Permeation of excised intestinal tissue by insulin released from Eudragit® L100/Trimethyl chitosan chloride microspheres

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“The only true wisdom is in knowing you know nothing” – Socrates 469 BC – 399 BC

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Abstract

The purpose of this research project was to develop and characterise matrix type microspheres prepared from Eudragit® L100, containing insulin as model peptide drug as well as an absorption enhancer, *N*-trimethyl chitosan chloride (TMC), to improve intestinal absorption via the paracellular route. Insulin loaded microspheres were prepared using a single water in oil emulsification/evaporation method in accordance with a fractional factorial design (2^3) and subsequently characterised in terms of morphology as well as internal structure. Also, insulin and TMC loading were determined using a high pressure liquid chromatography analysis (HPLC) and colorimetric assay, respectively.

Scanning electron microscopic characterisation revealed that most microsphere formulations showed a spherical shape and smooth surface with a sponge-like internal structure as well as relatively good homogeneity in terms of size distribution. Insulin loading ranged from 27.9 ± 14.25 – 52.4 ± 2.72% between the different formulations. TMC loading was lower than for insulin and ranged from 29.1 ± 3.3 - 37.7 ± 2.3% between the different formulations. The pronounced difference in insulin and TMC loading between the microsphere formulations is probably the result of the multitude parameters involved as well as the complex physicochemical processes which govern emulsification/solvent evaporation. Based on the microsphere characterisation results, two formulations were selected (i.e. B and F) for further characterisation (i.e. particle size distribution, dissolution behaviour, and enteric nature) and for *in vitro* evaluation of insulin transport across excised Fischer (FSR) rat intestinal tissue using a Sweetana-Grass diffusion chamber. Particle size analysis by means of laser light diffraction of the two selected microsphere formulations revealed that the mean particle size (based on volume) ranged from 135.7 ± 41.05 to 157.3 ± 31.74 μm. Dissolution results for microsphere Formulations B and F revealed that both insulin and TMC were released from the microsphere formulations in an alkaline environment (pH 7.4). The mean dissolution time (MDT) for insulin ranged from 34.5 ± 4.01 to 42.6 ± 9.06 min, while the MDT for TMC ranged from 1.2 ± 1.73 to 6.8 ± 6.42 min. Statistical analysis revealed no significant differences in the MDT of either insulin or TMC (p-value > 0.05) between the two formulations, although the difference between insulin and TMC of each formulation was significant (p-value < 0.05). Microsphere formulations B and F released 36.92 and 48.21% of their total drug content over a period of 1 h in 0.1 M HCl.

Microsphere Formulation B showed 8.3 ± 0.52% and formulation F 8.9 ± 2.26% transport of the initial insulin dose after a period of 120 min across excised rat intestinal tissue. The increase in

insulin transport by the microsphere formulations compared to that of the control group (i.e. insulin alone) correlated well with the decrease in transepithelial electrical resistance (TEER) caused by the microsphere formulations. The transport of insulin from Formulations B and F represented transport enhancement ratios of 10.67 and 9.68, respectively.

Insulin loaded Eudragit® L100 microspheres containing TMC were successfully prepared by emulsification/solvent evaporation that demonstrated promising potential to serve as oral drug delivery systems for insulin. The microspheres exhibited improved insulin permeability across intestinal epithelial tissue; however, its enteric properties should be improved and clinical effectiveness need to be confirmed by future *in vivo* studies.

Keywords: Eudragit® L100; Excised rat jejunum; Insulin; Microspheres; N-trimethyl chitosan chloride; Oral peptide delivery; Paracellular transport.

Uittreksel

Die doel van hiedie studie is om mikrosfere te berei vanaf Eudragit® L100 wat insulien as modelgeneesmiddel asook *N*-trimetielkitosaanchloried (TMC) as parasellulêre absorpsiebevorderaar bevat. Die insulienbevattende mikrosfere was berei deur middel van 'n enkel water in olie emulsifisering-oplosmiddelverdampingsmetode waarvan die samestelling met 'n fraksionele faktoriale (2^3) ontwerpbepaal is en daarna was die mikrosfere gekarakteriseer met betrekking tot morfologie en interne struktuur (skanderingselektronmikroskopie), deeltjiegrootte, insulien en TMC lading.

Karakterisering met elektronmikroskopie het getoon dat die meeste mikrosfeerformules ‘n sferiese voorkoms gehad het terwyl die interne struktuur van die mikrosfere ‘n sponsagtige voorkoms gehad het. Na aanleiding van die mikroskoopfotos lyk dit ook asof die insulien en TMC homogeen in die Eudragit® L100-matriks gedispergeer was, aangesien geen kristalle sigbaar was nie. Beide die insulien en die TMC was suksesvol in die mikrosfere geïnkorporeer. Die insulien lading het gewissel van 27.9 ± 14.25 – 52.4 ± 2.72% tussen die verskillende formules, terwyl TMC lading laer was en gewissel het tussen 29.1 ± 3.3 - 37.7 ± 2.3% vir die verskillende formules. Die duidelike verskil in lading tussen insulien en TMC was moontlik as gevolg van die menige parameters betrokke by emulsifisering-oplosmiddelverdampingsmetode. Na aanleiding van die resultate verkry, was twee Formules geselekteer (B en F) vir verdere karakterisering (naamlik deeltjiegrootteverspreiding, dissolusiegedrag en enteriese gedrag) en *in vitro* evaluering van insulien transport oor uitgesnyde Fischer (FSR) rot intestinale weefsel deur gebruik te maak van ‚n Sweetana-Grass diffusie apparaat. Die gemiddelde deeltjiegroote (volgens volume) van Formules B en F het gewissel van 135.7 ± 41.05 tot 157.3 ± 31.74 μm. Elektronmikroskopie sowel as deeltjiegrootte-analise toon dus dat die bereide produkte mikrosfere was. Dissolusieresultate het getoon dat beide insulien en TMC vrygestel is by ‘n pH van 7.4. Die gemiddelde dissolusietyd (GDT) vir insulien het gewissel van 34.5 ± 4.01 - 42.6 ± 9.06 min, terwyl die GDT vir TMC gewissel het van 1.2 ± 1.73 - 6.8 ± 6.42 min. Statistiese analise het geen verskille in insulien- en TMC-vrystelling tussen die formulerings getoon nie (p-waarde > 0.05), maar wel tussen insulien en TMC vir beide formules (p-waarde < 0.05). Evaluering van die enteriese gedrag van Formules B en F het getoon dat 36.92 en 48.21% onderskeidelik van hul insuliendosis vrygestel is na blootstelling aan 0.1 M HCl vir ‘n periode van 1 uur.

Die kumulatiewe getransporteerde insuliendosis vir Formules B en F was 8.3 ± 0.52% and 8.9 ± 2.26%, onderskeidelik na 'n periode van 120 min. Hierdie vehoogde transport van insulien wanneer vergelyk word met die kontrole groep (insulien alleen) korreleer goed met ‘n verlaging in transepiteelelektriese weerstand. Die absorpsiebevorderingsverhoudings vir Formules B en F is bereken as 10.67 en 9.68, onderskeidelik. Dus is dit duidelik dat insluiting van TMC ‘n voorvereiste is vir betekenisvolle parasellulêre transport van insulien wat in mikrosfere berei is.

Ten slotte kan gesê word dat Eudragit® L100 mikrosfere wat beide insulien en TMC bevat het, met sukses berei is. *In vitro* evaluering van hierdie mikrosfere het belowende resultate gelewer wat daarop dui dat mikrosfere ‘n suksesvolle afleweringsisteem vir die parasellulêre transport van insulien is, alhoewel dit nog *in vivo* bevestig moet word.

Sleutelwoorde: Eudragit® L100; Insulien; Mikrosfere; N-trimetielkitosaanchloried; Orale peptiedaflewering; Parasellulêre transport; uitgesnyde rot intestinale weefsel.

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List of abbreviations

ABC	Adenosine triphosphate-binding cassette
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
ANOVA	One-way analyses of variance
AUC	Area under curve
CLSM	Confocal laser scanning microscopy
DQ	Degree of quaternisation
DGAVP	8-arginine vasopressin
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
ESEM	Environmental scanning electron microscope
FD-4	Fluorescein isothiocyanate labled dextran
FDA	Food and Drug Administration
FSR	Fischer
GALT	Gut associated lymphoid tissue
GSH	Reduced glutathione
HIM2	Hexyl-insulin monoconjugate-2
HPLC	High pressure liquid chromatography analysis
IDDM	Insulin dependent diabetes mellitus
IgA	Immunoglobulin A
KRB	Krebs-Ringer bicarbonate
LHRH	Hormone-releasing hormone
M-cells	Microfold cells
MDCK	Madin-darby canine kidney

MDR	Multidrug resistance
MDT	Mean dissolution time
MRP	Multidrug resistance-associated protein
NMR	Nuclear magnetic resonance
PAMPA	Parallel artificial membrane permeation assay
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PepT1	Peptide transporter 1
PepT2	Peptide transporter 2
P-gp	P-glycoprotein
PVA	Polyvinyl alcohol
REAL	Reversible aqueous lipidisation
RESS	Rapid expansion of supercritical solutions
rhG-CSF	Human recombinant granulocyte-colony stimulating factor
SAS	Supercritical antisolvent crystallisation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TEER	Transepithelial electrical resistance
TMC	*N*-trimethyl chitosan chloride
TMC-H	High degree of quaternisation
ZO-1	Zonula occludens 1
ZO-2	Zonula occludens 2

CHAPTER ONE

Introduction and aim of study

1.1 Background and justification

1.1.1 Challenges with oral peptide drug delivery

Administering drugs orally is by far the most widely and preferred route of administration ensuring greater compliance and convenience to patients. This is because oral administration avoids the pain and discomfort as well as the possibility of infections associated with injections. However, the oral route is generally not practical for efficient peptide and protein drug delivery due to poor bioavailability which is typically less than 1-2% (Pauletti *et al*., 1996:3). The poor bioavailability of protein and peptide drugs can be attributed to their large molecular size, hydrophilicity and their susceptibility to enzymatic degradation (Hamman *et al.,* 2005:166; Morishita & Peppas, 2006:905). Apart from the intrinsic properties of peptide and protein drugs, the gastrointestinal tract contributes to the low bioavailability through the presence of various physical and biochemical barriers such as the unstirred water layer, the physical barrier of the lipid-bilayer membranes of the intestinal epithelia, enzymes localised at different locations and efflux systems (Daugherty & Mrsny, 1999:144).

1.1.2 Strategies to overcome challenges

Advances in biotechnology, chemistry and molecular biology led to the production of large quantities of structurally diverse peptides and proteins possessing a broad spectrum of pharmacological effects. These developments increased the need for novel delivery systems (Pauletti *et al*., 1996:3). To overcome the obstacles associated with oral peptide/protein delivery, various strategies have been investigated. The following approaches have been applied to increase the bioavailability of proteins and peptides: chemical modifications such as pro-drug strategies, structural modifications such as PEGylation and lipidisation; targeting of transporters or tissues such as receptor-mediated endocytosis and gut associated lymphoid tissue (GALT) and formulation technologies such as particulate carriers, use of absorption enhancers as well as enzyme inhibitors, mucoadhesive/bioadhesive systems and systems aimed at targeting the paracellular transport pathway. Only limited success has been achieved

thus far with these approaches, which highlights the need for further basic research in this field (Park *et al.,* 2010:66-75).

Promising results have been obtained with combinations of some of these strategies. For instance, Ziv *et al*. (1980:1035–1039) demonstrated that a combination of bile acid (i.e. sodium cholate) as permeation enhancer and aprotinin as proteinase inhibitor was more effective in improving the oral bioavailability of insulin as well as pancreatic RNase than either of the agents alone. Hosny *et al*. (2002:71-76) demonstrated that the inclusion of sodium salicylate as an absorption enhancer into enteric-coated insulin-containing capsule formulations resulted in a 25 - 30% reduction in plasma glucose levels when surgically delivered to the stomachs of streptozotocin-induced diabetic rats.

1.1.3 Microsphere formulations for effective drug delivery

Microencapsulation represents a range of techniques for the entrapment of solids or liquids within polymer coats or matrices. Microparticles in particular provide benefits such as rapid emptying from the stomach as well as more reproducible transit through the small intestine and colon due to their small size. The increased surface area facilitates rapid drug release, and more reproducible absorption and bioavailability (Nilkumhang & Basit, 2009:135). Additional versatility is provided by the use of various materials which provides further advantages when used in microsphere drug delivery systems, including: effective protection of encapsulated drugs against degradation, increase drug solubility, reduced adverse or toxic effects, and provides site-specific and controlled drug release profiles required for therapeutic effect (Singh *et al*., 2010:65). However, even with these significant advances certain challenges need to be addressed, such as the development of cheaper biopolymers and the development of universally acceptable evaluation methods for microspheres.

1.1.4 Paracellular drug absorption enhancement by *N*-trimethyl chitosan chloride

When considering drug transport across the intestinal epithelium two main routes exist namely between adjacent epithelial cells through the intercellular spaces (paracellular transport) and through the epithelial cells (transcellular transport). Transport via the paracellular pathway surmounts intracellular enzyme degradation and is the main target for absorption of hydrophilic drugs not recognized by a carrier, which makes it an attractive approach for peptide and protein drugs (Ward *et al.,* 2000:347). However, paracellular transport requires the controlled and reversible opening of the tight junctions. Modulation of the tight junctions can be achieved by

absorption enhancers such as chitosan and its derivatives, for example *N*-trimethyl chitosan chloride (van der Merwe *et al.,* 2004:228). The surface area of the paracellular route in the gastrointestinal tract is estimated to be about 200 to 2000 cm^2 and should not be underestimated for peptide and protein drug delivery since even minute quantities (pM - nM range) may be sufficient to produce the required therapeutic effect (Salamat-Miller & Johnston, 2005:203).

Chitosan and chitosan salts are only soluble in acidic environments and is therefore incapable of enhancing absorption in the small intestine. Kotzé *et al*. (1999:253-257) demonstrated that only TMC with a high degree of quaternization (TMC-H) was able to cause a decrease in the Transepithelial electrical resistance (indicator of paracellular transport) of Caco-2 cells at pH 7.40, compared to TMC with a low degree of quaternization (DQ) of 12.28% and chitosan hydrochloride. At this concentration a 35% reduction in transepithelial electrical resistance (TEER) was obtained. Transport studies with [^{14}C]-mannitol gave results in agreement with the TEER results. Increases of between 31-48-fold were obtained for TMC-H at a concentration range of 0.05–1.5% w/v (Kotzé *et al*., 1999b:253-257).

In addition, Hamman *et al*. (2003:161-172), investigated the effect of six different TMC polymers (DQ between 12% and 59%) on the TEER of Caco-2 cell monolayers and on the transport of hydrophilic and macromolecular model compounds ([^{14}C]-mannitol and [^{14}C] PEG 4000). It was found that only TMC with a DQ higher than 22% was able to reduce TEER in a neutral environment (pH 7.4). The maximum reduction in TEER (47.3 ± 6.0% at a concentration of 0.5% w/v and pH 7.4) was reached with TMC with a DQ of 48%, and this effect did not increase further with a higher DQ. In agreement with the TEER results, the transport of the model compounds increased with an increase in the DQ of TMC reaching a maximum at a DQ of 48% (25.3% of the initial dose for [^{14}C]-mannitol and 15.2% of the initial dose for [^{14}C] PEG 4000), and this effect did not increase further with a higher DQ of TMC.

In conclusion, the physiological barriers to absorption, the low bioavailability due to degradation and unfavourable physicochemical properties as well as high inter-patient variability are challenges that researchers need to overcome before the oral route can be viable for effective administration of peptide and protein drugs.

1.2 Aim and objectives

The aim of this research project is to develop, characterise and evaluate the drug delivery properties of matrix type microspheres prepared from Eudragit® L100 containing insulin as well as an absorption enhancer, *N*-trimethyl chitosan chloride (TMC).

To reach this aim, the following objectives needed to be achieved:

- To prepare different microsphere formulations from Eudragit® L100 containing insulin and TMC by means of a solvent evaporation method based on a fractional factorial design.
- To characterise the microspheres by means of scanning electron microscopy and insulin as well as TMC loading in order to select optimal formulations for further characterisation.
- To characterise selected formulations by means of particle size and size distribution, dissolution profiles and enteric nature.
- To conduct *in vitro* transport studies of insulin across excised rat intestinal tissues released from the microspheres.

1.3 Structure of dissertation

The introductory chapter describes the need for the research project by placing it in the context of current literature as well as the aim and objectives of this study. It is followed by a review of the relevant literature (Chapter two) regarding the oral delivery of peptide and protein drugs, the barriers which impedes their absorption and the strategies employed to overcome these barriers. In chapter three, the preparation of a range of microsphere formulations based on a factorial design and characterisation thereof in terms of scanning electron microscopy, particle size and size distribution and dissolution is described. Chapter four describes the *in vitro* transport studies across excised rat intestinal tissues of two optimal microsphere formulations compared to that of insulin alone (control group). Chapter five gives the final conclusions and future recommendations.

CHAPTER TWO

Oral delivery of protein and peptide drugs

2.1 Introduction

The gastrointestinal tract is designed in such a way that digestion and absorption of nutrients such as dipeptides, amino acids, vitamins and cofactors occur in parallel with its ability to prevent the entry of pathogens, toxins and undigested macromolecules. The intestinal mucosa encompasses biochemical as well as physiological mechanisms to complement its physical barrier against uptake of unwanted xenobiotics (Daugherty & Mrsny, 1999a:144). As a result of the susceptibility of protein and peptide drugs to degradation by the strong acidic environment of the stomach and proteolytic enzymes, these compounds exhibit low bioavailability. Unfavourable physicochemical properties such as high molecular weight and hydrophilic properties of protein and peptide drugs contribute to their poor permeability across the intestinal epithelium (Hamman *et al*., 2005:166).

There are two main routes by which a molecule can move across intestinal epithelia, namely between adjacent epithelial cells (paracellular transport) and through epithelial cells (transcellular transport) (Daugherty & Mrsny, 1999a:147), as illustrated in Figure 2.1. The major pathway for intestinal absorption of a drug depends on its physicochemical characteristics as well as the membrane features. In general, lipophilic drugs cross the intestinal epithelia transcellularly, while small hydrophilic drugs cross the epithelia mainly paracellularly (Salama *et al*., 2006:16). The absorption of amino acids, di- and tripeptides are believed to be carrier mediated. Proteins are very poorly absorbed by passive diffusion, if at all (Lee & Yamamoto, 1990:177-178).

To overcome the obstacles of oral peptide/protein absorption, various strategies have been implemented including modification of the physicochemical properties, addition of functional excipients (e.g. permeation enhancers, enzyme inhibitors) and the use of specially adapted drug delivery systems (e.g. use of mucoadhesive and pH responsive polymers). However, only limited success has been achieved thus far with these approaches due to problems such as inherent toxicities of absorption-enhancing excipients, variation in absorption between individuals and potential high manufacturing costs, which highlights the need for research in this field (Park *et al.,* 2010:286).

The barriers hampering intestinal peptide and protein absorption as well as techniques to overcome them are discussed in the following sections.

2.2 Transport of molecules across plasma membranes

Drug absorption across the intestinal epithelial cell membrane is a complex multi-path process as illustrated schematically in Figure 2.1. There are two major transport pathways of drug transport across the gastrointestinal epithelium, i.e. the transcellular pathway and paracellular pathway (Daugherty & Mrsny, 1999a:147; Ashford, 2007:271). Passive diffusion is an energy independent, non-specific absorption mechanism that occurs through the cell membrane of the enterocytes or via the tight junctions between the cells. Alternatively, carrier-mediated absorption involves specific membrane-associated channels or transporters that can be classified as active (energy dependent) or facilitated (energy independent) transport. Various efflux transporters such as P-glycoprotein (P-gp) can limit absorption of compounds that are recognised as substrates. In addition to these mechanisms for the transport of small individual molecules, cells may engage in endocytosis. During endocytosis the plasma membrane extends or invaginates to surround a particle, a foreign cell or extracellular fluid, which then closes into a vesicle that is released into the cytoplasm (Smith, 2005:164-166).

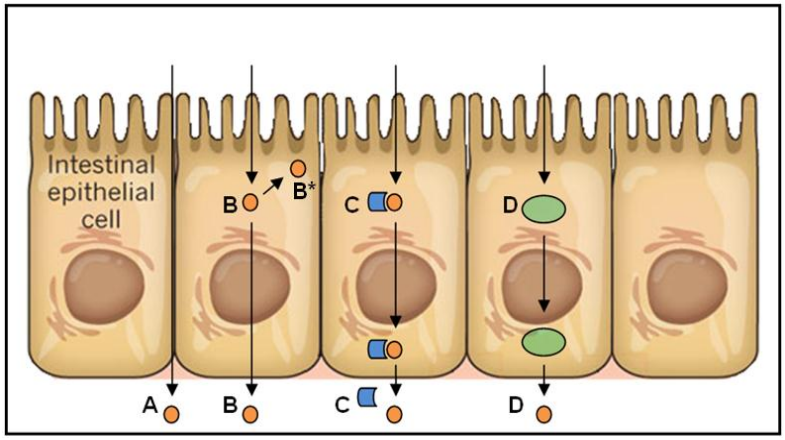


Figure 2.1: A schematic representation of transport pathways across the intestinal epithelium: (A) passive paracellular diffusion; (B) passive transcellular diffusion, (B*) intracellular metabolism; (C) carrier mediated transcellular transport; (D) transcellular vesicular endocytosis (adapted from Flint, 2012:602)

2.2.1 Transcellular pathway

The transcellular pathway is the transport of a molecule across the epithelial cells, which requires partitioning of the compound through both the apical and the basolateral membranes (Hochman *et al*., 1994:253). This route includes transport through channels, pumps and transporters as well as vesicles (endocytosis of a macromolecule at one side of a monolayer and exocytosis at the other side) (Tsukita *et al*., 2001:287). The most important transcellular transport processes will be briefly discussed in the following sections.

2.2.1.1 Passive diffusion

Passive diffusion is an energy independent process, which occurs through non-specific permeability pathways and includes simple diffusion (free diffusion) as well as facilitated diffusion. Simple diffusion is differentiated from facilitated transport, which requires the transported molecule to bind to a specific carrier or transport protein in the membrane (Daugherty & Mrsny, 1999a:148; Smith, 2005:164-166). The driving force involved in passive diffusion is the difference in concentration and charge across a membrane and transport occurs from a region of high concentration to a region of low concentration, in order to equilibrate between the two sides of the membrane (Shargel & Yu, 1999:101-103; Smith., 2005:164-166). Passive diffusion is the transport process for most drugs. The gastrointestinal membrane appears to contain small water-filled pores through which small molecules of radii less than about 4 Å can be transported. However, drugs generally are larger molecules with molecular weights in excess of 100 Da and therefore cannot permeate these pores (Notari, 1987:134-135). Passive diffusion of drugs can quantitatively be described by Fick's law of diffusion (Equation 1.1).

$$\frac{\mathrm{dQ}}{\mathrm{dt}} = \frac{\mathrm{DAK}}{\mathrm{h}}\left(\mathrm{C_{gi}} - \mathrm{C_p}\right) \qquad \text{Equation 1.1}$$

Where:

dQ/dt is the rate of diffusion; *D* is the diffusion coefficient; *K* is the lipid-water partition coefficient of the drug in the biologic membrane; *A* is the surface area of the membrane; *h* is the membrane thickness; and $C_{gi} - C_p$ is the difference between the concentrations of drug in the gastrointestinal tract and in the plasma (Shargel & Yu, 1999:102).

From Equation 1.1 it is apparent that a multitude of factors determine the diffusion of drugs across biological membranes. For instance, the lipophilicity of drugs is directly proportional to the partition coefficient (*K*), which will influence the extent and route of drug absorption. Lipid-soluble drugs tend to traverse the cell membranes more easily than water soluble molecules (Shargel & Yu, 1999:101-103).

2.2.1.2 Carrier-mediated transport (facilitated and active transport)

Although the majority of drugs are absorbed across cells by means of simple diffusion, certain compounds and many nutrients are absorbed transcellularly by a carrier-mediated transport system. This form of transcellular transport occurs through specific membrane-associated channels or transporters and is usually the transport mechanism by which amino acids, di- and tripeptides, monosaccharides, nucleosides and water-soluble vitamins are absorbed (Daugherty & Mrsny, 1999a:148). Many orally active peptide drugs share structural features with physiological substrates of the peptide transport system (for example, PepT1) including ß-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors, bestatin, thrombin inhibitors, renin inhibitors as well as thyrotropin releasing hormone and play an essential role in their absorption (Yang *et al*., 1999:1332; Lee, 2000:S41). Carrier-mediated transport can be classified into two main groups, i.e. facilitated transport and active transport.

Both facilitated transport and active transport are mediated by protein transporters (carriers) in the cell membrane. However, facilitated transport is an energy independent process in which the compound is transported down an electrochemical gradient, from a high concentration to a low concentration, to attain balance in concentration and charge across the two sides of the membrane. In contrast to facilitated transport, active transport can take place against a concentration gradient from regions of low concentration to regions of high concentrations. For this reason, energy (e.g. ATP hydrolysis by Na^+, K^+-ATPase) is applied directly to a transporter (primary active transport) or used to establish an ion gradient (secondary active transport) for the transport to take place (Smith, 2005:164-166). During this specialised process, a drug molecule first binds a specific carrier prior to transport to from a carrier-drug complex, which shuttles the drug across the membrane where it dissociates to render the drug free at the other side of the membrane. The protein transporters are highly specific for binding to molecules with a certain chemical structure; therefore drug transport can be competed for by molecules that resemble the structure of the compounds normally transported. Since there is only a limited number of a carrier, they exhibit saturation kinetics when all the binding sites on all of the

transporter proteins in the membrane are occupied. When the system is saturated, the rate of transport reaches a plateau (the maximum velocity) as demonstrated in Figure 2.2. (Shargel & Yu, 1999:105-106; Smith, 2005:164-166).

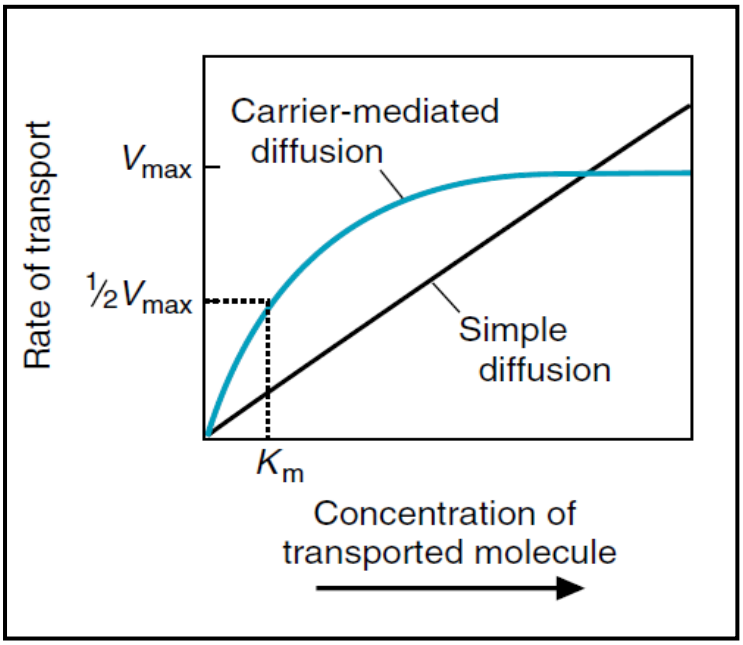


Figure 2.2: Relationship between rate of transport and concentration of transported molecule when comparing carrier-mediated transport and simple diffusion (reproduced from Smith, 2005:165)

2.2.1.3 Vesicular transport across the plasma membrane

Transport by means of simple diffusion or mediated by carriers is usually only possible for small organic molecules due to the restricted size of the pores (4 Å) located in the apical membrane (Washington *et al.*, 2001:15). In contrast, vesicular transport occurs when a membrane completely surrounds a compound dissolved in extracellular fluid, particles or cells and encloses it into a vesicle. Several mechanisms of vesicular transport are illustrated in Figure 2.3. When the vesicle fuses with another membrane system, the entrapped compounds are released. Endocytosis refers to vesicular transport into the cell, and exocytosis to transport out of the cell. Endocytosis has a number of mechanisms which enable the cell membrane to uptake macromolecules including pinocytosis, phagocytosis and receptor mediated endocytosis (Shargel & Yu, 1999:107; Washington *et al*., 2001:15).

During pinocytosis (fluid-phase endocytosis), a small cavity forms on the membrane surface and a vesicle develops around extracellular fluid containing dispersed molecules which is taken into the cell (Shargel & Yu, 1999:107; Smith., 2005:168). Phagocytosis is when the vesicle forms around particulate matter such as whole bacterial cells or metals and dyes from a tattoo. Receptor-mediated endocytosis is the internalisation of membrane-bound receptors into vesicles in response to the binding of a specific macromolecule to a surface receptor on the cell membrane (Washington *et al*., 2001:16). Epidermal growth factor, transferrin and vitamin B_{12}-intrinsic protein complex enter cells by means of receptor-mediated endocytosis (Daugherty & Mrsny, 1999a:149).

An additional receptor-mediated edocytotic pathway that should be mentioned due to its potential use for the mucosal transport of peptide and protein drugs is transcytosis (Baker *et al*., 1991:371; Hamman *et al*., 2005:101). During transcytosis some endosomes can avoid fusion with lysosomes thus preventing degradation of the enclosed material, which is then released in the extracellular space on the basolateral side. Transcytosis is characteristic of polarized cells such as intestinal epithelial cells.

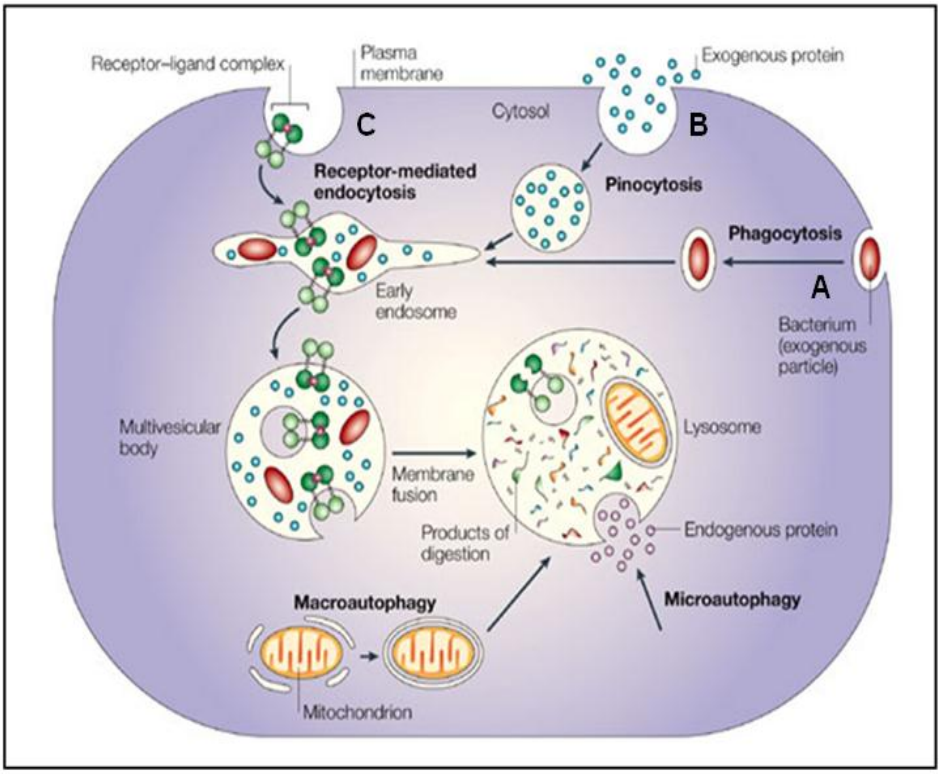


Figure 2.3: Schematic illustration of different mechanisms of vesicular transport: A) Phagocytosis and other processes involved in digestion such as autophagy (the engulfment of intracellular proteins (microautophagy) and organelles (macroautophagy), B) Pinocytosis, C) Receptor-mediated endocytosis (reproduced from Ciechanover, 2005: 79-87)

2.2.2 Paracellular pathway

In general, hydrophilic molecules that are not recognised by a carrier cannot partition into the hydrophobic membrane and therefore have to traverse the epithelial barrier via the paracellular pathway. The paracellular pathway constitutes movement of molecules through the aqueous intercellular spaces between the cells which are joined together by tight junctions (zonula occludens) at their apical sides (Daugherty & Mrsny, 1999a:147; Ward *et al*., 2000:346). These intracellular spaces occupy only about 0.01% of the total surface area of the epithelium and become less significant as you move down the length of the gastrointestinal tract. The controlled and reversible opening of the tight junction represents an attractive approach to increase the absorption of hydrophilic drugs like peptide and protein drugs, especially in view of the fact that degradation by intracellular enzymes is circumvented (Pauletti *et al*., 1996:5-6; Ward *et al*., 2000:347; Salama *et al*., 2006:15-17).

2.3 Barriers to oral delivery of protein and peptide drugs

Oral bioavailability requires absorption of drugs across the intestinal epithelium. The gastrointestinal tract is primarily designed for the digestion and selective absorption of essential nutrients, vitamins, cofactors and electrolytes, but simultaneously has to impede systemic entry of pathogens, toxins and undigested macromolecules (Daugherty *et al.,* 1999:144). This is accomplished through physical and biochemical barriers which also affect drug absorption (Hamman *et al.,* 2005:166). These barriers also hamper the intact absorption of proteins. Obstacle to protein and peptide absorption include the hostile acidic environment of the stomach, the metabolism by luminal digestive enzymes or by luminal microorganisms, the poor intrinsic permeability of peptides and proteins across the intestinal mucosa as well as biological membranes due to their hydrophilic nature and large molecular size, rapid post-absorptive clearance, tendencies to aggregate, non-specific adsorption to a variety of physical and biological surfaces (Fix *et al.,*1996:1282) and first-pass metabolism (Daugherty *et al.,*1999:144).

2.3.1 Physical barrier

Absorption of peptide and protein drugs are severely hampered by physical barriers as represented by the mucus layer, the epithelial cell membranes and the tight junctions between the apical ends of adjacent epithelial cells (Verhoef *et al*., 1990:85). The physical barrier of the epithelium can be attributed primarily to the cell lining itself, which is a tightly bound collection of cells with minimal leakage, including the cell membrane, tight junctions and mucus layer which restrict peptide flux to paracellular and transcellular pathways.

2.3.1.1 Unstirred water layer

The epithelial cells of the entire intestine are covered by a stagnant aqueous boundary layer consisting of mucus and glycocalyx (Hamman *et al*. 2005:167). The mucus layer consists of water, glycoproteins (mucins), enzymes as well as nucleic acids and is bound to the apical cell surface by the glycocalyx (Verhoef *et al*., 1990:85). These layers are separated from the mixing forces created by luminal flow (peristalsis) which results in an unstirred water layer, illustrated in Figure 2.4. This unstirred layer provides the first line of defence against physical and chemical injury caused by ingested food, microbes and the microbial products (Norris *et al*., 1998:136; Turner, 2009:800; Kim & Ho, 2010:319-320).

The unstirred layer of the small intestine slows nutrient absorption by reducing the rate at which nutrient molecules reach the transporting protein-rich microvillus brush border, but may also contribute to absorption by limiting the extent to which small nutrients are lost by the activities of brush border digestive enzymes (Turner, 2009:800). In addition, due to the presence of sialic acid residues and sulphate groups, the mucins of the mucus layer is negatively charged which can result in electrostatic binding or repulsion of the charged peptide or protein molecules (Verhoef *et al*., 1990:85; Aoki *et al*., 2004:99). Research by Fagerholm and Lennernäs (1995:247-252) demonstrated that the unstirred water layer is negligible compared to the intestinal cell membrane as the rate-limiting barrier to the intestinal uptake of highly permeable solutes like glucose and antipyrine. However, the absorption of fluorescein isothiocyanate labled dextran (FD-4), a poorly absorbed hydrophilic compound, was markedly improved when the drug was co-administered with certain penetration enhancers and *N*-acetylcysteine (a mucolytic drug). The results of this study confirmed the findings of Schipper *et al*. (1999:335) and support the hypothesis that the unstirred water layer might be a significant barrier to the intestinal absorption of hydrophilic compounds like peptide and protein drugs.

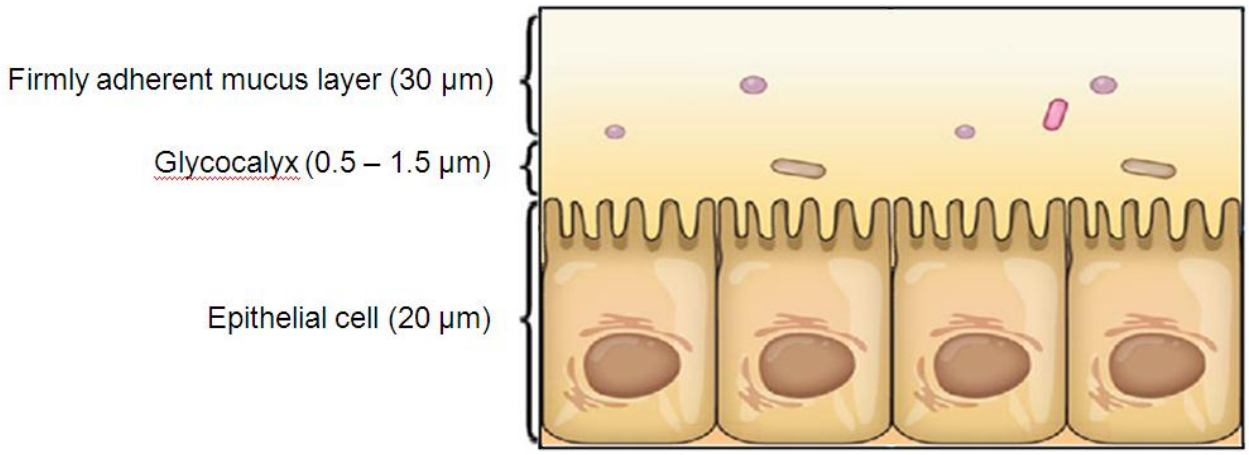


Figure 2.4: Diagram illustrating the mucus layer, glycocalyx and mucosal barrier (adapted from Flint, 2012:602)

2.3.1.2 Membranes of the intestinal epithelial cells

The human intestinal mucosa is composed of a simple layer of columnar epithelial cells, which include a mixture of enterocytes, goblet cells, endocrine cells and Paneth cells, as well as the underlying lamina propria and muscular mucosa (Turner, 2009:800). The cells are tightly bound to one another by the tight junctions (zona occludens), a component of the apical junctional

complex which limits passage of molecules through the intercellular spaces between cells (paracellular transport). Furthermore, the epithelium folds to form villi and also possess microvilli (the brush border), which are uniform 1 µm finger-like projections on the apical surface of the epithelial cells. These structures increase the absorptive area of the intestinal tract by approximately two orders of magnitude (Carino *et al*., 1999:251).

The phospholipid bilayer structure of the plasma membrane renders it semi-permeable allowing lipid-soluble molecules to cross by way of passive diffusion; however, the passage of charged and large molecules is prevented. Therefore, drugs need appropriate physicochemical properties in terms of size, charge, lipohilicity, hydrogen bonding potential and solution conformation to cross the lipophillic barriers of the apical and basolateral membranes. In general, the large size and hydrophilic characteristics of proteins and peptides prevent them from partitioning into the cell membrane and if they are not recognised by an active transport carrier system their absorption is limited to diffusion through the intercellular spaces which is prevented by the tight junctions (Hamman *et al*., 2005:167; Salama *et al*., 2006:16)

2.3.1.3 Tight junctions

Located between adjacent intestinal epithelial cells are intercellular junctional complexes consisting of three parts including the tight junctions (zonula occludens), underlying adherens junctions (zonula adherens) and the most basally spot desmosomes (or macula adherens), illustrated in Figure 2.5. As implied by the name, the adherens junctions along with desmosomes provide the strong adhesive bonds that maintain cellular proximity and are also a site of intercellular communication. Loss of adherens junctions results in disruption of cell–cell and cell–matrix contacts, ineffective epithelial cell polarisation and differentiation as well as premature apoptosis (Turner, 2009:801). The tight junctions are multi-protein complexes composed of transmembrane proteins, peripheral membrane (scaffolding) proteins and regulatory molecules that include kinases which play an important role in modulating many of the signaling pathways by phosphorylation of the tight junction proteins or the displacement of the perijunctional actin-myosin ring (Ward *et al.*, 2000:350-351). The most important of the transmembrane proteins are members of the claudin family, which define several aspects of tight junction permeability. Peripheral membrane proteins such as zonula occludens 1 (ZO-1) and ZO-2 are crucial to tight junction assembly and maintenance, partly owing to the fact that these proteins have multiple domains for interaction with other proteins including claudins, occludin and actin (Figure 2.5) (Turner, 2009:801).

The specific barrier properties of the tight junction can be defined in terms of size selectivity and charge selectivity. At least two routes allow transport across the tight junction. One route, the leak pathway, allows paracellular transport of large solutes including limited flux of proteins and bacterial lipopolysaccharides. The size at which particles are excluded from the leak pathway has not been precisely defined although it is clear that particles as large as whole bacteria cannot pass. A second pathway is characterised by small pores that are thought to be a high-capacity, size and charge-selective paracellular route that appear to be defined by the tight junction-associated claudin proteins expressed (Shen *et al*., 2010:285). These pores have a radius that excludes molecules larger than 4 Å. Thus, tight junctions show both size selectivity and charge selectivity and these properties may be regulated individually or jointly by physiological or pathophysiological stimuli.

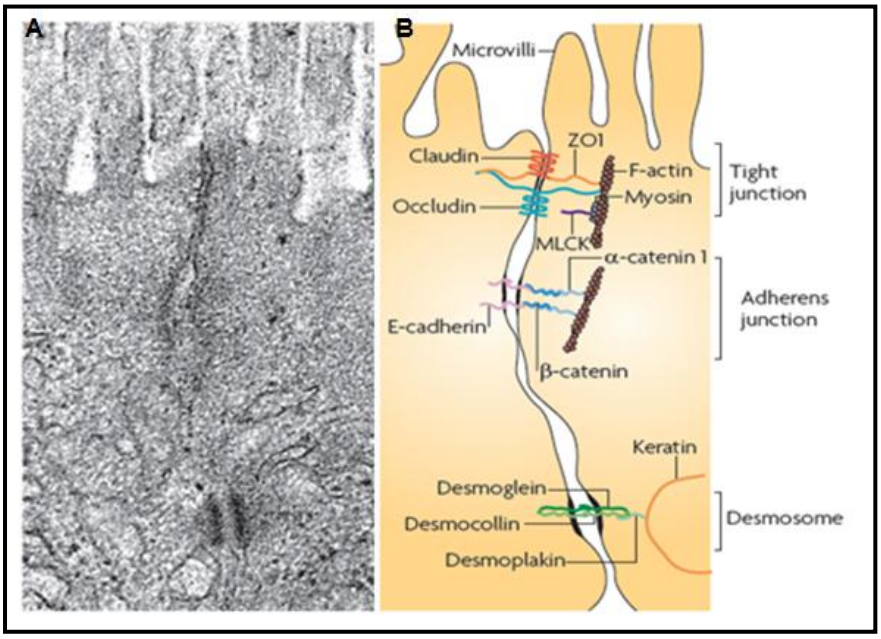


Figure 2.5: (A) An electron micrograph and (B) corresponding line drawing of the junctional complex of an intestinal epithelial cell (reproduced from Turner, 2009:800)

2.3.1.4 Efflux systems

Drugs that cross the apical membrane may be substrates for apical efflux transporters, which shuttle compounds back into the lumen and in combination with intracellular metabolism, may contribute significantly to low bioavailability of certain drugs such as proteins. These apical efflux transporters are principally adenosine triphosphate-binding cassette (ABC) proteins such as P-gp, multidrug resistance (MDR) and multidrug resistance-associated protein (MRP) (Chan

et al., 2004:26). The polarised expression of these efflux systems suggests that their physiological role is to restrict transcellular flux of some molecules, for instance, limiting the absorption of potentially harmful foreign substances (Pauletti *et al.*1996:6-7; Chan *et al.*, 2004:26).

2.3.2 Biochemical barriers

In addition to the physical barrier, degradation of peptides and proteins in the gastrointestinal track may be due to instability in an acidic environment, metabolism by digestive enzymes and luminal microorganisms (Hamman *et al.,* 2005:168). The digestion of proteins and peptides begins in the stomach and is completed in the intestine. Proteins and peptide, stability, conformation, integrity and solubility are dependent on the constituent amino acids state of ionisation, rendering them susceptible to the strong acidic environment of the stomach (Mahato *et al*., 2003:167). This inactivates the proteins and partially unfolds them such that they are better substrates for proteases (Banga & Chien, 1988:19; Smith., 2005:688-689).

The enzymatic barrier is considered to be one of the most important barriers to oral peptide delivery due to specific features and characteristics, for example, proteolytic enzymes are ubiquitous with wide substrate specificity (Pauletti *et al*., 1996:4; Hamman *et al*., 2005:168). Therefore, peptides and proteins are susceptible to degradation at more than one anatomical site as well as at more than one linkage within the peptide backbone. Proteolysis starts in the stomach with pepsin, and is continued throughout the gastrointestinal tract by various enzymes located in the intestinal lumen, the brush border membrane, the cytoplasm and the lysosomes (Zhou & Li Wan Po, 1991b:119; Langguth *et al*., 1997:40-41; Carino & Mathiowitz, 1999:251). The proteolytic enzymes produced by the pancreas (serine endopeptidases trypsin, α-chymotrypsin, elatase, and the exopeptidases carboxypeptidases A and B) act in the lumen of the small intestine. Also, the enzymes associated with the enterocytes such as those in the brush border membrane, the cytoplasm and the lysosomes also contribute to the pre-systemic degradation of peptides into their constituent amino acids either by hydrolysis of peptide bonds or by chemical modification such as oxidation, phosphorylation, or deamidation (Zhou & Li Wan Po, 1991b:117-130; Langguth *et al*., 1997:40-41; Mahato *et al*., 2003:167). Furthermore, a substantial amount of proteolytic activity is present within the colon, largely because of enzymatic secretions by microorganisms capable of reactions such as deglucuronidation, decarboxylation, reduction of double bonds, amide hydrolysis and dehydroxylation reactions (Mahato *et* al., 2003:167 and Hamman *et al*., 2005:168).

2.3.3 Protein drug stability

To successfully develop and administer peptide and protein therapeutics, an intimate understanding of their physicochemical as well as biological characteristics (chemical and physical stability) is needed. Examples of peptides and proteins known to be unstable along with some mechanisms of breakdown are listed in Table 2.1. During processing, formulation and presence in tissue fluid, the protein is exposed to conditions that could have significant effects on its chemical and physical stability, which may lead to aggregation and ultimately to precipitation (Frokjaer & Otzen, 2005:301). It is the primary structure which determines protein behaviour, however, many external factors take part in controlling/affecting protein aggregation, including temperature, pH, ionic strength, surface adsorption, shearing, shaking, presence of metal ions, organic solvents and additives, protein concentration, purity and morphism, pressure, freezing and drying. There are many analytical techniques available to monitor protein aggregation, although differences in accuracy, sensitivity, and operation difficulty require careful consideration (Wang, 2004:22).

Table 2.1: Instability of protein and peptide drugs with some mechanisms of breakdown (Zhou & Li Wan Po, 1991a:100)

Effect factor	Protein and peptide drugs
Physical instability	
Aggregation	Interferon-γ
	Bovine growth hormone
Precipitation	Insulin
Chemical instability	
β Elimination	Lysozyme
	Phosvitin
Deamidasion	Bovine growth hormone
	Human growth hormone
	Insulin
	r-Immunoglobulin
	Epidermal growth factor
	Prolactin
	Gastric releasing peptide
	ACTH*
Disulphide exchange	Lysozyme
	Ribonuclease A
Racemization	ACTH*
Oxidation	Corticotropin
	α-, β-Melanotropins
	Parathyroid hormone
	Gastrin
	Calcitonin
	Corticotropin releasing factor

* Adrenocorticotropic hormone

2.4 Strategies for effective oral delivery of peptide drugs

It is clear from the previous section that the oral route is not feasible for the delivery of intact peptide and protein therapeutics unless the multiple barriers impeding absorption are circumvented. There have been numerous pharmaceutical strategies that have been proposed

to maximise peptide drug bioavailability, which include chemical modification, special drug delivery systems, targeted delivery and co-administration of enzyme inhibitors as well as absorption enhancers. These strategies or approaches will be discussed briefly in the following sections.

2.4.1 Chemical modification

Structural manipulation or chemical modification of pharmacologically active peptides and proteins provides several opportunities to improve both their pharmacokinetic profile and pharmacodynamic properties (Hamman *et al*., 2005:168). Different chemical modifications have been employed in order to render derivates that are structurally recognised by transporters or transported via receptor-mediated endocytosis to minimise immunogenicity, increase enzymatic stability and/or membrane penetration (Mahato *et al*., 2003:173; Hamman *et al*., 2005:168).

2.4.1.1 Prodrug approaches

A prodrug can be defined as a pharmacologically inactive chemical derivate of a parent drug molecule that requires enzymatic or non-enzymatic transformation within the body in order to become active (Pauletti *et al*., 1996:10). The aim of the prodrug approach is to overcome the limitations of the parent drug such as poor solubility, stability, permeability, and/or short biological half-life (Hamman *et al*., 2005:168). Protein modification can be achieved either by direct modification of exposed amino acid side-chains or through the modification of the carbohydrate moiety of glycoproteins and glycoenzymes. Examples of peptide prodrugs include *N*-hydroxymethyl derivatives of *N*-acetyl-L-phenylalanine amides, which were found to protect the C-terminal amide bond against cleavage by α-chymotrypsin. These derivatives are readily bioreversible, undergoing spontaneous hydrolysis at physiological pH, converting them to the parent molecule (Kahns *et al*., 1993:809; Hamman *et al*., 2005:168). Additionally, prodrugs of the naturally occurring methionine and leucine encephalin (morphinomimetic pentapeptides), have also been successfully synthesised (Roemer *et al*., 1977:268). Low oral bioavailability of these peptides has been attributed to their susceptibility to peptidase (Mahato *et al*., 2003:173). At present, prodrug strategies have been employed successfully for small organic-based drugs and some short-chain peptides, however, its application to peptides and proteins in general might be limited due to their structural complexity and the lack of novel methodology (Hamman *et al*., 2005:169)

2.4.1.2 Structural modification

The unique structural features of peptides such as the primary amino acid sequence, secondary structures stabilised by hydrogen bonds (alpha helix and the beta-sheets) and overall three-dimensional conformation determines their affinity/specificity for receptors. However, this structural complexity is also responsible for undesirable physiochemical properties that hamper intestinal absorption. Therefore, although structural modification is potentially useful to optimise the biopharmaceutical and the pharmacokinetic properties, it should not adversely affect the pharmacological properties of the drug (Hamman *et al*., 2005:169).

A widely used structural modification is the linking of one or more polyethylene glycol (PEG) chain(s) to a protein, peptide or non-peptide molecule, which is termed PEGylation and has been shown to improve both their pharmacologic and biologic properties. The main advantages of PEGylated proteins include increased size to reduce kidney filtration, increase in solubility, decreased accessibility for proteolytic enzymes and antibodies as well as the fact that it has been FDA (Food and Drug Administration) approved for human intravenous, oral, and dermal applications. Therefore PEG conjugation is an attractive strategy for delivery of therapeutic peptides (Veronese & Pasut, 2005:1452).

A PEG-conjugated insulin, known as hexyl-insulin monoconjugate-2 (HIM2; NOBEX Corporation, North Carolina, USA), is one of the first successful orally delivered insulin forms to show acceptable bioavailability (± 5%) and acceptable glucose-lowering effects. For HIM2, a short-chain PEG is linked to an alkyl group to form an amphiphilic oligomer, which is in turn attached to the free amino acid group on lysine at position 29 in the β chain of recombinant human insulin via an amide bond (see Figure 2.6). The altered physicochemical characteristics of this modified form increases solubility, resist enzymatic degradation, and facilitate absorption. HIM2 was administered to type 1 and type 2 diabetic patients in two subsequent doses. The orally administered HIM2, showed potential to maintain steady glucose levels in these patients. However, low bioavailability (estimated 5%) continued to be a problem (Hamman *et al*., 2005:169; Morishita & Peppas 2006:907).

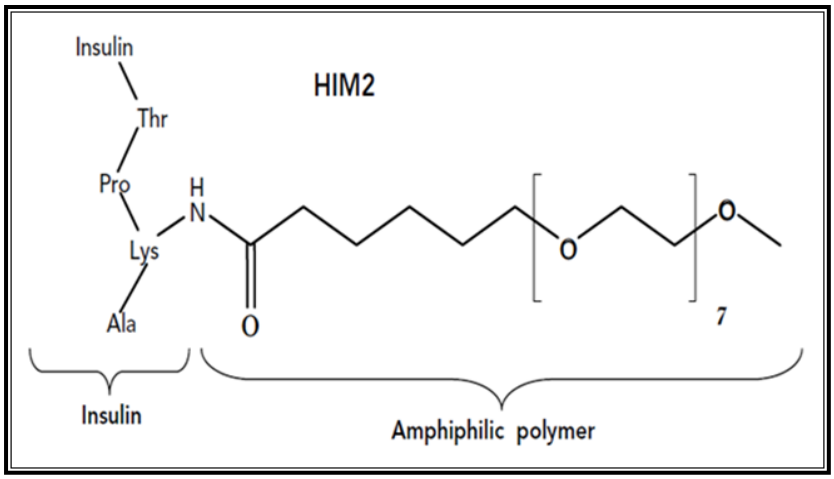


Figure 2.6: Chemical structure of hexyl insulin monoconjugate 2 (HIM2) (Kipnes *et al*., 2003)

Another approach employed is lipidisation or fatty acid-polypeptide conjugation, a chemical modification of the polypeptide molecule that increases its lipophilicity. This method of fatty acid conjugation can be carried out in aqueous solutions and allows regeneration of the original active polypeptide in tissues and the blood and is known as 'reversible aqueous lipidisation' (REAL). Several therapeutic peptide drugs including desmopressin and calcitonin have been lipidized using this technology. In general, REAL-modified peptides exhibited an increase in epithelial absorption, gastrointestinal stability, and plasma half-life (Hamman *et al*., 2005:169).

2.4.1.3 Peptidomimetics

A peptidomimetic is a molecule that mimics the biologic activity of a peptide, but is not completely peptidic in nature, such as pseudo-peptides, semi-peptides, and peptoids. The design of peptidomimetics includes strategies ranging from replacement of only one atom to the design of more complex mimetics, which show secondary structural elements of peptides (Pauletti *et al*., 1996:11). The development of small peptidomimetic molecules with selective actions at peptidergic G-protein-coupled receptors is growing quickly. Non-peptidic drugs targeting angiotensin receptors (e.g. losartan), dothelin receptors (e.g. bosentan), and neurokinin NK1 receptors (e.g. aprepitant) have already reached the marketplace, and other candidates have been identified which is already involved in preclinical testing (Hamman *et al*., 2005:170).

2.4.2 Targeting membrane transporters, receptors and tissues

2.4.2.1 Peptide transporters

As mentioned in section 2.2.1.2, there are specific intestinal peptide transporters with broad substrate specificity which facilitate the uptake of natural di- and tripeptides from the gastrointestinal lumen to the blood circulation as well as a variety of orally active peptide drugs that share structural features with physiological substrates (Yang *et al*., 1999:1332). As a result, this carrier-mediated pathway has received much attention as a potential transport mechanism for the delivery of small peptide-derived drugs. Two oligopeptide transporters, designated peptide transporter 1 (PepT1) and peptide transporter 2 (PepT2) were the first mammalian nutrient transporters to be identified, and utilises an electrochemical proton gradient, maintained by an active sodium/proton exchange pump, as their driving force (Pauletti *et* al., 1997:247 and Hamman *et al*., 2005:170). Some peptidomimetics recognised by these peptide transporters include, β-lactam antibiotics, ACE inhibitors, renin inhibitors and ubenimex (bestatin) (Pauletti *et* al., 1997:247; Rubio-Aliaga & Daniel 2002:434). In order to facilitate transport, the oligopeptide transporters recognise several structural features of di- and tri-peptides, including charge stereochemistry and size. It was suggested that by attaching a drug to an enzymatically stable dipeptide, which is recognised by a peptide transporter, its oral absorption could be increased. PepT1 has been utilised to increase the bioavailability of drugs such as zidovudine and acyclovir through dipeptide prodrug derivitisation (Hamman *et al*., 2005:170). However; this approach seems promising for di- and tri-peptidomimetics as well as for small drug molecules but seems to be of limited value for the delivery of larger peptides or macromolecules (Steffansen *et al*., 2005:245).

2.4.2.2 Receptor-mediated endocytosis

Receptor-mediated endocytosis is a tightly regulated transport pathway by which a select group of macromolecules are capable of moving through the phospholipid bilayer. Examples of compounds that are known to be transported by receptor-mediated endocytosis include epidermal growth factor, low-density lipoprotein, immunoglobulins, transferrin, and cyanocobalamin (vitamin B_{12})-intrinsic protein complex (Wagner *et al*., 1993:115). It has been shown that it is possible to utilise the receptor-mediated endocytosis of vitamin B_{12} by covalently linking a cyanocobalamin molecule to various peptide and protein pharmaceuticals such as luteinising hormone-releasing hormone (LHRH) analogs, α-interferon, erythropoietin, and

granulocyte colony-stimulating factor (Russell-Jones & Walker, 1999:248). Although permeation can be increased by means of this approach, stability of the peptide drug still remains a problem. In an effort to stabilise the protein drug and simultaneously maximise the potential of this delivery system, drug-loaded nanoparticles were coated with cyanocobalamin. A higher level of uptake was observed for these cyanocobalamin modified nanoparticles than uncoated particles in intestinal epithelial cells *in vitro* (Hamman *et al*., 2005:170).

Another route utilised for the site specific delivery of therapeutic agents is the receptor-mediated endocytosis of the non-heme iron-binding glycoprotein, transferrin, which plays an important part in cellular iron uptake. This cellular uptake pathway has been exploited for the site specific delivery of anticancer drugs (e.g. doxorubicin), proteins and therapeutic genes into proliferating malignant cells that overexpress the transferrin receptors. This is achieved chemically by conjugation of transferrin with therapeutic drugs, proteins, or genetically by infusion of therapeutic peptides or proteins into the structure of transferrin. The resulting conjugates significantly improved the cytotoxicity and selectivity of the drugs (Qian *et al*., 2002:562).

2.4.2.3 Gut-associated lymphoid tissues

Gut-associated lymphoid tissues (GALT) are represented by the Peyer's patches, the appendix, and small solitary lymphoid nodules. Peyer's patches are follicles of lymphoid tissue covered by a specialised epithelium containing microfold cells (M-cells). These M-cells play an antigen sampling role through uptake of microparticles via endocytosis and transcytosis (Mahato *et al*., 2003:195; Hamman *et al*., 2005:170). Furthermore, lysosomal degradation is normally associated with endocytotic uptake of microspheres, but because this could interfere with the antigen sampling role of M-cells, Peyer's patches are deficient of lysosomes. Studies using different animal models indicated that M-cells selectively transport antigens and microorganisms complexed with immunoglobulin A (IgA) to the underlying macrophages and lymphoid cells. These favourable characteristics of the GALT stimulated research to target the Peyer's patches for peptide and protein delivery. The Peyer's patches are usually targeted by means of particulate carriers which can be grouped into, lipid particles (e.g. liposomes), and polymeric particles, and will be discussed further in section 2.4.3.4.1 (Yeh *et al*., 1998:130). It was shown by van der Lubben *et al*. (2001:201-207) that chitosan microparticles loaded with albumin were successfully taken up by the Peyer's patches after intragastrical feeding to mice (Hamman *et al*., 2005:170). However, their distribution, number, and size vary considerably with age and species (Mahato *et al*., 2003:195). In addition, continuous absorption of particles by M cells into

Peyer's patches may result in toxicity problems, which could induce an immune response (Khafagy *et al*., 2007:1526).

2.4.3 Formulation technologies

Various formulation approaches have been explored in order to improve bioavailability of orally administered peptide and protein drugs with varying degrees of success. Delivery systems that have been investigated include those designed to protect the drug from the harsh environment of the gastrointestinal tract and/or the inclusion of absorption enhancing excipients which inhibits proteolytic degradation or utilises natural transport pathways. Other approaches include those that focus on their ability to be retained at the site of absorption (e.g. bioadhesive systems), or being actively phagocytosed (Hamman *et al*., 2005:171). A number of these approaches will be discussed in the following sections.

2.4.3.1 Mucoadhesive/bioadhesive systems

Bioadhesion describes the process whereby synthetic and natural macromolecules adhere to mucosal surfaces in the body. In instances where the bond forms between the macromolecule and the mucus layer, the terms mucoadhesion is used synonymously. Another term used in bioadhesion is referred to as cytoadhesion and is a cell-specific bioadhesion which involves highly specific interactions between an adhesive agent and the cell surface (Hamman *et al*., 2005:171; Steenekamp, 2007:30). The original intent of bioadhesive drug delivery systems were to extend the residence time at the site of drug adsorption, intensify contact with the mucosa to increase the drug concentration gradient, ensure immediate absorption without dilution or degradation in the luminal fluids, and localise the drug delivery system at a certain site. Bioadhesive polymers frequently used include polyacrylates, cellulose derivates, and chitosan derivates which adhere to biological surfaces by means of hydrogen bonds and ionic interactions (Hamman *et al*., 2005:172).

The richly glycosylated luminal surface of the gastrointestinal tract presents considerable advantages for the use of formulations with bioadhesive properties (Mahato *et al*., 2003:193). Lectins are carbohydrate binding proteins or glycoproteins that bind specific sugar residues such as those of the glycocalyx (Hamman *et al*., 2005:173). *In vitro,* tomato lectin has been shown to bind both specific and avidly to small intestinal tissue as well as individual glycoproteins isolated from enterocyte brush border membranes (Naisbett & Woodly, 1995:227-236). These properties of tomato lectin *in vitro* suggested that it might have potential for

increasing the intestinal residence time of drugs. However, this effect was less clear *in vivo*. Results indicated that although tomato lectin adhered to the gastrointestinal tissues, the transit time showed little difference when compared to the control. It was suggested that this was due to interactions of tomato lectin with the intestinal mucus, preventing the lectin from reaching the cell surface (Naisbett & Woodley, 1995:227-228)

2.4.3.2 Enzyme inhibitors

The use of protease inhibitors provides a viable means to circumvent the enzymatic barrier so as to increase peptide and protein drug bioavailability. The choice of protease inhibitor will depend on the structure of the particular therapeutic peptide or protein, and the specificity of the enzymes involved in their degradation. Table 2.2 lists some examples of protease inhibitors which have been used to improve the delivery of peptide and protein drugs after oral administration to the gastrointestinal tract.

Table 2.2: Examples of enzymes that cause degradation of protein and peptide drugs and their inhibitors

Peptide/protein studied	Compound	Enzyme affected
insulin	aprotonin	trypsin/chymotrypsin inhibition
	FK-448	
rhG-CSF*	chicken egg white trypsin inhibitor	trypsin inhibition
	soybean trypsin inhibitor	

* Human recombinant granulocyte-colony stimulating factor

Yamamoto *et al*. (1994:1496-1500) evaluated the effects of five protease inhibitors including sodium glycocholate, camostat mesilate, bacitracin, soybean trypsin inhibitor and aprotinin on the intestinal absorption of insulin in rats. Among the co-administered protease inhibitors, soybean trypsin inhibitor (1.5, 10 mg/ml) marginally promoted insulin absorption from the large intestine, whereas aprotinin (10 mg/ml) did it to a moderate degree. A significant hypoglycemic effect was obtained following large intestinal administration of insulin with 20 mM of Na-glycocholate, camostat mesilate and bacitracin, when compared to the controls. In contrast, a slight hypoglycemic effect following small intestinal co-administration of insulin with these protease inhibitors was seen. This may be the result of the number and wide substrate specificity of the enzymes secreted in this area.

Various other *in vivo* studies have also demonstrated significantly improved oral bioavailability of peptide and protein drugs after co-administration with enzyme inhibitors. However, the use of enzyme inhibitors may also affect the normal absorption and/or degradation of other intestinal peptides or proteins (Khafagy *et al*., 2007:1524). Furthermore, the chronic non-site-specific intestinal application of such compounds may cause toxicity or change the metabolic pattern in the gastrointestinal tract, due to reduced digestion of nutritive proteins (Khafagy *et al*., 2007:1524). It has been suggested that these shortcomings and adverse effects can be overcome by using delivery systems that provides simultaneous release of both the drug and inhibitor while maintaining them concentrated at the target area, immobilisation of an enzyme inhibitor on the delivery system, and/or intimate contact of the system with the mucosa (Bernkop-Schnürch, 1998:1-16).

2.4.3.3 Absorption Enhancers

Absorption enhancers are compounds that temporarily disrupt the intestinal barrier without causing significant damage or exerting toxic effects, thus allowing a drug to permeate the epithelial cells and enter the blood and/or lymph circulation (Mahato *et al*., 2003:185; Hamman *et al*., 2005:171). Absorption enhancers can be grouped into several classes such as chelating agents, surfactants, bile salts, fatty acids and non-surfactants (Lee, 1990:215; Mahato *et al*., 2003:185). These absorption enhancers have different mechanisms of action which improves the permeation of therapeutic agents at either the apical cell membrane (transcellular pathway) or the tight junctions between cells (paracellular pathway), or both (Lee, 1990:216). These mechanisms include a change in membrane fluidity, alteration of mucus rheology, leakage of proteins through the membrane, and increasing paracellular transport by opening the tight junctions (Mahato *et al*., 2003:185; Hamman *et al*., 2005:171). A more comprehensive list of the different classes of penetration enhancers as well as examples and proposed mechanism of action are given in Table 2.3.

Table 2.3: Classification of penetration enhancers (Lee *et al*., 1991:92 and Hamman *et al*., 2005:175)

Class with examples	Examples	Mechanism of action
Chelating agents	Ethylene diamine tetra acetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), citric acid and salicylates	Increasing membrane fluidity, decreasing concentration of non-protein thiols, prevention of protein aggregation or self-association
Surfactants	Sodium lauryl sulphate and sodium dioctyl sulfosuccinate	Membrane damage by extracting membrane proteins or lipids, phospholipids acyl chain perturbation
Fatty acids and derivatives	Medium chain glycerides, long chain fatty acid esters	Paracellular: e.g. sodium caprate dilates tight junctions
		Transcellular: epithelial cell damage or disruption of cell membrane
Bile salts and derivatives	Sodium taurocholate, sodium taurodi-hydrofusidate, sodium deoxycholate	Disruption of membrane integrity by phos-pholipid solubilisation and cytolytic effects, reduction of mucus viscosity
Complexation	Cyclodextrins	Increase aqueous solubility and dissolution rate
Ion paring	Counterion	Ionised drug and counterion form a more lipophilic ion pair that can partition into the membrane.
Toxins and venom extracts:	Zona occludens toxin.	Interaction with the zonulin surface receptor induces tight junction opening
	Melittin (bee venom extract)	α-helix ion channel formation, bilayer micellization and fusion.
Efflux pump inhibitors	Verapamil	Blocking drug binding site on P-gp, interfere with ATP hydrolysis and altering cell membrane integrity
	Biricodar (VX-710)	
	LY335979	
Anionic polymers	Poly(acrylic acid) derivatives	Combination of enzyme inhibition and extracellular calcium depletion (tight junction opening).
Cationic polymers	Chitosan salts, *N*-trimethyl chitosan chloride	Combination of mucoadhesion and ionic interactions with the cell membrane (tight junction opening)

Several studies have established that absorption enhancers are required in oral formulations of peptide and protein drugs, otherwise little, if any of the drug would be absorbed across the epithelial surface (Muranishi & Yamamoto, 1994:66-100).

In a study by Bernkop-Schnürch *et al*. (2003:95-103), the paracellular pathway was targeted using thiolated polymers (thiomers) such as poly(acrylic acid)-cysteine and chitosan-4-thio-butylamine in combination with reduced glutathione (GSH) as an enhancer, in order to improve the oral absorption of hydrophilic macromolecules such as heparin, insulin and calcitonin from the gastrointestinal tract. Using poly(acrylic acid)-cysteine combined with GSH the oral bioavailability of heparin was 19.9 ± 9.3% and the pharmacological efficiency of orally administered insulin was approximately 7% when compared to subcutaneous injections. Orally administered calcitonin with chitosan-4-thio-butylamine combined with GSH reduced plasma calcium levels by 1.3% when compared to intravenous injection.

However, certain critical issues need to be addressed before a compound can be considered as an acceptable absorption enhancer, such as the degree of bioavailability enhancement, possible long term toxic effects (local inflammation), and knowledge of the precise mechanism of permeation enhancement (Aungst, 2000:429-432; Kalra *et al*., 2010:2). Unfortunately, most proposed absorption enhancers lack sufficient clinical data in humans. These issues need to be addressed.

2.4.3.3.1 Chitosan and *N*-trimethyl chitosan chloride as absorption enhancers

The controlled and reversible opening of the tight junction represents a potential approach to increase the absorption of hydrophilic drugs via the paracellular route across the intestinal epithelium. This can be achieved by using paracellular absorption enhancers as a practical basis for developing novel dosage forms for poorly absorbable drugs such as peptides, peptide analogues or other hydrophilic macromolecules via the oral route (Ward *et al*., 2000:347; Cano-Cebrián *et al*., 2005:20). Chitosan and its derivatives (e.g. *N*-trimethyl chitosan chloride or TMC) are examples of paracellular acting absorption enhancers, which open tight junctions and show acceptable toxicity profiles. Furthermore, as TMC was chosen as absorption enhancer for this study, chitosan and TMC will be discussed in more detail in the following sections.

Chitosan is a linear cationic polysaccharide derived from full or partial alkaline *N*-deacetylation of chitin (Figure 2.7), an abundant naturally occurring polysaccharide found in the exoskeleton and the internal structure of invertebrates (Dutta *et al*., 2004:20). Chitosans comprise copolymers of randomly distributed glucosamine and *N*-acetylglucosamine residues linked entirely in the β-1,4-configuration, ensuing a rigid and unbranched structure (Dutta *et al*., 2004:20). Chitosan (pKa 5.5 – 6.5) is a weak base and is soluble under acidic conditions due to

the protonation of the amino groups of D-glucosamine monomeric units resulting in the positively charged water-soluble form. Chitosan's properties (e.g. solubility, ionisability, reactivity) and functionality (e.g. bioadhesive, paracellular transport enhancer) rely on two fundamental parameters namely the degree of deacetylation and degree of polymerisation (i.e. molecular weight) (Rege *et al.,* 1998:50).

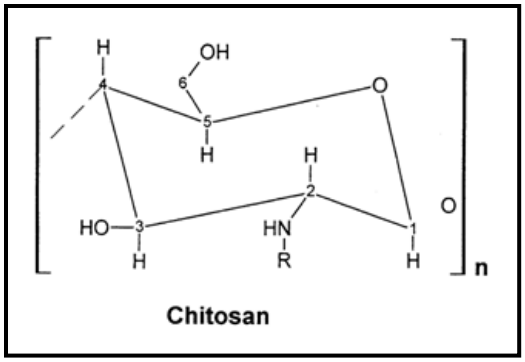


Figure 2.7: Chemical structure of chitosan $(C_6H_{11}O_4N)_n$

It was Illum *et al*. (1994:1186-1189) who first reported that chitosan is able to promote the transmucosal absorption of small polar molecules as well as peptide and protein drugs such as calcitonin, insulin and buserelin across nasal and intestinal epithelia (Illum *et* al., 1994:1186-1189; Luessen *et al*., 1996)**.** Subsequently, Arthursson *et al*. (1994:1358-1361) showed that chitosan can increase the paracellular permeability of [^{14}C] mannitol (a marker for the parcellular route) across Caco-2 intestinal epithelia. Furthermore, using human intestinal Caco-2 cell monolayers and ^{14}C-mannitol as model drug, Schipper *et al*. (1997:923-929) demonstrated that the absorption-enhancing as well as the dose-dependent cytotoxic effects of the chitosans are independently determined by the structural features of the polymer, i.e. the degree of deacetylation and size of the polymer chains.

In the studies mentioned thus far, absorption enhancement was found only at pH values lower than or closes to the pKa value of chitosan (5.5 – 6.5). The pH dependency of chitosan's effect on epithelial permeability was further demonstrated by Kotzé *et al*. (1997:243-253) who concluded that the chitosan salts (i.e. chitosan hydrochloride and chitosan glutamate) at pH-values of 4.4 and 6.2 are potent absorption enhancers as these chitosan salts were able to increase the transport of the peptide drugs buserelin, 9-desglycinamide, 8-arginine vasopressin (DGAVP) and insulin across Caco-2 cell monolayers. The increases in peptide transport were in agreement with the decreases in TEER of the Caco-2 cell monolayers. Measurement of TEER

is considered to be an accepted indication of the tightness of the tight junctions (Kotzé *et al*., 1997:250). However, at pH 7.4 both chitosan salts failed to increase the permeability due to solubility problems. This is due to their charge loss in neutral and basic environments, which caused chitosan to precipitate from solution rendering it unsuitable as an absorption enhancer (Mourya & Inamdar, 2009:1059)

Schipper *et al*. (1997:923-929) concluded that the absorption enhancing effects of chitosan is mediated by electrostatic interaction between the positively charged amino groups on the C-2 position of the chitosan molecule with the negatively charged sites on the cell surfaces at acidic pH values. This results in F-actin depolymerisation and disbandment of the tight junction protein ZO-1 (Schipper *et al*. 1997:923-929; Kotzé *et al*. 1998b:35-46). Therefore, the positive charge of chitosan is able to interact with anionic components of the glycoproteins such as sialic acid. The binding of the chitosan to the membrane resulted in enhanced permeability of the apical membrane and tight junctions. Given that no increase in basolateral membrane permeability was observed, it was concluded that the enhancement of absorption occurred through the paracellular pathway, a finding corroborated by the demonstration of paracellular transport of marker molecules of different molecular weights during confocal laser scanning microscopy (CLSM) and earlier studies using transmission electron microscopy (Schipper *et al*., 1997:928).

As mentioned before, chitosan and chitosan salts lack solubility at basic and neutral pH values. Chitosan aggregates in solutions at pH values above 6.5 and only protonated chitosan (i.e. in its uncoiled configuration) can trigger the opening of tight junctions, thereby facilitating the paracellular transport of hydrophilic compounds. This property implies that chitosan can only be effective as an absorption enhancer in specific areas of the gastrointestinal track where the pH values are close to or lower than its pKa-value. To overcome this problem, the chitosan derivative TMC (see Figure 2.8) has been synthesised (Thanou *et al*., 2001:91-101). This is achieved by reductive methylation of chitosan's amino groups with methyl iodine at elevated temperatures in a strong alkaline environment, which binds the acid being generated during the reaction and avoids protonation of the unreacted primary amino groups. The degree of quaternization (DQ) can be altered by increasing the number of reaction steps, by increasing the reaction time, by controlling the reaction steps or by using different deacetylated grades of chitosan. At higher DQ, evidence of O-methylation on the 3 and 6 hydroxyl groups of chitosan is found. In general, O-methylation leads to less soluble products. It is thus desirable to produce TMC polymers with a high DQ but with a low degree of O-methylation (Mourya & Inamdar, 2009:1057-1079).

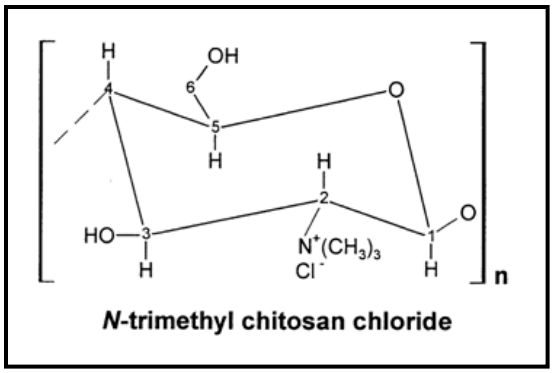


Figure 2.8: Chemical structure of *N*-trimethyl chitosan chloride

Kotzé *et al*. (1999:253-257) proposed that TMC with higher DQ might be more effective as an absorption enhancer for the increased paracellular transport of hydrophilic compounds in neutral environments, after concluding that the charge density and structural features of chitosan salts and TMC play an essential role in their absorption enhancing properties (Kotzé *et al*., 1998b:44). It was found that only TMC with a high DQ (TMC-H) at concentrations as low as 0.05% w/v was able to cause a decrease in the TEER of Caco-2 cells at pH 7.40, compared to TMC with a low DQ of 12.28% and chitosan hydrochloride. At this concentration a 35% reduction in TEER was obtained. Transport studies with [^{14}C]-mannitol gave results in agreement with the TEER results. Increases of between 31 - 48-fold were obtained for TMC-H at a concentration range of 0.05 – 1.5% w/v (Kotzé *et al*., 1999b:253-257). In addition, Hamman *et al*. (2003:161-172), investigated the effect of six different TMC polymers (DQ between 12% and 59%) on the TEER of Caco-2 cell monolayers and on the transport of hydrophilic and macromolecular model compounds ([^{14}C]-mannitol and [^{14}C] PEG 4000). All the TMC polymers were able to decrease the TEER markedly in a slightly acidic medium (pH 6.2), with only a DQ higher than 22% able to reduce TEER in a neutral environment (pH 7.4). The maximum reduction in TEER (47.3 ± 6.0% at a concentration of 0.5% w/v and pH 7.4) was reached with TMC at a DQ of 48%, and this effect did not increase further with a higher DQ. In agreement with the TEER results, the transport of the model compounds increased with an increase in the DQ of TMC reaching a maximum at a DQ of 48% (25.3% of the initial dose for [^{14}C]-mannitol and 15.2% of the initial dose for [^{14}C] PEG 4000), and this effect did not increase further with a higher DQ of TMC. These observations agree with previous results obtained by Jonker *et al*. (2002:205-213), which investigated the influence of the DQ of TMC on the absorption of [^{14}C]-mannitol *in vitro* and *in situ*, using everted intestinal sacs and single pass intestinal perfusion. TMC polymers with a DQ of 22 - 48% were used in the study. Results from this study indicated

that TMC was able to enhance the intestinal permeation of this hydrophilic radioactive marker. The extent of absorption enhancement obtained was dependent on the DQ of the TMC polymers used. In both models the best enhancement was obtained with TMC with a DQ of 48%. From these studies it is clear that TMC is an effective absorption enhancer in neutral and alkaline environments, especially TMC with a high DQ.

Furthermore, Snyman *et al*. (2003:59-67) found that the mucoadhesive properties of TMC decreased with an increase in DQ between 22.1 and 48.8%. This is probably the result of fixed positive charges present and their interaction with the negative sialic groups, which is part of the mucus protein structure. Thus it is clear that TMC, in contrast to the chitosan salts, is a promising absorption enhancer in neutral and basic environments, although a low DQ is not sufficient to produce noticeable absorption enhancement.

2.4.3.4 Particulate carrier systems

To overcome the various biological barriers within the gastrointestinal tract, delivery systems in the form of micro- or nano carrier systems have been investigated. These particulate systems are based on polymeric, solid lipid or liposomal technologies. The type of material and technique used to prepare the particles define the size and surface properties of such a carrier system to alter its bio-distribution (Stolnik & Shakesheff, 2009:4). The aim of using these microparticulate systems for drug delivery include protection of the incorporated constituents from degradation, controlling the release rate and targeting the drug delivery to specific intestinal sites (Mahato *et al*., 2003:180; Hamman *et al*., 2005:173). Also, in some instances these approaches are combined with other strategies such as the inclusion of enzyme inhibitors and/or absorption enhancers.

2.4.3.4.1 Liposomes

Liposomes are self-assembling colloidal particles usually 0.05 - 5.0 µm in diameter, which form when natural and/or synthetic lipids are hydrated resulting in an aqueous volume entrapped by one or more lipid bilayers (Sharma *et al*., 1997:124). Liposomes have been investigated as carriers of various pharmacologically active agents such as antineoplastic and antimicrobial drugs, chelating agents, steroids, vaccines and genetic material. Takeuchi *et al*. (2001:39-54) prepared a mucoadhesive liposomal system by coating a negatively charged liposome suspension (L-α-dipalmitoylphosphatidylcholine and diacetyl phosphate) with a mucoadhesive polymer solution, such as chitosan, poly(vinyl alcohol) or poly(acrylic acid) bearing cholesterols.

A positively charged liposome suspension prepared from stearyl amine was coated with Carbopol® (polymethacrylic acid). The results showed significant absorption of the model protein drugs investigated (insulin and calcitonin).

Although some successes have been achieved, liposome-based drug formulations have not entered the market in great numbers thus far. This is probably due to certain problems associated with their manufacturing, which include stability issues, batch to batch reproducibility, sterilisation methods, low drug entrapment, particle size control and production of large batches. Also, liposomes is fast eliminated from the blood and captured by cells of the reticulo-endothelial system, primarily the liver (Sharma *et al*., 1997:124; Torchilin, 2005:145).

2.4.3.4.2 Polymeric nano- and microparticles

Different polymers have been utilised for the production of micro-and nanoparticles. Polymers generally used include polystyrene, poly(lactic-co-glycolic acid), chitosan, cellulose, and other materials. Orally administered particulate drug delivery systems have several advantages over conventional dosage forms such as protection against enzymatic degradation, improved drug absorption and possible uptake of the particles themselves into the system. However, reproducible absorption in humans should first be proven to ensure their feasibility as an alternative system (Hamman *et al*., 2005:174).

A variety of methods have been developed for microencapsulation thus far, but each method faces challenges for effective encapsulation and delivery of peptide drugs. Each available method has its own advantages as well as limitations, which are listed in Table 2.4. Thus, it is important to understand the strengths and drawbacks of each method in order to apply as well as improve them.

Table 2.4: Several advantages and disadvantages associated with microencapsulation methods used for protein drug delivery (Yeo *et al*., 2001:213-230)

Method	Advantages	Limitations
Emulsification/solvent evaporation	Versatile and straight forward method	Drug encapsulation efficiency tends to be low
	Enables drugs with a wide range of physicochemical properties to be encapsulated	Possible protein denaturation during processing steps (e.g. homogenisation, and sonication)
Phase separation (Coacervation)	High encapsulation efficiency of water soluble drugs (non-solvent addition method)	Microspheres tend to aggregate
	Enables efficient control of particle size and distribution	Solvents hard to remove (non-solvent addition method)
		Limited pH range and the use of cross-linking agents (complex coacervation)
Spray Drying	Time effective (yield results equivalent to conventional methods in short preparation time)	Considerable amounts of material is lost during process
	One-stage closed process (ideal for production of sterile materials)	Possible change in polymorph
		Fluid of high viscosity cannot be sprayed, limiting the amount of polymer and/or drug that can be used
Ionotropic gelation/Polyelectrolyte complexation	System is simple fast and cost-effective	The matrix and membrane formed is incapable of controlling the release rate for a long period of time
	Proteins can be encapsulated without the use of organic solvents or elevated temperature	Potential biocompatibility problems with certain polyelectrolytes
Interfacial polymerization	High encapsulation efficiency	Proteins may participate in polymerisation reaction changing their biological activity
		Multiple washing steps are required and may lead to loss of water soluble drugs.
Supercritical fluid precipitation	Process does not use organic solvents nor produce a water/oil (w/o) interface	RESS* method limited to solutes soluble in supercritical fluids
	Relatively non-toxic, environmentally acceptable, non-flammable and inexpensive	Polymers with low glass transition temperature often agglomerate during SAS[#] method

* Rapid expansion of supercritical solutions, [#] Supercritical antisolvent crystallisation

Furthermore, as the emulsification/solvent evaporation method was selected for the preparation of insulin containing microspheres, it will be discussed in more detail in the following section.

The emulsification/solvent evaporation technique is often selected as preparation method owing to its versatility and straight forward approach. With the selection of a suitable polymer and preparation parameters, drugs with a wide range of physicochemical properties can be successfully encapsulated ranging from small hydrophilic or hydrophobic molecules to proteins and genetic material (Watts *et al*., 1990:255-256; Rosca *et al*., 2004:272; Singh *et al*., 2010:73-74). The properties of the materials and the type of emulsion produced (single or double) during the preparation strongly influences the properties of the microspheres and the resultant release (Yeo *et al*., 2001:213-215). Nonetheless, it is possible to give a basic experimental procedure for this technique. Microencapsulation via emulsification/solvent evaporation is essentially a two-step process: the emulsification of a polymer solution containing the drug (either dissolved or in suspension) into a second, immiscible liquid phase, followed by hardening of the microspheres through organic solvent evaporation at the water/air interface by application of heat, vacuum, or by allowing evaporation at room temperature. During emulsification, the polymer solution is broken up into microdroplets due to the shear stress applied either by a homogeniser, sonification or whirl mixer in the presence of an emulsifying agent. The microspheres can then be separated by centrifugation or filtration and subsequently washed and dried (Watts *et al*., 1990:236; O'Donnell & McGinity 1997:26).

Microsphere preparation using the emulsification/evaporation method involves a sequence of complex interfacial phenomena in which several parameters can affect the morphology and the physicochemical properties of the final product (Benoit *et al*., 1996:43). These parameters include the type of organic solvent or solvent mixture, the solvent/polymer/non-solvent interactions, the nature of the emulsifying agents, pressure and the rate of solvent evaporation.

It is well established that the morphology and the physicochemical properties of microspheres prepared by using the emulsification/evaporation method is governed by a sequence of complex interfacial phenomena. The particle size of the microspheres, for instance, is primarily controlled by the method and rate of agitation, pressure, temperature and the concentration of the polymer in the dispersed phase. Usually, an increase in agitation such as mixing speed, results in a decrease in particle size. Increases in polymer concentration or temperature usually result in an increase in particle size (Watts *et al*., 1990:241; Li *et al*., 2008:31). A more detailed description of parameters and processing conditions affecting microsphere properties is summarised in Table 2.5.

Table 2.5: Parameters and processing conditions affecting microsphere properties (Reproduced from Li *et al*., 2008:31)

	Factors	Effect on microsphere properties		
		Size	**Surface morphology**	**Encapsulation of drug**
Increase of parameters	Viscosity of internal phase	Increased diameter	Smoother surface	Increased efficiency; slower release rate
	Internal/continuous phase ratio	No influence or decreased diameter		Increased efficiency
	Drug concentration in internal phase		More porous and irregular shape	High quantity of drug can decrease efficiency due to large pores
	Concentration of emulsifier	Smaller diameter		
Processing conditions	Increased agitation rate	Smaller diameter and size distribution		
	Increase in temperature	Increased diameter	Coarser surface	Decreased efficiency
	Reduced pressure (relative to atmospheric pressure	No influence or decreased diameter	Smoother surface	slower release rate

2.4.3.5 Carrier mediated and site specific delivery

Protein and peptide absorption is not uniform throughout the gastrointestinal tract owing to differences in the composition and thickness of the mucus layer, pH, surface area and enzyme activity. These properties have been utilised to increase absorption after oral administration by releasing a peptide within a specific region of the gastrointestinal tract where enzyme activity is low or where uptake into the lymph system is high. This should allow for increased efficiency and decreased toxicity (Daugherty & Mrsny, 1999: 144-148; Hamman *et al*., 2005:174).

2.4.3.5.1 Targeting the small intestine and colon

The small intestine is the principle site of absorption of any ingested compound. The ileum is metabolically more active with a lower surface area when compared to the duodenum and is an

important site for oral peptide delivery because it houses highly specialised transport mechanisms (such as the vitamin B_{12} uptake pathway) (Mahato *et al*., 2003:188-190). Particulate carrier systems are the most widely used approach to protect fragile macromolecules against acid-catalysed and enzymatic degradation in the harsh environment of the stomach. This is accomplished by the use of pH-responsive polymers or the inclusion of excipients such as enzyme inhibitors.

The most commonly used polymers employed to protect peptide and protein drugs against the hostile environment of the stomach are the methacrylic acid-methyl methacrylate copolymers. These polymers are commercially available as Eudragit® products (Rubinstein *et al*., 1997:60). It has been shown that more than 90% of the insulin content of a Eudragit® L100 microsphere formulation could be protected from degradation in simulated gastric fluid (Morishita *et al*., 1992:1). In a follow-up study, it was found that the highest hypoglycemic effect in male Wistar rats was seen after the oral administration of Eudragit® L100-insulin containing microspheres. This was in comparison with a Eudragit® S100 and a 1:1 Eudragit® L100: Eudragit® S100 microsphere formulation. The higher hypoglycemic effect of the Eudragit® L100 formulation was attributed to the release of the insulin to the optimum site of absorption. It was concluded that optimum absorption was from the lower intestinal region (lower jejunum and ileum). In another study by Jain *et al*. (2005:E100-E107) it was found that Eudragit® S100 entrapped insulin microspheres resulted in a 24% blood glucose reduction after oral administration to albino rabbits, thus emphasising the need of an enteric coating.

Drug delivery to the colon has several attractive features including a prolonged residence time, reduced enzymatic activity, increased tissue responsiveness to absorption enhancers, and natural absorptive characteristics. For colon specific delivery approaches such as prodrugs, pH-dependent, time-dependent, and microflora-activated systems have been employed, but with limited success (Yang *et al*., 2002:235). For example, Saffram *et al*. (1986:1081) coated insulin as well as vasopressin with styrene and hydroxy-ethylmethacrylate crosslinked with azo-aromatic groups to form an impervious film that protects orally administered peptide drugs from digestion within the stomach and small intestine. In the colon, the natural microflora reduces the azo-bonds, breaking the cross-links, allowing degradation of the polymer film, thereby releasing the drug into the lumen of the colon for absorption. Oral administration to rats of the microparticles resulted in a biological response (i.e. antidiuresis and hypoglycemia), however, the responses occurred at variable times.

2.5 *In vitro* models used for the evaluation of intestinal permeability and absorption

The physicochemical properties of a drug, the physiological functions of the gastrointestinal tract, the biochemical and the physical properties of the epithelial cell layer influence intestinal drug absorption. There is a variety of methods that have been developed to assess drug permeation across the gastrointestinal tract wall. These methods include membrane based models such as a parallel artificial membrane permeation assay (PAMPA), cell culture-based models, and animal tissue based models (Deferme *et al*., 2008:182; Volpe, 2010:672). Each assay has its own advantages and limitations to be considered when developing models for evaluating permeability, which is summarised in Table 2.6.

Table 2.6: Advantages and limitations of different permeability models

Absorption model	Advantages	Limitations
In situ perfusion	Closest to *in vivo* anatomy	Not a screening tool
e.g. Single pass and recirculating methods	Retains blood flow and innervation	Implies anesthesia and surgery
Ex vivo tissue diffusion	Evaluate regional differences	Limited tissue viability
e.g. Ussing chamber and everted gut	Retains gut architecture	Suboptimal stirring conditions
	Directional and mechanistic transport	
In vitro cell monolayers	Reduction in number of laboratory animals	Inter-laboratory variability due to culture conditions
e.g. Caco-2 and Madin-darby canine kidney (MDCK) cells	Good screening model	Lack of mucus layer
	Mechanistic studies	
Artificial membranes	Tolerates wide pH ranges	Dependent upon lipid composition and pH
e.g. Parallel artificial membrane assay (PAMPA)	Relatively simple and high throughput	No active transport

The non-cellular parallel artificial membrane assay is comprised of two aqueous buffer solution chambers separated by a porous artificial lipid membrane barrier that mimics the intestinal epithelium. The artificial lipid membrane is a hydrophobic filter material coated with a mixture of lecithin and phospholipids dissolved in an inert organic solvent such as dodecane (Balimane *et al*., 2000:304; Volpe, 2010:676). Drug transport is assessed by adding the drug substance to a donor chamber and measuring the appearance of the drug in the receiver chamber over time.

The assay is usually performed in a 96-well microtiter, coupled with a spectrophotometric plate reader allowing for high-throughput screening of passive permeability. The flux measured through the membrane is compared to known drug absorption in humans (Volpe, 2010:676).

Various cell lines are grown on a semi-porous filter to form monolayers that morphologically and functionally resemble the intestinal epithelium with barrier properties. Therefore, the cell culture model provides an ideal system for the rapid assessment of the intestinal permeability of drug candidates (Balimane & Chong, 2005:337; Volpe, 2010:676). The Caco-2 cell model (derived from a human colon adenocarcinoma) has been the most extensively characterised cell-based model in examining the permeability and absorption of drugs. (Deferme *et al.*, 2008:193; Balimane & Chong, 2005:337). Caco-2 cells undergo spontaneous enterocytic differentiation to become polarised cells (i.e. having an apical and basolateral surface) with well-established tight junctions, and exhibit many functions of normal enterocytes. However, it is very difficult to compare the absolute permeability coefficient value of individual compounds reported in the literature, particularly with compounds that primarily permeate via the paracellular route. The variability may be attributed to differences in culture conditions and composition of cell subpopulation. Also, these tight junctions are more like those in the colon than the tight junctions in the leakier small intestine (Balimane *et al*., 2000:306; Ashford, 2002:257).

The Ussing chamber model develop by Ussing and Zerahn is frequently used in evaluating ion and water transport. The method involves the isolation of the intestinal tissues, cutting it into tissue sheets of appropriate size, clamping it on a suitable device and then the rate of drug transport across the tissue is measured (Balimane *et al*., 2000:305). This technique was refined for the measurement of excised tissue permeability by Sweetana and Grass. This method is widely used for evaluating the transport of compounds through mucosal/epithelial barriers (e.g. excised gastrointestinal tissue). In addition, active transport, permeability enhancers, enzymatic degradation, and absorption in different tissue sections can be evaluated using this technique (Grass & Sweetana, 1988:372). The method allows for the measurement of drug transport across the tissue as a function of time in the absorptive (apical to basolateral) and secretory (basolateral to apical) directions. This technique is also well suited to study the differences of drug transport in different parts of intestine (Berggren, 2006:18; Volpe, 2010:673).

2.6 Summary

The oral administration of drugs is by far the most widely used route of administration, although it is generally not feasible for peptide and protein drugs. This is due to their unfavourable physicochemical properties and their susceptibility to enzymatic/chemical degradation resulting in poor oral bioavailability (i.e. generally less than 2%). In addition, the gastrointestinal tract possesses considerable barriers that preclude absorption of peptide drugs. Several approaches have been used in order to improve the oral bioavailability of peptide and protein drugs. Some of the more promising approaches include the use of absorption enhancing excipients, chemical modification and specific targeting of transporters, receptors, and absorption sites.

Although different strategies have been employed with regard to the oral delivery of peptide and protein drugs, limited success have been achieved and many drawbacks still exist that need to be addressed. For example, most absorption enhancers show possible long term toxic effects, although natural polymers such as chitosan and its derivatives exhibit potentially safe profiles. Inclusion of enzyme inhibitors may affect the normal absorption and/or degradation of nutritional peptides with increased enzyme release due to positive feedback mechanisms. Chemical modifications showed promising results with the development of PEG-conjugated insulin, namely hexal-insulin-monoconjugate-2 (HIM2), which had acceptable bioavailability (± 5%) and acceptable glucose-lowering effects. Approaches that target peptide transport systems showed promise for di- and tri-peptidomimetics as well as for small drug molecules but seem to be of limited value for the delivery of larger peptides or macromolecules.

Varying degrees of success have been obtained for oral peptide delivery with formulation techniques such as the use of enteric coatings in combination with absorption enhancing excipients. Significant improvement in the bioavailability of various peptide drugs such as calcitonin, insulin and heparin have been obtained after targeting the paracellular pathway. It seems that although significant progress has been made with respect to the oral delivery of peptide and protein drugs, the end goal in terms of sufficient and safe absorption for most peptide and protein drugs is far from being reached.

CHAPTER THREE

Formulation and characterisation of a Eudragit®/TMC microsphere delivery system for insulin

3.1 Introduction

In terms of oral administration, single unit dosage forms (e.g. tablets and capsules) present problems such as erratic gastric emptying or incomplete drug delivery in the gastrointestinal tract. Multiple unit dosage forms (e.g. granules, pellets or microspheres) have been employed to address these drug delivery issues. Microparticles in particular, provide benefits such as rapid emptying from the stomach as well as more reproducible transit through the small intestine and colon due to their small size. The increased surface area of microparticles facilitates rapid drug release and they provide more reproducible absorption and bioavailability (Nilkumhang & Basit, 2009:135).

Microencapsulation techniques are widely used in the development and production of improved drug and food delivery systems. Versatility is provided by the availability of various materials for the production of microparticles, which provides advantages such as effective protection of encapsulated drugs against degradation, increase drug solubility, reduced adverse or toxic effects, site-specific drug delivery and controlled drug release profiles (Singh *et al*., 2010:65). However, even with these significant advantages of microparticles, certain challenges need to be addressed such as the development of cheaper biopolymers and the development of universally acceptable evaluation methods for microspheres.

3.2 Selection of model active pharmaceutical ingredient and polymer

Insulin is a hydrophilic, polypeptide drug with a moderately high molecular weight and is used in the treatment of diabetes mellitus. At present, insulin is mainly administered via the parentaral route, mainly subcutaneously (Wong, 2010:80). The administration of insulin by means of the

oral route has been the goal of many researchers since its discovery; however, the typical bioavailability of orally administered insulin which enters the systemic circulation is reported to be less than 0.5% (Peppas & Kavimandan, 2006:188). Insulin is an example of a polypeptide drug of which oral absorption is effectively hampered by the natural absorption barriers and remains the main therapy for insulin dependent diabetes mellitus (IDDM or Type 1 diabetes). It can therefore be considered as a good model peptide drug for inclusion in an oral dosage form.

As mentioned in chapter 2 (section 2.4.3.5), the most frequently used polymers for development of protein and peptide drug formulations are those which protect the drug against chemical degradation as well as polymers that target specific areas for absorption in the gastrointestinal tract. The methacrylic acid or Eudragit® range of polymers, in particular three grades namely Eudragit® L, S and FS (Degussa, 2005:3.1e) are intended to be used as enteric coatings or for pH-dependent drug delivery. These properties make Eudragit® suitable for use as an excipient in peptide delivery systems. Eudragit® consists of copolymers derived from esters of acrylic and methacrylic acid whose properties are determined by their functional groups. These polymers are pharmacologically inactive and are excreted unchanged. Furthermore, these polymers are compatible with skin as well as mucous membranes (Degussa, 2005:1e). Eudragit® L in particular dissolves at pH values above 6.0, making it ideal for drug release within the ileum and jejunum.

Eudragit® L100 is an anionic copolymer in a 50:50 ratio of methacrylic acid and methyl methacrylate (Degussa, 2005:3.1e). The structure of Eudragit® L100 is shown in Figure 3.1. The ratio of free carboxylic acid groups to ester groups is approximately 1:1 and the average molecular weight of Eudragit® L100 is approximately 135000 g/mole (Degussa, 2005:7.3e).

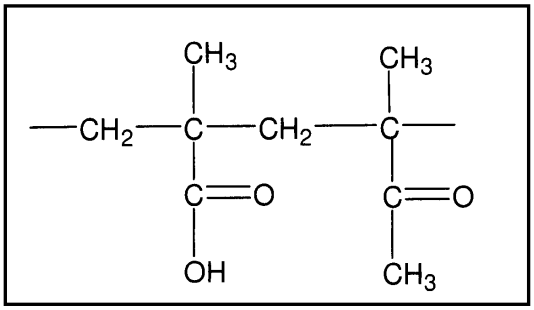


Figure 3.1: Chemical structure of Eudragit® L100 (Degussa, 2005:7.3e)

3.3 Selection of solvents

A key factor for successful application of the emulsification/solvent evaporation technique to produce microspheres is the selection of suitable solvents to constitute the two immiscible liquid phases (i.e. the internal phase and continuous phase). The internal phase should contain the active ingredient (dissolved or suspended) as well as the coating/matrix polymer. The other liquid forms the continuous phase. Liquids to be used for the internal phase should be able to dissolve the polymer and ideally the active ingredient and must be immiscible with, and possess a lower boiling point (more volatile) than the continuous phase. The polymer as well as drug should be insoluble in the continuous phase. The continuous phase should allow easy recovery of the microspheres (e.g. should have a low viscosity). Furthermore, all solvents used in the solvent evaporation technique to produce microparticles should have a low toxicity (Watts *et al*., 1990:237; Li *et al*., 2008:29). Frequently used solvents for the emulsification/solvent evaporation technique include chloroform, dichloromethane and ethyl acetate. However, chloroform has a low vapour pressure and toxic effects, dichloromethane is a confirmed carcinogenic (according to Environmental Protection Agency data), while ethyl acetate is less toxic but produced microspheres with a less acceptable morphology as well as encapsulation efficiency compared to other solvents. As a result of low toxicity and the high solubility of Eudragit® in ethanol, it was selected as the solvent for the internal phase in this study.

3.4 Emulsifying agent

An emulsifying agent is essential for the production of microspheres by the emulsification/solvent evaporation technique. The role of the emulsifier is to reduce the surface tension of the continuous phase, to avoid the coalescence and agglomeration of the polymer droplets and stabilising the emulsion. The stabilisation is only a temporary requirement, because once adequate solvent evaporation has taken place to cause some hardening of the drug-polymer droplets, coalescence and aggregation are usually prevented. The inclusion of a suitable emulsifier contributes to the production of microspheres with a uniform size and narrow size distribution resulting in reproducible drug release. In order to determine the suitability and concentration of the emulsifier, certain factors should be considered such as the polarity of the two immiscible phases, desired size as well as morphology of the microspheres. Frequently employed emulsifiers include non-ionic surfactants (e.g. partially hydrolyzed polyvinyl alcohol (PVA), methylcellulose, polysorbate 80 (Tween), sorbitan esters (Span), anionic surfactants

(e.g. sodium dodecyl sulphate (SDS)) and cationic surfactants (e.g. cetyltrimethyl ammonium bromide (CTAB)) (Watts *et al*., 1990:240; Li *et al*., 2008:30).

In general, the use of non-ionic emulsifiers such as the sorbitan esters and polysorbates result in fewer toxic effects or irritation when compared to their anionic and cationic counterparts. It is for this reason that non-ionic emulsifiers are preferred for preparations intended for oral administration (Billany, 2002:345). Emulsifier concentrations generally employed range from 0.5 - 3% v/v (Watts *et al*., 1990:240; Morishita *et al*., 1991:312; Jain *et al*., 2005:E101-E102).

3.5 Materials and methods

3.5.1 High pressure liquid chromatography

3.5.1.1 Preparation of standard solutions

An accurately weighed mass of approximately 50 mg of insulin raw material was transferred to a 250 ml volumetric flask followed by the addition of 100 ml of a 0.01 mol/l aqueous hydrochloric acid (HCl) solution. The solution was then agitated until the insulin was completely dissolved. Subsequently, the volumetric flask was filled to volume with phosphate buffered saline (PBS) to render a stock solution with a concentration of 200 µg/ml. Two dilutions were prepared from the stock solution by pipetting volumes of 5 and 10 ml to a 500 ml and 100 ml volumetric flask respectively. The volumetric flasks were filled to volume with PBS to render two standard solutions with concentrations of 2 and 20 µg/ml, respectively. From these three insulin solutions with different concentrations, different injection volumes were used during HPLC analysis to render a concentration range as shown in Table 3.1. Calibration curves were constructed by plotting peak area as a function of concentration.

Table 3.1: Different injection volumes used for construction of insulin standard curves

Concentration of standard (µg/ml)	Injected volume (µl)	Injected insulin concentration (µg/ml)
2	20	2
20	5	5
20	10	10
20	15	15
20	20	20
200	5	50
200	10	100
200	15	150
200	20	200

3.5.1.2 High performance liquid chromatography analysis method

Samples as previously described were analysed using an Agilent 1100 series high pressure liquid chromatograph equipped with a gradient pump, autosampler and UV detector. All measurements were evaluated by Chemstation Rev. A.08.03 data acquisition and analysis software (Agilent Technologies, Japan). A Jupiter C_{18}, 250 x 4.6 mm column, 5 µm spherical particles, with a 300 Å pore size, 13.3% carbon load, and endcapped (Phenomenex, Torrance, California, USA) was used. The flow rate was set to 1.0 ml/min and an injection volume of was 50 µl. The gradient elution performed is summarised in Table 3.2. The wave length for UV detection was 210 nm. The retention time for insulin was approximately 5.9 min.

Table 3.2: Gradient elution schedule with a mobile phase that consisted of acetonitrile (A) and 0.1% w/v of orthophosphoric acid (B)

Time (min)	Acetonitrile (%)	0.1% orthophosphoric acid (%)
0.0	20	80
6.0	60	40
8.0	60	40
8.2	20	80
12.0	20	80

3.5.2 Development of a microsphere delivery system

3.5.2.1 Materials

Eudragit® L100 was supplied as a gift by Evonik Industries (Midrand, South Africa). The TMC was synthesised according to the method of Sieval *et al*. (1998:158) from chitosan (Chitoclear™ Primex, Iceland). The synthesised TMC was placed into microcentrifuge tubes, followed by the addition of a carbide tungsten bead (Retsch GmbH & co. KG) where after it was subjected to a MM 400 vibration mill (Retsch GmbH & co. KG, Haan, Germany) for 5 min at 30 Hz to produce a fine powder. The TMC was then sieved (Aperture 45 µm) in order to acquire a powder with uniform particle size, which is suitable for maximum encapsulation in the microspheres from a dispersion. Recombinant human insulin, ethanol (absolute) and Span®80 (Sorbitan mono-oleate) were purchased from Sigma (Kempton Park, South Africa). Liquid paraffin was purchased from Merck (Johannesburg, South Africa). All other reagents including *n*-hexane and Tween® 80 (polyoxyethylenesorbitan mono-oleate) were purchased from (Associated Chemical Enterprises, Johannesburg, South Africa). All chemicals purchased were used as received and of analytical grade.

3.5.2.2 Microsphere preparation

Microspheres were prepared using a single water-in-oil (w/o) emulsification technique followed by solvent evaporation as described in Chapter 2 (section 2.4.3.4.2). The w/o solvent evaporation method used was adapted from the technique described by Morishita *et al*. (1991:309-319).

In this study, a 2^3 fractional factorial design was used to investigate the combined influence of three variables namely concentration of Eudragit® L100, insulin as well as TMC on microsphere morphology and encapsulation efficiency. The formulation variables investigated with the factorial design are summarised in Table 3.3.

Table 3.3: Fractional factorial design used to optimise the microsphere delivery system

		TMC 5% w/w		TMC 10% w/w	
		Eudragit® L100 (% w/w)		Eudragit® L100 (% w/w)	
		7.5%	3.5%	7.5%	3.5%
Insulin (% w/w)	2 %	Formula A	Formula B	Formula E	Formula F
	1%	Formula C	Formula D	Formula G	Formula H

Variables such as stirring rate, solvents, concentration of emulsifiers and drop-rate were kept constant in the experimental design. A schematic representation of the apparatus used to prepare all microsphere formulations is shown in Figure 3.2.

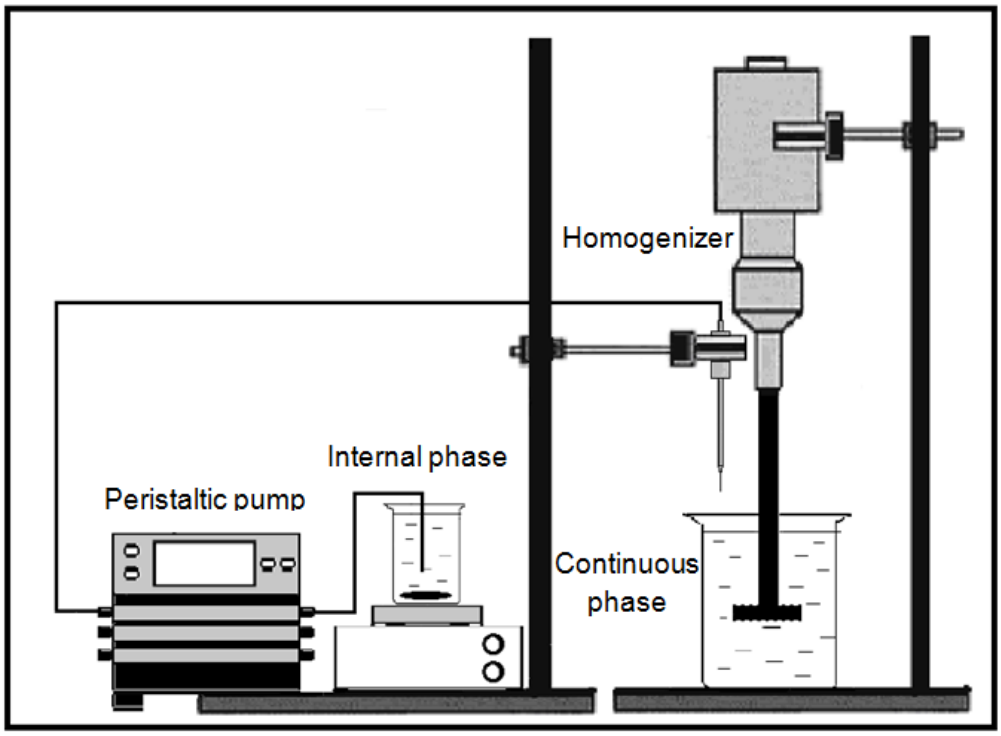


Figure 3.2: Schematic representation of the experimental setup used for the preparation of insulin containing microspheres

3.5.2.2.1 Preparation of the internal and continuous phase

The internal phase consisted of absolute ethanol (30 g) in which insulin (1 - 2% w/w), Eudragit® L100 (3.5 - 7.5% w/w), TMC (5 - 10% w/w) and Tween® 80 (1% w/w) were dissolved/dispersed

based on the composition of the microsphere formulation to be prepared as outlined in Table 3.3. All ingredients were accurately weighed on an analytical balance (Precisa 202A, Precisa Balances, UK). Firstly, Eudragit® L100 was dissolved in 38 ml of absolute ethanol followed by the dispersion of the TMC and insulin within the Eudragit® solution, which was facilitated by a magnetic stirrer (Labcon, Laboratory Marketing Services, South Africa). Liquid paraffin (100 ml) containing Span® 80 (1.5% v/v) was used as the continuous phase for the emulsion.

3.5.2.2.2 Preparation of microspheres

A volume of 10 ml of the internal phase was added drop-wise at a rate of 20 rpm (± 1ml/min) to the continuous phase via an 18 gauge needle using a peristaltic pump (Watson Marlow Ltd., Falmouth England). The mixture was stirred constantly by means of a 4 cm diameter, ten finned stirring disc at a stirring rate of 1000 rpm with an overhead stirring apparatus (Heidolph, Schwabach, Germany) to assist the formation of the microspheres by accelerating solvent evaporation. After the addition of the internal phase to the continuous phase was completed, the agitation of the emulsion was continued for approximately 15 h at 25°C. The microspheres were separated by means of filtration and washed tree times with *n*-hexane to remove the residual liquid paraffin. The microspheres were stored at -8°C until further evaluation.

3.5.3 Methods for microsphere evaluation

The Eudragit® L100/TMC microspheres loaded with insulin were characterised in terms of morphology, internal structure and encapsulation efficiency of both insulin and TMC. The insulin loading was determined using a validated high performance liquid chromatography (HPLC) method (Annexure A), while that of TMC was determined by means of a colorimetric assay. Based on these results, the best formulations were selected and further characterised in terms of particle size distribution, enteric nature, dissolution behaviour and *in vitro* transport of insulin across excised rat intestinal tissue.

3.5.3.1 Surface morphology and internal structure

3.5.3.1.1 Scanning electron microscopy

To investigate the morphology as well as the internal structure of the microsphere formulations, scanning electron microscope (SEM) images were captured with an FEI Quanta 250 FEG environmental scanning electron microscope (ESEM) with integrated Oxford x-max EDS system

(FEI Company, Netherlands). Prior to capturing the images, all samples were mounted on aluminium sample mounts covered with a double-sided carbon tape. Samples were then coated under vacuum using an IB-2 Ion-Sputter-coater (Eiko engineering, Japan) with gold-palladium (70/30%), to minimise surface charging, followed by ESEM analysis at 5 or 10 kV. For each microsphere formulation (i.e. formulation A-H as outlined in Table 3.3) images of both the surface and internal structure were captured. In order to visualise the internal structure, a sample of the microsphere formulations were embedded in resin (LR white, medium grade) and left for 16-24 h at 70 °C to set. After hardening of the resin occurred, the resin embedded samples were fractured using a blunt object to expose the internal structure of the microspheres.

3.5.3.2 Insulin loading

For microsphere preparations to be efficient and cost-effective, it is essential that the drug is incorporated in an adequate amount into the microsphere matrix. Drug loading was determined using a validated HPLC analysis method. The HPLC method used is described in section 3.5.1 and the validation thereof is given in Annexure A.

3.5.3.2.1 Determination of Insulin loading

Based on the quantities of insulin weighed and included in the microsphere formulations, all microsphere formulations prepared in this study (i.e. Formulation A-H) theoretically contained either an insulin concentration of 1% or 2% w/w.

The drug loading or encapsulation efficiency of each microsphere formulation was calculated using Equation 3.1 (Jain & Majumdar, 2006:201).

$$\%\mathrm{DL} = \frac{\text{Experimental insulin content}}{\text{Theoretical insulin content}} \times 100 \qquad \text{Equation 3.1}$$

Where:

%DL is drug loading as a percentage, which refers to the quantity of insulin encapsulated into the microspheres. The percentage drug loading is thus the amount of insulin relative to a theoretical quantity of 1% or 2% w/w effectively incorporated into the microsphere matrix.

The experimental insulin content of each microsphere formulation was calculated using Equation 3.2

$$\mathrm{DC} = \frac{PA_{exp-c}}{\mathrm{m}}$$

Equation 3.2

Where:

DC is the drug content expressed in µg/ml, PA_{exp} is the experimental peak area obtained from samples and *c* as well as *m* is the Y-intercept and slope values obtained from the calibration curve.

An accurately weighed mass of approximately 50 mg of each insulin containing microsphere formulation (A-H) were transferred to 25 ml volumetric flasks and made up to volume with PBS (pH 7.4). The samples were agitated by means of magnetic stirrers as well as placed in an ultrasonic bath (Julabo, Labotec, South Africa) and sonicated for 15 min to ensure complete dissolution of the microspheres. A sample of each volumetric flask was placed in HPLC vials and analysed according to the method described in section 3.5.1. The insulin content as well as drug loading or encapsulation efficiency was calculated for each sample in triplicate.

3.5.3.3 TMC loading

As mentioned in chapter 2 (section 2.4.3.3.1), the microsphere delivery system developed in this study was formulated to target the paracellular transport pathway for which opening of the tight junctions is a prerequisite. It was therefore necessary to determine whether TMC, a known paracellular acting permeation enhancer, was effectively incorporated into the microsphere formulations. The method employed to determine the TMC content was based on a colorimetric assay developed by Muzzarelli *et al*. (1998:255), for the determination of chitosan in aqueous solutions, where a specific colour dye (i.e. Cibacron brilliant red 3B-A) is added to the TMC containing solution of which the absorbance of light at a wavelength of 572 nm is measured.

3.5.3.3.1 Preparation of a calibration curve

Preparation of Cibacron brilliant red 3B-A solution

A quantity of approximately 37.5 mg of Cibacron brilliant red 3B-A was weighed accurately and transferred to a 500 ml volumetric flask and filled to volume with deionised water to render a solution with a concentration of 75 µg/ml.

Preparation of a calibration curve

A weight of approximately 112.5 mg of the TMC used in the microsphere formulations was accurately weighed and transferred to a 250 ml volumetric flask with approximately 50 ml of deionised water to dissolve the TMC. After the TMC was completely dissolved, the volumetric flask was filled to volume with PBS (pH 7.4) to render a stock solution with a concentration of 450 µg/ml. Different volumes of this stock solution were pipetted to volumetric flasks to render calibration solutions with concentrations of 90, 135, 180, 225 and 315 µg/ml. A volume of 1 ml of each of these standard solutions as well as the stock solution was pipetted to a suitable container for use with the cartridge sipper system, where after a volume of 2 ml of a Cibacron brilliant red 3B-A solution (75 µg/ml) was added before measurement of each standard solution to render standard concentrations of 30, 45, 60, 75 and 105 µg/ml. The absorbance of the TMC/Cibacron brilliant red 3B-A solutions was determined colorimetrically at a wavelength of 572 nm within 2 min of preparation, using a UV VIS SPECORD® 200 spectrophotometer (Analytik Jena AG, Germany) equipped with a cartridge sipper system and WinASPECT PLUS version 4.1 spectroanalytical software.

3.5.3.3.2 Determination of TMC content in microspheres

A weight of approximately 50 mg of each microsphere formulation (A-H) were weighed and transferred to 25 ml volumetric flasks and made up to volume with PBS (7.4). A volume of 1 ml of each solution was then pipetted to a suitable container for use with the cartridge sipper system where after 2 ml of a Cibacron brilliant red 3B-A solution (75 µg/ml) was added. The absorbance was then determined colorimetrically at a wavelength of 572 nm and the corresponding TMC concentrations were determined from the standard curve. Calibration curves were constructed prior to each analysis and the TMC encapsulation for each sample was determined in triplicate on different days.

Formulations A-D theoretically contained 5% w/w TMC and formulation E-H theoretically contained 10% w/w. The TMC loading of the microsphere formulations was calculated in a similar manner as insulin by means of Equation 3.3.

$$\%\mathrm{TL} = \frac{\text{Experimental TMC content}}{\text{Theoretical TMC content}} \times 100 \qquad \text{Equation 3.3}$$

Where:

%TL is TMC loading as a percentage, which refers to the quantity of TMC encapsulated into the microsphere matrix. The percentage TMC loading is thus the amount of TMC relative to a theoretical quantity of 5% or 10% w/w effectively incorporated into the microsphere matrix.

The TMC content of each microsphere formulation was calculated using Equation 3.4.

$$\mathrm{TC} = \frac{Abs_{exp-c}}{\mathrm{m}} \qquad \text{Equation 3.4}$$

Where:

TC is the TMC content expressed in µg/ml, Abs_{exp} is the experimental absorbance obtained from samples and *c* as well as *m* is the Y-intercept and slope values obtained from the calibration curve.

3.5.4 Selection of microsphere formulations for further characterisation and evaluation

When considering the morphology (Figures 3.7 - 3.14, Section 3.6.1) and relatively high drug (Section 3.6.2) and TMC (Section 3.6.3) loading, Formulations B and F were selected for further characterisation in terms of particle size distribution, enteric nature, dissolution behaviour and *in vitro* transport of insulin across excised rat intestinal tissue in a Sweetana-Grass diffusion apparatus (Chapter 4).

3.5.4.1 Particle size distribution

It is clear from the SEM images (Figures 3.7 - 3.14) that the microspheres of all formulations fall in the micrometer size range. However, it was necessary to conduct a particle size analysis to quantify the size of the selected microsphere formulations more accurately.

Particle size analysis of the selected formulations was conducted by means of laser diffraction with a Malvern Mastersizer 2000 instrument fitted with a Hydro 2000SM small volume dispersion unit (Malvern Instruments, Malvern, UK). Deionised water was used as dispersion medium at a stirring rate of 1500 rpm. The small volume dispersion unit was filled with 60 ml of deionized water and a background measurement was taken to compensate for electrical interference as well as possible interference from the dispersion medium. Upon completion of the background measurement, a sample of the appropriate microsphere formulation was added to the dispersion unit. Samples were dispersed in 5 ml of deionised water prior to addition to the small volume dispersion unit. A sufficient quantity of the microsphere formulation was added to obtain an obscuration of between 1 and 10%. After a suitable obscuration was obtained, the particle size distribution of the sample was measured. Each measurement consisted of 12000 sweeps. The particle size distribution of each formulation was measured in triplicate.

3.5.4.2 Dissolution studies

Intestinal absorption of drugs given in a solid dosage form is usually determined by, the available surface area, intestinal transit time, membrane permeability and the concentration time profile of the drug in the lumen. The free drug concentration available for transport across the intestinal mucosa is determined by solubility, dissolution rate, degradation, metabolism, and complex binding of the drug. Studies of solubility and dissolution rate are therefore crucial for a better understanding of the absorption and bioavailability of drugs (Bønløkke *et al*., 1997: 1490).

Dissolution studies were conducted to investigate the release characteristics of insulin as well as TMC from the microsphere formulations, and will be discussed separately in the following sections.

3.5.4.2.1 Comparison of dissolution profiles

The quantitative interpretation of the data obtained in dissolution tests is facilitated by the use of a general equation that mathematically translates the dissolution curve in function of some parameters related with the pharmaceutical dosage form (Costa & Lobos, 2001:123). To compare the dissolution profiles of the selected microsphere formulations (i.e. Formulations B and F), a model independent approach was followed. The area under curve (AUC), the mean dissolution time (MDT) as well as a similarity factor (f_2) was calculated and a correction factor was applied for both formulations.

Area under curve

The AUC is a ratio test procedure widely used in assessing bioavailability. In terms of plasma-time profiles, AUC values reflect the total amount of drug that reaches the systemic circulation (Shargel & Yu, 1999:253). Similarly, the AUC of the dissolution-time profile reflects the extent of drug dissolution. The AUC can be calculated for each dissolution profile as the sum of the areas of the trapezia formed by the points by means of Equation 3.5 (Shargel & Yu, 1999:9).

$$[\mathrm{AUC}]\frac{t_n}{t_{n1}} = \frac{C_{n1}+C_n}{2}t_n - t_{n1} \qquad \text{Equation 3.5}$$

Where:

AUC is the area under curve from time, t_n to time, t_{n-1}, C_n is the concentration at time *n*, and C_{n-1} is the concentration at time, *n-1*.

Mean dissolution time

Mean dissolution time (MDT) is the first statistical moment for the cumulative dissolution process and is defined by Equation 3.6. Mean dissolution time is the mean time taken for the drug to dissolve under *in vitro* dissolution conditions (Reppas & Nicolaides, 2000:231-232). A graphic representation of the parameters used to estimate the mean dissolution time is given in Figure 3.3.

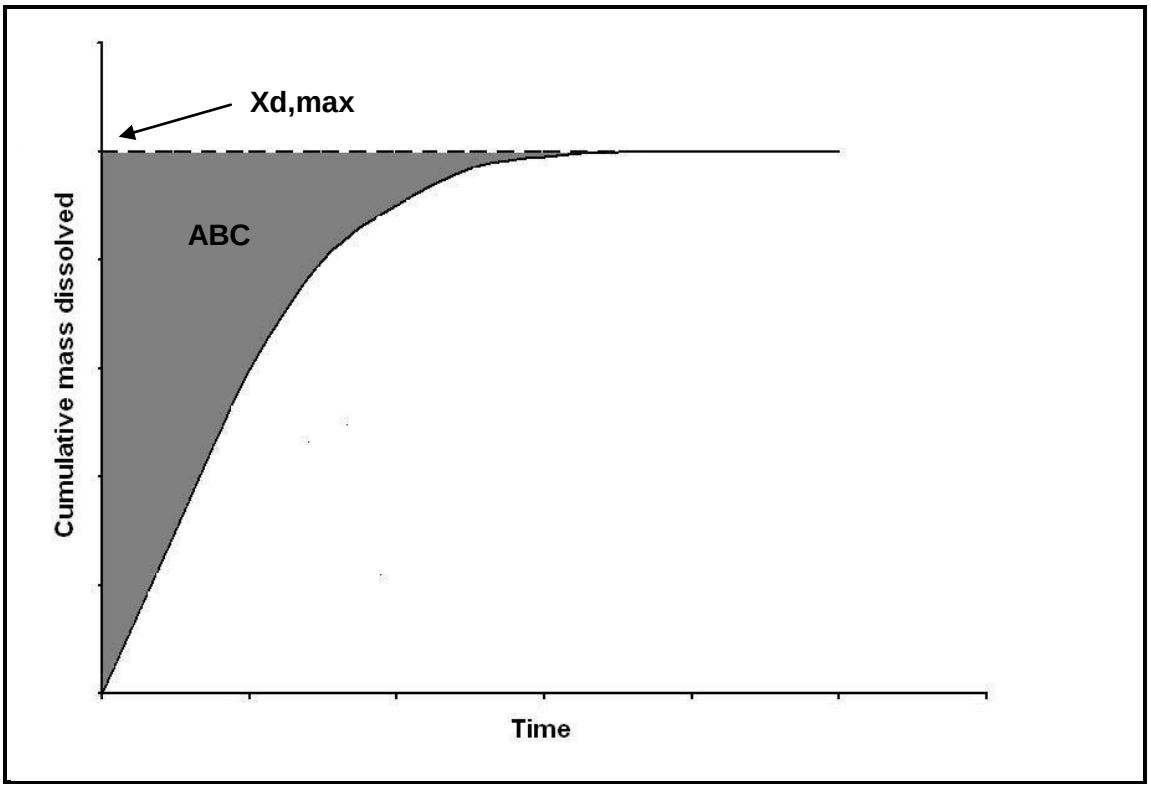


Figure 3.3: Graphic representation of the parameters used to estimate the mean dissolution time (MDT): $X_{d,max}$ is the actual maximum cumulative mass dissolved and ABC is the shaded area (Reproduced from Reppas & Nicolaides, 2000:232)

$$MDT = \frac{ABC}{X_{dmax}} \qquad \text{Equation 3.6}$$

MDT was estimated from the cumulative mass of drug dissolved versus time profile by employing Equation 3.7.

$$MDT = \frac{\sum_{i=1}^{n} t_{mid} \Delta X_d}{\sum_{i=1}^{n} \Delta X_d} \qquad \text{Equation 3.7}$$

Where:

MDT is the mean dissolution time, *i* the sample number, n the total number of sampling times, t_{mid} the time at the midpoint between *i* and *i – 1*, and ΔX_d is the additional mass of drug dissolved between *i* and *i – 1*.

Similarity factor

The similarity factor (f_2) compares the difference between the percentage drug dissolved per unit time for a test and reference formulation (Moore & Flanner, 1996:64-74). The similarity factor was calculated by using Equation 3.8.

$$f_2 = 50\log\left\{\left[1 + \frac{1}{n}\sum_{t=1}^{n} w_t(R_t - T_t)^2\right]^{-0.5} \times 100\right\} \quad \text{Equation 3.8}$$

Where:

f_2 is the similarity factor, R_t the reference assay at time point *t,* T_t the test assay at time point *t, n* the number of pull points, and, w_t the optional weight factor.

The value of the similarity factor is 100 when two dissolution profiles are identical and approaches 0 as similarity decreases (Moore & Flanner, 1996:66). The FDA considers two dissolution profiles similar when f_2-values obtained range between 50 and 100 (Costa & Lobos, 2001:130). Similarity factors provide a straightforward approach for comparing dissolution profiles, although this factor does not take into account the within-batch variability or the correlation between data (Moore & Flanner, 2000:74; Adams *et al*., 2001:43).

Correction factor

The dissolution of a solid in a solvent can be quantitatively expressed using the Noyes-Whitney equation calculated by using Equation 3.9.

$$\frac{dM}{dt} = \frac{DS}{h}(C_s - C) \quad \text{Equation 3.9}$$

Where:

M is the mass of solute dissolved in time *t*, *dM*/*dt* is the dissolution rate, *D* is the diffusion constant, *S* is the surface area of the exposed solid, *h* is the thickness of the diffusion layer, C_s is the solubility of the solid and *C* is the concentration of the solute in the bulk solution at time *t* (Martin *et al*., 1993:331). According to mass transfer theory, a static (or stagnant) liquid layer of thickness *h* exists on the surface of a dissolving solid, with solute concentrations ranging from *C* to C_s. As long as *Cs* >> *C*, the system can be represented by sink conditions, however, as *C* approaches C_s the dissolution rate will decrease until equilibrium solubility is established (Figure 3.4).

In small volume dissolution studies, replenishment of the sampled volume with fresh dissolution medium can lead to dilution of the system, resulting in a negative slope on the dissolution curve as the system approaches equilibrium solubility. Early in the dissolution study, while *Cs* >> *C*, this dilution is negligible because the system is operating under sink conditions. However, as the solute concentration in the bulk solution approaches that of C_s in the diffusion layer, the effect of the dilution becomes more apparent. Since the static diffusion layer is nearly saturated, any dilution of the bulk solution can be expressed as a fraction relative to the static diffusion layer's concentration. If the last data point on the dissolution curve displaying a positive slope is designated as the concentration where the system approximates equilibrium solubility, $C_{sapprox}$, a correction factor, α, can be calculated for each diluted concentration using Equation 3.10).

$$\alpha = \frac{\mathrm{Cs}^{\mathrm{approx}}}{\mathrm{C_{dil}}} \qquad \text{Equation 3.10}$$

By multiplying C_s^{approx} with the correction factor, a corrected concentration (C_c) can be calculated (Equation 3.11).

$$\mathrm{C} = \alpha \times \mathrm{C_s}^{\mathrm{approx}} \qquad \text{Equation 3.11}$$

This corrected concentration represents the undiluted concentration. To test the effectiveness of the correction factor it was applied to several dissolution studies, performed at different temperatures and with different equilibrium solubility values (Figure 3.5). In each of the dissolution curves, the correction factor managed to accurately convert the diluted concentrations into concentrations obtained experimentally in large volume dissolutions.

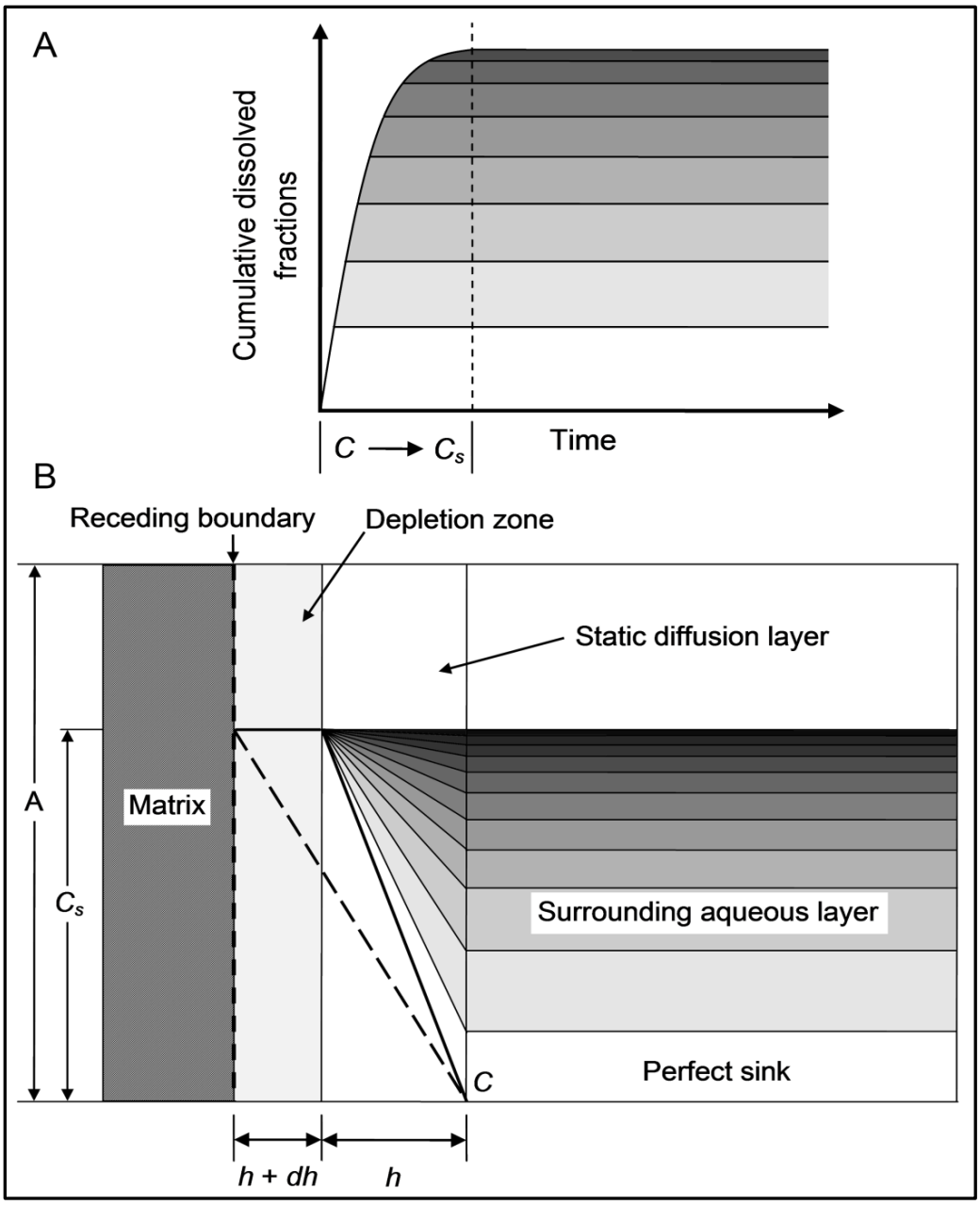


Figure 3.4: Schematic representations of a generalised dissolution curve (A) and the diffusion of a drug from a polymer matrix (B), adapted from Martin *et al*. (1993; 335)

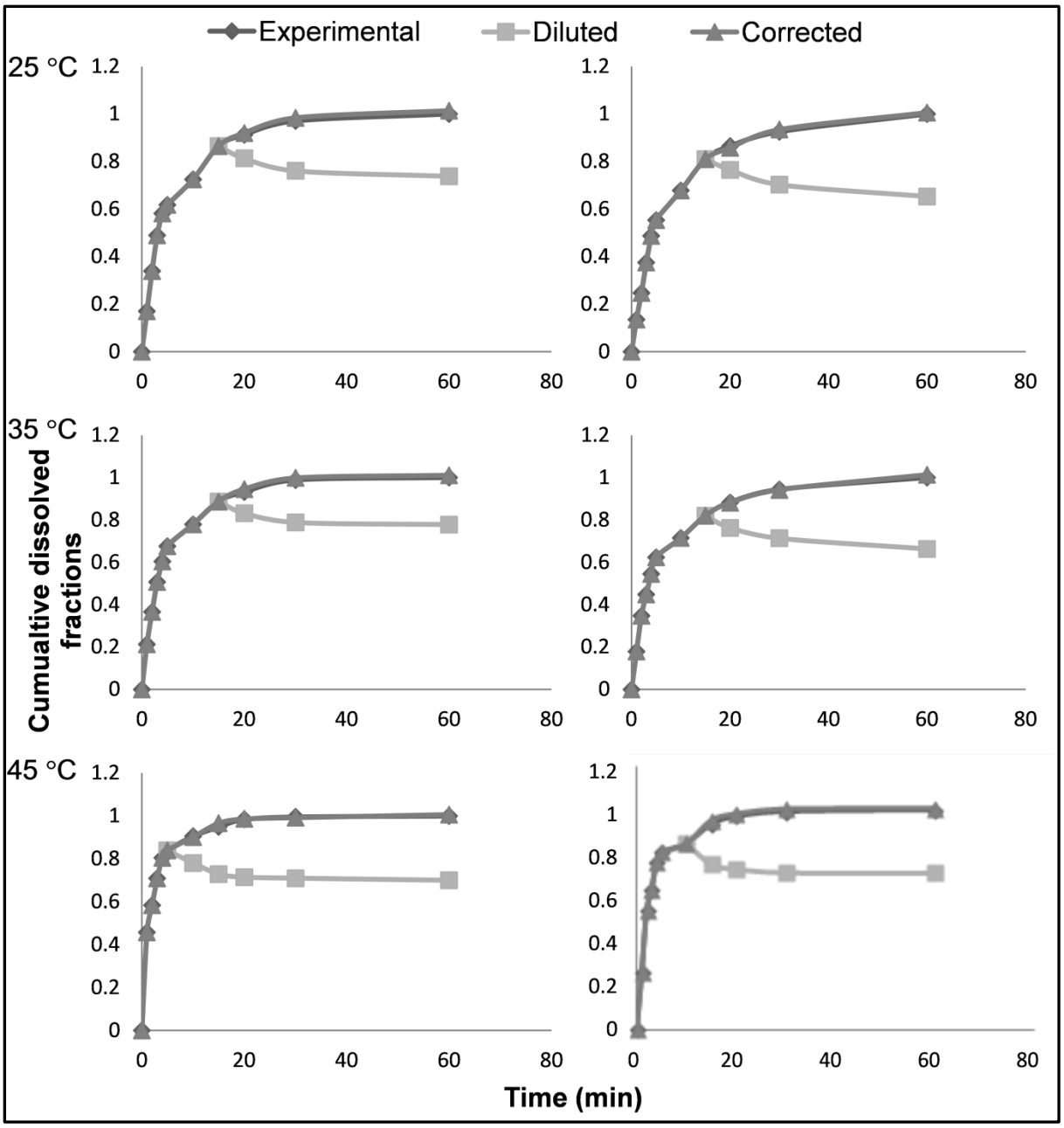


Figure 3.5: Fitting of the corrected concentration data to experimentally obtained dissolution data

3.5.4.2.2 Apparatus used for dissolution studies

As a result of the small amount of sample available, dissolution studies were conducted using a rotating bottle apparatus (see figure 3.6). The rotating bar is designed to hold 50 ml amber glass bottles. This apparatus was also used for the evaluation of the enteric nature of the microspheres, changing only the rotating bar.

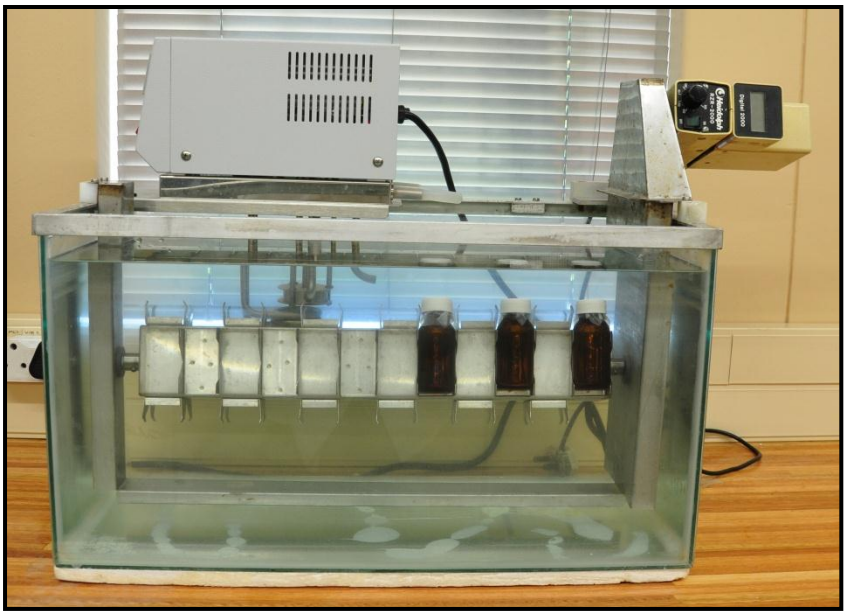


Figure 3.6: Rotating bottle apparatus used for dissolution

3.5.4.2.3 Dissolution method

Samples of approximately 100 mg of each microsphere formulation were accurately weighed and transferred to a 50 ml amber glass bottle containing 40 ml of PBS (pH 7.4) at a temperature of 37 °C. The glass bottles were sealed with parafilm as well as a screw cap, where after the bottles were secured on the rotating bar. The samples were rotated at a speed of 25 rpm and a volume of 2 ml from each sample were withdrawn at intervals of 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180 and 240 min following the start of the experiment, using a filter unit fitted with a glasfibre pre-filter (Sartorius Stedim Biotech GmbH, Germany). The volume withdrawn was replaced with the same volume of blank PBS (pH 7.4) at a temperature of 37 °C to keep the dissolution volume in the glass bottles constant. From the volume withdrawn, 1 ml was used to determine the TMC released and a sample was transferred to HPLC vials for determining the amount of drug released. After withdrawal of the sample at 240 min, the bottles were sonificated for 15 min to determine the total insulin content. Dissolution studies were performed in triplicate for each microsphere formulation.

3.5.4.2.4 Determining the amount of insulin released

Samples withdrawn from the dissolution studies were analysed by means of HPLC (described in section 3.5.1) to determine the insulin concentration. The insulin concentration corresponding to the obtained peak area was determined from a calibration curve. Corrections for the quantity of

insulin lost through withdrawal were made by means of equation 3.12. In addition, One-way analyses of variance (ANOVA) were done to determine if statistical significant differences exist between the MDT and the AUC values of the selected formulations.

$$CP_{(t)} = P_{(t)} + \frac{V_m}{V_d}\sum_{t-1} CP \qquad \text{Equation 3.12}$$

Where:

$CP_{(t)}$ is the corrected peak area of sample at time *t;* $P_{(t)}$ the peak area of sample at time *t;* V_d the sampling volume; V_m the dissolution medium volume and; $\sum_{t-1}$CP the sum of all the corrected peak areas prior to the sample at time *t*.

3.5.4.2.5 Determining the amount of TMC released

In order to determine the quantity of TMC in the dissolution medium, withdrawn samples were analysed by the colorimetric assay described in section 3.5.3.3.2. Corrections for the amount of TMC lost through withdrawal were done by means of Equation 3.13.

$$CA_{(t)} = A_{(t)} + \frac{V_m}{V_d}\sum_{t-1} CA \qquad \text{Equation 3.13}$$

Where:

$CA_{(t)}$ is the corrected absorbance of sample at time *t,* $A_{(t)}$ the absorbance of sample at time *t,* V_d the sampling volume, V_m the dissolution medium volume, and $\sum_{t-1}$CA the sum of all the corrected absorbance values prior to the sample at time *t*.

3.5.4.3 Enteric nature

The major aim of enteric coatings is protection of drugs that are sensitive or unstable at acidic pH values. This is particularly important for peptide and protein drugs, due to their rapid hydrolysis and inactivation in acidic environments (Schreier, 2001:11) The method used to evaluate the enteric nature of the microspheres was based on the method of Jain *et al*. (2005:E100-E107).

The apparatus used for the evaluation of the dissolution studies of the microspheres was also used for the evaluation of the enteric nature, changing only the rotating bar. Approximately 25 mg of Formulations B and F were dispersed in 15 ml centrifuge tubes containing 5 ml of 0.1 M HCl. The samples were rotated at 25 rpm at 37 °C for 1 hour where after the samples were centrifuged at 3000 rpm for 5 min. Samples of the supernatant of each formulation were withdrawn and filled in HPLC vials and analysed for insulin content. The experiment was conducted in triplicate for each formulation. The insulin released in acidic medium expressed as a percentage was calculated by means of Equation 3.14.

$$\%\mathrm{IR} = \frac{\text{insulin released}}{\text{Experimental insulin content}} \times 100 \qquad \text{Equation 3.14.}$$

Where:

%IR is insulin content obtained in the supernatant as a percentage, which refers to the quantity of insulin released into the acidic medium. The percentage insulin released is thus the amount of drug relative to the total quantity effectively incorporated into the microsphere matrix.

3.6 Results and discussions

3.6.1 Scanning electron microscopy

SEM images of microsphere F

Formulations A to H showing both the surface morphology and the corresponding internal structure are shown in Figures 3.7 - 3.14.

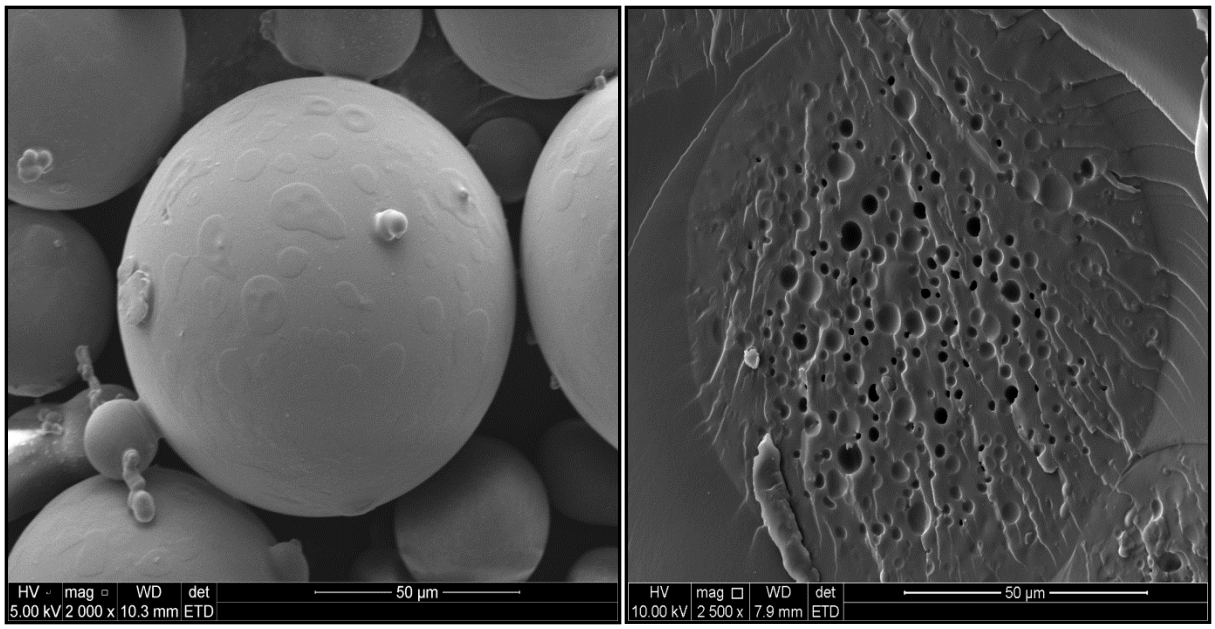


Figure 3.7: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation A (Eudragit® L100, 7.5% w/w; TMC, 5% w/w; insulin, 2% w/w)

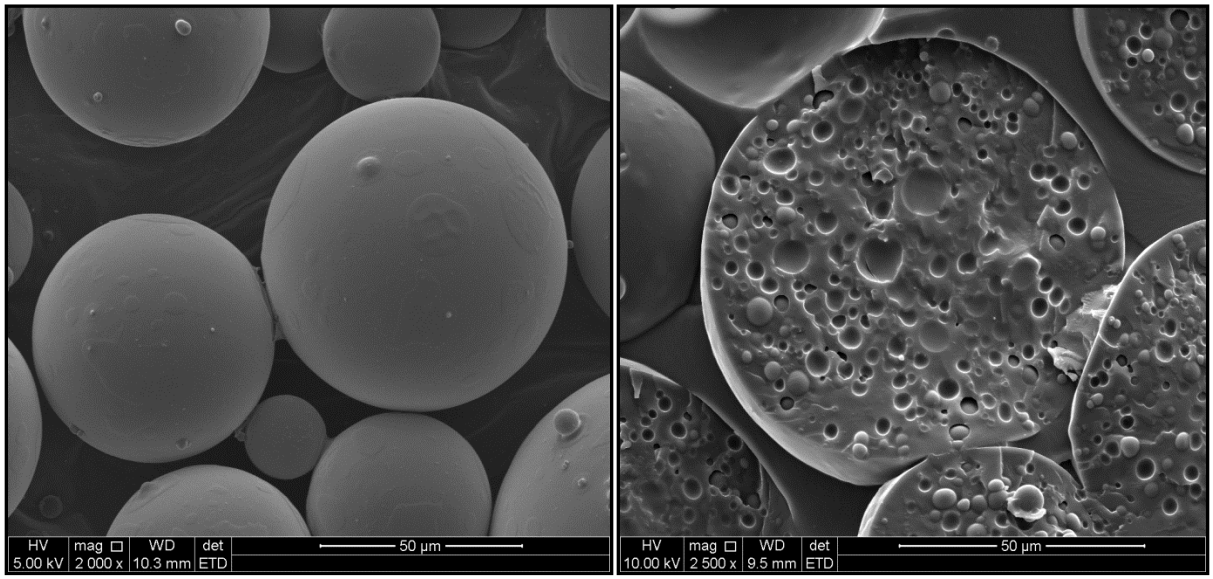


Figure 3.8: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation B (Eudragit® L100, 3.5% w/w; TMC, 5% w/w; insulin, 2% w/w)

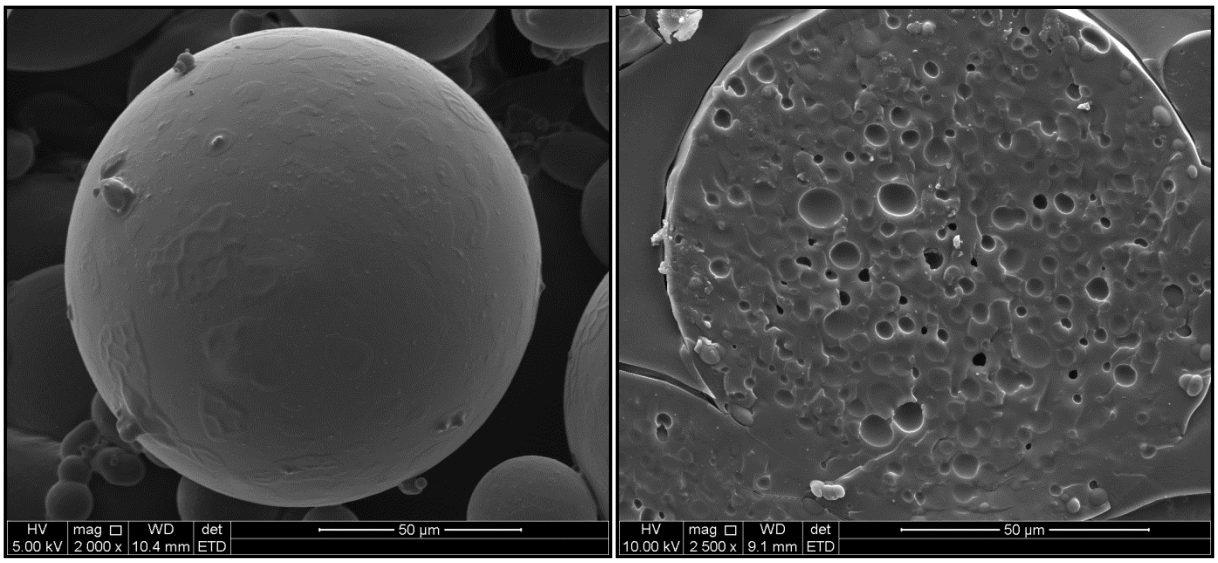


Figure 3.9: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation C (Eudragit® L100, 7.5% w/w; TMC, 5% w/w; insulin, 1% w/w)

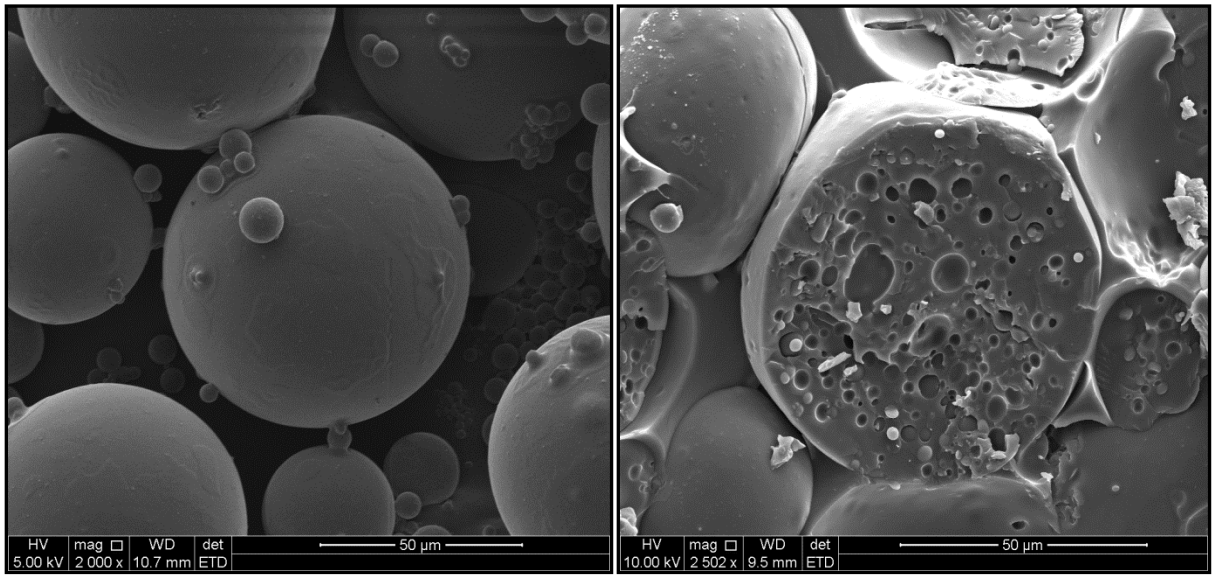


Figure 3.10: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation D (Eudragit® L100, 3.5% w/w; TMC, 5% w/w; insulin, 1% w/w)

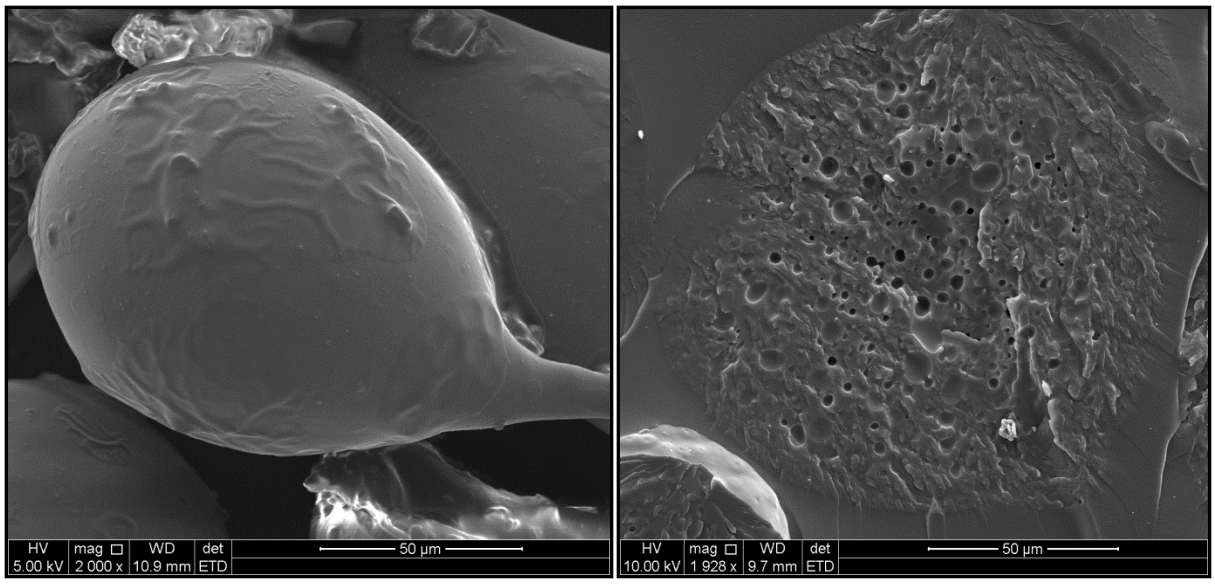


Figure 3.11: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation E (Eudragit® L100, 7.5% w/w; TMC, 10% w/w; insulin, 2% w/w)

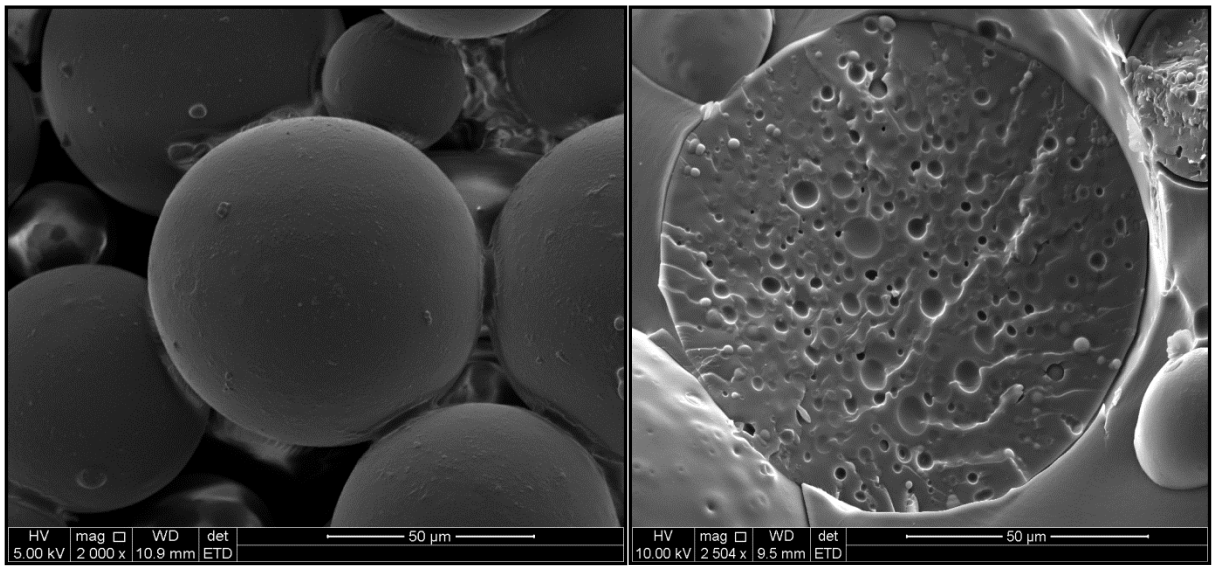


Figure 3.12: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation F (Eudragit® L100, 3.5% w/w; TMC, 10% w/w; insulin, 2% w/w)

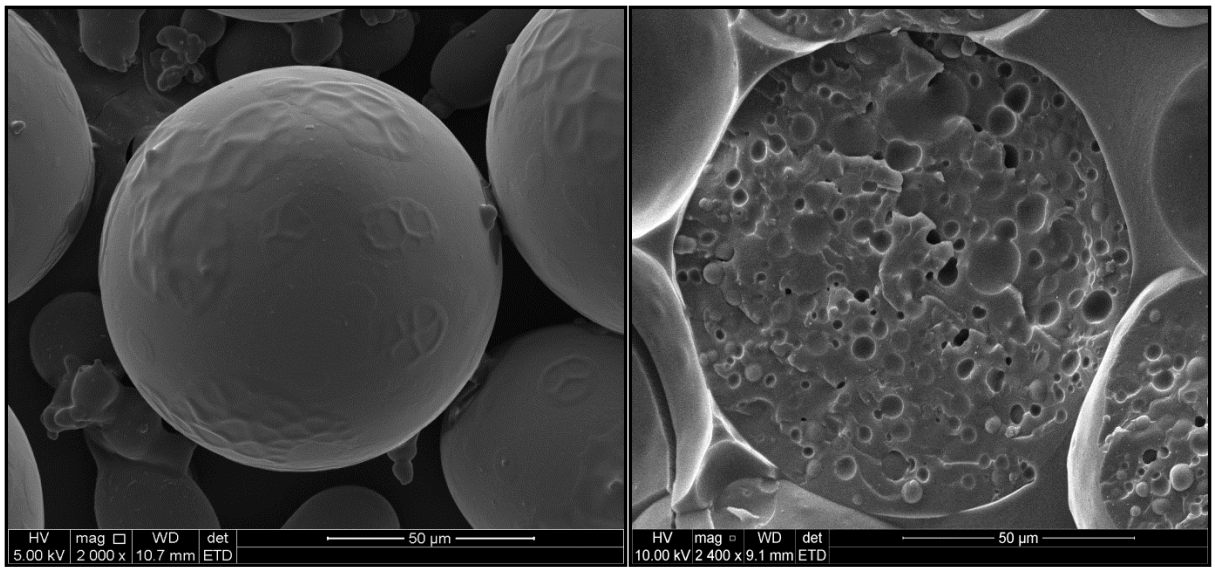


Figure 3.13: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation G (Eudragit® L100, 7.5%w/w; TMC, 10% w/w; insulin, 1% w/w)

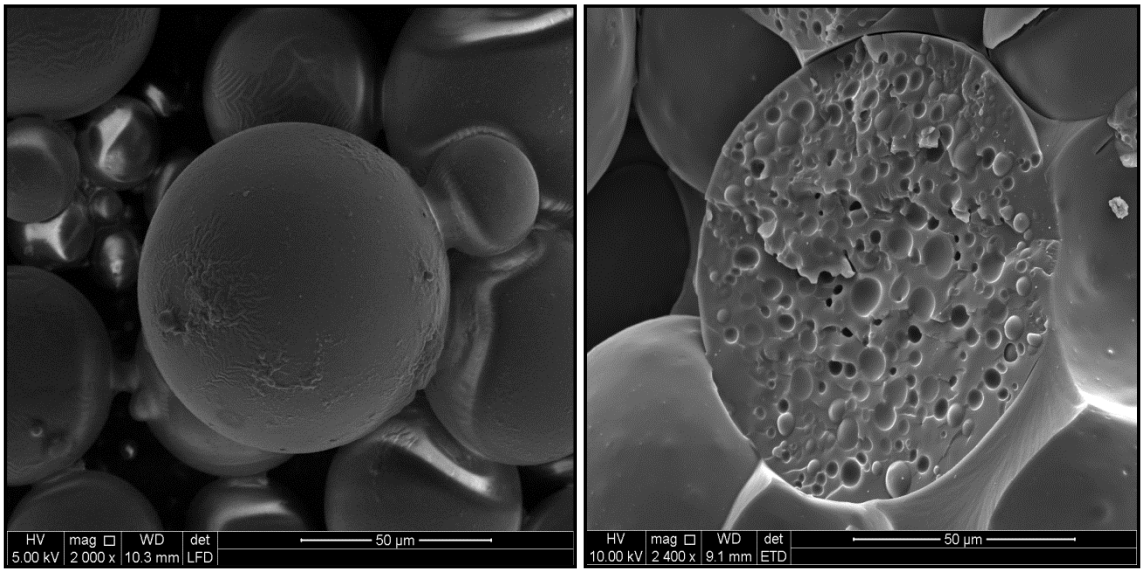


Figure 3.14: SEM micrographs of A) surface morphology (2000X magnification) and B) internal structure (2500X magnification) of microsphere Formulation H (Eudragit® L100, 3.5%w/w; TMC, 10% w/w; insulin, 1% w/w)

From Figures 3.7 - 3.14 it is clear that all the formulations from the factorial design produced spherical particles in the micrometer range with smooth surfaces, with the exception of Formulation E. This is possibly due to the high drug concentration and/or TMC concentration

included, which is known to cause more porous and irregular shaped microspheres (Li *et al*., 2008:32). Furthermore, the internal structure of all formulations showed a pitted or porous internal structure with small particles imbedded. The embedded particles may originate from the suspended TMC/insulin particles in the internal phase. Furthermore, no crystals or crystalline material can be observed in the internal structure of any of the microsphere formulations.

3.6.2 Insulin loading

The drug loading (%) of the microsphere formulations are listed in Table 3.4.

Table 3.4: Drug loading (%) and content obtained for Formulations A-H

Formula	Drug loading (%)*	Insulin content (µg/ml)*
A	29.1 ± 3.28	11.6 ± 1.31
B	49.8 ± 5.64	19.9 ± 2.26
C	39.1 ± 7.23	7.8 ± 1.65
D	39.3 ± 29.2	7.9 ± 5.28
E	45.5 ± 24.15	12.7 ± 0.94
F	52.4 ± 2.72	21.0 ± 1.09
G	37.4 ± 4.19	7.5 ± 0.93
H	27.9 ± 14.25	5.6 ± 2.85

*Mean ± standard deviation

From the results obtained it is clear that insulin was successfully encapsulated within al microspheres, with the mean percentage drug loading varying from 27.9 ± 14.25 – 52.4 ± 2.72%. The significant difference in %DL between the different formulations is probably the result of the multitude parameters involved as well as the complex physicochemical processes which govern emulsification/solvent evaporation. It should be mentioned that with the exception of microsphere Formulation A, that the rest of the formulations prepared with an insulin concentration of 2% w/w (Formulation, B, E and F) achieved the highest drug encapsulation. Also, the formulations prepared using a higher Eudragit® concentration (7.5% w/w) did not increase the encapsulation as would be expected, which demonstrated that increased viscosity of the internal phase negatively affected encapsulation efficiency.

3.6.3 TMC loading

The TMC loading and content of the different microsphere formulations are shown in Table 3.5.

Table 3.5: The calculated TMC loading (%) and content obtained for Formulations A-H

Formula	Mean TMC loading (%)*	TMC content (µg/ml)*
A	29.1 ± 3.3	36.5 ± 10.64
B	36.0 ± 3.4	42.3 ± 16.20
C	30.3 ± 3.8	30.3 ± 3.77
D	35.0 ± 8.3	35.0 ± 8.27
E	31.2 ± 2.0	31.2 ± 2.02
F	37.7 ± 2.3	32.3 ± 1.14
G	32.49 ± 1.9	32.5 ± 1.88
H	36.60 ± 0.9	36.6 ± 0. 88
*Mean ± standard deviation		

The results clearly indicate that TMC was successfully incorporated into all microsphere formulations. The mean TMC loading ranged from 29.1 ± 3.3 - 37.7 ± 2.3%. The TMC loading obtained is less than that for insulin, despite adding a much higher concentration (5 - 10% w/w). The formulations prepared by using a higher concentration of TMC (10% w/w) did not result in the highest TMC encapsulation. This could be related to the difference in particle size between the insulin and the TMC, which was milled and subsequently sieved (40 µm) prior to encapsulation. Thus, it can be concluded that the specific formulation and processing conditions used resulted in a limited TMC encapsulating capacity.

3.6.4 Particle size distribution

The particle size distribution plot for the two microsphere formulations (i.e. Formulations B and F) is shown in Figure 3.15. The mean d(0.1) value, mean median d(0.5), value mean d(0.9) and mean particle size (D[4,3]) for each formulation are shown in Table 3.5.

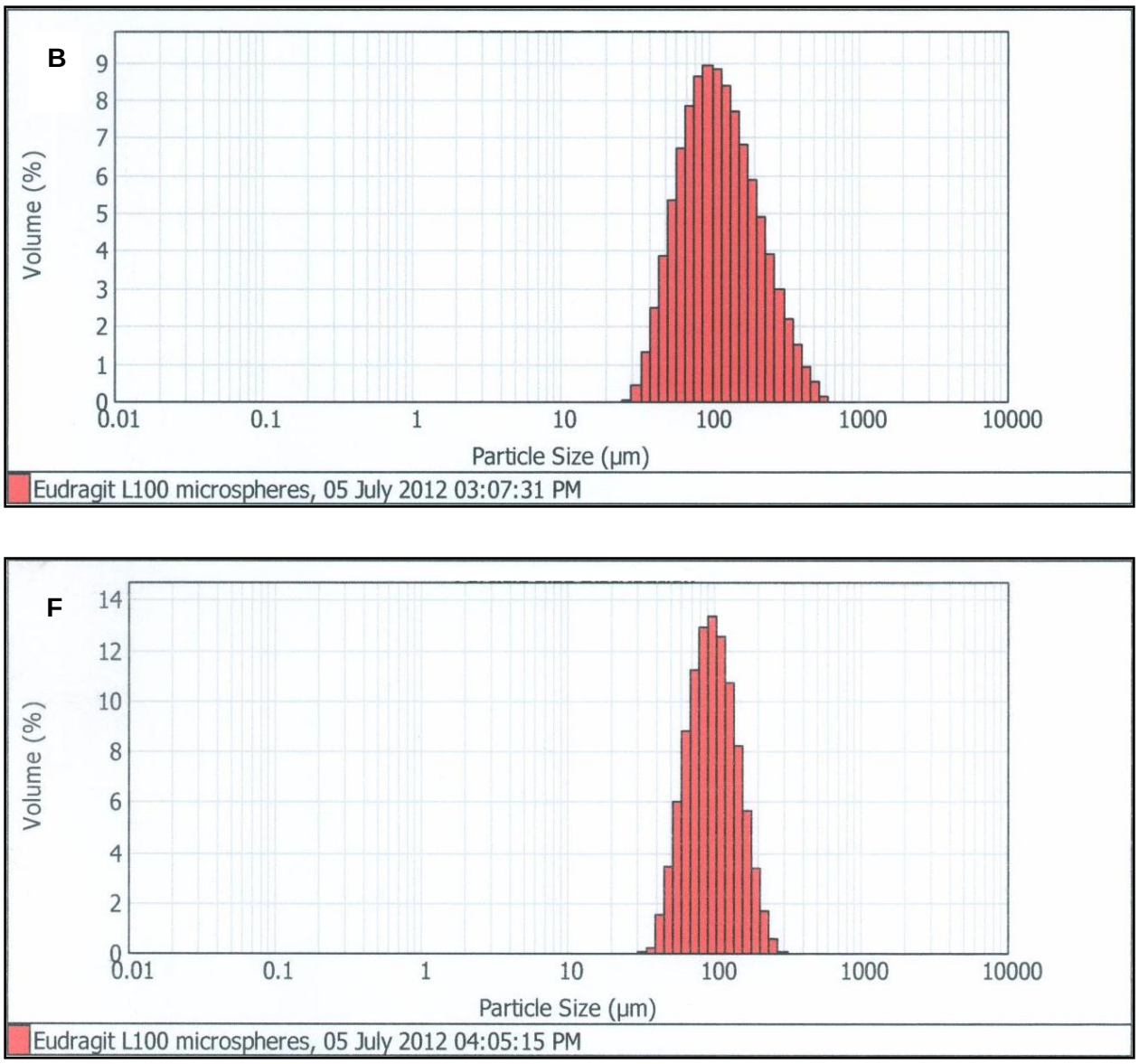


Figure 3.15: Particle size distribution plots for Formulations B and F

Table 3.6: mean d(0.1), mean median d(0.5), d(0.9) and mean particle size (D[4,3]) of the particle size distribution for microsphere Formulations B and F

Formulation	d(0.1)	d(0.5)	d(0.9)	D[4.3]
B	64.8 ±12.61	131.7 ± 29.50	273.2 ± 48.95	157.3 ± 31.74
F	58.0 ± 22.09	121.5 ± 30.73	221.8 ± 84.98	135.7 ± 41.05

Microsphere Formulation B had a larger mean particle size based on D[4.3] (157.3 ± 31.74 µm) compared to Formulation F (135.7 ± 41.05 µm) The particle size difference can be explained by the influence of the different concentrations of the components included in each formulation. Since the final structure and size of a microsphere result from a complex interplay between polymer, drug, solvent, continuous phase, emulsifier and other excipients it is not possible to explain the exact reason for the particle size difference.

3.6.5 Insulin dissolution

Dissolution profiles of Formulation B and F for insulin release, after correction factors were applied are shown in Figure 3.16. The mean AUC values and MDT-values for the formulations are reported in Table 3.7 and Table 3.8, respectively. The similarity factor values for Formulation B in comparison to Formulation F are reported in Table 3.9.

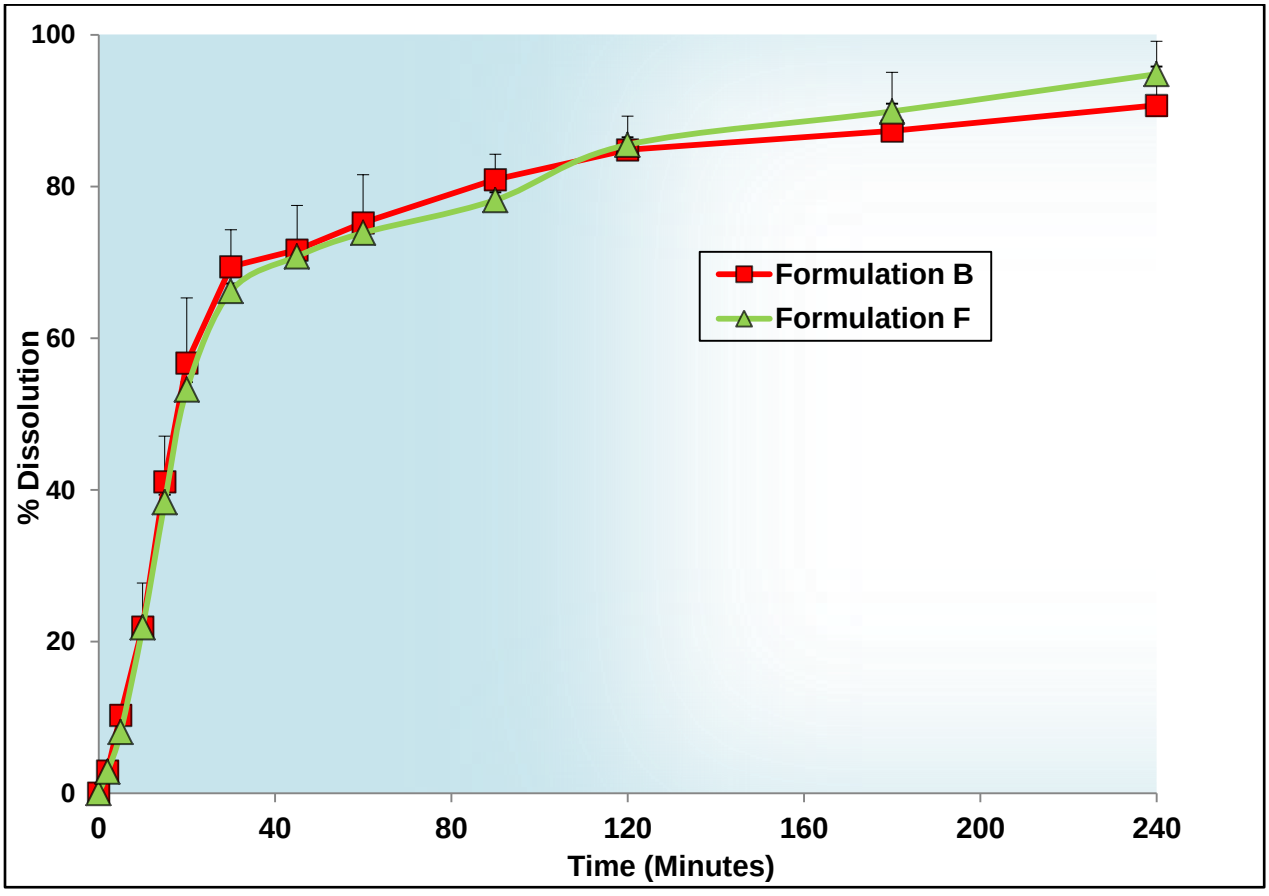


Figure 3.16: Dissolution profiles of microsphere Formulations B and F

From Figure 3.16 it is clear that both Formulations B and F released their insulin content at a pH of 7.4 to a relative large extent over a period of 240 min (B = 90.69%, F = 94.81%) Both formulations showed similar release profiles which indicates that the difference in the composition of the formulations (i.e. TMC concentration was 5 and 10% w/w, respectively) did not influence the release of insulin. In Table 3.7 the mean AUC-values of the dissolution profiles are reported.

Table 3.7: Mean AUC values for microsphere Formulations B and F

Formulation	AUC_{0-240} (µg/ml.min)
B	6412.6 ± 735.74
F	5999.6 ± 647.61

The AUC values did not differ statistically significantly (p-value > 0.05), which indicates that the extent of insulin release from the formulations was similar.

Table 3.8: Mean dissolution time (MDT) values for Formulations B and F

Formulation	MDT (minutes)
B	34.5 ± 4.01
F	42.6 ± 9.06

Mean dissolution time ranged from 34.5 ± 4.01 minutes for Formulation B to 42.6 ± 9.06 minutes for Formulation F. These MDT results, in conjunction with the dissolution profiles depicted in Figure 3.16 thus indicate that the mean dissolution time taken for the drug to dissolve under *in vitro* dissolution conditions was less than 45 minutes. The relatively fast insulin dissolution rates are illustrated by the steep slope of the dissolution profiles for the first 5-10 min of the dissolution experiment. Mean dissolution time is the mean time taken for the drug to dissolve under *in vitro* dissolution conditions

Table 3.9: Similarity factor value for Formulation B in reference to Formulation F

Formulation	f_2-value
B vs. F	68.4 ± 8.18

The f_2-value obtained were significantly higher than 50 (68.4 ± 8.18) and therefore the release profiles of Formulations B and F can indeed be considered similar.

3.6.6 TMC dissolution

The dissolution profiles of Formulation B and F in terms of TMC release are shown in Figure 3.17. The mean AUC values and MDT values for the formulations in terms of TMC release are reported in Table 3.10 and Table 3.11, respectively. The similarity factor value for Formulation B in comparison to Formulation F is reported in Table 3.12.

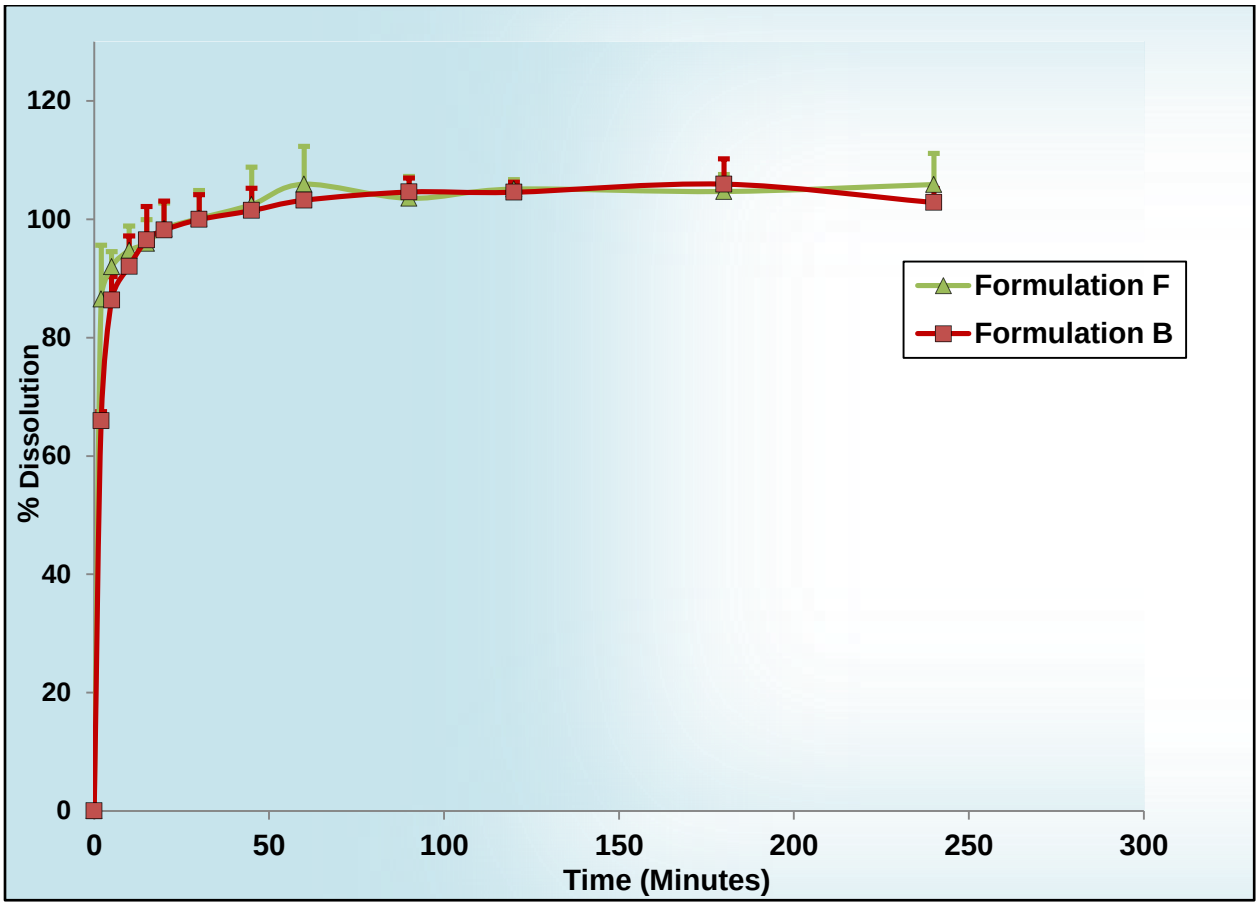


Figure 3.17: TMC dissolution profiles of Formulations B and F.

From Figure 3.17 it is clear that both Formulations B and F released TMC in an alkaline environment (pH 7.4). Both formulations showed similar release profiles as demonstrated by no statistically significant difference between the mean AUC values (p-value > 0.05).

Table 3.10: Mean AUC values for Formulations B and F

Formulation	AUC_{0-240} (µg/ml.min)
B	24248.9 ± 547.89
F	24668.0 ± 1941.78

The calculated MDT values for the two profiles shown in Table 3.11 on average is less than 7 min indicating a relatively fast release rate of the TMC from the microsphere formulations. When comparing the MDT results of insulin and TMC for Formulation B and F, the difference in release rate is apparent. The MDT for insulin ranges from 34.47 – 42.63 min whereas TMC ranges from 1.22 – 6.82 min. This may be beneficial, since TMC will be able to open the tight junctions prior to insulin release, which may minimise insulin lost through intestinal transit.

Table 3.11: Mean dissolution time (MDT) values for TMC release from microsphere Formulations B and F

Formulation	MDT (minutes)
B	1.2 ± 1.73
F	6.8 ± 6.42

The mean similarity factor shown in Table 3.12, calculated for the dissolution profile of Formulation B in reference to Formulation F was above 50 (61.00 ± 10.85) indicating that the release profiles of Formulations B and F can indeed be considered similar.

Table 3.12: Mean similarity factor value for Formulation B in reference to Formulation F

Formulation	F_2-value
B vs. F	61.0 ± 10.85

Thus, when considering the TMC dissolution profiles as well as the mean AUC values and MDT values, the TMC release and dissolution from Formulations B and F are similar. Although the preparation of Formulations B and F differed in terms of TMC concentration (5 and 10 %w/w respectively) only, the mean TMC as well as insulin loading, the particle size distribution and morphology were similar, which could explain the similar TMC release and dissolution.

3.6.7 Enteric nature

The percentage insulin released in 0.1 M HCl after one hour for each microsphere formulation is shown in Table 3.13

Table 3.13: Percentage insulin released in 0.1 M HCl after one hour

Formulation	% Insulin released
B	51.79%
F	63.08%,

From Table 3.13 it is clear that Formulations B and F lost a significant amount of insulin within the acidic medium, 51.79% and 63.08%, respectively. To achieve enteric protection of the encapsulated materials a precise amount of film coating with Eudragit® is needed, which also depends on the type of Eudragit® applied. Furthermore, no pores or cracks are visible on the surface of the spheres, thus it is possible that insulin close to the microsphere surface could have leached out into the acidic medium or the use of the centrifuge for separating solid particles could have facilitated additional release giving rise to the high percentage insulin released, however, this needs to be confirmed.

3.7 Summary of results and conclusions

A method based on emulsification/solvent evaporation was successfully employed for the preparation of insulin loaded microspheres. Processing conditions included Eudragit® L100 (3.5 and 7.5% w/w) at different concentrations dissolved in ethanol as solvent system; a binary emulsifier system consisting of Tween® 80 (1% w/w) and Span® 80 (1.5% w/v), and a stirring rate of 1000 rpm. Different microsphere formulations denoted A-H was prepared in accordance with a fractional factorial design to investigate the combined influence of three variables namely concentration of the Eudragit® L100, insulin as well as TMC.

The prepared microsphere formulations were characterised in terms of morphology and internal structure by scanning electron microscopy as well as encapsulation efficiency of both insulin as well as TMC. In general, most microsphere formulations showed a spherical shape and smooth surface, with the exception of Formulation E containing high concentrations of Eudragit® L100, insulin and TMC. The internal structure of all formulations showed a pitted or porous internal structure with small particles imbedded; this is possibly the result of deposition of TMC and

insulin particles in the microsphere matrix cores during the solvent evaporation step during the solidification of the microspheres. Both insulin and TMC were successfully encapsulated with mean insulin drug loading values varying from 27.9 ± 14.25 – 52.4 ± 2.72%, while mean TMC loading ranged from 29.1 ± 3.3 - 36.6 ± 0.9%. Based on the morphology and encapsulation results, two formulations (B and F) were selected. These formulations were further characterised in terms of particle size distribution, dissolution behaviour, enteric nature and *in vitro* transport of insulin across excised intestinal tissue.

Formulation B had a larger mean particle size (157.3 ± 31.74 µm) when compared to that of Formulation F (135.7 ± 41.05 µm). In general, the inclusion of TMC at a higher concentration resulted in lower D[4.3] and d(0.5) values. Both insulin and TMC were released at a relatively rapid rate from both formulations. The MDT values and AUC values (i.e. the extent of insulin release) from these formulations were similar as no statistically significant differences (p-value > 0.05) in these two parameters were found. Also, the similarity factor values confirmed the similarity of the dissolution profiles of Formulations B and F with regard to insulin as well as TMC release. The MDT of insulin were on average less than 45 min and for TMC less than 7 min illustrating the faster release of TMC compared to insulin. With regard to enteric behaviour it has been shown that both microsphere Formulations B and F released a significant amount of insulin, 51.79% and 63.08%, respectively after 1 hour of exposure to 0.1 M HCl. Although no pores or cracks are visible on the surface of the spheres, it is thus possible that insulin close to the microsphere surface could have leached out into the acidic medium or the use of the centrifuge for separating solid particles could have facilitated additional release giving rise to the high percentage insulin released.

CHAPTER FOUR

In vitro transport across excised intestinal tissue

4.1 Introduction

For the purpose of this study, Sweetana-Grass side-by-side diffusion cells were used to evaluate the release of insulin from two selected microsphere formulations (i.e. B & F) and the transport behaviour across excised intestinal tissue of Fischer (FSR) rats. The effect of the microsphere formulations on the transepithelial electrical resistance (TEER) of the excised rat intestinal tissue was also determined to indicate their ability to open tight junctions to allow for paracellular transport.

The selected formulations as well as a control (insulin only) were evaluated by determining the cumulative transport (% of initial dose) of insulin in the apical to basolateral direction across the excised intestinal tissue as a function of time. The TEER was measured in parallel with withdrawals as an indicator to determine paracellular transport as well as the viability of the tissue. Additionally, the results obtained were processed using calculations and statistical analysis to compare the results of the test groups with the control in order to indicate significant differences.

4.2 Sweetana-Grass diffusion apparatus

The Sweetana-Grass diffusion apparatus consists of twelve half-cells that are clamped together side-by-side to hold the tissue membrane in between, forming six cells each with an apical and basolateral chamber. A circulation path by means of gas flow was provided in each half-cell for disturbance of the fluid to prevent stagnant layer formation and for maximum exposure of the fluid to both sides of the membrane. The assembled chambers were placed vertically in a heating block, which consists of a front and back plate in order to maintain the cell blocks at a desired temperature (Sweetana *et al*., 1993:1). A NaviCyte Snapwell vertical diffusion chamber system (Costar catalogue no 3436) was used for the transport studies (see Figure 4.1).

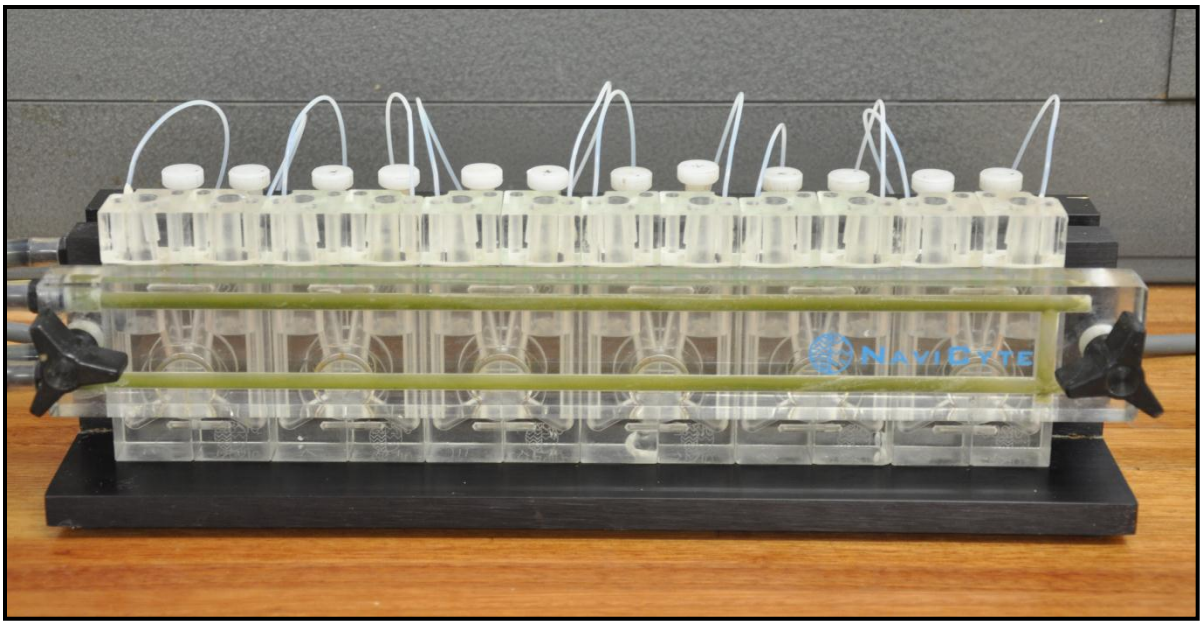


Figure 4.1: Picture of the assembled Sweetana-Grass diffusion chambers in the heating block and gas manifold

4.2.1 Reagents and chemicals

Krebs-Ringer bicarbonate (KRB) buffer and recombinant human insulin were purchased from Sigma-Aldrich (Kempton Park, South-Africa). Halothane was purchased from Safeline Pharmaceuticals (Roodepoort, South Africa). Sodium bicarbonate was purchased from Labchem (Edenvale, South Africa). The KRB buffer was prepared prior to each transport study by adding the entire contents of the KRB mixture (Sigma-Aldrich, South Africa) to 90% of the final required volume of water and stirred until dissolved. In addition, 1.26 g sodium bicarbonate purchased was added and stirred until dissolved, followed by pH adjustment to 7.4 (if needed) and the addition of water to bring the solution to the final volume (1000 ml). The KRB buffer was then refrigerated until needed.

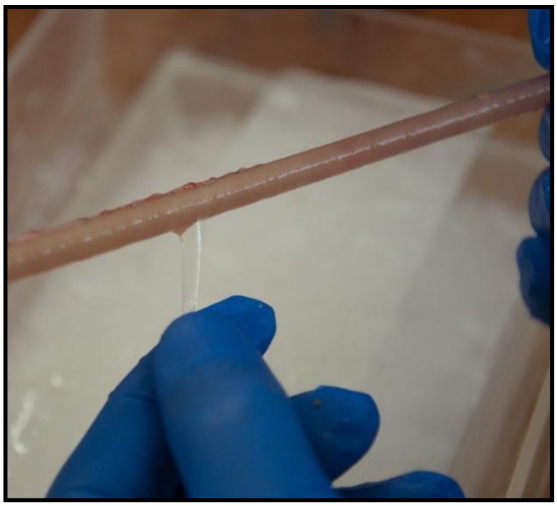
4.3 Preparation of rat intestinal tissue

Adult male FSR rats (400-450 g) were obtained from the Animal Research Centre (North-West University) and approved under protocol number NWU-0018-09-A5. All animals had free access to food and water prior to sacrifice and were selected at random. For preparation of tissue segments, rats were anaesthetised with halothane and an incision was made in the thorax, to ensure death. Furthermore, an incision was made in the abdomen to expose the small intestine and a 20-30 cm piece of proximal jejunum (10 cm from the pylorus sphincter of the stomach) was excised and washed thoroughly with ice-cold KRB buffer (±10 °C). Thereafter, the intestinal tissue was pulled over a glass rod (Figure 4.2a and b).

(a) (b)

Figure 4.2: Pictures illustrating excised piece of rat jejunum. a) Flushing out intestinal contents with cold KRB and b) puling the intestinal tissue onto a glass rod

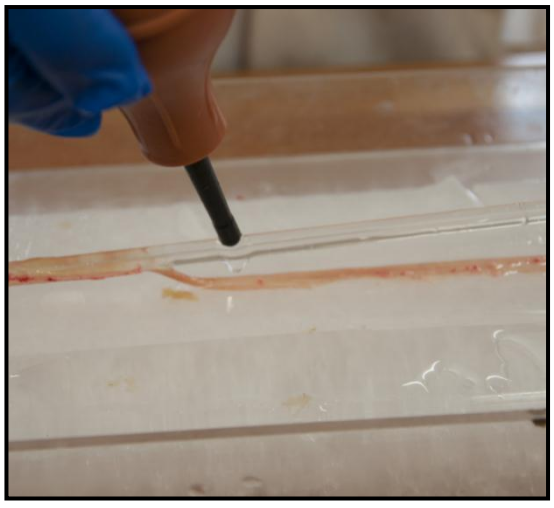
The excised tissue was then gently scoured along the mesenteric border by blunt dissection in order to remove the serosa and muscle layer by using the index finger along the length of the jejunum (Figure 4.3c and d).



(c) (d)

Figure 4.3: Pictures illustrating c) scouring of the tissue by blunt dissection and d) removal of the serosal layer from the rat jejunum with the index finger and thumb

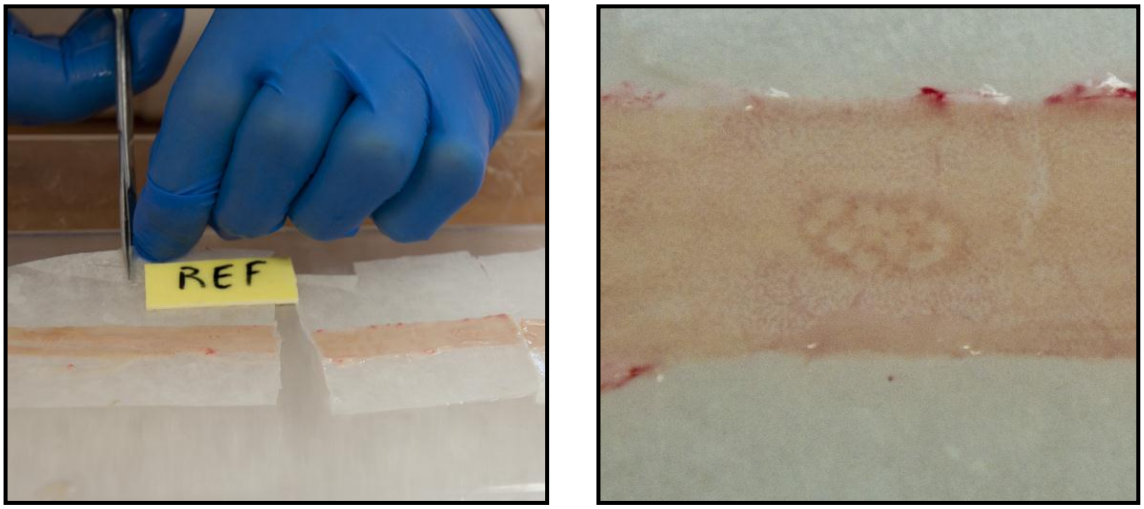
Throughout the procedure, the intestinal tissue was kept moist with cold KRB buffer and was kept in an ice bath. The excised jejunum was then cut open along the mesenteric border with a scalpel blade and washed off the glass rod with KRB onto a strip of filter paper to form a flat epithelial sheet (Figure 4.4e and f).



(e) (f)

Figure 4.4: Pictures illustrating e) cutting of the jejunum along the mesenteric border and d) washing the tissue off the glass rod onto a strip of filter paper

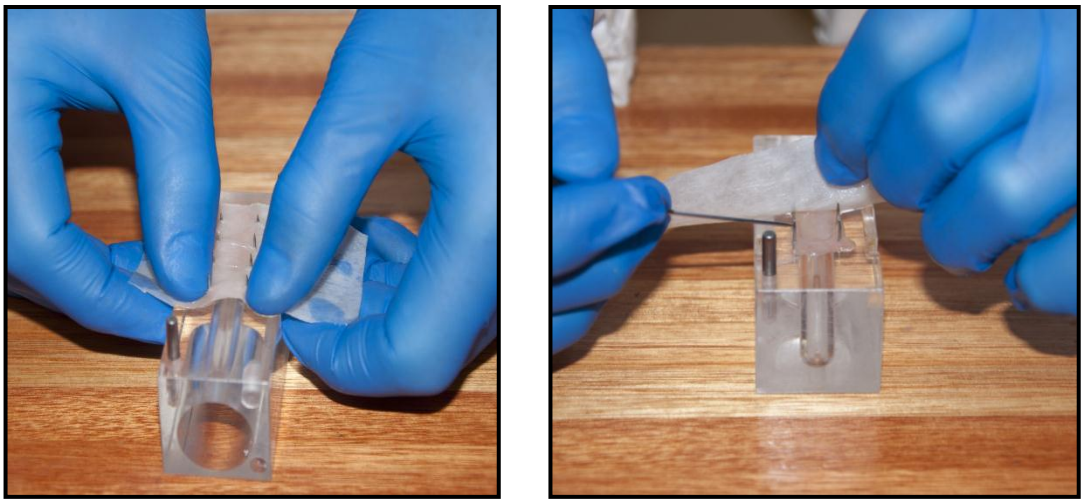
The excised tissue and filter paper was then cut simultaneously into pieces of approximately 3 cm in length (Figure 4.5h). Segments containing Peyers patches were identified visually and avoided (Figure 4.5i) as their morphology and thickness may cause greater variation in the rates of transport between compartments.



(h) (i)

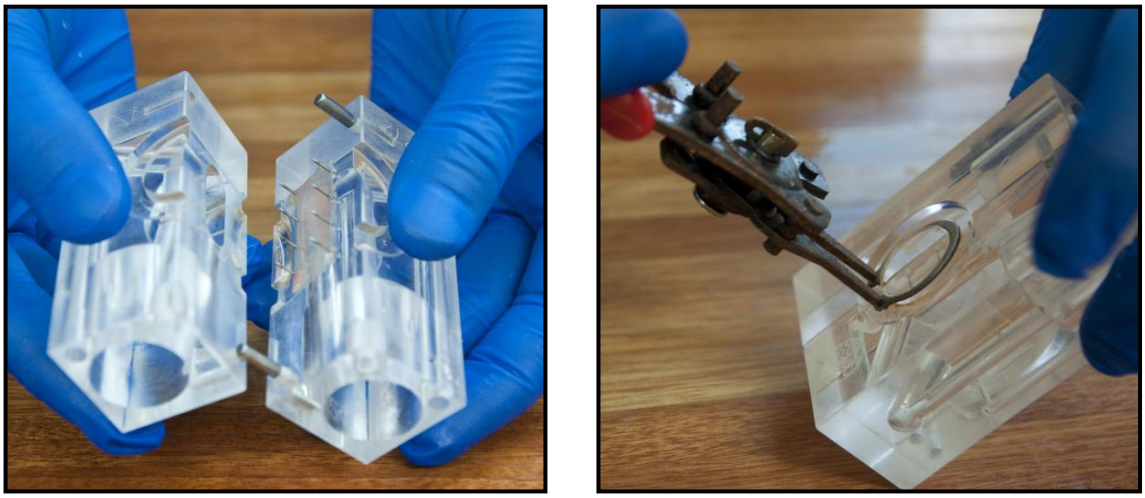
Figure 4.5: Pictures illustrating h) the cutting of the flat sheet of jejunum into pieces and i) an example of a segment containing Peyers patches

The segments were then carefully mounted onto the pins of the preheated (37 ºC) half-cells with the filter paper facing upwards and the apical side downwards (Figure 4.6j). The filter paper was used to minimise direct contact so as to maintain tissue viability, where after it was carefully removed and the matching half-cells were clamped together using a metal ring (Figure 4.5k). Assembled cells were placed into the heating block (preheated to 37 °C) and 7 ml of preheated (37 °C) KRB buffer were added to each compartment. Circulation as well as oxygenation of buffer was maintained by a gas lift of 95% O_2 : 5% CO_2 at a flow rate of approximately 15 ml/min. After the cells were placed in the heating block with their gas supply, it was left for 15 min for the tissue to adapt to the environment and reach a state of equilibrium. The entire procedure from the first incision till the system reached a state of equilibrium was performed within 45 min.



(j) (k)

Figure 4.6: Pictures illustrating the j) mounting of tissue onto the pins of the half cell and k) removal of the filter paper



(l) (m)

Figure 4.7: Pictures illustrating the assembling of the matching half cells

4.4 Permeation studies

The transport of insulin across excised rat jejunum was determined in the apical to basolateral (AP-BL) direction as a function of time. The KRB buffer in the chambers was completely removed from both sides using an Integra vacusafe aspiration system (Labotec, South Africa) and replaced by a volume of 7 ml of the microsphere suspensions in KRB buffer (pH 7.4) at a insulin concentration of 170 µg/ml on the apical side and 7 ml KRB buffer on the basolateral side. The microsphere dispersion remaining after the transport study was sonicated and used as 100% concentration (time 0 or t_0) to calculate the percentage of the dose transported across the excised tissue at each time point. Furthermore, samples of 200 µl were withdrawn every 20 min from the basolateral side from all six chambers over a period of 120 min. The TEER was measured along with each sample withdrawal using a Millicell® ERS-2 epithelial volt-ohm meter (Millipore Corporation, Germany) in order to evaluate the integrity of the excised tissue as well as the effect of the TMC (i.e. opening of tight junctions). The samples withdrawn from the basolateral side were immediately replaced with an equal volume of KRB buffer (preheated to approximately 37 °C).

A negative control group was included in the experimental design to determine the transport of insulin without the polymer microparticulate delivery system or TMC as permeation enhancer. The control experiment was used to indicate that the effects seen with the TMC and polymer delivery system were caused by their presence and not by chance interferences or external factors. After each procedure, samples were placed in HPLC vials and frozen at -86 °C until analysed by means of a validated HPLC method (section 3.5.1). In previous studies it was found that a period of 120 min is the optimum period over which *in vitro* transport experiments could be conducted using excised stripped intestinal tissue (Hattingh, 2002:63). The concentration of the transport samples was corrected for dilution and expressed as cumulative transport (% of initial dose). To further compare the absorption enhancement obtained, apparent permeability coefficient values (P_{app}) for insulin as well as transport enhancement ratios were calculated according to equation 4.1 and 4.2 respectively. The P_{app} is an index widely used as part of a general screening process to study drug absorption and is defined as the initial flux of a compound through the membrane, normalised by membrane surface area and donor concentration (Palumbo *et al.,* 2008:235).

$$P_{app} = \frac{dQ}{dt}\left\{\frac{1}{(A.60.C_0)}\right\}$$ Equation 4.1

Where:

P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), $\frac{dQ}{dt}$ is the permeability rate (amount permeated per minute), A the diffusion area of the excised tissue (cm^2), and C_0 the initial concentration of the marker molecule.

Transport enhancement ratios (R) were calculated from P_{app} values by:

$$R = \frac{P_{app(Sample)}}{P_{app(Control)}}$$ Equation 4.2

4.5 Results and discussion

The percentage cumulative transport of insulin released from the two optimised microsphere formulations (i.e. Formulation B and F) and insulin alone (control group) across the excised rat jejumum is presented in Figure 4.8.

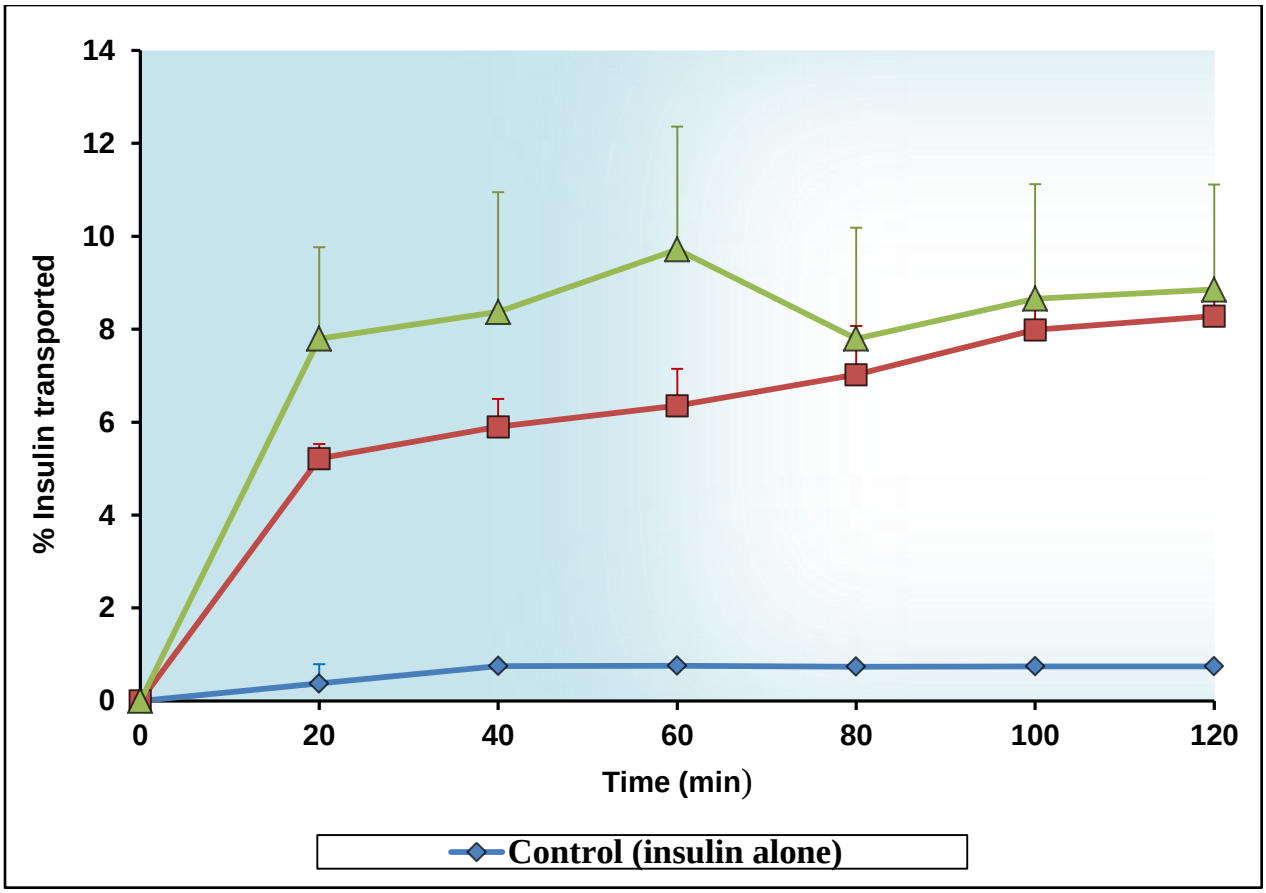


Figure 4.8: Cumulative transport (% of initial dose) of insulin across excised rat tissue plotted as a function of time

From Figure 4.8 it is clear that both formulations showed increased transport of insulin across the excised rat intestinal tissue compared to the control group. Formulation B showed 8.3 ± 0.52% and Formulation F 8.9 ± 2.26% transport of the initial insulin dose (t_0) after a period of 120 min. Insulin transport from the TMC containing microsphere formulations when compared to each other showed no statistical significant difference (p-value > 0.05). The insulin transport from the microsphere formulations was statistical significant higher (p-value < 0.05) compared to the 0.7 ± 0.02% transport of the control group (insulin only). The improved transport of insulin can be attributed to the inclusion of TMC in the microspheres. As mentioned in chapter 2, TMC acts as an absorption enhancer by the reversible opening of the tight junctions thereby enhancing paracellular transport. To confirm the effectiveness of the TMC from the microspheres, the TEER was measured which is a well-established indicator for the tightness of the tight junctions between cells. TEER is also widely accepted for monitoring the viability and

integrity of intestinal tissue segments in the Ussing chamber (Kotzé *et al*., 1997:250, Polentarutti *et* al., 1999:446-454; Thanou *et al*., 2000a:21-22). The percentage TEER from the two optimised microsphere formulations and control group plotted as a function of time is presented in Figure 4.9, with corresponding values in Table 4.1.

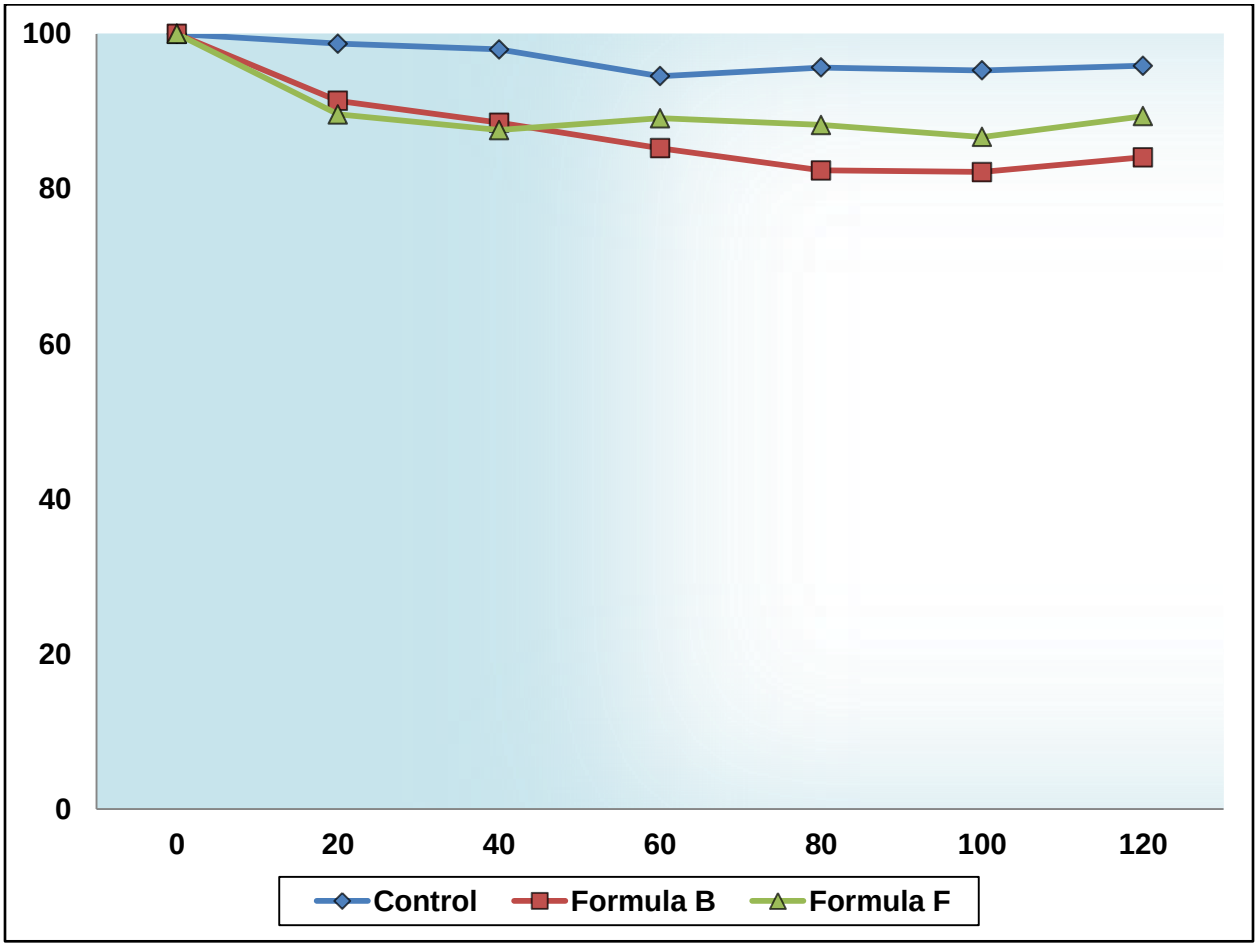


Figure 4.9: The transepithelial electrical resistance (TEER) of excised rat intestinal tissue treated with microsphere Formulations B and F as well as insulin alone (control group) plotted as a function of time

From Figure 4.9 and Table 4.1 it is evident that the percentage reduction in TEER caused by the TMC containing microsphere formulations is more pronounced than that of the control group. Formulation B showed a 15.9 ± 2.22% and Formulation F a 10.6 ± 5.29% reduction in TEER after a period of 120 min. In contrast, the control group lowered the TEER by only 4.11 ± 2.11%. Therefore, it is possible to associate the higher transport of insulin from Formulations B

and F with the reduction in TEER indicating the opening of the tight junctions, thus enhancing paracellular transport of insulin.

Table 4.1: Reduction in TEER of excised rat intestinal tissue treated with insulin only (control group) and microsphere Formulations B and F

TEER			
Time (min)	**Control (insulin only)**	**Formulation B**	**Formulation F**
0	100	100	100
20	98.7 ± 4.30	91.4 ± 3.95	89.6 ± 4.92
40	98.0 ± 2.35	88.5 ± 2.41	87.6 ± 5.32
60	94.6 ± 3.88	85.3 ± 5.60	89.1 ± 3.45
80	95.7 ± 3.40	82.4 ± 3.86	88.3 ± 4.82
100	95.3 ± 1.94	82.2 ± 2.42	86.7 ± 6.39
120	95.9 ± 2.11	84.1 ± 2.22	89.4 ± 5.29

The charge density of TMC, as determined by the degree of quaternization, plays an important role in its ability to reduce TEER as well as its absorption-enhancing properties. In this regard, Thanou *et al*. (2000b:27-31) evaluated TMC of high degrees of quaternization (39% and 63%) as intestinal permeation enhancers for the peptide drug buserelin *in vitro* using Caco-2 cell monolayers and *in vivo*, in rats. Transport of buserelin across Caco-2 cell monolayers was increased 17- and 46-fold by the co-administration of TMC (1% w/v) with a degree of quaternization (DQ) of 40 and 60%, respectively. Intraduodenal co-administration of buserelin with the same polymers resulted in an 8- and 16-fold increase respectively, in the absolute bioavailability in comparison to the buserelin control group. Furthermore, Hamman *et al*. (2003:161-172) studied the effect of six different TMC polymers (DQ between 12 and 59%) on the TEER and transport of hydrophilic as well as macromolecules ([^{14}C]mannitol and [^{14}C]PEG 4000 respectively) across Caco-2 cell monolayers. It was found that TMC polymers with a DQ higher than 22% were able to reduce TEER at neutral pH values. Also, the maximum reduction in TEER (47.3 ± 6.0%) as well as the maximum transport of model compounds (25.3% of initial dose for [^{14}C]mannitol and 15.2% of the initial dose for [^{14}C]PEG 4000) was obtained for TMC with a DQ of 48%, and these effects did not increase at higher DQ values. The DQ (i.e. 42%) of

TMC incorporated in the microsphere formulations in this study was sufficient to increase the transport of insulin across rat intestinal tissue at a neutral pH value of 7.4.

Apart from the DQ, the concentration of TMC also has a significant effect on the absorption of a peptide drug. This has been demonstrated by Thanou *et al.* (2001b:823-828) which investigated the enhancing absorption effect of TMC (DQ = 60%) on the absorption of intrajejunal administered octreotide as model drug. Co-administration of octreotide with 5 and 10% (w/v) TMC at pH 7.4 resulted in a 7.7- and 14.5-fold increase of octreotide absorption, respectively. This corresponded to an absolute bioavailability of 13.9 ± 1.3% and 24.8 ± 1.8%, respectively. Administration of an octreotide solution without TMC resulted in an absolute bioavailability of 0.5 ± 0.6%. This illustrates the significant effect of including TMC and the effect of the selected concentration on the absorption of peptide drugs. Microsphere Formulations B and F provided TMC at concentrations of 61.25 µg/ml and 64.07 µg/ml, respectively based on percentage loading. These concentrations proved to be sufficient to cause substantial enhancement in the paracellular transport of the insulin across excised rat jejunum. The calculated P_{app} for insulin is presented in Figure 4.10, with corresponding values in Table 4.2 including calculated transport enhancement ratios.

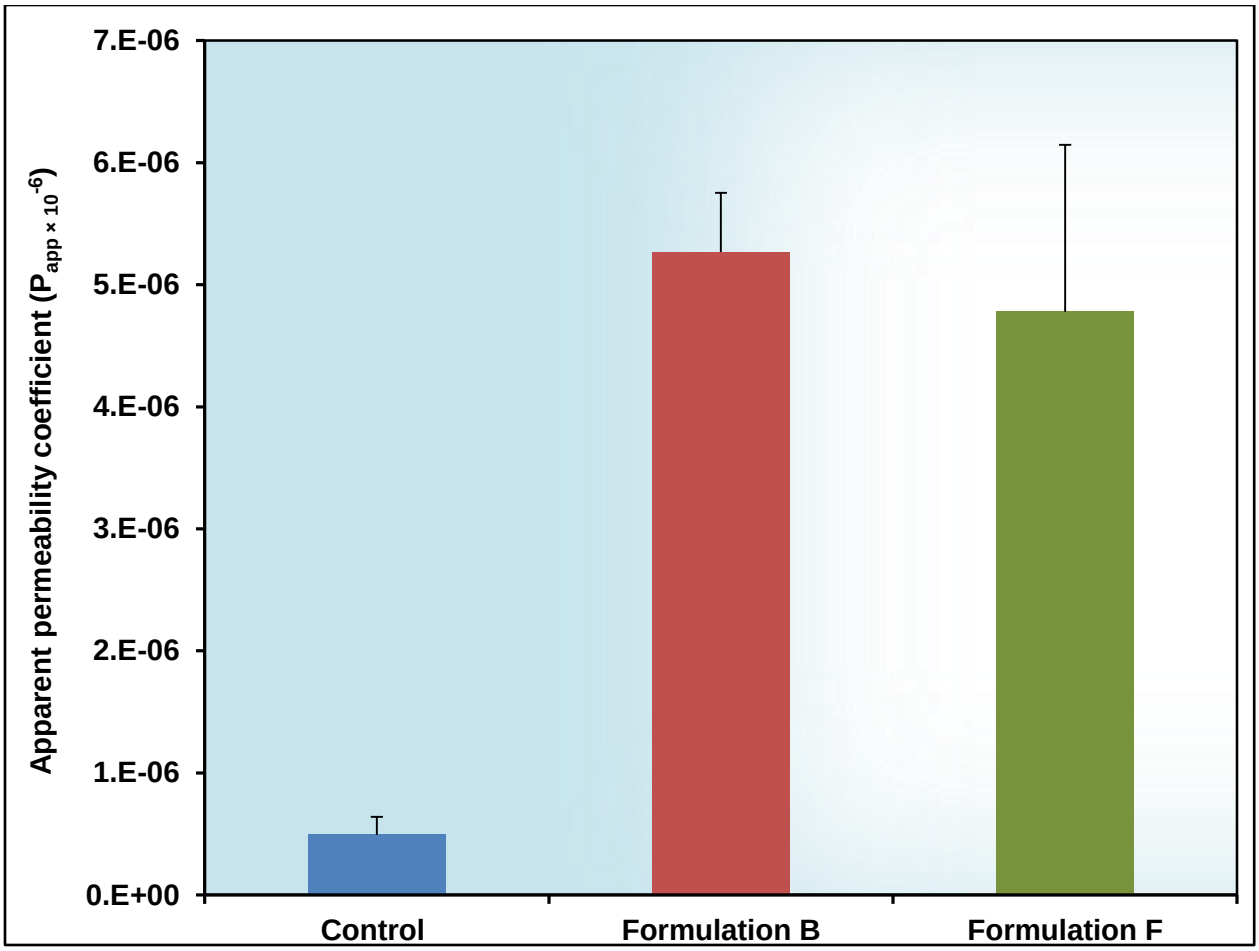


Figure 4.10: P_{app} values calculated from insulin transport of the control group (insulin only) and TMC containing microsphere formulations

Table 4.2: Apparent permeability coefficients (P_{app}) and transport enhancement ratios

Group	P_{app} value ($cm.s^{-1}$)	Enhancement ratios
Control group	$4.9 \pm 1.46 \times 10^{-7}$	1.00
Formulation B	$5.3 \times 10^{-6} \pm 4.85 \times 10^{-7}$	10.67
Formulation F	$4.8 \pm 1.37 \times 10^{-6}$	9.68

From Figure 4.10 and Table 4.2 it is clear that the P_{app} values as well as the enhancement ratios demonstrated the significant improvement in the transport of insulin released by the TMC containing microsphere formulations across excised rat intestinal tissue, when compared to the control group. The apical to basolateral flux of Formulations B and F, with P_{app} values of 5.3 x

10^{-6} ± 4.85 x 10^{-7} cm.s^{-1} and 4.8 ± 1.37 x 10^{-6} cm.s^{-1}, respectively was approximately 10-fold higher than that for the control group (P_{app} = 4.9 ± 1.46 x 10^{-7} cm.s^{-1}).

4.6 Summary and conclusion

The permeation studies on excised rat jejunum illustrated that both microsphere formulations (i.e. Formulation B and F) caused a significant enhancement in the transport of insulin in comparison to the control (insulin only). The microsphere formulations also reduced the TEER of the excised rat intestinal tissues and thereby exhibited the ability to open tight junctions and thereby allow for paracellular transport of insulin. The improved insulin transport could be attributed to the inclusion of TMC in the microsphere formulations. Therefore, not only was TMC successfully released from both formulations, the DQ as well as the concentration used was sufficient to cause an increase in the intestinal transport of insulin.

CHAPTER FIVE

Summary and future recommendations

5.1 Summary

Oral ingestion is by far the most widely and preferred route of drug administration ensuring good compliance and convenience to patients. This is because oral administration avoids the pain and discomfort of daily injections as well as the possibility of infections associated with the parentarel route of drug administration. Unfortunately, the oral route is generally not feasible for protein and peptide delivery due to the unfavourable properties associated with these drugs. These unfavourable properties include susceptibility to enzymatic and chemical degradation, low lipophilicity and large molecular size. Consequently, the oral bioavailability of many peptide drugs is low and typically ranges between 1 and 2% of the administered dose. Furthermore, low bioavailability leads to high inter-subject variability and poor control over plasma concentration as well as pharmacological effects (Pauletti *et al*., 1996:3).

Advances in biotechnology, chemistry and molecular biology led to the production of large quantities of structurally diverse therapeutic peptides and proteins on a commercial scale. These developments increased the need for novel oral delivery systems (Pauletti *et al*., 1996:3). To overcome the obstacles associated with oral peptide/protein delivery, various strategies have been investigated to increase the bioavailability of proteins and peptides such as the use of absorption enhancers; enzyme inhibitors; the development of pro-drugs and peptidomimetics; controlled structural modifications such as PEGylation and lipidisation; and targeting of transporters, tissues and absorption through the paracellular route depending on the drug (Hamman *et al*., 2005:168-174)

The controlled and reversible opening of the tight junctions represents an attractive approach to increase the paracellular absorption of hydrophilic compounds such as peptide and protein drugs, especially in view of the fact that degradation by intracellular enzymes is avoided. The paracellular route is an aqueous-filled channel with an estimated surface area of approximately 200-2000 cm^2 and should not be underestimated for peptide and protein delivery since even minute quantities (pM - nM range) may be sufficient to produce the required biological effect (Salamat-Miller & Johnston, 2005:203). Significant improvement in the oral bioavailability of certain peptide drugs such as heparin, insulin and calcitonin have been achieved so far by

targeting the paracellular route. An attractive group of compounds which enhances paracellular drug transport is chitosans and its derivatives, which opens epithelial tight junctions reversibly in a concentration- and pH-dependent manner. Also, chitosan and its derivatives such as TMC are considered safe, biodegradable and non-toxic which make them ideal for pharmaceutical use (van der Merwe, 2004:226-234).

In this study, microspheres were prepared from Eudragit® L100, containing insulin as model peptide drug as well as an absorption enhancer, TMC, using a single water-in-oil (w/o) emulsification/evaporation method in accordance with a 2^3 fractional factorial design. The factorial design was used to investigate the combined influence of three variables: concentration of the Eudragit® L100 polymer, insulin as well as TMC. Formulation conditions included Eudragit® L100 (3.5 and 7.5% w/w) at different concentrations dissolved in ethanol as solvent system; a binary emulsifier system consisting of Tween® 80 (1% w/w) and Span® 80 (1.5% w/v), and a stirring rate of 1000 rpm. Microspheres were subsequently characterised in terms of morphology and internal structure by means of scanning electron microscopy, encapsulation efficiency of insulin as well as TMC, which were determined using a validated HPLC method and colorimetric assay respectively.

Electron microscopic characterisation revealed that most formulations showed a spherical shape and smooth surface with a sponge-like internal structure as well as relatively good homogeneity in terms of size distribution. Although, when high concentrations of Eudragit® L100 (7.5% w/w), TMC (10% w/w) and insulin (2% w/w) were used in a single formulation (Formulation E), the shape of the spheres was irregular, probably due to the increased viscosity of the internal phase. Insulin as well as TMC was successfully incorporated into the microsphere matrix of all formulations. Insulin encapsulation ranged from 27.9 ± 14.25 – 52.4 ± 2.72% between the different formulations with higher drug loading corresponding to the use of 2% w/w insulin. TMC loading was lower than that of insulin and ranged from 29.1 ± 3.3 - 37.7 ± 2.3% between the different formulations.

From the results obtained with the characterisation of the microsphere formulations, two formulations were selected (i.e. B and F) for further characterisation in terms of particle size distribution, dissolution behaviour, enteric nature, and *in vitro* transport of insulin across excised rat intestinal tissue in a Sweetana-Grass diffusion chamber.

Particle size analysis of the selected formulations revealed that the mean particle size (based on volume) was 135.7 ± 41.05 for Formulation F and 157.3 ± 31.74 μm for Formulation B. Dissolution results revealed that both insulin and TMC released from the microsphere formulations in a neutral environment (pH 7.4). MDT for insulin ranged from 34.5 ± 4.01 to 42.6 ± 9.06 min, while the MDT for TMC ranged from 1.2 ± 1.73 to 6.8 ± 6.42 min for Formulations B and F, respectively. ANOVA revealed no significant differences in the MDT of either insulin or TMC (p-value > 0.05) between the two formulations, although the difference between insulin and TMC of each formulation was significant (p-value < 0.05). This may be beneficial, since TMC will be able to open the tight junctions prior to insulin release, which may minimise insulin lost through intestinal transit.

Evaluation of the enteric nature revealed that a significant amount of the drug content is lost after one hour of exposure to 0.1 M HCl. Formulation B and F released 51.79% and 63.08% of their total drug content respectively. This was probably due to superficially located insulin which leached through the thin enteric coating into the acidic medium or due to agitation during the use of the centrifuge. From the results it is clear that the Eudragit® L100 microspheres possessed some enteric properties although the extent of protection will not be sufficient to overcome the harsh environment of the stomach with an amount of protein drug sufficient to cause a therapeutic effect.

Transport studies across excised rat jejunum demonstrated that the inclusion of TMC is a prerequisite for insulin transport, indicating paracellular transport. The cumulative transport of insulin from Formulation B was 8.3 ± 0.52%, while it was had 8.9 ± 2.26% for Formulation F of the initial insulin dose after a period of 120 min. The insulin transport from the microsphere formulations was statistical significant higher (p-value < 0.05) compared to the 0.7 ± 0.02% transport of the control group (insulin only). The transport of insulin from Formulations B and F represented transport enhancement ratios of 10.67 and 9.68, respectively. The increase in cumulative transport correlated with a decrease in transepithelial electrical resistance (TEER). Formulation B showed a 15.9 ± 2.22% and Formulation F a 10.6 ± 5.29% reduction in TEER after a period of 120 min. In contrast, the control group lowered the TEER by only 4.11 ± 2.11%. Therefore, it is possible to associate the higher transport of insulin from Formulations B and F with the reduction in TEER indicating the opening of the tight junctions, thus enhancing paracellular transport of insulin.

In conclusion, microspheres was successfully produced by means of emulsification/solvent evaporation consisting of Eudragit® L100 containing both TMC and insulin. The microsphere delivery system lacked sufficient protection of insulin against a hostile acidic environment, but proved to be effective in targeting the paracellular transport pathway *in vitro* for insulin delivery.

5.2 Future recommendations

This study gave promising results with regard to the delivery of a peptide drug using an enteric microsphere delivery system in combination with an absorption enhancer. It is recommended that parameters during preparation affecting encapsulation, release and enteric properties should be further explored to improve the performance of the microspheres. These parameters include the volume and viscosity of the internal phase, the use of higher concentrations as well as different grades or mixtures of Eudragit® (L, S and FS) for matrix formation and for coating of the microspheres, the extent and method of agitation and the effect of drug and/or excipient particle size. The developed microsphere delivery system should be evaluated as a delivery system for other peptide drugs (e.g. vasopressin, calcitonin or buserelin). Due to the multivariate processes involved in drug absorption from the intestine, it is difficult to use just one *in vitro* model to accurately predict the *in vivo* absorption characteristics, it is therefore recommended to use different *in vitro* models as well as different animal tissues to confirm the results obtained in this study (Balimane *et al.*, 2000:301). Furthermore, the results obtained were *in vitro* only, but *in vivo* evaluation is necessary to determine if inclusion of TMC into the microspheres can clinically significantly increase insulin bioavailability. When conducting *in vivo* pre-clinical experimentation, it is suggested that the microspheres are filled into hard gelatine capsules for administration purposes as the mechanical stress of tableting could possibly damage the microspheres in terms of morphology and negatively impact the enteric properties.

Annexures

Annexure A

A.1 Validation of HPLC method

Validation of the HPLC method was necessary to demonstrate that the analytical procedure is simple to use and capable of detecting and quantifying insulin in PBS. A number of parameters were determined to validate the analytical procedure namely specificity, linearity, accuracy and precision.

A.1.1 HPLC analysis of insulin

The analysis of Insulin was done with an Agilent high pressure liquid chromatograph. An analytical method for insulin quantification was reproduced from the method used by Steenekamp, (2007:64). The apparatus and conditions are summarised in Table A.1.

Table A.1: HPLC system and conditions

Hardware and analytical conditions	Specifics
Software	Chemstation Rev. A.08.03 data acquisition and analysis software (Agilent Technologies, Japan).
HPLC pump	Agilent 1100 Series, G1310A Isopump, Serial # DE14904334
HPLC fluorescence detector	RF-551 Spectrofluorometric detector
Detection wavelength	UV at 210 nm
HPLC autosampler	Agilent 1100 Series, G1313A ALS, Serial # DE14918029
HPLC column	Jupiter C18-column, 250 x 4.6 mm, 5µm spherical particles, 300 Å pore size, 13.3% carbon load, endcapped (Phenomenex, Torrance, California, USA).
Sample volume injected	50 µl
Flow rate	1 ml/min

Mobile phase	Gradient elution with a mobile phase that consisted of acetonitrile (A) and 0.1% w/v of orthophosphoric acid (B) was employed.
Temperature	23 °C (Room temperature)

A.2 Method validation

A.2.1 Specificity

The specificity of the analytical procedure can be defined as the ability of the procedure to access unequivocally the analyte in the presence of components expected to be present, such as impurities, degradation products and matrix components (USP, 2007:681).

Insulin was determined in the presence of all the possible excipients. An example of a chromatogram showing the insulin peak (retention time approximately 5.9 minutes) in the presence of the formulation excipients (Eudragit® L100 and TMC) is shown in Figure A.1.

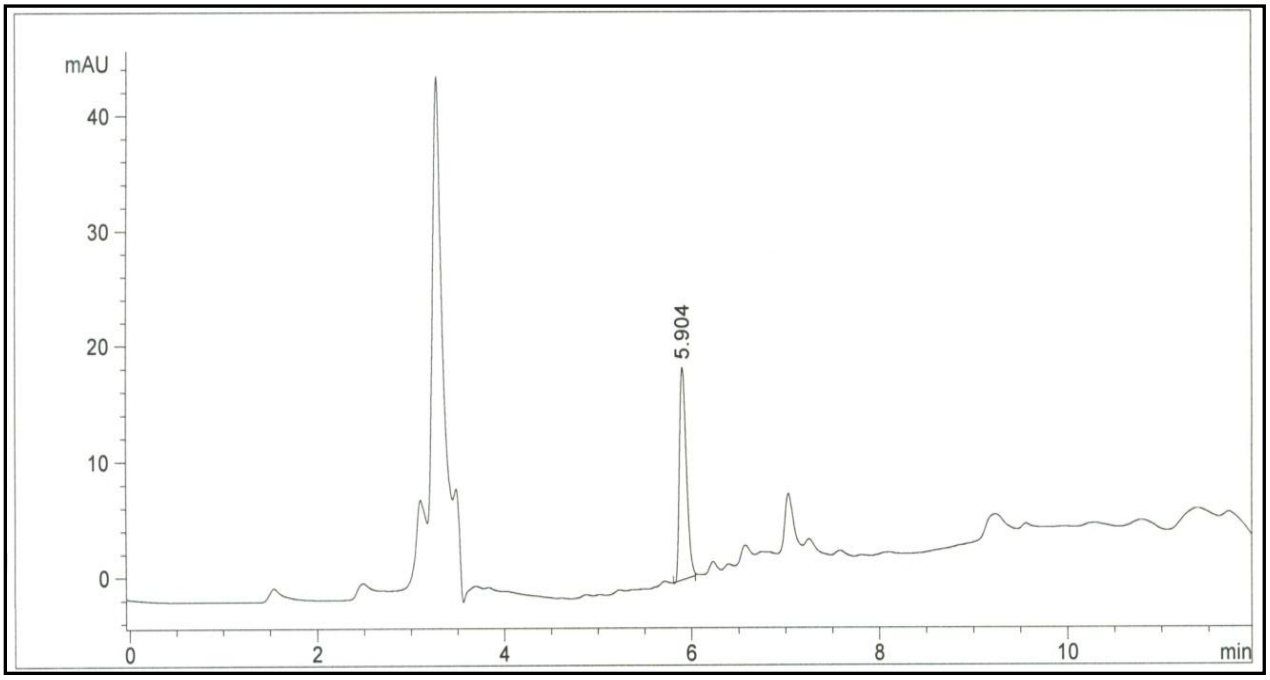


Figure A.1: Chromatogram showing the insulin peak in the presence of formulation excipients

From figure A.1 it is evident that the formulation excipients did not interfere with the insulin peak, and this proves the specificity of the analytical method for insulin.

A.2.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of an analyte in the sample (ICH guidelines, 1996). To determine linearity over the expected concentration range of different insulin concentrations were analysed and the instrument response (peak area) versus concentration injected was evaluated for a linear relationship between insulin concentration and peak area by using linear regression. The preparation of standard solutions is described in section 3.5.2.3.

An example of a standard curve obtained during the validation of the analytical method is shown in Figure A2. In Table A.2 the regression results of three standard curves are given.

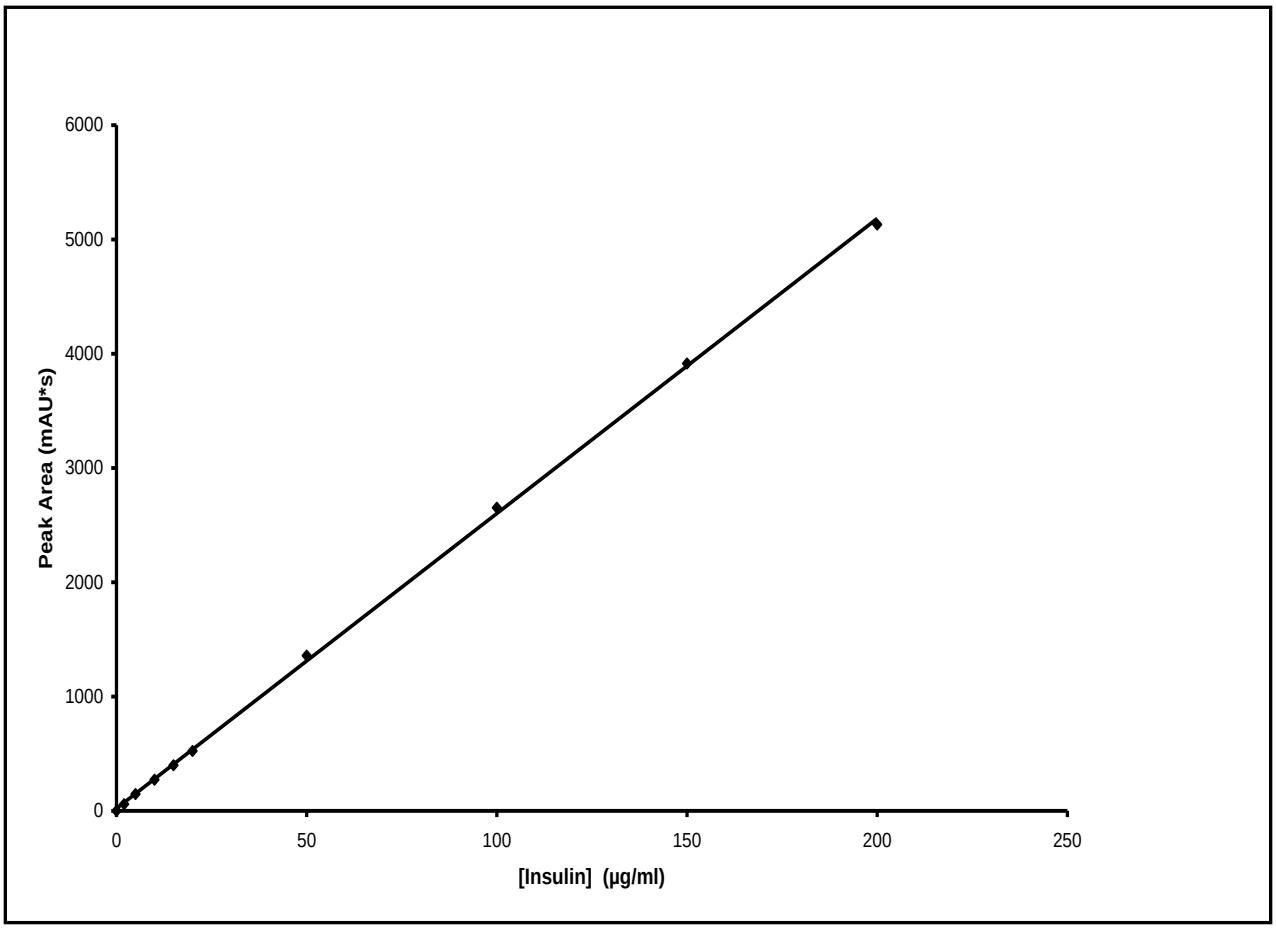


Figure A.2: Example of a standard curve obtained during validation of the analytical method

Table A.2: Regression results obtained for three standard curves during the validation of the analytical method

	Curve 1	Curve 2	Curve 3
Slope	25.775	25.5255	25.807
Y-intercept	25.653	-10.1372	21.361
R-squared value (R^2)	0.9997	0.9999	0.9997

From Figure A.2. and the regression results in Table A.2 it is evident that a linear relationship exists between insulin concentration and the instrument response (peak area) over the tested concentration range of 2-200 µg/ml

A.2.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (USP, 2007:681). Accuracy was determined by analysing three solutions with a low (2 µg/ml), intermediate (20 µg/ml) and a high (200 µg/ml) insulin concentration respectively. Three solutions of each concentration were prepared giving a total of nine samples. The spiked concentration values (reference values), obtained concentration values as well as the percentage insulin recovered are shown in Table A.3. The mean insulin recovery and relevant statistics for each spiked insulin concentration are shown in Table A.4.

Table A.3: Spiked concentration values (reference values), obtained concentration values as well as the percentage insulin recovered

	Low concentration (µg/ml)			Intermediate concentration (20 µg/ml)			High concentration (200 µg/ml)		
Spiked conc.	2	2	2	20	20	20	200	200	200
Obtained conc.	1.98	1.98	2.05	19.91	19.83	19.79	200.31	200.87	200.53
% Recovery	99.67	99.57	103.08	99.54	98.96	98.88	100.15	100.44	100.26

Table A.4: The mean insulin recovery for each spiked insulin concentration and coefficient of variation (%RSD) for each spiked insulin concentration

Statistics	Low concentration (2 μg/ml)	Intermediate concentration (20 μg/ml)	High concentration (200 μg/ml)
Mean insulin recovery	100.8 ± 2.00 %	98.99 ± 0.13 %	100.28 ± 0.15 %
%RSD	1.98	0.13	0.15

From the results it can be seen that the mean recovery ranged from 98.99 - 100.8% and the coefficient of variation was less than 2% and therefore the accuracy of the analytical method was considered acceptable

A.2.3 Precision

Precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (USP, 2007:681).

A.2.3.1 Intra-day precision

Intra-day precision was determined by preparing 3 sets of 3 samples with known insulin concentration at a low (2 µg/ml), intermediate (20 µg/ml) and high (200 µg/ml) concentration. The mean insulin recovery is shown in Table A.5.

Table A.5: Mean insulin recovery as percentage and coefficient of variation (%RSD)

	Low concentration (2 µg/ml)	Intermediate concentration (20 µg/ml)	High concentration (200 µg/ml)
Mean	101.6 ± 6.01%	103.5 ± 0.13%	103.2 ± 0.15%
%RSD	5.91	0.13	0.14

From the results it can be seen that the mean recovery ranged from 101.55 - 103.7% and the %RSD was not more than 6% and therefore the intra-day precision of the analytical method was considered acceptable.

A.2.3.2 Inter-day precision

Inter-day precision was determined by preparing one set of 3 samples with a known insulin concentration (200 µg/ml) on three different days. The mean insulin recovery (%), standard deviation and coefficient of variation are shown in Table A.6.

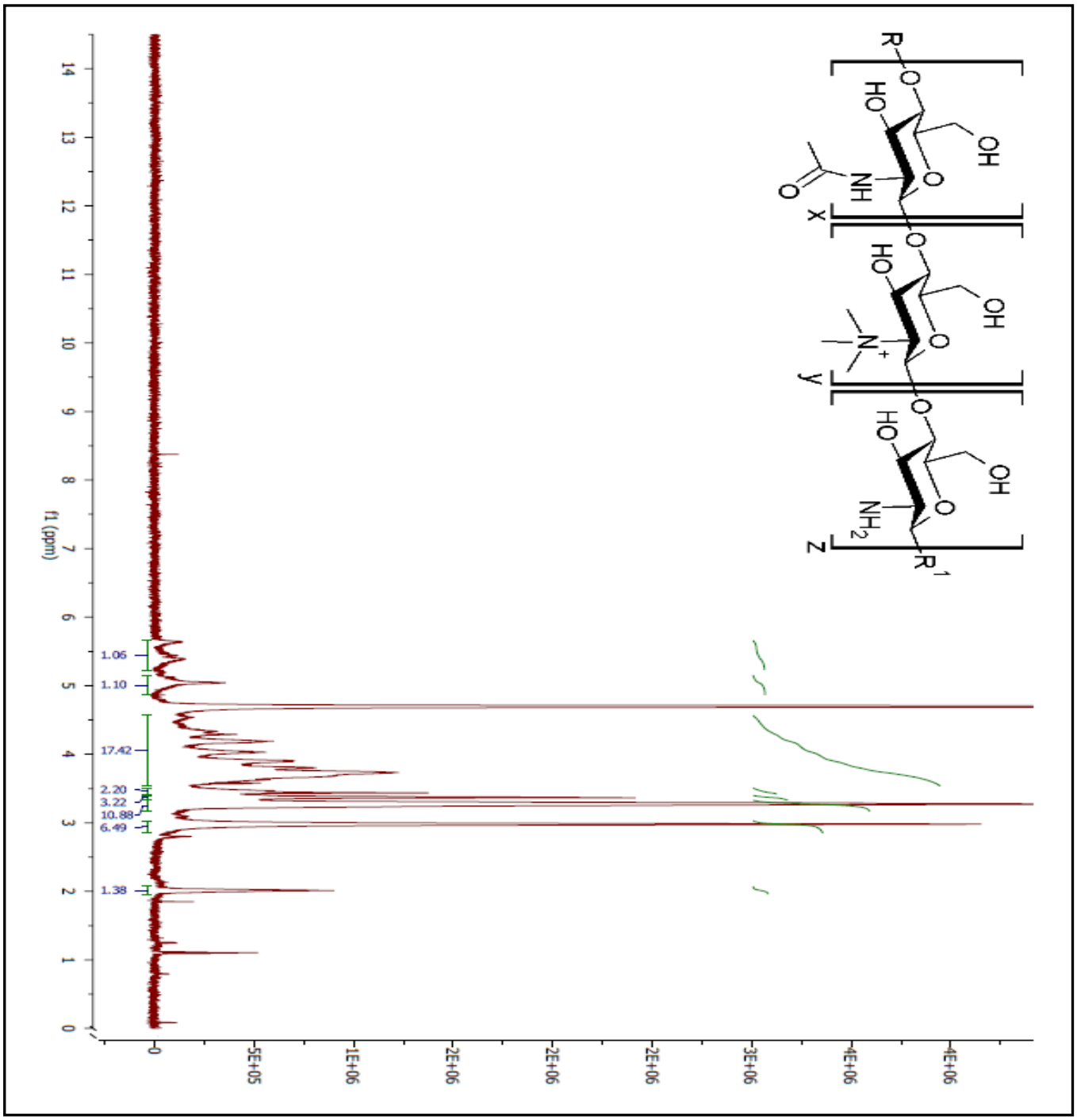
Table A.6: Mean insulin recovery expressed as a percentage, (%RSD) for three different days

	Day 1	**Day 2**	**Day 3**
Mean	103.21 ± 0.15%	99.81± 0.54%	97.85± 0.44%
%RSD	0.14	0.54	0.43

From the results it can be seen that the mean recovery ranged from 97.85 - 103.21% and the %RSD was not more than 1% and therefore the inter-day precision of the analytical method was considered acceptable.

Anexure B

^{1}H-Nuclear magnetic resonance (NMR) spectra of *N*-trimethyl chitosan chloride



Anexure C

primex

Certificate of analysis

Reanalysis 22.07.2010

Product name	ChitoClear® (Chitosan)
Source	*Pandalus borealis*
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine
Lot number	TM2832
Revision number	Rev. 03
Date of manufacture	5.1.2007
Retest date	5.1.2010
Extended esp. Date	5.1.2011

Manufactured by:
Primex ehf
Oskarsgata 7
580 Siglufjordur
Iceland
Ph: +354 460 6900
Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	86,9 %	
Ash	CP-002	0,4 %	
Turbidity	CP-003	126 NTU	
Viscosity	CP-004 (1% chitosan)	1066 cP (mPa·s)	
Solubility	CP-006	99,5 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	92 %	
Sieve Analysis	CP-008	100% through 18 mesh	
Tap Density	CP-009	0,3 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<1000 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<100 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	1,0 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,20 ppm
Lead	SW-846 6020 ICP/MS	<1,0ppm	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,01 ppm

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 22. 7. 2010.

Methods of analysis available upon request

NOTE: *Lack of colored logo indicates this document is a copy and is not controlled*

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>CERTIFICATE OF ANALYSIS <

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Evonik Pharma Polymers
Date 2012-02-08
Customer order item/date / 2012-02-08
Delivery item/date
33896141 000001 / 2012-02-16
Order item/date
23261467 000001 / 2012-02-08
Invoice number/date

Contact person/phone/fax/email
Silke Bernjus
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Product: EUDRAGIT L 100 / 400 G
Lot No.: B110603006
Quantity: 2 KG
Manufacturing Date / Production Date: 2011-07
Stability at least until end of: 2015-07

Test	Result	Units	Specification			
			INFO 7.3	Ph.Eur.	NF	JPE
Dry substance / Residue on evaporation	98,5	%	min. 95.0	N/A	N/A	N/A
Loss on drying	1,5	%	max. 5.0	max. 5.0	max. 5.0	max. 10.0
Methacrylic acid units, based on DS	48,8	%	46.0-50.6	46.0-50.6	46.0-50.6	38.0-52.0
Acid value	318	mgKOH/gDS	300-330	N/A	N/A	N/A
Viscosity / Apparent viscosity	80	mPa.s	60-120	50-200	60-120	N/A
Viscosity	18	mm2/s	10-24	N/A	N/A	10-24
Monomers	80	ppm	max. 500	max. 0.1 %	max. 0.05 %	N/A
Methyl methacrylate	4	ppm	N/A	N/A	N/A	max. 0.2 %
Methacrylic acid	76	ppm	N/A	N/A	N/A	N/A

Test methods are as prescribed in Specifications (Evonik Pharma Polymers (INFO 7.3) or referenced pharmacopoeias). Tests not listed above are performed on selected lots. Relevant data are available on request. The customer is not released from the obligation to conduct careful inspection and testing of incoming products.

Certificate of Analysis

Catalog Number: 91077C
Lot Number: 11D762-Z
Mfg Date: N/A
Exp Date: N/A

Reference:

Insulin, Human Recombinant

Test Name	Specification	Result
Activity (potency)	>/= 27.5 IU/mg (d.b.)	28.9 IU/mg (d.b.)
Appearance	White, crystalline powder	White, crystalline powder
Bioburden	</= 300 CFU/g	None detected CFU/g
Certificate of Analysis	Present, material complies to USP and EP	Present, material complies to USP and EP
Endotoxin	</= 1.0 EU/mg	<0.003 EU/mg
Growth Promotion/Cytotoxicity	>/= 75% Control; w/ Serum-free adapted cell line	101% Control; w/ Serum-free adapted cell line
Identification	Passes	Passes
ID-Peptide mapping	Passes	Passes
Limit of High Molecular Weight Protein	</= 0.4% (total of a-component + b-component)	0.1% (total of a-component + b-component)
Loss on Drying	</= 10.0%	8.3%
Related Compounds	</= 2% of the total insulin plus other related compounds	0.6% of the total insulin plus other related compounds
Solubility	Soluble @ 10 mg/mL in pH 2.0 - 2.5	Soluble @ 10 mg/mL in pH 2.0 - 2.5
Zinc	</= 1.00% (d.b.)	0.49% (d.b.)

Intended Use

For Research or Further Manufacturing Uses Only.
NOT INTENDED FOR DIRECT USE IN HUMANS OR ANIMALS.

Storage

-10 to -40 C

Signature Jennifer Cosens
Title Quality Assurance Associate
Date 26 JAN 12

Ref#: 91077C.00000004 20120126 150742

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