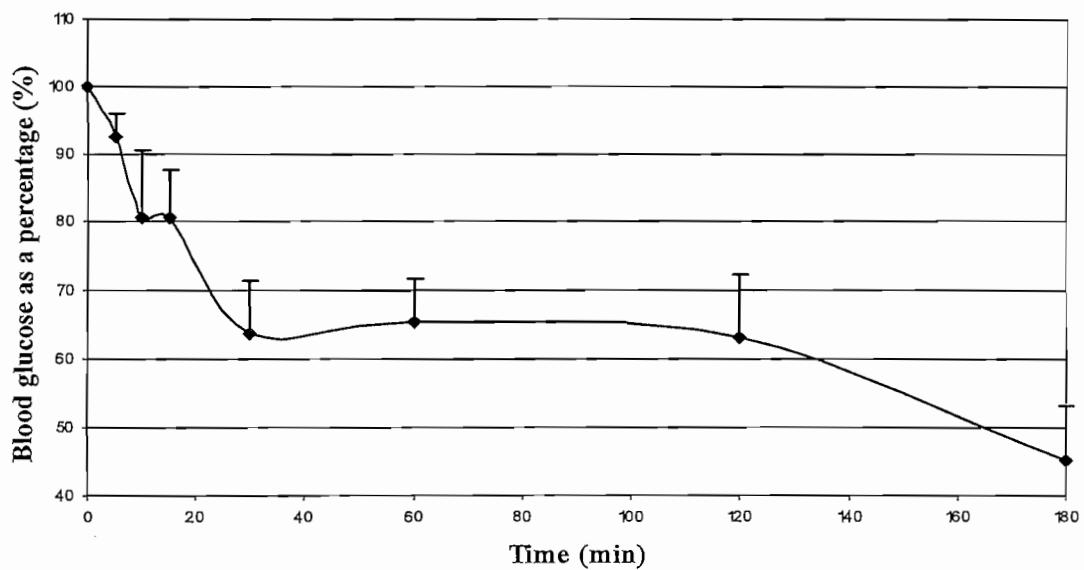


Figure 6.54 Blood glucose levels after intra-nasal administration of insulin (8.0 IU/kg) in Pheroid micro-sponges.

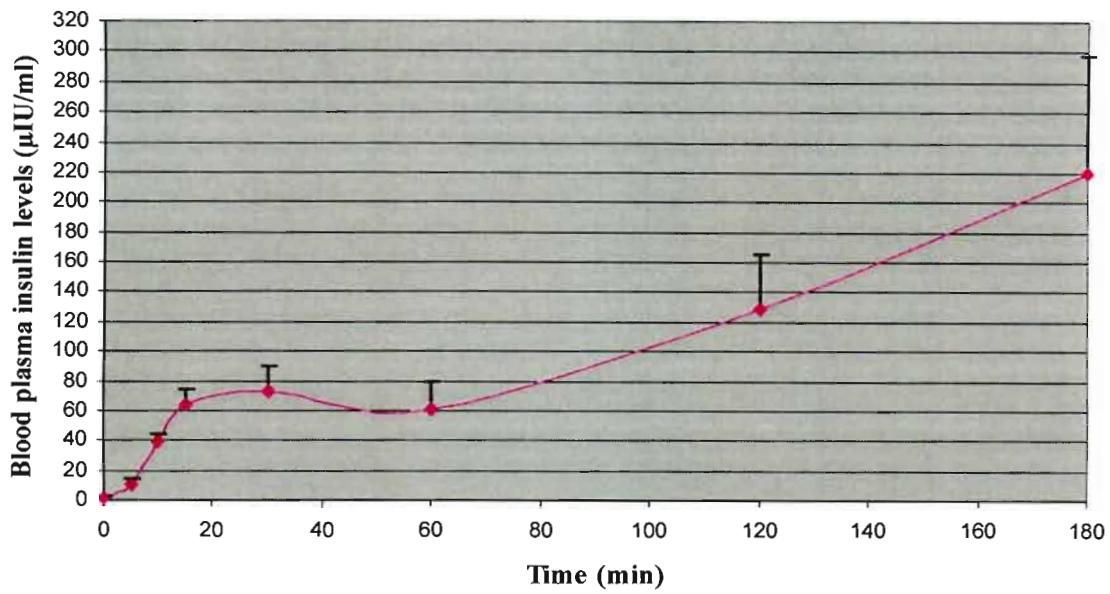


Figure 6.55 Blood plasma insulin levels after intra-nasal administration of insulin (8.0 IU/kg) in Pheroid micro-sponges.

There is a clear resemblance between the blood plasma insulin profile of the two Pheroid formulations, vesicles and micro-sponges after intra-nasal administration of insulin at 8.0 IU/kg. There is a shear incline of plasma insulin levels after the first 15 minutes where it reaches a concentration of 64.2 IU/ml. At 30 minutes it reaches 73.1 IU/ml after which there is a steady decline to 61.8 IU/ml. After 60 minutes it shows a linear increase up to the3-hour mark where it reaches a high of 220.0 IU/ml.

6.3.6.5 Intra-nasal administration of insulin (12.0 IU/kg) in Pheroid micro- sponges

Again, for comparison reasons, insulin was entrapped at a concentration of 12.0 IU/kg in Pheroid micro-sponges and administered intra-nasally. The results that were obtained are illustrated in figures 6.56 and 6.57 respectively.

Except for the steep decline between 5 and 10 minutes, the decrease in blood glucose levels show to be linear up to the 3-hour mark. The decrease is vast with a maximum low of 27.6% at 180 minutes after administration. The decline seems to still be continuing 3 hours after administration.

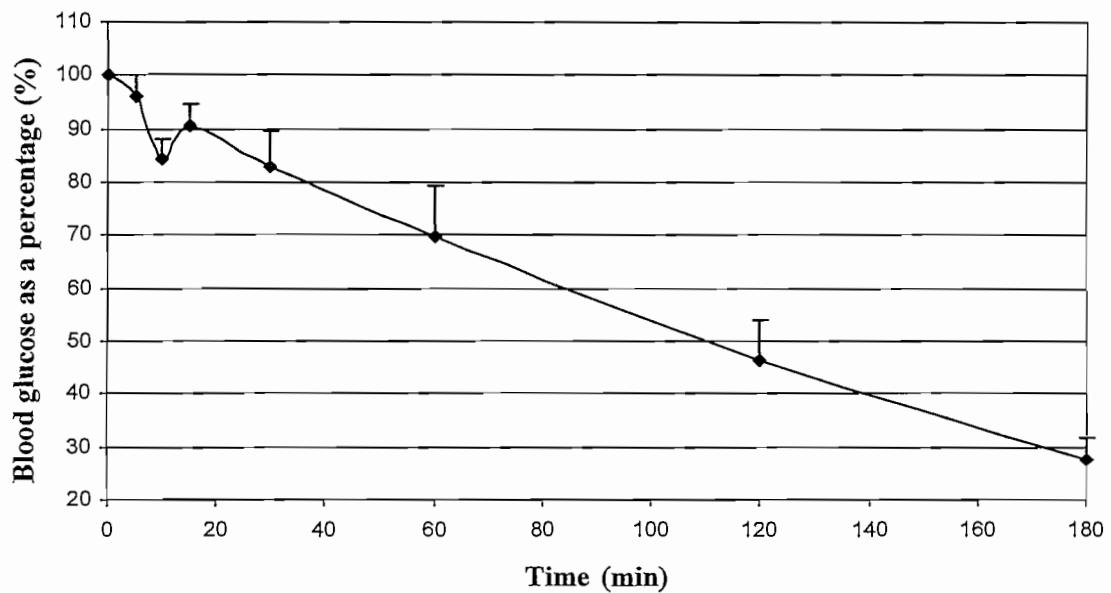


Figure 6.56 Blood glucose as a percentage after intra-nasal administration of insulin (12.0 IU/kg) in Pheroid micro-sponges.

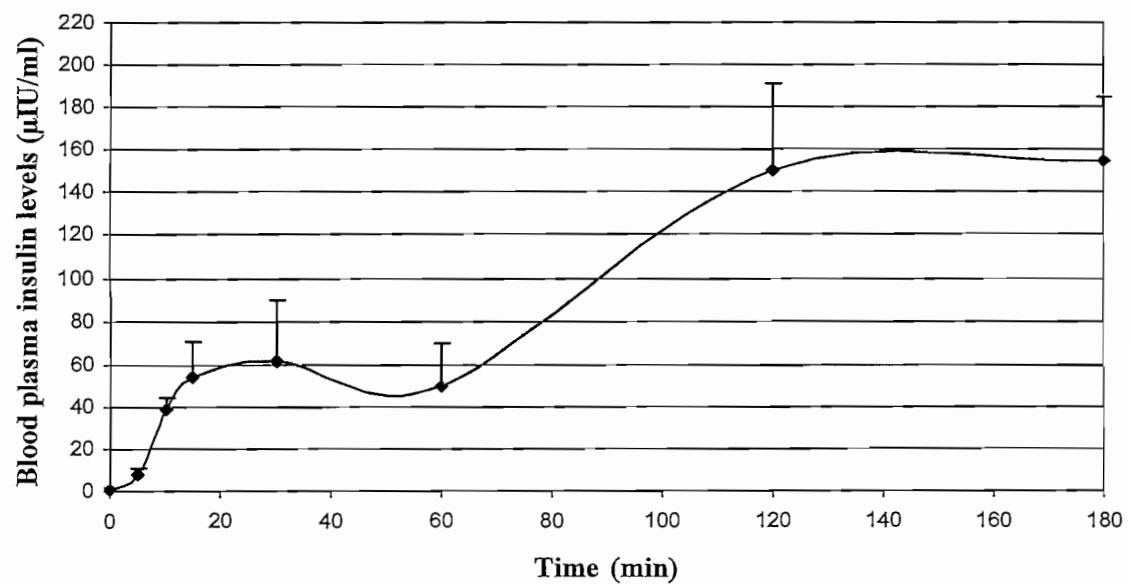


Figure 6.57 Blood plasma insulin levels after the intra-nasal administration of insulin (12.0 IU/kg) in Pheroid micro-sponges.

Blood plasma insulin levels show a sharp increase to 54.5 IU/ml in the first 15 minutes from administration after which it shows a diminutive increase to 61.2 IU/ml for the next 10 minutes. At the 60-minute mark blood glucose levels decline to 50.0 IU/ml where after blood glucose levels rise to 150.0 IU/ml at the 2-hour mark and remains fairly steady for the remaining hour in which it only increases by 4.8 IU/ml.

6.3.6.6 Comparison between insulin at 8.0 and 12.0 IU/kg in Pheroid vesicles and Pheroid micro-sponges

To clearly illustrate the effects of the different insulin and Pheroid formulations on blood glucose- and plasma insulin levels two comparative graphs have been compiled and is depicted in figures 6.58 and 6.59.

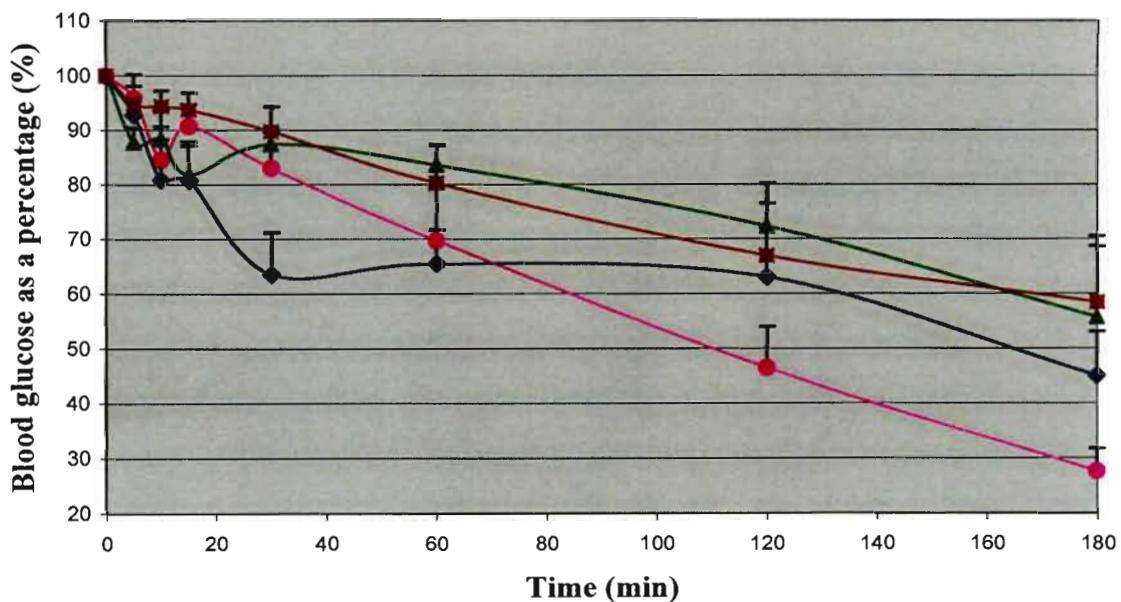


Figure 6.58 A comparison between the blood glucose values of insulin (8.0 IU/kg) in Pheroid vesicles (▲) and Pheroid micro-sponges (◆), and insulin (12.0 IU/kg) in Pheroid vesicles (■) and Pheroid micro-sponges (●).

From figure 6.58 it can clearly be seen that Pheroid micro-sponges triggers a faster onset of action than Pheroid vesicles, when loaded with insulin. The therapeutic effect, namely the reduction in blood glucose, is also much more pronounced with the Pheroid micro-sponge formulation resulting in a total decline of 54.9% and 72.4% for the 8.0 and 12.0 IU/kg administered concentrations respectively. The Pheroid vesicle formulation only managed a maximum decline of 44.4% and 40.4% for the 8.0 and 12.0 IU/kg administered concentrations respectively.

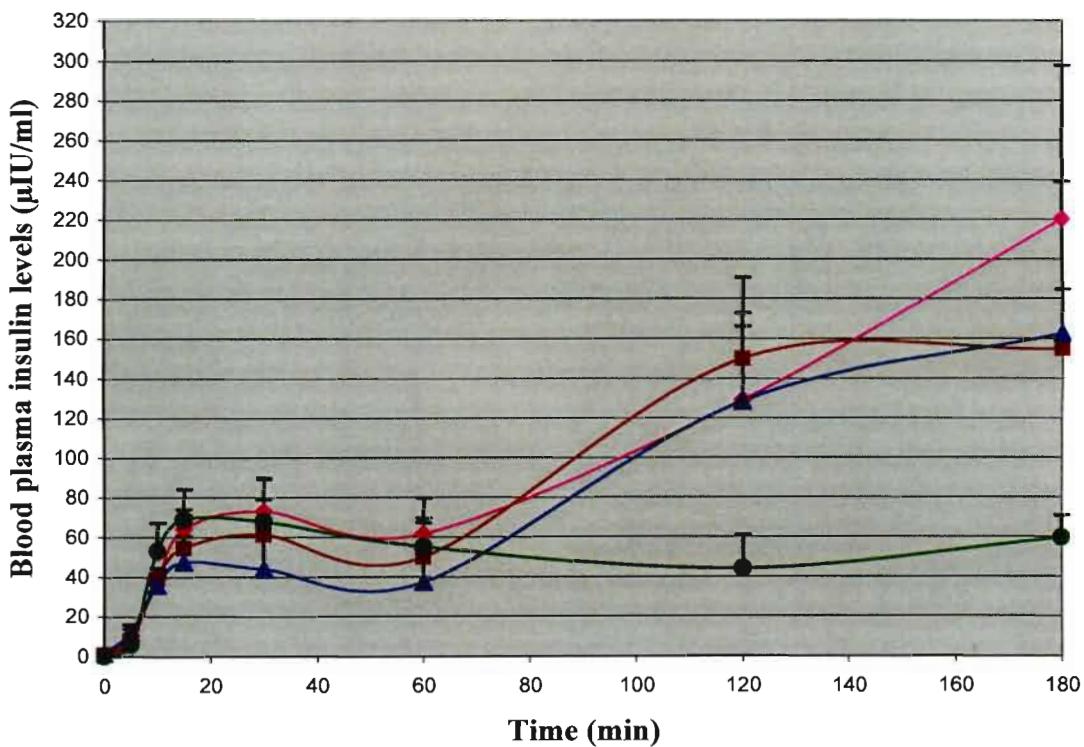


Figure 6.59 A comparative graph of blood insulin levels after the administration of insulin (8.0 IU/kg) in Pheroid vesicles (Δ) and micro-sponges (\blacklozenge), and insulin (12.0 IU/kg) in Pheroid vesicles (\bullet) and micro-sponges (\blacksquare).

It is clear in figure 6.59 that all four Pheroid formulations present with the same tendency to produce an initial rise in plasma insulin levels followed by a decrease at time 60 minutes. Except for Pheroid vesicles (12.0 IU/kg), the Pheroid formulations

results in a steep increase in blood plasma levels which still continues at 3 hours after administration with Pheroid micro-sponges (8.0 IU/kg) giving the highest concentration at 220.0 µIU/ml. Pheroid vesicles (12.0 IU/kg) shows the fastest initial absorption of insulin in the first 15 minutes but however does not seem to have any significant insulin releasing effect after 60 minutes such as the other Pheroid formulations. Pheroid micro-sponges (8.0 IU/kg) show the highest plasma concentrations at 30, 60 and 180 minutes.

Pheroid formulations containing 12.0 IU/kg of insulin does not seem to be so much more efficient than those containing 8.0 IU/kg in terms of plasma values. The reasons for this phenomenon may be concluded in further studies.

6.3.7 Absolute availability

Bioavailability is defined as the rate and extent to which the intact drug is absorbed from the drug product and becomes available at the sight of action. For a product that is not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extend to which the active ingredient becomes available at the site of action (Shargel *et al.*, 2005:453).

Absolute availability of a drug can thus be said to be the systemic availability of the drug after extravascular such as oral, nasal, rectal, transdermal and subcutaneous administration compared to IV dosing of such a drug. This is generally measured by comparing the respective area under the curves (AUC) after extravascular and IV administration. Absolute availability, F, after oral administration using plasma concentration data can be determined as follows:

Equation 6.1

$$F = \frac{[AUC]_{PO} / dose_{PO}}{[AUC]_{IV} / dose_{IV}}$$

Absolute availability is also expressed as a percentage by multiplying F by a 100.0 (Shargel *et al.*, 2005:458). The absolute availability of oral and nasal administration of insulin with Pheroid and TMC formulations in correlation with intra-venous

administration is given in table 6.8. Calculating the AUC of each individual rat and dividing the sum thereof by n of that specific group determined the AUC.

Table 6.8. Absolute availability of insulin after the administration in the different Pheroid and TMC formulations at different routes of administration.

Drug formulation	Dose (IU/kg)	AUC ($\mu\text{g} \cdot \text{hr}/\text{ml}$)	Absolute availability (%)
IV bolus injection	0.5	1146.2	100,0
Oral solutions:			
Stomach - Control	50,0	491.4	0.43
Stomach - Pheroid vesicles	50,0	2972.8	2.59
Ileum - Control	50,0	-223.8	-0.2
Ileum - Pheroid vesicles (1.0 hr)	50,0	727.4	0.63
Ileum - Pheroid vesicles (24.0 hr)	50,0	4603.9	4.02
Ileum - Pheroid microsponges	50,0	1475.7	1.29
Colon - Control	50,0	302.4	0.26
Colon - Pheroid vesicles	50,0	1221.8	1.07
Ileum - TMC	10,0	-238	-1.04
Ileum - TMC	25,0	5047.5	8.81
Ileum - TMC	50,0	10350	9.03
Nasal solutions:			
Control	8,0	545.1	2.97
Pheroid vesicles	8,0	24283.8	132.4
Pheroid microsponges	8,0	21291.6	116.1
Control	12,0	1188.7	4.32
Pheroid vesicles	12,0	8337	30.31
Pheroid microsponges	12,0	24823	90.24

Where AUC and absolute availability are negative values it means that blood plasma insulin levels declined to below the initial values at time 0, such as in the case of ileum control (50.0 IU/kg) and ileum TMC (10.0 IU/kg). These concentrations remained below the initial blood plasma value for extended periods of time or showed such a vast decline in blood plasma levels that it resulted in a negative sum and is thus deemed ineffective in increasing blood plasma insulin levels in any way.

Both nasal administrations of Pheroid vesicles and micro-sponges at 8.0 IU/kg show to have absolute availabilities of more than 100 %, which is theoretically impossible and must in this case be examined to find the cause thereof. As AUC is mainly

indicative of the amount of drug absorbed and detected in the blood plasma over the time period it may be that by some means the detection of the amount of drug is “mirrored” or artificially replicated to some degree. As Pheroid delivery systems are fairly new and not yet extensively researched, the reason for this may be much more complex and complicated and can for the time being not be concluded.

What can be concluded is that insulin administered orally and nasally only in a saline solution does not deliver enough insulin to the blood stream to give a significant therapeutic effect. Insulin trapped in Pheroid vesicles and micro-sponges however, vastly increases the amount of insulin absorbed, coupled by a significant lowering of blood glucose levels, especially after nasal administration.

6.3.8 Conclusion

From the data of blood glucose levels obtained it is evident that there exists a clear and distinct blood glucose lowering profile after intravenous administration of insulin (0.5 IU/kg) followed by a gradual increase in blood glucose. It was also clear that absorption of insulin from Pheroid vesicles was by far the most prominent after interileal administration, which indicated that absorption in the ileum is optimum that pledges a suitable environment for insulin release and absorption. Insulin in Pheroid vesicles decreased blood glucose levels to a great extent and maintained lower blood glucose levels for up to 3 hours and did not produce an obvious glucagon response. Pheroid micro-sponges did not prove to be as successful as it did not have such a distinct effect on blood glucose levels and produced an unwanted glucagon response.

TMC and insulin formulations lowered blood glucose levels to a great extent and maintained low blood glucose levels for up to 3 hours. The effect seems to be insulin dose dependent as 50.0 IU/kg of insulin showed to have lower average blood glucose levels than insulin 10.0 and 25.0 IU/kg. Administration of normal saline also showed inconsistent blood glucose levels over a period of 3 hours, which may be due to physiological stress factors, that affects hormonal levels.

By measuring the plasma insulin levels it can clearly be seen how effective the various formulations are by comparing the amount of insulin absorbed. The amount and extent of insulin absorption is an indication of to what extent the formulation protects the hormone from degradation and how effectively it transports the hormone across epithelial cells in order for it to be available for therapeutic action.

Both intra-gastric and -colonic administration of insulin in Pheroid vesicles seem to enhance the absorption of insulin only to a small extent with no prolonged release of insulin. Insulin absorption is drastically enhanced, however, when administered in Pheroid vesicles at a dose of 50.0 IU/kg compared to the control. Pheroid micro-sponges showed no absorption enhancing effect after intra-ileal administration.

Absorption enhancement of insulin co-administered with TMC 0.5% seemed to be dependent on the concentration of insulin. Insulin administered at 25.0 and 50.0 IU/kg showed large increases in the amount of insulin absorbed over time and had a distinct absorption enhancing effect, with insulin 50.0 IU/kg being even more pronounced.

Both Pheroid vesicles and TMC has proven to enhance the absorption of insulin in the ileum with much success and may be considered as potential absorption enhancers in the oral administration of insulin.

Insulin in Pheroid formulations does not show satisfying results after subcutaneous administration and does not seem suitable for such dosage forms as it presents with higher blood glucose levels and lower plasma insulin levels than the control group.

Nasal administration of Pheroid formulations seems to be extremely effective in both delivering insulin to the blood stream and producing a positive therapeutic response at fairly low insulin concentrations. Pheroid micro-sponges containing an insulin concentration of 12.0 IU/kg produced the largest glucose lowering effect (lowering blood glucose by 54.9 % after 180 minutes) and the highest blood plasma insulin levels (220.0 μ IU/ml after 180minutes).

Compared to subcutaneous and oral administration, nasal administration of insulin in Pheroid formulations is by far the best route of administration for these formulations. Even at low concentrations blood glucose levels were lowered to such an extent and in such a short time that it complies with the requirements of such a formulation intended to lower blood glucose levels. Delivering insulin to the blood stream does not seem to be hampered nor troublesome as it is seen that insulin levels increase within the first five minutes after administration. Vast amounts of insulin are absorbed into the blood stream and long after the formulation was administered (> 3 hours) the levels are still rising and blood glucose levels are still declining. It may also be concluded that even lower concentrations of insulin would be used in the further study of these formulations when nasally administered.

It is also noted however, that by increasing the dose from 8.0 IU/kg to 12.0 IU/kg did not show such a dramatic increase in the therapeutic effect nor amount of insulin absorbed. It was thus deemed necessary to measure the effect of each formulation in terms of bioavailability, by calculating absolute availability to give a more accurate indication of the efficacy of each formulation.

Part IV

Summary and future prospects

Even though still in its infancy of development, Pheroid technology has demonstrated to have numerous possibilities as an efficient drug carrier, synergist and absorption enhancing excipient. Its success in the study of its different therapeutic and preventative uses, such as those for tuberculosis and vaccines for rabies and hepatitis B (Grobler, 2004:19), has paved the way for this drug delivery system in the field of research and development.

Consisting mainly of essential fatty acids and nitrous oxide it can be manipulated in many ways to entrap, transport and deliver pharmacologically active compounds and other useful molecules to the sight of action. Depending upon the clinical indication it can also act in synergism with such compounds or molecules, resulting in an enhanced therapeutic action of such compound (Saunders et al., 1999:99). The different types and sizes of Pheroid make its applications so much more diverse and functional.

Researching the possibilities of increasing the bioavailability of several problematic peptide hormones for oral delivery with Pheroid technology seems inevitable. These peptide hormones present to be the “problem children” of oral and nasal drug delivery as they have low bioavailabilities due to enzymatic and chemical degradation and they have poor absorption qualities as they are too big to diffuse or being carried across membranes. It seems therefore only natural to utilise the excellent qualities of the Pheroid delivery system in improving the bioavailability of these drugs when administered orally or nasally.

Desperately in need of a proper, more practical and accepted form of administration it seemed priority to research the possibilities for such a dosage form for insulin. When testing the effect of Pheroid on the bioavailability of insulin the results were more than

pleasing. Firstly it was important to determine the optimum site of absorption for insulin after oral administration, which was found to be the ileum. Pheroid-insulin formulations significantly reduced blood glucose levels after intraileal administration. Plasma insulin levels of 244.0 μ IU/ml were reached only 5 minutes after administration, lowering blood glucose by 18.7 % and had a maximum blood glucose lowering effect of 42.0 % one hour after administration. Nasal administration of insulin in Pheroid microsponges showed an even more significant effect, lowering blood glucose by 72.4 %, three hours after administration. Administration of insulin in Pheroid vesicles yielded a decline of 44.4 % in blood glucose levels three hours after administration. This proved to be adequate when compared to what is needed in treating Type 1 diabetes mellitus effectively.

Another absorption enhancer named *N*-trimethyl chitosan chloride, or TMC, was also tested to determine its effect on the bioavailability of insulin when administered orally. It showed that TMC readily increased the absolute bioavailability of insulin to above 9.0 % when administered in the ileum in a 0.5 % TMC solution.

Compared to subcutaneous and oral administration, nasal administration of insulin in Pheroid formulations seems to be the best route of administration for these formulations. Even at low concentrations blood glucose levels were lowered to such an extent, and in such a short time, that it complies with the requirements of such a formulation intended to lower blood glucose levels effectively. Delivering insulin to the blood stream does not seem to be hampered nor troublesome as it is seen that insulin levels increase within the first five minutes after administration. Vast amounts of insulin are absorbed into the blood stream and long after the formulation was administered (> 3 hours) the levels are still rising and blood glucose levels are still declining. It may also be concluded that even lower concentrations of insulin could be used in the further study of these formulations for nasal administration as concentrations as low as 8 IU/kg yielded sufficient results.

The nasal route of administration has many advantages as opposed to the oral and parental route (Arora *et al.*, 2002:968; Behl *et al.*, 1998:96;). These advantages were discussed in detail in Chapter 2. It would be elementary to assume that this route of

insulin administration needs to be researched and explored further in order to reach the goal of a safe, adequate and effective nasal formulation for insulin. The ideal focus would be to formulate a dosage form that is- a) non-irritating; b) non-toxic; c) fairly pleasant to administer; d) effective at low dosage volumes and concentrations; e) remains stable at room temperature; f) practical to transport easily and safely from place to place; and g) has an acceptable expiry date. There may be aspects to be added to the list, in the process where the outcomes from research will dictate the direction to be followed in order to manufacture such a dosage form.

Several attempts have been made to produce a successful nasal insulin preparation, of which Exubera™ from Pfizer pharmaceuticals was one of the first to reach the market successfully. After years of research and clinical study (Skyler *et al.*, 2007:84; Rosenstock *et al.*, 2004:1318) the product was successfully launched to the market. Not long after, however, Exubera™ was taken off the market. Even though the reasons given from Pfizer all had to do with financial implications (Bailey & Barnett, 2007:1156) it was warned by the Food and Drug Administration (FDA) in the USA that several cases of primary pulmonary malignancies had been reported in patients using Exubera™. It had been added in the product labelling for Exubera™ that there is a possibility for lung cancer if the product is used (Waknine, 2008).

Pheroid technology, being unique in its kind, present great characteristics to produce a nasal insulin formulation, not discarding oral insulin formulations, that is both safe and effective. Essential steps to be taken in order to achieve this would be to do further *in vivo* studies in mammals, such as dogs or baboons. If these studies are successful it should be followed by clinical trials in humans. Extensive testing on the toxicity of the formulation is also required in order to ensure patient safety and to establish any predicted side effects or occurring ailments, if any, and to find ways to overcome them.

ANNEXURE 1



Prof AF Kotze
Postal Cubicle 36
North-West University
(Potchefstroom Campus)

Ethics Committee
Tel (018) 299 2558
Fax (018) 297 5308
Email drvearl@puk.ac.za

4 July 2005

Dear Prof Kotze

APPROVAL FOR EXPERIMENTING WITH HUMAN SUBJECTS

Your application for project "*Enhancement of insulin absorption after nasal and peroral administration with Emzaloid™ technology*" has been approved with reference number 05D17. Please quote this number in all correspondence regarding your project. According to a decision by the Senate (4 November 1992, Art. 9.13.2) approval of a project is valid for a period of five years. There after you have to re-apply.

A report regarding ethical aspects of this project, as well as possible publications resulting from this study, has to be submitted during June of each year. Such document will be forwarded during May 2006.

Wishing you every success

Yours sincerely

PROF NT MALAN
CHAIRMAN



HEAD OFFICE
Private Bag X6001, Potchefstroom, South Africa, 2520
Tel: (018) 299-4900 • Fax: (018) 299-4910
Internet: <http://www.nwu.ac.za>



In accordance with section 23(1) of the Higher Education Act, 1997 (Act No. 101 of 1997), as amended, the Potchefstroom University for Christian Higher Education and the University of North-West merged to form the North-West University on 1 January 2004. In accordance with section 24(1) of the Higher Education Act, 1997 (Act No. 101 of 1997), as amended, the staff and students of the Bekkersdal Campus of the Vista University were incorporated into the North-West University on 2 January 2004.

ANNEXURE 2



SIGMA-ALDRICH

Certificate of Analysis

Product Name Insulin human,
recombinant, expressed in yeast
Product Number I2643
Product Brand Sigma-Aldrich
CAS Number 11061-68-0
Molecular Formula C₂₅₇H₃₈₃N₆₅O₇₇S₆
Molecular Weight 5807.57
Storage Temp -20°C

TEST	SPECIFICATION	LOT 055K1321 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF-WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 10 MG PLUS 0.5 ML OF 0.01 M HYDROCHLORIC ACID	CONFORMS
LOSS ON DRYING	NMT 10.0%	5.0% *
ZINC	NMT 1.0% (DRY BASIS)	0.4% *
PURITY BY HPLC	>98%	≥99.9% *
POTENCY	NLT 27.5 INSULIN UNITS PER MG (DRY BASIS)	28.5 INSULIN UNITS PER MG (DRY BASIS) *
QC ACCEPTANCE DATE		* SUPPLIER'S INFORMATION MAY 2005

A handwritten signature in black ink, appearing to read "Lori Schulz".

Lori Schulz, Manager
Analytical Services
St Louis, Missouri USA

ANNEXURE 3

Table 1 Average blood glucose levels as a percentage (%) and plasma insulin levels ($\mu\text{IU}/\text{ml}$) after intravenous administration of insulin (0.5 IU/kg in saline ($n=4-6$))

Time (min)	Blood glucose levels (%)	Plasma insulin levels ($\mu\text{IU}/\text{ml}$)
0	100 \pm 0.0	1.56 \pm 0.7
5	87.7 \pm 0.79	28.9 \pm 11.8
10	61.4 \pm 1.59	11.46 \pm 4.7
15	47.8 \pm 2.46	5.25 \pm 2.35
30	34.9 \pm 1.76	4.19 \pm 1.87
60	44.15 \pm 1.92	2.34 \pm 0.96
120	76.97 \pm 9.11	0.76 \pm 0.3
180	109.72 \pm 8.03	0.77 \pm 0.32

Table 2 Average blood glucose levels as a percentage (%) and plasma insulin levels ($\mu\text{IU}/\text{ml}$) after intravenous administration of normal saline ($n=4-6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels ($\mu\text{IU}/\text{ml}$)
0	100.0 \pm 0.0	4.9 \pm 1.2
5	88.1 \pm 3.5	3.39 \pm 1.23
10	92.6 \pm 3.32	2.33 \pm 0.9
15	92.6 \pm 3.73	13.98 \pm 2.92
30	99.7 \pm 4.36	2.99 \pm 3.82
60	106.13 \pm 9.62	9.57 \pm 4.8
120	102.62 \pm 8.35	5.92 \pm 2.19
180	150.34 \pm 13.24	6.87 \pm 7.34

Table 3 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intragastric administration of insulin (50.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	12.95 \pm 3.58
5	96.2 \pm 4.08	19.09 \pm 3.21
10	87.7 \pm 2.93	15.3 \pm 3.85
15	84.7 \pm 3.4	11.1 \pm 1.13
30	87.92 \pm 7.33	13.1 \pm 2.81
60	96.05 \pm 7.62	11.8 \pm 2.12
120	105.62 \pm 15.5	10.48 \pm 1.54
180	118.9 \pm 15.95	13.51 \pm 1.69

Table 4 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intragastric administration of insulin (50.0 IU/kg) in Pheroid vesicles ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	8.3 \pm 2.49
5	100.98 \pm 2.56	49.37 \pm 8.36
10	101.23 \pm 2.03	18.85 \pm 6.83
15	96.12 \pm 4.17	19.13 \pm 5.0
30	95.9 \pm 4.46	19.59 \pm 10.1
60	96.12 \pm 5.31	16.06 \pm 10.15
120	101.23 \pm 19.32	26.48 \pm 10.08
180	131.92 \pm 19.59	32.56 \pm 10.91

Table 5 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intracolonic administration of insulin (50.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	10.72 \pm 3.72
5	95.32 \pm 3.64	29.85 \pm 5.89
10	97.54 \pm 4.82	22.2 \pm 7.1
15	100.46 \pm 5.19	14.4 \pm 4.1
30	97.96 \pm 5.29	14.7 \pm 2.8
60	107.56 \pm 7.95	17.97 \pm 5.68
120	144.82 \pm 16.19	12.67 \pm 3.2
180	141.75 \pm 22.03	8.9 \pm 1.39

Table 6 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intracolonic administration of insulin (50.0 IU/kg) in Pheroid vesicles ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	30.74 \pm 2.43
5	91.37 \pm 4.64	38.09 \pm 9.45
10	90.03 \pm 4.59	29.25 \pm 7.64
15	85.8 \pm 6.56	21.75 \pm 5.58
30	94.08 \pm 2.94	28.3 \pm 10.07
60	93.22 \pm 8.24	34.23 \pm 20.72
120	105.66 \pm 6.36	40.94 \pm 15.4
180	132.08 \pm 9.6	36.5 \pm 9.84

Table 7 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (50.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0	20.84 \pm 2.88
5	91.28 \pm 10.25	39.28 \pm 6.76
10	95.48 \pm 10.58	31.56 \pm 12.85
15	86.62 \pm 10.73	15.85 \pm 3.04
30	95.4 \pm 12.88	19.87 \pm 5.77
60	104.67 \pm 14.29	17.83 \pm 4.2
120	121.98 \pm 15.63	17.31 \pm 4.31
180	122.12 \pm 12.64	13.17 \pm 2.65

Table 8 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (24 hour entrapment) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	7.22 \pm 2.56
5	81.28 \pm 4.92	243.77 \pm 39.28
10	71.38 \pm 4.87	164.93 \pm 50.07
15	61.05 \pm 5.03	57.42 \pm 25.66
30	64.7 \pm 2.43	35.08 \pm 13.42
60	57.67 \pm 7.71	24.85 \pm 8.48
120	67.9 \pm 10.9	26.24 \pm 10.78
180	77.63 \pm 12.16	20.04 \pm 7.11

Table 9 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (50.0 IU/kg) in Pheroid vesicles (1 hour entrapment) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	15.82 \pm 2.15
5	99.88 \pm 1.39	34.24 \pm 7.66
10	95.97 \pm 3.69	22.34 \pm 1.51
15	108.13 \pm 4.73	24.32 \pm 3.04
30	103.45 \pm 4.96	23.89 \pm 3.99
60	103.0 \pm 9.42	22.46 \pm 2.12
120	110.87 \pm 8.61	14.4 \pm 0.72
180	155.4 \pm 18.5	20.53 \pm 4.89

Table 10 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (50.0 IU/kg) in Pheroid micro-sponges ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	16.08 \pm 4.21
5	98.83 \pm 5.12	30.7 \pm 4.79
10	85.75 \pm 2.57	43.43 \pm 5.84
15	84.65 \pm 3.12	29.14 \pm 5.48
30	87.83 \pm 3.0	22.36 \pm 5.39
60	93.67 \pm 5.66	18.63 \pm 5.07
120	121.57 \pm 8.19	15.94 \pm 2.77
180	137.15 \pm 7.44	17.61 \pm 2.2

Table 11 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (10.0IU/kg) and TMC (0.5% w/v) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	4.71 \pm 3.0
5	93.1 \pm 2.34	14.15 \pm 6.89
10	94.63 \pm 3.8	17.44 \pm 7.85
15	88.9 \pm 2.55	10.27 \pm 3.68
30	92.92 \pm 5.26	4.5 \pm 2.03
60	82.18 \pm 4.69	3.37 \pm 1.34
120	85.17 \pm 10.87	2.16 \pm 0.99
180	45.23 \pm 4.85	1.4 \pm 0.4

Table 12 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (25.0IU/kg) and TMC (0.5% w/v) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	9.95 \pm 4.26
5	91.42 \pm 4.83	238.3 \pm 94.18
10	78.2 \pm 4.24	124.01 \pm 53.58
15	73.27 \pm 3.96	86.25 \pm 35.23
30	72.45 \pm 6.66	98.18 \pm 53.36
60	73.78 \pm 10.55	40.62 \pm 11.03
120	74.32 \pm 11.3	16.39 \pm 8.27
180	62.82 \pm 10.19	15.99 \pm 10.99

Table 13 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intraileal administration of insulin (50.0IU/kg) and TMC (0.5% w/v) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	12.82 \pm 7.99
5	92.92 \pm 2.76	86.95 \pm 30.39
10	89.78 \pm 3.6	150.99 \pm 39.38
15	85.3 \pm 4.08	277.56 \pm 105.66
30	64.5 \pm 6.38	278.68 \pm 105.19
60	69.56 \pm 6.3	129.48 \pm 65.88
120	61.6 \pm 6.19	21.75 \pm 13.88
180	58.68 \pm 4.55	17.21 \pm 11.65

Table 14 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after subcutaneous administration of insulin (4.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	48.05 \pm 42.51
5	91.75 \pm 2.94	301.19 \pm 32.53
10	81.82 \pm 4.36	419.68 \pm 56.11
15	76.92 \pm 6.29	491.82 \pm 55.3
30	55.98 \pm 4.19	499.8 \pm 73.41
60	32.92 \pm 3.4	468.29 \pm 46.3
120	28.27 \pm 3.44	364.73 \pm 36.44
180	25.05 \pm 3.31	254.01 \pm 69.02

Table 15 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after subcutaneous administration of insulin (4.0 IU/kg) in Pheroid vesicles ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	9.15 \pm 3.51
5	95.58 \pm 2.13	11.9 \pm 5.13
10	94.3 \pm 7.95	37.02 \pm 13.2
15	90.55 \pm 5.39	55.82 \pm 13.75
30	86.78 \pm 5.43	89.97 \pm 11.19
60	67.47 \pm 5.05	109.58 \pm 15.87
120	70.82 \pm 11.36	70.88 \pm 29.06
180	97.3 \pm 10.61	25.76 \pm 6.76

Table 16 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after subcutaneous administration of insulin (4.0 IU/kg) in Pheroid micro-sponges ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	14.14 \pm 5.1
5	95.75 \pm 5.3	31.29 \pm 4.84
10	98.78 \pm 6.03	81.81 \pm 32.63
15	93.18 \pm 7.86	100.14 \pm 35.63
30	72.83 \pm 5.49	250.86 \pm 64.57
60	47.08 \pm 2.07	248.05 \pm 57.79
120	35.63 \pm 5.99	192.7 \pm 36.2
180	39.73 \pm 9.89	115.96 \pm 42.78

Table 17 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (8.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0±0.0	0.65±0.1
5	101.18±21.69	2.0±1.35
10	94.42±16.4	1.1±0.29
15	89.97±11.87	3.64±1.53
30	94.73±11.68	6.73±2.26
60	96.53±13.5	4.14±1.04
120	97.95±22.33	2.67±1.41
180	126.58±35.22	3.84±1.19

Table 18 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (8.0 IU/kg) in Pheroid vesicles ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0±0.0	1.77±1.22
5	87.65±2.74	12.13±4.03
10	88.28±5.28	35.72±5.3
15	81.78±5.29	47.36±13.0
30	87.32±3.03	44.04±16.43
60	83.48±3.63	37.48±15.09
120	72.33±7.85	128.58±43.96
180	55.62±12.98	162.47±76.49

Table 19 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (8.0 IU/kg) in Pheroid micro-sponges ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	1.74 \pm 0.6
5	92.73 \pm 3.28	10.89 \pm 3.1
10	80.83 \pm 9.74	39.05 \pm 5.16
15	80.78 \pm 7.06	64.19 \pm 9.78
30	63.53 \pm 7.69	73.15 \pm 16.1
60	65.47 \pm 6.2	61.76 \pm 17.63
120	63.05 \pm 9.02	129.33 \pm 36.52
180	45.06 \pm 8.04	220.21 \pm 77.45

Table 20 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (12.0 IU/kg) in saline (control) ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	2.28 \pm 0.87
5	93.52 \pm 3.72	2.35 \pm 1.2
10	100.08 \pm 6.59	0.76 \pm 0.15
15	104.1 \pm 6.74	1.28 \pm 0.51
30	108.32 \pm 7.25	2.0 \pm 0.57
60	113.98 \pm 9.98	2.56 \pm 0.62
120	130.7 \pm 11.8	3.82 \pm 1.41
180	140.55 \pm 10.57	17.45 \pm 5.43

Table 21 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (12.0 IU/kg) in Pheroid vesicles ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	0.16 \pm 0.1
5	94.8 \pm 3.22	6.07 \pm 2.19
10	94.35 \pm 2.84	52.94 \pm 14.07
15	93.7 \pm 3.11	68.79 \pm 15.17
30	89.68 \pm 4.56	67.76 \pm 11.2
60	80.2 \pm 7.08	55.1 \pm 11.98
120	66.84 \pm 9.73	44.01 \pm 16.8
180	58.4 \pm 12.1	59.63 \pm 10.91

Table 22 Average blood glucose levels as a percentage (%) and plasma insulin levels (μ IU/ml) after intra-nasal administration of insulin (12.0 IU/kg) in Pheroid micro-sponges ($n= 4 - 6$)

Time (min)	Blood glucose levels (%)	Plasma insulin levels (μ IU/ml)
0	100.0 \pm 0.0	1.3 \pm 0.4
5	95.92 \pm 4.31	8.2 \pm 2.4
10	84.54 \pm 3.66	39.24 \pm 5.22
15	90.7 \pm 4.04	54.55 \pm 16.27
30	83.0 \pm 6.53	61.21 \pm 28.58
60	69.68 \pm 9.75	49.96 \pm 19.43
120	46.46 \pm 7.57	150.02 \pm 40.58
180	27.63 \pm 4.1	154.77 \pm 29.81

ANNEXURE 4



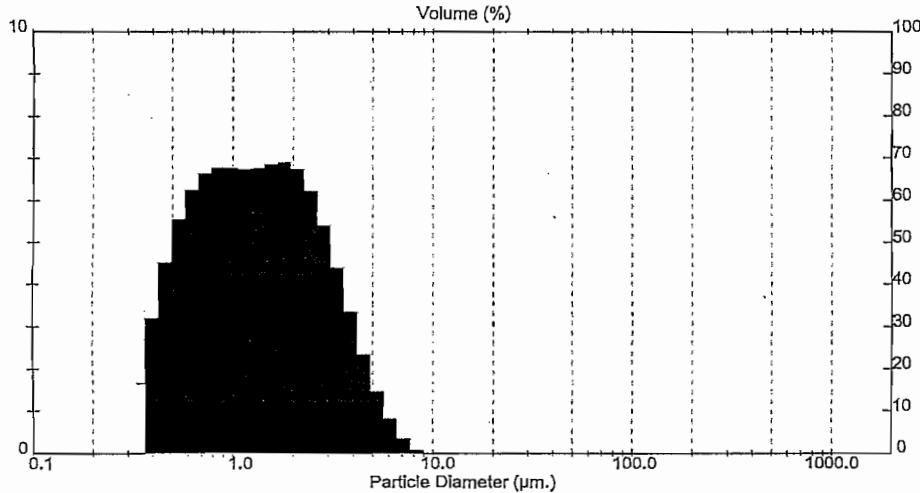
Result: Analysis Report

Sample Details			
Sample ID: Pheroids	Run Number: 2	Measured: 8 Mar 2006 08:50PM	
Sample File: IO08_03	Record Number: 2	Analysed: 3 Apr 2006 10:48AM	
Sample Path: E:\EKSPER-1\DEELTJ-1\ANDERIAN		Result Source: Analysed	
Sample Notes: Pheroids sonder geneesmiddel berel op 07_03, bepaal op 08_03			

System Details		Measured Beam Obscuration: 10.6 %
Sampler: Internal		
Presentation: 40HD	[Particle R.I. = (1.5295, 0.1000); Dispersant R.I. = 1.3300]	Residual: 0.692 %
Analysis Model: Polydisperse		
Modifications: None		

Result Statistics			
Distribution Type: Volume	Concentration = 0.0025 %Vol	Density = 1.000 g / cub. cm	Specific S.A. = 5.8573 sq. m / g
Mean Diameters:	D (v, 0.1) = 0.50 um	D (v, 0.5) = 1.29 um	D (v, 0.9) = 3.40 um
D [4, 3] = 1.68 um	D [3, 2] = 1.02 um	Span = 2.250E+00	Uniformity = 7.111E-01

Size Low (um)	In %	Size High (um)	Under%	Size Low (um)	In %	Size High (um)	Under%
0.31	1.67	0.36	1.57	10.48	0.00	12.21	100.00
0.36	3.21	0.42	4.87	12.21	0.00	14.22	100.00
0.42	4.53	0.49	9.40	14.22	0.00	16.57	100.00
0.49	5.55	0.58	14.96	16.57	0.00	19.31	100.00
0.58	6.26	0.67	21.21	19.31	0.00	22.49	100.00
0.67	6.64	0.78	27.86	22.49	0.00	26.20	100.00
0.78	6.79	0.91	34.64	26.20	0.00	30.53	100.00
0.91	6.78	1.06	41.42	30.53	0.00	35.56	100.00
1.06	6.74	1.24	48.17	35.56	0.00	41.43	100.00
1.24	6.77	1.44	54.93	41.43	0.00	48.27	100.00
1.44	6.86	1.68	61.79	48.27	0.00	56.23	100.00
1.68	6.92	1.95	68.71	56.23	0.00	65.51	100.00
1.95	6.75	2.28	75.45	65.51	0.00	76.32	100.00
2.28	6.23	2.65	81.68	76.32	0.00	88.91	100.00
2.65	5.41	3.09	87.10	88.91	0.00	103.58	100.00
3.09	4.42	3.60	91.52	103.58	0.00	120.67	100.00
3.60	3.36	4.19	94.87	120.67	0.00	140.58	100.00
4.19	2.35	4.88	97.22	140.58	0.00	163.77	100.00
4.88	1.49	5.69	98.71	163.77	0.00	190.80	100.00
5.69	0.82	6.63	99.53	190.80	0.00	222.28	100.00
6.63	0.37	7.72	99.90	222.28	0.00	258.95	100.00
7.72	0.10	9.00	100.00	258.95	0.00	301.68	100.00
9.00	0.00	10.48	100.00				



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