

**PERORAL DELIVERY OF CALCITONIN WITH PHEROID
TECHNOLOGY**

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***Praise be to the God and Father of
our Lord Jesus Christ,
Who has blessed us in the heavenly realms
with every Spiritual blessing in Christ
Eph 1:3***

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UITTREKSEL

Doel: Die doel van die studie was om die absorpsie verbeterende vermoë van Pheroid tegnologie en TMC (*N*-trimetiel chitosan chloried), 'n derivaat van chitosan, op die orale absorpsie van salmkalsitonien te evalueer.

Metodes: Die navorsingstrategie was om salmkalsitonien, 'n groot polêre peptiedgeneesmiddel met 'n bekende stabiliteitsprofiel en kwanitatiewe terapeutiese effek, alleen en in kombinasie met twee verskillende Pheroid formulerings en twee derivate van chitosan naamlik TMC en TMO, in die jejunum van Sprague-Dawley rotte toe te dien. Insluiting van kalsitonien in Pheroids is bevestig en geanaliseer met konfokale laser skanderingsmikroskopie. *In vivo* studies is gedoen met Sprague-Dawley rotte. Rotte is 24 ure voor die tyd gevas. Die *carotis communis* is gekanuleer vir die versameling van bloedmonsters op 0, 5, 10, 15, 30, 60, 120 en 180 minute. 'n Insnyding is gemaak in die abdomen van die rotte om die jejunum af te punt waar die verskillende formulerings van Pheroid mikrosponsies en druppeltjies en TMC en TMO met salmkalsitonien (500 IU/kg) toe gedien is. Bloedmonsters is versamel en gesentrifugeer. Serum is gestoor by -40° C totdat dit geanaliseer is. Analises is gedoen met 'n Radio Immuno Essai.

Resultate: Met beide Pheroid tegnologie en TMC is hoër konsentrasies van kalsitonien waargeneem as met salmkalsitonien in fisiologiese sout oplossing. Piek plasma konsentrasie waardes is waargeneem net na 15 minute.

Gevolgtrekking: Daar is bevind dat Pheroid tegnologie definitiewe potensiaal besit om die absorpsie van salmkalsitonien te verbeter na orale toediening. Pheroid tegnologie het 'n gunstige vrystellingsprofiel wat tot gevolg het dat salmkalsitonien 'n groter biobeskikbaarheid het. Die studie het weereens bewys dat TMC 'n polimeer is wat nie weggelaat kan word in die orale toediening van peptiedgeneesmiddels nie.

ABSTRACT

Purpose: The purpose of this study was to evaluate the absorption enhancing potential of Pheroid technology and TMC (*N*-trimethyl chitosan chloride), a chitosan derivative, on the peroral administration of salmon calcitonin.

Methods: The research strategy was to administer salmon calcitonin, a large polar peptide drug with a known stability profile and quantitative therapeutic effect, alone and in combinations with two different Pheroid formulations and two derivatives of chitosan namely TMC and TMO to the jejunum mucosal surface of laboratory rats. Entrapment of calcitonin in Pheroids were confirmed and analyzed with CLSM. *In vivo* studies were conducted on Sprague-Dawley rats. Rats were fasted for 24 hours. The *carotis communis* was cannulated for the collection of blood samples after 0, 5, 10, 15, 30, 60, 120 and 180 minutes. An incision was made to the abdomen of the rats to ligate the jejunum where the different formulations of Pheroid microsponges and vesicles and TMC and TMO with salmon calcitonin (500 IU/kg) were injected. Blood samples were collected immediately and centrifuged. Serum was stored at -40 °C until analysis. Analyses were done with a Radio Immuno Assay.

Results: With both Pheroid technology and TMC greater concentrations of salmon calcitonin were observed as with salmon calcitonin in saline. Peak plasma concentration values were found just after 15 minutes.

Conclusion: It was found that Pheroid technology shows definite potential for enhancing absorption of salmon calcitonin after peroral administration. Pheroid technology has a favourable release profile which results in a greater bioavailability of salmon calcitonin after oral administration. This study once again confirmed TMC is not an excipient that can be omitted in the peroral administration of peptide and protein drugs in the future.

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INTRODUCTION AND AIM OF STUDY

Oral delivery is by far the easiest and most convenient way for drug delivery, especially when chronic administration is needed. However, the clinical development of orally active peptide drugs has been restricted by their unfavourable physicochemical properties, which limit their intestinal mucosal permeation and their stability against degradation. Successful oral delivery of peptides depends therefore on strategies designed to alter the physicochemical characteristics of these potential drugs, without changing the biological activity, in order to overcome the physical and biochemical barrier properties of the intestinal cells.

Some of the difficulties which are associated with limited peptide drug absorption are enzymatic degradation, low permeability, mucus binding capacities, efflux pathways, first path metabolism, poor stability of peptides in the GIT and pH changes throughout the intestine. Unfortunately the GIT is designed to break down peptides into amino acids which are small enough to be absorbed across the intestinal mucosa. The enzymatic barrier is by far the most aggressive and important obstacle. Digestive processes for proteins are catalyzed by various enzymes which specialize in the hydrolysis of peptide bonds.

The absorption of peptide drugs are mostly limited to the paracellular transport pathway, which mainly allow only certain molecules through depending on their size and hydrophilicity. The major barrier in the paracellular transport pathway is structures known as tight junctions.

Previous studies have recognized that chitosan, a mucoadhesive polymer, and its quaternized derivative (*N*-trimethyl chitosan chloride, TMC) are powerful enhancers of drug transport across mucosal surfaces by opening the tight junctions of the paracellular pathway. Research also indicated that chitosan's absorption enhancement capabilities are limited to the more acidic parts of the gastro intestinal tract. TMC on the other hand have superior water solubility compared to chitosan, and is therefore also effective as an absorption enhancer in neutral and basic environments

In recent years a fairly new absorption enhancing technology namely Pheroid technology has also shown potential for increased peptide drug absorption. This absorption enhancer consists mainly of essential fatty acids. Pheroid can entrap, transport and deliver pharmacologically active compounds, most probable through the transcellular transport pathway.

The aim of this study was to evaluate the absorption enhancing capabilities of TMC, a quaternised chitosan derivative, and Pheroid technology for intestinal salmon calcitonin absorption.

The specific objectives of this study were to:

- a. Conduct a literature study on peroral peptide delivery.
- b. Conduct a literature study on salmon calcitonin as peptide drug.
- c. Conduct a literature study on Pheroid technology and *N*-trimethyl chitosan chloride (TMC).
- d. Evaluate the absorption enhancing properties of TMC and Pheroids with a selected intestinal *in vivo* method.

Chapter 1 is an overview of difficulties associated with the oral administration of peptide and protein drugs and some strategies to overcome these difficulties. The potential use of chitosan and TMC as absorption enhancers are discussed in chapter 2. Chapter 3 provides present information available on Pheroid technology and considers the potential use of Pheroid as an absorption enhancer. Chapter 4 describes salmon calcitonin as peptide drug. Chapter 5 contains the procedures followed in the *in vivo* experiments to evaluate the absorption enhancing capabilities of Pheroid technology and TMC. Results obtained are also presented and discussed in chapter 5.

CHAPTER 1

PERORAL DELIVERY OF PEPTIDE AND PROTEIN DRUGS

1.1 INTRODUCTION

For many years, the lack of industrial manufacturing processes for peptides and proteins have restricted their use as therapeutic agents (Torres-Lugo & Peppas, 2000:1191). However, in recent years rapid advances in the field of biotechnology and genetic engineering have made it possible for peptide drugs to become a very important class of therapeutic agents.

Research created a wide diversity of biomedical peptide hormones, synthetic peptides, enzyme substrates and inhibitors. The growing interest can be ascribed to the increased understanding of their role in physiology and therapy as well as the established capability of producing large quantities by sophisticated biotechnology processes (Sarciaux *et al.*, 1995:129). The majority of these drugs are not suitable for oral administration and are commonly administered by parenteral routes which are often complex, difficult, painful and sometimes dangerous (Guggi *et al.*, 2003:125). Peptides also presents with a very short biological half life when administered parenteral and repeated injections are often needed. Frequent injections result in a rapid increase and subsequent rapid decrease of the blood serum concentration levels that could lead to side effects (Torres-Lugo & Peppas, 2000:1191).

Other routes of drug administration have also been explored. The rectal route has, in spite of not being well accepted by patients, presented certain advantages for peptide and protein drug delivery. The rectal environment is quite invariable with respect to the amount and viscosity of the rectal fluid. Moreover the proteolytic activity is significantly lower than in the upper intestine. (Ingemann *et al.*, 2000:190).

The nasal route composes of a mucosa which is highly vascularized, and the absorptive epithelium consists of only a single barrier. Therefore, the possibility of rapid delivery of the peptide or protein drug exists, despite the existence of an often limited and variable residence time (Ingemann *et al.*, 2000:190). The buccal route is much less efficient with respect to transport due to a higher epithelial thickness of the multilayer, presumably due to keratinisation. Prospective advantages to transdermal peptide or protein delivery are the ease of discontinuing drug administration, the possibility of long-term application, and excellent patient compliance. Although the skin is extremely impermeable, the transdermal administration of peptide and protein drugs is expected to be important in the future, e.g. by the use of iontophoresis or electrostimulus-induced absorption processes (Ingemann *et al.*, 2000:190-191).

Until recently, the pulmonary route has not been explored in detail as a possible route of administration of peptide and protein drugs for systemic effects. The potential of this route relies on the large area available for absorption and the thin epithelial barrier. The pharmaceutical challenges of pulmonary delivery lie in the inefficient deposition of aerosol particles in the alveoli (Ingemann *et al.*, 2000:190-191).

Vaginal application of peptide and protein drugs gained some attention in the last 10 to 15 years. Conversely, in spite of a rich blood supply, good permeability and a relatively large surface area, the cyclic changes and aesthetic concerns do make the route unrealistic for widespread use in drug delivery (Ingemann *et al.*, 2000:190-191). Thus, establishing an oral delivery system for peptide and protein drugs is of great importance because of the resulting poor patient compliance during chronic treatment, resulting in limited clinical utility (Lee & Sinko, 2000:226). Oral delivery offers one of the most adequate ways to administer drugs (Hamman *et al.*, 2005:2), but there are several difficulties associated with delivering peptides in oral formulation (Lee & Sinko, 2000:226).

These obstacles include the high enzymatic activity of the small intestine, the inherently low permeability of the intestinal mucosa, the binding capacities of resident mucus and luminal contents, the efflux pathways back into the gut lumen following uptake into

epithelial cells, first-pass metabolism (Daugherty & Mrsny, 1999:144), poor stability in the gastro intestinal tract (GIT) (Hamman *et al.*, 2005:2), the hostile acidic environment of the stomach, pH changes in the small intestine and proteolytic enzymes in the intestinal secretions which rapidly destroy most orally administered proteins (New *et al.*, 1997:2). These physical and biochemical barriers severely hamper intestinal absorption of peptides into the systemic circulation (Hamman *et al.*, 2005:2).

Physiological considerations such as gastric transit time, dilution and interaction with intestinal debris also influence peptide contact with the absorptive epithelium of the most appropriate intestinal region. Even if survival and delivery to the hepatic portal vein is achieved, a sufficient amount of viable peptide must then negotiate first-pass metabolism as well as the enterohepatic shunt (Brayden & O'Mahony, 1998:291).

Most of the important factors, leading to low bioavailability of peptide drugs, are shown in table 1.1. Bioavailability is clinical important because pharmacologic and toxic effects are proportional to both dose and bioavailability. When bioavailability is very low as in the case of most peptides, inter and intra subject variability in bioavailability is magnified and incomplete (Sarciaux *et al.*, 1995:128).

Physiological	Physiochemical
Gastric pH	Dissolution
Gastrointestinal transit and dilution	Solubility
Peptidases	Molecular weight
Intestinal permeability	Log octanol/water coefficient
GI site-specific uptake	Hydrogen bonding
P-glycoprotein efflux	Polar molecular surface area
Cytochrome P450 in GI wall	Aggregation
Colonic bacterial degradation	Interaction with food/mucus
Liver first-pass effect	

Table 1.1 Factors affecting oral delivery of peptides (Brayden & O'Mahony, 1998:292).

The major reason for trying to maximize oral bioavailability is to control drug concentrations and the effect of analysis. Cost may be another good reason (Sarciaux *et*

al., 1995:128). Various strategies have been employed and several advantages have been made in terms of increased bioavailability. Even though successes have been achieved with individual peptide drugs, no single oral dosage form has yet been entirely developed for the delivery of peptide and protein drugs as in a whole (Hamman *et al.*, 2005:2).

1.2 ANATOMY OF THE STOMACH

The stomach wall is composed of the similar basic layers as the rest of the GIT with certain differences. When the stomach is empty the mucosa lies in large folds called rugae (fig. 1.1) (Tortora & Anagnostakos, 1984:746).

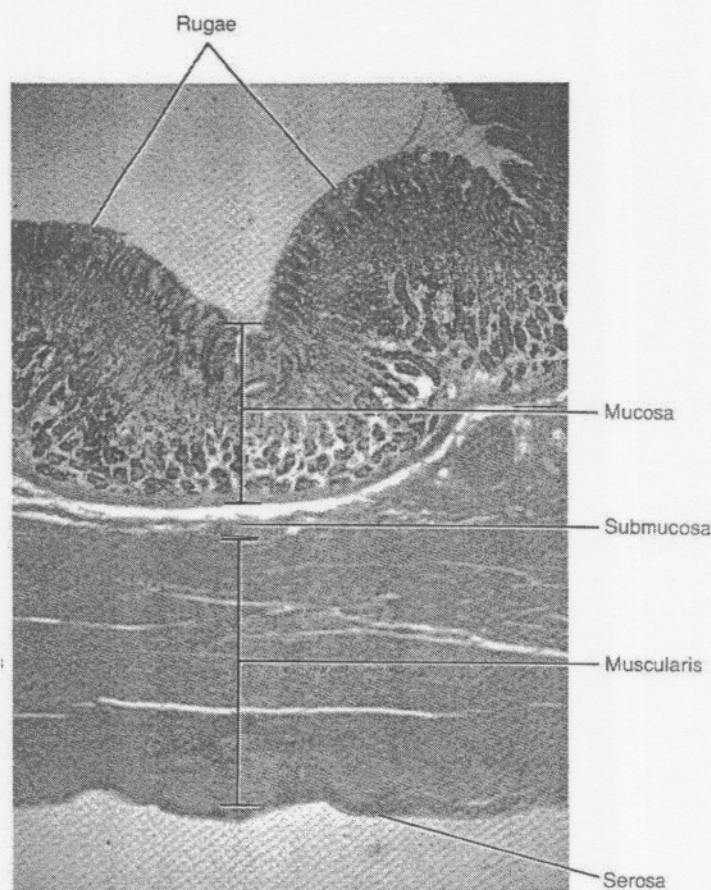


Figure 1.1 Photomicrograph of a portion of the fundic wall of the stomach showing the rugae (Tortora & Anagnostakos, 1984:748).

Micro inspection exposes a layer of simple columnar epithelium (surface mucus cells) containing many narrow openings that extend down into the lamina propria called gastric pits (fig. 1.2). At the bottom of the pits are the orifices of gastric glands.

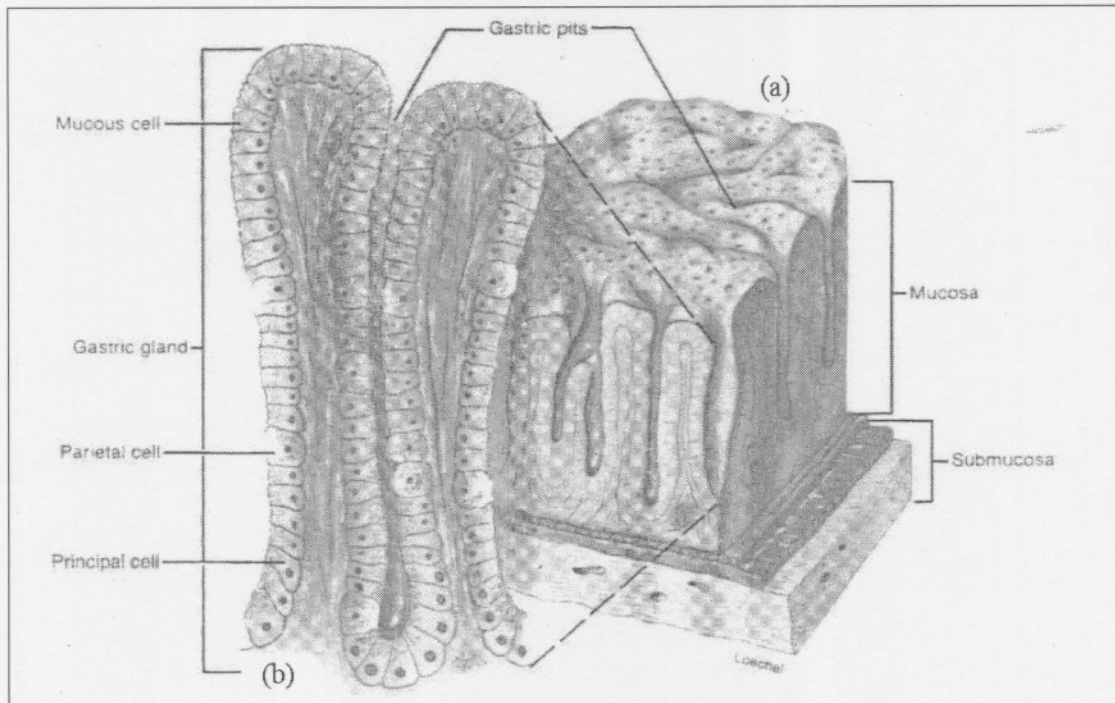


Figure 1.2 Gastric pits and gastric glands of the stomach. (a) Gastric pits are the openings of the gastric glands. (b) Gastric glands consist of mucus cells, principal cells, and parietal cells each type producing a specific secretion (Van der Graaff, 2000:631).

Each gland consists of four types of secreting cells: zymogenic, parietal, mucus and enteroendocrine. The zymogenic (peptic) cells secrete the primary gastric enzyme precursor, pepsinogen. The parietal cells produce hydrochloric acid. Hydrochloric acid is involved in the conversion of pepsinogen to the active enzyme pepsin. The mucus cells secrete mucus. Secretions of the zymogenic, parietal and mucus cells are collectively called gastric juice. The enteroendocrine cells secrete stomach gastric juice, a hormone that stimulates secretion of hydrochloric acid and pepsinogen, contracts the lower oesophageal sphincter, slightly increases motility of the GIT and relaxes the pyloric sphincter. The submucosa is composed of loose connective tissue, which

connects the mucosa to the muscularis. The muscularis, unlike that in other areas of the GIT, has three layers of smooth muscle: an outer longitudinal layer, a mobile circular layer and an inner oblique layer. The oblique layer is limited mostly to the body of the stomach. The serosa covering the stomach is part of the visceral peritoneum over the intestines (Tortora & Anagnostakos, 1984:747-748).

The most important chemical activity of the stomach is the digestion of proteins. The digestion of proteins is primarily achieved by pepsin. Pepsin breaks certain peptide bonds between the amino acids making up proteins in smaller fragments called peptides. Pepsin is most effective in the very acidic environment of the stomach (pH 2). It becomes inactive in an alkaline environment. Pepsin is secreted in an inactive form called pepsinogen, so it cannot digest the proteins in the zymogenic cells that produces it. It is not converted into active pepsin until it comes in contact with the hydrochloric acid secreted by the parietal cells. The stomach cells are protected by alkaline mucus, especially after pepsin has been activated. The mucus coats the mucosa to form a barrier between it and the gastric juices. Another enzyme of the stomach is gastric lipase. Gastric lipase splits the short chain triglycerides in butterfat molecules found in milk. This enzyme operates best at a pH of 5 to 6. The pancreas also secretes an enzyme into the small intestine, called pancreatic lipase. The principal activities of gastric digestion are summarized in table 1.2. (Tortora & Anagnostakos, 1984:749).

When partially digested proteins leave the stomach and enter the duodenum, they stimulate the duodenal mucosa to release enteric gastrin, a hormone that stimulates the gastric glands to continue their secretion (Tortora & Anagnostakos, 1984:750). The surface area of the small intestine is increased by circularis, villi and microvilli. The small intestine is primarily responsible for the uptake of nutrients and consists of the duodenum, jejunum and ileum (Van der Graaff, 2000:632-633).

Table 1.2 Summary of gastric digestion (Tortora & Anagnostakos, 1984:749).

STRUCTURE	ACTIVITY	RESULT
MUCOSA		
Zymogenic (peptic) cells.	Secretes pepsinogen.	Precursor of pepsin is produced.
Parietal (oxyntic) cells.	Secretes hydrochloric acid. Secrete intrinsic factor.	Converts pepsinogen into pepsin, which digests proteins into peptides. Required for absorption of vitamin B ₁₂ , which is required for normal erythrocyte formation.
Mucus cells	Secretes mucus.	Prevents digestion of stomach wall.
Enteroendocrine cells.	Secretes stomach gastrin.	Stimulates gastric secretion, contracts lower esophageal sphincter, increases motility of the stomach and relaxes pyloric sphincter.
MUSCULARIS	Mixing waves.	Macerate food, mix it with gastric juice, reduce food to chyme and force chyme through pyloric sphincter.
PYLORIC SPHINCTER	Opens to permit passage of chyme into duodenum.	Prevents backflow of food from duodenum to stomach.

1.3 BARRIERS LIMITING THE ORAL BIOAVAILABILITY OF PEPTIDE AND PROTEIN DRUGS

The most fundamental step necessary in mucosal membrane permeation is achieving oral absorption and systemic availability (Lipka *et al.*, 1996:122). The GIT is physiologically designed to break down dietary proteins into subunits that are adequately small (e.g., di/tri-peptides, amino acids) to be absorbed across the intestinal mucosa. Digestive processes for peptides and proteins are catalyzed by a range of enzymes that specializes in the hydrolysis of peptide bonds. Due to the wide substrate specificity of these proteases and peptidases, it is not surprising that the metabolic activity in the intestinal lumen is a major barrier limiting the absorption of peptide based drugs (Pauletti *et al.*, 1997:236).

At the same time the GIT must also protect humans against systemic invasion of harmful agents such as toxins, antigens and pathogens. Regrettably, these protective mechanisms, compromising physical and biochemical components, also counteract drug absorption after peroral administration. The physical barrier can mostly be attributed to the cell lining itself, as well as the cell membranes and the tight junctions between adjacent epithelial cells. Also playing a substantial role in regulating the absorption of substances is the mucus layer and efflux systems. The broad substrate specificities and variety of the luminal enzymes form a considerable enzymatic barrier, which is enlarged by the catabolic enzymatic activities in the brush border and intracellular cytoplasm (Hamman *et al.*, 2005:2). The degree of degradation also depends on the size and amino acid composition of the peptide (Pauletti *et al.*, 1997:236). However, over the past two decades, it has been recognized that the intestinal mucosa is not an absolute barrier that entirely prevents permeation (Pauletti *et al.*, 1996:4).

1.3.1 PHYSICAL BARRIERS

A specific peptide to be absorbed must bypass the unstirred water layer, mucus layer, apical and basal cell membranes and cell contents, tight junctions, basement membrane and the wall of lymph and blood capillaries (Sarciaux *et al.*, 1995:129).

1.3.1.1 THE UNSTIRRED WATER LAYER

The unstirred water layer is more or less a stagnant layer of water, mucus and glucocalyx adjunct to the intestinal wall. It is formed because it is practically impossible to stir the luminal contents so that complete mixing occurs right up to the intestinal mucosal surface. The rate limiting step in the transmucosal uptake of a low permeability compound is the transport across the apical membrane, rather than diffusion through the unstirred water layer. Therefore, the unstirred water layer can be considered as a insignificant barrier to the uptake of slowly absorbed drugs (Fagerholm & Lennernäs, 1995:247). The most important components of the mucus gel layer are mucins (high-molecular weight secretions) which may act as a barrier to drug absorption by stabilizing the unstirred water layer or by interactions between the diffusing molecules and components of the mucus layer (Hamman *et al.*, 2005:3).

1.3.1.2 EFFLUX SYSTEMS

The combination of biochemical and physical barriers alone can not adequately describe the barrier function of the intestinal mucosa. In cancer cells, it has long been recognized that polarized efflux systems are present that pose a major barrier to the absorption of a variety of chemotherapeutic agents (Pauletti *et al.*, 1997:244).

Efflux systems such as P-glycoprotein (P-gp) in combination with intracellular metabolism may contribute significantly to the poor bioavailability of certain drugs, including peptides (Hamman *et al.*, 2005:3-4). P-gp is expressed in high levels on the apical surface of the columnar epithelia cells in the jejunum (Benet *et al.*, 1996:140) and

actively pumps compounds from within the cell back into the intestinal lumen. P-gp was originally discovered in cancer cells as a membrane-bound, multi drug resistant transporter, although it is also present in normal intestinal tissues such as the blood-brain barrier, plasma, intestine, liver and kidney. Several clinical agents with diverse structures and functions have been found to be inhibitors, substrates or inducers of P-gp (Hamman *et al.*, 2005:3-4). The polarized expression of these efflux systems suggests that their physiological role is to restrict the transcellular flux of some molecules (Pauletti *et al.*, 1997:244).

1.3.1.3 MEMBRANES OF THE INTESTINAL PATHWAYS

The intestinal epithelium consists of a single layer of columnar cells, which includes a combination of enterocytes, goblet cells, endocrine cells and Paneth cells (Hamman *et al.*, 2005:3).

1.3.1.4 INTESTINAL PATHWAYS

The intestinal epithelium represents the largest interface ($>200\text{m}^2$) between the external environment and the internal host milieu, and constitutes a major barrier through which molecules can either be adsorbed or secreted (Fasano, 1998b:12-13).

Conceptually, two transepithelial pathways are available for the passage of molecules from the intestinal lumen to the bloodstream: namely the transcellular and paracellular pathways. Transcellular transport is carrier-mediated active, facilitated or passive transport (fig 1.4). Transcellular transport is when molecules are transported into and through epithelial cells and is then transferred into the systemic circulation (Sarciaux *et al.*, 1995:129). This involves movement of the solute across the apical cell membrane, through the cytoplasm of the cell and across the basolateral membrane by passive diffusion, or by a carrier- or vesicle mediated process. In general, transcellular flux by passive diffusion is negligible because of the predominantly hydrophilic nature of biologically active peptides (Pauletti *et al.*, 1997:237). Conceptually the phospholipid

bilayers of the plasma membrane of the epithelial cells that normally line the intestine are considered to be the major factor restricting the free movement of substances from the lumen to the bloodstream through the transcellular pathway (Fasano, 1998a:152). To permeate the intestinal mucosa via the transcellular pathway, the ability of the molecules to partition into the cell membrane is of critical importance. Therefore, most of the studies performed to date have focused on the individual contributions of physiochemical properties, including size and lipophilicity (hydrophobicity and hydrogen bonding potential) (Pauletti *et al.*, 1997:243) and solution conformation to cross the lipophilic barriers of the basolateral and apical membranes (Hamman *et al.*, 2005:3).

The absorption of peptides with their hydrophilic nature and macromolecular dimensions is mainly limited to the paracellular pathway (fig 1.4). However, there are exceptions, e.g. immunoglobulin G and epidermal growth factor which are absorbed by transcytosis (Ingemann *et al.*, 2000:191).

The paracellular pathway of drug absorption is an aqueous extracellular route through the intracellular spaces between adjacent epithelial cells (Gangwar *et al.*, 1997:149). Translocation through the paracellular pathway is passive and the flux of the molecule is driven by electrochemical potential gradients originating from differences in concentration, electrical potential and hydrostatic pressure between the two sides of the epithelium (Pauletti *et al.*, 1997:237). It has gained importance for the delivery of peptides because of the deficiency of proteolytic activity (Gangwar *et al.*, 1997:149). The primary barrier of the paracellular route is a structure known as the tight junction complex which exists in the apical neck where adjacent intestinal epithelial cells are observed to be very closely opposed. The tight junction complex completely circumnavigates each epithelial cell to form a continuous seal that segregates the apical and basolateral membrane. This restricts diffusion of molecules in a charge-specific and molecular-size manner (Daugherty & Mersny, 1999b:281).

Only molecules with molecular radii smaller than 11 Å are accessible (Fasano, 1998a:152). This number represents the maximum hydrodynamic radius of a spherical

rigid molecule which is small enough to diffuse into the intercellular space. For peptides possessing a high degree of conformational flexibility, it might be possible that even larger molecules can permeate the tight junction (Pauletti *et al.*, 1997:242). Certain smaller peptides such as dipeptides and tripeptides have been shown to have affinity for active nutrient transporters and to be absorbed by this transport mechanism in the intestine (Ingemann *et al.*, 2000:192). Since the paracellular pathway is an aqueous extracellular route across the epithelium, adequate hydrophilicity is the most important requirement for a peptide to pass through the cell barrier via this pathway (Pauletti *et al.*, 1997:241).

Another important biological process is cellular internalization of polypeptides by endocytosis whereby peptides that are too large to be absorbed by di- and tripeptides transport systems may be taken up into intestinal mucosal cells. Fluid-phase endocytosis (pinocytosis) does not require any interaction between the polypeptide and the apical membrane. In contrast, receptor mediated or absorptive endocytosis involves the binding of peptides and proteins to the plasma membrane before being incorporated into the endocytic vesicles. Finally, some polypeptides can be carried in endosomes directly to the basolateral side (i.e., bypassing the lysosomes), where they are released into the extracellular space. This process is known as transcytosis. Though there is some evidence that mucosal peptide / protein uptake is mediated by endocytic processes, in most cases this does not lead to transcytosis (Pauletti *et al.*, 1997:237).

1.3.1.5 TIGHT JUNCTIONS

A century ago, tight junctions were thought to be secreted extracellular cement forming an absolute and unregulated barrier inside the paracellular space. There is now bountiful evidence that tight junctions are dynamic structures that readily adapt to a variety of developmental, physiological and pathological circumstances. The assembly of tight junctions is the result of cellular interactions that trigger a complex cascade of biochemical events that ultimately leads to the formation and modulation of an organized

network of tight junction elements, the composition of which has been only partially characterized (Fasano, 1998b:13).

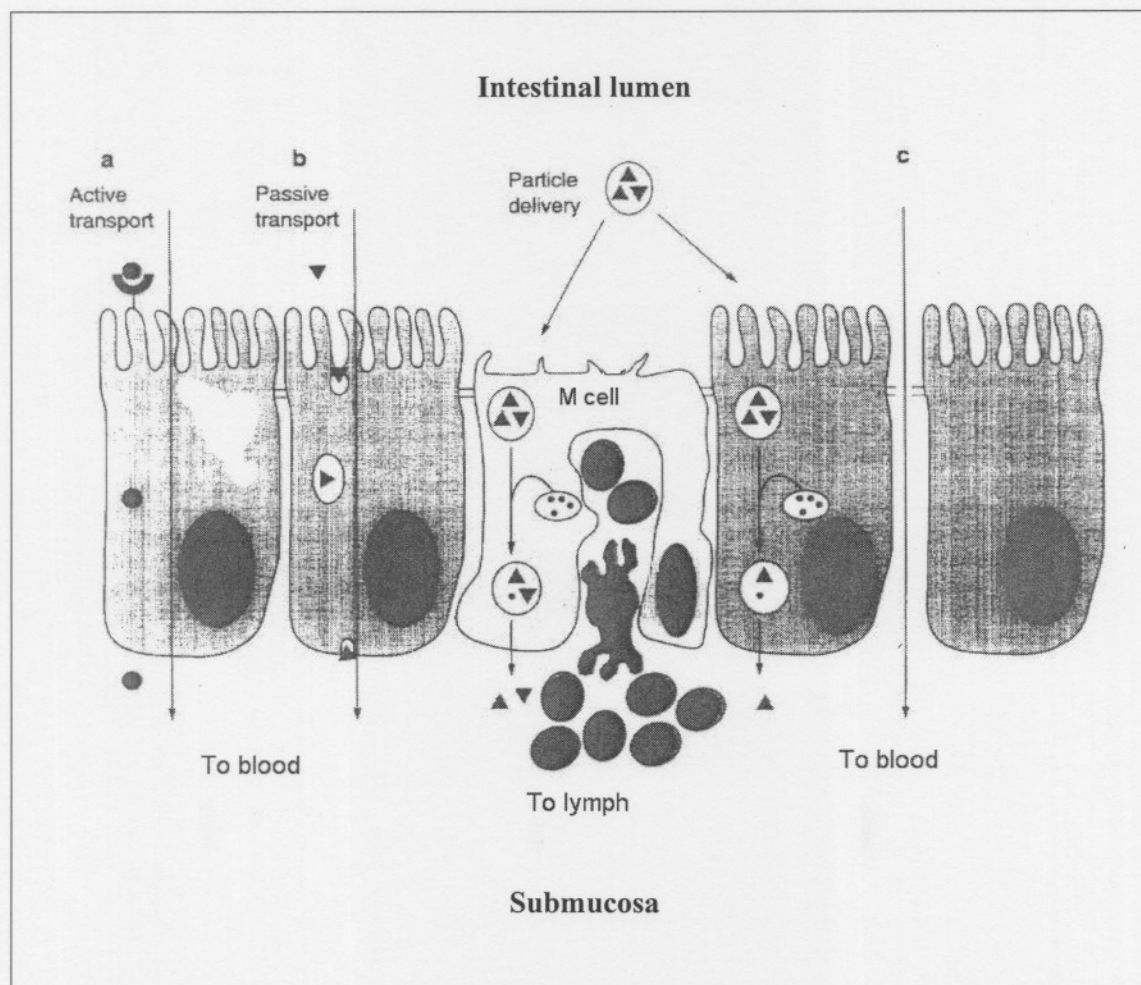


Figure 1.3 Schematic presentation of the three intestinal pathways: (a) transcellular active transport; (b) transcellular passive transport; (c) paracellular transport (Fasano, 1998a:153).

1.3.2 BIOCHEMICAL BARRIERS

The biochemical barrier reduces transport of peptides through the intestinal mucosa by brush-border and / or intracellular metabolism and by apically polarized efflux systems (Gangwar *et al.*, 1997:151).

Luminal micro-organisms, digestive enzymes and an acidic environment may cause degradation of peptides within the lumen of the GIT (Hamman *et al.*, 2005:4). Figure 1.4 shows a general overview of degradation and transport of peptides and proteins.

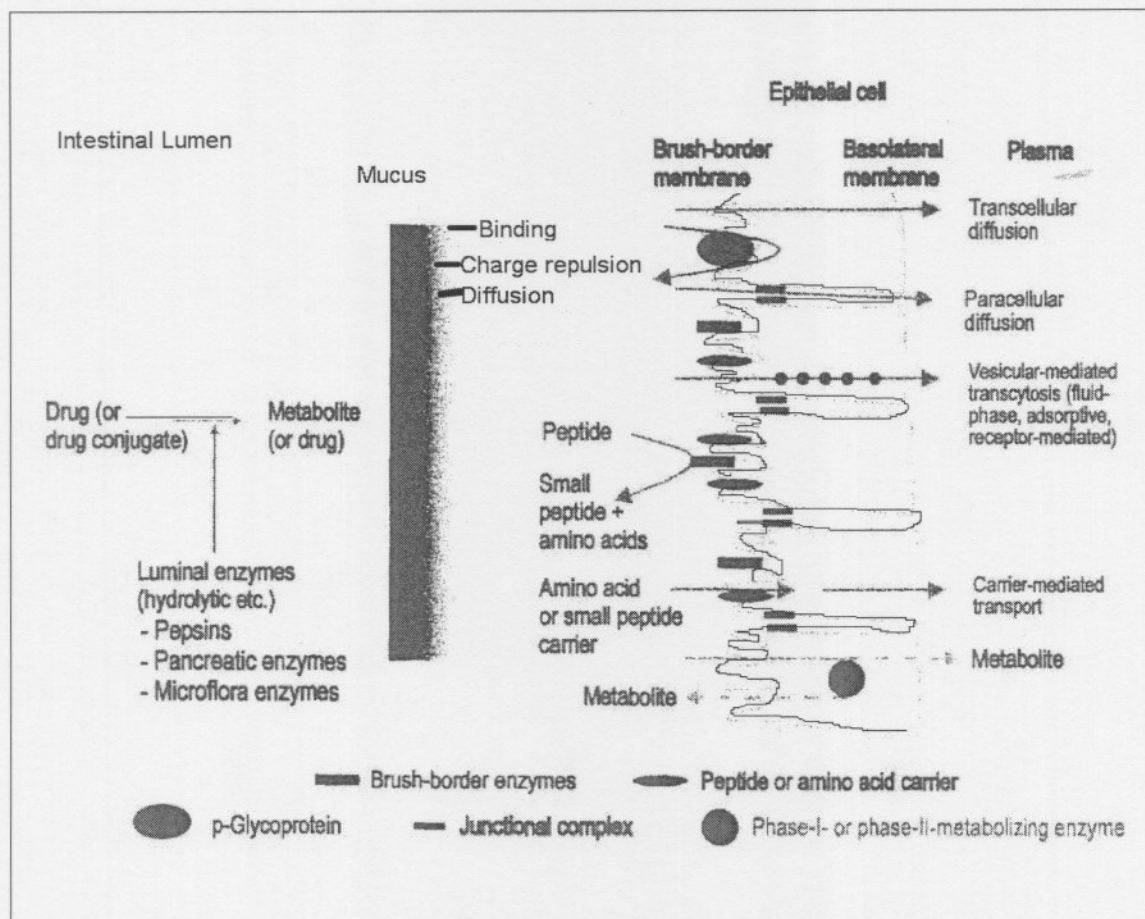


Figure 1.4 A general overview of the multiple degradation and transport events for proteins and peptides in the intestine (Langguth *et al.*, 1997:41).

The biochemical barrier is shown in figure 1.5.

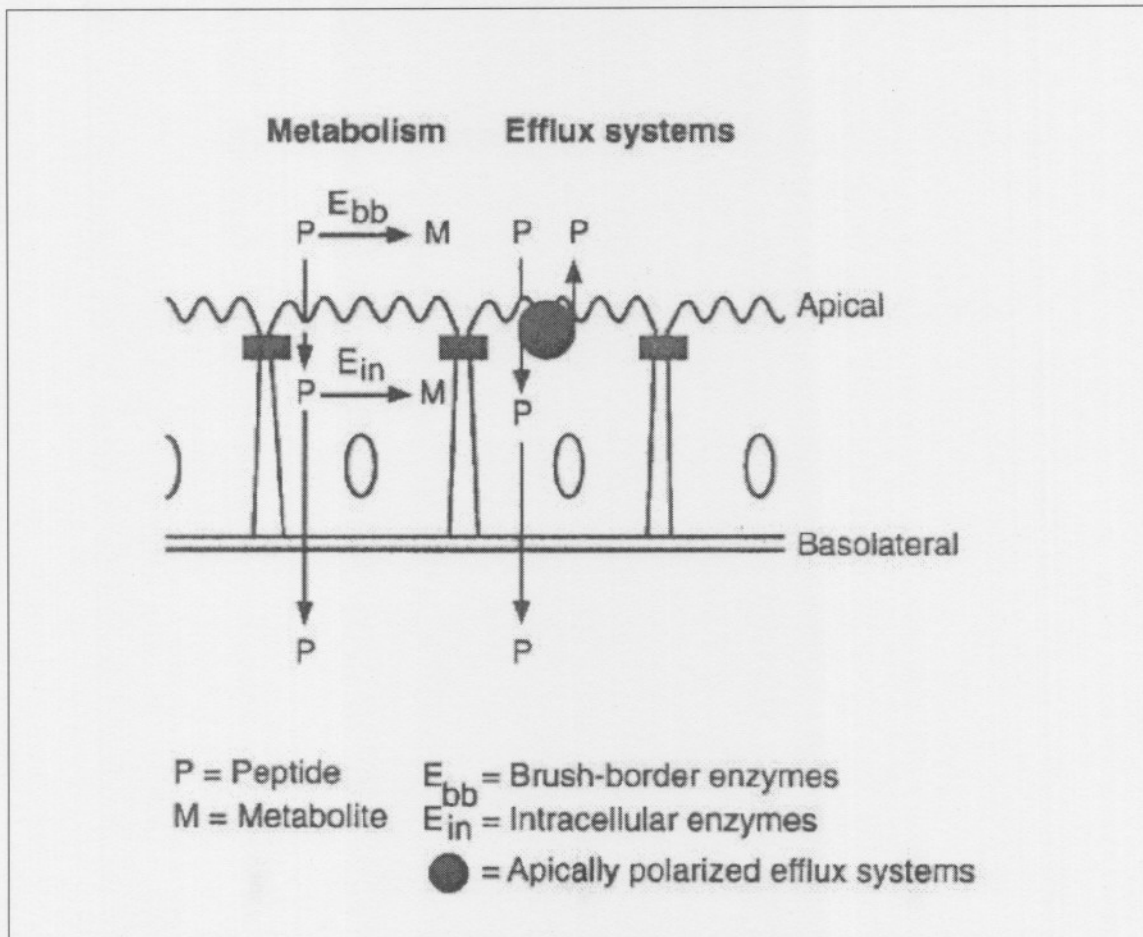


Figure 1.5 The biochemical barrier (Gangwar *et al.*, 1997:151).

1.3.2.1 THE ENZYMATIC BARRIER

The enzymatic barrier is by far the most aggressive and important obstacle for oral peptide delivery. The oral bioavailability of most peptides is less than 10%. The enzymatic barrier, composed of exo- and endopeptidases, is well designed for the digestion of peptides to a mixture of amino acids and thus it is not surprising that the oral absorption of intact peptides is difficult (Sarciaux *et al.*, 1995:129). Digestive processes for proteins are catalyzed by a variety of enzymes specialized in the hydrolysis of peptide bonds (Gangwar *et al.*, 1997:148-149). Hydrolysis of peptides occurs at several sites:

luminally, at the brush border and intracellular. Unlike most small drug molecules that are metabolized primarily in the liver, peptides are usually susceptible to degradation in the blood and the kidney. A given peptide is susceptible to degradation at more than one linkage within its backbone and each locus of hydrolysis is mediated by a certain peptidase (Sarciaux *et al.*, 1995:129). The digestive processes are catalyzed by enzymes through hydrolytic cleavage of peptide bonds such as phosphorylation (kinases) and oxidation (xanthine oxidase). Luminal degradation of peptides is due to exposure to enzymes released from the pancreas into the intestine. The most relevant pancreatic proteases are the serine endopeptidases namely trypsin; α -chymotrypsin; elastase and the exopeptidases carboxypeptidases A and B. All five proteases have a pH optimum of approximately 7 to 8 (Langguth *et al.*, 1997:41-42). Modification of one linkage in the backbone of a peptide still leaves the rest of the peptide drug vulnerable to proteolysis. A general tendency for proteins is to be degraded by luminal pancreatic enzymes, and for tripeptidases and larger peptides to be metabolized by intestinal cytosolic enzymes (Sarciaux *et al.*, 1995:129). Contact with enzymes associated with the enterocytes such as those in the brush border membrane, cytoplasm and lysosomes also contributes to the pre-systemic degradation of peptides (Langguth *et al.*, 1997:41-42). The GIT fluids differ in terms of pH in the different regions, which may influence the pH-dependant hydrolysis of drugs (Hamman *et al.*, 2005:4). Although lysosomes and other organelles may act as potential sites of peptide metabolism, peptidases in the brush-border membrane are probably the biggest deterrent to the absorption of small peptides across the intestinal mucosa. It appears that brush-border peptidases are mainly active against tri-, tetra- and higher peptides up to ten amino acids residues while intracellular peptidases are active predominantly against dipeptides. The lowest activity is in the ileo-caecal junction (Pauletti *et al.*, 1996:5).

1.4 DIFFERENT STRATEGIES FOR EFFECTIVE ORAL DELIVERY OF PEPTIDE AND PROTEIN DRUGS

One of the major barriers in the development of effective oral protein delivery systems, rests in the actual fabrication methods used for the formulation. Proteins have a complex

internal structure which helps define their biological activity. Any disruption in the protein structures can result in deactivation of a protein. The slightest changes in the environment (or even microenvironment) of the protein can cause disruptions. The most likely variables which can affect protein structure and stability are related to temperature, pH, solvent, other solutes and crystallinity states of the protein. Thus understanding the stability of proteins is just as important (Carino & Mathiowitz, 1999:251).

Many approaches have been investigated to reduce the effect of the intestinal barriers for the oral delivery of peptide drugs. Pharmaceutical strategies that have been proposed to enhance peptide drug bioavailability include the following:

- Co-administration of enzyme inhibitors
- Chemical modification of peptide and proteins
- Special drug delivery systems
- Targeted delivery
- Absorption enhancers (Hamman *et al.*, 2005:4).

More than one factor is often simultaneously responsible for the low bioavailability of peptides. Therefore, results of previous attempts in studies to overcome the enzymatic barrier were only with limited success (Hamman *et al.*, 2005:4).

New formulations consisting of different kinds of delivery vehicles are therefore being developed to attempt to overcome both the physical and enzymatic barrier. Systems that have been used are non-replicating systems such as polymeric particles and lipid-containing particles. These systems encapsulate the drugs within the particles and therefore protect them from the harsh environment in the GIT (Chen & Langer, 1998:338).

1.4.1 ENZYME INHIBITORS

Digestion of proteins begins in the stomach and is continued by many different enzymes located throughout the remainder of the GIT. Pepsins are situated in the stomach and

trypsin, chymotrypsin, elastase and carboxypeptidases are secreted from the pancreas into the small intestinal lumen. The above mentioned enzymes are responsible for only 20% of the enzymatic degradation of ingested proteins. The remainder of the degradation occurs at the brush-border membrane (by various peptidases) or within the enterocytes of the intestinal tract. Numerous efforts have been made to overcome enzymatic barriers, but this is certainly a difficult challenge. The presence of just one or two of these enzymes could lead to a total denaturation of a protein drug (Carino & Mathowitz, 1999:251). The easiest way to overcome these barriers is by the use of protease and peptidase inhibitors during peptide and protein delivery (Ingemann *et al.*, 2000:192).

Specific catabolic protease and peptide activities can be identified and a corresponding specific inhibitor can be employed to stabilize the sensitive protein or peptide (Van der Merwe, 2003:5). Examples of enzyme inhibitors which have been used in peroral peptide delivery in the past are aprotinin (trypsin/chymotrypsin inhibitor), chymostatin, amastatin, boroleucin, puromycin, EDTA, leupeptin, bestatin, carbomer and polycarbophil (Ingemann *et al.*, 2000:192; Van der Merwe, 2003:5).

The use of enzyme inhibitors has gained considerable interest to overcome the enzymatic barrier after peroral administration of peptides. Due to the side-effects caused especially after long term administration, the safety of these agents remains questionable. Even if intestinal damage and systemic toxic side effects can be excluded, enzyme inhibitors of pancreatic proteases still have a toxic potential based on the inhibition of these digestive enzymes themselves. Besides a disturbed digestion of nutritive proteins, an inhibitor-induced stimulation of protease secretion caused by a feed back regulation has to be expected. Studies on feed back regulation revealed that it leads to both hypertrophy and hyperplasia of the pancreas. Development of a drug delivery system, which keeps the inhibitor(s) concentrated on a restricted area of the intestine and where drug delivery and subsequent absorption takes place, might lead to a reduction or even exclusion of this feed back regulation (Bernkop-Schnürch, 1998:7). Additionally this may lead to a decrease in the amount of inhibitor needed for effective inhibition of the enzymes as well as increased absorption of the peptide drug (Langguth *et al.*, 1997:54).

1.4.2 CHEMICAL MODIFICATION

Several opportunities are provided when pharmacological active peptides and proteins are structurally manipulated to improve not only the pharmacokinetic profile, but also its pharmacodynamic properties. Prodrug formation, manipulation of the amino acid sequence to reduce immunogenic and proteolytic cleavage, conjugation of peptide drugs to natural or synthetic polymers and changes to the structure to be recognized by transporters or receptor-mediated endocytosis are examples of chemical modification (Hamman *et al.*, 2005:4).

The series of tools existing for restricting peptide bond hydrolysis *in vivo* by chemical modification extends from easy additions that chemically protect the targeted bond from attack, to its replacement altogether, to global changes that instead modify the peptide conformation in such a way that it is no longer recognized by the proteases of concern. The introduction of steric bulk in the form of an N-alkyl (usually methyl) group or a quaternary amino acid, as shown in fig 1.6, can significantly decrease the enzymatic rate at which a peptide is degraded. A more subtle introduction of a chemical bulk would entail the use of a D-amino acid or a residue containing an unnatural side chain, in the hope that the peptidase would not tolerate moving steric bulk from one face of the enzyme to the other. Consequently, a conformational change is introduced along with intended steric blockage (Pauletti *et al.*, 1997:239-240).

Although medicinal chemists have successfully circumvented the metabolic enzymes present in the intestinal mucosa through structural modification, chemical modification of peptides can additionally alter the physiochemical properties of peptides resulting in an increase in the absorption of peptides from the intestinal tract (Pauletti *et al.*, 1997:239).

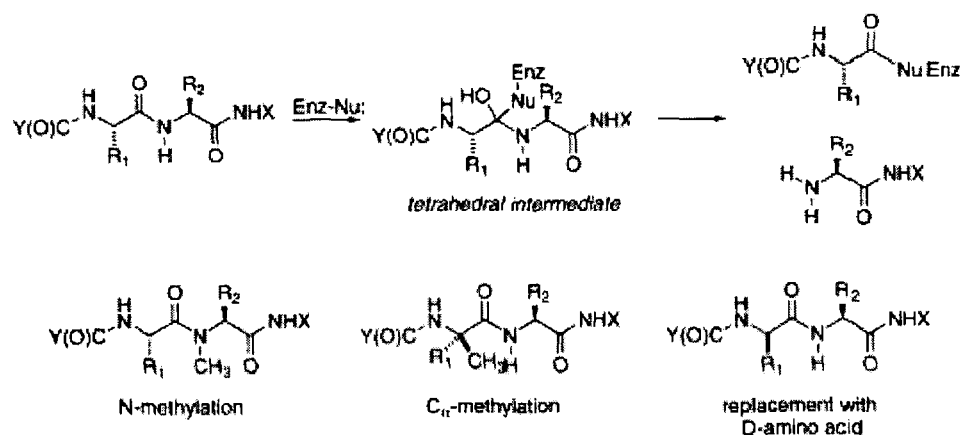


Figure 1.6 Examples of chemical modifications of the peptide bond leading to increased metabolic stability of peptide drugs against enzyme-mediated hydrolysis (Pauletti *et al.*, 1997:239).

1.4.2.1 PEPTIDOMIMETICS

Peptidomimetics are peptide-derived substances in which amide functions are replaced by non hydrolysable bonds that mimic the original structures of peptides. This strategy solves the problem of peptide instability towards rapid proteolysis (Allemann *et al.*, 1998:183). Some peptidomimetics also mimic the biological activity of a peptide, but is no longer peptidic in nature, such as pseudo-peptides, semi-peptides and petoids (Pauletti *et al.*, 1996:11). It is important to realize that chemical modification and peptidomimetics are never completely unrelated as even modest structural changes in the peptide can result in significant conformational changes.

1.4.2.2 PRO-DRUG APPROACHES

Another important approach is the use of pro-drugs for chemical modification of peptide drugs (Pauletti *et al.*, 1996:10). Pro-drugs are molecules that must undergo chemical or biochemical conversion to the active drug before exerting pharmacological effects. The main objective in designing pro-drugs is to overcome some of the limitations of the parent drug, including poor stability, poor chemical and / or enzymatic stability, poor

membrane permeability, rapid elimination by the liver or kidney, and lack of targeted delivery. The pro-drug concept is illustrated in figure 1.7. Unfortunately, the syntheses of pro-drugs are limited, due to their structural intricacy and the lack of methodology for their efficient synthesis (Gangwar *et al.*, 1997:152). Bioreversible cyclization of the peptide backbone is one of the most promising and intriguing approaches in the development of peptide pro-drugs (Pauletti *et al.*, 1997:245). Recent examples clearly demonstrate that the oral bioavailability of peptide drugs can be significantly improved by using the pro-drug approach.

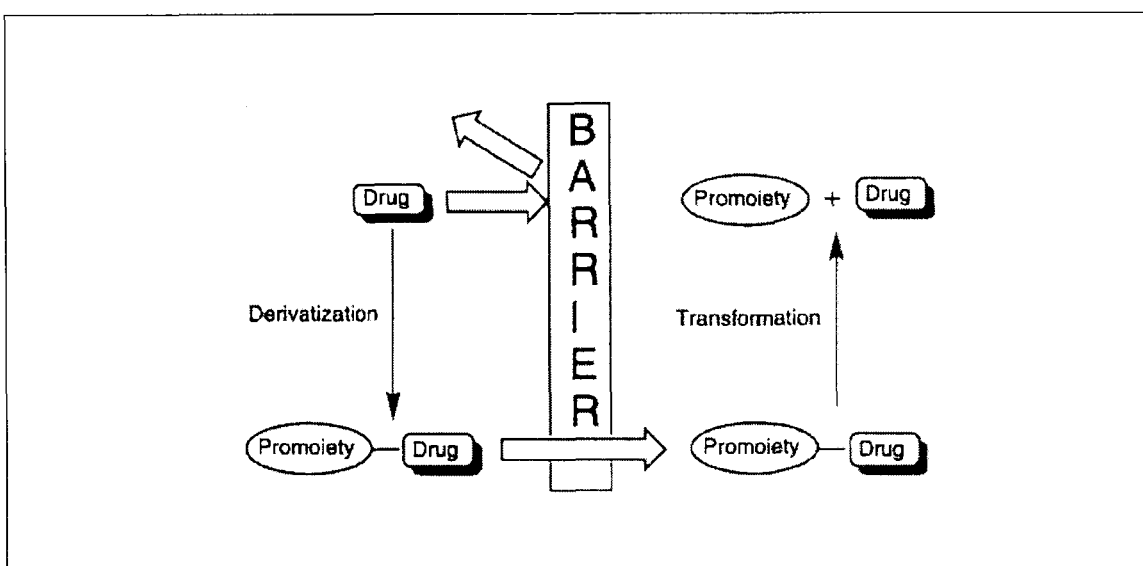


Figure 1.7 The pro-drug concept (Gangwar *et al.*, 1997:152).

1.4.3 ABSORPTION ENHANCERS

Absorption enhancers are compounds (usually formulation components) which allow a drug to penetrate the epithelial cells and enter the blood and / or lymph circulation by temporarily disrupting or reversibly removing the intestinal barrier with minimum tissue damage. Occasionally intestinal permeation enhancement has been correlated with acute epithelial damage (Hamman *et al.*, 2005:7). Enhancers must act in a reversible way and should furthermore be effective from the low pH in the stomach up to the very basic pH in the colon, being most effective in the small intestine (Kotze *et al.*, 1999b:343). Ideal

absorption enhancers should show no toxicity, high efficacy, high specificity (Hamman *et al.*, 2005:7) and achieve reproducible intestinal permeation (Aungst *et al.*, 1996:19). Absorption enhancers have different mechanisms of action which is often complex (Hamman *et al.*, 2005:7). Absorption enhancers can either act directly on the mucus layer, the membrane components of the epithelial cells or the tight junction (Junginger & Verhoef, 1998:371). Mucoadhesive polymers are thought to interfere with the mucus layer, first by covering the mucus surface and then by interpenetration of the mucus network (Junginger & Verhoef, 1998:372).

The desired reaction of enhancers should be a rapid reaction with fast recovery of the normal barrier functions. A fast onset and offset (a matter of a few minutes) of the enhancement action in the GIT means that a poorly absorbed drug will be exposed to the epithelial cells with constant amounts of absorption enhancers. Best results would be obtained when the simultaneous release occurs under “closed compartment conditions” (Rubinstein *et al.*, 1997:62). Absorption enhancement is usually by facilitated chelating agents and surfactants in the GIT (Van der Merwe, 2003:7). Chelators are believed to exert their action by complexation with calcium ions and by facilitating leaching of proteins from the membrane. The result is a concentration of proteins in the cell followed by an increase in paracellular transport due to opening of tight junctions and an increase in membrane fluidity (Ingemann *et al.*, 2000:193). EDTA and sodium lauryl sulphate also promote the intestinal absorption of impermeable drugs. However they also showed significant cell damage and their use is considered rather impractical due to the fact that their toxicity are directly related to the mechanism of enhanced absorption (Van der Merwe, 2003:7). Examples of selected compounds that have been investigated are listed in table 1.3.

Table 1.3 Examples of some absorption enhancers and their mechanism of action (Aungst *et al.*, 1996:21; Van der Merwe, 2003:8; Hamman *et al.*, 2005:8).

Absorption enhancer	Mechanism of action
Bile salts	
Na-taurocholate	Disruption of membrane integrity by phospholipid solubilization and cytolytic effects, reduction of mucus viscosity.
Na-taurodeoxycholate	
Na-taurodihydrofusidate	
Surfactants	
Na-dodecyl sulfate Na-dioctylsulfosuccinate Na-laurylsulfate Dioctyl sulfosuccinate Polysorbates Polyoxyethylene-9-laurylether Nonyl phenoxy (polyoxyethelene)	Membrane damage by extracting membrane protein or lipids, phospholipid acyl chain perturbation.
Fatty acids	
Medium chain glycerides Long chain fatty acid esters (Palmitoylcarnitine)	Paracellular (e.g. Na-caprate dilates TJ) and transcellular (epithelial cell damage or disruption of cell membrane).
Salicylates	
Na-salicylate Na-methoxysalicylate Salicylate ion	Increasing cell membrane fluidity, decreasing concentration of non-protein thiols.
Chelating agents	
EDTA	Complexation of Calcium and Magnesium (TJ opening). Opening of tight junctions.
Polyacrylates	
Toxins and venom extracts	
Zonula occludens toxin Melittin (bee venom extract)	Interaction with the zonulin surface receptor induces actin poly-merization (TJ opening) ?-helix ion channel formation, bilayer micellization and fusion.
Cyclodextrins	
α -, β - and γ cyclodextrins Methylated β -cyclodextrin	Inclusion of membrane compounds.
Cationic polymers	
Chitosan salts N -trimethyl chitosan chloride	Combination of mucoadhesion and ionic interactions with the cell membrane (TJ opening).
Anionic polymers	
Poly(acrylic acid) derivatives	Combination of enzyme inhibition and extracellular calcium depletion (TJ opening).

(TJ = Tight junctions)

Numerous studies have shown that absorption enhancers can increase the permeability of membranes by affecting biological membranes consisting of proteins and lipids. The most likely mechanism by which these absorption enhancers, which act on the membrane components, enhance drug absorption is by solubilising the phospholipids and membrane proteins, resulting in an increase of membrane permeability. Paracellular transport of peptides and proteins has been of great interest due to proteolytic activity, which is thought to be deficient in the paracellular space. Absorption enhancers improve both paracellular and transcellular transport, albeit in different ratios. Several studies have shown that paracellular transport of poorly absorbed drugs can be increased if the absorption enhancer is capable of loosening tight junctions located between adjacent epithelial cells. Some of the absorption enhancers, which act in this way are EDTA, the polyacrylates and methylated B-cyclodextrins. The poly (acrylic acid) derivatives and chitosans are high molecular weight absorption enhancers that exclusively trigger paracellular transport with no toxic effects (Junginger & Verhoef, 1998:372). The efficacy of poly (acrylic acid) derivatives and chitosan as absorption enhancers has been increased by the covalent attachment of thiol groups to form the so-called thiolated polymers or thiomers.

These thiomers in combination with reduced glutathione (GSH) showed very high *in vitro* permeation enhancement ratios and their relatively high absorption enhancement effects were confirmed by various *in vivo* studies (Bernkop-Schnürch, 2003:95). Lately it was also discovered that *Vibrio cholera*, a bacteria which infects the intestinal tract and causes serious disease, produces a protein known as the Zonula occludens toxin (ZOT) which is able to increase the permeability of tight junctions. ZOT acts specifically on the actin filaments of the zona occludens without compromising the overall intestinal integrity or function. This regulation of the paracellular pathway has been shown to be safe, reversible, time- and dose-dependent and limited to the ileum and jejunum (Carino & Mathiowitz, 1999:252). The most important criteria to be met are: (1) how effective the absorption enhancer is for the drug of interest; (2) the potential to cause toxicity, and the mechanisms by which absorption is enhanced (Aungst *et al.*, 1996:20-21).

1.4.4 PEPTIDE TRANSPORTERS

Carrier-mediated drug transport is a fairly new and unexplored field in comparison with passive transcellular and paracellular drug transport. Yet, there are a multitude of transporter proteins that can be targeted for improving epithelial drug absorption (Lee, 2000:S41). Intestinal peptide transporters are physiologically involved in facilitating the uptake of natural di- and tripeptides from the gut lumen to the blood circulation (Pauletti *et al.*, 1997:247). Two peptide transporters, PepT1 and PepT2, have been recognized in mammals. Peptide transporters are proton-coupled, energy dependent transporters with extensive substrate specificities (Hamman *et al.*, 2005:6) and it has been recognized that various peptide mimetics, such as β -lactam antibiotics, ACE-inhibitors and renin inhibitors are also efficiently transported across the intestinal mucosa via these transporters (Pauletti *et al.*, 1997:247).

Stabilized peptides have also been successfully coupled to bile acids to exploit the uptake mechanism of the ileal bile acid transporters (Brayden & O'Mahony, 1998:294). Based on the high concentration and transport capacity of these peptide transporters in tissues such as the small intestine, they appear to be good targets for the delivery of drugs (Hamman *et al.*, 2005:6). They are also destined to play a more visible role in peptide drug absorption in the years to come (Lee, 2000:S41).

1.5 FORMULATION PRINCIPLES FOR PEPTIDE AND PROTEIN DRUGS

The general strategy for improving peptide or protein drug delivery is to incorporate the drug substance in a delivery system designed to protect the peptide or protein drug from contact with the proteolytic enzymes present at the site of administration and further to release the drug substance only upon reaching an area favourable for its absorption (Ingemann *et al.*, 2000:194).

Different dosage forms have been studied through the years, which include the following:

- Mucoadhesive polymers.
- Liposomes.
- Micro-emulsions.
- Polymeric nanoparticles.
- SPH & SPHC based drug delivery systems (Van der Merwe, 2003:9-16).

1.5.1 MUCOADHESIVE POLYMERS

Mucoadhesive polymers adhere to the mucus layer in the intestine. A relatively new concept in bioadhesion (mucoadhesion) is referred to as cytoadhesion and is based on highly specific interactions between an adhesive and the cell surface that are comparable to a receptor-ligand interaction (Hamman *et al.*, 2005:8). The transit of the polymeric carriers in the GIT is slowed down by the interaction between the polymeric particles and the mucus layer in the intestine. This results in prolonged residence time (Chen & Langer, 1998:346), intensify contact with the mucosa to increase the drug concentration gradient, guarantee immediate absorption without dilution or degradation in the luminal fluids and restrict the drug delivery system at a certain site (Hamman *et al.*, 2005:9). The transit time is dependent on the physiological turnover time of the mucus layer. Interaction with food and other intestinal contents may also significantly reduce the effectiveness of the mucoadhesive systems (Chen & Langer, 1998:346). Some examples of mucoadhesive polymers are carbomer, polycarbophil and chitosan and its derivatives. These polymers have been used in the past to deliver peptide drugs perorally (Van der Merwe, 2003:10).

1.5.2 LIPOSOMES

Lipids are ideal carrier systems for the administration of therapeutic agents because they are natural biodegradable materials which are taken up by the intestinal cells, and for the processing of which the gut has developed many different and complementary strategies. Among these is the use of lipase to break down lipids into surface active products such as

monoglycerides and lyso-lecithin which together with bile salts, help convert bulk oil phase into micelles which can interact readily with the gut wall (New & Kirby, 1997:59).

The most frequent used form of lipid particles is liposomes (Chen & Langer, 1998:343). Liposomes are vesicles in which an aqueous core is enclosed by phospholipid bilayers. Liposomes are broadly classified by size and lamellarity as small unilamellar vesicles (SUVs; size 25-50 nm), large unilamellar vesicles (LUVs; 100 nm) and multilamellar vesicles (MLVs). Liposomes present unique therapeutic opportunities because both hydrophilic and hydrophobic drug molecules can be incorporated, they are biodegradable, they can be made in a variety of sizes, and they can be used for controlled release and targeted delivery (Ingemann *et al.*, 2000;197). Some disadvantages are their susceptibility towards dissolution by intestinal detergents and degradation by phospholipases (Chen & Langer, 1998:344).

1.5.3 MICRO-EMULSIONS

To incorporate water-soluble molecules such as peptides into oils, water-in-oil emulsions and micro-emulsions have been employed, with the peptide dissolved in the aqueous phase, which is dispersed throughout the bulk oil (New & Kirby, 1997:59). Emulsions are two immiscible phases dispersed in one another; micro-emulsions are more complex quaternary or ternary systems, containing a surfactant / co-surfactant blend added to a two-phase hydrophilic / lipophilic mixture (Ingemann *et al.*, 2000:198). In micro-emulsion systems which have shown the greatest efficacy in delivery of hydrophilic molecules, it is clear that the incorporated material is released rapidly from the oil phase when the bulk oil is dispersed in water (New & Kirby, 1997:59). Micro-emulsions form spontaneously at room temperature and is a stable, optically clear, isotropic, colloidal system apparently thermodynamically stable with ultra low interfacial tension and consists of micro-droplets of a size less than 100 nm (Sarxiaux *et al.*,1995:132).

1.5.4 POLYMERIC NANOPARTICLES

Particles with sizes ranging from submicron to several hundred microns have been made from various polymeric materials, most of which are degradable *in vivo*. Biodegradable polymers are biocompatible and have been used in pharmaceutical and medicinal applications for years (Chen & Langer, 1998:343). 'Nanoparticles' is a collective name for nanospheres and nanocapsules. Nanospheres have a matrix type structure, where active compounds (e.g. peptides) can be adsorbed at their surface and then be entrapped or dissolved in the matrix. Nanocapsules have a polymeric shell and an inner core. The active compound can be dissolved in the core or adsorbed at the surface (Alleman *et al.*, 1998:174).

The use of such a particulate system is also a way to improve the peroral delivery of peptides. Incorporating or encapsulating peptides in polymeric particles should have at least one effect: protection against degradation by substances found in the GIT. Depending on the nature of the particles used (type of polymer, size, adjuvant substances added), these systems should also enhance oral absorption. Translocation of intact peptides from the intestinal mucosa to the systemic circulation after oral administration is qualitatively possible, but remains quantitatively low. Particles can also be designed as site-specific delivery systems, with polymers dissolving at defined values of pH (corresponding to the pH of specific parts of the GIT). Polymers used can also have bioadhesive properties, contain a protease inhibitor or penetration enhancers (Alleman *et al.*, 1998:173).

Similarly, 'microparticles' is a collective name for microspheres and microcapsules. The above mentioned differences between spheres and capsules are similar, but the size ranges from 1 to 250 μm (and in exceptional circumstances up to 1000 μm) for these carriers.

Some examples of particulate systems are:

- Polyacrylamide nanospheres
- Poly(alkyl cyanoacrylate) nanoparticles

- Derivatized amino acid microspheres
- Poly(capro,actone) nanospheres (Alleman *et al.*, 1998:174).

1.5.5 SPH & SPHC BASED DRUG DELIVERY SYSTEMS

SPH (superporous hydrogel) and SPHC (superporous hydrogel composition) is a new concept in drug delivery and is different from mucoadhesion (Dorkoosh *et al.*, 2001:308). SPH was initially synthesised as gastric detention devices (Van der Merwe, 2003:16). This drug delivery system is called a shuttle system, which is made of two distinct parts: (1) a core consisting of the drug within an appropriate formulation, and (2) a conveyor system consisting of a superporous hydrogel (SPH) and SPHC. The enteric coated conveyor system takes the core to the desired intestinal site of drug absorption depending on the release time of the enteric coating. After the dissolving of the enteric coated and gelatine layers, the SPHC conveyor systems will swell rapidly and be mechanically attached at the desired part of the gut wall. Two different delivery systems have been developed: core inside the shuttle system (fig 1.8A) and core attached to the surface of the shuttle system (fig 1.8B).

For the core inside the shuttle system, the core (including the drug of interest) is incorporated inside the conveyor system, which is made of SPHC. The system is closed with a plug made of SPH (Dorkoosh *et al.*, 2001:315). SPH and SPHC are able to swell very quickly due to their highly porous structure. The difference between SPH and SPHC is their swelling ratio and mechanical stability. SPH swells more quickly, but it is mechanically less stable, whereas SPHC has less swelling, but is mechanically more stable (Dorkoosh *et al.*, 2001:308). The use of SPHC as a conveyor system guarantees resistance to peristaltic movement of the intestine (Dorkoosh *et al.*, 2001:315). SPH and SPHC are able to keep the dosage form mechanically at the site of drug absorption. Since the mucus layer of the human intestine is regenerated with a turn-over time of 12-24 hours, mucoadhesive dosage forms will stay attached to the mucosal layer for a specific period at specific sites of the intestine, in contrast to mechanical attachment of SPH and SPHC.

In addition to the use of this novel system it is possible to get an appropriate time-controlled release profile, necessary for intestinal absorption of peptide drugs. For a normal release profile from the dosage form, drug release will be started at time zero, indicating that from the moment the dosage form is in the intestinal lumen the drug release is started. However, for the delivery of peptide drugs a lag time of 20-30 min is necessary to inactivate proteolytic enzymes and to open tight junctions. Thereafter a burst release is required in which the whole amount of drug should be released from the dosage form in a short period of time. This type of drug release is called a time-controlled release profile (Dorkoosh *et al.*, 2001:308).

The SPH based delivery systems were placed in a gelatin capsule and the capsules were enteric coated with Eudragit® S 100. These developed systems were able to pass through the acidic environment of the stomach and after the dissolution of the enteric coating and gelatin in the small intestine, the polymers were able to swell rapidly and mechanically attach to the gut wall. Absorption studies with octreotide in pigs, using enteric-coated gelatin capsules containing SPH and SPHC-based delivery systems, revealed that peroral administration of core inside and core attached to the surface of the conveyor systems resulted in absolute bioavailabilities of 12.7 % and 8.7 % respectively, whereas for the control group a bioavailability of 1.0 % was found. The increased bioavailabilities were attributed to opening of the tight junctions thereby increasing absorption via the paracellular route (Van der Merwe, 2003:17).

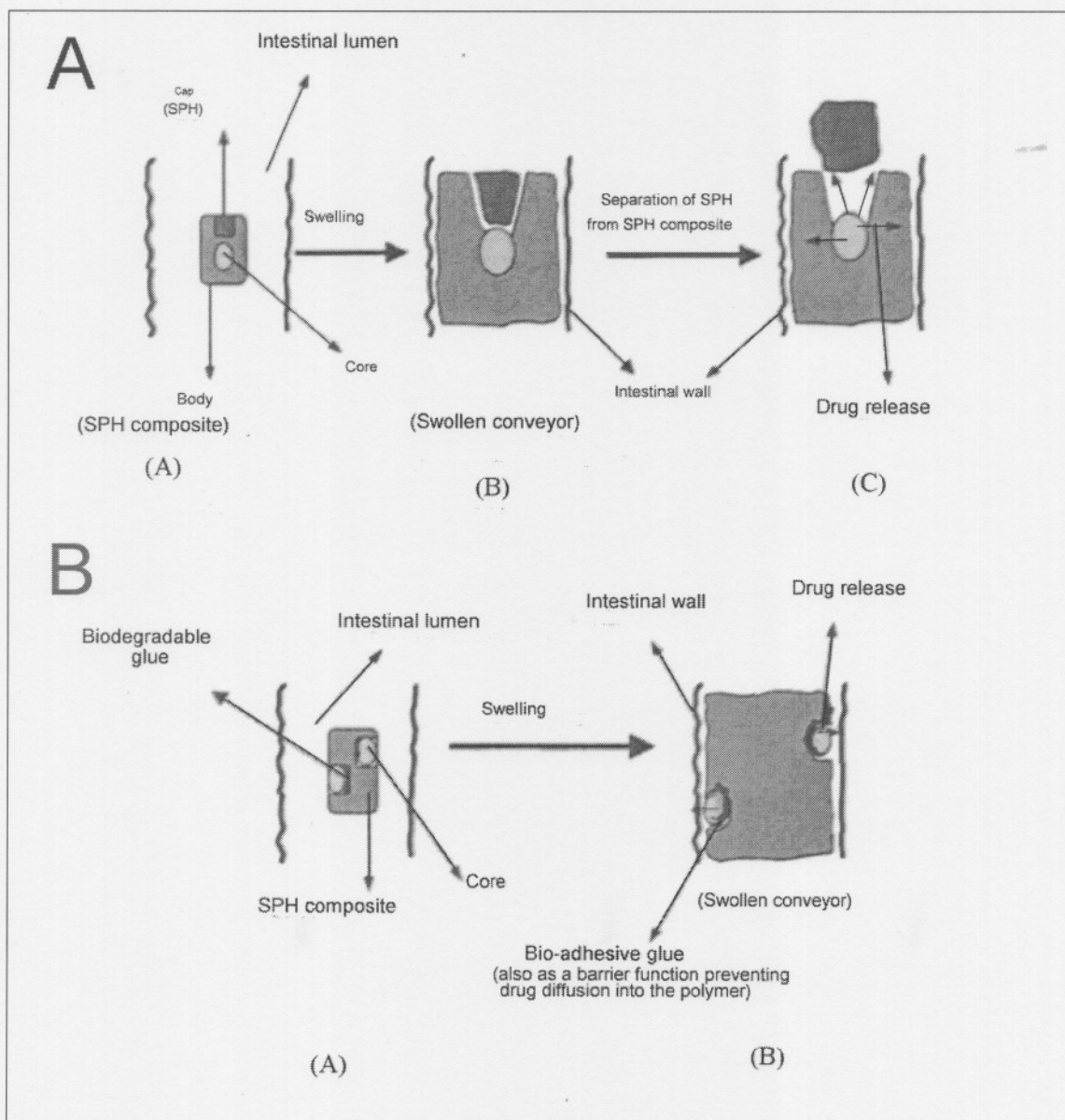


Figure 1.8 Schematic view of the mechanism of action of the SPH and SPHC based drug delivery system. (A) Core inside the shuttle system; drug is being released from the core after swelling of the conveyor system (SPHC / SPH). (B) Core attached to the surface of the shuttle system, drug is released after attachment of the delivery system to the intestinal wall (Dorkoosh *et al.*, 2001:310).

1.6 CONCLUSION

From the studied literature it can be concluded that peptide and protein drugs are inadequately absorbed from the GIT. This is because of their high molecular weight and hydrophilicity. It was also stated that there are numerous obstructions in the transport pathways for the delivery of peptide and protein drugs which cause poor bioavailability. Numerous researchers therefore have attempted to enhance the peroral absorption of hydrophilic compounds such as peptides and proteins by using different formulation approaches. Some of the approaches used included enzyme inhibitors, chemical modification (pro-drugs & peptidomimetics), absorption enhancers and targeted drug delivery systems. However it can be concluded that the necessity for an appropriate peroral delivery system for peptide and protein drugs still exist.

CHAPTER 2

CHITOSAN AND N-TRIMETHYL CHITOSAN CHLORIDE AS ABSORPTION ENHANCING AGENTS

2.1 INTRODUCTION

Chitosan [α (1 $\rightarrow$ 4) 2-amino 2-deoxy β -D-glucan] (fig 2.1), a natural linear bio poly amino saccharide, is acquired by the alkaline deacetylation of chitin (Sinha *et al.*, 2004:1), which is the second most abundant polysaccharide next to cellulose (Sinha *et al.*, 2004:1; Junginger & Verhoef, 1998:374).

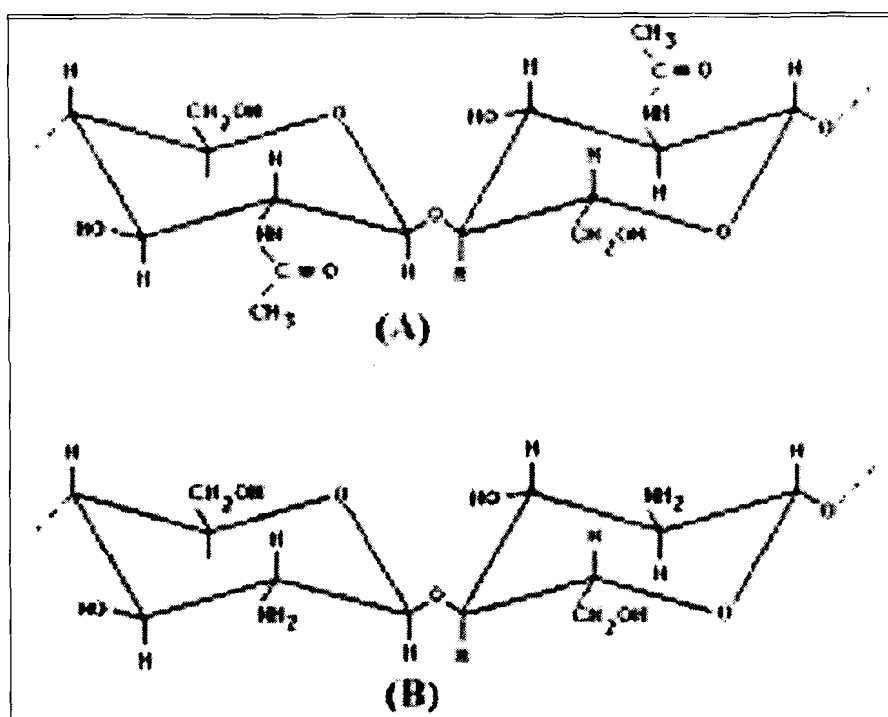


Figure 2.1 Chemical structures of chitin (A) and chitosan (B) (Paul & Sharma, 2000:7).

Chitin was first described by Braconnet in 1811, as a distinct substance present in hyphae or spores of molds. Chitosan, the primary derivative of chitin was later discovered by Rouget in 1859. He found that when chitin was boiled in a concentrated potassium hydroxide solution, a product was obtained that dissolved in dilute iodine and acids, unlike chitin that only stained brown (Paul & Sharma, 2000:5). But it took until 1894 before it was formally named by Hoppe-Seyler (Van Der Lubben *et al.*, 2001:202). Chitosan is easily obtainable because chitin is widely distributed in nature (Dodane & Vilivalam, 1998:246). Chitin is the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and cell walls of some fungi such as *Aspergillus* and *mucor* (Sinha *et al.*, 2004:1; Koide, 1998:1092). The term chitosan is used to describe a series of polymers which is available in a range of molecular weights (50 kDa – 2000 kDa) and degrees of deacetylation between 40 – 98 % (Jonker, 2000:2). Chitosan, a weak base (Sinha *et al.*, 2004:2) is insoluble in alkaline and neutral pH values but form salts with inorganic and organic acids such as hydrochloric acid, lactic acid, acetic acid and glutamatic acid (Illum *et al.*, 2001:83) of which the pH is lower than 6.5 (Sinha *et al.*, 2004:2). In solution, the amine groups of chitosan are protonated and the resultant soluble polysaccharide is positively charged (Illum *et al.*, 2001:83). Chitosan is biocompatible, biodegradable (Dodane, 1998:246; Junginger & Verhoef, 1998:374; Ravi Kumar, 2000:1-2), hydrophilic and of low toxicity and commercially available in different types of salts (Junginger & Verhoef, 1998:374). It neither is an irritant nor allergen for humans (Seferian & Martinez, 2000:661).

Over the last decade, chitosan has been attracting increasing attention as a biomaterial and as pharmaceutical excipient for drug delivery (Vila *et al.*, 2004:123). Chitosan possesses absorption enhancing, controlled release and bioadhesive properties (Dodane & Vilivalam, 1998:246). Chitosan acts by opening the tight junctions between epithelial cells to allow macromolecular compounds such as peptide drugs through the paracellular pathway (Jonker, 2000:3). The mechanism underlying this permeation enhancing effect seems to be based on the positive charges on the polymer, which interact with the cell membrane resulting in a structural reorganization of the tight junction associated proteins (Bernkop-Schnürch, 2000:2).

2.2 CHEMICAL STRUCTURE OF CHITOSAN

Chitosan, [α (1 $\rightarrow$ 4) 2-amino 2-deoxy β -D-glucan] comprises of co-polymers of glucosamine and *N*-acetyl-glucosamine (Van Der Lubben *et al.*, 2001:202; Paul & Sharma, 2000:5). Thus, chitosan is poly-(*N*-acetyl-2-amino-2-deoxy-D-glucopyranose), where the *N*-acetyl-2-amino-2-deoxy-D-glucopyranose (or Glu-NH₂) units are linked by (1 $\rightarrow$ 4)- β -glycosidic bonds (Ravi Kumar, 2000:1). Chitosan has one primary amino and two free hydroxyl groups for each building unit (fig 2.2).

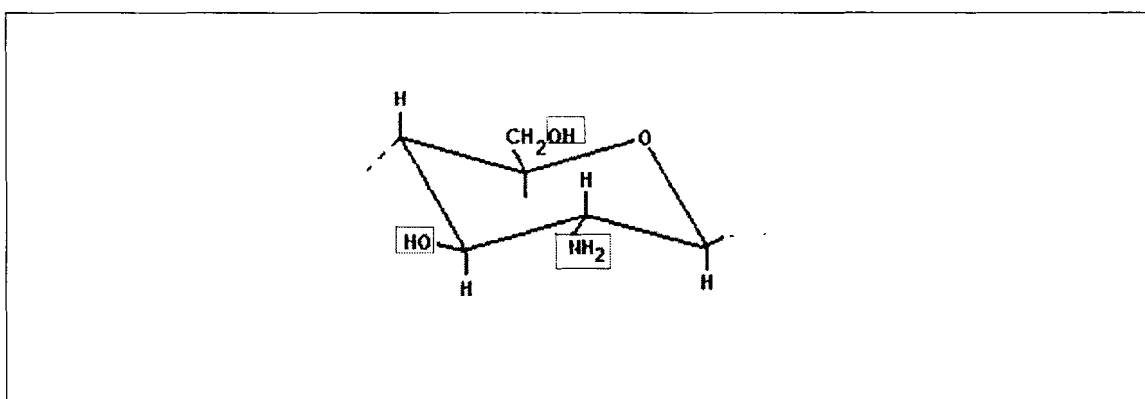


Figure 2.2 Structure of chitosan with one amino group and two hydroxyl groups (Paul & Sharma, 2000:7).

Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus in turn reacts with many negatively charged surfaces / polymers and also undergoes chelation with metal ions (Sinha *et al.*, 2004:1). Further chemical modifications are easily feasible; the features of chitosan can be optimized according to a given task in delivery systems focusing on specific pharmaceutical – technological challenges (Bernkop-Schnürch, 2000:2).

2.3 AVAILABILITY OF CHITOSAN

Chitin is the second most abundant polysaccharide in nature, cellulose being the most abundant (Hejazi *et al.*, 2003:151). Chitosan is produced by the alkaline hydrolysis of chitin, a process which randomly deacetylates and shortens the chain lengths of chitin molecules. Chitosan is commercially available in a different range of grades with different molecular weights and degrees of deacetylation (Aspden *et al.*, 1996:23). Chitin is effortlessly obtained from crab or shrimp shells (Ravi Kumar, 2000:3). In the world, several million tons of chitin is harvested annually (Hejazi *et al.*, 2003:151). Potentially the biggest source of chitin is the vast shoals of krill found in the oceans. However, it is more economical and ecologically sound to commercially utilize the discarded shells of crabs and shrimp caught for human consumption (Aspden *et al.*, 1996:23). Commercially chitosan is available in the form of dry flakes, solutions and fine powders with an average molecular weight ranging between 3800 and 2,000,000 Da and is 66 % to 95 % deacetylated (Sinha *et al.*, 2004:1-2). The abundance of chitin and resulting chitosan leads to the great popularity of this polymer in pharmaceutical applications.

2.4 SYNTHESIS OF CHITOSAN

To prepare chitin, crab and shrimp shells are treated in boiling aqueous sodium hydroxide solution (3-5 % w/v) at 80-90°C for a few hours after decalcification in dilute hydrochloric acid (3-5 % w/v) giving a white to beige coloured sample of chitin and then deproteinized in a dilute sodium hydroxide solution. Chitin is then deacetylated to become chitosan in a concentrated sodium hydroxide solution at boiling point (fig 2.3).

The conditions used for the deacetylation will determine the degree of deacetylation and the molecular weight of chitosan. Biomedical grade purified chitosan is prepared by repeating the deacetylation process. Pharmaceutical grade chitosan is deacetylated between 90% and 95% and the food grade between 75% and 80% (Paul & Sharma, 2000:5; Hejazi *et al.*, 2003:152).

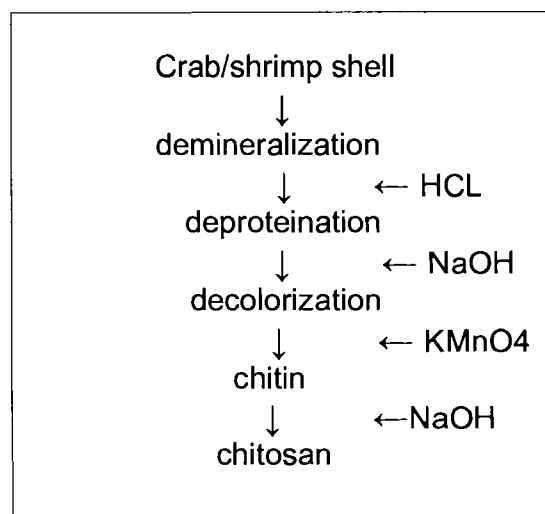


Figure 2.3 Production flow chart of chitosan from chitin (Paul & Sharma, 2000:5).

2.5 PHARMACEUTICAL APPLICATIONS OF CHITOSAN

Chitosan has been used for a range of applications ranging from a food additive, to a water purification agent (when spread over the surface of water it adsorbs the impurities) as well as in numerous pharmaceutical applications (Aspden *et al.*, 1995:70). The popularity of chitosan can be attributed to its low production costs, biodegradability, biocompatibility and FDA approval. The pharmaceutical and food applications of chitosan have increased extremely over recent years (Van der Lubben *et al.*, 2001:202).

Chitosan is also being used as a biomaterial in tissue engineering and in controlled drug release systems for various routes of delivery (Thanou *et al.*, 2001a:S92). Some applications of chitosan polymers that are being pharmaceutically applied are (a) excipients in granules, tablets, gels and microspheres (Junginger & Verhoef, 1998:374) (b) as binders, (c) disintegrants and (d) coating materials (Issa *et al.*, 2005:1). The same properties that made chitosan popular in water purification have also made it a powerful fat-absorber in the digestive system. The strong positive charge of chitosan causes it to bind negatively charged substances such as fats, lipids and bile (Paul & Sharma, 2000:6). Further unique properties of chitosan include polyoxysalt formation, ability to form films,

chelate metal ions (as mentioned), it has optical structural characteristics, the ability to form a gel (Ravi Kumar, 2000:4), wound healing, antimicrobial and hemostatic properties (Şenel *et al.*, 2000:2067).

Seferian & Martinez (2001:661) also reported that chitosan suspension or microparticles have immune stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cells. Chitosan also shows strong mucoadhesive properties (Junginger & Verhoef, 1998:374).

2.5.1 MUCOADHESIVE PROPERTIES

The mucoadhesive properties of chitosan was first described by Lehr *et al.* (1992:43) and demonstrated that chitosan in its swollen state is an excellent mucoadhesive excipient for porcine intestinal mucosa and also suitable for repeated adhesion. Polycarbophil failed in the repeated adhesion test. At neutral pH values (artificial intestinal fluid), chitosan underwent minimal swelling, proposing that substitution of the free $-NH_2$ groups with short alkyl chains changes the solubility and thus the mucoadhesion profile of chitosan (Thanou *et al.*, 2001a:S94).

The requirements for efficient adhesion are at least one of the following: (I) high molecular weight, (II) adequate quantities of hydrogen bonding chemical groups ($-OH$ and $-COOH$), (III) anionic surface charges, (IV) adequate chain flexibility and (V) surface energy properties supporting spreading onto the mucus. These characteristics favor the formation of bonds with mucus which are either mechanical or chemical in origin (Lehr *et al.*, 1992:43).

Though chitosan, with significant mucoadhesive properties, does not have any anionic charges it forms a positively charged hydrogel in acidic environments that could develop extramolecular attraction forces by electrostatic interactions with negatively charged mucosal surfaces (Lehr *et al.*, 1992:43). Mucoadhesive properties are also subjective to

molecular weight; the best results are obtained at higher molecular weights (Lehr *et al.*, 1992:46).

Because of the solubility of the pure polymer in acidic solutions, chitosan shows weak, short-lived mucoadhesive properties in artificial gastric fluid. Nevertheless, cationic polymers are likely to be superior mucoadhesives in a slightly alkaline or neutral medium. It would be desirable for adhesion in the small or large intestines. It was also noted that chitosan underwent minimal swelling in artificial intestinal fluid, which is elucidated by poor water solubility of the free base. Swelling can be improved by substitution of free amino groups with short alkyl chains in order to increase the pKa and therefore the ionization of these groups at a higher pH, thus possibly also increasing mucoadhesion (Lehr *et al.*, 1992:48).

2.5.2 CHITOSAN AS ABSORPTION ENHANCER FOR PEPTIDE DRUGS

The mucoadhesive polymer chitosan, has attracted a lot of attention as a potent enhancer of drug transport across mucosal surfaces (Kotze *et al.*, 1999a:269; Kotze *et al.*, 1999b:343), especially in the absorption enhancement of hydrophilic macromolecular drugs such as peptide drugs. Chitosan, when protonated (pH<6.5), is able to increase the paracellular permeability of peptide drugs across the mucosal epithelia (Thanou *et al.*, 2001a:S91). By modifications of the primary amino group at the 2-position of this poly-(14 D-glucosamine) the features of chitosan can even be optimized according to a given task in the drug delivery systems. For peroral peptide delivery these tasks focus on overcoming the absorption and enzymatic barrier of the digestive system.

Though even unmodified chitosan displays a permeation enhancing effect for peptide drugs. A protective effect for polymer embedded peptides towards degradation by intestinal peptidases can be achieved by the immobilization of enzyme inhibitors on the polymer (Bernkop-Schnürch, 2000:1).

Chitosan shows some favorable properties, namely:

- due to its high molecular weight, it is not absorbed and therefore are not expected to display systemic toxicity;
- it intensifies the contact between the dosage form and the site of absorption due to its mucoadhesive properties;
- it improves peptide transport across the epithelial barrier (Van der Merwe, 2003:23).

Lueßen *et al.* (1996:1668) showed that the intestinal absorption of buserelin, a synthetic nonapeptide with a molecular weight of 1240 Da was improved in rats by chitosan hydrochloride. In this study, the following polymers were tested: carbomer 934P (C934P), it's a freeze-dried neutralized sodium salt (FNaC943P) and chitosan hydrochloride. Of all the polymers tested chitosan hydrochloride (1.5 % w/v) in a gel formation resulted in the highest absolute bioavailability for intraduodenally administered buserelin in rats (5.1 ± 1.5 %), even superior to the value reported for the nasal formulation Suprecur[®] (3.3 %) in men.

Other poorly absorbed polypeptide drugs, insulin and calcitonin's absorption also have been enhanced by using chitosan (Schipper *et al.*, 1999:335). Chitosan's mucoadhesiveness, favorable toxicological properties and ability to open tight junctions between epithelial cells makes it an important potential absorption enhancer for future use.

2.6 MECHANISM OF ACTION OF CHITOSAN

The increase in the absorption of hydrophilic compounds could be ascribed to an interaction of a positively charged amino group on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions of the mucosal epithelial cells. Chitosan induces a redistribution of cytoskeletal F-actin (from filamentous to globular) and the tight junction protein ZO-1, thereby changing the integrity of the tight junctions which is related directly or indirectly with the F-actin filaments (Kotze *et al.*, 1999a:270; Van der Lubben *et al.*, 2001:203).

Thus chitosan interpenetrate the mucus layer, reaching the physical barrier and facilitating transport of hydrophilic molecules across the mucus layer. Furthermore, mucoadhesive polymers are suitable for the design of delivery systems that would adhere to the mucus for a period of two to four hours and allow local interactions with the gut wall (Junginger & Verhoef, 1998:373). Confocal laser scanning microscopy (CLSM) confirmed that chitosan is able to open tight junctions to allow for the paracellular transport of large hydrophilic compounds (Kotze *et al.*, 1999a:270).

A disadvantage of chitosan and chitosan salts is its insufficient solubility at 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. This implies that chitosan can be effective only in a limited area of the GIT where the pH values are close to its pKa (Thanou *et al.*, 2001a:S94). To overcome this problem, chitosan derivatives have been synthesized and characterized. This will be discussed in detail in section 2.9.

2.7 SAFETY OF CHITOSAN

Van der Lubben *et al.* (2001:202) stated that chitosans with a low degree of deacetylation, which are active as absorption enhancers at both low and high molecular weights, display an obvious dose dependent toxicity. On the other hand, chitosans with a high degree of deacetylation are only active enhancers at high molecular weights, and shows a very low toxicity. Thus toxicity is dependent on the structural features of the polymer, but not always linked to its absorption enhancing effect.

Schipper *et al.* (1999:341) also showed in studies using Caco-2 cells that the absorption enhancing and dose dependent cytotoxic effects of chitosan were independently determined by the structural features of the polymer, i.e., the degree of deacetylation and molecular weight.

Aspden *et al.* (1996:28) conducted an investigation on chitosan salts of different molecular weights and degrees of deacetylation on their ability to enhance nasal perfusion. The chitosan salts produced minimal membrane and cellular damage when compared to laurth-9 solutions. They also investigated the effect of the chronic application (28 days) of chitosan to nasal epithelia of guinea pigs. By measuring the ciliary beat frequency (CBF) of excised nasal tissue, it was proven that all chitosans tested did not influence the ciliary activity (Aspden *et al.*, 1997:137).

The US Food and Drug administration (FDA) has approved chitosan as food additive in animal feeds. Due to its safe toxicity profile, chitosan is an interesting and promising excipient for the pharmaceutical industry (Paul & Sharma, 2000:7).

2.8 FACTORS INFLUENCING THE ABSORPTION ENHANCING PROPERTIES OF CHITOSAN

2.8.1 MOLECULAR WEIGHT AND DEGREE OF DEACETYLATION

Both the degree of deacetylation (number of GlcNAc units) and the molecular weight of chitosans have been shown to influence the physical and biological properties of the polymer (Schipper *et al.*, 1999:335). Chitosans with a high degree of deacetylation and high molecular weight display the relatively highest increase in epithelial permeability (Bernkop-Schnürch, 2000:2) and showed low toxicity (Schipper *et al.*, 1997:923). Toxicity and absorption enhancement can thus be controlled by selecting a chitosan with the most favorable degree of deacetylation and molecular weight.

2.8.2 CHARGE DENSITY

Chitosan is a weak base and a certain quantity of acid is necessary to alter the glucosamine units into a positively charge, water-soluble form. Most chitosan molecules will loose their charge and precipitate from the solution in neutral and basic environments. This limits the use of chitosan as a absorption enhancer to acidic

environments. Macromolecular peptide drugs are mainly indicated for chronic administration and therefore possibilities are restricted for the potential use of chitosan in the more neutral and basic environments of the small intestine and colon (Kotze *et al.*, 1997b:1197). Since chitosan is most effective on the transport of molecules when the pH is below its pKa of 6.5, it can be concluded that the charge density plays a vital role in enhancement of mucosal absorption (Schipper *et al.*, 1996:1686).

2.9 N-TRIMETHYL CHITOSAN CHLORIDE (TMC)

As mentioned earlier, chitosan is an effective penetration enhancer for hydrophilic drugs in acidic environments as it is only soluble at low pH values. Therefore chitosan's use is limited to the more basic environment of the large and small intestine (Jonker, 2000:8). It was the theory of Kotze *et al.* (1999b:341) that polymers such as unmodified chitosan with a primary amino group may not be the most favourable, but that polymers or derivatives with different substituents, basicities or charge densities will have the same or even increased efficacy in opening tight junctions. A perfect approach would be to modify the solubility while still retaining the biodegradability and absorption enhancing properties of chitosan.

Several chitosan derivatives have been synthesized and characterized. The chitosan derivative N-trimethyl chitosan chloride (TMC) is perfectly soluble at neutral and basic pH values (Van der Lubben *et al.*, 2001:204). TMC has been intensely studied and described for its absorption enhancing effects. Thus the potential use of TMC, in neutral and basic environments where regular chitosan salts are unsuccessful as absorption enhancers, could contribute considerably to the efficient delivery of hydrophilic compounds, for instance peptide drugs.

2.9.1 CHEMICAL STRUCTURE AND SYNTHESIS OF TMC

The chemistry of chitosan has been explored by various researchers, including those reactions that would result in a product soluble in basic or neutral aqueous medium.

During the synthesis of TMC the methylation of amino groups in the C-2 position of the chitosan subunit forms quaternary amino groups with permanent positive charges in the TMC structure (Snyman, 2003:30.) as shown in figure 2.4.

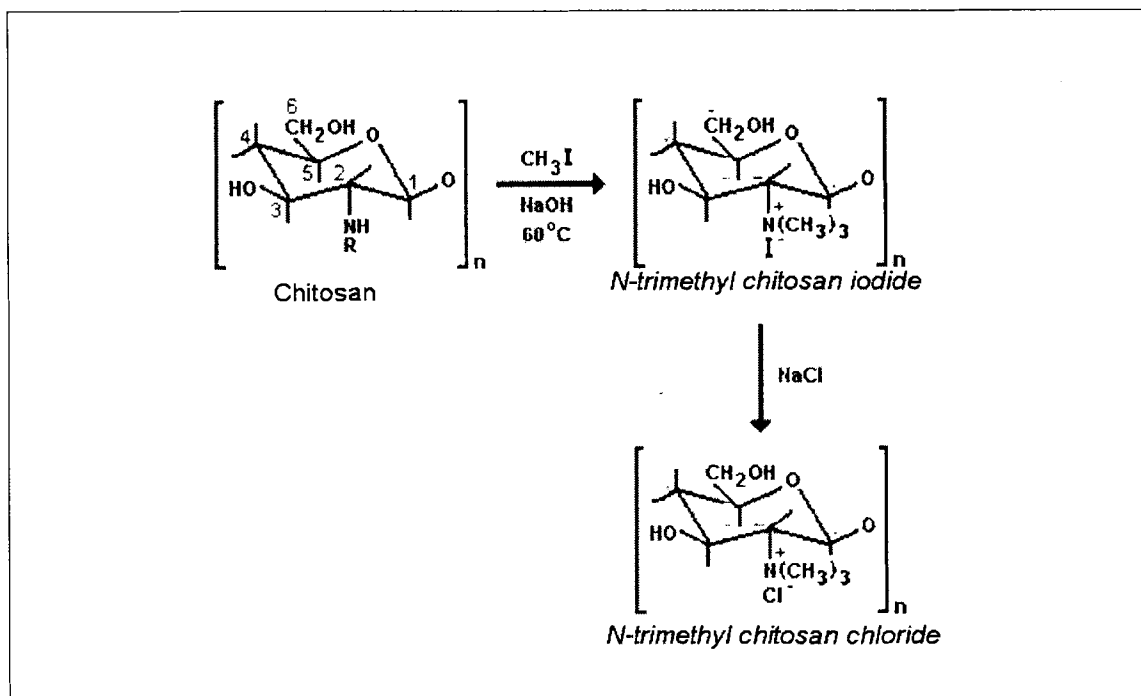


Figure 2.4 Synthesis and structure of *N*-trimethyl chitosan chloride (Kotze *et al.*, 1999b: 353).

The number of positive charges on the polymer is increased, causing the polymeric molecule to enlarge in solution due to repelling forces between the functional groups. The methylation of the primary amine to the quaternary stage is accomplished by using a base to bind to the acid, being generated during the reaction taking place and to avoid protonation of the unreactive primary amino groups. Although a high degree of substitution on the amino group of chitosan is complicated due to the presence of the acetyl group, the hydroxyl group of chitosan can be substituted in the methylation process (Polnok *et al.*, 2004:78-79).

The degree of quaternisation can be changed by increasing the number of reaction steps or by increasing the reaction time (Hamman, 2001:76; Sieval *et al.*, 1998:157) during the reductive methylation of chitosan with methyl iodide in the presence of a strong base (NaOH) (Sieval *et al.*, 1998:157).

Absolute quaternisation of chitosan will probably be complicated due to the presence of some acetyl groups on adjoining quaternary amino groups. Additional proof of this is obvious from ¹H-NMR spectra that shows that a high percentage of the amino groups are mono- and dimethylated and can still be protonated in acidic environments. However, even at low degrees of quaternisation (12 %) of this polymer, it becomes soluble at all pH values and an increase in basicity has been found with an increase in the degree of quaternisation (Sieval *et al.*, 1998:158).

2.9.2 MUCOADHESIVE PROPERTIES OF TMC

As mentioned earlier chitosan possesses mucoadhesive properties and the same is true for TMC with different degrees of quaternisation. The mucoadhesive properties of TMC polymers can be used to increase the contact period of the active ingredients with the epithelium and its absorption via the paracellular pathway through the tight junctions. Mucoadhesive properties of TMC polymers are affected by their molecular weight up to a value of 100,000 g/mol, where after any further increase had no visible effect. Molecular weight is influenced by the synthesis procedure used to produce TMC, thus the procedure followed is just as important (Snyman *et al.*, 2002:145).

Snyman *et al.* (2003:67-68) also reported that the mucoadhesive properties of TMC are also influenced by their degree of quaternisation. Mucoadhesion decreases as the degree of quaternisation increases. However, it was also observed that chitosan salts possessed stronger mucoadhesive properties than the TMC polymers. This was explained by a change in the conformation of the TMC polymers due to interactions between the fixed positive charges on the C-2 position of each polymer. These interactions may force the polymer to change its conformation with a decrease in polymer flexibility. The

interpenetration into the mucus layer by TMC polymers is influenced by a decrease in flexibility resulting in a subsequent decrease in mucoadhesivity. Nevertheless, the TMC polymer with the highest degree of quaternisation (48.8%) was still sufficiently mucoadhesive to be included into a mucoadhesive dosage form.

2.9.3 MECHANISM OF ACTION OF TMC

TMC most likely has a similar mechanism of action as chitosan and chitosan salts. Chitosan primarily acts by an interaction between the positively charged amino groups on the C-2 position with negatively charged sites on the cell membranes and tight junctions to allow for opening of the tight junctions (Kotze *et al.*, 1997a:251). This interaction could be inhibited by heparin, indicating that the positive charge is essential for the binding properties of chitosan. TMC, at all degrees of quaternisation, has positive charges, regardless of the environmental pH (Van der Merwe *et al.*, 2004:232). Opening of the tight junctions by TMC to allow increased transport of hydrophilic compounds along the paracellular transport pathway was confirmed with confocal laser scanning microscopy (CLSM) (Thanou *et al.*, 2001b:122).

2.9.4 THE EFFECT OF TMC ON THE TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER) OF HUMAN INTESTINAL EPITHELIAL CELLS (CACO-2)

Tight junctions serve as barriers to paracellular diffusion and the measurement of the TEER is believed to be a good indication of the tightness of the junctions between epithelial cells. Boulenc *et al.* (1995:14) also suggested that the measurement of TEER could be used to predict the paracellular transport of hydrophilic molecules.

The effects of two types of TMC polymers on the TEER of Caco-2 cell monolayers were measured by Kotze *et al.* (1999a:272). The results found clearly showed that both polymers were able to decrease the TEER of the Caco-2 monolayers. Incubation on the apical side of the monolayers with 1.5-2.5 % w/v of the polymers resulted in an obvious

decrease in TEER values compared with the control group. Both polymers, especially at concentrations of 2.0 and 2.5 %, caused instantaneous reduction in TEER. The reduction of TEER thus seems concentration dependent, with the highest reduction measured at 2.5 % concentrations (Kotze *et al.*, 1999a:272).

The effect of two chitosan salts, chitosan hydrochloride and chitosan glutamate, and TMC (with a degree of quaternisation of 12.8 %) on the TEER of Caco-2 cell monolayers were compared and the results revealed once again that all of these compounds were able to decrease the TEER significantly. The decrease in TEER at 0.25 % (w/v) concentrations, 20 minutes after incubation on the apical side, was in the following order; chitosan hydrochloride (71 ± 4 % reduction) > chitosan glutamate (56 ± 1 % reduction) > TMC (28 ± 1 % reduction), indicating that the chitosan salts were more effective than TMC at the similar weight concentrations (Kotze *et al.*, 1998:41). Solutions above 1.5 % w/v concentrations of the chitosan salts could not be prepared at a pH of 6.5 due to its low solubility and high viscosity. In contrast with TMC, which easily dissolves at this pH in much higher concentrations, a further decrease in TEER was found for these higher concentrations.

According to the authors, since the degree of quaternisation was the same, the difference in effect of these polymers could be explained in terms of the equivalent weights of each repeating unit in the polymer backbone of the respective polymers (theoretically 197.62 for chitosan hydrochloride, 308.20 for chitosan glutamate and 239.80 for TMC), thus determining the amount of free chitosan base and therefore the density of the amino groups available for protonation at similar weight concentrations. About 50 % of chitosan glutamate by weight is glutamate salt whereas for chitosan hydrochloride the salt part only constitutes a small fraction (5 – 10 %).

Additionally, the attached methyl groups on the C-2 position of TMC probably causes steric effects and also partially hide the positive charge on the quaternary amino groups, thereby altering the time needed for interaction with the negatively charged cell membranes and tight junctions. Nevertheless, at higher concentrations of TMC (2.0 – 2.5

% w/v) similar effects on the TEER, as seen with chitosan hydrochloride, were observed. The better solubility of TMC may as a result compensate for its lower effect at similar weight concentrations (Kotze *et al.*, 1998:41).

2.9.5 THE EFFECT OF TMC ON THE ABSORPTION ENHANCEMENT OF PEPTIDE DRUGS

Metabolically inert substances such as [^{14}C]-mannitol and [^{14}C]-polyetheleneglycol (PEG-4000) are highly hydrophilic in nature. Mannitol has been used previously to follow changes in the epithelial integrity of mucosal cell membranes. Both compounds do not diffuse to a great degree into the cell membranes, but are absorbed through the alternative aqueous paracellular pathway (i.e. through the tight junctions) and are therefore perfect substances to detect changes in permeability in studies of paracellular absorption enhancement (Borchard *et al.*, 1996:131). The compared effects of TMC (degree of quaternisation 12.28 %), chitosan hydrochloride and chitosan glutamate on the permeability of Caco-2 cells at pH 6.20 for the hydrophilic marker [^{14}C]-mannitol are shown in table 2.1.

Exposure of the apical side of the monolayers to 0.25 % (w/v) of the polymers resulted in a 34-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 11-fold (TMC) increase in the absorption rate of [^{14}C]-mannitol, compared to the control group as indicated by the P_{app} values and absorption enhancement ratios (R). This changes to a 36-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 10-fold (TMC) increase in the presence of 1.5 % (w/v) concentrations of the respective polymers. Similar results were obtained for [^{14}C]-PEG-4000. At higher concentrations TMC was able to increase the P_{app} and R-values further for both [^{14}C]-mannitol and [^{14}C]-PEG-4000. A 17-fold and 21-fold increase in R were found for [^{14}C]-mannitol at 2.0 and 2.5 % (w/v) concentrations of TMC, respectively. The same tendency was also seen with [^{14}C]-PEG-4000 (Kotze *et al.*, 1998:41).

Table 2.1 The effect of TMC, chitosan glutamate and chitosan hydrochloride on the permeability of [¹⁴C]-mannitol at a pH of 6.20 (Kotze *et al.*, 1998:41).

Marker	Concentration (% w/v)	TMC		Chitosan glutamate		Chitosan hydro- chloride	
		$P_{app} \times 10^{-7}$ (cm/s) ^a	R	$P_{app} \times 10^{-7}$ (cm/s) ^a	R	$P_{app} \times 10^{-7}$ (cm/s) ^a	R
[¹⁴ C]-mannitol	Control	0.72 ± 0.08	1	0.72 ± 0.08	1	0.72 ± 0.08	1
	0.25	8.11 ± 0.21 ^b	11	18.25 ± 1.10 ^b	25	24.62 ± 2.13 ^b	34
	0.50	9.26 ± 0.35 ^b	13	14.17 ± 0.45 ^{bc}	20	23.28 ± 1.00 ^b	32
	1.00	14.00 ± 0.40 ^b	19	20.82 ± 0.30 ^b	29	25.56 ± 2.95 ^b	36
	1.50	7.52 ± 0.86 ^b	10	18.29 ± 1.53 ^b	25	26.16 ± 1.86	36
	2.00	12.35 ± 0.43 ^b	17	n.d.	n.d.	n.d.	n.d.
	2.50	15.21 ± 1.37 ^b	21	n.d.	n.d.	n.d.	n.d.

^a Each value represents the mean ± S.D. of 3 experiments

^b Significantly different from control (n < 0.05)

^c Significantly different from all other treatments in group (n < 0.05)

n.d. = not determined due to insolubility of chitosan salts.

From these results, it is evident that TMC was not as effective at similar weight concentrations as chitosan hydrochloride and chitosan glutamate. These results are also similar to the results obtained in the TEER studies referred to in section 2.9.4. The authors concluded that additional factors play a role in the absorption enhancement mechanism of TMC. This could possibly be explained in terms of the charge density, the equivalent weight of each repeating unit in the polymer backbone and possible steric effects of the attached methyl groups and partial hiding of the positive charge on the quaternary amino groups (Kotze *et al.*, 1998:43).

Kotze *et al.* (1997b:1197) also showed that TMC, with a degree of quaternisation of 12 % was able to increase the transport of fluorescein isothiocyanate-labelled dextran (FD-4) across Caco-2 cell monolayers. The transport of this large hydrophilic model compound (Mw = 4400 Da) was increased 167-, 274- and 373-fold with 1.5, 2.0 and 2.5 % (w/v) concentrations of TMC respectively.

Lueßen *et al.* (1996:1668) proved that the intraduodenal administration of buserelin with chitosan hydrochloride in a gel formation increased the absolute bioavailability of buserelin from 0.1 % to 5.1 %. In another study the nasal application of insulin with chitosan glutamate led to a significant reduction of blood glucose levels in rats and sheep. In the sheep model the addition of chitosan to a nasal formulation of insulin resulted in a seven fold increase in the peak plasma insulin levels (Illum, 2002:1187).

2.9.6 THE EFFECT OF THE DEGREE OF QUATERNISATION ON THE ABSORPTION ENHANCING PROPERTIES OF TMC

The degree of substitution of TMC plays a vital role in the ability to the open tight junctions of intestinal mucosa (Junginger & Verhoef, 1998:375). Kotze *et al.* (1998:41) studied the effect of TMC (degree of quaternisation 12.28 %), chitosan hydrochloride and chitosan glutamate on the permeability of Caco-2 cells at a pH of 6.60 for the hydrophilic marker [14C]-mannitol (as mentioned in section 2.9.5). From these results, it was

evident that TMC was not as effective at the similar weight concentrations as chitosan hydrochloride and chitosan glutamate.

Its lesser efficacy was explained by the degree of quaternisation of TMC, which determines the amount and density of the positive charges on the C-2 position of the polymer, and by partial hiding of the positive charge on the amino group by the attached methyl groups. The amount of the positive charges on TMC influences the amount of interactions with negative sites on the cell membranes and the opening of the tight junctions. Jonker *et al.* (2002:205) used both an *in situ* (single pass perfusion) and an *in vitro* (everted intestinal sacs) method in rats to study the effect of the degree of quaternisation on the absorption of [¹⁴C]-mannitol.

TMC polymers with a degree of quaternisation ranging from 22 to 48 % were used in these studies. It was clearly demonstrated that TMC enhances intestinal permeation in a neutral pH environment and that the extent of absorption enhancement was dependant on the degree of quaternisation of TMC. In both methods the best permeation enhancing results were obtained with the highest degrees of quaternisation. In general it was proposed that TMC with higher degrees of quaternisation might be more effective as an absorption enhancer for the increased paracellular transport of hydrophilic compounds in neutral environments (Jonker *et al.*, 2002:205 & Kotze *et al.*, 1999a:274).

2.9.7 CYTOTOXIC EVALUATION OF TMC

Chitosan is considered to be a biocompatible and non-toxic polymer, but the properties of cationic polymers can also exhibit damaging effects on the epithelial cells as some dose dependent toxic effects for certain chitosans were reported by Schipper *et al.* (1996:1686). Therefore, safety studies are required for novel absorption enhancers to assure the absence of tissue damaging effects of the compound under research. Thanou *et al.* (1999:77) studied the effects of TMC on the ciliary beat frequency (CBF) and its possible membrane damaging effects. The effect of 1.0 % (w/v) TMC (degree of quaternisation 60 %) on the CBF of chicken embryo trachea resulted in a slight reduction

in this frequency. This reduction in frequency was even less pronounced than the reduction observed after incubation with physiological saline (0.9 % NaCl) (Thanou *et al.*, 1999:80).

In the presence of TMC a fluorescent probe was used for a cytotoxic study in selected Caco-2 cell monolayers. The probe only emits fluorescence upon binding with the nuclei of cells and those cells which did not take up this fluorescent probe were considered viable. Horizontal cross-sections of cell monolayers treated with 1.0 % (w/v) TMC for 4 hours confirmed no nuclei staining when visualized with confocal laser scanning microscopy (CLSM). It was therefore concluded by Thanou *et al.* (1999:81) that TMC is a safe absorption enhancer for hydrophilic macromolecules such as peptide and protein drugs across nasal and other mucosal tissues.

Kotze *et al.* (1997b:1199) also confirmed that Caco-2 cell monolayers, after prolonged incubation with TMC, remained undamaged and functionally intact with the use of trypan blue (a staining marker which identifies damaged cells).

Kean *et al.* (2005:650) showed that the degree of trimethylation also influences the toxicity of chitosan derivatives. If trimethylation increased, the toxicity of both oligomer and TMC also increased, but to a greater extent in TMC polymers. Overall, in comparison to PEI (the most popular “gold standard”), TMC was relatively non-toxic.

2.10 CONCLUSION

In this chapter a broad overview of chitosan and its quaternary derivative (TMC) were given. The ability of chitosan and TMC to enhance the absorption of hydrophilic compounds across the GIT has led to great interest in these polymers for the development of bioadhesive dosage forms and drug delivery systems. The mechanism of action of chitosan and TMC (partially quaternized derivative of chitosan) is by means of opening the tight junctions between epithelial cells to allow paracellular transport of peptide and protein drugs. However, chitosan is insoluble in an alkaline medium, thus the potential

use of chitosan as absorption enhancer in the small intestine at higher pH values is limited.

TMC on the other hand have shown to be much more soluble at neutral pH levels and can act as a potential absorption enhancer of hydrophilic compounds in the small intestine. These absorption enhancing capabilities were confirmed in studies conducted on Caco-2 cell monolayers where a decrease in TEER was observed as well as an increase in transport of hydrophilic compounds across these monolayers at neutral pH values. Similar results were found in *in vivo* studies. It was also shown that the degree of quaternisation of TMC plays an important role in its ability to act as an absorption enhancer. Cytotoxic studies on the polymer also revealed that it was safe to use as an absorption enhancer in the delivery of peptide and protein drugs. Therefore TMC may acts as a potential delivery system for poorly absorbable peptide and protein drugs.

CHAPTER 3

PHEROID TECHNOLOGY AS A DRUG DELIVERY SYSTEM

3.1 PHEROID TECHNOLOGY

Pheroid technology (further referred to as Pheroid or Pheroids; previously known as Emzaloid™ technology) is a patented system comprised of a unique submicron emulsion formulation. A Pheroid is a stable structure within a system that can be manipulated in terms of morphology, structure, size and function. Pheroids was first discovered when it was used as a basic formulation which led to the remission of psoriasis. One of the basic ingredients of this first formulation was present in banana peel extract and was later identified as essential fatty acids (Schlebusch, 2002:7).

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

3.2 PHEROID TYPES, CHARACTERISTICS AND FUNCTIONS

There are many potential barriers for effective drug delivery. Pheroids entrap drugs and deliver them to target sites in the body. The Pheroid penetrates keratinized tissue, skin, intestinal lining, nasal epithelium, vascular system, fungi, bacteria and parasites.

Figure 3.1 shows confocal laser scanning micrographs of various formulations of the Pheroid delivery system (Grobler, 2004:4). While there are many existing delivery systems, the Pheroid is unique in that its components are manipulated in a specific

manner to ensure its high entrapment capabilities, fast rate of transport and delivery and stability.

Various types of Pheroids exist:

- Lipid bilayer vesicles in both the nano and micrometer size range.
- Micro sponges.
- Depots / reservoirs that contain pro Pheroids.

Each type consists of a specific formulation and size and shape of vesicles, which can be reproduced within the same size size vesicles of: 80 – 30 nanometer; micro sponges of 0.5-5 μm . Sizes of depots / reservoirs are determined by the amount pro Pheroid contained in the reservoir. The absorption capabilities and drug release characteristics of the Pheroid can thus be controlled (Schlebusch, 2002:8). Pheroids consists mainly of 3 phases namely an aqueous phase, an oil phase and nitrous oxide. The aqueous phase is mainly water while the oil phase is a unique combination of essential fatty acids. The entrapment of drugs within the Pheroid generally creates a safer, more effective formulation than a formulation containing only the drug (Grobler, 2004:4).

In addition, these essential fatty acids are required for many cell functions, but cannot be manufactured by human cells and therefore have to be ingested. It has been shown that western diets lack these basic lipid molecules. Some of the functions of the fatty acid components of the Pheroid system are the maintenance of membrane integrity of cells, energy homeostasis and modulation of the immune system through leukotrienes and prostaglandins and some regulatory aspects of programmed cell death. The Pheroid system therefore has inherent therapeutic qualities that afford it significant advantages over various delivery systems (Grobler, 2004:4).

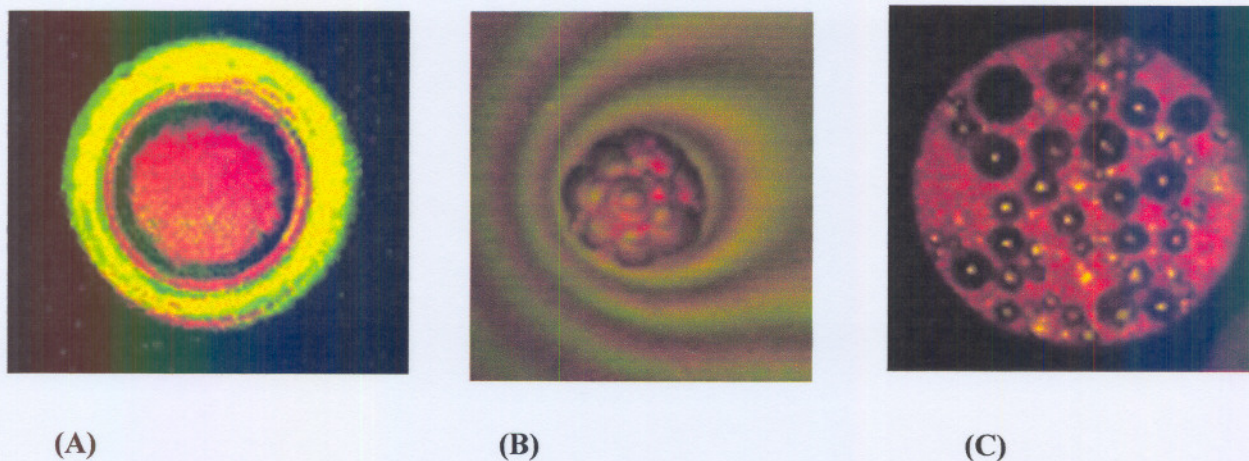


Figure 3.1: Confocal laser scanning micrographs of some of the basic Pheroid types.

(A) A bilayer membrane vesicle containing Rifampicin. (B) The formation of small pro-Pheroids that are used in oral drug delivery. (C) A reservoir that contains multiple particles of coaltar (Grobler, 2004:5).

3.3 PHEROID VERSUS OTHER LIPID BASED DELIVERY SYSTEMS

Although Pheroid systems are often confused with lipid based delivery systems, the fundamentals of the Pheroid system clearly shows that the system differs substantially from conventional macromolecular carriers, such as the liposomal delivery systems. The following table provides a comparison of the variation and key advantages of the Pheroid and other lipid-based delivery systems.

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

Pheroid	Lipid- based delivery systems
Consists mainly of essential fatty acids, a natural and essential ingredient of the human body.	Delivery systems generally contain a proportion of substances foreign to the human body.
Cytokine studies demonstrated that Pheroid elicits no immune response in man.	Some liposomal formulations have been shown to elicit immune responses in man.
By using different combinations of fatty acids and / or other substances, the Pheroids can be targeted at subcellular level to some extent.	Since phospholipids are metabolized in the cell membrane, it is difficult to envisage how subcellular organelles can be targeted by this approach.
Since it is part of the natural biochemical pathways, Pheroids cause no cytotoxicity and assists with cell maintenance.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body. Liposomal systems may decrease the cytotoxicity of compounds or may cause cytotoxicity.
It is polyphonic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Due to its composition is is sterically stabilised without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be sterically stabilised. This generally leads to an increase in size and rigidity of the carrier.

It contains no cholesterol, the interior volume remains stable.	Most lipid-based delivery systems contain phospholipids and cholesterol to stabilise the interior of the vesicles.
Due to Pheroid composition, it is able to inhibit the drug efflux mechanism in the intestinal lumen and can thereby enhance the bioavailability of a compound.	Liposomal systems containing this feature have not been described. A separate compound (e.g. Cremophor) is co-administered to achieve the same effect.
The entrapment efficiency of compounds inside the Pheroid is high (between 85 % and 100 %).	Due to the charge and steric limitations of liposomal delivery systems, entrapment efficiencies may be problematic.
The type of Pheroid formulated for a specific compound determines the loading capacity of the Pheroid.	The loading capacity of most lipid-based delivery systems is dependant on the interior of the intramembrane volume and is therefore limited.
Pheroid micro-sponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other drug in the sponge spaces. Geographical separation of active compounds into different interior spaces minimizes drug interactions or interactions between drug compounds.	Combination therapies are problematic in most liposomal drug delivery systems.
Batch-to-batch reproducibility and stability has been proven with existing products containing Pheroid, such as the registered product Exorex®.	Large scale manufacturing of other liposomal delivery systems sometimes shows low batch-to-batch reproducibility as well as problems with size control.

It is designed to show a high degree of elasticity and fluidity, with a relatively high phase transition temperature.	Because of the general use of stabilising compounds and cholesterol, liposomal systems lose fluidity and elasticity. The phase transition temperatures of phospholipid groups are higher than that of essential fatty acids, resulting in loss of elasticity.
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3.4 PHARMACEUTICAL APPLICABLE FEATURES OF THE PHEROID SYSTEM

3.4.1 DECREASED TIME OF ONSET OF ACTION

Research has signified that the Pheroid delivery system rapidly transverses most physiological barriers and delivers the active compound intact. An active compound delivered with Pheroids has been shown to act considerably faster than the same active compound delivered via a conventional approach, therefore suggesting a potentially faster relief from target symptoms (Grobler, 2004:9).

3.4.2 INCREASED DELIVERY OF ACTIVE COMPOUNDS

Both *in vitro* and *in vivo* studies have revealed that by using Pheroid as a delivery system, the percentage active compound that is delivered to the target site can be dramatically increased by entrapment of the active compound in Pheroids (Grobler, 2004:9).

3.4.3 REDUCTION OF MINIMUM DRUG CONCENTRATION

Research performed with the Pheroid system has shown that, for certain active compounds, using as little as 2.5% of the active compound may result in an effective drug plasma concentration. In practice, this characteristic will result in a reduction of patient side effects as well as major savings in treatment cost (Grobler, 2004:10).

3.4.4 INCREASED THERAPEUTIC EFFICACY

It has been shown that using Pheroids as a delivery system increases the efficacy of the active compound incorporated in the Pheroid system (Grobler, 2004:10).

3.4.5 REDUCTION IN CYTOTOXICITY

Side effects of drugs are in most cases caused by cellular damage. The Pheroid system has the potential to minimise cellular damage that occurs as a result of membrane damage caused by active compounds. Given that Pheroids are also part of the natural biochemical pathway, it is not cytotoxic. Therefore, by incorporating the drug in the Pheroid system, the appearance of side effects can be dramatically reduced (Grobler, 2004:11).

3.4.6 IMMUNOLOGICAL RESPONSES

Some drugs, such as proteins and peptides, may induce an immunological response or adverse intolerance reactions. By masking these compounds using the Pheroid system, this may prohibit the human immune system from recognising these compounds. By doing this the dosage can be reduced without diminishing the potency, or the dosage can be increased to enhance therapeutic effects (Grobler, 2004:12).

3.4.7 TRANSDERMAL DELIVERY

Whilst the Pheroid system is not limited to topical application, research has indicated that many medications can be administered topically instead of orally. The digestive system seems to be the origin of many side effects. Therefore transdermal delivery will eradicate many of the unwanted side effects caused by oral medication (Grobler, 2004:12).

3.4.8 THE ABILITY TO ENTRAP AND TRANSFER GENES TO NUCLEI AND EXPRESSION OF PROTEINS

Experiments performed on the Pheroid delivery system demonstrate its applicability in DNA vaccines and gene therapy. *In vitro* studies have shown entrapment of human and viral DNA of various lengths into Pheroids. Reproducible expression of appropriate proteins was observed after transfection of cells by Pheroid-entrapped genes (Grobler, 2004:13).

3.4.9 REDUCTION AND ELIMINATION OF DRUG RESISTANCE

In vitro studies have demonstrated that by incorporating a drug into the Pheroid delivery system, drug resistance can be reduced or eliminated. Analysis of bacterial growth of multidrug resistant tuberculosis has shown that formulations containing the standard antimicrobial rifampicin entrapped in Pheroids, obviated pre-existing drug resistance. The ability to enhance the effectiveness of antibiotics such as penicillin can have widespread implications in the healthcare industry (Grobler, 2004:13).

3.4 THERAPEUTIC AND PREVENTATIVE USES OF PHEROID TECHNOLOGY

3.5.1 THERAPY OF TUBERCULOSIS

According to the World Health Organisation “*Tuberculosis kills 2 million people each year. Overall, one-third of the world’s population is currently infected with the TB bacillus*”. Previous *in vitro* and animal studies suggested that the Pheroid delivery system might have significant benefits when combined with TB medication. Therefore, a bio-equivalence study was performed to determine whether the Pheroid delivery system would remain as effective in oral administration as it was proven to be in topical use (Grobler, 2004:14).

The purpose of the study was to prove that the use of Pheroid after administration in a pro-Pheroid formulation could:

- 1) Increase the availability of the antimicrobials in human plasma.
- 2) Enhance the absorption of the formulated antimicrobials from the gastrointestinal tract without an increase in toxicity.
- 3) Increase the intracellular concentration of the antimicrobial in the target cells where the tuberculosis bacteria breed.
- 4) Extend the circulatory time of the active drugs.
- 5) Increase the bactericidal effect of the antimicrobials inside the target cells.
- 6) Decrease the side effects caused by the antimicrobials (Grobler, 2004:14).

Results of the bio-equivalence study, showed that:

- 1) The entrapment of the antimicrobials into Pheroids led to an increase in absorption of the antimicrobials after oral administration, with a resulting increase in plasma levels of these antimicrobials.
- 2) The entrapment of the antimicrobials led to an increased rate of absorption and cellular response.

- 3) The therapeutic concentrations of the drugs were maintained for longer and the circulatory time of the drugs was extended, indicating that the exposure of the bacteria to the antimicrobials was increased.
- 4) The entrapment of the antimicrobials in the Pheroid delivery system increased the delivery of the antimicrobials to the target cells, thereby decreasing the minimum inhibitory concentration.
- 5) A lower dosage can therefore be used to obtain similar effects.
- 6) A decrease in side effects was observed, with an increase in patient compliance, therefore suggesting that it could prevent the development of multi-drug resistance (Grobler, 2004:17).

3.5.2 PREVENTATIVE THERAPIES VACCINES

Historically, vaccination is the only strategy that has led to the elimination of the viral disease, smallpox. An indirect relationship has been observed for vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor; hence there is an urgent need for effective vaccine adjuvants to enhance the immunogenicity and immunostimulatory properties of vaccines (Grobler, 2004:17).

3.5.2.1 A VIRUS-BASED VACCINE: RABIES

The efficacy of a commercially available rabies vaccine and a rabies vaccine incorporated in a Pheroid delivery system was investigated and compared. An inactivated virus is used in the formulation of rabies vaccines. For the comparative animal studies, different formulations of the virus were used namely the inactivated virus, the inactivated virus with alum (aluminium hydroxide) as adjuvant and the inactivated virus incorporated in a Pheroid delivery system. The inactivated virus incorporated in a Pheroid delivery system showed a 9-fold increase in antibody response when compared to the other formulations (Grobler, 2004:18).

3.5.2.2 A PEPTIDE-BASED VACCINE:HEPATITIS B

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

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

3.5.2.3 PHEROID TECHNOLOGY FOR NASAL VACCINE DELIVERY

The hypothesis for the nasal delivery of vaccines using Pheroid as delivery system is based on the same principle than that of microparticulate systems, such as chitosan and *N*-trimethyl chitosan chloride microparticles.

The antigen is loaded into the Pheroids and administered where it is taken up by the microfold cells (M-cells) in the nasal epithelium which are responsible for the sampling of and transporting of antigens to the underlying nasal associated lymphoid tissue (NALT), germinal centers containing B cells and T cells, plasma cells and antigen presenting cells (APCs). These cells are involved in the regulation and induction of antigen-specific effector cells, which produce the protective humoral and cellular immune responses.

3.5.3 PHEROID TECHNOLOGY FOR PEPTIDE DRUG DELIVERY

A further hypothesis made was that the absorption of peptide drugs can be greatly improved by using Pheroids. Pheroids are polyphilic and is capable of encapsulating drugs that have different solubilities as well as insoluble drugs, many of which are highly toxic (Miconazole nitrate) (Schlebusch, 2002:12). Emzloids can actively transport these peptide drugs trough various physiological barriers and increase bioavailabilty (Schlebusch, 2002:13).

3.6 CONCLUSION

Although the similarities between Pheroid and lipid based delivery systems are ample, many advantages of Pheroids over these lipid base delivery systems are shown by research. Pharmaceutical applications of Pheroids are almost infinite. The most important of these advantages of Pheroids are the ability to enhance the absorption of peptide and protein drugs after peroral administration. This will result in higher plasma levels, faster cellular responses and lowering of the Tmax. Furthermore, therapeutic concentrations will be maintained longer, enhancing delivery and in return lowering dosage size and side effects. Therefore Pheroids acts as a potential delivery system for poorly absorbable peptide and protein drugs.

CHAPTER 4

SALMON CALCITONIN AS THERAPEUTIC PEPTIDE

4.1 INTRODUCTION

One of the most important developments in the field of calcium metabolism in the 1950's was the discovery of calcitonin. Studies perfusing the thyroid-parathyroid complex of canines with calcium concentrations exposed changes in blood calcium levels that were not explicable in terms of changes in secretion of parathyroid hormone (PTH) alone, drawing attention to a possibility of another hormone that could counteract the effect of PTH. The existence of calcitonin as a hypocalcemic principal was confirmed in a mammalian preparation, which made it possible to demonstrate that the thyroid was the source of calcitonin. These physiological studies culminated in the extraction of a highly potent hypocalcemic peptide, calcitonin, from parafollicular C cells in the thyroid. The purification and sequencing of various calcitonins soon followed. However, it was not until 1982 that Chambers and colleagues confirmed a direct effect of femtomolar calcitonin concentrations on osteoclast function (Zaidi *et al.*, 2002:655).

Calcitonin is a 32 amino acid long peptide with a N-terminal disulfide bridge and a C-terminal prolineamide residue. This peptide was shown to potently inhibit bone resorption however; a direct osteoclastic action of the peptide was confirmed only in the early 1980's. Calcitonin's potent anti-resorptive effect has led to its further use in treating Paget's disease of bone, osteoporosis and hypocalcaemia (Zaidi *et al.*, 2002:655).

Four calcitonins are currently being used clinically, namely: synthetic human calcitonin (hCT), synthetic salmon calcitonin (sCT), natural porcine calcitonin (pCT) and a synthetic analogue of eel calcitonin (eCT) (Lee & Sinko, 2000:226; Rochira *et al.*, 1996:19).

4.2 SYNTHESIS AND STRUCTURE OF SALMON CALCITONIN

Salmon calcitonin ($C_{145}H_{240}N_{44}O_{48}S_2$) is an endogenous polypeptide hormone composed of 32 amino acids (Lee & Sinko, 2000:226; Marcus, 2001:1732) (fig 4.1) with a molecular weight of 3431.9 Dalton. Eight of the amino acid residues of CT are invariant, including a carboxyl-terminal prolinamide and a disulfide bridge between cysteines at positions 1 and 7. Together these structural features are essential for biological activity. The residues in the middle portion of the molecule (positions 10 to 27) are variable and appear to influence potency and / or duration of action.

CTs derived from the ultimobranchial bodies of salmon and eel are more potent than the mammalian thyroidal CTs (both *in vivo* and *in vitro*) and they differ from the human hormone on 13 and 16 amino acid residues, respectively. Therapeutically sCT appears to be more potent than hCT per mg, because it is cleared more slowly from the circulation (Diaz *et al.*, 1999:55; Marcus, 2001:1732; Rochira *et al.*, 1996:19). Salmon calcitonins actions are identical to the actions of mammalian CTs (Novartis: 2005).

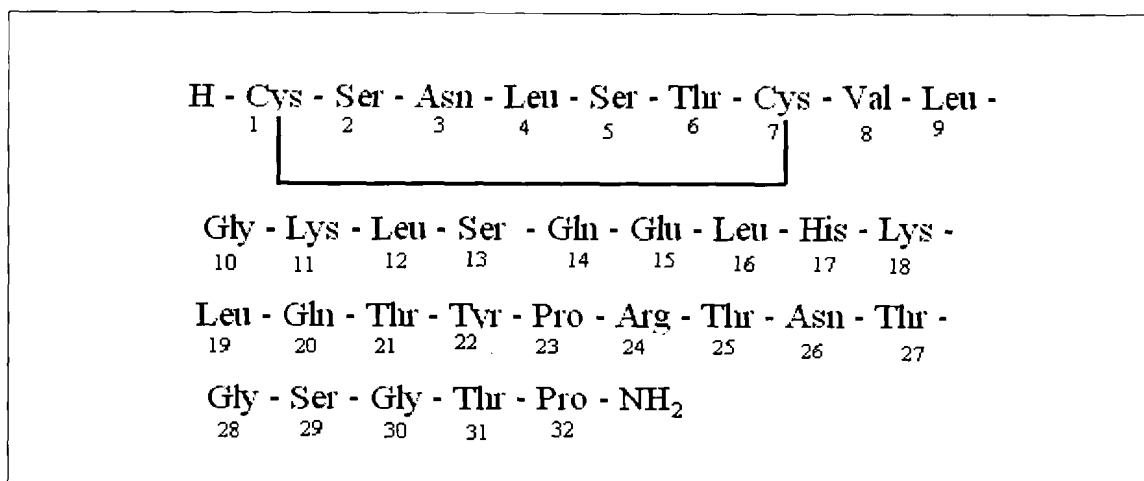


Figure 4.1 Amino acid sequence of salmon calcitonin (British Pharmacopoeia, 2002:277).

Salmon calcitonin is obtained by chemical synthesis and is available as an acetate. For labeling purposes 1 mg of salmon calcitonin is equivalent to 6000 IU of biological activity (British Pharmacopoeia; 2002:277).

4.3 THERAPEUTIC USES AND SIDE-EFFECTS OF SALMON CALCITONIN

The biosynthesis and secretion of CT are regulated by the concentration of Ca^{2+} in the plasma. When plasma Ca^{2+} is high, CT secretion is increased; when plasma Ca^{2+} is low, CT secretion is low or undetectable. Normal circulating CT concentrations in human beings are less than 10 pg/ml. The hypocalcemic and hypophosphatemic effects of calcitonin are caused predominantly by direct inhibition of osteoclastic bone resorption (Marcus, 2001:1733). Salmon calcitonin is one of the most therapeutically effective peptides available for the treatment of hypercalcemia, post menopausal osteoporosis and Paget's disease (Song *et al.*, 2002:27).

CT is used for patients with hypercalcaemia as initial therapy, after a few days of treatment, escape from the response is observed. CT is also effective in disorders of increased skeletal remodeling, such as Paget's disease and in osteoporosis. In Paget's disease, chronic use of CT produces long-term reduction in symptoms and in serum alkaline phosphatase activity (Marcus, 2001:1733). CT is a powerful inhibitor of osteoclastic bone resorption and produces a modest increase in bone mass in patients with osteoporosis (Marcus, 2001:1738). Osteoporosis is a disease which is characterized by low bone mass and architectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk as patients approach or fall below a bone mineral density associated with increased frequency of fracture. Vertebral fractures occur with highest frequency and are associated with back pain, spinal deformity and a loss of height (Novartis: 2005). CT reduces the incidence of vertebral compression fracture by about 40 % in women with osteoporosis (Marcus, 2001:1738). Development of antibodies to CT does occur with long-term therapy, but this is not necessarily associated with clinical resistance.

An additional advantage is the newly discovered analgesic effect of salmon calcitonin. Studies indicated that salmon calcitonin reduced pain associated with osteoporosis such as musculoskeletal pain. Thus salmon calcitonin has a binary action in osteoporosis (Lyritis & Trovas, 2002:71S).

Salmon calcitonin is approved for clinical use as Calcimar[®] or Miacalcin[®] (Marcus, 2001:1738). Calcimar[®] injection (Rhône-Poulenc, Rorer, USA), is a solution containing 200 IU/ml salmon calcitonin in 2 ml multidose vials. Injections are intended for subcutaneous or intramuscular administration. Miacalcin[®] injection (Sandoz, USA), is a solution containing 200 IU / ml salmon calcitonin in 2 ml multidose vials. Injections are intended for subcutaneous or intramuscular administration. Miacalcin[®] nasal spray (Sandoz, USA) is a solution containing salmon calcitonin 2200 IU/ml and delivers 200 IU per actuation. (Dollery, 1999:C13).

Some side effects of salmon calcitonin include nausea, vomiting, diarrhea, dizziness, flushing, hand swelling, urticaria and rarely intestinal cramping (Marcus, 2001:1732 & Martindale, 1993:655). These reactions are dose dependent, usually transient and occur more often with intravenous administration. Other adverse effects have included an unpleasant taste, skin rash, abdominal pain, urinary frequency and tremor. Transient hypocalcaemia may occur after injections of CT (Martindale, 1993:655). Direct renal effects of salmon calcitonin and actions on the gastrointestinal tract have also been observed (Novartis: 2005).

4.4 PHARMACOKINETICS OF SALMON CALCITONIN

Calcitonins are quickly inactivated when given by oral administration. Enzymes responsible for degradation of CT in the GIT are trypsin, α -chymotrypsin and elastase. After injection, hCT and salmon calcitonin are quickly metabolized primarily in the kidneys but also in blood and peripheral tissues. The inactive metabolites and a small proportion of unchanged drug are excreted in the urine (Martindale, 1993:655).

The half-life of salmon calcitonin is 15-20 minutes, due to its large systemic clearance and low stability in circulating blood (Song *et al.*, 2002:27). Salmon calcitonin's unique structure protects it against sequestration in the liver (Lee & Sinko, 2000:226). Salmon calcitonin has high solution stability around neutral pH (Baudyš *et al.*, 1996:146). Calcitonins are also absorbed through the nasal and rectal mucosa, but are generally given by subcutaneous or intramuscular injection or by intravenous infusion (Martindale, 1993: 655).

4.5 PERORAL DELIVERY OF SALMON CALCITONIN

Establishing an oral delivery system for peptide and protein drugs, such as salmon calcitonin, is of great importance because parenteral administration results in poor patient compliance during chronic treatment resulting in limited clinical utility. There are numerous difficulties associated with delivering peptide drugs in an oral formulation (discussed previously in Chapter 1). These same difficulties are also applicable for the various calcitonins (Lee & Sinko, 2000:226). Because of difficulties such as extensive proteolytic degradation in the GIT and low intrinsic intestinal membrane permeability, salmon calcitonin has insufficient oral bioavailability (Song *et al.*, 2002:27). This necessitates high doses of salmon calcitonin (4000 – 6000 IU/mg) in peroral administration, even though salmon calcitonin is 20 – 30 times more potent than hCT (Lee & Sinko, 2000:226).

For these reasons, researchers are trying to develop alternative formulations. Examples of a few of the alternatives explored so far are:

- hydrophilic polymers: polyacrylate derivatives, (Garcia-Fuentes *et al.*, 2005a:133) chitosan nanocapsules (Issa *et al.*, 2005:2) and poly(ethylene glycol) (Garcia-Fuentes *et al.*, 2005a:133).
- uncoated oily nanoemulsions (Issa, *et al.*, 2005:2).
- nanoparticles: poly(*N*-isopropylacrylamide) (Sakuma *et al.*, 2002:186)
- lipid nanoparticles coated with chitosan (Garcia-Fuentes *et al.*, 2005b:122).
- submicron emulsions with/without Carbopol®940 (Baluum *et al.*, 1997:235).

- liposomes (Diaz *et al.*, 1997:59 & Song *et al.*, 2002:27).

4.6 CONCLUSION

Up to now, calcitonin has not yet achieved its full market potential due to the difficulty and pain associated with the injectable dosages forms and the low patient acceptance of the nasal delivery system. Therefore establishing an oral delivery system for salmon calcitonin is of immense importance. An oral dosage form of the drug will allow full clinical exploration of the role of CT in osteoporosis or Paget's disease and might lead to a broader use of CT as a safe and efficacious adjunct to the treatment of these diseases. However, apart from poor absorption from the GIT and unfavourable physicochemical properties, the oral bioavailability of CT is strongly restricted by proteolytic degradation (Guggi & Bernkop-Schnürch, 2003:187-188). Potential approaches to enhance the absorption of salmon calcitonin include the use of formulation additives. Some of these additives modulate the intestinal environment and targets specific intestinal regions.

Previous studies concluded that satisfactory salmon calcitonin bioavailability can be obtained by using a suitable formulation. However, a successful formulation design for peptide-based drugs with acceptable oral bioavailability still remains a major challenge confronting the pharmaceutical researchers.

CHAPTER 5

***IN VIVO* EVALUATION OF PERORAL SALMON CALCITONIN ABSORPTION WITH PHEROID TECHNOLOGY AND TMC**

5.1 INTRODUCTION

Both quaternized chitosan derivatives, TMC and TMO, and Pheroid technology are absorption enhancers with the potential to contribute greatly to the development of selective and effective delivery systems for poorly absorbed peptide drugs, such as salmon calcitonin. This chapter describes the experimental procedures performed on rats to evaluate the intestinal absorption of salmon calcitonin after co-administration with these absorption enhancers.

5.2 EXPERIMENTAL DESIGN AND IN VIVO PROCEDURES

5.2.1 EXPERIMENTAL ANIMALS

Male Sprague Dawley rats weighing between 250 to 350 g were chosen as experimental animals to study the absorption enhancing effects of TMC, TMO and Pheroid. Sprague Dawley rats are readily available, breed successfully and quickly in captivity, and are easy to handle.

Direct administration into the jejunum was chosen as the *in vivo* experimental procedure for the absorption studies. The experimental procedures for using rats in the selected *in vivo* method are well illustrated in literature (Baudyš *et al.*, 1996:146; Garcia-Fuentes *et al.*, 2005a:136; Prego *et al.*, 2005:154; Rakhi & Khan, 2004:2; Sakuma *et al.*, 2002:185 and Wang *et al.*, 2003:372). An application for the use of Sprague Dawley rats in this study was compiled and was approved by the Ethical Committee of the North-West University (04D15) (Annexure 1). Six rats were used for each experiment to ensure

detection of any statistical significant differences in absorption between the experimental and control groups (Statistical Consultation Services, North-West University).

5.2.2 BREEDING CONDITIONS

Male Sprague-Dawley rats were bred and kept in a closed, controlled environment at the Animal Research Centre, North-West University. The animals were kept under controlled conditions to ensure an ideal growth environment with minimum exposure to pathogens, accomplished by a constant air flow. All the variables in the Animal Research Centre were kept constant. Conditions, to which the rats used in this study were exposed, are shown in table 5.1. Rats were fed with Epol® mice cubes (Epol Pty (Ltd), Pretoria, RSA) provided by the Research Animal Centre of the North-West University.

Table 5.1 Conditions under which rats were kept.

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

*Values recommended according to international standards.

5.2.3 EXPERIMENTAL DESIGN

For each formulation six Sprague-Dawley rats were used, in accordance with previous literature (Baluom *et al.*, 1997:237; Baudyš *et al.*, 1996:146; Garcia-Fuentes *et al.*,

2005a:137; Lamprecht *et al.*, 2004:3; Prego *et al.*, 2005:154; Sakuma *et al.*, 2002:185; Takeuchi *et al.*, 2003:235 and Wang *et al.*, 2003:372.) to determine the differences and extent of salmon calcitonin absorption. The experimental design was a parallel design where the animals were arranged according to treatment groups and one treatment was administered per experimental animal. A control group of four animals also received salmon calcitonin in saline to determine absorption without any additive to enhance absorption.

5.2.4 PREPARATION OF FORMULATIONS CONTAINING SALMON CALCITONIN

5.2.4.1 MATERIALS

Salmon calcitonin was supplied by Merck (South Africa) (Annexure 2). *N*-Trimethyl chitosan chloride (TMC), *N*-Trimethyl chitosan oligosaccharide (TMO). TMC and TMO were kindly donated by the North-West University (Potchefstroom). The degree of quaternisation of TMC was given as 48.0 % and of TMO as 54.6 %. The molecular weight of each of the polymers was given as 166000 g/mole for TMC and 6600 g/mole for TMO. Pheroids were donated by the North-West University (Potchefstroom). Heparin Sodium 5000 IU / ml was supplied by Intramed (South Africa). A Ultra-Sensitive Salmon Calcitonin Radio Immuno assay was supplied by Diagnostic Systems Laboratories (Laboratory Specialties, Johannesburg).

5.2.4.2 METHOD

A dose of 500 IU/kg salmon calcitonin was administered into the jejunum of the rats. The five different experimental formulations used, are shown in table 5.2.

Table 5.2 The formulations used for oral administration of salmon calcitonin.

FORMULATION	ROUTE OF ADMINISTRATION	DOSE ADMINISTERED
A: Pheroid microsponges	Intestinal	500 IU / kg
B: Pheroid vesicles	Intestinal	500 IU / kg
C: Control	Intestinal	500 IU / kg
D: TMC	Intestinal	500 IU / kg
E: TMO	Intestinal	500 IU / kg

Formulation A:

⇒ 500 µg of salmon calcitonin was weighed and dissolved in 10.0 ml of Pheroid microsponges at 36 °C. The formulation was then shaken for 30 minutes in a IKA VIBRAX VXR basic at 700 MOT/min. The mean particle size of the Pheroid microsponges was measured before and after entrapment of salmon calcitonin with a Malvern, Mastersizer (Malvern Instruments, UK). The mean particle size before addition of salmon calcitonin was 3.32 µm and with the addition of salmon calcitonin it was 3.48 µm. (Confocal laser scanning microscopy images of this formulation are shown in Annexure 3).

Formulation B:

⇒ 500 µg of salmon calcitonin was weighed and dissolved in 10 ml of Pheroid vesicles at 36 °C. The formulation was then shaken for 30 minutes in a IKA VIBRAX VXR basic at 700 MOT/min. The mean particle size of the Pheroids vesicles was measured before and after entrapment of salmon calcitonin with a Malvern, Mastersizer (Malvern Instruments, UK). The mean particle size before addition of salmon calcitonin was 2.19 µm and with the addition of salmon calcitonin it was 2.32 µm. (Confocal laser scanning microscopy images of this formulation are shown in Annexure 4).

Formulation C:

- ⇒ 500 µg of salmon calcitonin was weighed and dissolved in 10.0 ml saline. This solution was administered as the control.

Formulation D:

- ⇒ 500 µg of salmon calcitonin was weighed and dissolved in 5.0 ml of distilled water and added to 5.0 ml of 1.0 % w/v TMC solution. The final polymer concentration of this formulation was 0.5 % w/v.

Formulation E:

- ⇒ 500 µg of salmon calcitonin was weighed and dissolved in 5.0 ml of distilled water and added to 5.0 ml of 0.1 % w / v TMO solution. The final polymer concentration of this formulation was 0.5 % w/v.

5.2.5 INDUCTION AND MAINTENANCE OF ANAESTHESIA

The rats were anesthetized with inhalation anaesthesia consisting of 4.0 % v/v halothane (Fluothane®, Zebeca SA (Pty.) Ltd., Woodmead, RSA) in medicinal oxygen and 2.0 % v/v halothane in medicinal oxygen was used for the maintenance of anaesthesia. Anaesthesia was induced with 4.0 % v/v halothane, where each rat was placed in a closed plastic container with a metal grid floor above a cotton wool layer containing liquid halothane. Direct contact between the rat and halothane in the cotton wool layer, was not possible. The rat inhaled the halothane, and was removed upon loss of consciousness.

Mixtures of 2.0 and 4.0 % halothane in medical oxygen were used alternatively as needed. The apparatus for this procedure consisted of two 5 liter plastic bags containing 0.12 ml (2.0 %) and 0.24 ml (4.0 %) halothane respectively and medical oxygen. Each bag was connected to one end of a three-way valve. A latex rubber sheath was fitted to the remaining end and fitted securely over the head of the rat to supply one of the two halothane mixtures. A small amount of soda-lime was placed in the plastic bags near the connecting valve to absorb CO₂. Anaesthesia was maintained by connecting the rat to the 2.0 % halothane bag. The 4.0 % halothane was used to deepen the anaesthesia when

necessary. Each rat was placed on a small thermal electric blanket to maintain body temperature at 37 °C for the duration of the experiment.

Euthanasia was performed at the end of each experiment before the rat regained consciousness, deepening the anaesthesia to 4.0 % halothane until breathing ceased.

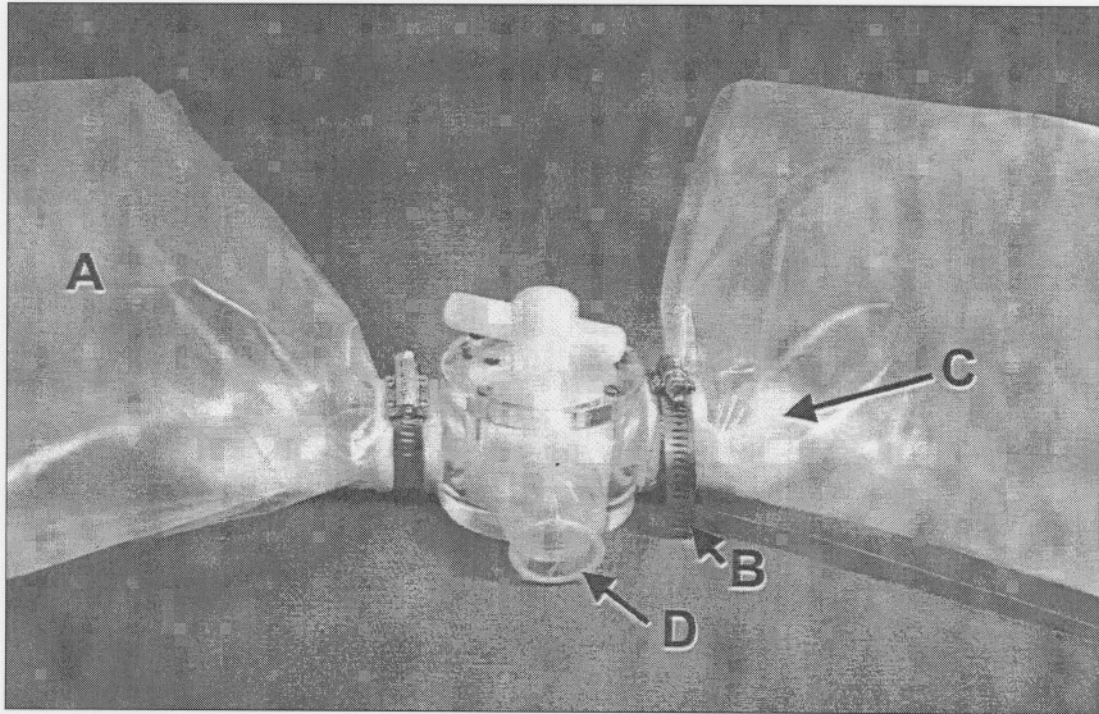


Figure 5.1 Apparatus needed to perform anaesthesia: A – Plastic bag; B – Clamp; C – Sodium lime; D – Latex rubber sheath.

5.2.6 SURGICAL PROCEDURES

5.2.6.1 CANNULATION OF THE ARTERY CAROTIS COMMUNIS

All surgical procedures were carried out on rats while they were under anaesthesia, and were performed by a veterinary technician of the Animal Research Centre. The rats were fasted 12 hours prior to the experiments, with water available *ad lib*. The hair was

shaved in the area of the ventral neck and the skin disinfected. The rat was placed prostrate in a supine position with the head stretched in slight opisthotonus. A mid ventral incision of 1 cm was made in the neck skin. Between the two bilateral *m. stemohyoideus* to the area lateral to the trachea a blunt dissection was made to expose the *a. carotis communis* beneath the *m. stemohyoideus*.

The *a. carotis communis* was freed, and lifted out of the operation wound and was kept wet with physiological saline at body temperature. The rostral part of the artery was ligated out with silk and tension was placed on the artery by taping the loose ends of the ligature in front of the rat. Another ligature was preplaced loosely at the caudal part of the artery. The artery was temporarily clamped with a mosquito artery clamp proximal to the loosely preplaced ligature. A "V" incision was made with a pair of scissors between the two ligatures in the artery wall. A sterile PVC cannula (Fine Bore Polythene tubing, 0.58 mm ID (0.96 mm OD) REF 800/100/200/100, UK) which was filled with a saline-heparin solution at body temperature and connected to a syringe was guided through the artery. A 5.0 % heparin solution was used to avoid blood clotting in the cannula. The cannula was threaded so that 2 cm of the cannula was inserted into the artery and the clamp was then released. The loose ligature around the artery with the cannula inside was tied. Another ligature was tied to ensure that the cannula would not come loose. The wound was then covered with gauze and wetted with saline-heparin (5.0 %) solution which was at body temperature.

5.2.6.2 INTESTINAL SURGICAL PROCEDURES

The hair was shaved in the area of the ventral abdomen and the skin disinfected. A midline abdominal incision (laparotomy) of 1 cm through the linea alba caudal to the sterno was performed and the jejunum was identified. Gauze wetted with saline was placed on the incision to keep the wound damp. During these surgical procedures rats were kept in a controlled environment. Rats were placed on small thermal electric blankets in a supine position. Depth of anaesthesia was monitored continuously using the pedal reflex and depth and rate of breathing.

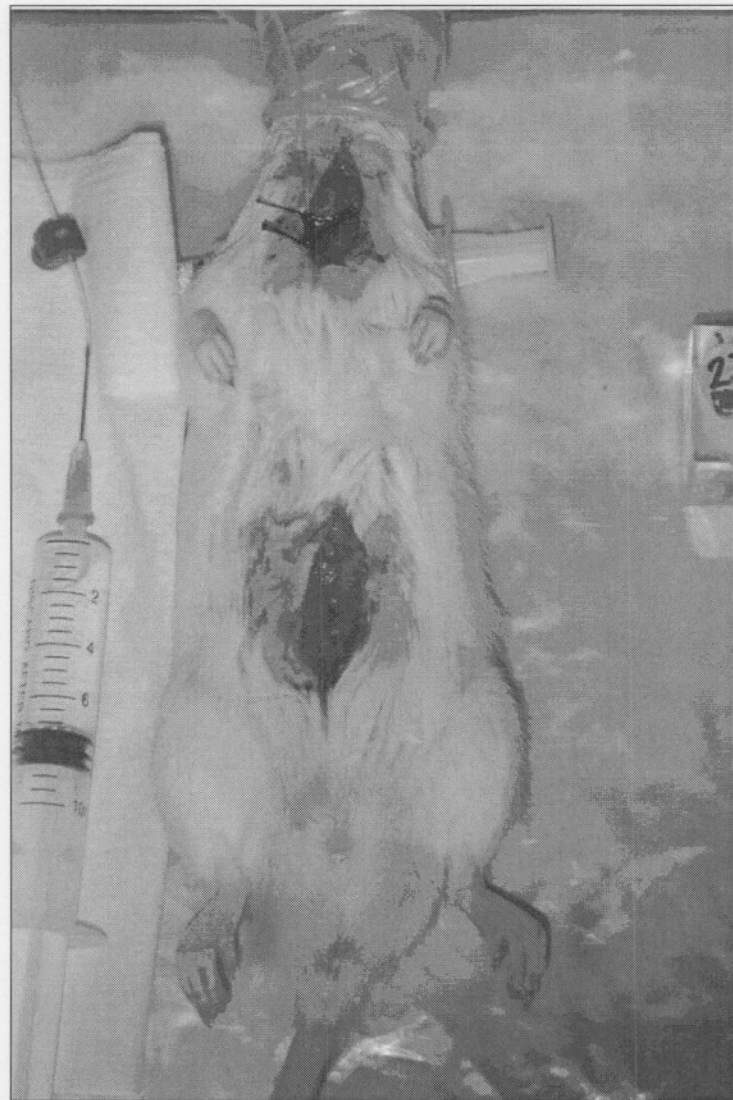


Figure 5.2 Rat after cannulation and laparotomy.



Figure 5.3 Rat during experimental procedure.

5.2.7 ADMINISTRATION OF FORMULATIONS

Between 350 and 600 μL (500 IU salmon calcitonin/kg bodyweight) of the different formulations was directly injected with a syringe into the jejunum. The volume administered depended on the weight of the rats but all rats received a final salmon calcitonin concentration of 500 IU/kg body weight.

5.2.8 BLOOD SAMPLE COLLECTION

Blood samples, with a volume of 800 μL were collected from the cannula in Eppendorf[®] micro test tubes (Merck, RSA). Blood samples were collected at 0 (2 minutes before administration), 5, 15, 30, 60, 120 and 180 minutes into test tubes and kept at 0 °C (crushed ice). Thus, eight samples were taken over a period of 3 hours. Samples were centrifuged (Eppendorf[®] centrifuge 5415C) within 20 minutes of collection at 7000 min^{-1} rpm for 7

minutes and the collected plasma was stored at -40°C until the samples were analyzed. After the collection of all the samples, euthanasia was performed by an overdose of halothane.

5.2.9 QUANTITATIVE MEASUREMENT OF SALMON CALCITONIN IN PLASMA

Samples were analyzed with an Ultra-Sensitive Salmon Calcitonin Radio Immuno Assay (Diagnostic Systems Laboratories, Laboratory Specialties, Johannesburg). The principle of this test is based on the competition between a radioactive and a non-radioactive antigen for a fixed number of antibody sites. The amount of labeled salmon calcitonin bound to the antibody is inversely proportional to the concentration of unlabeled salmon calcitonin present. The separation of the free and bound antigen was achieved by using a double antibody system. A standard assay, controls and samples were prepared as stipulated in the information sheet received with the RIA. Each test tube was then counted for 1 minute in a gamma counter (Auto-Gamma[®] 5000 series, Gamma counting system, Packard).

5.3 RESULTS AND DISCUSSION

5.3.1 INTRODUCTION

Several studies have been done on salmon calcitonin (Baluom *et al.*, 1997:235; Garcia-Fuentes *et al.*, 2005a:136; Guggi *et al.*, 2003:125; Lamprecht *et al.*, 2004:1; Prego *et al.*, 2005:151; Sakuma *et al.*, 2002:185; Takeuchi *et al.*, 2003:235 and Wang *et al.*, 2003:369.) to improve its absorption after oral administration. These studies indicate that the oral route may be an alternative to the parenteral route for administering peptide drugs intended for a systemic therapeutic effect. The objective of this study was to compare the absorption enhancing capabilities of the different formulations of TMC, TMO, Pheroid vesicles and Pheroid microsponges after oral administration in Sprague-Dawley rats.

5.3.2 TMC

Table 5.3 gives the plasma salmon calcitonin concentrations obtained after intestinal administration of salmon calcitonin with TMC. In figure 5.4 these results are graphically depicted.

Table 5.3 Plasma salmon calcitonin concentrations after administration of salmon calcitonin with TMC (0.5 % w/v).

Time	sCT concentration (pg/ml)	
	Control	TMC (0.5 % w/v)
0	153.78 ± 83.86	98.30 ± 60.77
5	236.96 ± 30.93	685.68 ± 278.87
10	249.09 ± 21.53	738.96 ± 277.09
15	236.88 ± 49.33	641.15 ± 200.51
30	206.60 ± 57.50	602.39 ± 200.51
60	182.02 ± 47.62	354.42 ± 128.80
120	151.23 ± 62.24	252.27 ± 152.94
180	176.10 ± 53.33	164.45 ± 84.50

(n=6)

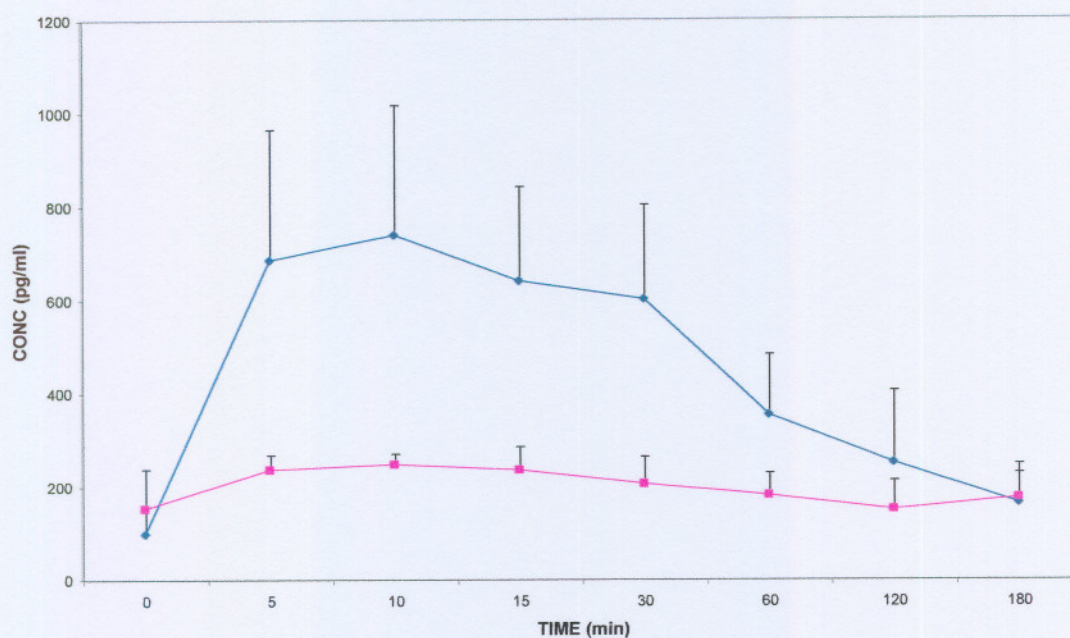


Figure 5.4 Salmon calcitonin concentrations (pg/ml) in plasma after administration in the jejunum with TMC (♦) (500 IU/kg sCT) against the control (■).

As evident from table 5.3 and figure 5.4 there was negligible absorption of salmon calcitonin in the control formulation. In contrast to the administration of the formulation with TMC which lead to major salmon calcitonin absorption. At time $t = 0$ minutes the concentration of salmon calcitonin was 98.30 pg/ml. After 5 minutes the salmon calcitonin concentration rose to 685.68 pg/ml, after which the concentration of salmon calcitonin increased even more to reach a peak concentration of 738.96 pg/ml after 10 minutes. The concentration of salmon calcitonin remained high up until 30 minutes and then slowly started to decrease to 354.42 pg/ml at time $t = 60$ minutes. Even after 120 minutes the concentration was still high at 252.27 pg/ml after which it gradually decreased towards the initial value obtained before salmon calcitonin administration. The results are in agreement with the results obtained in previous studies where TMC was also used as an absorption enhancer for several experimental drugs (Kotze et al., 1998:41 & Lueßen et al., 1996:1668). TMC acts by opening of the tight junctions thereby allowing paracellular transport of the drug molecules. This study with salmon calcitonin

confirmed that TMC are a potent absorption enhancer for peptide drugs and that it may play an important role in peptide drug delivery systems in the future.

5.3.3 TMO

Table 5.4 gives the plasma salmon calcitonin concentrations obtained after intestinal administration of salmon calcitonin with TMO. In figure 5.5 these results are graphically depicted.

Table 5.4 Plasma salmon calcitonin concentrations after administration of salmon calcitonin with TMO (0.5 % w/v).

Time	sCT concentration (pg/ml)			
	Control		TMO (0.5 % w/v)	
0	153.78	± 83.86	186.59	± 50.74
5	236.96	± 30.93	379.71	± 105.66
10	249.09	± 21.53	337.31	± 83.48
15	236.88	± 49.33	364.05	± 57.40
30	206.60	± 57.50	197.49	± 44.90
60	182.02	± 47.62	180.71	± 41.54
120	151.23	± 62.24	187.03	± 64.61
180	176.10	± 53.33	198.55	± 115.17

(n=6)

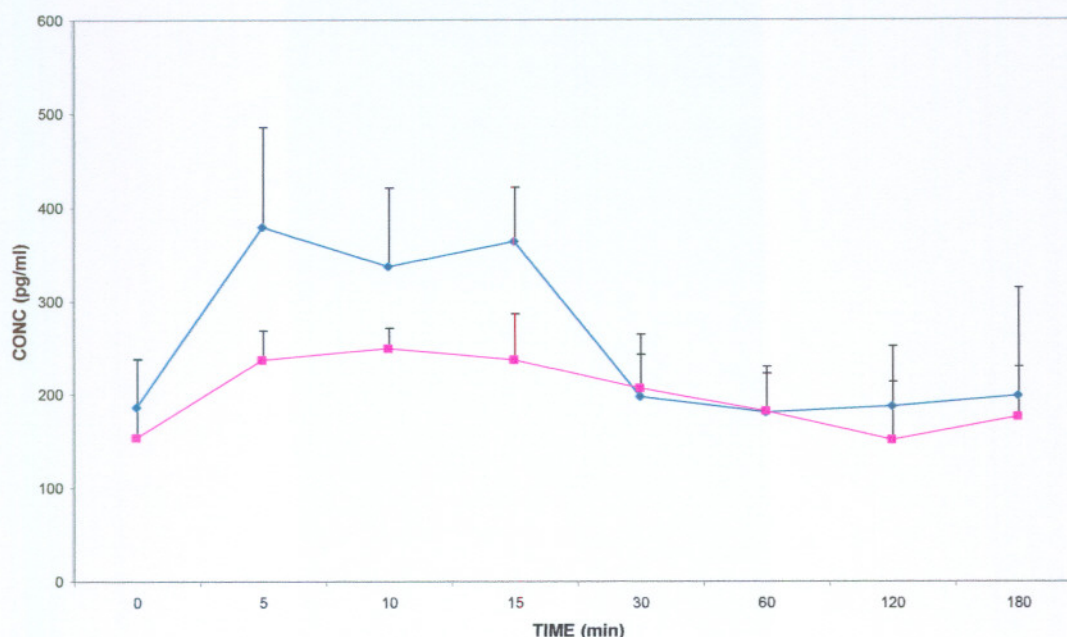


Figure 5.5 Salmon calcitonin concentrations (pg/ml) in plasma after administration in the jejunum with TMO (♦) (500 IU/kg sCT) against the control (■).

As evident from table 5.4 and figure 5.5 there was negligible absorption of salmon calcitonin in the control formulation. In contrast to the administration of the formulation with TMO which lead to an increase in salmon calcitonin absorption. At after time $t = 0$ minutes the concentration of salmon calcitonin was 185.59 pg/ml. After 5 minutes the salmon calcitonin concentration rose to 379.71 pg/ml, after which the concentration of salmon calcitonin remained relatively constant until 15 minutes post administration. The salmon calcitonin concentration then decreased gradually towards the initial concentration obtained before drug administration. Similar results were obtained as with TMC although the increase in plasma salmon calcitonin concentrations was not as high as obtained with TMC [685.68 pg/ml (TMC) versus 379.71 pg/ml (TMO), 5 minutes post administration]. TMO probably also open tight junctions to allow for paracellular transport. The obtained results indicate that the molecular weight of quaternised chitosan polymers play an important role in the absorption enhancing properties [166000 g/mole for TMC and 6600 g/mole for TMO].

5.3.4 PHEROID VESICLES

Table 5.5 gives the plasma salmon calcitonin concentrations obtained after intestinal administration of salmon calcitonin with Pheroid vesicles. In figure 5.6 these results are graphically depicted.

Table 5.5 Plasma salmon calcitonin concentrations after administration of salmon calcitonin with Pheroid vesicles.

Time	sCT concentration (pg/ml)			
	Control		Pheroid vesicles	
0	153.78	± 83.86	136.56	± 19.59
5	236.96	± 30.93	386.25	± 45.55
10	249.09	± 21.53	343.27	± 51.90
15	236.88	± 49.33	314.43	± 47.90
30	206.60	± 57.50	247.48	± 79.27
60	182.02	± 47.62	200.86	± 45.50
120	151.23	± 62.24	149.73	± 56.63
180	176.10	± 53.33	153.58	± 40.45

(n=6)

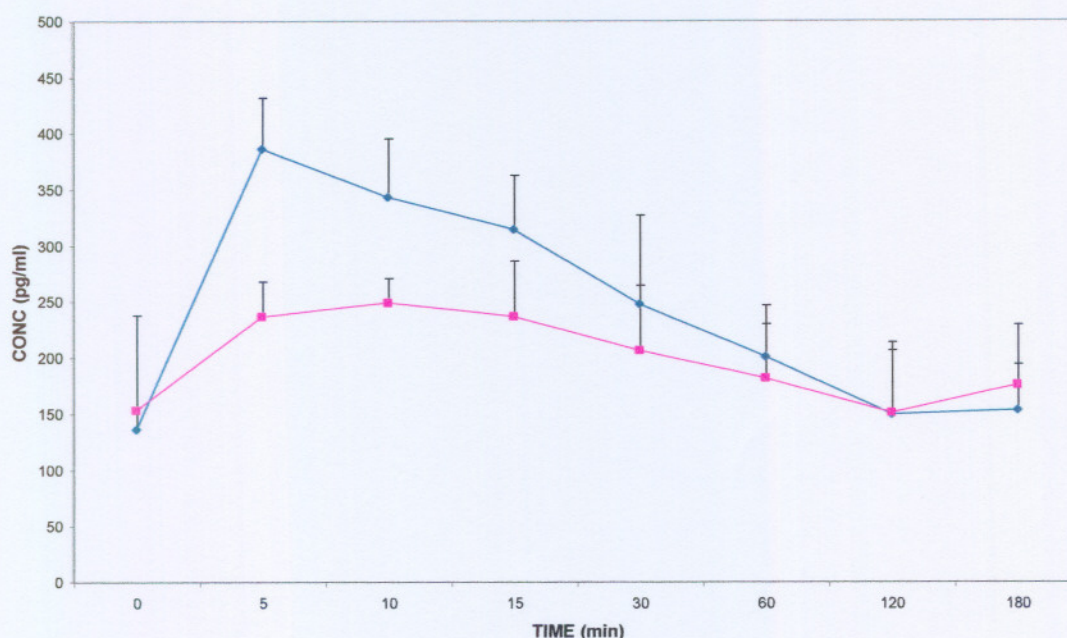


Figure 5.6 Salmon calcitonin concentrations (pg/ml) in plasma after administration in the jejunum with Pheroid vesicles (◆) (500 IU/kg sCT) against the control (■).

As evident from table 5.5 and figure 5.6 there was negligible absorption of salmon calcitonin in the control formulation. In contrast to the administration of the formulation with Pheroid vesicles which lead to an increase in salmon calcitonin absorption. At time $t = 0$ minutes the concentration of salmon calcitonin was 136.56 pg/ml. After 5 minutes the salmon calcitonin concentration rose to 385.25 pg/ml, after which the concentration of salmon calcitonin gradually declined towards the initial value obtained before drug administration. Although the plasma salmon calcitonin concentrations after administration of salmon calcitonin entrapped in Pheroid vesicles, were not as profoundly increased as obtained after salmon calcitonin administration with TMC, it is clear that Pheroid vesicles are able to increase the intestinal absorption of salmon calcitonin. This difference in outcome obtained between TMC and Pheroid vesicles could also be explained by different mechanisms of action in enhancing permeation. It has already been shown in several studies that TMC opens the tight junctions between epithelial cells to allow for the paracellular transport of several classes of compounds. Salmon calcitonin is a hydrophilic peptide with a molecular weight of 3431.9 Da and has probably been

absorbed paracellularly after administration with TMC. The exact mechanism of action of Pheroid vesicles is not yet apparent but is believed that there are a group of fatty acid transporters present on cell membranes which are responsible for the absorption of the Pheroid vesicles inside the cell contents, thereby allowing the entrapped salmon calcitonin to enter the cells through the transcellular route of absorption. This is, however, still a hypothesis and needs to be clarified in detail in future studies.

5.3.5 PHEROID MICROSPONGES

Table 5.6 gives the plasma salmon calcitonin concentrations obtained after intestinal administration of salmon calcitonin with Pheroid microsponges. In figure 5.7 these results are graphically depicted.

Table 5.6 Plasma salmon calcitonin concentrations after administration of salmon calcitonin with Pheroid microsponges.

Time	sCT concentration (pg/ml)			
	Control		Pheroid microsponges	
0	153.78	± 83.86	114.96	± 53.88
5	236.96	± 30.93	416.58	± 76.74
10	249.09	± 21.53	432.14	± 118.90
15	236.88	± 49.33	357.34	± 64.69
30	206.60	± 57.50	309.04	± 125.59
60	182.02	± 47.62	240.22	± 128.16
120	151.23	± 62.24	147.97	± 84.75
180	176.10	± 53.33	195.14	± 132.81

(n=6)

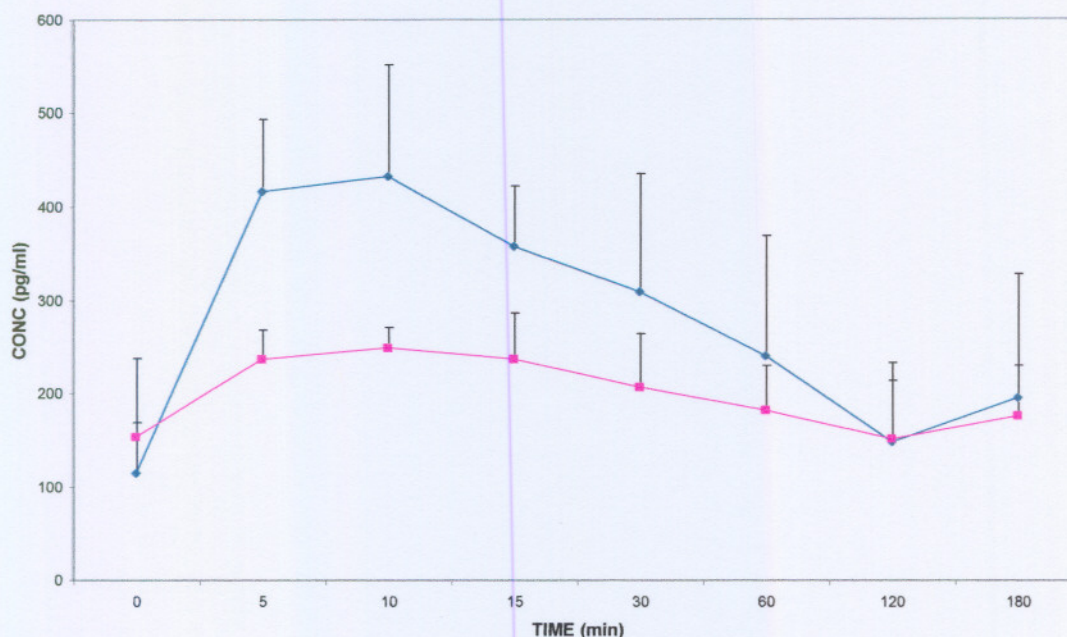


Figure 5.7 Salmon calcitonin concentrations (pg/ml) in plasma after administration in the jejunum with Pheroid microsponges (♦) (500 IU/kg sCT) against the control (■).

As evident from table 5.6 and figure 5.7 there was negligible absorption of salmon calcitonin in the control formulation. In contrast to the administration of the formulation with Pheroid microsponges which lead to an increase in salmon calcitonin absorption. At time $t = 0$ minutes the concentration of salmon calcitonin was 114.96 pg/ml. After 10 minutes the salmon calcitonin concentration rose to 432.14 pg/ml, after which it gradually declined towards the initial value obtained before drug administration. The obtained results clearly indicated that Pheroid microsponges were also able to increase the intestinal absorption of salmon calcitonin. A comparison of the results obtained with Pheroid microsponges and Pheroid vesicles indicate that Pheroid microsponges lead to a slightly higher salmon calcitonin absorption compared to Pheroid vesicles [416.58 pg/ml (microsponges) versus – 386.25 pg/ml (vesicles) 5 minutes post administration].

5.3.6 COMPARISON OF OBTAINED RESULTS

In table 5.7 the plasma salmon calcitonin concentrations obtained after intestinal administration of 500 IU/kg bodyweight with various formulations to rats are given. Figure 5.8 graphically depicts the plasma salmon calcitonin against time while figure 5.9 graphically depicts the plasma salmon calcitonin concentrations obtained 5 minutes after intestinal administration.

As noted from the previous sections and also apparent from figures 5.8 and 5.9 intestinal administration of salmon calcitonin with TMC lead to a major increase in plasma concentrations. Both the Pheroid microsponges and Pheroid vesicles also lead to an increase in salmon calcitonin absorption although not as profound as acquired with TMC. The differences in effect have been explained in the pervious sections.

Table 5.7: Salmon calcitonin concentrations (pg/ml) found in plasma after administration in the jejunum of rats with different formulations as a function of time (n=6).

Time (minutes)	Concentration in plasma (pg/ml)									
	Microsponges		Pheroid vesicles		Control		TMC		TMO	
0	114.96	± 53.88	136.56	± 19.5899	153.78	± 83.86	98.3	± 60.77	186.59	± 50.74
5	416.58	± 76.74	386.25	± 45.5517	236.96	± 30.93	685.68	± 278.87	379.71	± 105.66
10	432.14	± 118.90	343.27	± 51.8998	249.09	± 21.53	738.96	± 277.09	337.31	± 83.48
15	357.34	± 64.69	314.43	± 47.9021	236.88	± 49.33	641.15	± 200.51	364.05	± 57.40
30	309.04	± 125.59	247.48	± 79.2694	206.6	± 57.50	602.39	± 200.51	197.49	± 44.90
60	240.22	± 128.16	200.86	± 45.4984	182.02	± 47.62	354.42	± 128.80	180.71	± 41.54
120	147.97	± 84.75	149.73	± 56.6278	151.23	± 62.24	252.27	± 152.94	187.03	± 64.61
180	195.14	± 132.81	153.58	± 40.4484	176.1	± 53.33	164.45	± 84.50	198.55	± 115.17

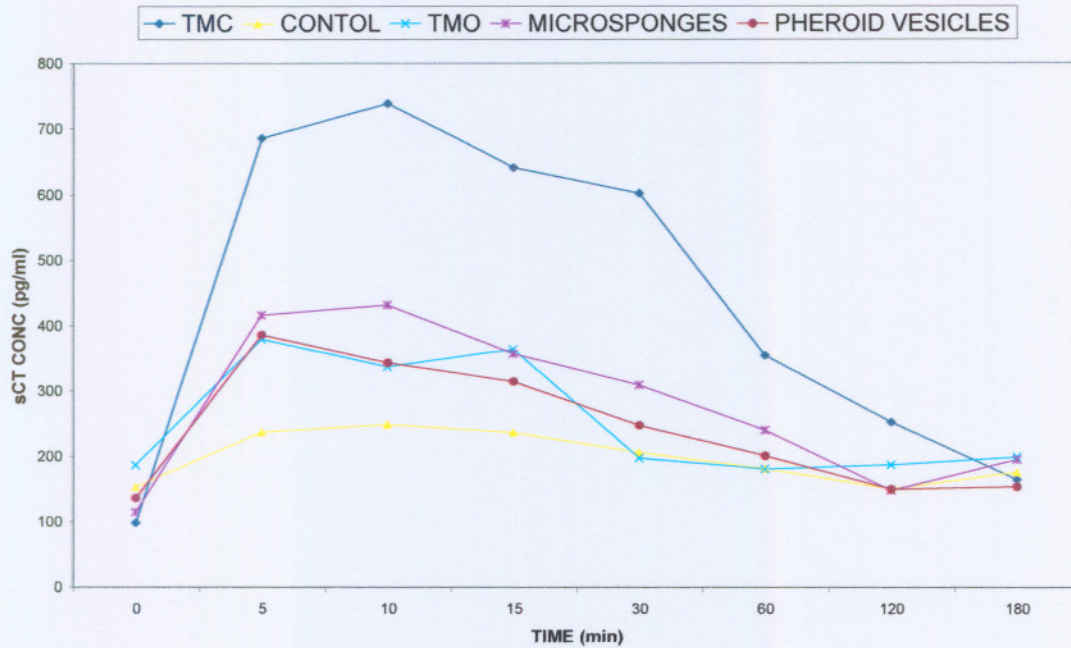


Figure 5.8 Plasma salmon calcitonin concentrations after administration into the jejunum of rats with different formulations as a function of time (Error bars omitted for clarity).

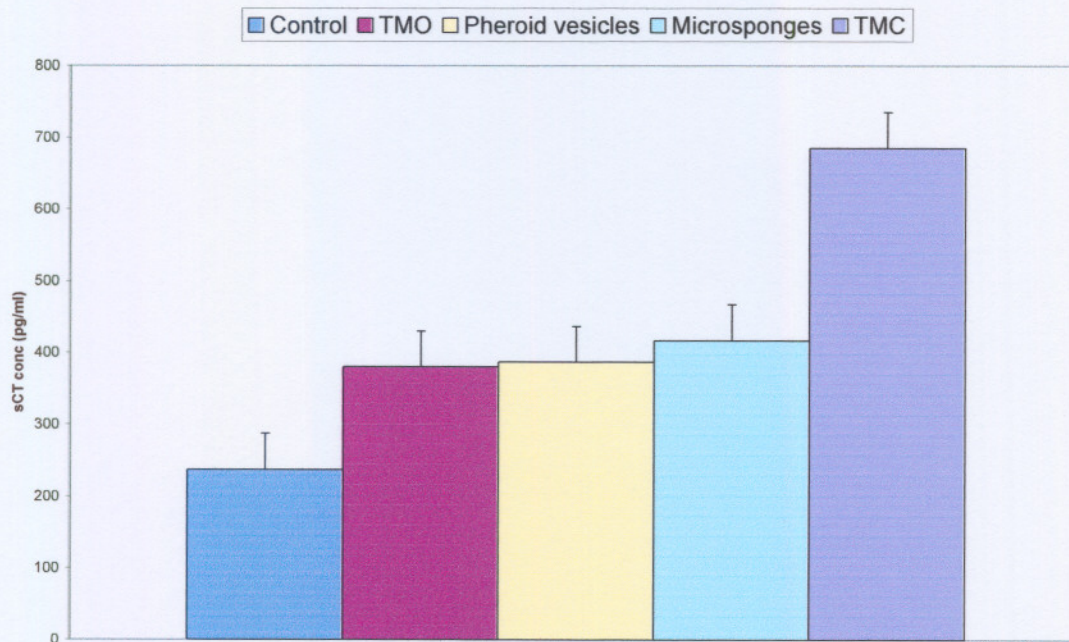


Figure 5.9 Plasma salmon calcitonin concentrations 5 minutes after intestinal administration with various formulations.

5.4 CONCLUSION

From the obtained results it can be clearly seen that TMC is the most potent absorption enhancer. Pheroid microsponges are the second best absorption enhancer. Pheroid vesicles and TMO shows similar results. Pheroid microsponges enhance absorption of salmon calcitonin rapidly to show a peak plasma concentration after just 10 minutes. Pheroid vesicles shows a peak plasma concentration of salmon calcitonin after 5 minutes, but the peak concentration remains lower than the peak plasma concentration obtained when using Pheroid microsponges. Salmon calcitonin concentrations also decrease more rapidly with Pheroid vesicles than with Pheroid microsponges, thus indicating that a lower concentration of salmon calcitonin is absorbed with Pheroid vesicles. This correlates well with the lower peak concentration observed with Pheroid vesicles than with Pheroid microsponges (386.25 pg/ml versus 432.14 pg/ml). The use of TMO resulted in salmon calcitonin being rapidly absorbed and shows a peak plasma concentration just after 5 minutes. Plasma concentrations decline very slowly over a period of time. In comparison with TMC, TMO shows a significant lower absorption enhancement capability. After just 10 minutes TMC enhances the concentration of salmon calcitonin to 738.96 pg/ml, almost double the peak concentration as seen with TMO (379.71 pg/ml). The control used (salmon calcitonin in saline) clearly shows none or very low absorption of salmon calcitonin. The obtained results indicates that absorption enhancement of salmon calcitonin did take place with all 4 formulations used.

SUMMARY AND FUTURE PROSPECTS

Due to the current progress in the manufacturing of peptide and protein drugs, large quantities are now available, making it possible to use it as therapeutic drugs. However, despite of these advantages in manufacturing the bioavailability of peptide and protein drugs after peroral administration remains low. Low bioavailability is also frequently associated with variable plasma concentrations and poorly controlled pharmacological and toxic effects. Bioavailability of peptide drugs is low because of their insufficient stability in the gastro intestinal fluids and their poor absorption pattern. This is due to the presence of pancreatic proteases, such as trypsin, chymotrypsin, elastase and carboxypeptidase. Furthermore, in the intestinal tract peptides are exposed to brush border proteases and also finally have to cross the epithelium. During this absorption process, degradation by enzymes is also highly possible. The absorption barrier in the intestine is represented by epithelial cell membranes, interconnected by proteinaceous tight junctions.

The development of a pharmaceutically acceptable drug delivery system for a peptide or protein drug is therefore more complicated and requires more time for research and development than for traditional drug compounds. This is primarily due to a greater structural complexity of the peptides and proteins. Many peptide and protein drugs have molecular weights greater than 500 Da, low lipophilicity, charged functional groups and tend to form hydrogen bonds with water. During the past few decades, much effort has been put into research and much knowledge regarding non-parenteral administration of peptides and proteins has been achieved. The problems with peptide and protein delivery are not trivial and will not be overcome by trivial solutions. The use of peptidase inhibitors, absorption enhancers and specialized delivery systems may improve peptide and protein drug delivery into the systemic circulation.

It is unlikely that any of these approaches would by itself enable the controlled delivery of peptide and protein drugs, or that any single system is going to be universally

applicable. Formulations will have to be developed for the needs and requirements as well as the properties of each individual peptide or protein drug. The potential usefulness of formulations depends on whether or not the enzymatic and absorption barriers existing for the non-parenteral route of delivery can be surmounted. It is important, however, that the natural barriers remain and are altered only momentarily, although only a small amount of peptide or protein drug needs to be absorbed to get an effect due to its high potency.

Co-administration of protease inhibitors or absorption enhancers, chemical modification (peptidomimetics and pro-drug approaches) and special drug delivery systems are possible ways of solving the problems. The use of the formulation approach by shielding the peptide or protein drug in a polymer or lipid drug delivery system is another possibility. Another solution is the use of micro-emulsions, polymeric nanoparticles and hydrogels for the delivery of peptide and protein drugs.

There are a number of diseases that requires long-term treatment with peptide drugs. The usual parenteral administration of these substances is tedious and patients often have difficulty accepting them. The same is true for patients receiving salmon calcitonin in the treatment for osteoporosis and Paget's disease. The possibility of delivering these peptides perorally would be a much appreciated alternative.

Over the past years chitosan and its derivatives have been studied quite intensively. Because of their permeation enhancing effect (I), enzyme inhibitory capabilities (II), and mucoadhesive properties (III), chitosan and its quaternised derivative, TMC, are able to reduce there barriers, which make these polymers important excipients for peroral peptide delivery systems. Mucoadhesive properties offer the advantage of remaining concentrated at the absorption site of the drug.

TMC is thereby able to enhance the paracellular absorption, which is important for the transport of peptide and protein drugs across the intestinal epithelium. The mechanism underlying this absorption enhancing capability is based on the positive charges of the

polymer, which interact with the cell membrane resulting in structural reorganisation of tight junctions-associated proteins. TMC is able to act in the more neutral and basic environments of the GIT, thus making it more favourable to use as chitosan which is only active in the more acidic stomach. These unique features make TMC a valuable excipient for the peroral administration of peptide and protein drugs.

The Pheroid system, based on nano- and microtechnology, has been investigated by researchers and proved to be an important technology in the delivery of peptide and protein drugs. The most important advantages include increased delivery of active compounds, penetration of most known barriers in the body and cells and increased therapeutic efficiency.

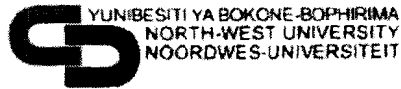
The results obtained in this study indicated that TMC with its mucoadhesive properties and ability to open tight junctions produced the highest absorption enhancement of salmon calcitonin. Pheroid microsponges were also able to enhance the absorption of salmon calcitonin. This absorption enhancement of Pheroids was not as high as obtained with TMC, but Pheroids definitely have the potential of enhancing peptide absorption more.

A defined need to study Pheroid technology further is clearly noticed. Determination of the possible mechanism of action must be confirmed. Cytotoxicity studies must also be further investigated. Quantitative release studies of the entrapped active compounds must also be determined. Nevertheless in spite of these shortcomings, Pheroids still produced a high absorption enhancement of salmon calcitonin. This formulation must definitely be kept under the magnifying glass.

Chitosan and TMC are well known polymers and numerous researchers have confirmed its absorption enhancing capabilities. This study once again confirmed that TMC is not an excipient that can be absent in the peroral administration of peptide and protein drugs in the future.

ANNEXURE 1

APPROVAL OF ETHICAL COMMITTEE



YUNIBESITHI YA BOKONE-BOPHIRIMA
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT

Etiëkkomitee

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26 Oktober 2004

Prof AF Kotze
Bussie 36
Noordwes-Universiteit
(Potchefstroomkampus)

Geagte prof Kotze

GOEDKEURING VIR EKSPERIMENTERING MET DIERE

Hiermee wens ek u in kennis te stel dat u projek getiteld "*Enhancement of calcitonin absorption after nasal and peroral administration with Emzaloid™ technology*" goedgekeur is met nommer 04D15.

Gebruik asseblief die nommer genoem in paragraaf 1 in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekteleiers verwag word om jaarliks in Junie aan die Etiëkkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het. U sal in Mei 2005 die dokumentasie hieroor ontvang.

Goedkeuring van die Etiëkkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring verkry word.

Die Etiëkkomitee wens u alle voorspoed met u werk toe.

Vriendelike groete

ESTELLE LE ROUX
NAMENS SEKRETARIAAT



POTCHEFSTROOMKAMPUS
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ANNEXURE 2

SERTIFICATE OF ANALYSIS FOR SALMON CALCITONIN



Certificate of Analysis

Calcitonin, Salmon

Product Number: 05-23-2401 Lot Number: B61334
Molecular Formula: $C_{145}H_{240}N_{44}O_{48}S_2$ RTECS Number: EV8000000
Molecular Weight: 3431.9 CAS Number: 47931-85-1

TEST	RESULTS
Appearance	White lyophilized powder
Solubility	Soluble in 5% AcOH
HPLC	100%
Peptide content	87%

Storage and Handling	FREEZER (-20°C) Hygroscopic
----------------------	--------------------------------

Ying Peng, Ph.D, Director, QA/QC

20-Aug-2004

Date

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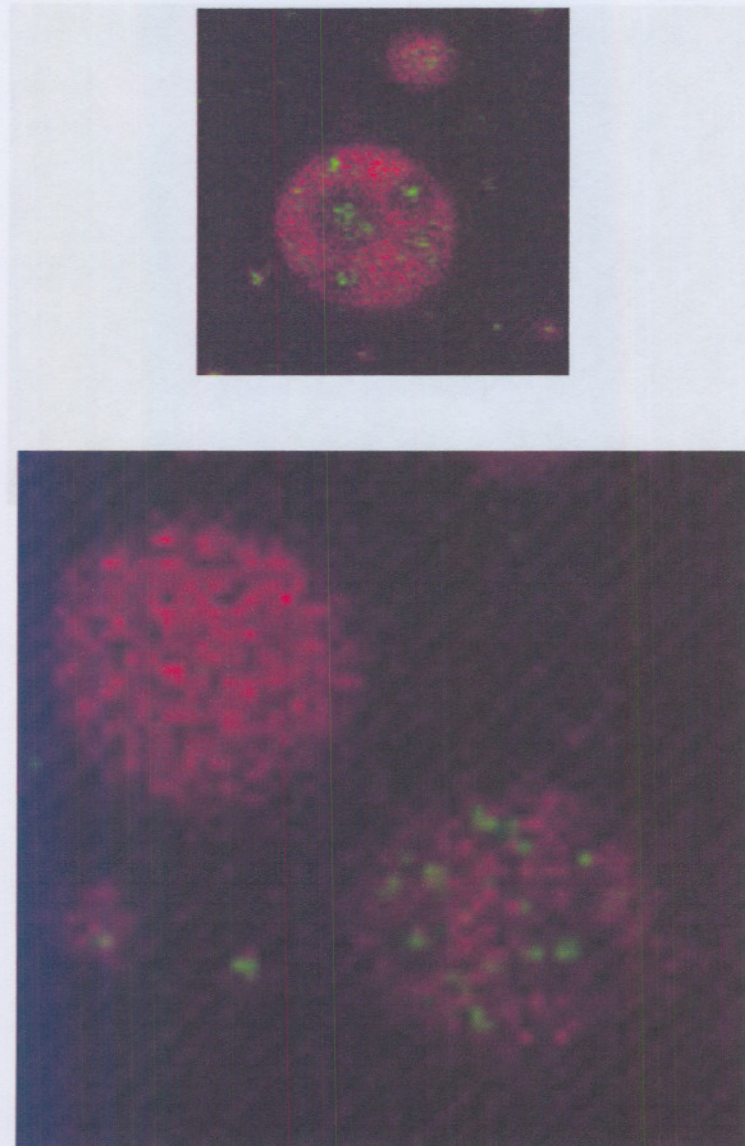
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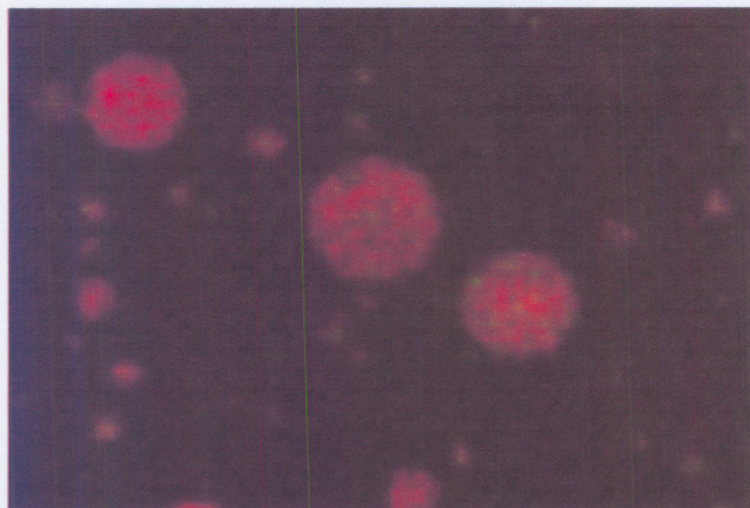
A Brand of EMD Biosciences, Inc., an Affiliates of Merck KGaA, Darmstadt, Germany
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ANNEXURE 3

PHEROID MICROSPONGES

Confocal laser scanning micrographs of Pheroid microsphere (red) with salmon calcitonin inside (green).

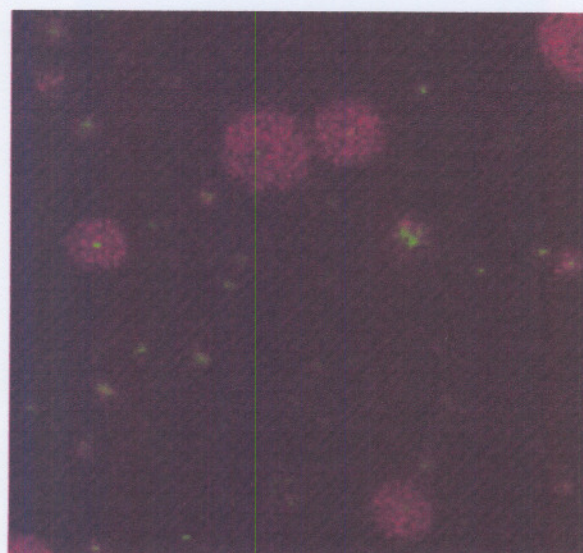
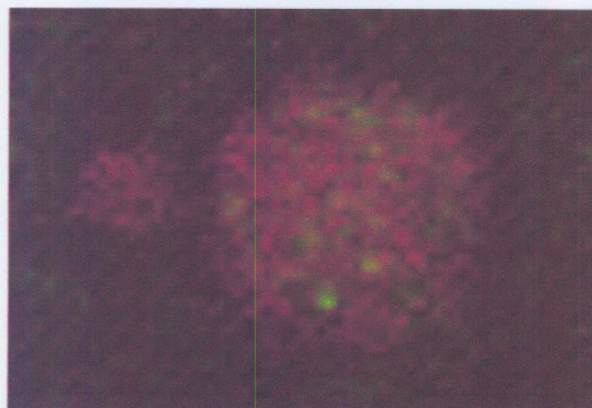




ANNEXURE 4

PHEROID VESICLES

Confocal laser scanning micrographs of Pheroid vesicles (red) with salmon calcitonin inside (green).



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