Nasal Drug Delivery of Calcitonin with Pheroid technology

Jeanéne Celesté Kotzé

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Supervisor: Prof. A. F. Kotzé

Co-supervisor: Mr. J. Lubbe

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I dedicate this thesis to my mother, who was and will continue to be an inspiration to me:

Petro C. Kotzé

With Love

The future belongs to those who believe in the beauty of their dreams.

- Eleanor Roosevelt

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ABSTRACT

Advances in biotechnology and recombinant technologies have lead to the production of several classes of new drugs such as peptide and protein drugs. These compounds are mostly indicated for chronic use but their inherent characteristics such as size, polarity and stability prevent them from incorporation in novel dosage forms. The bioavailability of nearly all peptide drugs is very low due to poor absorption from the administration site. Several challenges confront the pharmaceutical scientist in developing effective and innovative dosage forms for these classes of drugs. A lot of attention has been given to the nasal route of drug administration for delivery of peptide drugs. The availability of several promising classes of absorption enhancers and new drug delivery technologies has also prompt scientists to develop new delivery systems for nasal administration of peptide drugs.

It has been shown in recent years that *N*-trimethyl chitosan chloride (TMC), a quaternary derivative of chitosan, is effective in enhancing the absorption of several peptide drugs, both in the peroral route and in the nasal route of drug administration. Early indications are that new drug delivery technologies such as Pheroid technology will also be able to enhance peptide drug absorption in the nasal route. The aim of this study was to evaluate and compare the absorption enhancing abilities of TMC and Pheroid technology in the nasal delivery of calcitonin, a peptide hormone with low bioavailability.

Pheroid vesicles and Pheroid microsponges were prepared and characterized for their morphology and size distribution. Calcitonin was entrapped into these vesicles and microsponges and TMC and TMO solutions (0.5 % w/v), containing calcitonin, was also prepared. These formulations were administered nasally to rats in a volume of 100 µl/kg body-weight to obtain a final concentration of 10 IU/kg body-weight of calcitonin. Plasma calcitonin and calcium levels were determined over a period of 3 hours.

The results of this study clearly indicated that both Pheroid formulations and the TMC formulation increase the nasal absorption of calcitonin with a resulting decrease in plasma calcium levels, indicating an increased absorption of calcitonin. The highest increase in calcitonin absorption was obtained with the TMC formulation and this was explained by the difference in the mechanism of action in enhancing peptide absorption between TMC and Pheroid technology. It was concluded that Pheroid technology is also a potent system to enhance peptide drug delivery and that the exact mechanism of action should be investigated further.

Key words: Calcitonin, Pheroid vesicles, Pheroid microsponges, *N*-trimethyl chitosan chloride (TMC), nasal drug delivery.

HITTREKSEL

Die vooruitgang in biotegnologie en genetika het onder andere ook gelei tot die ontwikkeling van proteïen en peptiedgeneesmiddels. Die aard van hierdie geneesmiddels maak dit egter moeilik om nuwe doseervorme te ontwikkel. Peptiedgeneesmiddels beskik oor eienskappe soos hoë molekulêre massas, swak stabiliteit en lae biobeskikbaarheid wat talle uitdagings bied aan die formuleerder van nuwe doseervorme daarvan. Alternatiewe roetes van toediening, verbindings wat die absorpsie van geneesmiddels bevorder en nuwe afleweringssisteme, maak dit egter moontlik om wel peptiedgeneesmiddels toe te dien.

TMC ('n kwaternêre derivaat van kitosaan) is al met groot sukses in die verlede gebruik om die absorpsie van verskeie peptiedgeneesmiddels, wat nasaal en oraal toegedien word, te verbeter. Daar is eksperimentele bewyse dat die unieke Pheroid afleweringssisteem die absorpsie van peptiedgeneesmiddels kan verbeter. Die doel van hierdie studie was om die absorpsiebevorderende eienskappe van TMC en Pheroids, na nasale toeniening van kalsitonien, te evalueer en met mekaar te vergelyk.

Pheroid druppeljies en Pheroid mikrosponsies is voorberei en gekarakteriseer ten opsigte van morfologie en druppelgrootteverspreiding. Pheroid druppeltjies en Pheroid mikrosponsies en TMC en TMO (0.5 % m/v) oplossings is voorberei en kalsitonien is daarby ingesluit. Hierdie formulerings is in 'n volume van 100 µl/kg ligaamsmassa, om 'n finale kalsitonien konsentrasie van 10 IU/kg liggaamsmassa te gee, nasaal toegedien in rotte. Die plasmakalsitonien en plasmakalsiumvlakke is bepaal oor 'n tydperk van 3 ure.

Die resultate wat behaal is met beide die Pheroid formulerings en die TMC oplossing toon duidelike verhogings in die nasale absorpsie van kalsitonien, met 'n ooreenkomstige verlaging in plasmakalsiumvlakke. Die TMC formule het die grootste verhoging in kalsitonien absorpsie getoon, wat moonlik

verduidelik kan word deur die verskil in die werkingsmeganisme tussen TMC en Pheroidtegnologie. Die gevolgtrekking van hierdie studie is dat Pheroidtegnologie ook as 'n goeie afleweringssisteem vir peptiedgeneesmiddels beskou kan word en dat die werkingsmeganisme daarvan verder ondersoek moet word.

Sleutelwoorde: Kalsitonien, Pheroid druppeltjies, Pheroid mikrosponsies, *N*-trimetiel kitosaan chloried (TMC), nasale geneesmiddelaflewering.

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INTRODUCTION AND AIM OF STUDY

Over the years, the development of improved drug formulations with effective drug transport and drug absorption has remained a major challenge for the pharmaceutical scientist. The demands to increase transport and bioavailability have become more essential and the search for appropriate delivery systems continues. New classes of drugs such as proteins and peptides have opened an area of ongoing research in discovering ways of improving the delivery of drugs. Nasal drug administration has become an important and specialized field of study. The nasal route, with its rich blood supply as a first-rate advantage, can be regarded as safe and convenient, making this route even more suitable for drug administration.

Because several major permeation barriers in the nasal route of drug administration have to be overcome, the potential of absorption enhancers have captured the attention of many researchers. The ideal absorption enhancer should be non-toxic, non-irritable, effective and be able to act reversible on tight junctions. Furthermore, there should be no membrane damage to the epithelial cells.

N-trimethyl chitosan chloride (TMC), a partially quaternised derivate of chitosan, has shown great potential as an absorption enhancer across mucosal surfaces and could be of great importance in the development of effective delivery systems for hydrophilic drugs, especially in neutral environments where chitosan is ineffective as an absorption enhancer. TMC has been used with success in the nasal route and it has been shown that it is able to enhance the absorption of peptide drugs after nasal administration.

Pheroid technology is a unique delivery system with a stable structure, which can be manipulated in terms of morphology, structure, size and function. Pheroid vesicles show high entrapment capabilities, rapid transport and delivery with reduced side effects and minimal membrane damage, which provide the ideal opportunity to use this technology to investigate the possible

enhancement of calcitonin absorption after entrapment in Pheroid vesicles and nasal administration.

Although the development and progress in the formulation of the peptide hormone calcitonin, are important to researchers and because of its great potential in remarkable modulation of osteoclast activity and the direct reduction in osteoclast numbers, limitations such as poor bioavailability and low permeation as well as inherent characteristics, such as molecular size and polarity, reduce its possibilities for effective non-parenteral administration. The possible solution of adding absorption enhancers to calcitonin is evaluated in this study to overcome these obstacles and increase the permeability of calcitonin. Therefore, the aim of this study is to evaluate the potential application of Pheroid technology and TMC calcitonin for nasal delivery.

The specific objectives of this study can be summarized as:

- A literature study on nasal drug delivery, calcitonin, Pheroid technology and *N*-trimethyl chitosan chloride (TMC).
- Preparation and characterisation of Pheroid vesicles and Pheroid microsponges.
- Entrapment of calcitonin into Pheroid vesicles and Pheroid microsponges.
- Evaluation and determination of the effects of the Pheroid vesicles and microsponges and TMC on calcitonin absorption after nasal administration in rats.

Chapter 1 gives a detailed description of the anatomy and morphology of the human nose with the advantages and limitations of nasal drug delivery. In chapter 2, information on calcitonin, as a peptide, is given and Pheroid technology and TMC, are discussed. In chapter 3 the preparation and characterisation of Pheroid vesicles and Pheroid microsponges are given and the procedure for *in vivo* nasal permeation studies on the rat model is described in detail. The results of these studies are presented in chapter 4.

CHAPTER 1

NASAL DRUG DELIVERY

1.1 HISTORICAL AND BEHAVIOURAL USAGE

Many important contributions are to be found which have improved the science of nasal drug delivery from a pharmaceutical and technological point of view (Colombo, 1999:592). The delivery of drugs, for the local treatment of diseases such as nasal allergy, congestion and infections, through the nasal route has been used for centuries, but recent research has shown that the nasal route can also be exploited for systemic delivery of drugs, such as small molecular weight polar drugs, peptides and proteins, which are not easily administered via other routes, or where rapid onset of action is required (Illum, 2003:187). The therapeutical potential of synthetic, biologically active peptides increases the need for acceptable non-parenteral and non-oral routes of drug delivery (Donovan *et al.*, 1990:808).

New materials and new technologies led pharmaceutical researchers to identify and develop alternatives to the classical oral and injectable routes which include the nasal route. The nasal mucosa provides a large surface area and good blood supply, an efficient filtering system and systemic effects when substances are absorbed through the nasal mucosa, which makes it more suitable for therapeutic drug delivery (Colombo, 1999:592).

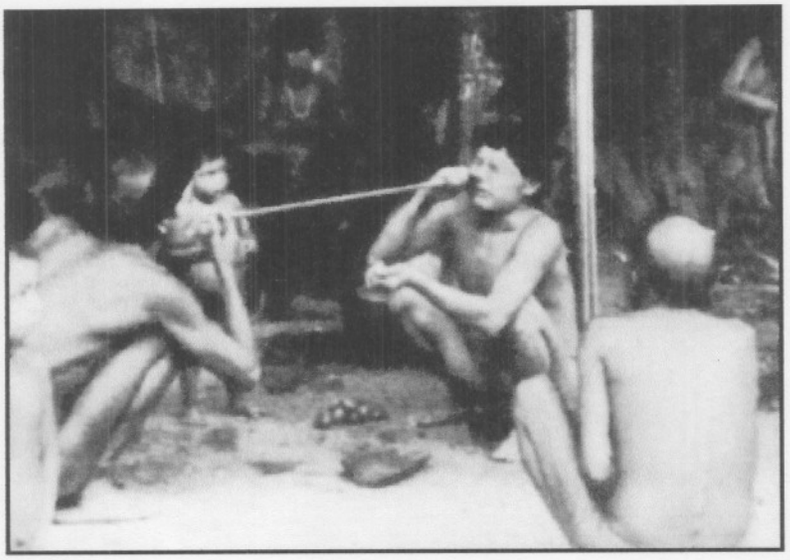


Figure 1.1 Yanomano Indian tribe using the insufflator pipe for *epenà* nasal administration (Colombo, 1999:593).

In Ancient Tibet, inhalers containing extracts of sandalwood and aloe wood were used as anti-emetics, which illustrate the practice of intranasal drug delivery since ancient times (Quraishi *et al.*, 1997:289).

An instructive behavioural employment of the nose for drug absorption is the religious rite of the Amazonian population of Yanomano to consume *epenà*. They inhaled a powdered mixture of different plant-parts like *Mimosa acacioides, Piptadenia peregrina,* and others not identified, which cause hallucinations or more general mental effects (Colombo, 1999:592). An insufflator with a long linear pipe, activated by an assistant, or a Y-shaped curved tube for self administration, is used by the natives to deposit the powdered drug in the nose (Figure 1.1) and is of interest to pharmaceutical researchers. These "medical devices" focalize the typical aspect of nasal delivery, which is the need for a nebulizer or an insufflator for depositing solid or liquid formulations into the nose (Colombo, 1999:593).

During nasal administration there is a rapid onset of the effect, linked to high mucosal permeability. The possibility of reaching the brain directly after deposition on the olfactory mucosa is increased and if drugs are successfully

administered in powder form, it increases the formulation possibilities for designing an appropriate dosage form (Colombo, 1999:593).

1.2 ANATOMY AND PHYSIOLOGY OF THE NOSE

The human nasal cavity is approximately 12 cm long, extending from the nostrils (external nares) to the junction with the nasopharynx with a volume of 15 ml and a total surface area of 150 cm^2 (Illum & Fisher, 1997:139). The nose opens to the environment through nostrils in the front and is connected to the pharynx and via the larynx to the trachea in the back (Illum, 1999:508) as shown in figure 1.2. The nose is divided into two nasal cavities separated by a nasal septum (Colombo, 1999:593).

The nasal cavities are located in the skull between the base of the cranium and the roof of the mouth, in front of the nasopharynx (Greisheimer & Wiedeman, 1972:406).

Every nasal cavity is enclosed by a roof, floor and medial and lateral wall. The middle of the roof is made up by the lamina cribrosa of the ethmoid bone (figure 1.2 and figure 1.3), the nasal bone and the spine of the frontal bone form the roof in front, as illustrated in figure 1.3; and the posterior part consists of the body of the sphenoid, the sphenoidal concha, the ala of the vomer, and the sphenoidal process of the palatine bone. The floor is formed by the palatine process of the maxilla in the front, and by the horizontal process of the palatine bone in the back. The medial wall (nasal septum) is formed by the crest of the nasal bones in the front, the frontal spine in the middle by the perpendicular plate of the ethmoid and in the back, by the vomer end-part of the sphenoid. The nasal septal cartilage completes the septum. The frontal process of the maxilla and the lacrimal bone forms the front of the lateral wall, the middle is created by the ethmoid, the maxilla and the inferior nasal concha while the back is structured by the vertical plate of the palatine bone and a part of the sphenoid (Greisheimer & Wiedeman, 1972:407).

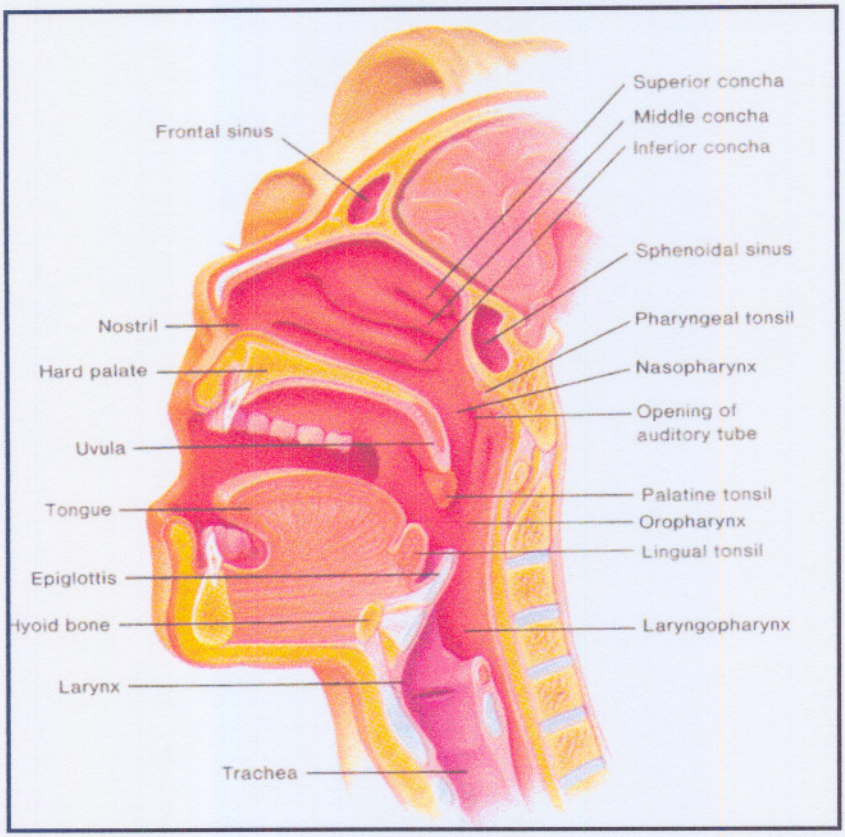


Figure 1.2 Features of the upper respiratory tract (Hole, 1993:583).

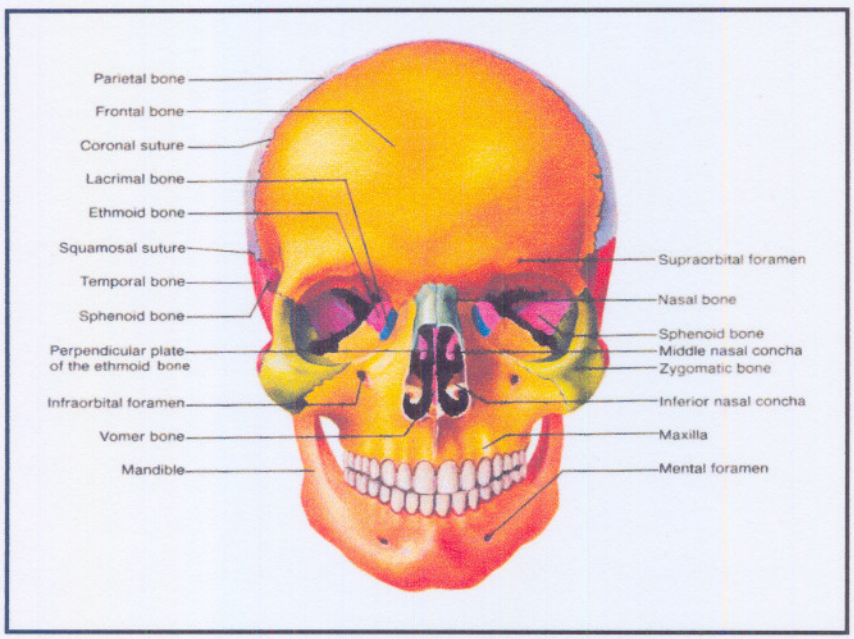


Figure 1.3 Anterior view of the skull (Hole, 1993:188).

Each nasal cavity is again divided into five different regions namely the vestibule (nostril), the atrium region, the respiratory region, the olfactory region and the nasopharynx (Colombo, 1999:593) as shown in figure 1.4.

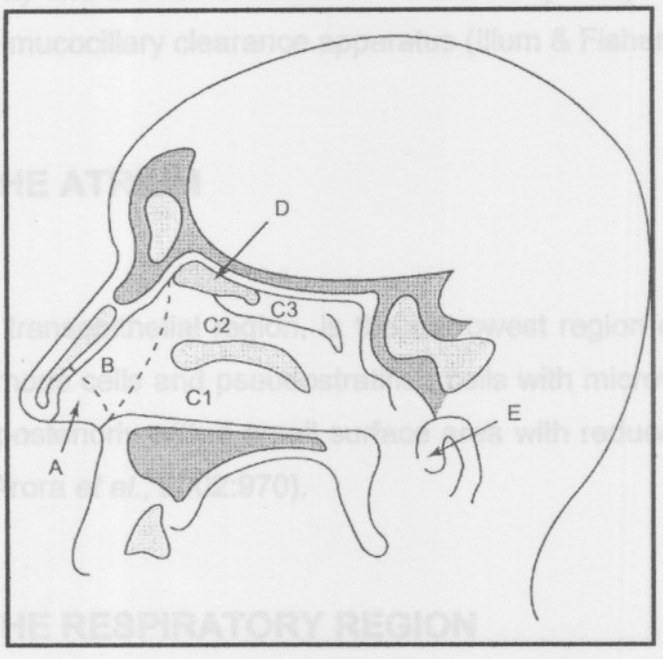


Figure 1.4 Schematic view of a saggital section of the nasal cavity showing the nasal vestibule (A), atrium (B), respiratory area: interior turbinate (C1), middle turbinate (C2) and the superior turbinate (C3), the olfactory region (D) and nasopharynx (E) (Arora *et al.*, 2002:969).

1.2.1 THE VESTIBULE

The vestibule is the widened portion just behind the external nares in each nasal cavity (Greisheimer & Wiedeman, 1972:408) and has a surface area of 0.6 cm^2 and is about 1.5 cm from the nares in the nasal valve (the narrowest part of the airway with a cross section of 0.3 cm^2) (Illum & Fisher, 1997:139). The frontal part is lined with stratified squamous keratinizing epithelium and contains hair follicles with coarse hair, sebaceous and sweat glands. The

adjoining part is also lined with stratified squamous epithelium and beyond this lining there is pseudostratified ciliated columnar epithelium with goblet cells (Greisheimer & Wiedeman, 1972:408). Inhaled particles are trapped by hairs in the cavity and are removed either mechanically or by the action of the well-developed mucociliary clearance apparatus (Illum & Fisher, 1997:139).

1.2.2 THE ATRIUM

The atrium, or transepithelial region, is the narrowest region of nasal cavity. Stratified squamous cells and pseudostratified cells with microvilli are present anteriorly and posteriorly with a small surface area with reduced permeability in this region (Arora *et al.*, 2002:970).

1.2.3 THE RESPIRATORY REGION

The biggest part of the nasal cavity is the respiratory region, which contains the nasal turbinates (or conchae) divided in the superior-, middle- and inferior turbinate (Illum & Fisher, 1997:139) as shown in figure 1.5. The two nasal cavities are dominated by these three turbinates, which are mainly responsible for heating and humidification of the nose (Arora *et al.*, 2002:969).

The turbinates are arranged, one above the other in the lateral wall of each nasal cavity, to create the idea of sagging shelves. The portions of the nasal cavity under and lateral to the respective nasal turbinates are called the superior-, middle- and inferior meatuses. The recess of the nasal cavity above the superior turbinate is called the spheno-ethmoidal recess (Greisheimer & Wiedeman, 1972:407).

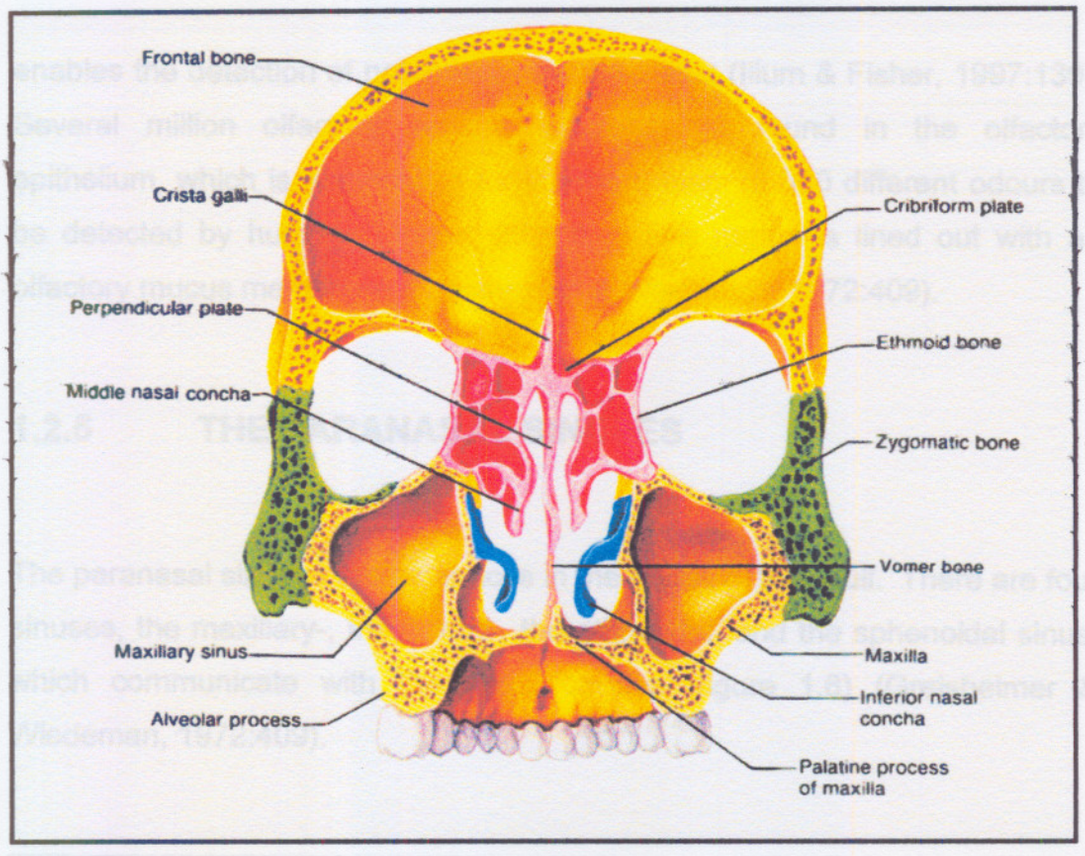


Figure 1.5 Frontal section of the skull (Hole, 1993:197).

The respiratory region is lined with a mucus membrane consisting of pseudo-stratified, ciliated columnar epithelium with goblet cells and supporting tissues (lamina propria) with collagenous, elastic fibers and serous glands. The mucus membrane is adherent to the periosteum of the bone or perichondrium of cartilage beneath it. Consequently, the two together is called the mucoperiosteum (Greisheimer & Wiedeman, 1972:408).

1.2.4 THE OLFACTORY REGION

The olfactory region covers an area of approximately 10 to 20 cm^2 in the roof of the nasal cavity, the upper portions of the nasal cavity and the lateral walls. Furthermore the olfactory function of the nose is a protective mechanism that

enables the detection of potentially noxious gases (Illum & Fisher, 1997:139). Several million olfactory sensory neurons are found in the olfactory epithelium, which is responsible for the more than 10 000 different odours to be detected by humans (Jones, 2001:6). This region is lined out with an olfactory mucus membrane (Greisheimer & Wiedeman, 1972:409).

1.2.5 THE PARANASAL SINUSES

The paranasal sinuses are air spaces in the bones of the skull. There are four sinuses, the maxillary-, the frontal-, the ethmoidal- and the sphenoidal sinus, which communicate with the nasal cavities (figure 1.6) (Greisheimer & Wiedeman, 1972:409).

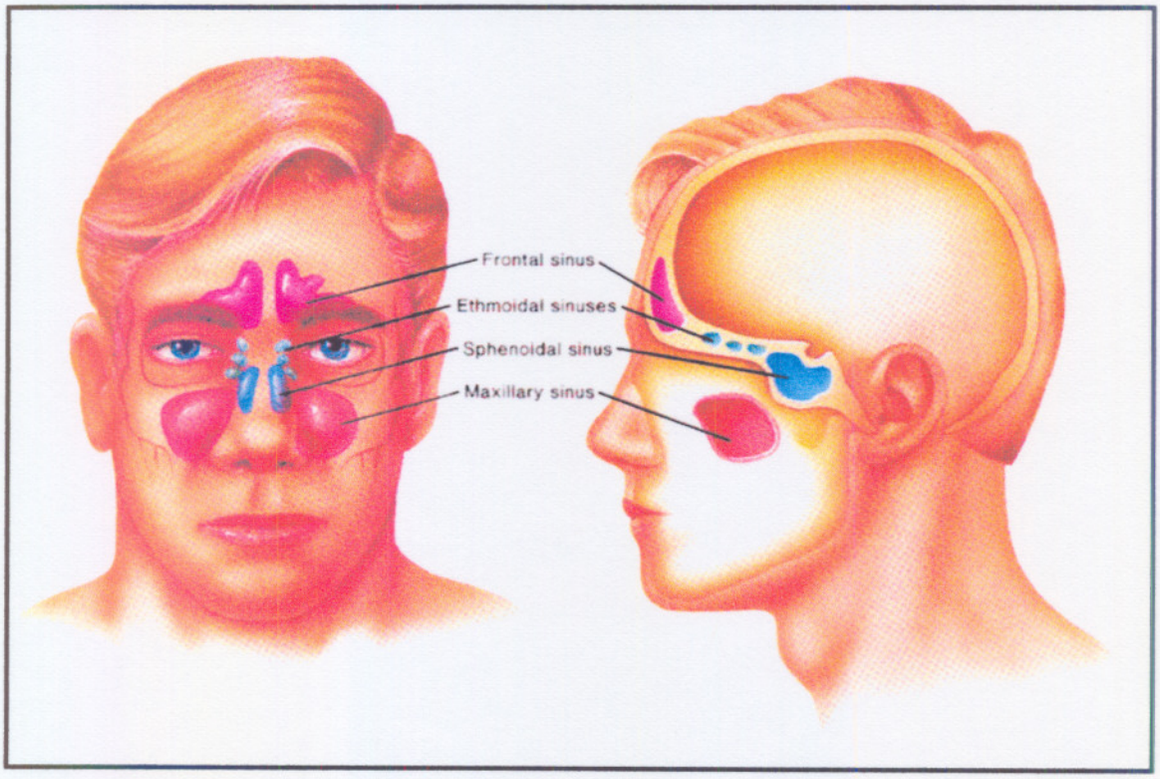


Figure 1.6 Locations of the sinuses (Hole, 1993:195).

The maxillary sinus lies in the body of the maxillae, opens in the middle meatus and is the largest paranasal sinus with a capacity of about 15 ml. There are two frontal sinuses with a capacity of 3-8 ml that enter the middle meatus of the nasal cavity. The ethmoidal sinuses consist of air cells and the two sphenoidal sinuses open into the spheno-ethmoidal recess with a capacity of 7.5 ml (Greisheimer & Wiedeman, 1972:409).

The sinuses are lined with mucus membranes similar to that of the nasal cavity. Mucus secretions can cause inflammation if the sinuses are blocked by secretion of the mucus membranes. The main function of the sinuses is to reduce the weight of the skull, but they also serve as a resonant chamber to affect the quality of the voice (Hole, 1993:583).

1.2.6 NASAL BONES

The nasal bones are short and make up a third of the length of the nose. Laterally the nasal bones are attached to the maxilla by the sydesmosis. The piriform aperture, which is smaller in women than in men, is the bony inlet of the nasal bones and the maxilla (Jones, 2001:11).

1.2.7 NASAL NERVE SUPPLY

The nasal blood vessels and glands have a rich nerve supply from both the autonomic and somatic nervous system. The nasal mucus membrane derives its sensory supply from the cranial nerve and contains sympathetic and parasympathetic fibres of the autonomic nervous system. Figure 1.7 shows the origin of the autonomic innervation of the nose. The autonomic innervation of about three-quarters of the nasal mucus membrane reaches the nose via the vidian nerve and follows the distribution of the second division of the trigeminal nerve to the nose. The vidian nerve consists of parasympathetic and also sympathetic cholinergic fibres. The capillary vessels receive the primary alpha-adrenergic sympathetic fibres (constrictor),

but also receive small infections and other environmental insults occurring during childhood (Chien *et al.*, 1989:7-9).

The olfactory nerve supplies the olfactory mucus membrane. The sensory nerve of the nasal mucosa is the trigeminal and the glands of the lamina propria and the smooth muscle of the arterioles are supplied by autonomic nerves (Greisheimer & Wiedeman, 1972:410).

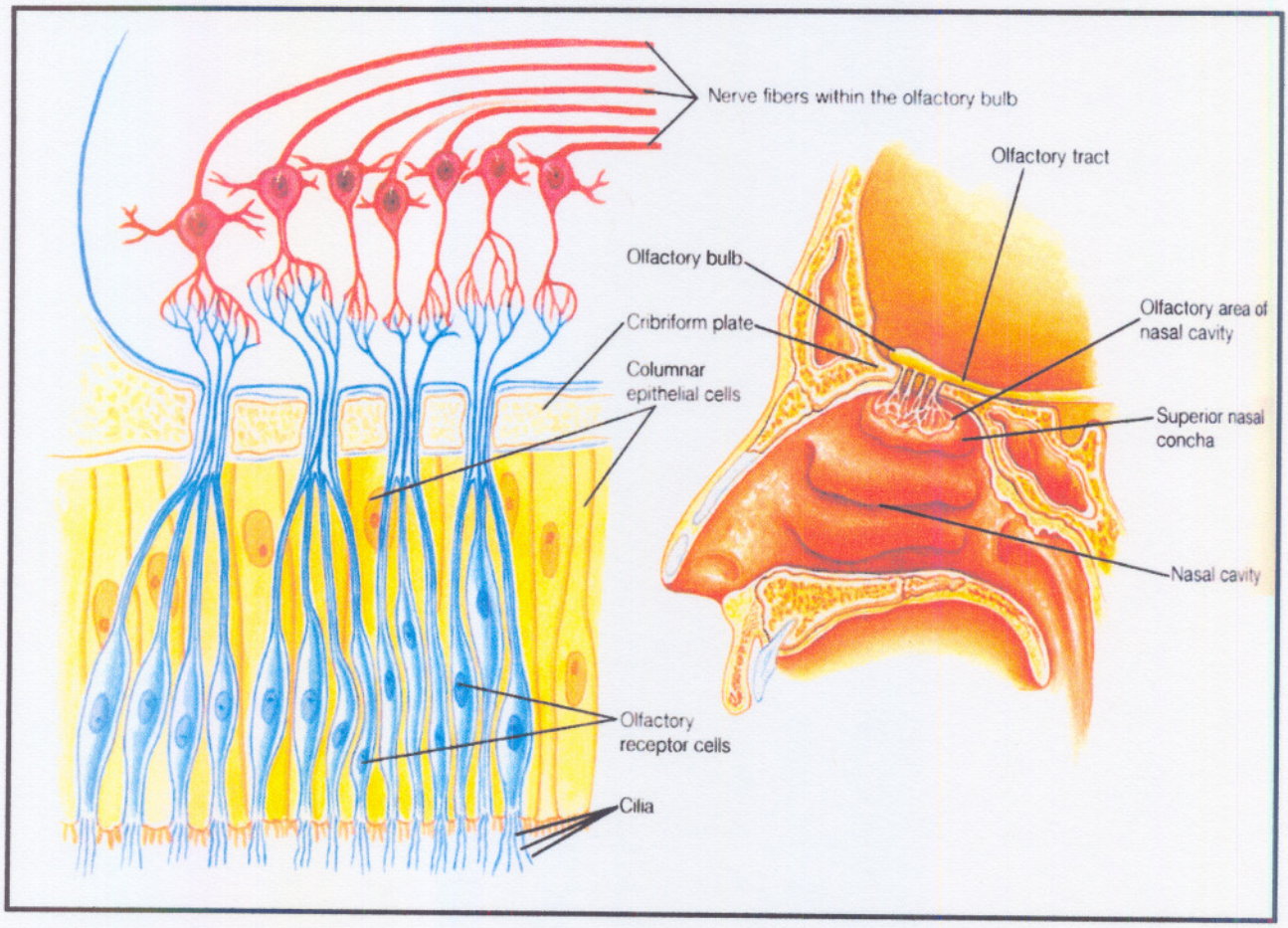


Figure 1.7 The olfactory receptor cells with cilia, which are supported by columnar epithelial cells, at the distal ends. The olfactory area is associated with the superior nasal concha (Hole, 1993:424).

1.2.8 THE NASOLACRIMAL DUCT

The nasolacrimal duct (figure 1.8) is a canal that transports the lacrimal secretion (tears) from the nasolacrimal sac into the inferior meatus of the nose (Tortora & Anagnostakos, 1990:468). It is lined with mucus membranes, which are continuous with the lining of the nasal cavity and opens into the inferior meatus (Greisheimer & Wiedeman, 1972:409).

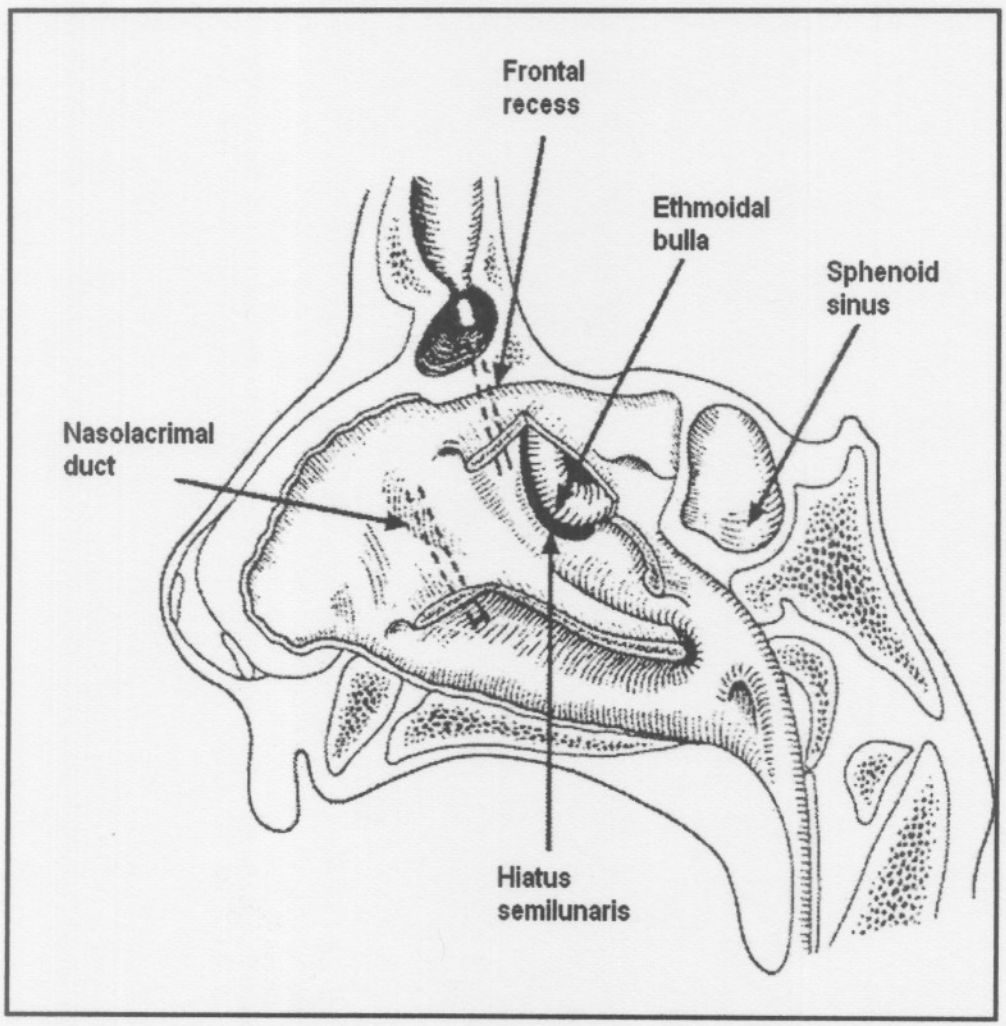


Figure 1.8 A line diagram of the lateral nasal wall (Jones, 2001:16).

1.3 MORPHOLOGY OF THE NASAL CAVITY

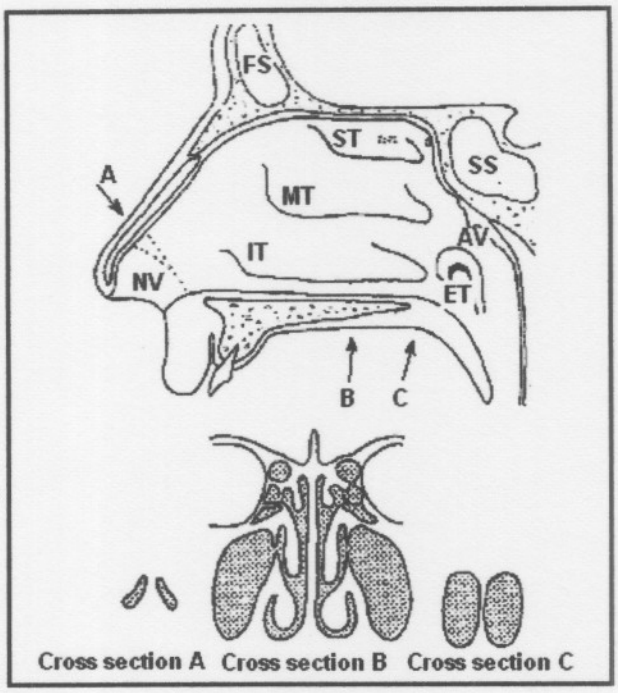


Figure 1.9 The general morphology of the human nasal cavity. Lateral wall of the human cavity and cross section through (A) the internal ostrium, (B) the middle of the nasal cavity, and (C) the choanae. Hatched area in upper figure: olfactory region. NV = nasal vestibule; IT = inferior turbinate and orifice of the nasolacrimal duct; MT = middle turbinate and orifices of frontal sinus, anterior ethmoidal sinus and maxillary sinus, mentioned in anteroposterior direction; ST = superior turbinate and orifices of posterior ethmoidal sinuses; FS = frontal sinus; SS = sphenoidal sinus; AV = adenoid vegetations; ET = orifice of the Eustachian tube (Illum & Fisher, 1997:140).

The nasal cavity (figure 1.9) is lined with various epithelial cells, which is of great importance in nasal drug delivery. The major types of epithelia in the human nasal cavity are stratified squamous, olfactory and respiratory epithelium. A continuous sheet of mucus, secreted by various mucosal or

submucosal glands, covers the epithelia of the nasal cavity (Illum & Fisher, 1997:141).

1.3.1 STRATIFIED SQUAMOUS EPITHELIUM

A keratinized, stratified, squamous epithelium lines the nasal vestibule. This epithelium occurs in several overlapping layers, is flattened and has neither cilia nor villi on its surface. This area contains the stiff hairs that form the first defense against inspiration of large particles (Illum & Fisher, 1997:139).

1.3.2 OLFACTORY EPITHELIUM

The Olfactory epithelium (figure 1.10 and figure 1.11) is specialized pseudostratified epithelium that lines the olfactory region. It is composed of three main cell types namely: 1) the olfactory cells also known as the receptor cells 2) the sustentacular cells or supporting cells, which bear numerous microvilli and 3) the basal cells, close to the basal lamina (Illum & Fisher, 1997:141).

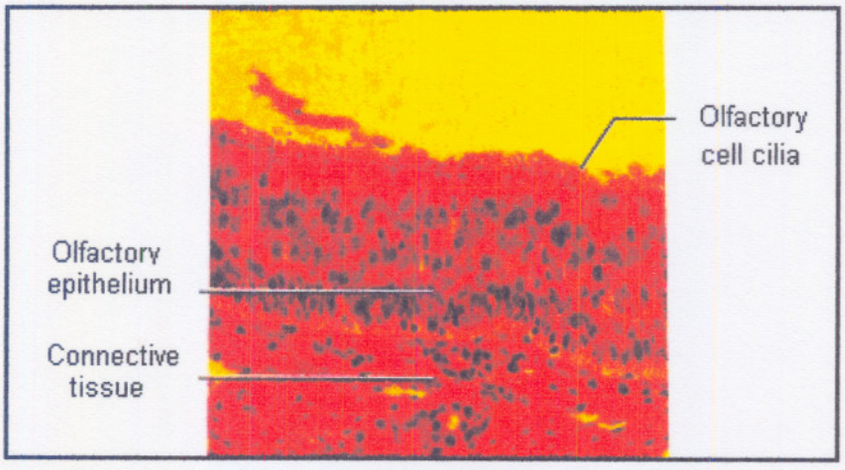


Figure 1.10 Olfactory epithelium (Hole, 1993:425).

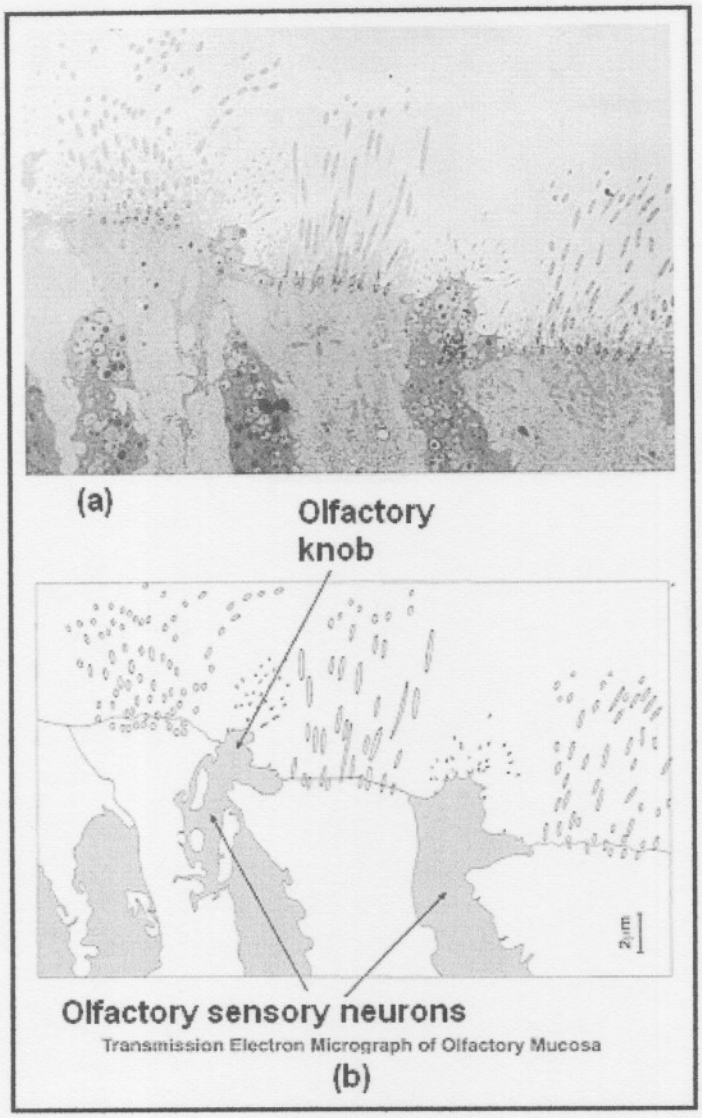


Figure 1.11 Electron micrograph of olfactory mucus (a) with the line diagram (b) (Jones, 2001:7).

Bowman's glands are found in the connective tissue below the olfactory epithelium and are responsible for continuously discharging secretions onto the epithelial surface via the nasolacrimal ducts. The connective tissue also contains blood vessels, lymphatic vessels and nerves (Illum & Fisher, 1997:142).

1.3.3 RESPIRATORY EPITHELIUM

The respiratory epithelium is generally considered to be the site of absorption when drugs are administered via the nasal cavity and covers most of the nasal cavity. The epithelium consists of a basement membrane of four major cell types with a thickness of approximately 100 µm (Illum & Fisher, 1997:142) as illustrated by figure 1.12.

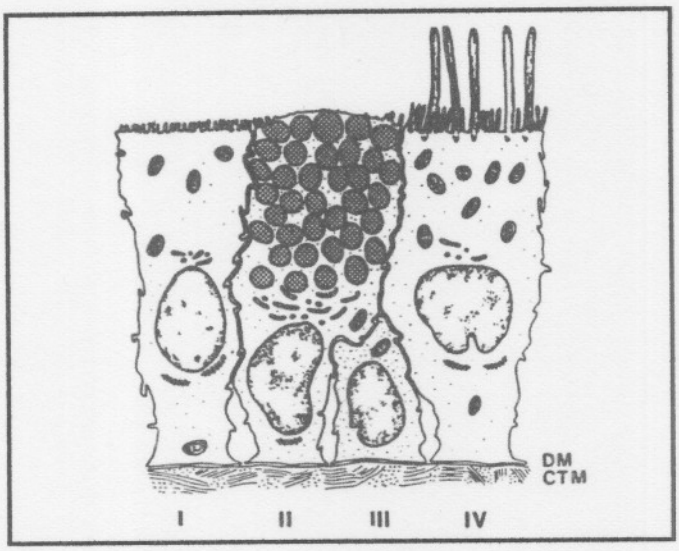


Figure 1.12 Illustration of types of cells in the respiratory epithelium. I = non-ciliated columnar cell, covered by microvilli of uniform length; II = goblet cell; III = basal cell; IV = ciliated columnar cell, covered by cilia and microvilli of uniform length (Illum & Fisher, 1997:143).

1.3.3.1 BASAL CELLS

Basal cells can differentiate into other cells and are known as replacement cells (Illum & Fisher, 1997:142). Basal cells lie on the basement membrane. It has an electron-dense cytoplasm and bundles of tonofilaments and is

believed to be active in helping with the adhesion of columnar cells to the basement membrane (Mygind & Dahl, 1998:4).

1.3.3.2 CILIA

Cilia are hair-like structures on the free surface of the epithelial cells and move to facilitate the flow of mucus. Every cilium consists of two central protein microtubules enclosed by nine microtubule pairs and is anchored below the luminal cell surface to structures called basal bodies (figure 1.13) (Illum & Fisher, 1997:142).

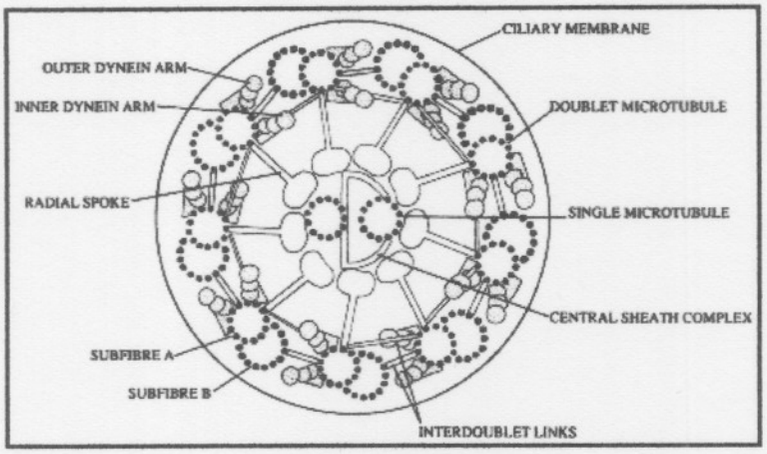


Figure 1.13 Cross-section of a cilium (Marttin *et al.*, 1998:15).

This nine microtubules and the central pair is known as an axoneme and is surrounded by a specialized extension of the cell membrane, the cilia membrane. Nexin, a highly extensible protein, connects the adjacent doublets and the microtubules are connected to the pair of microtubules in the center with radial spokes. By sliding movements of the microtubules, movement of the cilium is created. Cilia have a length of 5-10 µm with a width of 0.1-0.3 µm and the number of cilia is approximately 200 per cell (Marttin *et al.*, 1998:15).

1.3.3.3 GOBLET CELLS

Goblet cells (figure 1.14) are unicellular, mucus-secreting gland cells (Illum & Fisher, 1997:142). Compared to submucosal glands, the contribution of goblet cells to the volume of nasal secretion is small (Mygind & Dahl, 1998:5).

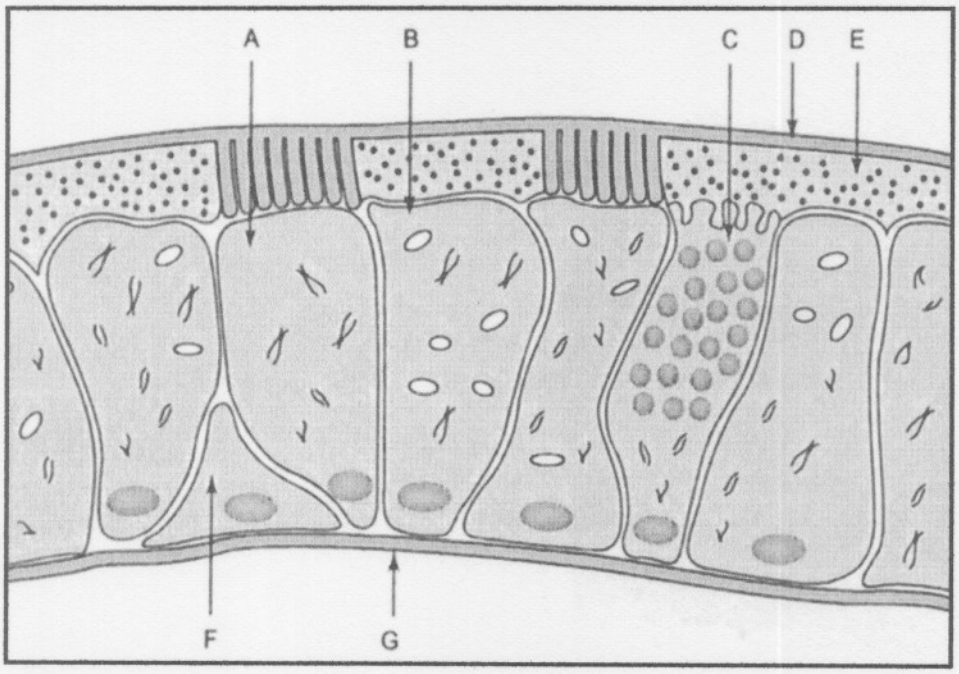


Figure 1.14 Cell types of the nasal epithelium showing ciliated cell (A), non-ciliated cell (B), goblet cells (C), gel mucus layer (D), sol layer (E), basal cell (F) and basement membrane (G) (Arora *et al.*, 2002:970).

In table 1.1 a comparison of respiratory and olfactory epithelia is given.

Table 1.1 Comparison of respiratory and olfactory epithelia (Mathison *et al.*, 1998:418).

CHARACTERISTIC	RESPIRATORY EPITHELIA	OLFACTORY EPITHELIA
Mucus layer	Dense, high renewal rate. Pinkish tinge.	Thicker, viscous, low renewal rate. Yellowish tinge.
Principal epithelial cell types	Columnar cells, ciliated and non-ciliated with microvilli. Goblet cells with microvilli. Basal cells.	Neural receptor cells, ciliated. Supporting cells with microvilli. Basal cells.
Cilia	Motile. Co-ordinated movement.	Mature cilia are immotile. No co-ordinated movement.
Principal contributors	Goblet cells.	Bowman's glands with mucus and serous cells.
Mucus secretions	Mucus and serous glands. Lacrimal glands.	Supporting cells.
Epithelial pH	5.5-6.5 (adults).	Not available.
Innervation	Trigeminal (cranial nerve V) vidian, terminal (cranial nerve 0), autonomic nerves.	Olfactory (cranial nerve I) trigeminal, terminal, autonomic nerves.

1.4 NASAL MUCUS AND NASAL SECRETIONS

1.4.1 NASAL MUCUS

The respiratory mucosa (figure 1.15), lining the posterior two thirds of the nasal cavity, is covered by the mucus layer and supported by a basement membrane (Cornaz & Buri, 1994:263).

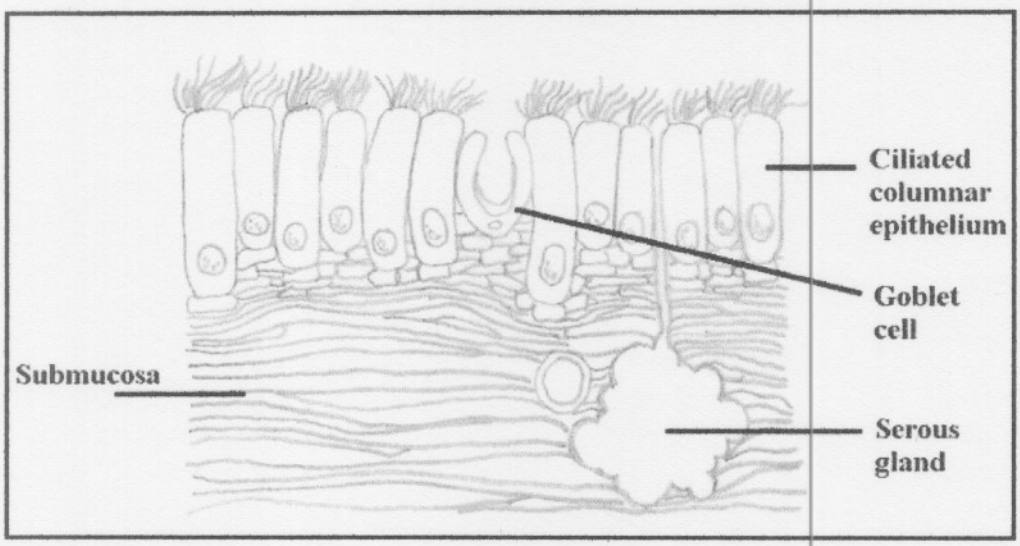


Figure 1.15 Diagrammatic representation of the microscopic appearance of the nasal mucosa.

Mucus, in the airways, is composed of 95% water, 2% mucus glycoproteins, other proteins (1%) including albumin, immunoglobulins, lysozyme and lactoferin, 1% inorganic salts and < 1% lipids. The normal 'resting' nose secretes approximately 20-40 ml of mucus every day (Jones, 2001:8).

The mucus layer is the first barrier that must be crossed before a solute comes in contact with the epithelia. Secretory materials are produced by goblet cells, nasal glands and lacrimal glands and along with a transudate from plasma forms the nasal secretions and it contains a variety of

electrolytes, glycoproteins and proteins (Rossen *et al.*, 1966:369; Lorin *et al.*, 1972:275; Ogawa *et al.*, 1979:73 and Shelhamer *et al.*, 1984:30).

The glycoproteins consist of a 20% protein core with an 80% oligosaccharide side chain, crosslinked by disulphide and hydrogen bonds. Because of the viscoelastic properties of mucus, it can cross-link and produce a gel which forms a mechanical coupling with cilia. The nasal secretions have a lower viscosity than tracheobronchial secretions but comparable elasticity which is more important than viscosity for mucus transport (Mygind & Dahl, 1998:7).

Nasal mucus has the following functions:

- It acts as a retainer for the substances in the nasal duct.
- The mucus has the ability to retain water.
- It behaves as an adhesive.
- It transports particulate matter.
- It exhibits surface electrical activity.
- It acts as a mesh with permeability.
- It allows heat transfer (Chien *et al.*, 1989:7).
- It protects the nasal mucosa from cold and low humidity (Dondeti *et al.*, 1996:116).

The mucus layer is possibly a double layer and is divided into an aqueous periciliary sol phase, in which the cilia beat, and the more superficial blanket of gel, which is moved forwards by the tips of the cilia. Both layers are about 5 µm thick. The thickness and the composition of the double layer are essential for mucociliary transport. The viscous surface layer will inhibit the ciliary beating if the sol layer is too thin, and if it is too thick the gel layer looses its contact with the cilia and the mucociliary clearance is impaired (Mygind & Dahl, 1998:8). The mucus layer is removed and replaced about every ten minutes (Fabricant, 1964:60).

A denser layer of mucus with a slight yellow colour covers the olfactory epithelia. Bowman's glands and the supporting cells comprise the major secretory components of the olfactory epithelia. Unlike the respiratory epithelia, the mucus layer in the olfactory epithelia does not have motile cilia to facilitate flow, but this mucus layer is viscous and stationary. The excess mucus slowly flows into the respiratory region where it is swept away with the respiratory mucus layer by the motile cilia located there. Few solute particles enter the olfactory region due to the lack of airflow in the region. Once a solute particle comes in contact with the olfactory mucus layer, it will not be removed quickly due to the lack of motion of the mucus and it may be able to come in contact with the olfactory epithelia itself (Mathison *et al.*, 1998:418).

1.4.2 THE MUCOCILIARY CLEARANCE MECHANISM

Maintenance of the health and defense of the nose is made possible through nasal mucociliary clearance (Jones, 2001:8). The nasal mucociliary clearance is one of the most important physiological defense mechanisms of the respiratory tract (Warner & Satir, 1974:35). The mucociliary clearance mechanism, as shown in figure 1.16, is the movement of the cilia that causes the movement of the mucus layer covering the epithelial cells, backwards to the throat (Illum & Fisher, 1997:146).

The function of this system is to remove foreign substances and particles from the nasal cavity, preventing them from reaching the lower airways. The efficiency of the mucociliary clearance system depends on the physiological control of the ciliated cells and on the rheological properties of the mucus blanket (Marttin *et al.*, 1998:17).

The nasal mucociliary clearance takes the airway secretions towards the nasopharynx. A cilium moves upwards and penetrates the mucus during the stroke. The energy of the cilia is transmitted to the mucus, causing the mucus to move forwards. The mucus blanket is transported to the nasopharynx, where it is swallowed (Marttin *et al.*, 1998:17).

Factors affecting the mucociliary clearance are:

- Disruption of cilia by viruses and bacteria by producing specific toxins.
- Changes in cilia structure and secreted mucus due to long-standing allergic rhinitis.
- Chronic rinosinisitis causes areas of ciliary denudement.
- Nasal polyps (oedematous swelling of the nasal mucosa) (Jones, 2001:9).

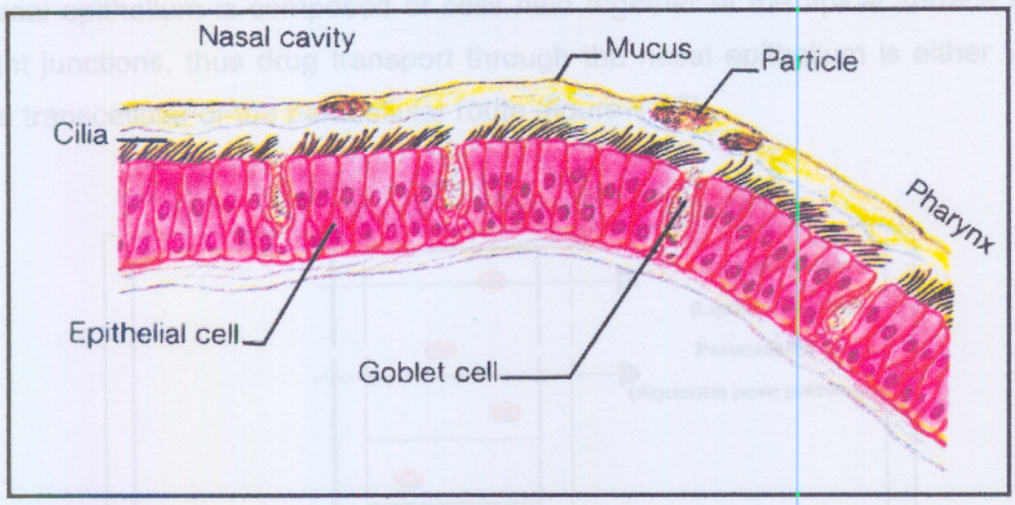


Figure 1.16 Cilia move mucus and trapped particles from the nasal cavity to the pharynx (Hole, 1993:583).

Under normal conditions, inhaled substances or delivery systems are cleared from the nose within 15 to 20 minutes (Dondeti *et al.*, 1996:116). This is the normal defense mechanism of the nasal cavity, which clears mucus and is responsible for increasing or decreasing drug permeation. Clearance of substances from the nasal cavity by mucociliary clearance (MCC) is approximately 21 minutes. Reduction in MCC increases the contact time between the drug and the mucus membrane and increases the drug

permeability and thus an increase in MCC decreases drug permeability (Arora *et al.*, 2002:971).

Apart from the mucociliary clearance mechanism, which removes particles and secretions from the nose, sniffing, nose blowing and sneezing also help to clear the airway secretions. Nasal washing and spraying can be used to remove secretions and trapped particles (Mygind & Dahl, 1998:8).

1.5 DRUG PERMEABILITY IN THE NOSE

1.5.1 PERMEABILITY OF THE NASAL BARRIER

The absorption of foreign material in the nose is prevented by different barriers namely:

1) a physical barrier namely the mucus and epithelium,

2) a temporal barrier also known as mucociliary clearance and

3) a chemical (enzymatic) barrier.

These barriers may influence drug permeation as nasally administered drugs have to pass through these barriers. Intranasally administrated drugs intended for systemic delivery have to pass through the epithelial layer and reach their site of pharmacological action via the bloodstream (Cornaz & Buri, 1994:261-269). The permeation of nasally administrated drugs are favoured by the relatively large surface area because of the large number of microvilli, a porous endothelial membrane and a highly vascularised epithelium (Arora *et al.*, 2002:967).

1.5.2 MECHANISM OF PERMEATION

A drug can permeate epithelial membranes either passively by the paracellular pathway or both passively and actively via the transcellular pathway. Some other transport mechanisms include carrier-mediated

transport. The method of transport depends on the lipophilicity of the compound: if the lipophilicity is increased the absorption of the compound increases through the nasal mucosa via the transcellular passive diffusion pathway (Arora *et al.*, 2002:969).

Water-soluble substances, with a small molecular size and a radius less than 0.4 nm, are absorbed by simple diffusion through water-filled channels. Larger molecules cannot pass through these channels and cross the membrane mostly through passive diffusion (Proudfoot, 1988:142).

Nasal epithelium is composed of cells held together at the apical surface by tight junctions, thus drug transport through the nasal epithelium is either via the transcellular or the paracellular route (figure 1.17).

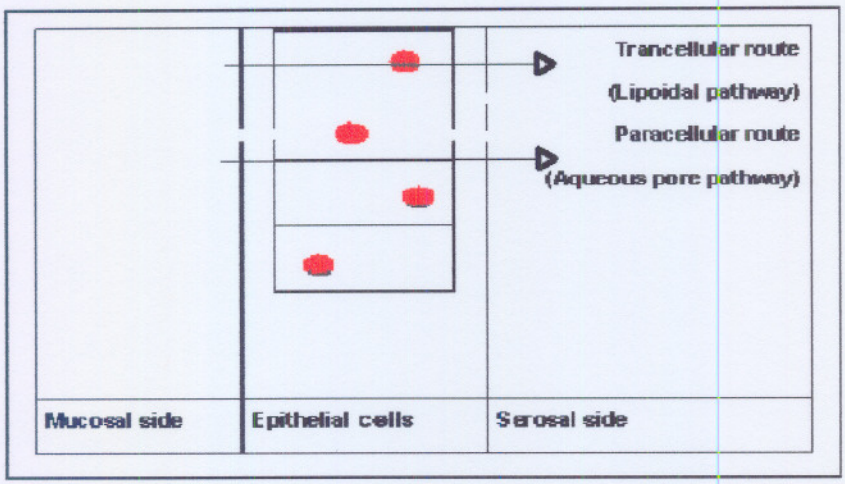


Figure 1.17 Passage through the nasal epithelium (Cornaz & Buri, 1994:264).

The following equation is used mathematically to express the effective permeability coefficient P_{eff} under steady state conditions across excised mucosa:

$$P_{eff} = (dc/dt)_{ss}\ V/\ (AC_D)$$

where $(dc/dt)_{ss}$ is the time-dependent change of concentration in the steady state, **A** is the permeation area, **V** is the volume of the receiver compartment and C_D is the initial concentration in the donor compartment. Fluorophore-labelled markers and drugs, together with sophisticated microscopy techniques such as confocal laser scanning microscopy have been used in visualizing the permeation pathways (Arora *et al.*, 2002:968).

1.5.2.1 PARACELLULAR TRANSPORT

With this pathway, diffusion takes place between the adjacent cells (through the extracellular spaces between the cells) of the epithelium (Vander *et al.*, 1994:137). This is the transport of molecules around or between the cells. Tight junctions exit between the cells. At these tight junctions, the cell membranes are brought into extremely close contact, but are not fused, to occlude the extra-cellular space. Consequently, ions or molecules may not be able to pass through the intercellular spaces (Vander *et al.*, 1994:138).

1.5.2.2 TRANSCELLULAR TRANSPORT

During transcellular transport, molecule movement across the plasma membrane of epithelial cells takes place through different ion channels and transport proteins and can be divided into two subgroups described below.

1.5.2.2.1 PASSIVE TRANSPORT

Facilitated diffusion or passive transport enhances spontaneous movement of involved substances down the electrochemical gradient without requiring energy supply (Wilkinson, 2001:4). Specifically dissolved molecules bind to carrier proteins which carry them across the lipid bilayer with changes in their

conformation occurring in such a way that the site binding the carrier molecule is first opened on the side of the membrane and then on the other side. Transport by means of carriers can be either passive or active but transport through channels is always passive (Szachowicz-Petelska *et al.*, 2001:171).

1.5.2.2.2 ACTIVE TRANSPORT

This transport mechanism differs from passive transport in the way that the pathway is mediated by membrane carrier proteins, directly consuming energy, working against an electrochemical gradient (Wilkinson, 2001:4). There are three main ways of active transport to and from the cell. 1) Coupled carriers combine the transport of a molecule across the membrane up the gradient with the transport of another one occurring down the gradient. 2) Transport with an adenosinetriphosphate (ATP) driven pump. 3) Transport up the gradient with ATP hydrolysis and with light-driven pumps, occurring mainly in bacterial cells. Transport up the gradient is coupled with absorption of the energy of light (Szachowicz-Petelska *et al.*, 2001:172).

1.5.2.3 ENDOCYTOSIS

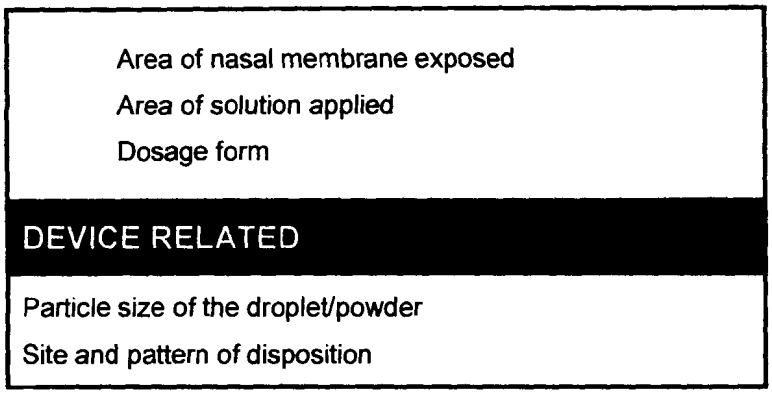
Endocytosis and exocytosis are other pathways by which molecules can enter or leave cells without passing through the structural matrix of the membrane. During endocytosis an intracellular membrane-bound vesicle encloses a small volume of extracellular fluid into the cell. Endocytosis are divided into three classes: (1) fluid endocytosis (2) adsorptive endocytosis and (3) phagocytosis. Exocytosis, on the other hand, occurs when membrane-bound vesicles in the cytoplasm fuse with the plasma membrane and release the contents outside the cell. The main functions of exocytosis are: 1) replacement of plasma membranes, 2) construction of new membrane material and 3) it provides a route by which membrane-impermeable molecules can be released into extracellular fluid (Vander *et al.*, 1994:137).

1.5.3 FACTORS AFFECTING NASAL PERMEABILITY

Permeability of drugs through the nasal mucosa is affected by various factors, broadly classified into three categories as shown in table 1.2. Some of these factors will be explained in more detail in the next sections.

Table 1.2 Various factors affecting the permeability of drugs through the nasal mucosa (Arora *et al.*, 2002:969).

BIOLOGICAL
Structural features
Biochemical changes
Physiological factors
Blood supply and neuronal regulation
Nasal secretions
Nasal cycle
pH of the nasal cavity
Mucociliary clearance and ciliary beat frequency
Pathological conditions
Environmental factors
Temperature
Humidity
FORMULATION
Physicochemical properties of the drug
Molecular weight
Size
Solubility
Lipophilicity
pK_a and partition coefficient
Physicochemical properties of the formulation
pH and mucosal irritancy
Osmolarity
Viscosity/Density
Drug distribution



1.5.3.1 BIOLOGICAL FACTORS

Numerous efforts are being made to modify and explore the mechanisms and structural features of nasal mucosa to increase permeability, which is not necessarily advisable because of anticipated alterations in the normal physiology of the nasal cavity (Arora *et al.*, 2002:969).

1.5.3.1.1 STRUCTURAL FEATURES

The five different regions that are responsible for permeability in the nasal cavity are the nasal vestibule, atrium, respiratory area, olfactory region and nasopharynx. Their impact on permeability are listed in table 1.3. Absorption in the two nasal cavities are both limited by the septum wall and dominated by the three tubinates (figure 1.4). The respiratory region with its rich blood supply, large surface area and nasal secretions, makes it the most suitable area for permeation of compounds. The type, density and number of cells in this region favour the permeability of drugs. The presence of the microvilli on the cells increases the area available for contact with the drug formulation, which further improves permeation of drugs. Permeation can also be improved by using various absorption enhancers in the drug formulation. The permeation increases as result of the following:

- Increasing of the membrane fluidity.
- Decreasing viscosity of the mucus layer.
- Inhibiting proteolytic enzymes.
- Disrupting the tight junctions.
- Increasing the paracellular or transcellular transport.
- Increasing the blood flow.
- Dissociating protein aggregation.
- Initiating the pore formation (Arora *et al.*, 2002:969).

Table 1.3 Structural features of different sections of the nasal cavity and their relative impact on permeability (Arora *et al.*, 2002:970).

REGION	STRUCTURAL FEATURES	PERMEABILITY
Nasal vestibule	Nasal hairs (vibrissae). Epithelial cells are stratified, squamous and keratinized. Sebaceous glands present.	Least permeable because of presence of keratinized cells.
Atrium	Transepithelial region. Stratified squamous cells present anteriorly and pseudostratified cells with microvilli present posteriorly. Narrowest region of nasal cavity.	Less permeable as it has a small surface area and stratified cells are present anteriorly.
Respiratory region (inferior, middle and superior turbinate)	Pseudostratified ciliated columnar cells with microvilli (300 per cell), large surface area. Receives maximum nasal secretions because of presence of seromucus glands, nasolacrimal duct and goblet cells. Richly supplied with blood for heating and humidification of inspired air, presence of paranasal sinuses.	Most permeable region because of large surface area and rich vasculature.

Olfactory region	Specialized ciliated olfactory nerve cells for smell perception. Receives ophthalmic and maxillary divisions of trigeminal nerve. Direct access to cerebrospinal fluid.	Direct access to cerebrospinal fluid.
Nasopharynx	Upper part contains ciliated cells and lower part contains squamous epithelium.	Receives nasal cavity drainage.

1.5.3.1.2 BIOCHEMICAL CHANGES

The nasal mucus, with all its enzymes, acts as an enzymatic barrier to deliver drugs. A pseudo-first-pass order is created, because of the enzyme responsible for degradation of drugs in the nasal mucus, which hamper the absorption of the drug. Various approaches have been used to overcome these degradations, including the use of protease and peptidase inhibitors. Other ways for increasing the stability and permeation of compounds include designing prodrugs of esters, steroids, peptides and amino acids (Quraishi *et al.*, 1997:290).

1.5.3.1.3 PHYSIOLOGICAL FACTORS

Possible physiological factors that influence the absorption of drugs are:

- The drug retention on the mucosal surface.
- The mucus layer clearance.
- The vascularity of the nasal mucosa.
- Conditions such as rhinitis and influenza.
- Mucociliary transport rates which determine the length of time that the drug is available for absorption through the nasal mucosa (Quraishi *et al.*, 1997:291).

1.5.3.1.3.1 BLOOD SUPPLY AND NEURAL REGULATION

The good blood supply and neuronal regulation contribute to the relatively high permeability of the nasal mucosa. The rise and fall in the amount of drug permeated is being regulated by nasal cycles of congestion and relaxation. The increased permeability results from parasympathetic stimulation (Arora *et al.*, 2002:969).

A dense network of cavernous tissue which is well developed over the turbinate and septum, covers the surface of the epithelium and provides a rich surface for drug absorption. This highly vascular network plays important physiological roles in the thermal regulation and humidification of the inspired air and for controlling the size of the lumen of the nasal passage. (Illum & Fisher, 1997:148).

A rich capillary network in the subepithelia as well as around the nasal glands and a cavernous plexus deep to the glandular zone forms the nasal vasculature and this is characterised by fenestrated endothelium. Blood reaches the cavernous plexuses from the capillary bed and from arteries by means of arteriovenous anastomoses and venous drainage occurs through a number of venous plexuses (Su, 1991:603).

The venous plexuses drain into the internal jugular vein by way of the cranial venous sinuses and into the external jugular vein by way of the facial vein (Greisheimer & Wiedeman, 1972:409).

Thus, drugs absorbed via the nasal mucosa enter the right side of the heart for distribution to the systemic arterial circulation prior to traversing the liver and this makes the nasal mucosa suitable for potential drug absorption (Su, 1991:603).

Nasal blood flow is affected by many factors which include:

- Trauma and compression of large veins in the neck.

- Emotions (fear, frustration, humiliation, anxiety).
- Changes in environmental temperature.
- Hyperventilation.
- Exercise (Illum & Fisher, 1997:148).

Certain local or systemic drug administrations increase nasal blood flow such as histamine, fenoterol, isoproterenol, phenylephrine, albuterol, prostaglandins PGE_1 and PGE_2, while other drugs decrease the nasal blood flow like oxymetazoline and clonidine (Illum & Fisher, 1997:149).

1.5.3.1.3.2 NASAL SECRETION

Nasal secretions can also cause variation in drug permeability for example in:

- the viscosity of nasal secretions,
- the solubility of nasal secretions,
- diurnal variation and the
- pH of the nasal cavity (Arora *et al.*, 2002:969).

Various pathological conditions such as the common cold or rhinitis may lead to mucociliary dysfunctioning hyposecretion or hypersecretion or irritation of the nasal mucosa which influence drug permeability (Arora *et al.*, 2002:969).

The influence of environmental conditions includes temperature and humidity. At a temperature of 24°C, the MCC reduces and thus increases the drug permeability, an increase in temperature decreases beat frequency and increases drug permeability. A humidity increase also decreases MCC and increases drug permeability (Arora *et al.*, 2002:971).

1.5.3.1.3.3 NASAL pH

Nasal secretion in adults has a normal pH that ranges between 5.5 – 6.5, whereas in infants and young children it ranges from 5.0 to 6.7. The pH of the nasal secretions was found to be more alkaline during acute rhinitis, acute sinusitis and in the more acute phases of allergic rhinitis, and shifted back to more acidic pH values when the stage of clinical resolution was reached. Cold and heat influence nasal pH to the extend that cold air causes a drift towards alkalinity, whereas heat yields a drift towards acidity. Sleep, rest, ingestion of food, emotion and infection also have an influence on the pH of the nasal secretions (Chien *et al.,* 1989:17).

1.5.3.1.3.4 NASAL PATHOLOGY

The nasal epithelia may be altered to produce excess mucus by different agents including gases, viruses, bacteria and certain diseases such as allergic rhinitis (Chien *et al.,* 1989:7). The nose, due to its protective function, can develop pathological conditions including allergic reactions, rhinitis, polyposis and colds (Illum & Fisher, 1997:149).

Previous nasal surgery may also affect the mucociliary transport rate and the capacity for nasal absorption. Purulent sinusitis may reduce ciliary beat frequencies while nasal polyposis delays the clearance of radio labelled nasal spray solutions. Bacteria destroy ciliated epithelium and produce ciliostasis in turbinate tissue. Allergic rhinitis increases the volume of nasal secretion, reduces viscosity and increases water transport towards the epithelial potential difference (Jones *et al.,* 1997:309).

Hay fever, with symptoms like tickling in the nose, sneezing and watery rhinorrhoea as well as itching eyes and asthmatic episodes, is the most common of all allergic diseases. Nasal polyps can also arise from the nasal sinuses and project into the nasal cavity, eventually blocking the nasal cavity if not treated. Polyps are round smooth, soft, yellow to pale structures that

are attached to the nasal mucosa by a narrow pedicle and, depending on their etiology, these growths can be removed with surgery or medication. Viral infections, from which most of us suffer from time to time such as, common colds, include symptoms like sneezing, coughing and thick and/or excessive nasal secretion (Illum & Fisher, 1997:149). Pathology may have a great influence on the rates of clearance of solutions from the nasal cavity and on the absorption of drugs from the nasal cavity (Pennington *et al.*, 1988:224).

1.5.3.2 FORMULATION FACTORS

1.5.3.2.1 PHYSICOCHEMICAL PROPERTIES OF DRUGS

1.5.3.2.1.1 MOLECULAR SIZE

The size of a molecule influences its permeability across the nasal membranes. An increase in the molecular size exponentially decreases the absorption through the nasal mucosa if the molecule is larger than 400 Da. The rate-limiting molecular weight for nasal administration was found to be 1000 Da, in comparison to the 300 Da for the oral route (Dondeti *et al.*, 1996:116).

Absorption of larger molecular weight compounds can be enhanced by:

- transport enhancers that modify the permeability of the nasal mucosa at a molecular level and
- by conversion of the drug into its salts or esters (Quraishi *et al.*, 1997:291).

1.5.3.2.1.2 DRUG LIPOPHILICITY

Nasal absorption of compounds increases when its lipophilicity is increased. Studies have shown that the nasal mucosa has the highest *in vitro* transport

of both the hydrophilic compound, mannitol, and the lipophilic compound, progesterone. The nasal mucosa is primarily lipophilic and plays an important role in the barrier function of the membranes (Arora *et al.*, 2002:972). The transport of drugs through nasal, rectal or vaginal membranes may be characterized by a lipoidal pathway and an aqueous pore pathway. Absorption of compounds with a hydrophilic or lipophylic nature through mucosal membranes may thus be increased (Corbo *et al.*, 1990:202).

1.5.3.2.1.3 SOLUBILITY

Solubility is an important factor in determining the absorption of drugs through biological membranes although few reports, regarding the relationship between the absorption of the drug via the nasal route and its solubility, are available. Unionized species are absorbed better compared to ionized species, according to the pH partition theory and this is also true in nasal absorption (Arora *et al.*, 2002:971).

1.5.3.2.2 PHYSICOCHEMICAL PROPERTIES OF THE FORMULATION

The drug's permeation might be affected by the pH of the formulation as well as the pH of the nasal surface. To avoid irritation, the pH of nasal formulations is kept at 4.5-6.5 to obtain efficient drug permeation and prevent the growth of bacteria. Isotonic solutions increase the permeability and are usually preferred for administration. The contact time between the drug and the nasal mucosa is increased by a higher viscosity of the formulation and thereby the time of permeation is increased (Arora *et al.*, 2002:972).

Hypotonic saline solutions tend to inhibit mucociliary activity, whereas hypertonic saline solutions will cause it to stop (Duchateau *et al.*, 1985:854). Drying of the nasal mucus membrane will cause the cessation of cilia activity,

whereas prompt moisture will restore its normal activity (Chien *et al.*, 1989:7).

1.5.3.3 PHARMACEUTICAL FACTORS AFFECTING NASAL PERMEABILITY

Some dosage form-related factors that can influence the rate and extent of drug absorption through the nasal mucosa include:

- The volume of the dose.
- The cone of drug applied in the nose.
- The dispersion of the drug in the nasal cavity.
- The density and viscosity of the compound and the vehicle of the dosage form.
- The pH of the dosage form.
- The toxicity of the drug and its additives.
- The pharmacological and immunological effects of the drug on the nasal mucosa (Quraishi *et al.*, 1997:289).

Nasal drug formulations must not alter the histology and physiology of the nose in the sense that the mucosa must retain its functionality as a barrier against external substances and micro-organisms. Neither should it interfere with the mucociliary clearance and changes induced must be reversible (Colombo, 1999:594). Histological toxicity refers to the alteration of mucosa, including membrane protein removal, cell loss, excessive mucus discharge, ciliotoxicity and disturbance of the normal enzymatic balance (Colombo, 1999:594).

1.6 NASAL ENZYMES

The nasal secretions contain many enzymes, which include cytochrome P450-dependant monooxygenases, lactate-dehydrogenases, oxidoreductases, hydrolases, acid phosphatase and esterase, NAD^+-dependent formaldehyde dehydrogenase and aldehyde dehydrogenase, leucine aminopeptidase, glucose-6-phosphate dehydrogenase, NAD^+ - dependent 15-hydroxyprostaglandin dehydrogenase, carboxylesterase, lysosomal proteinases and their inhibitors; B-glucosidase, B-fucosidase and lysozyme and steroid hydroxylases (Chien *et al.*,1989:16).

Cytochrome P450-dependant monooxygenase has been reported to metabolise many drugs delivered intranasally like cocaine, decongestants, nicotine and progesterone (Quraishi *et al.*, 1997:297). Cytochrome P450-dependant monooxygenase has also been observed to catalyze the metabolism of different xenobiotics (Chien *et al.*, 1989:16).

1.7 DOSAGE FORMS FOR NASAL APPLICATION

Nasal dosage forms consist of a squeeze- or spray activated container with preparations containing dispersed or dissolved drugs. The aim is to deliver the preparation on the mucosa and coat the available surface, in particular the major absorption site for drugs namely the respiratory part. The key factors to be considered during the development phase of the formulation are dispersion patterns and bioadhesion (Colombo, 1999:594).

1.7.1 NASAL SOLUTION/SUSPENSION SPRAYS

Currently, sprays are very elegant and acceptable and the aim of formulation is to obtain an aerosol useful for inspiration and deposition in the upper airways (Colombo, 1999:594).

In the early days, aerosol type systems using propellants were utilised, but mechanical actuators/pumps were later developed as the harmful effects of propellants became known. Presently, metered dose nasal actuators are of the more sophisticated drug delivery systems, in which solution formulations are packaged and they can deliver accurate volumes as low as 25 µl (Behl *et al.*, 1998:102)

The advancement of insufflator technology has made drops, which were often instilled in the nose in the past, obsolete and their use is limited to paediatric patients or to patients who are less able to perform the insufflative manoeuvres. Liquid preparations are frequently used as dosage forms for nasal delivery. Transnasal transport is related to the nasal mucosa tissue water partition coefficient, suggesting also an important role of the stereochemical conformation of the drugs during membrane transport. The nasal epithelium is essentially a lipophilic transport barrier and therefore aspects such as formula pH, ionic strength, surface active agents, viscosity and drug concentration have to be considered to facilitate drug transport (Colombo, 1999:595).

Liquid preparations may present problems linked to formulation stability, low drug concentration at the absorption site and short residence time in the nasal cavity. (Colombo, 1999:595).

1.7.2 NASAL DROPS

The advantage of nasal drops, when administering drug formulations into the nose, is that it is perhaps the simplest and most convenient form of nasal delivery, but the exact amount of formulation cannot be controlled. Before metered dose nasal devices were available, nasal drops were the most popular dosage form for nasal administration of drugs (Behl *et al.*, 1998:102). However, studies show that nasal drops provide better drug delivery compared to sprays over the whole surface area as seen in figure 1.18 (Rogerson & Parr, 1990:8).

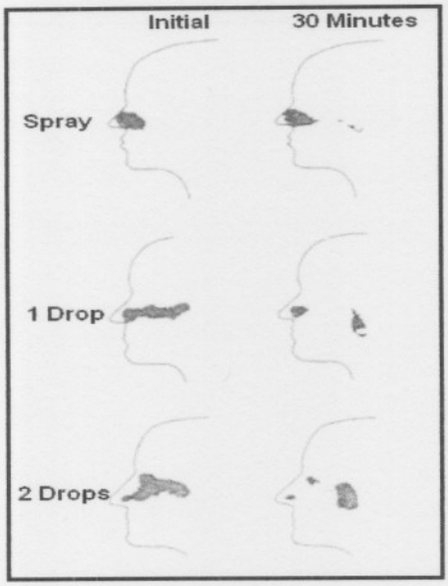


Figure 1.18 Sites of deposition and patterns of clearance after nasal sprays and drops were administered (Rogerson & Parr, 1990:9).

1.7.3 NASAL POWDERS

Disadvantages associated with nasal solutions or sprays led to the development of nasal powders as alternative nasal dosage forms with improved chemicophysical and microbiological stability. The superiority of nasal powders compared to nasal liquids was demonstrated, in particular with peptide drugs. A nasal insufflator is required for the administration of powders for dose emission and deposition of particles in the nose. The aerodynamic properties of the powder affect the site and pattern of the inhalator powder deposition in the respiratory tract and the formalities of dose delivery, control the deposition mechanisms like inertial impacting, sedimentation and diffusion. It could be assumed that efficient nasal delivery of powders from a spraying device needs impacting with adhesion and/or sedimentation of particles on the nasal mucosa; therefore, powder properties such as particle size, shape, surface, density and flow must be optimized to activate the proper mechanism for therapeutic treatment (Colombo, 1999:595).

A dry powder dosage form may be developed when a suitable solution or suspension cannot be found, and needs to be delivered into the nose in metered doses by specialized delivery devices. Powders may cause irritation of the nasal mucosa and are most suitable for drugs which act locally in the nasal cavity. Powders are also difficult and expensive to manufacture (Behl *et al.*, 1998:102).

1.7.4 NASAL GELS

Gels are thickened or gelled solutions or suspensions of drugs and offer advantages like the reduction of post nasal dripping and the minimizing of bad tastes and decreases the loss of drug formulation from the nasal cavity. Gels also reduce anterior leakage of the drug out of the nasal cavity and decrease irritation of certain drugs because of soothing agents and they help localization of the formulation on the mucosa and increase absorption of the drug (Behl *et al.*, 1998:102).

1.7.5 EMULSIONS AND OINTMENTS

Due to limited studies on the development of emulsions and ointments, the full potential of these dosage forms is not yet known but it seems to be more appropriate for local acting drugs. However, disadvantages include poor acceptance by patients, development requirements are high and there is also the need to deliver the precise doses from metered nasal actuators (Behl *et al.*, 1998:102).

1.7.6 METERING DEVICES AND INSUFFLATORS

The nature of the formulation to be dispensed determines the devices to be used for nasal delivery. Metered atomizing pumps, metered-dose pressurized

nasal inhalers, rhinal tubes for variable volume delivery and plastic spray or squeeze bottles are used for liquid solutions. The major factor is the quantity dispensed and its reproducibility. Pumps are very precise and accurate with a fast delivery rate because emission is usually completed in less than one second. Multidose preparations require preservatives in the solution which can alter the state of the mucosa. This problem can be solved by using metered unidose insufflator devices as shown in figure 1.19 (Colombo, 1999: 595).

The shape and size of the device used, determine the particle size of the droplet produced. Particle sizes < 10 µm are deposited in the upper respiratory tract whereas particle sizes < 0.5 µm will be exhaled. Particle sizes between 5-7 µm will be retained in the nasal cavity and subsequently permeated (Arora *et al.*, 2002:972).

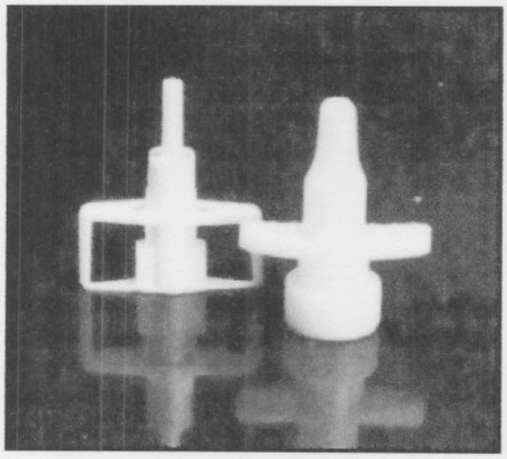


Figure 1.19 Monospray systems for nasal administration of monodoses of liquid formulations (Colombo, 1999:595).

Different types of insufflators are required for powder delivery. Mechanically designed devices consist of a rubbery bulb connected through the dose reservoir to a nasal adapter as shown in figure 1.20 (Colombo, 1999:595).

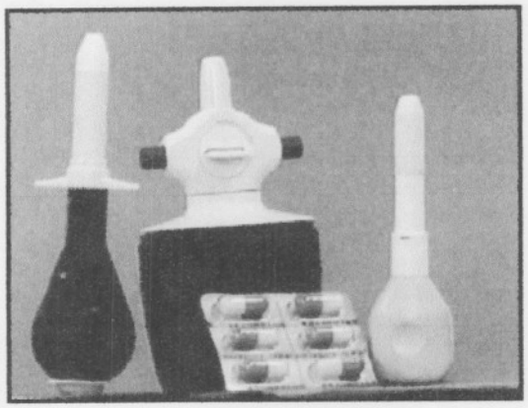


Figure 1.20 Different insufflators for nasal administration of powdered formulations (Colombo, 1999:595).

1.8 ADVANTAGES OF THE INTRANASAL ROUTE

The nasal cavity as a site for drug delivery has some very important advantages:

- The microvilli on epithelial cells create a relatively large surface area.
- The endothelial membrane is highly porous.
- A highly vascularised epithelial layer is present.
- A high total blood-flow per cm^2 absorption area is available.
- Avoidance of first-pass hepatic metabolism is possible.
- Nasal drug delivery is easy and improves patient compliance (Cornaz & Buri, 1994:261).
- For certain drugs, nasal administration achieves plasma levels comparable to those obtained through intravenous drug administration (Chien *et al.*, 1989:1).

- Degradation of drugs in the gastrointestinal tract resulting from acidic or enzymatic degradation can be avoided.
- Rapid absorption and onset of effect are possible.
- Higher bioavailability is possible.
- There is a lower risk of overdoses.
- No complex formulation requirements exist (Arora *et al.*, 2002:968).

1.9 LIMITATIONS OF INTRANASAL DRUG DELIVERY

Nasal drug delivery also has some limitations, which may inhibit drug absorption.

- Rapid mucociliary clearance of the therapeutic agent from the site of deposition resulting in a short contact time available for absorption is possible.
- Low permeability of the nasal membrane for larger macromolecules.
- The presence of proteolytic enzymes that may cause drug degradation in the nasal cavity.
- Limited formulation manipulation for changing drug delivery profiles.
- The possible presence of pathological conditions such as colds or allergies, which may alter nasal bioavailability.
- A relative small area available for drug delivery (Dondeti *et al.*, 1996:115).
- Volume of delivery restricted to 25-200 µl.

- Potential irritation of the nasal mucosa by drugs (Arora *et al.*, 2002:968).

Strategies to overcome these limitations include:

- The use of bioadhesive polymers that increase the residence time of the formulation in the nasal cavity thereby improving absorption.
- The use of non-toxic absorption enhancers to improve the permeability for high molecular weight compounds.
- The use of enzyme inhibitors that prevent degradation of peptide drugs in the nasal cavity (Duchêne & Ponchel, 1993:112).

1.10 CONCLUSION

History and past research provide convincing evidence that the nasal route can be considered to improve the absorption of molecules. The intranasal route has excellent potential for drug delivery with a large surface area and rich blood-flow to maximize drug absorption. With the avoidance of the first-pass hepatic effect and no gastro-intestinal degradation of drugs, the nasal route offers an ideal alternative route for drug administration with possible increased bioavailability of drugs.

CHAPTER 2

PHEROID TECHNOLOGY AND *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC) AS POSSIBLE DELIVERY SYSTEMS FOR CALCITONIN

2.1 INTRODUCTION

Most peptide and protein drugs have low mucosal permeability due to inherent characteristics such as size, polarity, instability and susceptibility to enzymatic degradation (Lee *et al.*, 1991:92). The oral route is mostly unsuitable for peptide drug administration, due to digestion and inactivation in the gastrointestinal tract and first-pass metabolism in the liver (Pontiroli, 1998:81). In chapter 1 it was mentioned that the nasal route of drug administration would be an ideal delivery route for peptides and hormones. It is convenient and assures good patient compliance (Pontiroli, 1998:81). Salmon calcitonin (sCT), used for osteoporosis, has poor bioavailability after oral administration and the development of delivery systems with absorption enhancing properties would greatly improve its effectiveness, especially after nasal administration (Pontiroli, 1998:84).

2.2 CALCITONIN

2.2.1 BACKGROUND AND CLASSIFICATION

In the early 1960's, Copp and colleagues described the 32-aminoacid peptide, calcitonin, as a hormone from the parathyroids, which regulates calcium concentrations in body fluids and soon after that the association was made between the lowering in serum calcium and its inhibition of osteoclast activity. Calcitonin's potential as a treatment for osteoporosis was quickly realised (Colman *et al.*, 2002:885). This hypocalcemic hormone with effects, opposite to those of the parathyroid hormone (PTH), was named calcitonin by Copp in 1962 (Marcus, 2001:1732).

In the early 1970's, reports of calcitonin's bone sparing effect were published and by the late 1970's industry-sponsored studies were completed. A new drug application was submitted to the FDA to approve injectable calcitonin (Calcimar®) but without success due to incomplete clinical data. In 1981 the data was once again presented to the FDA's Endocrinologic and Metabolic Drug Advisory Committee and after the discussions, Calcimar® was approved for treatment of postmenopausal osteoporosis contingent on a phase-4 study (Colman *et al.*, 2002:885). The phase-4 fracture study took place in the next 3 years but failed due to unreliable results and the use of Calcimar® has since decreased. However, in the 1990's, a nasally administered calcitonin (Miacalcin® nasal spray, Novartis, East Hannock, NJ, USA) was registered for treatment of postmenopausal osteoporosis (Colman *et al.*, 2002:885).

Calcitonin, as part of the antiparathyriod hormone system, is a natural polypeptide hormone that regulates calcium concentrations in plasma together with parathyroid hormones. Calcitonin is found in three therapeutic forms such as porcine calcitonin which is the most immunogenic and not suitable for long-term use, human calcitonin which is less potent although less

immunogenic and synthetic salmon calcitonin suitable for use in long term treatment (Gibbon, 2000:239).

Calcitonin is produced in mammals, including humans and is secreted by the parafollicular or C cells of the thyroid gland. These cells are of neural crest origin. During embryonic development they migrate to be localized mainly in the thyroid gland in mammals, whereas in sub-mammalian vertebrates these cells remain as a separate gland, termed the ultimo-bronchial body (MacIntyre & Girgis, 2002:1).

2.2.2 CHEMISTRY

The molecular formula for human calcitonin is $C_{151}H_{226}N_{40}O_{45}S_3$ with a molecular weight of 2777.9 while salmon calcitonin has a molecular formula of $C_{145}H_{240}N_{44}O_{48}S_2$ (Lunn, 2000:948).

Calcitonin is a single chain peptide consisting of 32 amino acid residues with one disulfide bond at the cysteine residues at position 1 and 7 (figure 2.1). Together with the carboxy-terminal polinamide and five additional residues, these two cysteine residues are the only amino acids conserved among the calcitonins from various species. The complete sequence of 32 amino acids and the C-terminal proline amide are essential for full biological activity (MacIntyre & Girgis, 2002:3).



Figure 2.1 Amino acid sequence of human calcitonin with the disulfide bond (MacIntyre & Girgis, 2002:3).

2.2.3 PHARMACOKINETICS

The duration of action varies between 30 minutes and 12 hours after IV administration and after subcutaneous or IM injection, effects last for 8-24 hours (Gibbon, 2000:239). A decrease in bone resorption, after treatment with 200 IU of salmon calcitonin, was reported to be 60 % within 1 hour with a reduced risk of vertebral compression fractures (Stepan & Zikan, 2003:51).

2.2.3.1 DOSAGE AND ADMINISTRATION

The adult dose for hypercalcaemia is 5-10 IU/kg/day in 2-4 divided doses for slow IV injection or as a single dose IV infusion diluted in 500 mL normal saline over 6 hours. The dose for Paget's disease is 100 IU subcutaneous or IM daily or every second day with a maintenance dose of 50-100 IU three times a day. For osteoporotic vertebral fracture, a daily dose of 100 IU for 2 weeks is administered subcutaneously (Gibbon, 2000:239).

The highly purified synthetic salmon calcitonin preparations have high potency and superior analgesic effects and are available in several dosage forms including injections, a nasal spray or suppositories. The potency is assigned in international units (IU), defined by the International Standard for sCT established by the World Health Organization (WHO) (Rafferty *et al.*, 2001:85).

2.2.3.2 METABOLISM AND ELIMINATION

Calcitonin is rapidly metabolized in the kidneys, blood and peripheral tissues (Gibbon, 2000: 239).

2.2.4 BIOSYNTHESIS, SECRETION AND REGULATION OF SECRETION

The concentration of Ca^{2+} in plasma regulates the biosynthesis and secretion of calcitonin. When Ca^{2+} levels are high, calcitonin secretion increases and when the Ca^{2+} levels are low, the calcitonin secretion is low. The normal circulating calcitonin concentrations in women are lower than in men and the circulating half-life time for calcitonin is 10 min (Marcus, 2001:1733).

Calcitonin is produced as a precursor and then processed by cleavage and amidation before secretion. The human calcitonin precursor is a 141–amino acid peptide in which calcitonin is flanked by two peptides, C-terminal 21–amino acid peptide, called katacalcin, and an amino terminal 82–amino acid peptide. Calcitonin and katacalcin are both secreted from the same substance. The calcitonin gene is located on the short arm of chromosome 11. It is a simple gene and consists of six exons. It codes for two precursors, one for calcitonin and the other for a related peptide, calcitonin gene-related peptide (CGRP). The messenger RNA's for these two precursors are produced, in a tissue-specific manner, by differential processing of a single primary nuclear transcript. Calcitonin is expressed mainly in the thyroid gland, whereas CGRP is produced predominantly in the neural tissues (MacIntyre & Girgis, 2002:1).

A number of agents including catecholamines, glucagons, gastrin and cholecystokinin, are responsible for the stimulation of calcitonin (Marcus, 2001:1733).

2.2.5 BIOLOGICAL AND PHYSIOLOGICAL EFFECTS

Calcitonin has a variety of effects with the most noticeable effect on bone. Studies have shown that there is a remarkable fall in plasma calcium due to

the acute inhibition of osteoclastic activity as a direct result of calcitonin as well as a decrease of the flow of calcium to the bone. The two main actions of calcitonin on the modulation of osteoclast activity is firstly a direct inhibition of the resorptive activity of mature osteoclasts (figure 2.2) and secondly a progressive reduction in osteoclast numbers because of inhibition of osteoclast recruitment (MacIntyre & Girgis, 2002:2).

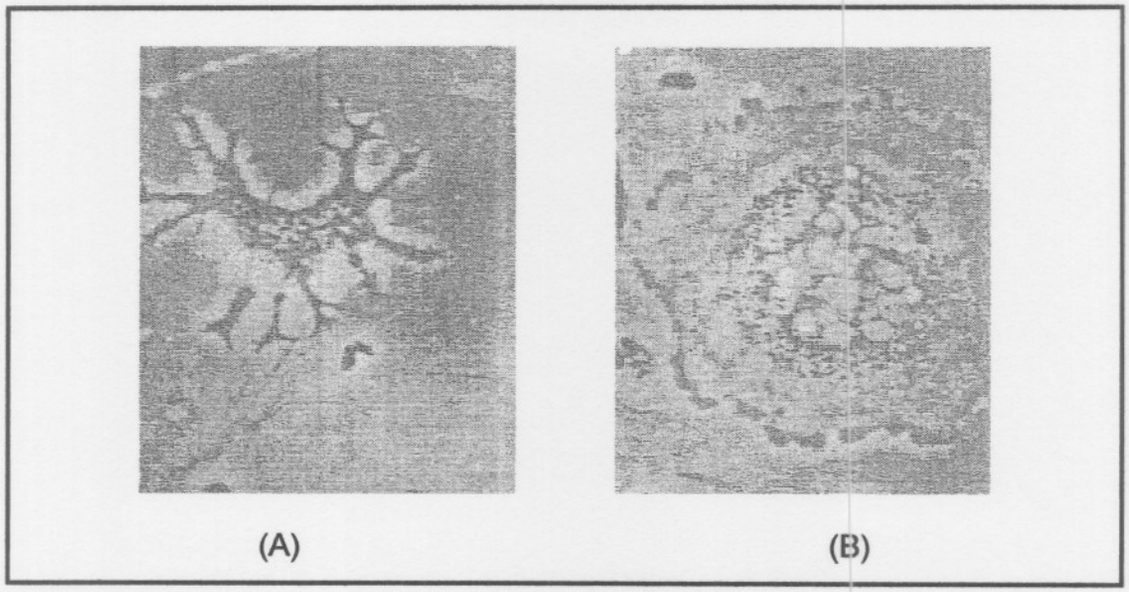


Figure 2.2 (A) An osteoclast after two hours in control medium showing lobulated periphery and (B) motility inhibited with physiological concentrations of calcitonin (MacIntyre & Girgis, 2002:2).

The physiological role of calcitonin is not yet clear but the hypothesis is that calcitonin has the major role of protecting the skeleton during calcium stress like growth, pregnancy and lactation (MacIntyre & Girgis, 2002:3).

2.2.6 PHARMACOLOGY

2.2.6.1 INDICATION

Calcitonin is used for treatment of Paget's disease associated with bone mineral loss such as hyperparathyroidism, malignancies and immobilisation and more specifically relieves pain in severe cases (Gibbon, 2000:239). Salmon calcitonin is indicated for treatment of some forms of hypercalcemia and osteoporosis in women who are more than five years past menopause and for whom oestrogen replacement therapy is not an option (Raffery *et al.*, 2001:84).

2.2.6.2 MECHANISM OF ACTION

Calcitonin acts by binding to a G-protein-coupled cell surface receptor of the PTH-secretin receptor family. Binding of calcitonin to its receptors results in the activation of either adenylate cyclase or phospholipase C. Several forms of the calcitonin receptors (CTR) have been described. These forms arise from alternative splicing of mRNA transcribed from a single gene. The gene for the human CTR is localized on chromosome 7. The CTR is robustly expressed in osteoclasts, the kidneys, and the brain. In the latter, the highest calcitonin-receptor densities are in the median eminence of the hypothalamus, which is believed to be involved with pain, perception and appetite regulation (MacIntyre & Girgis, 2002:2). Decrease in bone resorption by salmon calcitonin depends on binding on the hormone receptors on the basolateral membrane of osteoclasts (Stepan & Zikan, 2003:50).

2.2.6.3 BIOAVAILIBILITY

Intramuscular preparations show relative bioavailability of salmon calcitonin to be only 1.6 %, which is poor and various enhancers have been investigated to enhance nasal absorption efficacy without success due to nasal membrane damages (Sinswat & Tengamnuay, 2003:16). Lipophilic drugs are generally well absorbed from the nasal cavity and plasma levels are often typical to those obtained after an intravenous injection with bioavailabilities approaching 100 %. However, despite the large surface area and extensive blood supply of the nasal cavity, the permeability of the nasal mucosa is normally low for polar molecules and large molecular weight molecules, especially peptides and proteins (Illum, 2003:189).

2.3 PHEROID TECHNOLOGY AS A DRUG DELIVERY SYSTEM

2.3.1 THE PHEROID SYSTEM

Pheroid technology (previously Emzaloid™ technology) (further referred to as Pheroid or Pheroids) 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 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 (Saunders *et al.*, 1999:99).

2.3.2 PHEROID TYPES, CHARACTERISTICS AND FUNCTIONS

There are many barriers to drug delivery. Pheroids entrap drugs and deliver them to target sites in the body. The Pheroid penetrates keratinised tissue, skin, intestinal lining, nasal epithelium, the vascular system, fungi, bacteria and parasites. Figure 2.3 shows confocal laser scanning micrographs of various formulations of the Pheroid delivery system (Grobler, 2004:4).

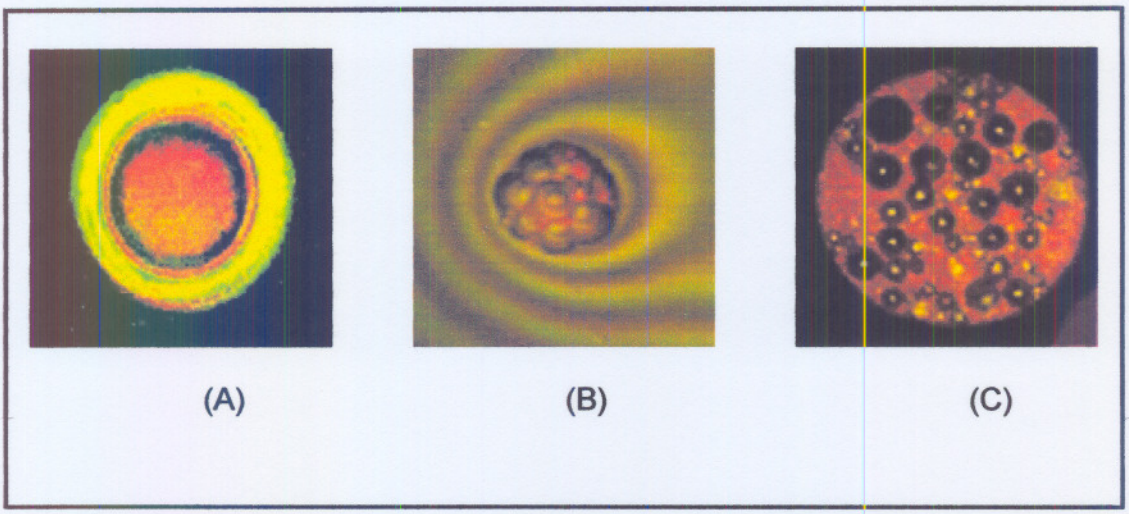


Figure 2.3 Confocal laser scanning micrographs of some of the basic Pheroid types. (A) A bilayer membrane vesicle containing Rifampicin. (B) The formation of small pro-Pheroids that are used in oral drug delivery. (C) A reservoir that contains multiple particles of coaltar (Grobler, 2004:5).

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, delivery and stability. The absorption capabilities and drug release characteristics of the Pheroid can thus be controlled. The entrapment of drugs within the Pheroid generally

creates a safer, more effective formulation than a formulation containing only the drug (Grobler, 2004:4).

Furthermore, the Pheroid contains essential fatty acids that are necessary for many cell functions, but cannot be manufactured by human cells and therefore have to be ingested. It was shown that the western diet lacks these essential fatty acids. 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 leukotrins and prostaglandins and some regulatory aspects of programmed cell death. The Pheroid system therefore has inherent therapeutic qualities that afford it significant advantages over some delivery systems (Grobler, 2004:4).

2.3.3 THE PHEROID VERSUS OTHER LIPID BASED DELIVERY SYSTEMS

The fundamentals of the Pheroid system show that the system differs substantially from conventional macromolecular carriers, such as liposomal delivery systems. The following table provides a comparison of the differences and key advantages of the Pheroid and other lipid-based delivery systems.

Table 2.1 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 the 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 metabolised in the cell membrane, it is difficult to envisage how subcellular organelles can be targeted by this approach.
The Pheroid is polyphonic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Although the Pheroid contains no cholesterol, the interior volume remains stable.	Most lipid-based delivery systems contain phospholipids and cholesterol to stabilise the interior of the vesicles.
The Pheroid 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 those of the essential fatty acids, resulting in loss of elasticity.
Due to the Pheroid's 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 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.
Batch-to-batch reproducibility and stability have been proven with existing products containing Pheroid, such as the registered product Exorex®.	Large scale manufacturing of other liposomal delivery systems sometimes show low batch-to-batch reproducibility as well as problems with size control.

2.3.4 PHARMACEUTICALLY APPLICABLE FEATURES OF THE PHEROID SYSTEM

2.3.4.1 DECREASED TIME TO ONSET OF ACTION

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

2.3.4.2 INCREASED DELIVERY OF ACTIVE COMPOUNDS

Both *in vitro* and *in vivo* studies have shown that by using the 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).

2.3.4.3 REDUCTION OF MINIMUM DRUG CONCENTRATION

Research conducted with the Pheroid system has shown that, for certain active compounds, using as little as $1/40^{th}$ 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 costs (Grobler, 2004:10).

2.3.4.4 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).

2.3.4.5 REDUCTION IN CYTOTOXICITY

Side effects of drugs are in most instances 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. Therefore, by incorporating the drug in the Pheroid system, the appearance of side effects can be dramatically reduced (Grobler, 2004:11).

2.3.4.6 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).

2.3.4.7 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).

2.3.4.8 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).

2.3.4.9 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 TB 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).

2.3.5 THERAPEUTIC AND PREVENTATIVE USES OF PHEROID TECHNOLOGY

2.3.5.1 THERAPY OF TUBERCULOSIS

"Tuberculosis kills 2 million people each year. Overall, one-third of the world's population is currently infected with the TB bacillus" (World Health Organisation). 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).

A previous study proved that the use of the Pheroid, after administration in a pro-Pheroid formulation, could increase the availability of the antimicrobials in human plasma, enhance the absorption of the formulated antimicrobials from the gastrointestinal tract without an increase in toxicity, increase the intracellular concentration of the antimicrobial in the target cells where the tuberculosis bacteria breed, extend the circulatory time of the active drugs, increase the bactericidal effect of the antimicrobials inside the target cells as well as decrease the side effects caused by the antimicrobials (Grobler, 2004:14).

The results of the bio-equivalence study, showed that:

- 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.
- The entrapment of the antimicrobials led to an increased rate of absorption and cellular response.
- 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.

- 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.
- A lower dosage can therefore be used to obtain similar effects.
- 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).

2.3.5.2 PREVENTATIVE THERAPIES: VACCINES

Historically, vaccination is the only strategy that has led to the elimination of a viral disease, namely 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).

2.3.5.2.1 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 ninefold increase in antibody response when compared to the other formulations (Grobler, 2004:18).

2.3.5.2.2 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).

2.3.5.2.3 PHEROID TECHNOLOGY FOR NASAL VACCINE DELIVERY

The hypothesis for the nasal delivery of vaccines using the Pheroid as delivery system is based on the same principle as 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 centres 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.

2.4 *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC) AS AN ABSORPTION ENHANCER FOR PEPTIDE DRUGS

N-trimethyl chitosan chloride (TMC) is a partially quaternised derivative of chitosan with enhanced water solubility and superior drug absorption enhancing properties, especially in neutral and alkaline environments where chitosan is insoluble (Kotzé *et al.*, 1999b:254). It displays muco-adhesive characteristics and decreased intrinsic viscosity, and reduces transepithelial electrical resistance (TEER) across Caco-2 cell-layers (Kotzé *et al.*, 1997a:1199), which contributes to its activity as a drug absorption enhancer.

2.4.1 SYNTHESIS OF TMC

TMC is synthesized from a sieved fraction (< 500 µm) of chitosan, based on the method of Domard *et al.*, (1986:105) as shown in figure 2.4. Briefly, the experimental conditions are reductive methylation of chitosan for 60 minutes with iodomethane in a strong basic environment at 60 °C. The counterion (I^-) is exchanged for Cl^- by dissolving the quaternised polymers in a sodium chloride solution (Kotzé *et al.*, 1997a:1197). Different TMC polymer with varying degrees of quaternisation can be prepared by repeating the reaction step for the reductive methylation of chitosan several times under the same conditions, with the polymer obtained from each reaction step (Hamman & Kotzé, 2001:374). The degree of quaternisation is increased significantly with an increase in the number of reaction steps (Hamman & Kotzé, 2001:379).

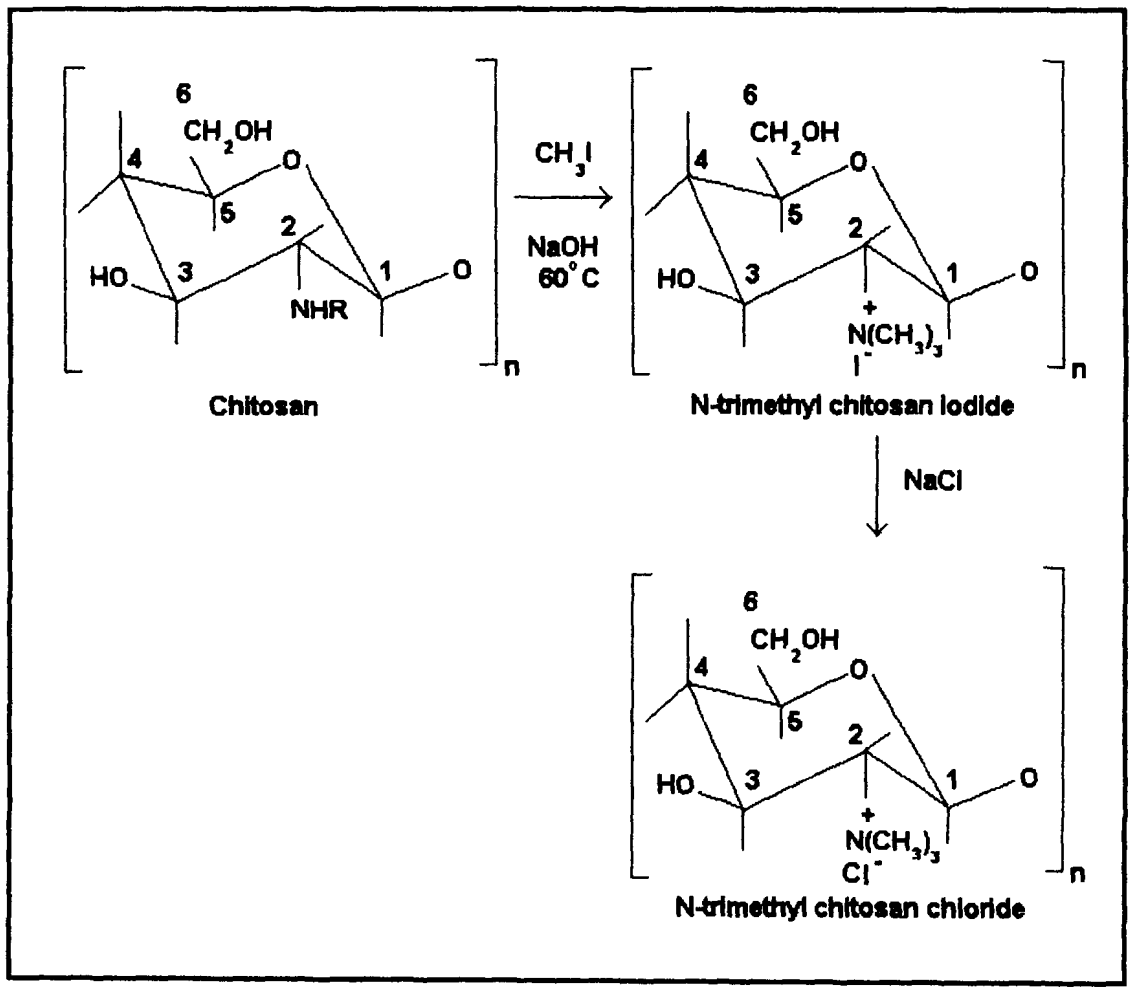


Figure 2.4 Synthesis of *N*-trimethyl chitosan chloride from chitosan by reductive methylation.

2.4.2 PHYSICOCHEMICAL PROPERTIES OF TMC

TMC displayed highly increased water solubility compared to chitosan and chitosan salts, especially at neutral and basic pH values (Kotzé *et al.*, 1997b:244). In general TMC with a degree of quaternisation as low as 10% is soluble in water (Kotzé *et al.*, 1998a:39). The pK_a values of TMC polymers with degrees of quaternisation ranging from 10.37 to 13.06 % were 5.9 to 6.0 respectively (Kotzé *et al.*, 1997a:1199).

TMC has a markedly decreased intrinsic viscosity, compared with chitosan, which indicates polymer degradation under reaction conditions in an alkaline medium. During the synthesis of TMC, the number of positive charges on the polymer chain is increased, causing the molecule to expand in solution due to repelling forces between the functional groups (Snyman *et al.,* 2002:145). TMC solutions of 10 % can be prepared in either acidic or basic mediums. Thus TMC is a derivative of chitosan with superior solubility and basicity, even at low degrees of quaternisation. The increase in solubility and basicity could be attributed to the replacement of the primary amino group on the C-2 position of chitosan with methylated quaternary amino groups (Kotzé *et al.*, 1998a:39). According to Kotzé *et al.* (1999a:273) the degree of quaternisation of TMC plays an important role in its absorption enhancing activity, especially in neutral environments.

2.4.3 EFFECT OF TMC ON THE TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER) OF INTESTINAL EPITHELIAL CELLS (CACO-2 CELL MONOLAYERS)

The measurement of TEER is believed to be a good indication of the tightness of the tight junction between cells and has been used in previous studies to predict the paracellular transport of hydrophilic compounds (Kotzé *et al.*, 1998b:149). The effect of TMC on the TEER of Caco-2 monolayers has been investigated by Kotzé *et al.* (1997a:1199) and Hamman *et al.* (2000:37). Incubation with TMC (degree of quaternisation 12 %) in concentrations of 1.5 % to 2.5 % (w/v) resulted in a pronounced and immediate reduction in TEER values in a concentration dependant way compared to the control group. In a concentration of 1 % and less, no significant reduction in the resistance of the cells was measured (Kotzé *et al.*, 1997a:1199).

In another study five TMC polymers with different degrees of quaternisation (12- 59 %) were tested to investigate the reduction in TEER of Caco-2 monolayers at a pH of 7.4. The results clearly indicated that the degree of TMC quaternisation plays an important role in the ability of this polymer to

reduce the TEER value. TMC-59 was the most effective in reducing the TEER, followed by TMC polymers with lower degrees of quaternisation. No reduction in the TEER values was observed for TMC with degrees of quaternisation of 22 % and lower (Hamman *et al.*, 2000:37). Thus higher degrees of TMC quaternisation showed to be more effective in reducing the TEER of the Caco-2 monolayers in neutral environments than lower degrees of quaternisation. These results can be explained by the amount of positive charges on the TMC molecule available for interaction with the negative sites on the cell surface. TMC polymers with higher degrees of quaternisation have more quaternary amino groups than those with lower degrees of quaternisation, and are therefore more effective in lowering the TEER values (Hamman *et al.*, 2000:37). An increase in the transport of peptide drugs across Caco-2 monolayers has also been found to be in good agreement with a decrease in the TEER values (Kotzé *et al.*, 1997b:250).

2.4.4 MUCOADHESIVE PROPERTIES OF TMC

Non-invasive drug delivery requires the administration of the drug delivery system to an epithelium that is a suitable site for absorption of the active compound. The use of mucoadhesive polymers as drug carriers has been investigated to control and/or increase the residence time at these absorption sites. The mucoadhesive properties of the dosage form seem to have an important influence on bioavailability, especially for peroral peptide delivery systems, from which the therapeutic agent is released in the intestines. If the delivery system is not adhesive, the released peptide drug will be degraded by luminally secreted proteases before reaching the absorption membrane. Such presystemic metabolism can be strongly reduced by mucoadhesive formulations providing an intimate contact with the intestinal mucosa. Additionally, a somewhat prolonged intestinal transit time and a higher concentration gradient should be provided by mucoadhesive delivery systems (Bernkop-Schnürch, 2000:8).

Negatively charged groups and hydrogen-bonding capabilities are common to presently known mucoadhesives. Positively charged polymers such as chitosan and TMC could possibly develop additional molecular attraction forces by electrostatic interaction with the negatively charged mucosal surfaces (Bernkop-Schnürch, 2000:8). The mucoadhesive properties of chitosan and TMC increase the contact time of the active ingredient in a formulation with the absorptive epithelium, and thus enhance its absorption via the paracellular transport pathway through the tight junctions (Snyman *et al.*, 2002:145).

2.4.5 EFFECT OF TMC ON THE ABSORPTION OF HYDROPHYLIC MODEL COMPOUNDS AND PEPTIDE DRUGS

The bioavailability of peptide and protein drugs are mostly incomplete due to resistance of the mucosal membrane to their penetration and additional factors such as enzymatic degradation by luminal and brush border enzymes. Apart from these factors, the size and polarity of peptide drugs limit their diffusion across biological membranes. Absorption of these compounds is limited to the alternative paracellular pathway that is primarily, restricted by the tight junctions (Lee *et al.*, 1991:182).

The effect of TMC (degree of quaternisation 12 %) on the cumulative transport of the hydrophilic and peptide compounds [^{14}C]-mannitol, FD-4 and buserelin across Caco-2 monolayers was investigated by Kotzé *et al.* (1997a:1197). Incubation with TMC solutions of 1.0 % to 2.5 % (w/v) resulted in a marked accumulation of all the compounds in the acceptor compartment. The permeability decreased with an increase in molecular weight of the compounds, with [^{14}C]-mannitol, having the lowest molecular weight, exhibiting the highest permeability, and FD-4 exhibiting the lowest permeability. This suggests that the permeation of these compounds across intestinal epithelial cells is, among other factors, dependent on molecular size.

In general, an increase in TMC concentration resulted in an increase in transport and permeability, with 2.5 % (w/v) concentrations of TMC showing the greatest effect (Kotzé *et al.*, 1997a:1200).

The effect of TMC on the absorption of insulin across Caco-2 cell monolayers was also investigated by Kotzé *et al.* (1997b:249). No transport in the control group could be detected up to 4 hours. Incubation for 4 h with TMC (1.5 % and 2.5 % (w/v)) increased the insulin transport, with the 2.5 % concentration exhibiting the greatest increase in transport at neutral and basic pH values. They showed that TMC with a degree of quaternisation of 61 % was a very potent absorption enhancer of the hydrophilic marker [^{14}C]-mannitol at neutral pH (7.4) where TMC with a 12 % degree of quaternisation was ineffective as absorption enhancer. The latter increased the transport of [^{14}C]-mannitol at a pH of 6.2. *In vivo* studies in rats showed similar results (Kotzé *et al.*, 1999b:256).

2.4.6 PROPOSED MECHANISM OF ACTION OF TMC

Tight junctions play a crucial part in maintaining the selective barrier function of cell membranes and in sealing cells together to form a continuous cell layer, through which even small molecules cannot penetrate. Furthermore, the paracellular route occupies only a very small surface area compared to the transcellular route. However, tight junctions are permeable to water, electrolytes and other charged or uncharged molecules up to a certain size. Tight junctions are known to respond to changes in calcium concentration, c-AMP, osmolarity, pH and the status of the cytoskeleton (Cereijido *et al.*, 1993:18).

Chitosan and TMC polymers have been found to enhance absorption across intestinal epithelia by opening the tight junctions. Because of their positive charge, cationic macromolecules such as chitosan and TMC can interact with the anionic components of the glycoproteins on the surface of epithelial cells. Cationic macromolecules can displace cations from electronegative sites on cell membranes, thereby disrupting their dimensional stability. It is also

known that the interior of the tight junction channel is hydrated and contains fixed negative sites. Changes in the concentration of certain ions in the pore could result in alteration in tight junction resistance, leading to opening of the pore with increased paracellular permeability. It has been reported that chitosan is able to induce redistribution in cytoskeletal F-actin, thereby resulting in a structural reorganization of tight junction-associated proteins such as ZO-1. TMC probably acts in a similar way to open the tight junctions (Artursson *et al.*, 1994:1359).

It has been proposed that chitosan salts open the tight junctions in a reversible way. According to Kotzé *et al.* (1997a:1199) and Thanou *et al.* (2000:23) a slight increase in the TEER values of Caco-2 cell monolayers was found towards initial values after removal of the TMC polymer solution, indicating reversibility of their effect.

Confocal laser scanning microscopy was used to visualise the transport pathway across the cell monolayers (Kotzé *et al.*, 1998a:44). Optical cross-sections of cell monolayers, after incubation with a control solution containing only FD-4, showed no evidence of any intercellular or intracellular fluorescence. Fluorescence was only detected on top of the monolayers. After 60 min of incubation with 0.5 % concentrations of chitosan and TMC polymers at pH 6.2, fluorescence was detected in the intercellular spaces as evident from vertical scans (XZ images) through the monolayers. Similar results were obtained in horizontal (XY) images. These confocal images clearly showed that the tight junctions were open and that FD-4 was able to permeate into the paracellular spaces. No fluorescence could be found within the cells, which indicated that incubation with any polymer did not damage the cell membranes (Kotzé *et al.*, 1998a:44).

2.4.7 TMC TOXICITY STUDIES

Various studies on the effect of TMC on Caco-2 cell monolayers have investigated their effect on the viability of the cells. Kotzé *et al.* (1997b:246) tested the viability of Caco-2 cell monolayers after completion of their transport experiments by incubating the cell monolayers apically with a solution of 0.1 % trypan blue in PBS (0,01 M phosphate-buffered solution, pH 7.4) for 30 minutes, while the basolateral medium was PBS. The medium was then removed from both sides, and the cell monolayers examined by light microscopy for exclusion of the marker. Cells excluding trypan blue were considered to be viable. There was no visible intracellular uptake of trypan blue after prolonged incubation with TMC polymers, implying that the Caco-2 cell monolayers remained undamaged and functionally intact and viability was not affected (Kotzé *et al.*, 1997b:246).

Similar tests were performed by Kotzé *et al.* (1997a:1199) in a separate study on the effect of TMC on the TEER of Caco-2 cell monolayers and by Kotzé *et al.* (1999a:271) after testing the effect of TMC polymers with different degrees of quaternisation on the permeability of Coca-2 cell monolayers. In both studies there was no visible uptake of trypan blue, indicating that the viability of the cell monolayers was not affected by incubation with TMC polymers with degrees of quaternisation ranging from 12.6 % to 19.9 % (Kotzé *et al.*, 1999a:273).

Thanou *et al.* (2000:18) tested the viability of Caco-2 cell monolayers after incubation with TMC polymers with degrees of quaternisation of 40 % and 60 %, by testing trypan blue exclusion as well as propidium iodide exclusion. At the end of their experiment, all monolayers excluded the trypan blue staining, indicating the retention of cell viability. The results of the propidium iodide test showed that the toxicity found on the Caco-2 cell monolayers was negligible, even at relatively high TMC concentrations (1.0 % w/v). These findings indicted that the absorption enhancing effects of TMC were not due to possible cytotoxic activities (Thanou *et al.*, 2000:23). The effect of TMC on the ciliary beat frequency of epithelia has also been tested to investigate local

toxicity. Thanou *et al.* (1999:82) showed that TMC solutions of 1 % (w/v) had minimal influence on ciliary beat frequency. It has also been shown that low molecular weight TMC polymers (oligomers) inhibited ciliary beat frequency to a lesser extent than high molecular weight TMC (Jordaan, 2001:79).

2.4.8 EFFECT OF THE DEGREE OF QUATERNISATION OF TMC ON ABSORPTION ENHANCEMENT

The degree of quaternisation of TMC plays an important role in determining the ability and effectiveness of TMC to open the tight junctions and regulate permeation through the paracellular transport pathway. The degree of quaternisation determines the amount and density of the positive charges on the C-2 position of this chitosan derivative (Kotzé *et al.*, 1999a:274). Highly quaternised TMC molecules have shown to be more effective than those with lower degrees of quaternisation in decreasing the TEER of Caco-2 cell monolayers, as well as increasing the permeation of hydrophilic drugs (Kotzé *et al.*, 1999b:256).

TMC polymers with degrees of quaternisation of 12.3 % to 61.2 % have been tested for enhancement of the absorption of [^{14}C]-mannitol across Caco-2 cell monolayers (Kotzé *et al.*, 1999b:255). At pH 7.2 only highly quaternised polymers enhanced absorption. It was proposed that at low degrees of quaternisation, the charge density had not reached the threshold concentration to induce interaction with the anionic components of the glycoproteins at the surface of the cells or with the fixed negative charges within the aqueous tight junctions. Additionally, the attached methyl groups may partially shield the positive charge from significant interaction with the cell membranes or tight junctions. Highly quaternised TMC, on the other hand, has a much higher proportion of quaternary amino groups that seems to be sufficient to interact with anionic components of the cell membranes or the negative sites within the tight junctions (Kotzé *et al.*, 1999b:256).

The enhancement of absorption of [^{14}C]-mannitol across Caco-2 monolayers, as well as in rat nasal epithelia by TMC, increased with an increase in degree of quaternisation (Thanou *et al.*, 2000:22; Hamman *et al.*, 2002:240), confirming that TMC polymers with higher degrees of quaternisation enhance paracellular absorption more effectively. The absorption enhancing effect seemed to reach a maximum value at a 48 % degree of quaternisation. Hamman *et al.* (2002:241) proposed that this was due to steric effects caused by the methyl groups and changes in the flexibility of the TMC molecules with an increase in the degree of quaternisation above an optimum value for absorption enhancement.

2.4.9 EFFECT OF THE MOLECULAR WEIGHT OF TMC ON ITS ABSORPTION ENHANCING PROPERTIES

Chitosan with low and high molecular weights, with varying degrees of deacetylation, have been investigated for their effect on epithelial permeability, using intestinal Caco-2 cell monolayers. It has been found that the molecular weight and degree of deacetylation of chitosan dictate the absorption enhancing properties and toxicity to a large extent (Schipper *et al.*, 1996:1689). Chitosans with a low molecular weight (22 000 g/mole) and low degree of deacetylation lacked absorption enhancing activity, whereas chitosan with a high molecular weight (98 000 g/mole to 190 000 g/mole) and/or high degree of deacetylation increased epithelial permeability. It was concluded that a high molecular weight and/or high degree of deacetylation are required for chitosan to increase epithelial permeability (Schipper *et al.*, 1996:1691).

TMC polymers with high and low molecular weights have also been tested for reduction in TEER values as well as transport of [^{14}C]-mannitol across Caco-2 cell monolayers. It was shown that both the high and the low molecular weight polymers reduced the TEER across Caco-2 cell monolayers (Swartz, 2002:69). It was however only the high molecular weight TMC that showed

an increase in transport of [^{14}C]-mannitol across the Caco-2 cell monolayers (Swartz, 2002:80).

2.5 CONCLUSION

As discussed in this chapter calcitonin is a suitable drug for further experimental studies due to low bioavailability and the potential of this peptide hormone in the treatment of various diseases. In an attempt to increase the bioavailability of calcitonin, an absorption enhancer (TMC) and a drug delivery vesicle (Pheroid technology) have been described and selected for this study.

The two groups of Pheroids (microsponges and vesicles) have been chosen in consideration of their characteristics based on their absorption ability to entrap, transport and deliver active drugs, as well as their enhancing potential. Research has indicated that the Pheroid technology system carries and delivers active compounds through most physiological barriers with reduced membrane damage and minimal side effects. The advanced absorption enhancing properties of *N*-trimethyl chitosan chloride (TMC) was also discussed.

CHAPTER 3

NASAL DELIVERY OF CALCITONIN WITH PHEROID TECHNOLOGY AND *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC): EXPERIMENTAL DESIGN.

3.1 INTRODUCTION

Nasal drug delivery has been reviewed in chapter 1. It has been concluded that the nasal route of drug delivery showed great potential for delivering various classes of drugs, especially peptide and protein drugs. There are however several permeation barriers which must be overcome for effective drug delivery. The absorption enhancing properties of both Pheroids and TMC have been discussed in chapter 2. Furthermore, the potential improvement of the bioavailability of calcitonin with these absorption enhancers has also been explained in chapter 2.

In this chapter the preparation and characterisation of Pheroid vesicles and Pheroid microsponges are described. The characterisation of the Pheroids will be described with regard to size and morphology.

Calcitonin was entrapped into these formulations and administered nasally in rats. Plasma levels of calcitonin and plasma calcium levels were obtained to evaluate the effect of the entrapment of calcitonin in Pheroids on its nasal permeability. These permeation properties were compared to permeation profiles where calcitonin and TMC were administered together.

3.2 *IN VIVO* STUDIES IN RATS

3.2.1 ROUTE OF ADMINISTRATION

In chapter 1 it was concluded that the nasal route of drug delivery offered advantages concerning the absorption of peptide and protein drugs. This route has been well described in literature and some information about absorption enhancement in this route is available. The nasal route is also easily accessible and for these reasons it was chosen as the route of choice for studying the absorption enhancing properties of Pheroids and TMC when administered with calcitonin. The following sections describe the experimental methods used to study the effect of these compounds.

3.2.2 ANIMALS

Rats were chosen as the *in vivo* model because they are easy to obtain, relatively low in cost to care for than other animals and easy to handle. The use of a specialised surgical procedure, cannulation of the *artery carotis communis*, ensured that sufficient blood volumes from the same rat at different time intervals could be obtained for analysis of drug content. In this study male Sprague Dawley rats with a body weight of approximately 250 g - 350 g were used. The recommendation of the Statistical Consultation Service (North-West University) was that statistical meaningful results could be obtained between different treatments, if a minimum of at least six rats were

used per experiment type performed. Rats were fasted 18 hours prior to nasal drug administration but water was supplied *ad libitum*.

3.2.2.1 BREEDING CONDITIONS

Rats were bred and kept at the Animal Research Centre (North-West University). The rats were kept under artificial conditions to create the ideal environment for the optimum growth and health of the animals. Infection with pathogen organisms was also minimised and variables were kept constant. The conditions at the Animal Research Centre under which the rats were bred and kept are shown in table 3.1.

Table 3.1 Conditions at the Animal Research Centre, North-West University (NWU), Potchefstroom.

Conditions	Recommended value*	Value in Animal Research Centre
Temperature	19 ± 2 °C	21 ± 2 °C
Relative humidity	55 ± 15 °C	55 ± 10 °C
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 by the Animal Research Centre (NWU) according to international standards

3.2.3 SURGICAL PROCEDURES

3.2.3.1 INDUCTION OF ANAESTHESIA

Anaesthesia was provided by halothane (Fluothane®, Zebeca SA (Pty) Ltd, Woodmead, RSA) and lasted for ± 3 hours. For the induction of anaesthesia rats were placed in a glass container with a metal grid floor above a cotton-wool layer containing liquid halothane. Rats were removed from the container at loss of consciousness.

3.2.3.2 MAINTENANCE OF ANAESTHESIA

Two mixtures of halothane (2 % and 4 % respectively) in medical oxygen were used alternatively as needed. An apparatus consisting of two 5-liter plastic bags, containing respectively 0.6 ml and 1.2 ml of halothane in medical oxygen, was used to perform this procedure. Each bag was connected to one end of a three-way valve. A rubber jacket attached to the remaining end of the valve was securely fitted over the head of the rat to supply one of the two halothane mixtures. The CO_2 exhaled by the rat was absorbed by a small amount of sodalime placed in the bags near the connecting valve.

3.2.3.3 CANNULATION OF THE *ARTERY CAROTIS COMMUNIS*

Cannulation of the *artery carotis communis,* to obtain several blood samples from the same animal at different time intervals, is a quick and convenient method, with a low mortality rate (Chandler *et al.,* 1994:1624; Illum *et al.,* 1989:50).

Blood samples were obtained from the rats by carrying out the following procedures (also illustrated in figures 3.1 - 3.8). The rat was placed prostrate

in a supine position. The hair in the area of the ventral neck was shaved and the skin disinfected as shown in figure 3.1.

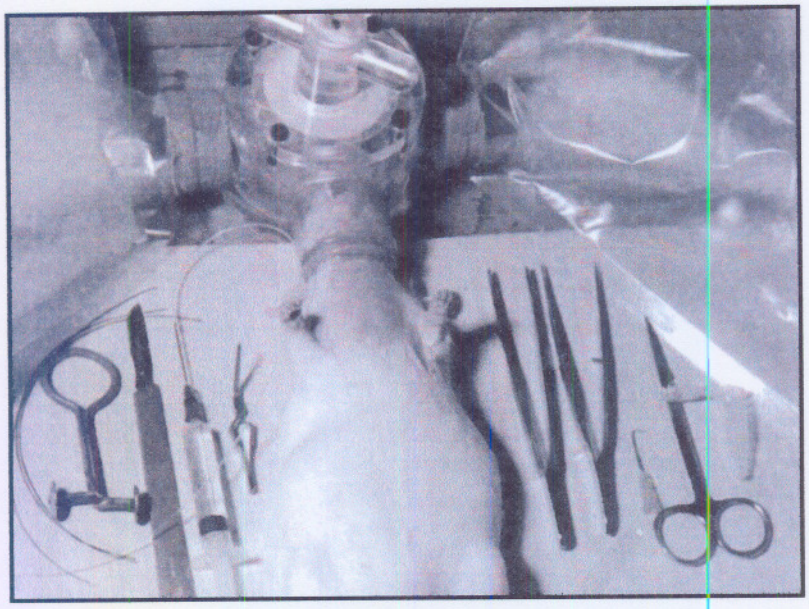


Figure 3.1 The rat was anaesthetised with the halothane mixtures in medical oxygen and placed prostrate in a supine position. The hair in the area of the ventral neck was shaved and the skin disinfected.

A midventral incision of 1 cm was made in the neck skin, illustrated in figure 3.2, and a blunt dissection between the muscles in the neck was made, to expose the *artery carotis communis,* illustrated in figure 3.3. The *artery carotis communis* was lifted out of the wound and kept wet with physiological saline at body temperature (figure 3.4). The rostal part of the artery was ligated 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 loosely pre-placed at the caudal part of the artery. The artery was temporarily clamped with a mosquito artery clamp proximal to the loosely pre-placed ligature, as shown in figure 3.5. A 'V' incision between the two ligatures in the artery wall was cut

with a pair of scissors.

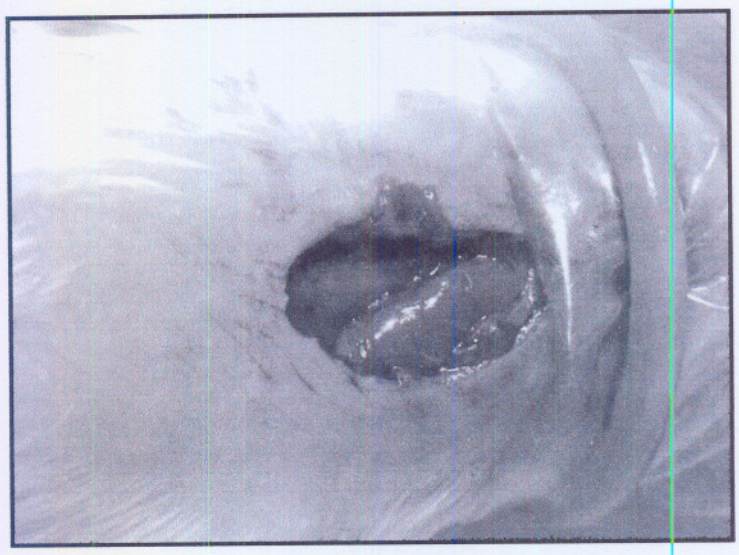


Figure 3.2 A 1 cm midventral incision in the neck skin was made.

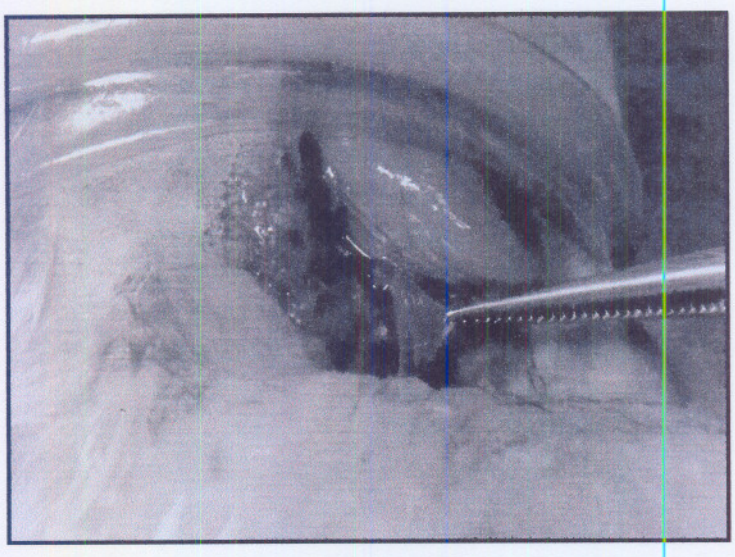


Figure 3.3 The *artery carotis communis* was exposed by a blunt dissection between the muscles in the neck.

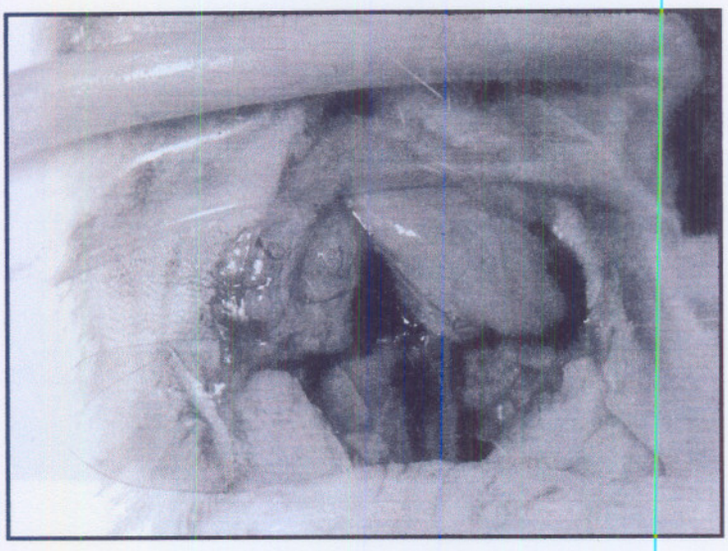


Figure 3.4 The *artery carotis communis* was lifted out of the wound and kept wet with physiological saline at body temperature.

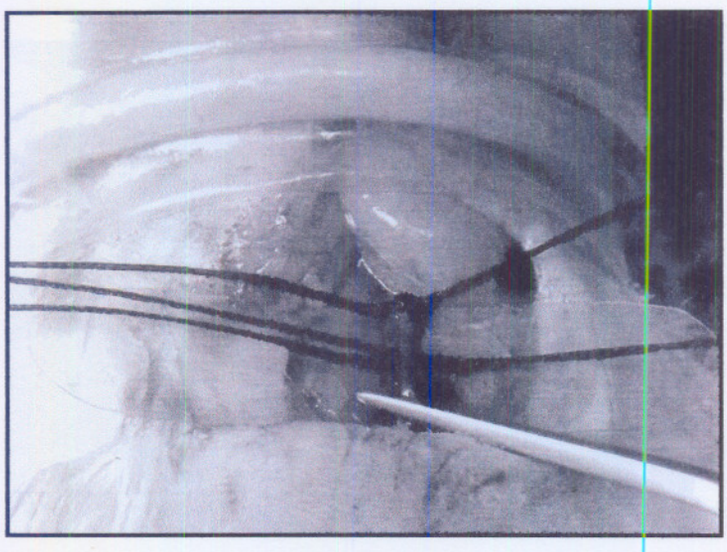


Figure 3.5 The rostal part of the artery was ligated with silk and two other ligatures were loosely pre-placed at the caudal part of the artery. The artery was temporarily clamped with a mosquito artery clamp proximal to the loosely pre-placed ligatures.

A sterile fine bore polythene cannula with an inner diameter of 0.58 mm (Portex Limited, Hythe, Kent, England) filled with sodium heparin (dissolved in sterile saline at a concentration of 10 IU/ml) at 37 °C and connected to a syringe was guided through the 'V' incision in the artery. The clamp was released and the cannula threaded so that 1 cm of the cannula was inserted into the artery. The loose ligature was tied around the artery with the cannula inside, as seen in figure 3.6. Another ligature was tied for safety. The skin was closed with sutures with the cannula penetrating (figure 3.7). The syringe and clamp were removed from the cannula to obtain blood samples as shown in figure 3.8. The integrity of the cannula was maintained with 10 IU/ml heparin in saline. Blood loss was compensated for with saline.



Figure 3.6 A 'V' incision between the two ligatures in the artery wall was cut with a pair of scissors. A sterile cannula filled with saline at body temperature was guided through the 'V' incision in the artery. The clamp was released and the cannula was threaded 1 cm into the artery. The loose ligature was tied around the artery with the cannula inside.



Figure 3.7 The skin was closed with sutures with the cannula penetrating.

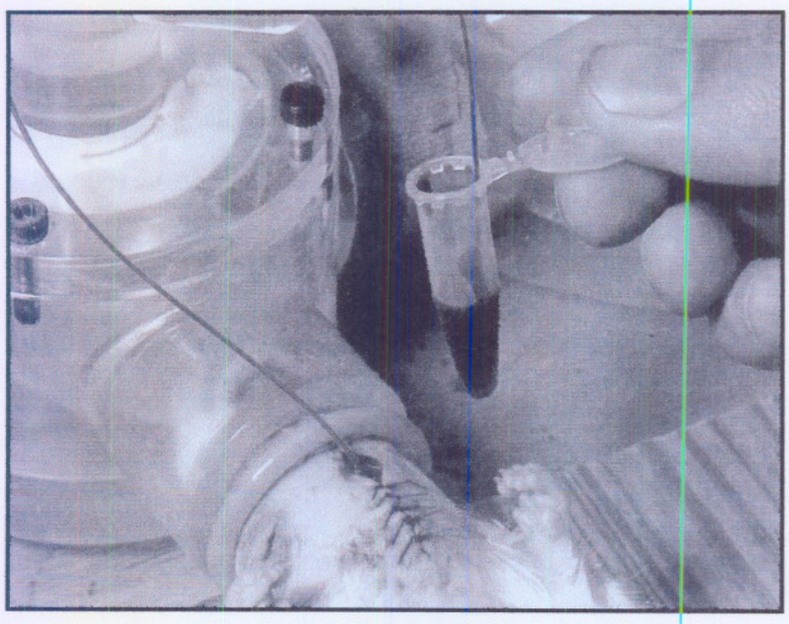


Figure 3.8 Blood samples were obtained by removing the syringe and clamp from the cannula.

3.2.4 NASAL ADMINISTRATION OF CALCITONIN

An Eppendorf® micropipet (10-100 µl) was used for the nasal administration of calcitonin with Pheroids and TMC polymers in solution. These test solutions were administered at a dose of 10 IU/kg body-weight in the right nasal cavities of the rats in a volume of 100 µl/kg body-weight. Care was taken not to induce epithelial injury in administration of the test solutions. Table 3.2 gives a summary of the different nasal formulations administered to the rats.

Table 3.2 Nasal formulations administered to the rats.

Formulation		IU/kg
Control		10
Pheroids	**Vesicles**	10
	Microsponges	10
Quaternized chitosan polymers	**TMC (0.5 % w/v)**	10
	TMO (0.5 % w/v)	10

3.2.5 BLOOD SAMPLING

Blood samples of 1000 µl were taken at 0, 5, 10, 15, 30, 60,120 and 180 minutes after nasal administration of the test solutions. Samples were collected in 1.5 ml Eppendorf® tubes (Merck, RSA) (see figure 3.8), centrifuged at 7000 rpm for 8 minutes and the plasma was kept on ice until further analysis. Blood withdrawn were immediately replaced with an equal volume of heparinised saline at 37 °C. After the completion of the

experiments rats were euthanased with CO_2 gas.

3.2.6 CALCITONIN AND CALCIUM ANALYSIS

A volume of 100 µl of each plasma sample was used for analysis of calcitonin. The plasma immunoreactive calcitonin was quantitated by radioimmunoassay using a commercial kit obtained from Diagnostic Systems Laboratories (Laboratory Specialties, Johannesburg, South Africa). All samples were assayed using the standard preparations and procedures provided by the kit. The radioactivity was detected with a Gammatec™ II gamma counter (A Canberra Company, South Africa). Results were presented as plasma calcitonin concentration (pg/ml) against time.

Plasma calcium levels were determined spectrophotometrically using a calcium assay kit (Roche Diagnostics, Germany). A volume of 50 µl of plasma of each sample was used for analysis. Results were presented as plasma calcium concentration (mmol/l) against time.

3.3 PREPARATION OF PHEROIDS

3.3.1 PHEROID VESICLES

3.3.1.1 MATERIALS

Vitamin F ethyl ester was obtained from Kurt Richer Pharma (Germany). Cremaphor RH-40 was obtained from BASF (Germany) and vitamin E was obtained from Chempure (South Africa). Purified NO_2 water was freshly prepared at the North-West University (South Africa) and amber glass bottles were obtained from Merck (South Africa).

3.3.1.2 METHOD

The Pheroid vesicles were prepared by weighing 2.8 g of vitamin F ethyl ester, 1 g of Cremaphor RH-40 and 0.2 g vitamin E in a closed glass container to create the oil phase of the preparation. This mixture was then melted for approximately 80 seconds in a microwave at 900 watt. 96.0 g of water saturated with nitrous oxide (N_2O) was placed in a glass beaker and the opening of the beaker was closed before heating the N_2O saturated water to ± 75 °C on a hotplate. While the temperature of the N_2O saturated water was maintained at 75 °C, the oil phase was added to the N_2O saturated water to form an emulsion. This preparation was then mixed with a Heidolph Diax 600 homogenizer at 8000 rpm for 2 minutes until homogenous, before being transferred to amber glass bottles. These bottles were shaken until its contents reached room temperature and then it was stored in a refrigerator at a temperature of ± 6 °C.

3.3.1.3 CHARACTERISATION

3.3.1.3.1 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

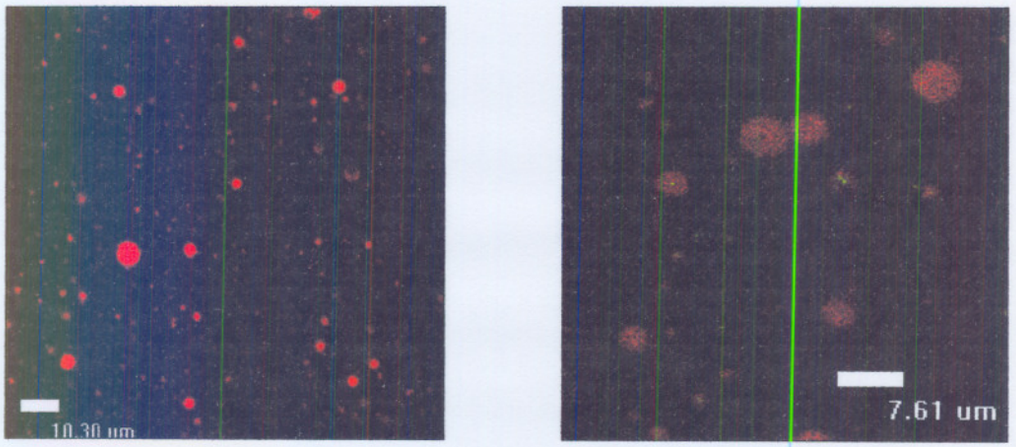
The approximate size and morphology of the Pheroid vesicles were determined with Confocal Laser Scanning Microscope (CLSM) (PCM 2000 CLSM with Nikon digital camera DXM 1200, realtime imaging, Nikon, Holland and He/Ne laser with a 60 x ApoPlanar oil immersion objective, excitation at 505 nm and oil emission at 568 nm). The vesicles were layered on a glass slide, covered with a glass cover-slip and sealed with adhesive to prevent the Pheroids from drying out. Digital images were obtained through capturing by photomultipliers.

3.3.1.3.2 PARTICLE SIZE ANALYSIS

The droplet sizes of the pheroid vesicles were measured with a Malvern Mastersizer (Malvern Instruments, United Kingdom). Milli Q water (800 ml) was used to wash the Malvern Mastersizer for 30 seconds. Fresh water was used for alignment of the laser. The background was measured and then 2 ml of the sample was added for measurement of volume sizes.

3.3.1.4 RESULTS AND DISCUSSION

The morphology of the Pheroid vesicles was analysed with the Confocal Laser Scanning Microscopy (CLSM). As shown in figure 3.9, the Pheroid vesicles are spherical in nature with a homogenous intensity. The studies showed that calcitonin, after entrapment, caused a decrease in the quantity of Pheroid vesicles in the preparation, and an increase in the size of the Pheroid vesicles in the preparation.



(A) (B)

Figure 3.9 Confocal Laser Scanning Micrographs of (A) Pheroid vesicles and (B) Pheroid vesicles entrapped with calcitonin.

In table 3.3 the results of the particle size analysis are given. Confocal Laser Scanning Microscopy (CLSM) was also used to analyse the approximate size of the Pheroid vesicles and the approximate size was found to be between 0.88 μm and 4.90 μm before entrapment of calcitonin and between 2.35 μm and 7.21 μm after calcitonin entrapment (see section 3.4). It was established that the Pheroid vesicles were reasonably consistent in size. The Malvern Mastersizer showed that the particle distribution varied mainly between 0.67 μm to 8.95 μm before entrapment with calcitonin and between 0.72 μm to 8.71 μm after calcitonin entrapment with a median volume diameter of 2.83 μm after calcitonin entrapment.

Table 3.3 The sizes (volume) of Pheroid vesicles before and after the entrapment of calcitonin.

	SIZE (μm)					
	D_1 = 0.1		**D_2 = 0.5**		**D_3 = 0.9**	
	CLSM	**Malvern**	**CLSM**	**Malvern**	**CLSM**	**Malvern**
Pheroid vesicles without calcitonin	0.88	0.67	2.66	3.04	4.90	8.95
Pheroid vesicles with calcitonin	2.35	0.72	4.57	2.83	7.21	8.71

3.3.2 PHEROID MICROSPONGES

3.3.2.1 MATERIALS

Vitamin F ethyl ester was obtained from Kurt Richer Pharma (Germany). Cremaphor RH-40 was obtained from BASF (Germany). Incromega was obtained from Croda Chemicals (South Africa) and vitamin E was obtained from Chempure (South Africa). Purified NO_2 water was freshly prepared at the North-West University (South Africa) and amber glass bottles were obtained from Merck (South Africa).

3.3.2.2 METHOD

The Pheroid microsponges were prepared by weighing 2.8 g of vitamin F ethyl ester, 1 g of Cremaphor RH-40, 0.5 g of Incromega and 0.2 g of vitamin E in a closed glass container. The oil phase was melted for approximately 80 seconds in a microwave at 900 watt. 96.0 g of water saturated with nitrous oxide (N_2O) was placed in a glass beaker and the opening of the beaker was closed before heating the N_2O saturated water to ± 75 °C on a hotplate. While the temperature of the N_2O saturated water was maintained at 75 °C, the oil phase was added to the N_2O saturated water to form an emulsion. This preparation was then mixed with a Heidolph Diax 600 homogenizer at 8000 rpm for 2 minutes until homogenous, before being transferred to amber glass bottles. These bottles were shaken until their contents reached room temperature and then they were stored in a refrigerator at a temperature of ± 6 °C.

3.3.2.3 CHARACTERISATION

3.3.2.3.1 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

The approximate size and morphology of the Pheroid microsponges were determined with a Confocal Laser Scanning Microscope (CLSM) (PCM 2000 CLSM with Nikon digital camera DXM 1200, realtime imaging, Nikon, Holland and He/Ne laser with a 60 x ApoPlanar oil immersion objective, excitation at 505 nm and oil emission at 568 nm). The microsponges were layered on a glass slide, covered with a glass cover-slip and sealed with adhesive to prevent the Pheroids from drying out. Digital images were obtained through capturing by photomultipliers.

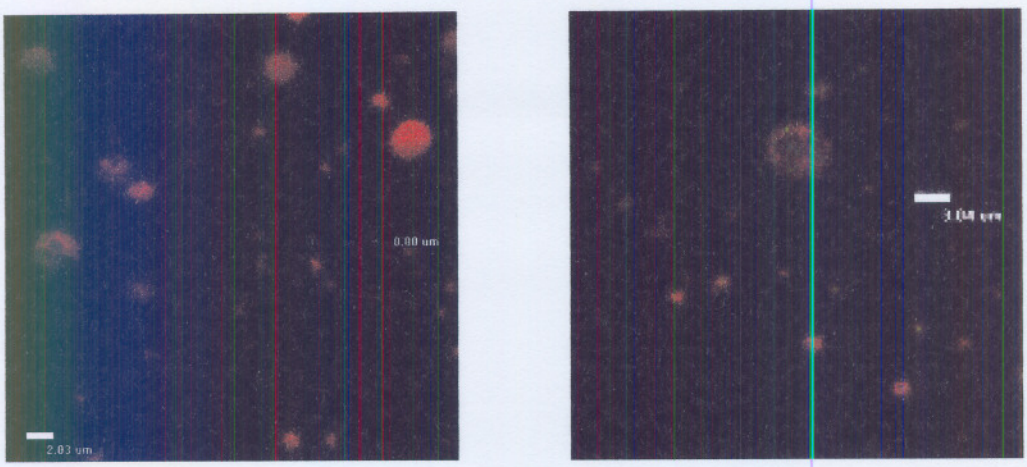
3.3.2.3.2 PARTICLE SIZE ANALYSIS

The droplet sizes of the pheroid microsponges were measured with a Malvern Mastersizer (Malvern Instruments, United Kingdom). Milli Q water (800 ml) was used to wash the Malvern Mastersizer for 30 seconds. Fresh water was used for alignment of the laser. The background was measured and then 2 ml of the sample was added for measurement of volume sizes.

3.3.2.4 RESULTS AND DISCUSSION

The morphology of the Pheroid microsponges was analysed by Confocal Laser Scanning Microscopy (CLSM). As shown in figure 3.10, the Pheroid microsponges are sponge-like with a homogenous intensity. The studies showed that calcitonin, after entrapment, caused a decrease in the quantity of

Pheroid microsponges in the preparation, and an increase in the size of Pheroid microsponges in the preparation.



(A) (B)

Figure 3.10 Confocal Laser Scanning Micrographs of (A) Pheroid microsponges and (B) Pheroid microsponges entrapped with calcitonin.

In table 3.4 the results of the particle size analysis are given. Confocal Laser Scanning Microscopy (CLSM) was also used to analyse the approximate size of the Pheroid microsponges and the approximate size was found to be between 0.90 µm and 5.76 µm before entrapment of calcitonin and between 1.04 µm and 5.91 µm after calcitonin entrapment (see section 3.4). It was established that the Pheroid microsponges were reasonably consistent in size. The Malvern Mastersizer showed that the particle distribution varied mainly between 0.78 µm to 5.77 µm before entrapment with calcitonin and between 0.80 µm to 5.91 µm after calcitonin entrapment with a median volume diameter of 2.21 µm after calcitonin entrapment.

Table 3.4 The sizes (volume) of Pheroid microsponges before and after the entrapment of calcitonin.

	SIZE (µm)					
	$D_1 = 0.1$		$D_2 = 0.5$		$D_3 = 0.9$	
	CLSM	Malvern	CLSM	Malvern	CLSM	Malvern
Pheroid microsponges without calcitonin	0.90	0.78	1.74	2.18	5.76	5.77
Pheroid microsponges with calcitonin	1.04	0.80	2.08	2.21	5.91	5.80

3.4 ENTRAPMENT OF CALCITONIN IN PHEROIDS

3.4.1 MATERIALS

Calcitonin was obtained from Merck (South Africa). Pheroid vesicles and Pheroid microsponges were freshly prepared as described in section 3.3.

3.4.2 METHOD

750 µg of calcitonin was weighted and dissolved in 45 ml of both the Pheroid vesicle and Pheroid microsponge formulations. The Pheroid preparations were brought to a temperature of 31 °C in a water-bath, before the calcitonin was dissolved and entrapped into the Pheroid solutions. These formulations were then slowly shaken for 30 minutes and afterwards kept on ice until

administration. The final preparations contained the equivalent amount of calcitonin of 10 IU/kg body-weight when administered in a volume of 100 µl/kg body-weight.

The control solution was prepared in the same way by dissolving 750 µg of calcitonin in 45 ml phosphate buffer solution (PBS; pH 7.3). All solutions were freshly prepared before nasal administration.

3.5 PREPARATION OF POLYMER SOLUTIONS

3.5.1 MATERIALS

Calcitonin was obtained from Merck (South Africa). *N*-trimethyl chitosan chloride (TMC) and *N*-trimethyl chitosan oligosaccharide (TMO) were a generous gift from the Department of Pharmaceutics at the North-West University (South Africa) and were used as received. 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.

3.5.2 METHOD

0.225 g of both the TMC and TMO polymers was dissolved in 22.5 ml phosphate buffer solution (PBS; pH 7.3) to obtain a 1 % (w/v) solution. Calcitonin (750 µg) was also dissolved in 22.5 ml phosphate buffer solution (PBS; pH 7.3). The calcitonin and polymer solutions were mixed together to obtain a final polymer concentration of 0.5 % (w/v). When administered at a volume of 100 µl/kg body- weight, rats received a dose of 10 IU/kg body-weight of calcitonin.

3.6 CONCLUSION

In this chapter the preparation and characterisation of Pheroid vesicles, Pheroid microsponges and the entrapment of calcitonin into these Pheroid formulations were described. The Pheroid formulations were characterized based on their size and morphology. Solutions of TMC and TMO with calcitonin were also prepared.

These different formulations will be administered in the right nostril of rats, based on the *in vivo* experimental procedures described in this chapter. Blood samples will be collected and the plasma concentrations of calcitonin and calcium will be determined as discussed in this chapter. The results of this study will be presented in chapter 4.

CHAPTER 4

NASAL DELIVERY OF CALCITONIN WITH PHEROID TECHNOLOGY AND *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC): RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter describes the effect of Pheroid vesicles and Pheroid microsponges as well as *N*-trimethyl chitosan chloride (TMC) and *N*-trimethyl chitosan oligosaccharide (TMO) on calcitonin absorption after nasal administration. The efficacy of these formulations was determined with *in vivo* studies in rats as described in chapter 3. The absorption enhancing properties of these compounds were discussed in chapter 2. Plasma calcitonin concentrations were determined with a radioimmunoassay while the plasma calcium concentrations were determined with a spectrophotometric calcium analysing kit. Plasma concentrations of both calcitonin and calcium were plotted against time.

4.2 NASAL DELIVERY OF CALCITONIN WITH PHEROID TECNOLOGY AND *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC): RESULTS AND DISCUSSION

4.2.1 *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC)

In table 4.1 the plasma calcitonin and plasma calcium concentrations obtained after nasal administration of 10 IU/kg body-weight calcitonin with TMC to rats are given. From the plasma calcium concentrations (mmol/l) the plasma levels in percentage (%) were also calculated. Figure 4.1 graphically depicts the plasma calcitonin concentrations against time while figures 4.2 and 4.3 graphically depict the plasma calcium concentrations and plasma calcium levels against time.

Table 4.1 The plasma concentration values of calcitonin and calcium after nasal administration of calcitonin with TMC.

Time (min)	Calcitonin (pg/ml)		Calcium concentration			
	Control	TMC (0.5 % w/v)	Control		TMC (0.5 % w/v)	
			mmol/l	%	mmol/l	%
0	62.29 ± 22.77	136.46 ± 31.29	3.38 ± 0.13	100.00 ± 0.00	3.61 ± 0.94	100.00 ± 0.00
5	66.92 ± 37.98	405.30 ± 308.10	3.45 ± 0.11	102.07 ± 5.44	3.36 ± 0.25	93.07 ± 13.32
10	74.89 ± 15.65	389.85 ± 152.76	3.43 ± 0.16	101.48 ± 6.87	3.22 ± 0.27	89.20 ± 14.87
15	70.29 ± 12.12	397.63 ± 98.83	3.42 ± 0.13	101.18 ± 6.61	3.15 ± 0.25	87.26 ± 15.54
30	79.26 ± 34.05	393.05 ± 247.95	3.34 ± 0.17	98.82 ± 4.26	2.94 ± 0.15	81.44 ±14.60
60	65.99 ± 47.52	163.24 ± 85.58	3.20 ± 0.15	94.67 ± 5.50	2.87 ± 0.29	79.50 ± 14.38
120	82.94 ± 42.71	100.55 ± 72.77	3.17 ± 0.28	93.79 ± 10.04	2.70 ± 0.35	74.79 ± 14.72
180	56.28 ± 20.10	61.52 ± 41.22	3.25 ± 0.31	96.15 ± 9.34	2.60 ± 0.31	72.02 ± 14.77

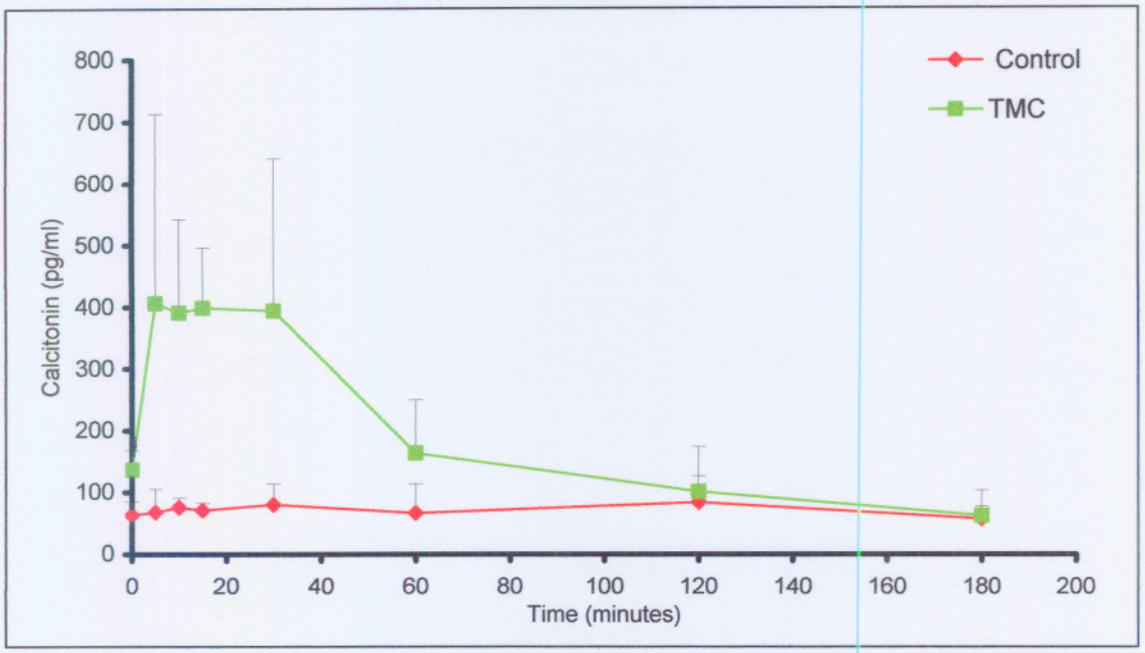


Figure 4.1 Plasma calcitonin concentrations against time after nasal administration of calcitonin with TMC (0.5 % w/v).

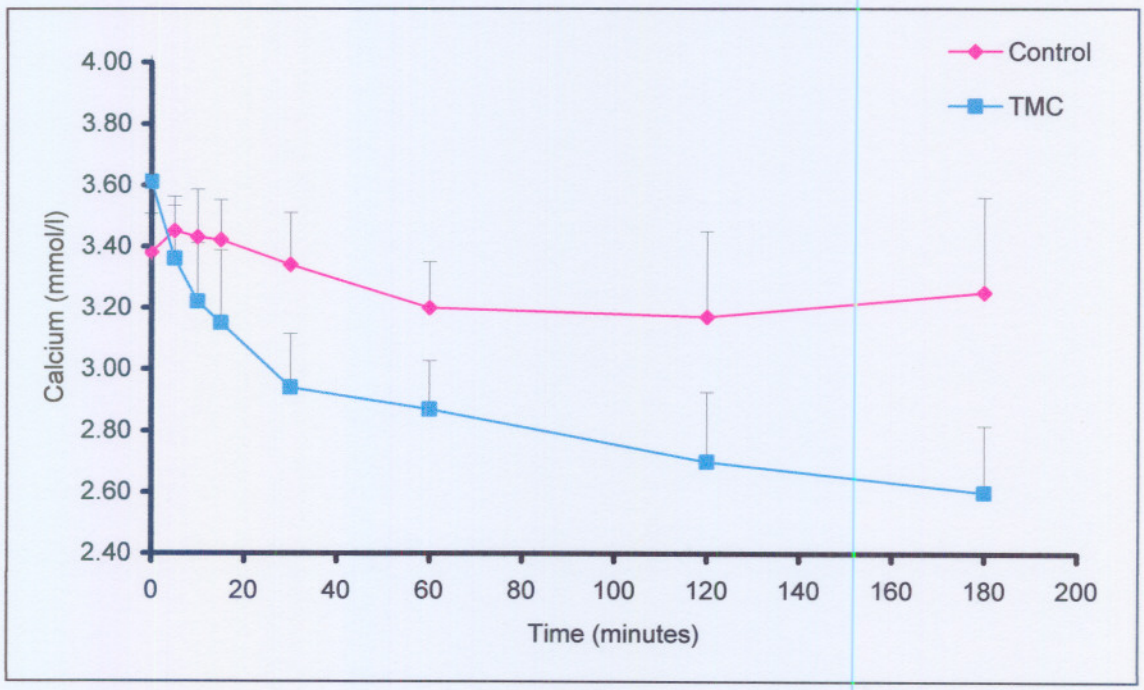


Figure 4.2 Plasma calcium concentrations against time after nasal administration of calcitonin with TMC (0.5 % w/v).

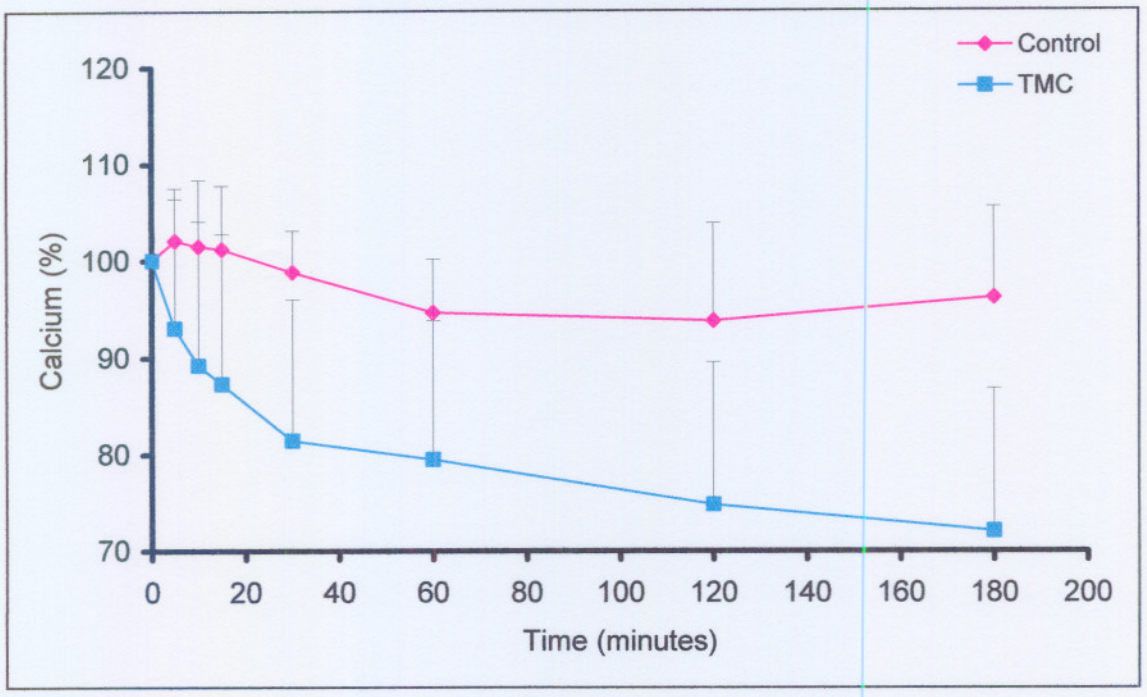


Figure 4.3 Calcium levels (%) after nasal administration of calcitonin with TMC (0.5 % w/v).

As evident from table 4.1 and figure 4.1 the absorption of calcitonin without TMC was extremely low and it can be accepted that calcitonin was not absorbed significantly, as also indicated by the diminutive differences in calcitonin levels over time (table 4.1). Similar results were obtained for the plasma calcium concentrations. No major differences in calcium concentrations were observed against time in the control group (calcitonin without TMC) and it can be concluded that nasal administration of calcitonin alone, in the dose given, did not lead to any changes in the plasma calcium levels. The plasma concentration levels of both calcitonin and calcium obtained, are probable the normal levels which are maintained in the experimental animals under normal conditions.

However, as evident from table 4.1 and figure 4.2, nasal administration of calcitonin with TMC (0.5 % w/v) lead to a major increase in plasma calcitonin concentrations. The plasma calcitonin concentration increased from 136.46 pg/ml at t = 0 min to 405.30 pg/ml at time t = 5 min and remained around this

level up to 30 min post administration before it started to decrease and reach the initial concentration 3 hours post administration.

It is also evident (figures 4.2 - 4.3) that the increased plasma calcitonin concentrations lead to a decrease in plasma calcium concentrations. The therapeutic effect of increased plasma calcitonin levels should be an increase in calcium absorption and this should correspond to lower calcium levels in the plasma, as was obtained in this experiment.

However, these results must also be judged in comparison to normal species levels of calcium in the blood, which is a function of numerous factors such as the calcium content of the daily diet, age and disease status. It must also be remembered that the rats used in these experiments were fasted for 18 hours before initiation of the experiments. However, it can safely be concluded that nasal administration of calcitonin with TMC did lead to an increase in calcium absorption.

4.2.2 *N*-TRIMETHYL CHITOSAN OLIGOSACCHARIDE (TMO)

In table 4.2 the plasma calcitonin and plasma calcium concentrations obtained after nasal administration of 10 IU/kg bodyweight calcitonin with TMO to the rats are given. From the plasma calcium concentrations (mmol/l) the plasma levels in percentage (%) were also calculated. Figure 4.4 graphically depicts the plasma calcitonin concentrations against time while figures 4.5 and 4.6 graphically depict the plasma calcium concentrations and plasma calcium levels against time.

Table 4.2 The plasma concentration values of calcitonin and calcium after nasal administration of calcitonin with TMO.

Time (min)	Calcitonin (pg/ml)		Calcium concentration			
	Control	TMO (0.5 % w/v)	Control		TMO (0.5 % w/v)	
			mmol/l	%	mmol/l	%
0	62.29 ± 22.77	168.15 ± 39.60	3.38 ± 0.13	100.00 ± 0.00	3.38 ± 0.25	100.00 ± 0.00
5	66.92 ± 37.98	157.95 ± 46.91	3.45 ± 0.11	102.07 ± 5.44	3.35 ± 0.30	99.11 ± 16.19
10	74.89 ± 15.65	159.58 ± 35.88	3.43 ± 0.16	101.48 ± 6.87	3.23 ± 0.27	95.56 ± 10.64
15	70.29 ± 12.12	164.16 ± 50.28	3.42 ± 0.13	101.18 ± 6.61	3.17 ± 0.19	93.79 ± 7.36
30	79.26 ± 34.05	154.32 ± 43.20	3.34 ± 0.17	98.82 ± 4.26	3.09 ± 0.20	91.42 ± 7.42
60	65.99 ± 47.52	163.96 ± 32.03	3.20 ± 0.15	94.67 ± 5.50	3.05 ± 0.07	90.24 ± 7.54
120	82.94 ± 42.71	152.20 ± 47.91	3.17 ± 0.28	93.79 ± 10.04	2.81 ± 0.22	83.14 ± 9.46
180	56.28 ± 20.10	125.62 ± 22.54	3.25 ± 0.31	96.15 ± 9.34	2.85 ± 0.17	84.32 ± 10.72

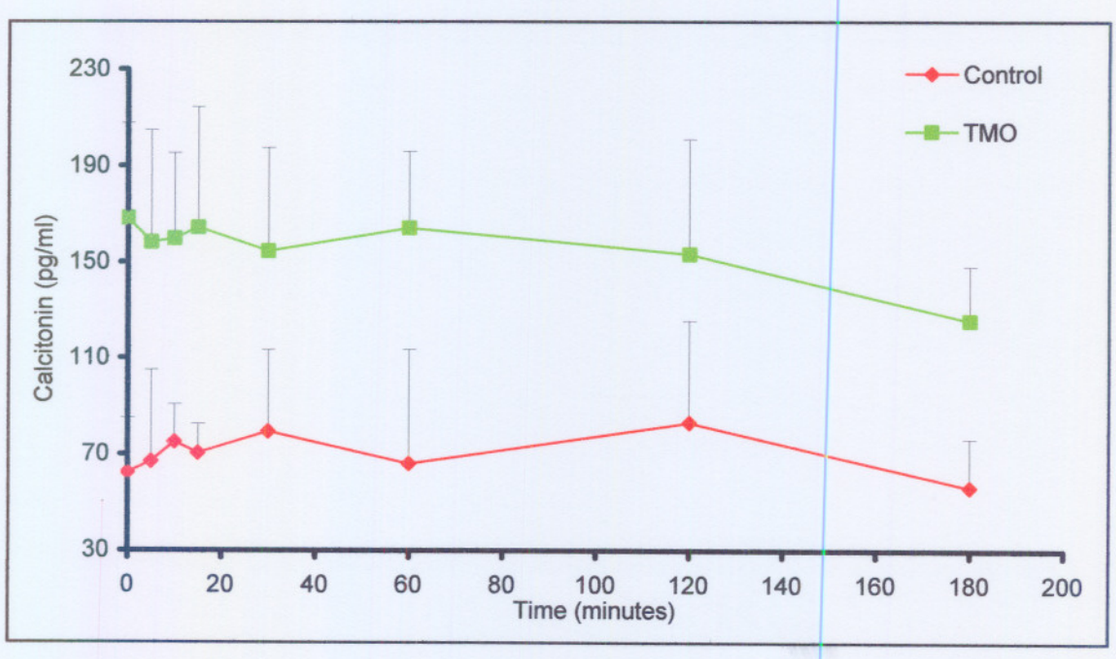


Figure 4.4 Plasma calcitonin concentrations against time after nasal administration of calcitonin with TMO (0.5 % w/v).

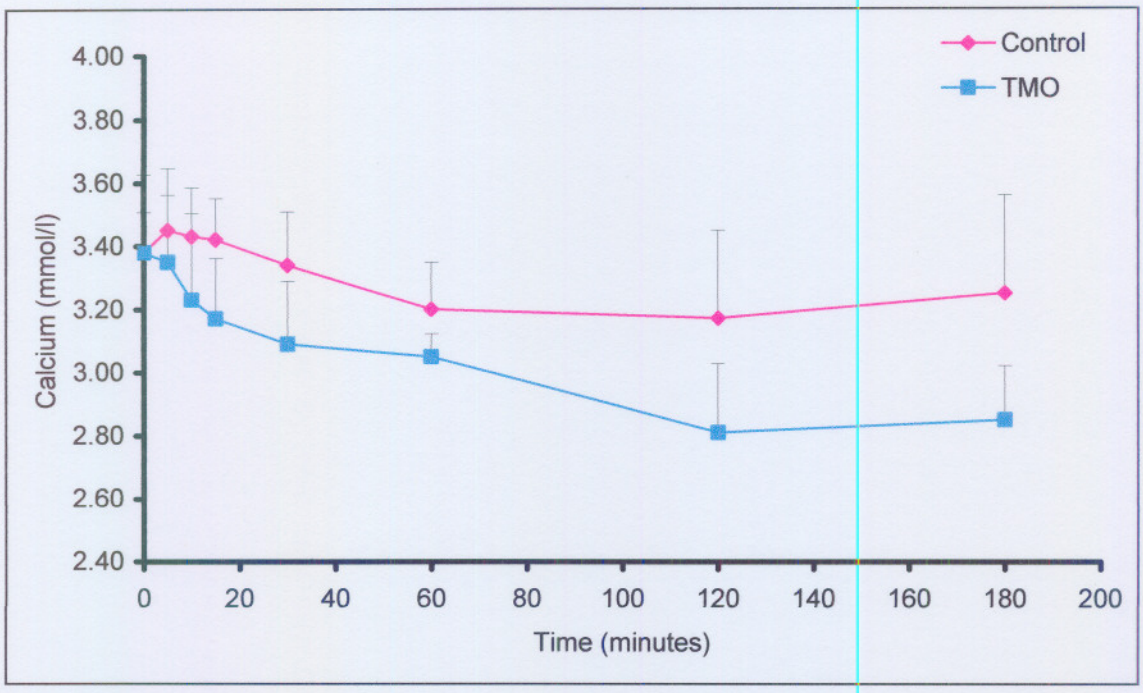


Figure 4.5 Plasma calcium concentrations against time after nasal administration of calcitonin with TMO (0.5 % w/v).

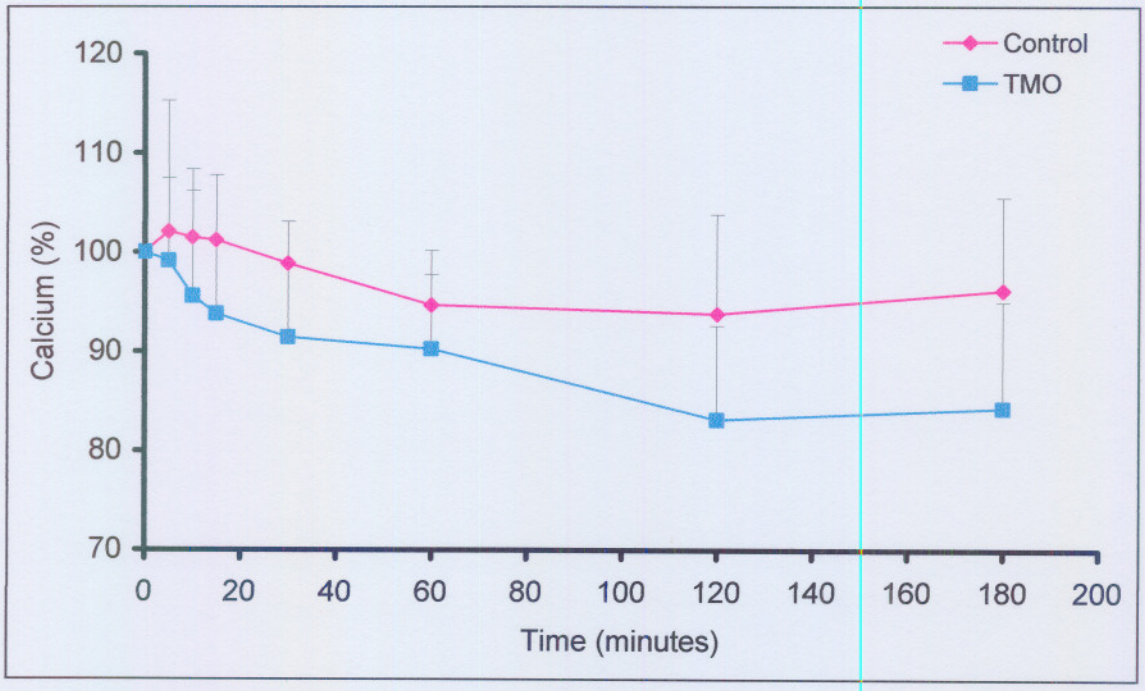


Figure 4.6 Calcium levels (%) after nasal administration of calcitonin with TMO (0.5 % w/v).

As evident from table 4.2 and figure 4.4 the absorption of calcitonin without TMO was extremely low and it can be accepted that calcitonin was not absorbed significantly, as also indicated by the diminutive differences in calcitonin levels over time (table 4.2). Similar results were obtained for the plasma calcium concentrations. No major differences in calcium concentrations were observed against time in the control group (calcitonin without TMO) and it can be concluded that nasal administration of calcitonin alone, in the dose given, did not lead to any changes in calcium levels. The plasma concentration levels of both calcitonin and calcium obtained, are probable the normal levels which are maintained in the experimental animals under normal conditions.

Although figure 4.4 illustrated that there were no major changes in the plasma calcitonin concentration against time when administered with TMO, this result is most probably not an accurate presentation of calcitonin absorption with TMO. The obtained results could probable be explained by human or experimental error in determining the plasma calcitonin concentration at time t = 0 min. Further justification of this is evident, when the plasma calcium concentrations are compared over time, as a marked decrease was observed against time. Although this decrease was not as profound as obtained with TMC, a clear trend can be seen in the reduction of plasma calcium concentrations after nasal administration of calcitonin with TMO. The obtained results however, will not be argued either for or against any further, as it was not possible at this time, to repeat this particular experiment. This experiment should be repeated in a future study.

4.2.3 PHEROID VESICLES

In table 4.3 the plasma calcitonin and plasma calcium concentrations obtained after nasal administration of 10 IU/kg bodyweight calcitonin with Pheroid vesicles to rats are given. From the plasma calcium concentrations (mmol/l) the plasma levels in percentage (%) were also calculated. Figure 4.7 graphically depicts the plasma calcitonin concentrations against time while

figures 4.8 and 4.9 graphically depict the plasma calcium concentrations and plasma calcium levels against time.

Table 4.3 The plasma concentration values of calcitonin and calcium after nasal administration of calcitonin with Pheroid vesicles.

Time (min)	Calcitonin (pg/ml)		Calcium concentration			
	Control	Pheroid vesicles	Control		Pheroid vesicles	
			mmol/l	%	mmol/l	%
0	62.29 ± 22.77	68,16 ± 31.60	3.38 ± 0.13	100.00 ± 0.00	3.56 ± 0.64	100.00 ± 0.00
5	66.92 ± 37.98	63.64 ± 50.61	3.45 ± 0.11	102.07 ± 5.44	3.29 ± 0.17	92.42 ± 11.70
10	74.89 ± 15.65	90.24 ± 45.87	3.43 ± 0.16	101.48 ± 6.87	3.22 ± 0.19	90.45 ± 12.83
15	70.29 ± 12.12	113.58 ± 37.22	3.42 ± 0.13	101.18 ± 6.61	3.18 ± 0.24	89.33 ± 14.31
30	79.26 ± 34.05	118.36 ± 34.41	3.34 ± 0.17	98.82 ± 4.26	3.12 ± 0.18	87.64 ± 14.78
60	65.99 ± 47.52	78.96 ± 31.02	3.20 ± 0.15	94.67 ± 5.50	2.99 ± 0.16	83.99 ± 14.93
120	82.94 ± 42.71	80.85 ± 37.74	3.17 ± 0.28	93.79 ± 10.04	2.80 ± 0.23	78.65 ± 15.01
180	56.28 ± 20.10	49.90 ± 16.35	3.25 ± 0.31	96.15 ± 9.34	2.79 ± 0.22	78.37 ±14.83

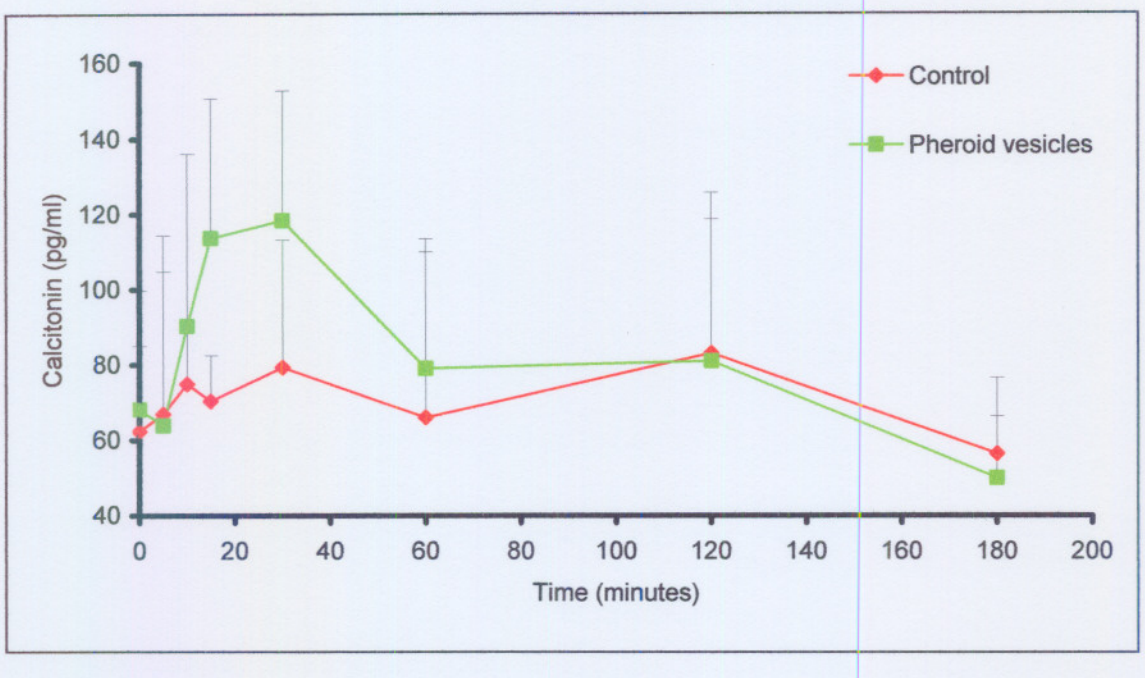


Figure 4.7 Plasma calcitonin concentrations against time after nasal administration of calcitonin with Pheroid vesicles.

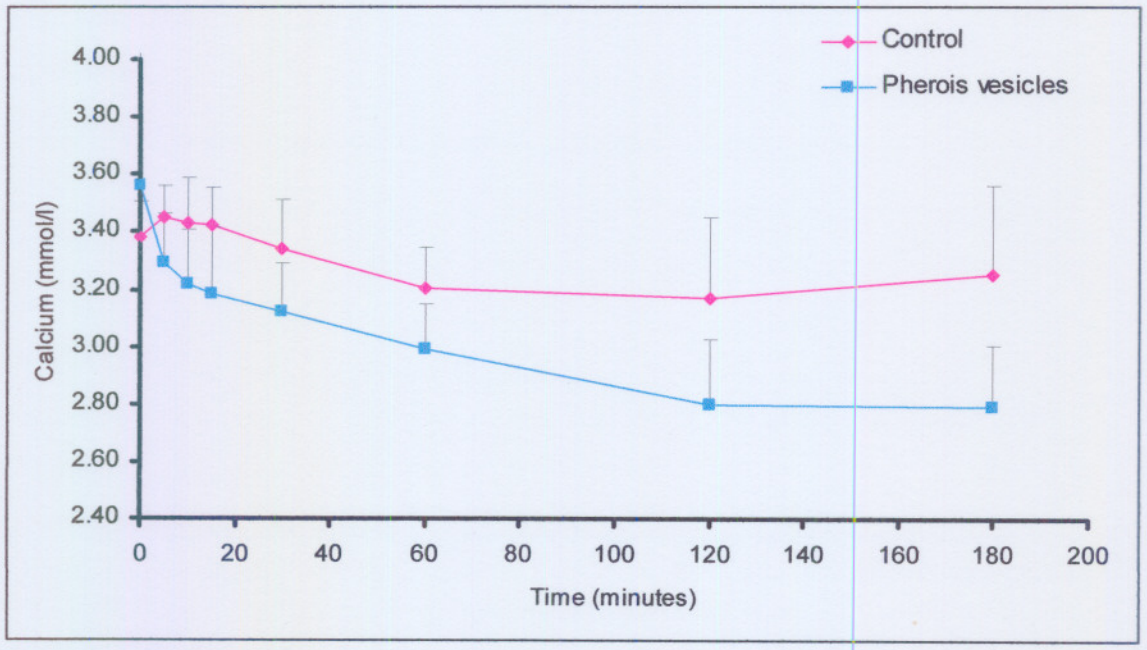


Figure 4.8 Plasma calcium concentrations against time after nasal administration of calcitonin with Pheroid vesicles.

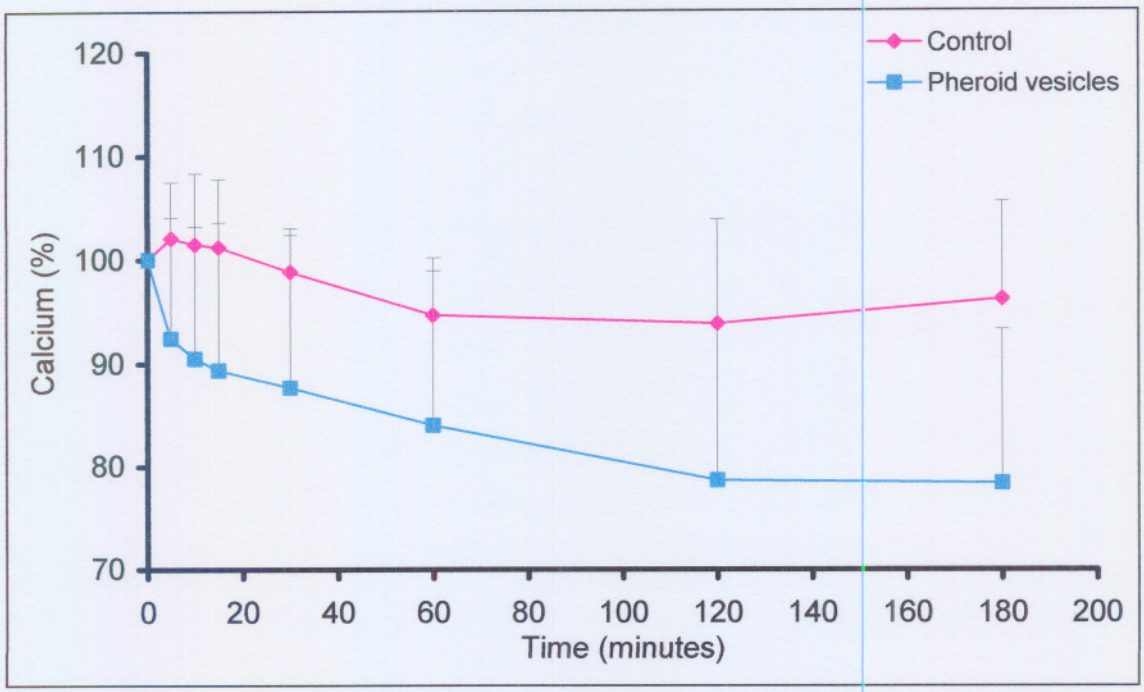


Figure 4.9 Calcium levels (%) after nasal administration of calcitonin with Pheroid vesicles.

As evident from table 4.3 and figure 4.7 the absorption of calcitonin without Pheroid vesicles was extremely low and it can be accepted that calcitonin was not absorbed significantly, as also indicated by the diminutive differences in calcitonin levels over time (table 4.3). Similar results were obtained for the plasma calcium concentrations. No major differences were observed against time in the control group (calcitonin without Pheroid vesicles) and it can be concluded that nasal administration of calcitonin alone, in the dose given, did not lead to any changes in the calcium levels. The plasma concentration levels of both calcitonin and calcium obtained, are probable the normal levels which are maintained in the experimental animals under normal conditions.

However, as evident from table 4.3 and figure 4.7, nasal administration of calcitonin with Pheroid vesicles did lead to an increase in plasma calcitonin concentrations. The plasma calcitonin concentration increased from 68.16 pg/ml at t = 0 min to 118.36 pg/ml at time t = 30 min before it started to decrease towards the initial concentration of calcitonin obtained at t = 0 min.

Although the plasma calcitonin concentrations after administration of calcitonin entrapped in Pheroid vesicles, were not as profoundly increased as obtained after calcitonin administration with TMC, it is clear that Pheroid vesicles are also able to increase the nasal absorption of calcitonin. It is also clear that the maximum effect obtained with Pheroid vesicles are around 15-30 min post administration while the maximum effect obtained with TMC already occurred 5 min after administration and was maintained up to 30 min after administration. This could be an important factor in nasal drug delivery as a fast onset of action is desirable due to the mucociliary clearance mechanism present in the nasal route of drug administration. This difference in effect obtained between TMC and Pheroid vesicles could also be explained by different mechanisms of action in enhancing permeation. It has already been shown in numerous studies that TMC open the tight junctions between epithelial cells to allow for the paracellular transport of several classes of compounds. Calcitonin is a hydrophilic peptide with a molecular weight of 3431.9 Da and has probable been absorbed paracellularly after administration with TMC. The exact mechanism of action of Pheroid vesicles is not yet clear but it 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 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.

4.2.4 PHEROID MICROSPONGES

In table 4.4 the plasma calcitonin and plasma calcium concentrations obtained after nasal administration of 10 IU/kg bodyweight calcitonin with Pheroid microsponges to rats are given. From the plasma calcium concentrations (mmol/l) the plasma levels in percentage (%) were also calculated. Figure 4.10 graphically depicts the plasma calcitonin concentrations against time while figures 4.11 and 4.12 graphically depict the plasma calcium concentrations and plasma calcium levels against time.

Table 4.4 The plasma concentration values of calcitonin and calcium after nasal administration of calcitonin with Pheroid microsponges.

Time (min)	Calcitonin (pg/ml)		Calcium concentration			
	Control	Pheroid microsponges	Control		Pheroid microsponges	
			mmol/l	%	mmol/l	%
0	62.29 ± 22.77	53.11 ± 19.97	3.38 ± 0.13	100.00 ± 0.00	3.63 ± 0.34	100.00 ± 0.00
5	66.92 ± 37.98	88.57 ± 51.21	3.45 ± 0.11	102.07 ± 5.44	3.48 ± 0.21	95.87 ± 8.36
10	74.89 ± 15.65	127.58 ± 50.59	3.43 ± 0.16	101.48 ± 6.87	3.48 ± 0.37	95.87 ± 14.77
15	70.29 ± 12.12	136.93 ± 29.95	3.42 ± 0.13	101.18 ± 6.61	3.16 ± 0.09	87.05 ± 8.71
30	79.26 ± 34.05	103.98 ± 32.85	3.34 ± 0.17	98.82 ± 4.26	3.00 ± 0.23	82.64 ± 7.99
60	65.99 ± 47.52	62.96 ± 17.56	3.20 ± 0.15	94.67 ± 5.50	2.93 ± 0.16	80.72 ± 6.80
120	82.94 ± 42.71	72.64 ± 49.79	3.17 ± 0.28	93.79 ± 10.04	2.94 ± 0.21	80.99 ± 4.13
180	56.28 ± 20.10	72.71 ± 43.81	3.25 ± 0.31	96.15 ± 9.34	2.77 ± 0.24	76.31 ± 11.53

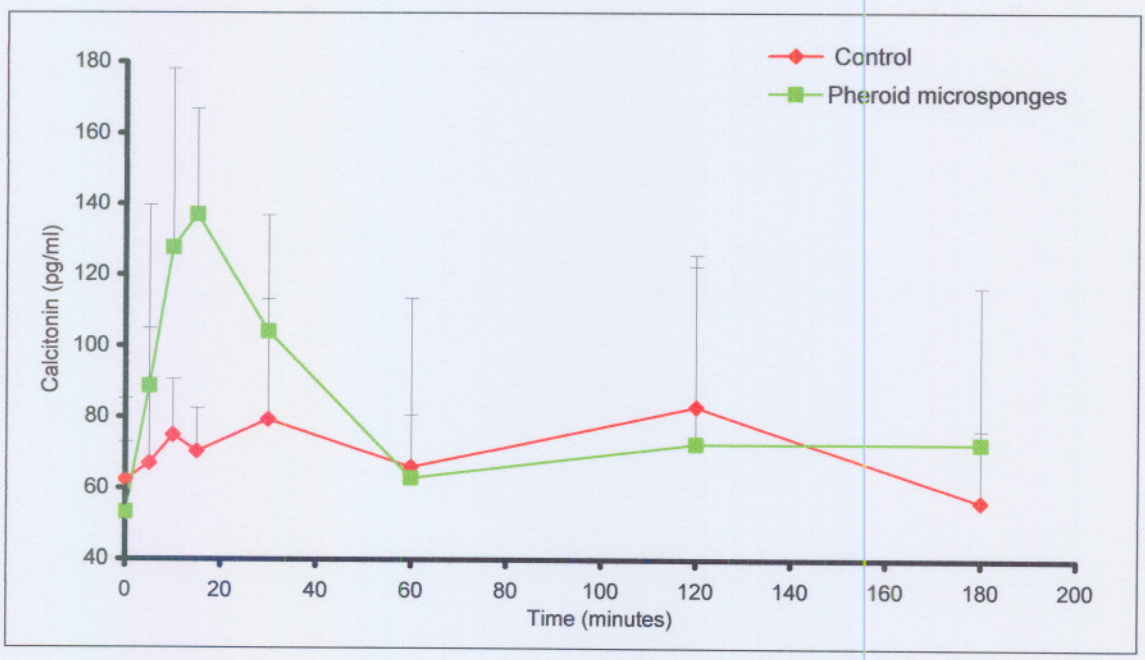


Figure 4.10 Plasma calcitonin concentrations against time after nasal administration of calcitonin with Pheroid microsponges.

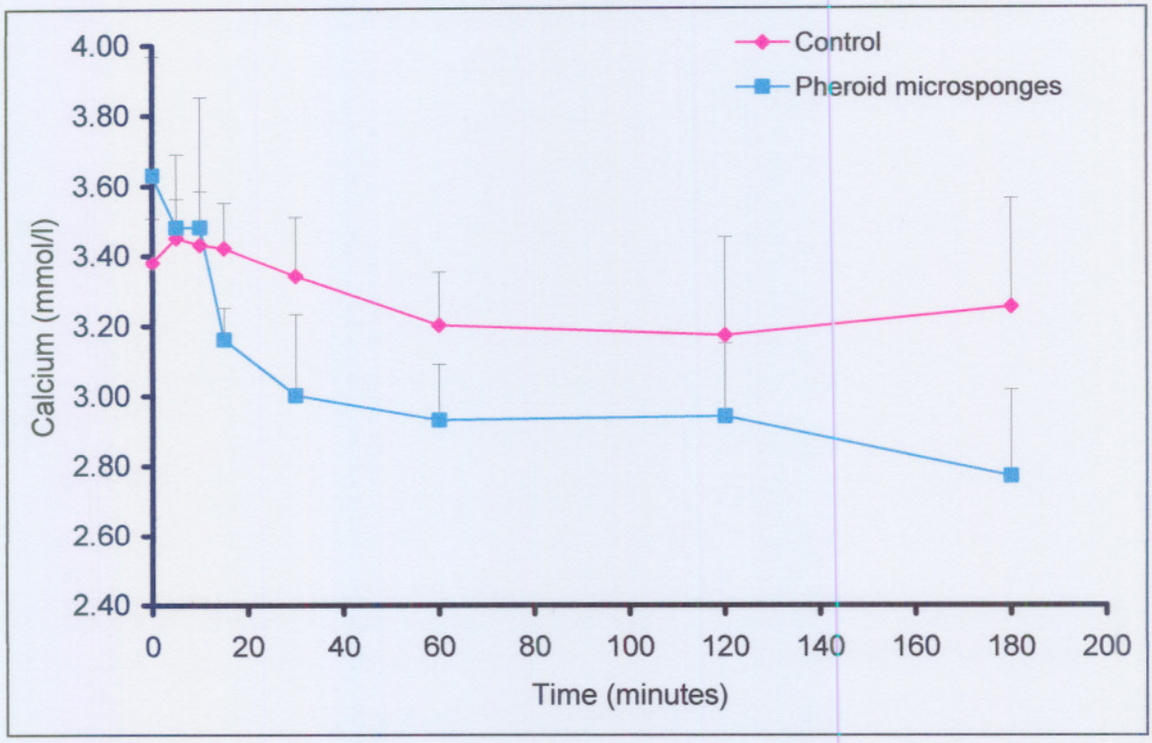


Figure 4.11 Plasma calcium concentrations against time after nasal administration of calcitonin with Pheroid microsponges.

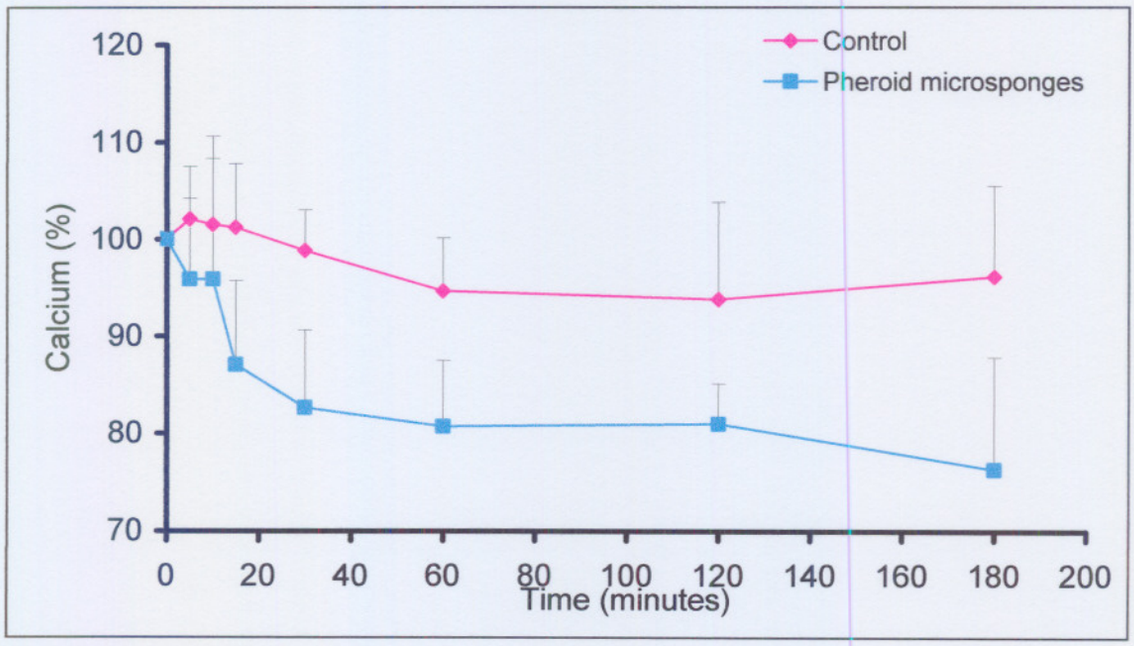


Figure 4.12 Calcium levels (%) after nasal administration of calcitonin with Pheroid microsponges.

As evident from table 4.4 and figure 4.10 the absorption of calcitonin without Pheroid microsponges was extremely low and it can be accepted that calcitonin was not absorbed significantly, as also indicated by the diminutive differences in calcitonin levels over time (table 4.4). Similar results were obtained for the plasma calcium concentrations. No major differences were observed against time in the control group (calcitonin without Pheroid microsponges) and thus it can be concluded that nasal administration of calcitonin alone, in the dose given, did not lead to any changes in the calcium levels. The plasma concentration levels of both calcitonin and calcium are probable the normal levels which are maintained in the experimental animals under normal conditions.

However, as evident from table 4.4 and figure 4.10, nasal administration of calcitonin with Pheroid microsponges did lead to an increase in plasma calcitonin concentrations. The plasma calcitonin concentration increased from 53.11 pg/ml at time t = 0 min to 136.93 pg/ml at time t = 15 min after which it sharply decreased towards the initial concentration of calcitonin obtained at time t = 0 min. This increase in plasma calcitonin concentration corresponds with a decrease in the plasma calcium concentration as evident from figure 4.11.

When the results obtained with Pheroid microsponges are visually compared to the results obtained with Pheroid vesicles (section 4.2.3) it is clear that similar results were obtained and that it appears that Pheroid microsponges lead to a slightly better absorption of calcitonin compared to Pheroid vesicles. The maximum effect with Pheroid microsponges was obtained 10-15 minutes post administration while the maximum effect of Pheroid vesicles was obtained 15-30 minutes post administration. It appears that Pheroid microsponges have a faster onset of action compared to Pheroid vesicles and that they lead to slightly better absorption of calcitonin, although the Pheroid vesicles maintained its effect for a larger period of time compared to the Pheroid microsponges. These differences are however not statistically proven and should be investigated in more detail before any sound conclusions could be made.

4.2.5 COMPARISON OF OBTAINED RESULTS

In table 4.5 the plasma calcitonin concentrations obtained after nasal administration of 10 IU/kg bodyweight calcitonin with various formulations to rats, are given. Figure 4.13 graphically depicts the plasma calcitonin concentrations against time while figure 4.14 graphically depicts the plasma calcitonin concentrations obtained 15 minutes after nasal administration.

As explained in the previous sections and as evident from figures 4.13 and 4.14 nasal administration of calcitonin with TMC lead to a major increase in plasma calcitonin concentrations. Both the Pheroid vesicles and Pheroid microsponges also lead to an increase in calcitonin absorption although not as profound as obtained with TMC. It has already been explained that the reason for these differences in calcitonin absorption is a result of different mechanisms of action in enhancing the permeation of calcitonin.

Table 4.5 The plasma concentration values of calcitonin after nasal administration of calcitonin with various formulations.

Time (min)	Calcitonin (pg/ml)				
	Control	TMC (0.5 % w/v)	TMO (0.5 % w/v)	Pheroid vesicles	Pheroid microsponges
0	62.29 ± 22.77	168.15 ± 39.60	168.15 ± 39.60	68.16 ± 31.60	53.11 ± 19.97
5	66.92 ± 37.98	157.95 ± 46.91	157.95 ± 46.91	63.64 ± 50.61	88.57 ± 51.21
10	74.89 ± 15.65	159.58 ± 35.88	159.58 ± 35.88	90.24 ± 45.87	127.58 ± 50.59
15	70.29 ± 12.12	164.16 ± 50.28	164.16 ± 50.28	113.58 ± 37.22	136.93 ± 29.95
30	79.26 ± 34.05	154.32 ± 43.20	154.32 ± 43.20	118.36 ± 34.41	103.98 ± 32.85
60	65.99 ± 47.52	163.96 ± 32.03	163.96 ± 32.03	78.96 ± 31.02	62.96 ± 17.56
120	82.94 ± 42.71	152.20 ± 47.91	152.20 ± 47.91	80.85 ± 37.74	72.64 ± 49.79
180	56.28 ± 20.10	125.62 ± 22.54	125.62 ± 22.54	49.90 ± 16.35	72.71 ± 43.81

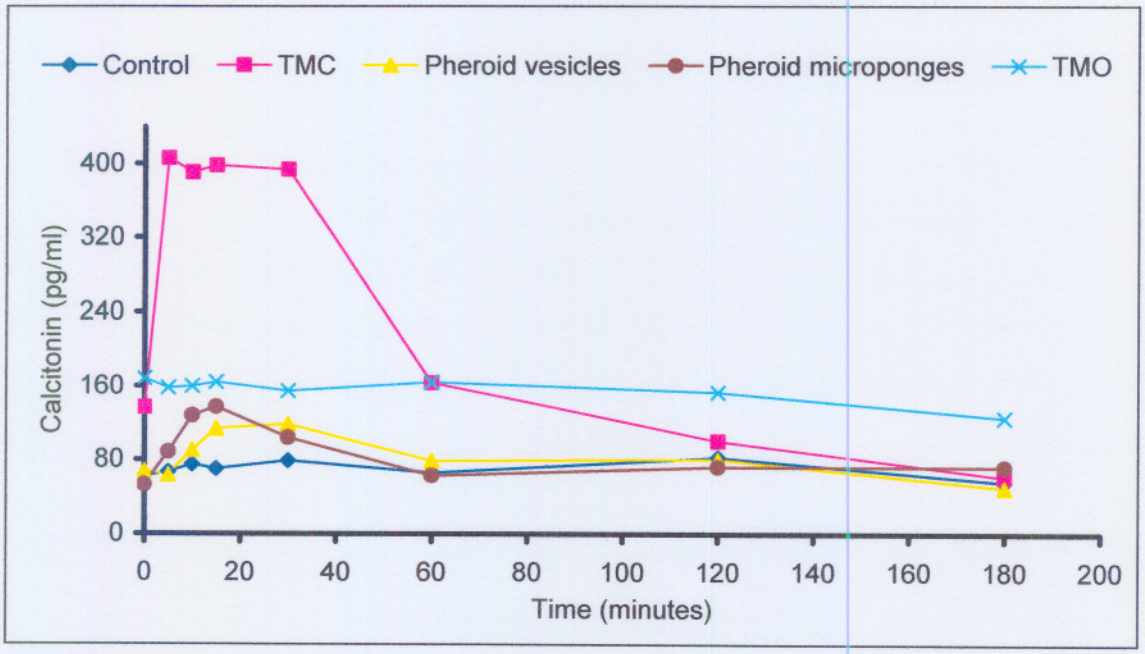


Figure 4.13 Plasma calcitonin concentrations after nasal administration of calcitonin with various formulations (error bars omitted for clarity).

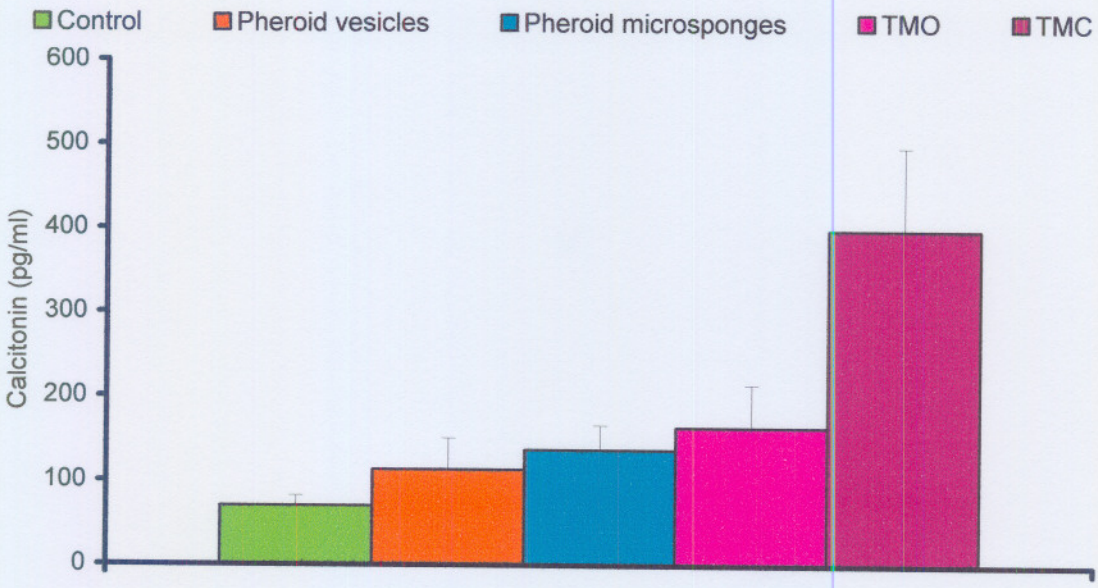


Figure 4.14 Plasma calcitonin concentrations 15 min after nasal administration with various formulations.

In table 4.6 the plasma calcium concentrations obtained after nasal administration of 10 IU/kg bodyweight calcitonin with the various formulations to rats, are given. From the plasma calcium concentrations (mmol/l) the plasma levels in percentage (%) were also calculated. Figures 4.15 and 4.16 graphically depict the plasma calcium concentrations and plasma calcium levels against time. Figure 4.17 gives a comparison of the plasma calcium concentrations obtained 180 minutes after nasal administration of calcitonin with the different formulations while figure 4.18 gives the corresponding reduction in plasma calcium levels 180 minutes after nasal administration of calcitonin with the different formulations.

These results are in agreement with the obtained plasma calcitonin concentrations. It is clear that administration of calcitonin with TMC lead to the highest reduction in plasma calcium levels. Administration of calcitonin with both the Pheroid vesicles and Pheroid microsponges also lead to a reduction (± 21 % and ± 24 % respectively) in plasma calcium levels although not as profound as obtained with TMC (± 28 %).

Table 4.6 The plasma concentration values of calcium after nasal administration of calcitonin with various formulations.

Time (min)	Calcium concentrations									
	Control		TMC (0.5 % w/v)		TMO (0.5 % w/v)		Pheroid vesicles		Pheroid microsponges	
	mmol/l	%	mmol/l	%	mmol/l	%	mmol/l	%	mmol/l	%
0	3.38 ± 0.13	100.00 ± 0.00	3.61 ± 0.94	100.00 ± 0.00	3.38 ± 0.25	100.00 ± 0.00	3.56 ± 0.64	100.00 ± 0.00	3.63 ± 0.34	100.00 ± 0.00
5	3.45 ± 0.11	102.07 ± 5.44	3.36 ± 0.25	93.07 ± 13.32	3.35 ± 0.30	99.11 ± 16.19	3.29 ± 0.17	92.42 ± 11.70	3.48 ± 0.21	95.87 ± 8.36
10	3.43 ± 0.16	101.48 ± 6.87	3.22 ± 0.27	89.20 ± 14.87	3.23 ± 0.27	95.56 ± 10.64	3.22 ± 0.19	90.45 ± 12.83	3.48 ± 0.37	95.87 ± 14.77
15	3.42 ± 0.13	101.18 ± 6.61	3.15 ± 0.25	87.26 ± 15.54	3.17 ± 0.19	93.79 ± 7.36	3.18 ± 0.24	89.33 ± 14.31	3.16 ± 0.09	87.05 ± 8.71
30	3.34 ± 0.17	98.82 ± 4.26	2.94 ± 0.15	81.44 ±14.60	3.09 ± 0.20	91.42 ± 7.42	3.12 ± 0.18	87.64 ± 14.78	3.00 ± 0.23	82.64 ± 7.99
60	3.20 ± 0.15	94.67 ± 5.50	2.87 ± 0.29	79.50 ± 14.38	3.05 ± 0.07	90.24 ± 7.54	2.99 ± 0.16	83.99 ± 14.93	2.93 ± 0.16	80.72 ± 6.80
120	3.17 ± 0.28	93.79 ± 10.04	2.70 ± 0.35	74.79 ± 14.72	2.81 ± 0.22	83.14 ± 9.46	2.80 ± 0.23	78.65 ± 15.01	2.94 ± 0.21	80.99 ± 4.13
180	3.25 ± 0.31	96.15 ± 9.34	2.60 ± 0.31	72.02 ± 14.77	2.85 ± 0.17	84.32 ± 10.72	2.79 ± 0.22	78.37 ±14.83	2.77 ± 0.24	76.31 ± 11.53

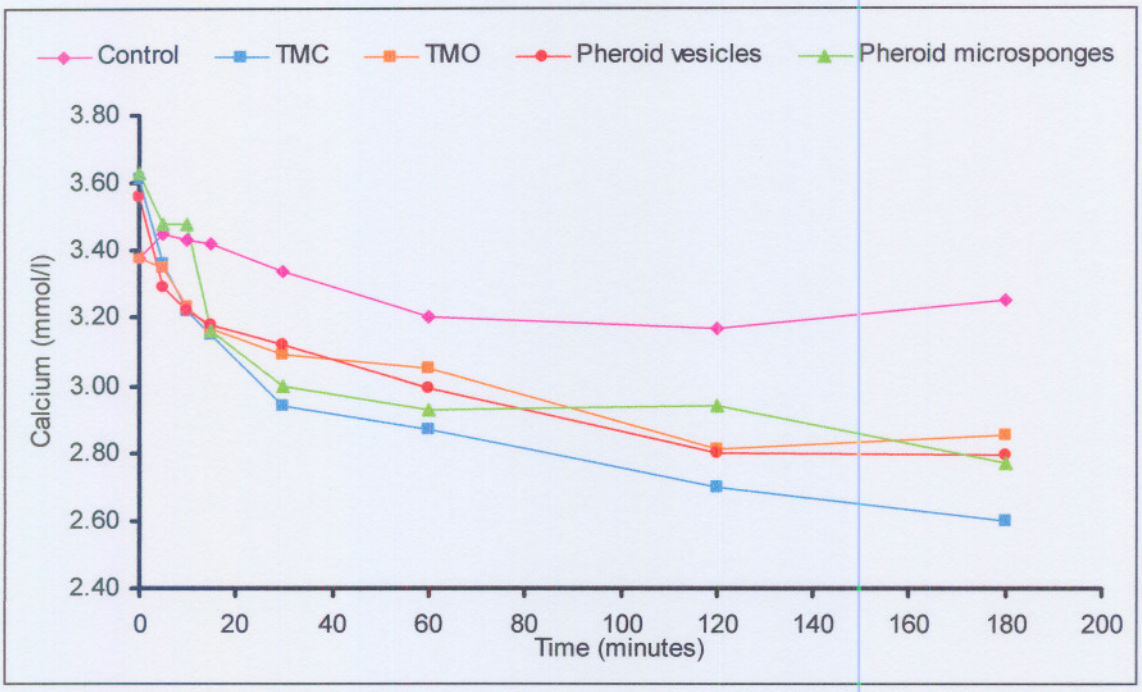


Figure 4.15 Plasma calcium concentrations after nasal administration of different calcitonin formulations (error bars omitted for clarity).

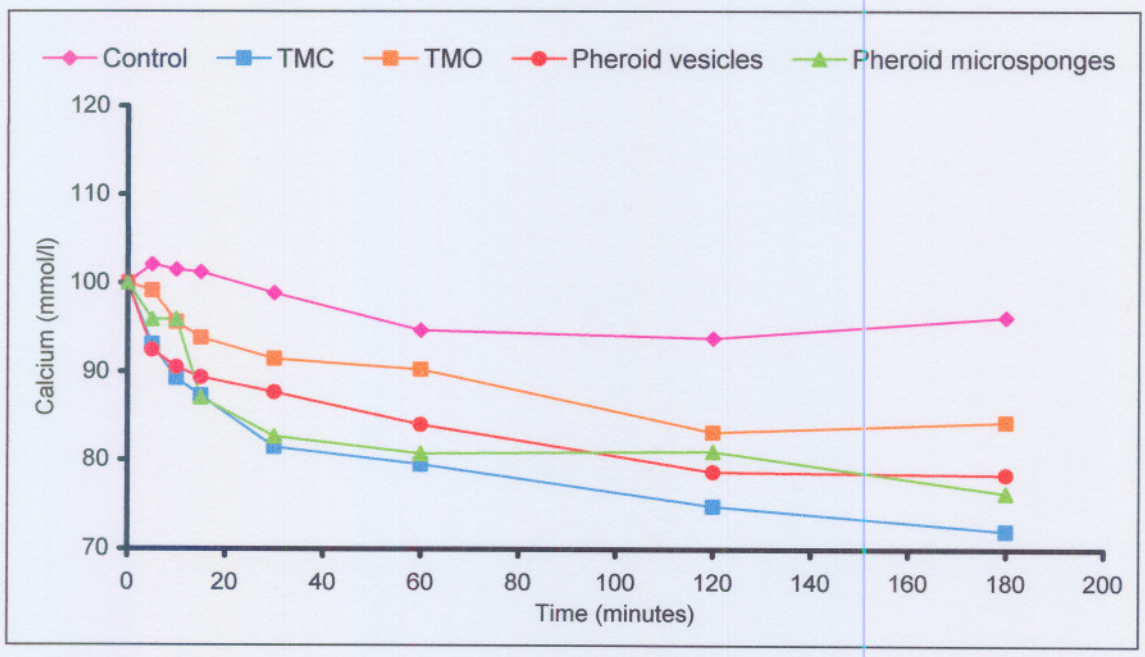


Figure 4.16 Plasma calcium levels after nasal administration of different calcitonin formulations (error bars omitted for clarity).

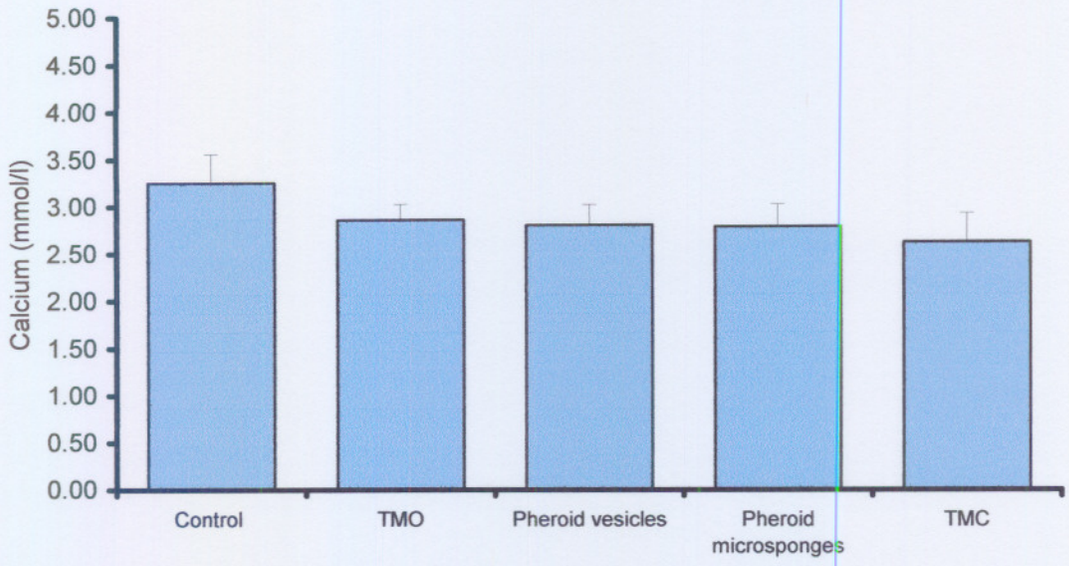


Figure 4.17 Plasma calcium concentrations 180 minutes after nasal administration of calcitonin with different formulations.

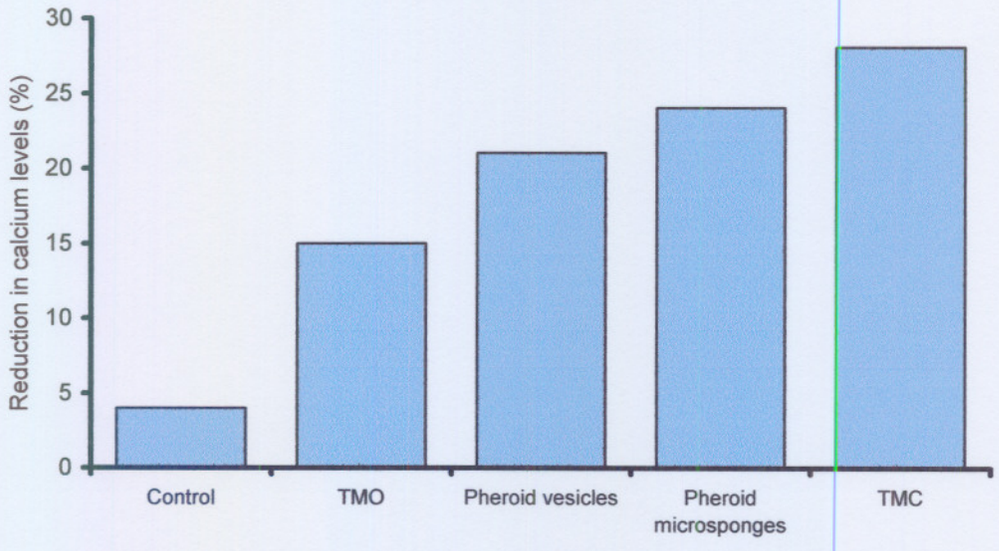


Figure 4.18 The reduction in calcium levels 180 minutes after nasal administration of calcitonin with different formulations.

4.3 CONCLUSION

In this chapter the nasal administration of calcitonin with different absorption enhancers was described in an attempt to evaluate the improvement in calcitonin absorption. It is evident from the results obtained, that the Pheroid formulations as well as the TMC formulation caused a decrease in calcium levels after nasal administration of calcitonin. The results of this study clearly illustrate that TMC proved to be the better absorption enhancer which gave the highest increase in plasma calcitonin concentrations after nasal calcitonin administration. It has been shown that TMC act, by increasing paracellular transport of peptide drugs, but further studies are needed to clarify the mechanism of action of the Pheroid formulations.

SUMMARY AND FUTURE PROSPECTS

Different studies have proved that peptide and protein drug molecules are not easily absorbed via any delivery route, because of inherent characteristics such as their high molecular size, polarity and instability which makes it difficult to cross the physiological barriers. Low permeability and bioavailability of peptide and protein drugs as well as rapid clearance of the delivery systems and possible enzymatic degradation are challenges which must be overcome by considering alternative routes of drug administration and by including absorption enhancers to the preparations.

The nasal route offers unique advantages as an administration site for drugs and recent years have shown that the intranasal route of drug administration can be exploited as an alternative route for delivering peptides and proteins. Although nasal delivery also has biological barriers, which inhibit the transport and absorption of drugs across the nasal cell membranes, low drug transport and targeting can be overcome by using non-toxic, non irritable and effective absorption enhancers. This approach to increase the poor absorption and bioavailability of peptide and protein drugs, has led to the investigation of possible absorption enhancers to entrap, transport and deliver these molecules effectively.

To enhance the absorption of calcitonin, a peptide hormone, several absorption enhancers was considered for nasal administration with calcitonin *in vivo* in rats. Pheroid microsponges and Pheroid vesicles were prepared for this study and calcitonin was entrapped into Pheroid vesicles and Pheroid microsponges. The size and morphology of the Pheroid vesicles and Pheroid microsponges were investigated before and after entrapment of calcitonin. TMC and TMO solutions containing calcitonin were also prepared and administered nasally to rats. These calcitonin formulations were administered nasally to male Sprague Dawley rats (250g - 350g) at a dose of 10 IU/kg

body-weight calcitonin. After blood samples were collected at different intervals over a period of 180 minutes, it was analysed to determine the plasma calcitonin concentrations and plasma calcium concentrations.

The results of this study showed that both Pheroid vesicles and Pheroid microsponges and TMC have the ability to enhance the nasal absorption of calcitonin, with a resulting decrease in the plasma calcium levels and that TMC proved to be better in enhancing the absorption of calcitonin compared to the other preparations that was used.

A few recommendations to consider for future studies can be summarised as follow:

- The exact mechanism of action of Pheroids should be investigated on a cellular and molecular level to explain their absorption enhancing activity.
- The effect of different concentrations of calcitonin with these absorption enhancers should be investigated.
- The differences in absorption efficacy, onset of action and duration of effect between Pheroid vesicles and Pheroid microsponges should be investigated further.
- The stability profile of calcitonin in Pheroid formulations should be investigated.
- Complete toxicity studies on different Pheroid formulations should be performed.
- Applicable dosage form devices should be developed for nasal administration of Pheroid formulations.

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