

***Nasal delivery of insulin
with Pheroid technology***

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*“Wherever you are, be all there.
Live to the hilt every situation you
believe to be the will of God.”*

~ Jim Elliot ~

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Approximately 350 million people worldwide suffer from diabetes mellitus (DM) and this number increases yearly. Since the discovery and clinical application of insulin in 1921, subcutaneous injections have been the standard treatment for DM. Because insulin is hydrophilic and has a high molecular weight and low bioavailability, this molecule is poorly absorbed if administered orally.

The aim of this study is to evaluate nasal delivery systems for insulin, using Sprague Dawley rats as the nasal absorption model. Pheroid technology and *N*-trimethyl chitosan chloride (TMC) with different dosages of insulin (4, 8 and 12 IU/kg bodyweight insulin) was administered in the left nostril of the rat by using a micropipette. Pheroid technology is a patented (North-West University) carrier system consisting of a unique oil/water emulsion that actively transports drug actives through various physiological barriers. These formulations were administered nasally to rats in a volume of 100 μ l/kg bodyweight in different types of Pheroids (vesicles, with a size of 1.71 – 1.94 μ m and microsponges, with a size of 5.71 – 8.25 μ m).

The systemic absorption of insulin was monitored by measuring arterial blood glucose levels over a period of 3 hours. The TMC formulation with 4 IU/kg insulin produced clinically relevant levels of insulin in the blood and as a result also the maximal hypoglycemic effect. TMC is a quaternary derivative of chitosan and is able to enhance the absorption of various peptide drugs by opening tight junctions between epithelial cells. Pheroid formulations were also effective in lowering blood glucose levels but only at higher doses (8 and 12 IU/kg) of insulin. This study indicated that Pheroid microsponges had a faster onset of action and a slightly better absorption of insulin when compared to Pheroid vesicles, but many more studies are needed in this field.

Although the results of this study with absorption enhancers are encouraging, nasal insulin bioavailability is still very low, and the Pheroid formulations and long-term safety of nasal insulin therapy have yet to be investigated.

Keywords: Nasal delivery; Insulin; Absorption enhancers; Pheroid vesicles; Pheroid microsponges; *N*-trimethyl chitosan chloride (TMC).

Ongeveer 350 miljoen mense wêreldwyd ly aan diabetes mellitus (DM) en dié getal neem jaarliks toe. Onderhuidse inspuitings is vandat hulle in 1921 ontdek en terapie toegegee is, die standaard behandeling vir DM. Omdat insulien hidrofiel is, 'n hoë molekulêre massa het en sy biobeskikbaarheid laag is, word dié molekule swak geabsorbeer wanneer dit oraal toegegee word.

Die doel van hierdie studie was om nasale insulientoedieningstelsels te evalueer. Sprague Dawley-rotte is as die absorpsiemodel gebruik. Pheroidtegnologie en *N*-trimetielkitosaanchloried (TMC) met verskillende doserings insulien (4, 8 en 12 IU/kg liggaamsmassa insulien) is met 'n mikropipet in die linker neusgat van die rot toegegee. Pheroidtegnologie is 'n gepatenteerde (Noord-Wes Universiteit) draersisteem, bestaande uit 'n unieke olie/water-emulsie wat aktiewe geneesmiddelbestanddele deur verskeie fisiologiese membrane vervoer. Hierdie formulering is nasaal toegegee in rotte in 'n volume van 100 μ l/kg liggaamsmassa in verskillende tipes Pheroids (mikrodruppeltjies met 'n grootte van 1.71 – 1.94 μ m en mikrosponsies met 'n grootte van 5.71 – 8.25 μ m).

Die sistemiese absorpsie van insulien is gemoniteer deur die arteriële bloedglukosevlakke oor 'n tydperk van 3 uur te meet. Die TMC-formulering met 4 IU/kg insulien het klinies relevante insulienvlakke in die bloed gegee en dus ook die maksimum hipoglukemiese effek. TMC is 'n kwaternêre derivaat van kitosaan, en kan die absorpsie van verskeie peptiedgeneesmiddels verhoog deur vaste heftings tussen eptieselle oop te maak. Verder kon Pheroidformulering die bloedglukosevlakke doeltreffend verlaag, maar slegs teen hoër (8 en 12 IU/kg) insulienindosisse. Hierdie studie het aangetoon dat Pheroidmikrosponsies – vergeleke met Pheroidmikrodruppeltjies – vinniger begin werk en insulien effens beter absorbeer, maar daar moet nog meer navorsing op dié gebied gedoen word.

Hoewel die uitslae van hierdie studie met absorpsiebevorderaars belowend is, bly die nasale biobeskikbaarheid van insulien steeds baie laag, en moet die Pheroidformulering en lang termyn veiligheid van nasale insulientherapie nog ondersoek word.

Sleutelwoorde: Nasale toediening; Insulien; Absorpsiebevorderaars; Pheroidmikrodruppeltjies; Pheroidmikrosponsies; *N*-trimetielkitosaanchloried (TMC).

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Introduction and Aim of Study

Since ancient times, the nasal administration of drugs received much attention to achieve systemic pharmacological effects. Intranasal drug delivery is being investigated as an interesting alternative to the parenteral administration of peptides and proteins, because of their low bioavailability. The nasal route offers advantages such as rapid absorption of drug molecules across the nasal membrane, with less enzymatic degradation; the blood supply in the nose is rich, which makes this a convenient method of administration.

Despite all the above advantages, nasal drug administration has some limitations, such as low permeability to high molecular weight and hydrophilic compounds, local enzymatic activity and rapid clearance by the actively beating cilia (Cornaz & Buri, 262:1994).

It was found in various studies that by incorporating bioadhesive polymers into a formulation the residence time of the drug in the nasal cavity is increased, which improves the absorption of the drug concerned (Harris & Robinson, 654:1990).

In an attempt to overcome the low permeability (of the nasal membrane) for large molecules, many different researchers investigated various absorption enhancers. However, many of these absorption enhancers may alter nasal membrane integrity irreversibly and cause damage to the nasal epithelium (Schipper *et al.*, 175:1992). Therefore, there is a constant search to find a non-toxic, non-irritable and effective absorption enhancer that enhances the nasal absorption of peptide drugs.

Recent studies indicate that *N*-trimethyl chitosan chloride (TMC) could enhance the absorption of poorly absorbed peptide drugs after nasal administration. TMC, a partially quarternised derivate of chitosan have mucoadhesive properties that enable the polymer to reduce the clearance rate of drugs from the nasal cavity and thereby prolong the contact time of the TMC delivery system in the nasal epithelium. TMC causes the opening of the tight junctions between epithelial cells, which allows the transportation of large hydrophilic compounds across the

epithelium. In previous studies, it was indicated that TMC is non-toxic, and therefore a potential absorption enhancer for peptide drugs even in chronic use.

Pheroid technology is a unique delivery system that can be manipulated in terms of size, morphology and structure. Confocal Laser Scanning Microscopy (CLSM) micrographs show high entrapment capabilities of both pheroid vesicles and pheroid microsponges. The rapid transport and delivery of insulin molecules and minimal adverse effects make pheroid formulations an ideal alternative to use as an absorption enhancing system for nasal administration. Critical factors that should be evaluated in optimising nasal absorption of drugs are a suitable dosage form, the delivery device and the selection of a suitable animal model.

The aim of this study is to investigate the absorption enhancing effects of pheroid technology and TMC for the nasal delivery of insulin.

The objectives of this study are:

- a) to do a complete literature study on nasal drug delivery, insulin, pheroid technology and TMC;
- b) to understand and gain more knowledge of the preparation, characterisation and ingredients of pheroid vesicles and pheroid microsponges;
- c) to become more secure in practical techniques used, for example weighing of very small amounts of insulin powder, using a glucometer, accurate usage of a micropipette and correct entrapment of insulin in pheroid vesicles and pheroid microsponges;
- d) to compare the absorption enhancing effects of pheroid vesicles, pheroid microsponges and TMC after nasal administration with insulin to rats.

Nasal drug delivery

1.1 Introduction

Nasal drug delivery is a very appealing approach in terms of drugs that are active in low doses and show no or minimal oral bioavailability. The route chosen to deliver a drug determines its bioavailability, which in turn influences its therapeutic effectiveness. Efficient absorption to accomplish greater bioavailability and administration are both essential for achieving maximum drug efficacy. The rich vascularity and permeability of the nasal epithelium makes the nasal route an ideal alternative to the parenteral route. These characteristics of the nasal route make it possible for drugs to bypass enzymatic or acidic degradation, as well as first-pass metabolism (Chien *et al.*, 1989:1).

A few potential advantages of nasal administration include the following: (1) It is a very easy and convenient method of administering drugs; (2) The nasal area is an essential absorption area; and (3) The nasal area has an excellent systemic blood supply (Taylor, 2002:489).

The nasal route is also ideal for drugs with (a) poor bioavailability, (b) high biosensitivity, or (c) a high molecular weight, for example peptides, proteins, vaccines and steroids. Rapid drug absorption rate and onset of therapeutic action is ensured with the nasal route (Arora *et al.*, 2002:967; Ugwoke *et al.*, 2000:3).

However, there are some factors that could potentially influence the efficiency of the intranasal administration of drugs, namely (a) the existence of any pathological conditions that may affect the nasal functions; (b) the rate of drug clearance; (c) techniques and methods of administration; and (d) the site of disposition (Chien *et al.*, 1989:1).

Although the nose is normally used for achieving local effects with compounds such as decongestants, antiallergic agents and local anesthetics, it can also be used for systemic effects such as the administration of dexamethasone for the treatment of sinovitis associated with osteo- and rheumatoid arthritis. The main reason for studying systemic nasal absorption is the

growing interest in the use of peptide or protein drugs, apart from the conventional parenteral injections, as an alternative route for drug delivery (Illum & Fisher, 1997:135-136). The nasal mucosa therefore provides an interesting route of administration for products with both local and systemic activity, because of its ideal surface and accessibility (Duchêne & Ponchel, 1993:102).

In this chapter the anatomy and physiology of the nasal route are discussed for a better understanding of the nasal delivery of drugs, especially peptide drugs.

1.2 Anatomy and physiology of the nose

The human nose is externally covered with skin, while muscle, cartilage and a framework of bone support the nose internally. Figure 1.1 illustrates the nasal bones, which form the bridge and pliable cartilage, which in turn form the distal portions (Tortora & Anagnostakos, 1990:690). Air enters and leaves the nasal openings (nostrils) and these openings are covered with a mucus membrane and internal hairs, which prevents large particles from entering the nose. The cribriform plate of the ethmoid bone separates the nasal cavity from the cranial cavity and the hard palate separates it from the mouth (Shier *et al.*, 1999:740).

The hollow space behind the nose is known as the nasal cavity, extending from the nostrils to the nasopharynx. The nasal cavity has a length of approximately 12 cm and has a volume of 15-20 cm³. The vertical midline that divides the cavity in two symmetrical halves is called the nasal septum (Illum & Fisher, 1997:139). Each wall of the cavity contains three folds known as nasal turbinates (or conchae), which means that the nasal cavity has a relatively large surface (i.e. absorption) area (approximately 160 cm²) (Taylor, 2002:489).

The turbinates make communication with the nasal passages possible (Ridley *et al.* 1992:14). This region is also called the respiratory region or the nasal conchae, which occupies the major part of the respiratory region, which is approximately 100 cm² (Illum & Fisher, 1997:139). The medial wall of each passage is smooth, whereas the lateral walls have three turbinates – the superior, middle and inferior – on each side (illustrated in figure 1.2).

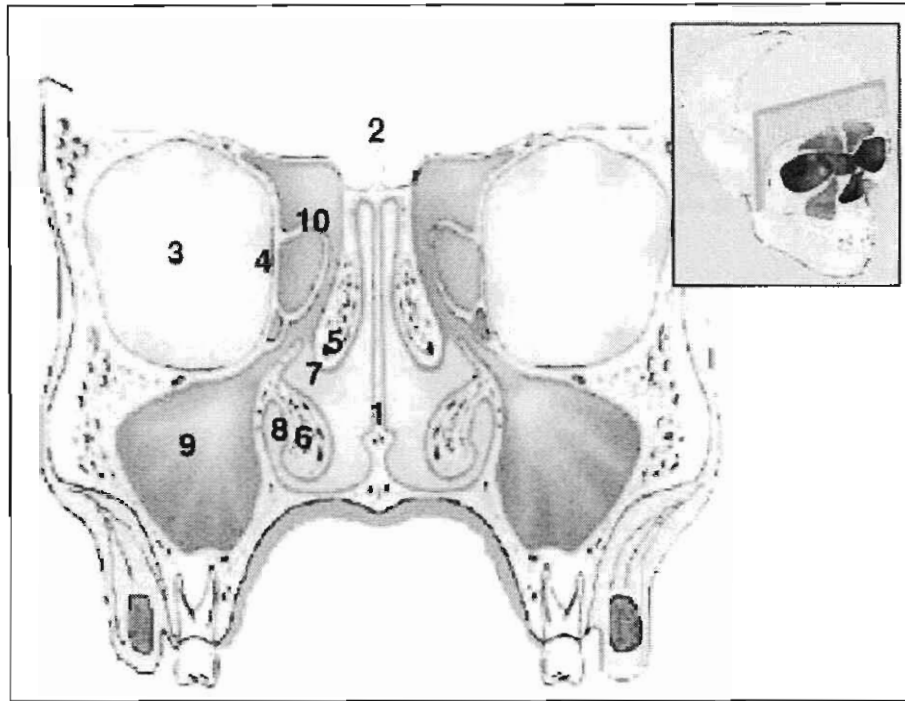


Figure 1.1 Anatomy of the nasal and paranasal cavities (frontal section). 1) Nasal septum, 2) crista galli, 3) orbit, 4) lamina papyracea, 5) middle turbinate, 6) inferior turbinate, 7) middle meatus, 8) inferior meatus, 9) maxillary sinus, 10) ethmoid sinus (Watelet & Cauwenberge, 1999:17).

The turbinates increase the total surface area. The narrow passages are responsible for an increase in the turbulent airflow, so that contact with the mucus surfaces for the efficient conditioning of inspired air is ensured.

Paranasal sinuses (ethmoidal, sphenoidal maxillary and frontal) are the large air-filled spaces within the facial bones. The sinuses open into the nasal cavity and are lined with the same lining as the nasal cavity, which is continuous with the nasal cavity, namely the mucus membranes. The anatomy of the paranasal cavities is illustrated in figure 1.1. Mucus secretions drain into the nasal cavity from the sinuses. Nasal infections or allergic reactions (sinusitis) may cause inflamed and swollen membranes, which may block the drainage and increase pressure within a sinus, causing a headache. The sinuses also serve as a resonant chamber that affects voice quality (Shier *et al.*, 1999:741).

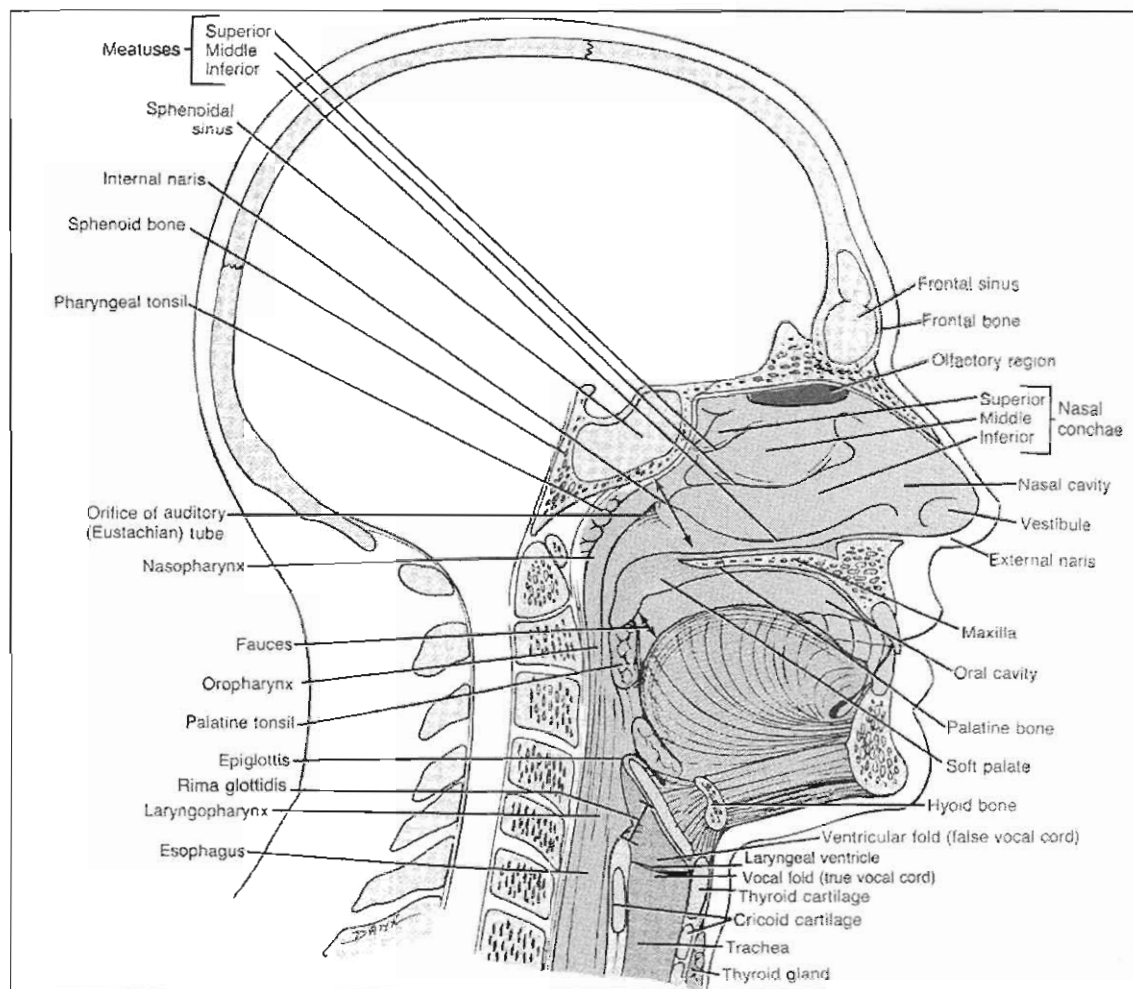


Figure 1.2 Diagram of the left side of the head and neck seen in sagittal section, with the nasal septum removed (Tortora & Anagnostakos, 1990:692).

1.2.1 Functions of the nose

The nose is not only a sensory organ but also conditions, heats and humidifies inspired air before it reaches the lungs. The normal function of the nose is closely related to its anatomy (Taylor, 2002:489). The three distinct functional zones in the nasal cavity are the vestibular, respiratory and olfactory regions (arranged anteroposteriorly in this order). In these three regions, the structure of the epithelial membranes varies considerably according to their function. This will be discussed in more detail in the following paragraphs.

The vestibular area is a short chamber inside the nostrils and its surface is lined with the pseudostratified epithelium with long hair. This makes it possible for airborne particles to be filtered (Marom *et al.*, 1984:36; Ridley *et al.*, 1992:15).

The respiratory mucosa consists of pseudostratified columnar epithelium, covered by dense layers of mucus. A powerful system of motile short cilia moves the mucus toward the posterior openings by sweeping the secretions of the goblet cells and mucus glands toward the nasopharynx (Chien *et al.*, 1989:2; Marom *et al.*, 1984:36). This region has a very large surface area and is vascular. The main function of this region is to condition inspired air and to warm, humidify and clear away large particles and watersoluble gases (Ridley *et al.*, 1984:15). It is also in this region where drug absorption is optimal (Ugwoke *et al.*, 2000:4).

The olfactory region is adjusted for the function of smell and has an area of about 10 cm² (Chien *et al.*, 1989:2; Ridley *et al.*, 1984:15). This region is situated above the superior nasal turbinate and possesses specialised ciliated olfactory nerve cells for smell perception. The central axons of these nerve cells pass through the cribriform plate of the ethmoid and into the olfactory bulb (Ugwoke *et al.*, 2000:4). This is the most superior-posterior and protected area of the nasal cavity which is normally free of inspiratory airflow, as the airway here has a width of only 1-2 mm. This surface is guarded with long, uncoordinated cilia and is washed out with a mucus secretion (Marom *et al.*, 1984:36).

1.2.2 Nasal epithelia

In the human nasal cavity, three types of epithelia can be found, namely stratified squamous, olfactory and respiratory epithelium (Illum & Fisher, 1997:141). Squamous epithelium occurs from the nasal vestibule to the turbinates, i.e. the anterior area of the nose (Taylor, 2002:491). The olfactory region (the upper part of the nasal cavity) is lined with olfactory epithelium (Illum & Fisher, 1997:141 and Taylor, 2002:491).

The basement membrane, which is a dense layer of protein polysaccharide and a rich intercellular substance, lies between the underlying connective tissue and any of the types of epithelia (Illum & Fisher, 1997:141). Sensory olfactory, serous- and mucosal cells are all located in the olfactory membrane. This makes it possible for a large proportion of inspired air to move over this region (Taylor, 2002:491). The different types of cells that constitute the nasal epithelium are shown in figure 1.3.

1.2.2.1 Stratified squamous epithelium

The stratified squamous epithelium lines the nasal vestibule (nostrils) and is a continuation of the facial skin (Illum & Fisher, 1997:141). It is characterised by a stratified keratinised and squamous epithelium that contains nasal hairs as well as sweat- and sebaceous glands (Duchêne & Ponchel, 1993:103). These cells are rounded or elongated in shape and does not have any cilia or villi in its rather rough surfaces. Only stiff nasal hairs (vibrissae) occur in this region, thus forming the first defense against inspiration of large particles. This type of epithelium is found over more exposed areas like the turbinate, but also in the posterior parts of the respiratory area (Illum & Fisher, 1997:141).

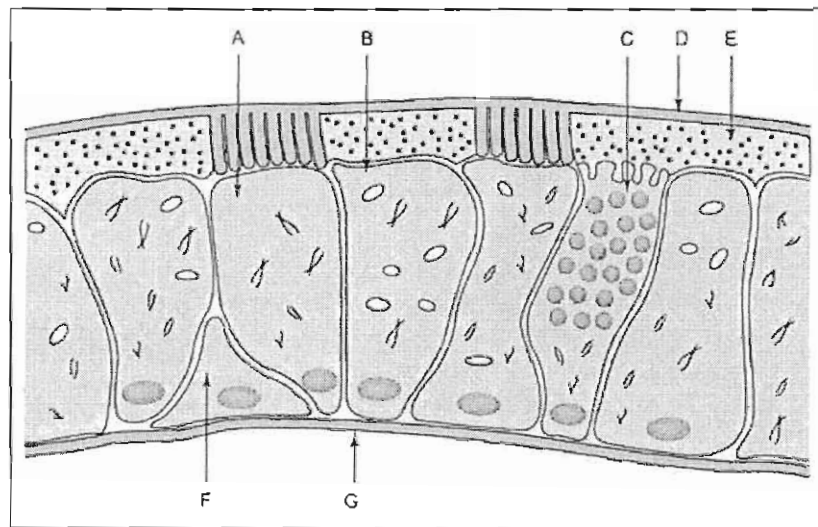


Figure 1.3 Cell types of the nasal epithelium showing ciliated cell (A), non-ciliated cell (B), goblet cells (C), gel mucus layer (D), sol layer (E), basal cell (F) and basement membrane (G) (Arora *et al.*, 2002:970).

The stratified squamous epithelium loses its keratine behind the ostium and becomes a mucosa. In the beginning this epithelium, which is progressively covered with microvilli in the turbinate, is without microvilli (Duchêne & Ponchel, 1993:103).

1.2.2.2 Olfactory epithelium

The olfactory mucosa contains the receptors for the sense of smell. These receptors are distributed in the upper region of the nasal septum and in the roof of the nasal cavity (Hinchcliffe & Illum, 1999:202). Olfactory receptors act as dendritic endings of the olfactory nerve that

respond to any chemical stimuli, which transmit the sensation of olfaction directly to the olfactory portion of the cerebral cortex (Van de Graaff, 2000:479). The olfactory receptor area within the roof of the nasal cavity is illustrated in figure 1.4. This is specialised pseudostratified epithelium, composed of three main cell types: (1) olfactory cells (bipolar primary olfactory neurons), (2) basal cells and (3) sustentacular (supporting) cells, which bear numerous microvilli (Illum & Fisher, 1997:141). The thickness of the olfactory epithelium varies from 60 to 70 μm (Duchêne & Ponchel, 1993:103).

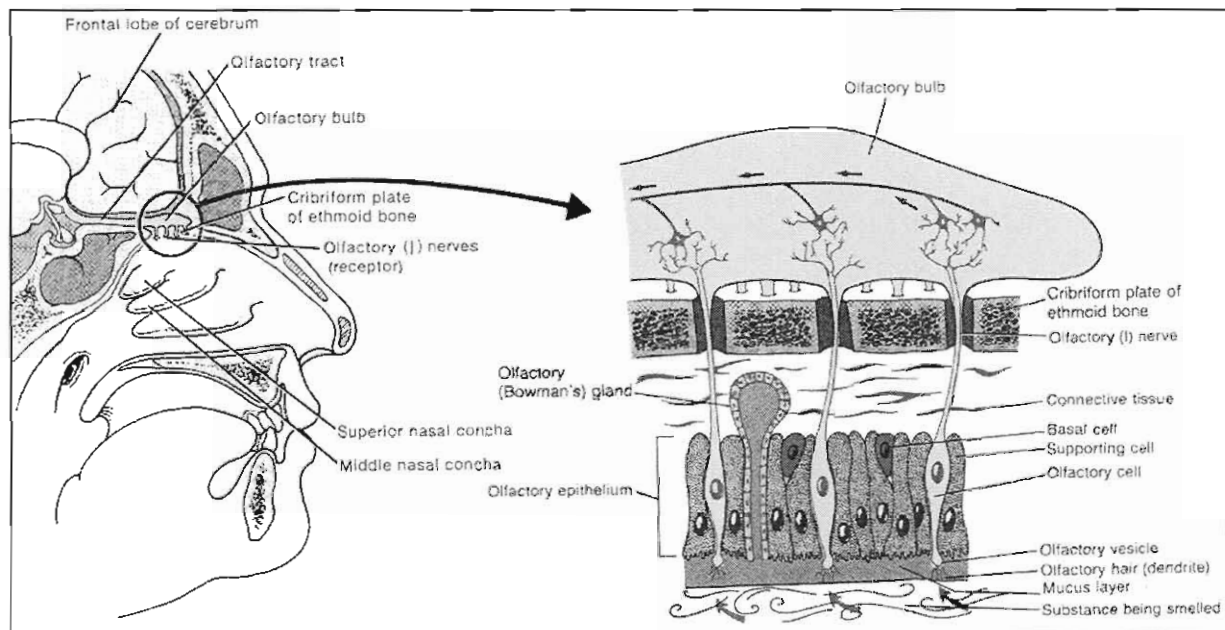


Figure 1.4 Olfactory receptor area within the roof of the nasal cavity (Tortora & Anagnostakos, 1990: 463).

1.2.2.3 Respiratory epithelium

This pseudostratified columnar epithelium covers most of the areas in the nasal cavity (Illum & Fisher, 1997:142). It is composed of the precursors of cylindrical cells (ciliated and non-ciliated) called basal cells, and of goblet cells (Duchêne & Ponchel, 1993:104). It is believed that basal cells help the adherence of columnar cells to the basement membrane. These respiratory epithelium cells are interdispersed between the columnar cells (Illum & Fisher, 1997:142). The goblet cells have microvilli on their surfaces, but the existence of mucus granules is a very specific characteristic.

Ciliated cells are the most commonly occurring type of cells. Microvilli occur on non-ciliated cylindrical cells, are 3.0 μm high, and have a diameter of 0.1 μm (Duchêne & Ponchel, 1993:104). Both ciliated and non-ciliated cells are covered with microvilli and there are about 300 of them per cell (Illum & Fisher, 1997:142). The respiratory epithelium is thinner than the olfactory epithelium (20-30 μm) (Duchêne & Ponchel, 1993:104). This is the region in the nasal cavity where drug absorption is optimal.

A continuous thin sheet of mucus, produced from the goblet cells, basal cells and the seromucous glands, covers the surface of nasal epithelia (Ugwoke *et al.*, 2000:4, Illum, 2002:491). The mucus is arranged in two layers – a viscous gel layer (epiphase) which floats on a less viscous sol layer (hypophase). This double layer is directly adjacent to the epithelial surface and is renewed every 10-15 minutes. Mucosal glands and goblet cells therefore continuously secrete new mucus (Duchêne & Ponchel, 1993:105, Hinchcliffe & Illum, 1999:203 and Illum & Fisher, 1997:146).

Goblet cells are unicellular, with microvilli on their surfaces (illustrated in figure 1.5). These microvilli have a short clublike appearance and each ciliated cell has approximately 500 microvilli on its surface (Chien *et al.*, 1989:5). They are referred to as gland cells, which are abundantly and unevenly distributed throughout the epithelium. The secretions of goblet cells are rich in carbohydrates, therefore these cells are essential to the protective surface mucus layer (Illum & Fisher, 1997:142).

The fine hairlike structures on the free surface of epithelial cells are called cilia. Every cilium is anchored to a so-called basal body and consists of two central protein microtubules. Each ciliated cell has approximately 500 cilia, is about 20 μm high and 70-80 % of them are superficial cells. Cilia move in such a way that the mucus flow across the epithelial surface is co-ordinated (Illum & Fisher, 1997:142). While making contact with the gel layer, the tips of the cilia push the gel layer, while the sol layer remains relatively motionless (Hinchcliffe & Illum, 1999:203).

Basal cells are multilateral and have various microvilli-like processes on their surfaces. These cells have wider intercellular spaces and looser connections in the lining epithelium than in the ciliated cells. A homogenous gelatine-like substance covers the subepithelial layer, which is

packed with fibrils. Individual fine fibrils form bundles, with branches in order to connect to the adjacent fibrils and form a network. (Chien *et al.*, 1989:5-6).

Figure 1.5, a histological section, shows the typical appearance of the nasal mucosa. Surface cells are mainly ciliated columnar cells, interspersed with goblet cells (Sarkar, 1992:2).

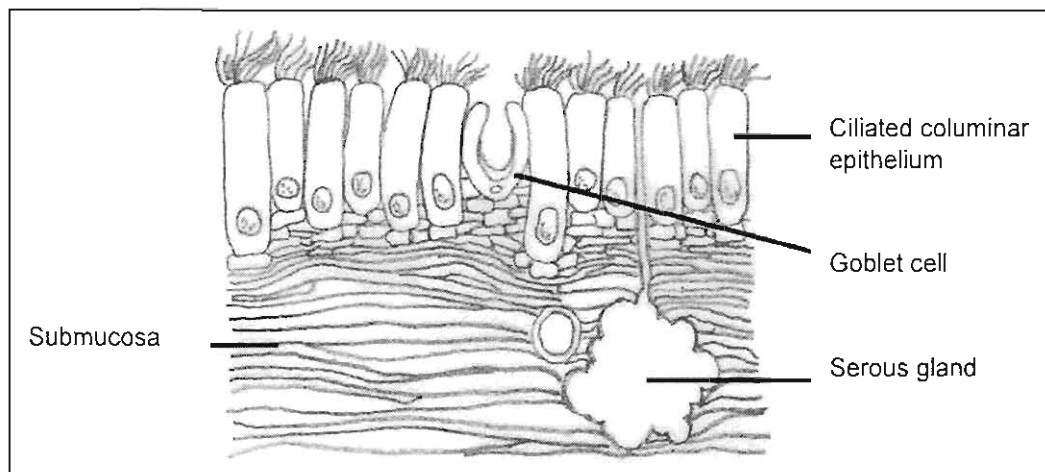


Figure 1.5 Diagrammatic representation of the microscopic appearance of the nasal mucosa (Sarkar, 1992:2).

In general epithelial cells are in close contact with their neighbouring cells, but not all the junctions between epithelial cells are the same. However, connections between ciliated cells are stronger than those between goblet cells and ciliated cells or between goblet cells, i.e. the epidermal region determines the protection of the epithelial barrier (Duchêne & Ponchel, 1993:104).

1.2.3 Nasal secretions

The respiratory area of the nasal cavity is covered with a blanket of visco-elastic fluid of approximately 5 μm thick and consists of two layers – a lower sol (watery) and an upper gel (mucus) layer. The nasal secretion is a complex mixture of secretory material from goblet cells, nasal- and lacrimal glands. However, a great quantity is secreted from the submucosal glands. The nasal mucus is covered by a thin layer of clear mucus, which is secreted from the nasal mucosa and submucosa. The cilia in the nasal cavity move the mucus via the posterior part of the nose at a rate of 1 cm/min in order to renew the nasal mucus about every 10 minutes. In the

nasal cavity of a healthy human being, a total of approximately 1500-2000 ml nasal mucosa is produced daily. The composition of this mucus is 90-95 % water; 2-3 % mucin and 1-2 % salt. Various electrolytes (sodium, potassium and calcium) as well as a great range of proteins are present in the mucus (Chien *et al.*, 1989:6-7).

The nasal mucus performs the following physiological functions:

- It acts as a carrier system for foreign particles and substances in the nasal duct;
- It is an adhesive and transports particles (e.g. drug molecules) in the direction of the nasopharynx;
- It can hold water;
- There is surface electrical activity on it;
- It is a permeable mesh (e.g. drug delivery); and
- It allows heat transfer (Chien *et al.*, 1989:6-7).

1.2.4 Nasal mucocilliary clearance

The most important physiological defence mechanism responsible for respiratory route clearance is known as mucocilliary clearance (MCC), i.e. the combined action of the cilia and the mucus layer in the nasal cavities (Chien *et al.*, 1989:7; Duchêne & Ponchel, 1993:105). A very important function of the upper respiratory tract is the prevention of allergens, bacteria, viruses and toxins from entering the lungs. When any of these noxious substances dissolve in or adhere to the mucus lining of the nasal cavity, they are transported in the direction of the nasopharynx. Factors that affect the mucus or cilia would obviously also influence the MCC (Ugwoke *et al.*, 2000:5).

Mucus is a mixture of glycoprotein (mucin), lipids, enzymes (proteolytic enzymes), bacterial products, antibodies and (of course) water. Although the temperature and relative humidity of the ambient air do not have any influence on the MCC (Chien, *et al.*, 1989:9), inhaled gases, locally applied drugs, bacterial or viral infections, tobacco smoke or environmental exposure to large amounts of wood, dust and chromium vapours are all factors that can severely impair the cilliary function (Watelet & Van Cauwenberge, 1999:17). Cilliary activity can also be blocked

by the nasal mucus getting dry. However, if the nasal mucosa is moistened, normal activity is immediately restored (Chien *et al.*, 1989:9).

As a result, MCC is a very essential defence mechanism for the entry respiratory tract. A mucus imbalance (i.e. too viscous or too watery, too little or too much) and impaired ciliary movement both affect the MCC. Enzymes or immunologically active materials deactivate any unwanted material in the mucus layer. These unwanted materials are removed from the respiratory system by transporting them to the external surface (mouth or nose) or the stomach (Illum & Fisher, 1997:147).

1.2.5 Nasal pH

How effectively a drug is absorbed is affected by the environmental pH. In adults, the pH of the nasal cavity varies between 5.5 and 6.5, whereas in young children it varies from 5.0 to 7.0. When nasal pH is lower than pka of the drug, greater drug permeation is achieved, because then the penetrant molecules exist as non-ionised species. Ionisation can be affected by pH-changes in the mucus, thus increasing or decreasing the degree of permeation of the drug (Arora *et al.*, 2002:971).

In certain pathological conditions such as acute sinusitis and rhinitis, the pH of nasal secretions is more alkaline. When the clinical resolution stage is reached, the pH of the nose becomes more acidic. Cold or heat can alter the course of nasal pH. Heat produces a drift to the more acidic side, while cold air on the other hand yields a drift towards alkalinity. Other factors such as emotions, food ingestion, sleep, rest and infections all influence the pH of nasal secretions (Chien *et al.*, 1989:17).

The pH of a formulation can alter the pH of the nose and vice-versa. It is therefore essential that the formulation should have an ideal pH between 4.5 and 6.5 to ensure optimal absorption and so that it has a buffering capacity if possible (Arora *et al.*, 2002:971).

1.2.6 Nasal vascularisation and innervation

Blood vessels in the nose have important physiological roles in the humidification and regulation of inhaled air. These blood vessels are also involved in the control over nasal resistance. They form a rich vascular network which is essential for drug absorption as well as for the exchange of heat and moisture (Chien, *et al.*, 1989:9). Blood reaches various tissues and organs before reaching the liver, in order to escape the first pass portal system (figure 1.9). This bypassing of the portal system prevents the degradation of drugs (Duchêne & Ponchel, 1993:104).

Autonomic innervations control the nasal blood flow. The stimulation and innervation of the predominant alpha-adrenoreceptors (constrictors) reduce the nasal blood flow. This stimulation aids the decongestion of the nasal venous erectile tissue. Beta-adrenergic receptors (dilators) can also be stimulated, and lead to an increase in blood flow. The parasympathetic innervation of glandular cholinergic receptors leads to vasodilatation and hypersecretion. As a result, the innervation of adrenergic (sympathetic) fibres is the most important in the vascular system, while the innervation of cholinergic (parasympathetic) fibres plays a dominant role in the nasal glands (Chien *et al.*, 1989:12).

1.2.7 Drug metabolism in the nasal cavity

There are various enzymes (for example conjugate mono-oxygenase enzymes, proteases and aminopeptidases) in the nasal mucosa, which act as an enzymatic barrier to the delivery of drugs (Arora *et al.*, 2002:969). Even though the hepatic first pass effect in the nose has been avoided, the enzymatic barrier of the nasal mucosa creates a pseudo-first-pass-effect. This pseudo-first-pass-effect is then responsible for the degradation of drugs (Sarkar, 1992:1).

The levels of mono-oxygenase enzymes such as cytochrome P-450-dependent mono-oxygenases are much higher in the nasal epithelium than in the liver, which may be due to the 3 to 4 fold higher NADPH-cytochrome P-450 reductase content (Hinchcliffe & Illum, 1999:204).

Protease and peptidase are responsible for drug degradation and consequently leads to a lower permeation level of peptide drugs (Arora *et al.*, 2002:970). Amino peptides with membrane bound amino peptidases account for more or less half of the total enzyme activity. These

proteolytic enzymes in the nasal cavity act as an important protective mechanism against any proteinaceous material penetrations (Hinchcliffe & Illum, 1999:204).

Various nasal mucosal enzymes deactivate the nasal delivery of drugs. Nasal enzymes should therefore be reduced in order to minimise the enzymatic degradation of the drug (Ugwoke *et al.*, 2000:9). Enzyme inhibitors improve the absorption of drugs and therefore act as absorption enhancers. Specific enzyme inhibitors such as bacitracin, puromycin, boroleucin and amastatin are used to overcome such degradations (Arora *et al.*, 2002:970).

The absorption enhancers have many disadvantages, such as (a) membrane protein removal, (b) surface changes in the nasal cavity, (c) excessive mucus discharge, (d) cell loss, and (e) cytotoxicity. This is why many compounds are excluded from nasal formulations indicated for chronic therapy (Chandler *et al.*, 1991:62).

1.3 Factors influencing nasal drug absorption

A drug molecule can cross the nasal epithelium by one of the following two main mechanisms: (1) the transcellular pathway (across cells) or (2) the paracellular pathway (between cells). The paracellular pathway is passive, whereas the transcellular pathway is both passive and active and can further be divided into (1.1) simple passive diffusion, (1.2) carrier-mediated transport (active transport and facilitated diffusion) and (1.3) endocytosis. These pathways are illustrated in figure 1.6.

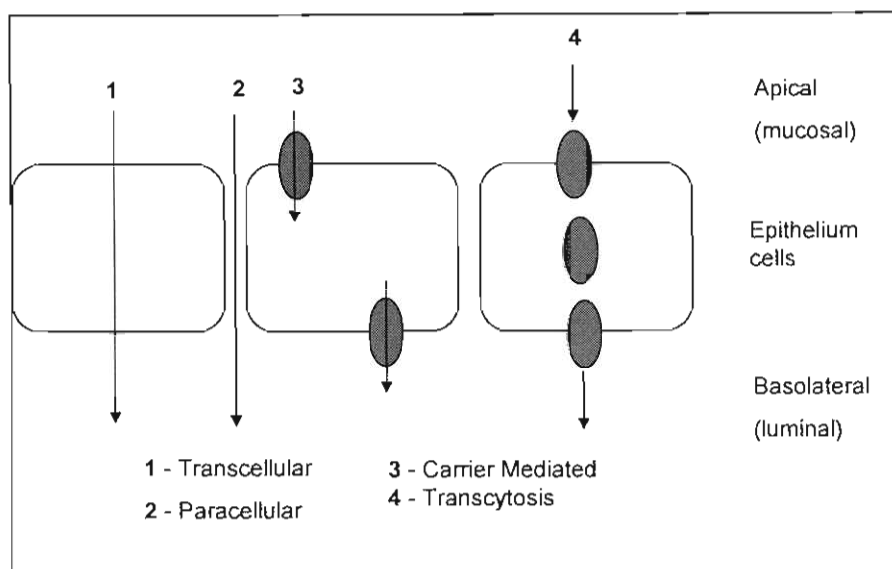


Figure 1.6 Mechanisms of transport across the nasal membrane (Ashford, 2002:227).

The passive diffusion of drugs is the major absorption process for most drugs. It is assumed that the same mechanism occurs in the nasal cavity as in the gastrointestinal tract. In this process drug molecules spontaneously cross the lipoidal membrane, from a region of higher concentration (the nasal cavity) to one of lower concentration (the blood) (Ashford, 2002:227).

Physicochemical properties of the drug, the nature of the membrane and the concentration gradient of the drug across the membrane determine the transportation rate of the drug molecule. This passive diffusion process involves the dividing of the drug between the mucus layer and the cell membrane in the nose. When the drug is in solution, it diffuses across the epithelial membrane. Drug molecules move in this manner through successive cell membranes, until they finally reach the capillary network in the lamina propria. To maintain a much lower drug concentration than at the absorption site, the drug will be rapidly distributed as soon as it reaches the blood (Ashford, 2002:227). The different stages of nasal drug absorption by means of passive diffusion can be seen in figure 1.7, if the mucus layer and the cell membrane (making up the nasal blood barrier) can be regarded as a single membrane, which divides the sol and gel layers in the capillary blood in the lamina propria (Ashford, 2002:228).

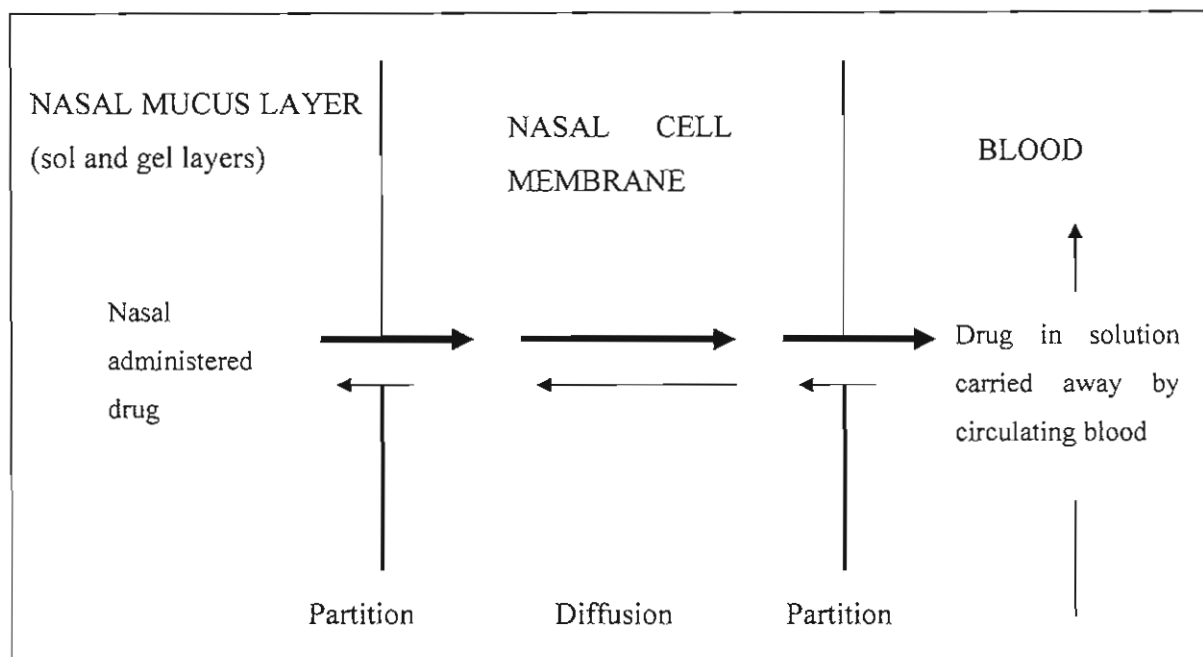


Figure 1.7 Schematic representation of drug absorption via passive diffusion in the nasal cavity (Ashford, 2002:228).

Fick's first law of diffusion, which mathematically expresses the passive diffusion of drugs across the nasal bloodbarrier, is explained by equation 1.1 (Shargel & Yu, 1999:102).

$$\frac{dQ}{dt} = \frac{DAK(C_n - C_p)}{h}$$

Equation 1.1

Where:	dQ/dt	→	Rate of diffusion
	D	→	Diffusion coefficient of the drug
	A	→	Surface area of membrane
	K	→	Lipid-water partition coefficient of drug in the biologic membrane that controls drug permeation
	$(C_n - C_p)$	→	Difference between concentrations of drug in the nasal cavity and in the plasma
	h	→	Thickness of the membrane

From equation 1.1 it is obvious that the constants; D, A, K and h influence the passive diffusion rate of a drug. For instance, the surface area of a membrane, A, depends on the diffusion rate of nasal absorption of a drug by passive diffusion. A large diffusion coefficient value, D, which indicates how much of a drug diffuses across the membrane of a particular area per unit time, also increases the diffusion rate of absorption. The more lipid-soluble a drug, the larger its K-value, which indicates the lipid-water-partitioning, and this increases the rate of diffusion. On the other hand, the membrane, h, must clearly be thin in order to increase the diffusion rate. Fick's first law of diffusion also states that the rate of diffusion across a membrane, dQ/dt , is proportional to the difference in concentration on both sides of the membrane.

Under normal absorption conditions D, A, K and h are constants, and therefore a permeability coefficient, P, may be defined in the following equation:

$$P = \frac{DAK}{h}$$

Equation 1.2

It is assumed that the drug concentration in plasma, C_p , is very small comparing to the drug concentration in the nasal cavity, C_n . Therefore, C_p is negligible, and P is substituted into equation 1.3.

$$\frac{dQ}{dt} = P(C_n)$$

Equation 1.3

Most drugs tend to be absorbed by means of a first-order absorption process. Equation 1.3 indicates an expression for a first-order process. The rate of drug absorption is more rapid than the rate of drug elimination, due to the large concentration gradient between C_n and C_p . Only the concentration of the drug in solution in the nasal-fluids at the absorption site influences the rate of passive absorption.

To formulate and design a unique device for intranasal administration, factors such as physiological conditions, the physicochemical properties of the drug and the properties of the dosage form are all essential in drug absorption via the nasal cavity.

The intranasal route is known for its excellent potential to deliver peptide drugs in order to achieve a systemic effect. On the other hand, many factors have been identified that could influence the nasal absorption of drugs. Figure 1.8 summarises various factors that affect the nasal absorption of drugs to some extent. The three main factors, namely physicochemical, formulation and anatomical and physiological factors (see below) will be discussed in detail.

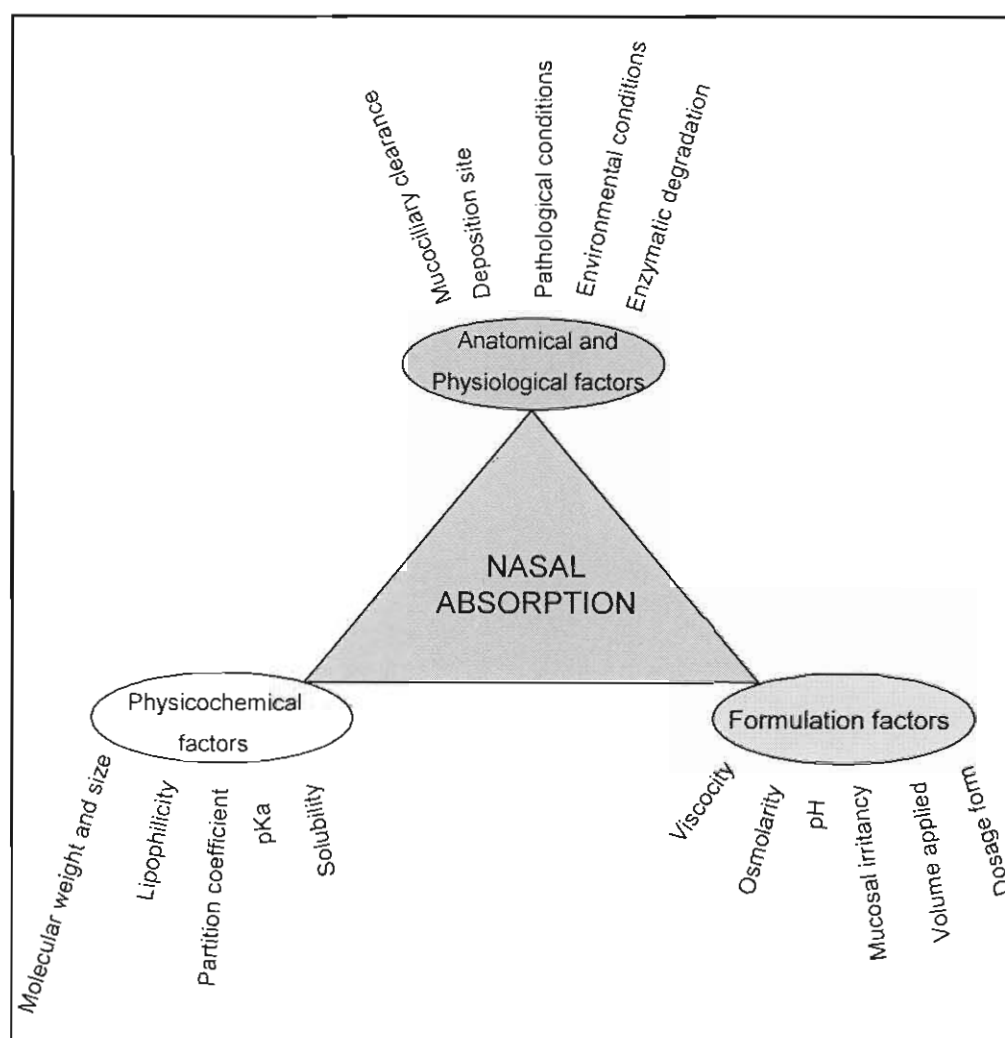


Figure 1.8 Factors influencing nasal absorption

1.3.1 Anatomical and physiological factors

1.3.1.1 Mucocilliary clearance and site of deposition

Two very important factors for the absorption of peptides and proteins in the nose, after the administration of a nasal formulation, are (1) the site of deposition and (2) mucocilliary clearance (MCC). The mucocilliary clearance mechanism rapidly clears particles deposited in the nasal cavity, with a half-life ($t_{1/2}$) of clearance of approximately 15 – 30 minutes (Illum, 1995:515).

Mucocilliary clearance is a normal defence mechanism that clears mucus as well as substances adhering to the nasal mucosa (e.g. bacteria and allergens). These substances are then drained into the nasopharynx, where they eventually discharge into the gastrointestinal tract. The mechanism clears substances from the nasal cavity within 21 minutes. The contact time between a drug and the mucus membrane increases with a reduced MCC, which enhances drug permeation. An increased MCC, on the other hand, leads to a decrease in drug permeation (Arora *et al.*, 2002:971).

The size of administered droplets or powder particles is very important, as it determines the absorption site. Small particles (diameter less than 1 μm) travel down to the lower respiratory tract (trachea) and are then deposited in the nasal cavity. Larger particles (greater than 10 μm), are trapped in the mucus layer and deposited in the nasal cavity (Hinchcliffe & Illum, 1999:205).

The larger the particle size the more anterior the deposition, so that smaller particles are deposited further back in the cavity. Additional factors such as the velocity of the air current and the turbulence of airflow influence the nasal absorption of particles smaller than 1.0 μm (Illum, 1995:515, Hinchcliffe & Illum, 1999:205 and Illum & Fisher, 1997:147).

1.3.1.2 Pathological conditions

Many pathological conditions, e.g. allergic or atrophic rhinitis, chronic sinusitis, nasal polyposis and virus infections such as the common cold, are associated with irritation of the nasal mucosa and the hypo- or hypersecretion of mucus. These pathological conditions tend to impair mucocilliary function. Excessive mucus production from rhinorrhoea and the abovementioned other conditions reduces the clearance of drug formulations from the nasal cavity (Arora *et al.*,

2002:971, Chien *et al.*, 1989:19 and Hinchcliffe & Illum, 1999:205). In short, pathological conditions cause inappropriate drug distribution in the nose, due to an adverse effect on MCC, which then affects nasal drug absorption.

1.3.1.3 Environmental conditions

The rate of MCC reduces moderately with temperatures in the range of 24°C whereas the ciliary beating frequency increases with an increase in temperature (Gizurason, 1993:329)

1.3.1.4 Enzymatic degradation

The nasal mucosa is an active enzymatic barrier and must be considered one of the reasons for low bioavailability. However, the metabolism in the nose is not as extensive as that in the gastrointestinal tract (Duchêne & Ponchel, 1993:111).

Drugs that are systematically delivered through a range of body cavities that contain absorptive mucosa (e.g. nasal route of administration) have the following two advantages: (1) They bypass the “first-pass“(hepato-gastrointestinal) clearance, following oral delivery; and (2) They avoid the health risks related to parenteral administration.

A study by Hirai *et al.* indicated that after being in contact with nasal tissue homogenate for 60 minutes insulin was rapidly degraded, with only 9.0 % remaining intact (Hirai *et al.*, 1981:173). Moreover, Kashi and Lee (1986:2020) found that methionine enkephalin, leucine enkephalin and (D-Ala2) met-enkephalinamide were all rapidly hydrolysed in homogenates from the nasal tissue with a degradation half life of about 25 minutes. It was discovered that after incubation thyrotropin-releasing hormone was extensively degraded in rabbit nasal homogenate, while there was no degradation in human nasal wash (Jørgensen & Bechgard, 1994:233). The same study also showed that degradation could be reduced by adding sodium glycocholate, which has an enzyme-inhibitory effect.

Consequently nasal enzymes present a significant although not major barrier to the intranasal absorption of peptide and protein drugs. Figure 1.9 illustrates a comparison between different routes of systemic drug delivery (Chien, 1991:44).

1.3.2 Physicochemical factors

The following basic physicochemical properties need to be determined in order to develop a successful nasal formulation: (1) molecular size and weight, (2) lipophilicity, (3) partition coefficient, pK_a , and (4) chemical stability.

1.3.2.1 Molecular weight and size

The nasal route is appropriate for the effective and rapid delivery of molecules with a molecular weight (MW) smaller than 1000 dalton. The MW of a compound can have a direct effect on its bioavailability (Arora *et al.*, 2002:971).

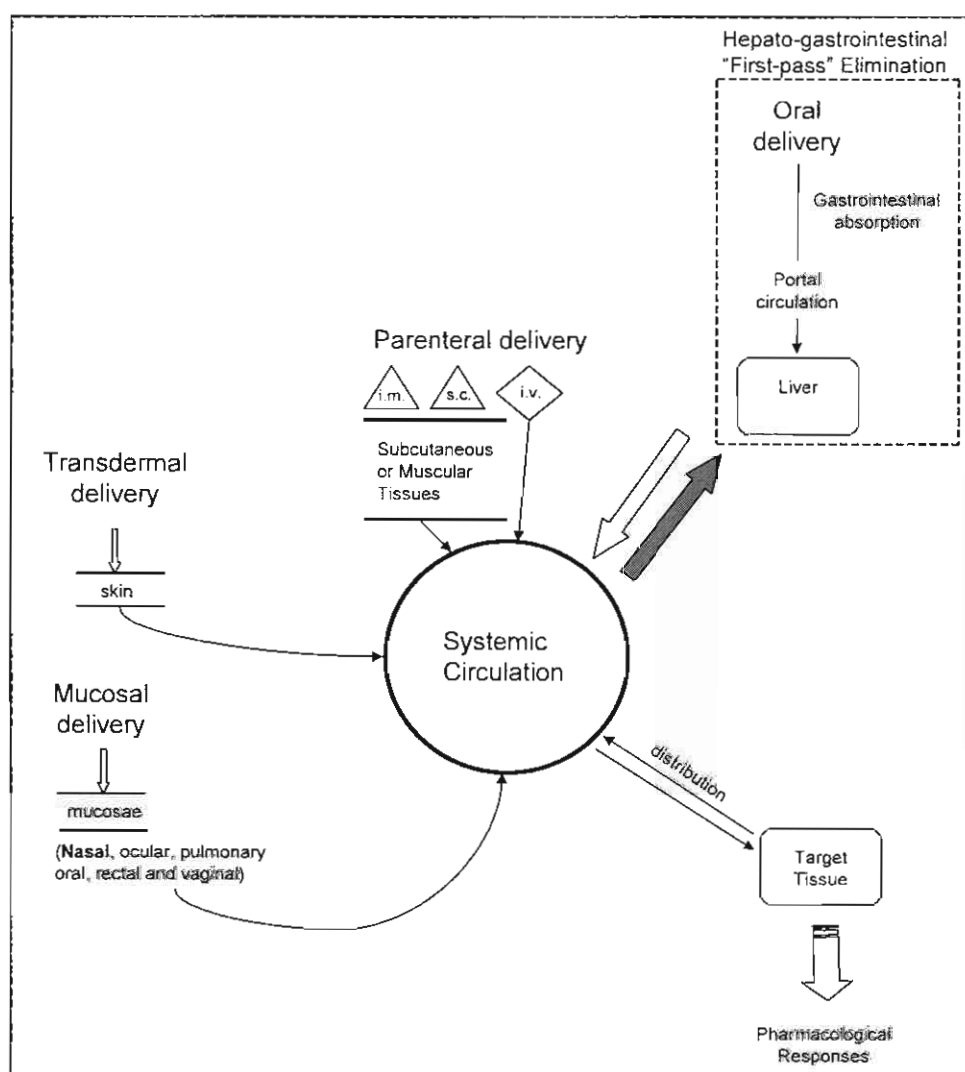


Figure 1.9 Systemic drug absorption through different routes of administration

When a drug is nasally delivered, the molecular weight cut-off point was more or less two orders of magnitudes greater (approximately 20 000 dalton) than for peroral delivery (about 200 dalton). Only molecules smaller than the channel can diffuse through a channel, which is why the so-called molecular cut-off occurs (Taylor, 2002:492).

Nasal drug absorption decreases exponentially, as the MW of the penetrant increases when the MW is >1000 dalton. The results indicate that nasal absorption is less dependent upon MW compared to oral absorption.

The relationship between nasal absorption is indicated in equation 1.4

$$\%absorption = 100[1 + a(MW)^b]$$

Equation 1.4

1.3.2.2 Lipophilicity

Drug lipophilicity plays a very important role in nasal drug delivery. When lipophilic drugs are absorbed from the nasal cavity, their pharmacokinetic profiles are often identical to those obtained after an intravenous injection, with a bioavailability of 100% (Illum, 2003:188).

A compound with a high lipophilicity also shows increased permeation through the nasal mucosa. Consequently, the higher the lipophilicity, the higher the permeation and thus the more rapidly and the better a drug is absorbed from the nasal cavity.

A study by Duchateau *et al.* (1986:110) indicated that compared to the hydrophilic drug metoprolol, the lipophilic compounds alprenolol and propranolol were well-absorbed from the nasal mucosa. An excess of hydrophilicity might decrease systemic bioavailability of many drugs (Arora *et al.*, 2002:972).

Hussain *et al.* (1985:925) studied the absorption of barbiturates at pH values at which these compounds exist entirely in their non-ionised lipophilic form. The extent of absorption was found to be closely related to the octanol / water partition coefficient.

1.3.2.3 Partition coefficient and pK_a

The partition coefficient is an indication of the lipophilic character of the drug. According to the pH partitioning theory, the non-ionised form of the drug is better absorbed (due to the greater lipid solubility) than the ionised form. The role of the partition coefficient was mainly recognised from the extent of the ionised and non-ionised forms of the drug (Taylor, 2002:493).

Equation 1.5 expresses the partitioning coefficient (P)

$$P = \frac{C_o}{C_w}$$

Equation 1.5

Where: P = Partition coefficient
 Co = Drug concentration in the organic phase
 Cw = Drug concentration in the watery phase

According to equation 1.5, the partition coefficient is an indication of the relative affinity of the drug for a watery and non-watery phase. Therefore, the higher the P-value, the greater the lipid solubility of the drug (Aulton, 2002:31).

In nasal drug delivery, not only the physicochemical properties of the drug, but also the stereochemical conformation during membrane transportation, determines the permeation of a drug molecule across the nasal mucosa (Chien, 1989:43).

Based on various observations, the partition coefficient is a major factor in controlling nasal absorption. This is because partitioning is rarely the only factor controlling absorption (Taylor, 2002:493).

1.3.2.4 Solubility

Solubility and the dissolution rate are extremely important when a drug exists as a solid dosage form (for instance a powder). This is because the drug must be able to cross the mucus layer before it can be absorbed by the epithelial cells in the nose (Taylor, 2002:493).

1.3.3 Formulation factors

Investigations into various formulation factors that influence absorption provide insight in the formulation of a drug for nasal delivery. The most important formulation factors are probably the viscosity, osmolarity, pH and mucosal irritancy of the formulation and of course the dosage form.

1.3.3.1 Viscosity

The higher the viscosity of the formulation, the longer the contact time between the drug and the nasal mucosa. This means that the time for permeation is increased, which enhances nasal absorption. The viscosity can also alter the permeability of drugs, by interfering with the normal defence mechanisms such as ciliary beating frequency and mucociliary clearance (Arora *et al.*, 2002:972).

1.3.3.2 Osmolarity

A study on the effect of osmolarity on the absorption of secretin in rats showed that absorption reached a maximum at a sodium chloride concentration of 0.462 M. Because the permeation of secretin decreases as a consequence, isotonic solutions are usually preferred for administration (Ohwaki *et al.*, 1985:550).

1.3.3.3 pH and mucosal irritancy

Many studies have shown that the extent of nasal absorption is pH dependent. Where the pH was lower than the pK_a , a greater nasal absorption was achieved. This is because the penetrant molecules exist as non-ionised species. As mentioned earlier, non-ionic species are better absorbed than ionic species. Due to the ionisation of the penetrant molecule, the rate of absorption in the nose decreases as the pH increases (Chien, 1989:41).

As previously mentioned, the pH of the nasal formulation should further be adjusted to 4.5 – 6.5 to avoid nasal irritation. This prevents the growth of bacteria and ensures efficient drug permeation (Arora *et al.*, 2002:972).

1.3.3.4 Volume of solution applied

The volume delivered to the nasal cavity is restricted to 0.05 – 0.15 ml. However, to use this volume effectively, various approaches were explored, such as the use of solubilisers, or gelling or viscofying agents (Abe *et al.*, 1995:2232).

Solubilisers (e.g. polyethylene glycol (PEG), polyols or surfactants) increase the aqueous solubility of insoluble compounds and can therefore enhance nasal absorption of the drug.

1.3.3.5 Dosage form

The dosage form in which a drug is incorporated is in fact a drug delivery system. The main objective of dosage form design is to reach a specific therapeutic area before the expected therapeutic response can be achieved. The choice of a suitable dosage form plays a very important role in achieving the clinical effect for a specific condition, and the type of drug delivered (York, 2002:1).

Different types of drug delivery systems for nasal drug delivery are: nasal drops, nasal sprays, powder formulations and metered-dose gel devices.

Nasal drops are the classic, simplest and most convenient form of drugs administered in the nasal route. The biggest disadvantage is that it is not easy to quantify exactly how much of the drug is delivered, which usually leads to overdose (Arora *et al.*, 2002:973).

The bioavailability of elcatonin (which is intranasally delivered in a powder dosage form) compared to a liquid dosage form, was investigated by Ishikawa *et al.* (2001:105). In this study, it was discovered that the powder formulation improves nasal bioavailability by increasing the residence time of elcatonin in the nose. However, powder may result in mucosal irritation, which may be a disadvantage.

Recent studies have developed metered-dose gel devices, which accurately deliver the drug in the nose. The metered-dose gel device has many advantages, such as localising the formulation in the mucosa and reducing the postnasal drip and anterior leakage (Arora *et al.*, 2002:973). Most studies deal with bioadhesive microspheres. The following drugs use this type of delivery system: Insulin, propranolol, human growth hormone, oxytocin and desmopressin. Microspheres offer better permeation of drugs, as they provide an intimate prolonged contact time between the drug and mucosal membrane (Duchêne & Ponchel, 1993:116).

1.3.4 Summary

A few challenges still need to be overcome for a drug to be successfully administered through the nasal cavity. The main factors that influence drug absorption in the nasal cavity are molecular size, membrane permeability, the enzymatic barrier of the nasal mucosa and mucociliary clearance. To support the optimal formulation for nasal delivered drugs and reduce the number of experimental efforts involved, it is necessary to establish a correlation between the physicochemical properties of the drug and formulation with those of the permeation rate.

It is possible to improve the nasal absorption of drugs by administering them in combination with an absorption enhancer, which promotes the transportation of the drug across the nasal membrane. However, many more efforts will be needed to make nasal drug delivery more popular and efficient.

1.4 Strategies to improve drug availability in nasal administration

Great possibilities have already been discovered for the utilisation of nasal administration to deliver numerous compounds. However, most peptides and proteins have insufficient nasal bioavailabilities. There are several possible approaches to enhancing the nasal absorption of peptide drugs. These are summarised in figure 1.10.

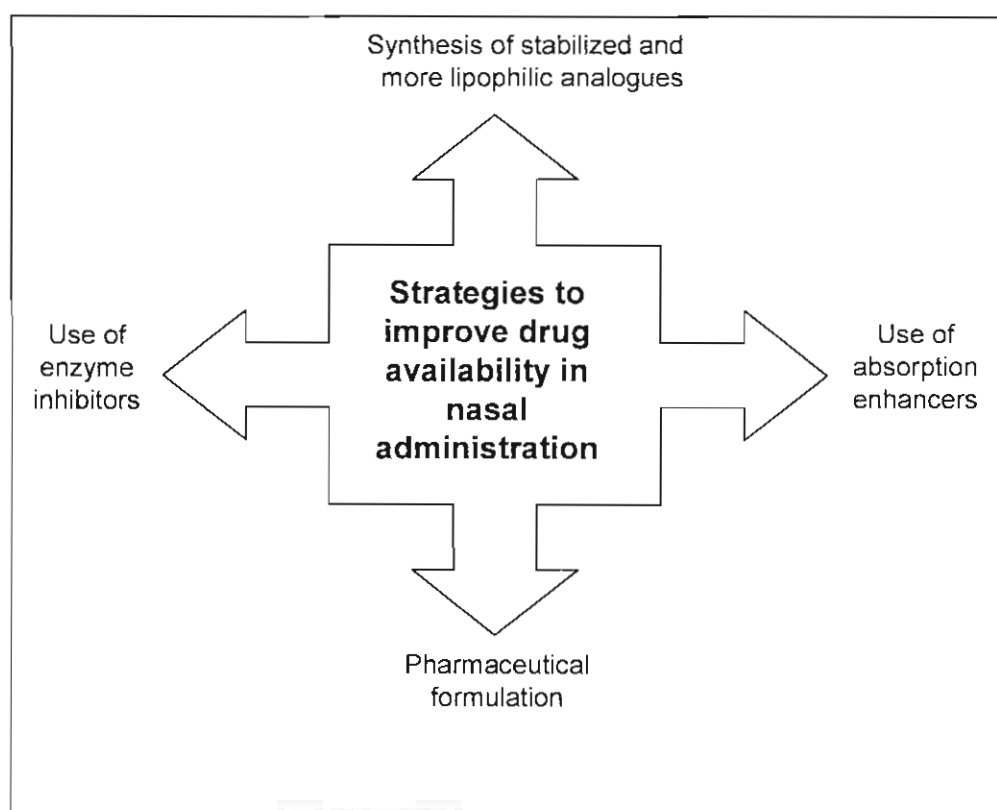


Figure 1.10 Summary of the different approaches to improving the nasal delivery of drugs

Many compounds have been investigated as absorption enhancers for protein and peptide drugs, which are usually classified according to the different chemical groups to which they belong. The main categories of absorption promoting systems are listed in table 1.1 (Hinchcliffe & Illum, 1999:209).

Table 1.1 Nasal absorption promoting excipients

<i>Type of compound</i>	<i>Examples</i>	<i>Mechanisms of action</i>
<i>Absorption enhancers</i>		
Bile salts (and derivatives)	Sodium deoxycholate, sodium glycocholate, sodium taurodihydrofusidate	Disrupt membranes, open tight junctions, enzyme inhibition
Surfactants	Sodium lauryl sulphate, saponin, polyoxyethylene-9-lauryl ether	Disrupt membranes
Chelating agents	Ethylenediaminetetraacetic acid, salicylates	Open tight junctions
Fatty acids (and derivatives)	Sodium caprylate, sodium laurate phospholipids	Disrupt membranes
Enzyme inhibitors	Bestatin, amastatin	Enzyme inhibition
Miscellaneous	Cyclodextrins	Disrupt membranes, open tight junctions
<i>Bioadhesive materials</i>		
Powders	Carbopol, chitosan, starch microspheres	Reduce nasal clearance, open tight junctions
Liquids	Chitosan, carbopol	Reduce nasal clearance, open tight junctions

The different strategies to enhance the systemic bioavailability of intranasal drugs will be discussed according to the four different approaches illustrated in figure 1.10.

1.4.1 Synthesis of stabilised and more lipophilic analogues

The two main approaches to improving the lipophilicity and metabolic stability of a drug will now be discussed in more detail.

Firstly, the synthesis of potent peptide analogues with increased lipophilicity and stability to enzymatic degradation was accomplished in four ways: (i) building in “unnatural” amino acids, (ii) cyclisation, (iii) derivatisation of the NH₂-terminus and (iv) using C-terminal blocking agents. This led to the development of metkephamid, the antidiuretic drug desmopressin and the luteinizing hormone (LH) agonist, buserelin (Verhoef & Merkus, 1994:128).

Nasally administered luteinizing hormone-releasing hormone (LHRH) analogues, used for the induction of ovulation, have a potency of 5-200 times more than the parent compound. However, the nasal bioavailability remains very low at 2-3 % (Sandow & Petri, 1985:183).

The design of prodrugs is a second interesting possibility in terms of improving the metabolic stability and lipophilicity of peptides. The prodrug would be rapidly absorbed and then converted to the parent peptide. Møss and Bundgaard (1990:40) found that the thyrotropin releasing hormone (TRH) enhances the percutaneous absorption of TRH. Although this method of absorption has not yet been studied in terms of nasal drug delivery, it holds great possibilities for improved nasal drug absorption in future.

1.4.2 Enzyme inhibitors

There are significant quantities of enzymes (for example peptidases and proteases) in the nasal epithelial tissue. These enzymes are able to degrade proteins, peptides and peptide drugs like enkephalins, insulin and substance P. Aminopeptidase is the predominant enzyme occurring in the nasal epithelia (Verhoef & Merkus, 1994:129).

Many studies have indicated that enzymatic degradation constitutes a major barrier, especially for smaller peptides in the nasal cavity. Several of the compounds mentioned in table 1.1, such as bile salts, surfactants and fatty acids, also possess enzyme inhibitory action, which will contribute to their general efficacy as absorption enhancers (Hinchcliffe & Illum, 1999:220).

Although enzyme inhibitors alone are not sufficient to increase the bioavailability of intranasal drugs, they are nevertheless an essential factor that contributes to the mechanism of action of several absorption enhancing compounds (Hinchcliffe & Illum, 1999:220).

1.4.3 Absorption enhancers

The use of absorption enhancers is the most frequently used method for improving the absorption efficacy of peptides and proteins for intranasal administration. The mechanism of an absorption enhancer is to increase the rate at which drugs pass through the nasal mucosa. This is done by altering the epithelial cell structure without causing any damage or permanent change (Taylor, 2002:494). An ideal absorption enhancer must measure up to the following requirements.

It should –

- ✓ give an effective increase in the absorption of the drug;
- ✓ not cause permanent damage or change to the tissues;
- ✓ not be an irritant or toxic, either to the local tissues or to the rest of the body;
- ✓ be effective in small quantities;
- ✓ ensure that the enhancing effect is temporary and reversible; and
- ✓ fulfil all other expectations of formulation excipients, for example stability and compatibility (Taylor, 2002:494).

According to Lee (1990:231), the majority of absorption enhancers are capable of increasing membrane fluidity. This is done either by creating disorders in the phospholipids in the membrane, or by facilitating the leaching of proteins and lipids from the membrane. Absorption enhancers such as bile salts, fatty acids and most phospholipids function by modifying the phospholipids bilayer structure of cells, leaching out proteins or stripping the outer layer of the mucosa, which promotes the transcellular transportation of drugs (Illum, 2002:1187). Figure 1.11 summarises the mechanisms of absorption enhancement.

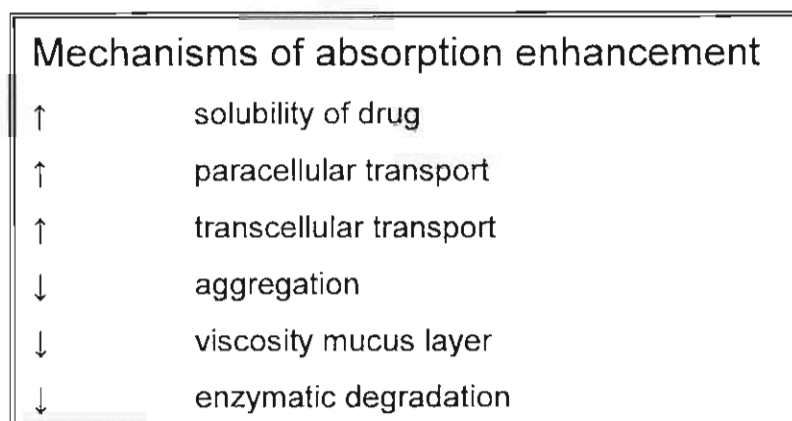


Figure 1.11 Mechanisms of absorption enhancement (Verhoef & Merkus, 1994:132)

The following is a discussion of various absorption enhancers, and of studies on their effect on certain drugs.

1.4.3.1 Bile salts

Bile salts appear to have an enhancing activity and claim to be safer than surfactants, but can still cause damage to epithelial cells. Examples of bile salts, which cause enhancement at concentrations of 10 – 20 mM, are sodium cholate, sodium deoxycholate, sodium glycocholate and glycodeoxycholate (Behl *et al.*, 1998:89).

The damaging potential of various bile salts and surfactants has been investigated. This investigation was done by using a measurement of ciliary beating frequency (CBF) and morphological damage as index. Measurements of CBF provide a relative easy quantitative measure of toxicity in time. Decreases in CBF indicate cellular damage and an increase in nasal residence time (Merkus *et al.*, 1993:201).

1.4.3.2 Surfactants

Various types of surfactants, for instance non-ionic ethers and esters, and anionic surfactants are particularly effective enhancers in the absorption of insulin (Hirai *et al.*, 1981). Surfactants are experimentally useful as reference compounds, which are guaranteed to cause enhancement (Hinchcliffe & Illum, 1999:220).

The efficiency of a nasal aerosol containing insulin and 1% laureth-9 (surfactant) in insulin dependent diabetes mellitus (IDDM) patients has been evaluated. In such patients, a formulation with a surfactant as an absorption enhancer has potential as an adjunct to subcutaneous insulin (Salzman *et al.*, 1985:1080).

1.4.3.3 Sodium taurodihydrofusidate (STDHF)

Sodium tauro-24, 25-dihydrofusidate (STDHF) has good aqueous stability (>10% w/v) and forms micelles at a critical micelle concentration of 2.5 mM. It is therefore surface active and has a structure similar to bile salts. The concentration of STDHF determines the degree of penetration enhancement at a maximum of 0.3% w/v (Taylor, 2002:495).

1.4.3.4 Phospholipids

Phospholipids are very similar to compounds that occur naturally as part of cell membranes. Phosphatidylcholines are part of the surface-active materials and the mechanism of action is the disruption of the cell membrane, in order to increase its permeability. It is also an effective protein absorption enhancer and does not cause the anticipated damage to the nasal lining (Taylor, 2002:495).

Hinchcliffe & Illum (1999:220) noted that dodecanoyl-L- α -phosphatidylcholine (DPPC) is one derivate, which has been developed with a high activity and low toxicity profile. Studies showed that DPPC increases the absorption of insulin in humans, with little or no irritation to the nose.

1.4.3.5 Cyclodextrins

Cyclodextrins are hollow cylindrical molecules that offer a wide range of pharmaceutical uses, from stability enhancement to taste masking, because it is able to form 'inclusion complexes'. The whole drug molecule or part thereof inserts itself in the hollow central cavity and takes on some of the physicochemical properties of the cyclodextrin molecule.

The bioavailability of lipophilic compounds is increased by cyclodextrins, by increasing their aqueous solubility, and therefore their availability. This is because cyclodextrins have polar outer surfaces and less polar interiors (Taylor, 2002:495).

1.4.4 Pharmaceutical formulation

The type of dosage form used for nasal drug delivery depends on a wide range of issues, such as (i) patient convenience, (ii) efficacy of drug delivery and (iii) formulation reasons. As mentioned earlier, drugs deposited in the anterior part of the nasal cavity will be better absorbed than those applied further back. It is for this reason that the specifics of the dosage form or delivery system are very important in the absorption of the drug by influencing its deposition (Taylor, 2002:496).

The time in which a drug molecule is in contact with nasal epithelia is rather short and exhibits high interindividual variability. Therefore, the choice of an appropriate formulation is very essential as it extends the residence time that will lead to the enhancement of the efficiency of nasal absorption (Verhoef & Merkus, 1994:134).

In a nasal dosage form, the drug will usually be in a liquid or powder formulation, delivered by a pressurised or pump system. The following is a comparison between sprays and drops, and between powders and solutions, and the addition of bioadhesives is discussed thereafter.

1.4.4.1 Sprays vs drops

The dosage form determines the site of drug deposition in the nose. Nasal sprays are deposited more anteriorly, compared to nasal drops, resulting in a slower clearance of sprays (Verhoef & Merkus, 1994:134).

In a study by Harris *et al.* (1986:1087) the nasal bioavailability of desmopressin was found to be significantly increased with spray administration, compared to nasal drops.

1.4.4.2 Powders vs solutions

Compared to liquid formulations, powders have the following advantages: (i) The drug is normally more stable; (ii) No preservative is needed in the formulation; and (iii) It is possible to administer larger quantities of drugs and excipients (Lee *et al.*, 1991:725).

Intranasal delivery of a nafarelin powder dosage form with high molecular weight dextrans presented increased peptide absorption, when compared to the liquid formulation (Verhoef & Merkus, 1994:134).

1.4.4.3 Bioadhesives

Bioadhesives have been used in nasal drug delivery in order to reduce MCC and consequently improve nasal drug absorption (Illum *et al.*, 1987:189). Polymeric materials are used to increase the residence time of drug formulations, by increasing solution viscosity and/or having bio- or mucoadhesive properties in solution or powder formulations. The nature of the compound and the surrounding media will determine the interaction of the compound with the mucus layer, which is exactly what bioadhesion involves (Hinchcliffe & Illum, 1999:222).

Carbopol is one of the bioadhesives used to increase the viscosity of solutions, and reveal mucoadhesive properties. The viscosity of a 1.0% carbopol gel is high, which contributes to the slower release of insulin from the 1.0% gel base (Morimoto *et al.*, 1985:134).

Chitosan's bioadhesive properties contribute to the mechanism of an absorption enhancer. Chitosan also has an effect on the gating properties of the epithelial tight junctions (Hinchcliffe & Illum, 1999:225). The residence time of the drug formulation in the nasal cavity may be increased by the mucoadhesive and viscosity enhancing properties of chitosan. Illum (1987:189) was first to report that the application of chitosan improves the nasal absorption of insulin.

1.5 Conclusion

The nasal route has proven especially suitable for the administration of compounds that are active at a low concentration (i.e. potent), are non-irritant and have very low oral bioavailability. The high vascularity of the nasal mucosa (allowing good bioavailability), the absence of the first pass effect through the liver and the low aminopeptidase activity all contribute to make the nasal route the ideal channel for the administration of certain drugs.

There are a number of factors that need to be borne in mind when designing a nasal formulation. These factors include the physicochemical properties of the drug, such as molecular weight and size, lipophilicity, solubility of the drug, partition coefficient and the pK_a of the drug. The anatomical and physiological properties of the nose, for example mucocilliary clearance, site of deposition, enzymes present and pathological conditions, are also important factors. Due to mucocilliary clearance and possible hydrolysis, the nasal dosage form needs to be designed so that the clearance effect is slowed down in order to increase the absorption rate of the active ingredient. Formulation factors such as viscosity, osmolarity, mucosal irritancy, volume applied and dosage form can also be contributory factors.

The use of absorption enhancers, enzyme inhibitors and bioadhesives seems to be a good solution to the poor absorption, potential enzymatic degradation and low bioavailability, after nasal administration, which leads to improved drug absorption.

From this point of view, the nasal route is clearly preferable to parenteral administration, as local discomfort, irritation, and inconvenience are associated with the parenteral route. Numerous studies have been carried out in order to improve the absorption enhancement of peptides and proteins (for example insulin, human growth hormone, oxytocin and desmopressin). The results allow the hope that it will be possible for the nasal route to replace the parenteral route, leading to more patient comfort and a higher degree of compliance.

Pheroid technology and N-trimethyl chitosan chloride (TMC) as possible delivery systems for insulin

2.1 Introduction

The intranasal delivery of peptide and protein drugs has been widely investigated to improve nasal bioavailability (Dondeti *et al.*, 1995:94). The oral route leads to low bioavailability of peptide drugs due to chemical and enzymatic degradation in the gastro-intestinal tract, low permeability across the gastrointestinal mucosa and first pass metabolism in the liver (Hinchcliffe & Illum, 1999:200).

There are, however, a number of factors that limit the intranasal absorption of peptide drugs for systemic delivery. These factors include (i) enzymatic degradation, (ii) deposition and clearance from the nasal cavity, and (iii) penetration of the mucus layer (Hinchcliffe & Illum, 1999:204).

As mentioned in the previous chapter, the vascular nature of the nasal mucosa, together with its high drug permeation, makes the nose an excellent route for the administration of many drugs, including proteins and peptides such as insulin. The nasal delivery route is very attractive, because of its convenience and its ability to absorb various drugs.

Insulin is a peptide drug, used for insulin dependent diabetes mellitus (IDDM). This drug has a high molecular weight and is barely absorbed after oral administration due to degradation by proteolytic enzymes (Hirai *et al.*, 1981:175).

Developing a safe and effective nasal delivery system for insulin with absorption enhancing properties would improve the effectiveness of insulin after intranasal administration (Hinchcliffe & Illum, 1999:226).

2.2 Insulin

2.2.1 Background and classification

The discovery of insulin was one of the greatest events in the history of medicine. Two scientists, Frederick G. Banting and Charles H. Best began testing insulin on dogs in 1921. John J. R. Macleod provided them with a laboratory at the University of Toronto. Their method consisted of tying a string around the pancreatic duct of a dog. After a number of weeks, they discovered that the pancreatic digestive cells of the dog had died and had been absorbed by the immune system, leaving thousands of islets. The protein was then isolated from the islets to produce what they called *isletin*. This protein, however, still needed purification. Macleod invited the brilliant biochemist James B. Collip to help with the task of purifying insulin (Brar, 2005:2; Davis & Granner, 2001:1679).

In December 1922 the fourteen-year-old Leonard Thompson became the first person with diabetes ever treated with an insulin injection (Davis & Granner, 2001:1679). Unfortunately he suffered a severe allergic reaction because the extract given was impure, and further injections were cancelled. Collip was able to improve the extract, which had no obvious side-effects. Although the extract remains impure, Best managed to improve this technique in 1922, in order to extract large quantities of insulin. Fortunately, the pharmaceutical company Eli Lilly became able to produce large quantities of pure insulin and this breakthrough made it possible to offer insulin for sale (Thayer, 2005:2).

Banting and Macleod were awarded the 1923 Nobel Prize for Medicine and Physiology for their discovery of insulin, which they shared independently and respectively with Collip and Best. They are known as heroes in the history of medicine, and saved millions of lives worldwide. It was only in 1923 that insulin was commercialised for the first time, in Great Britain. By October 1923, insulin was being sold in America and Canada as well (Thayer, 2005:2).

Insulin was characterised as a protein in 1928. It was the first protein in terms of which the molecular weight and chemical structure (consisting of 51 amino acids) were determined (Lin *et al.*, 2004:151). British molecular biologist, Frederick Sanger, determined the exact sequence of the amino acids and the so-called primary structure of the insulin molecule. Sanger was awarded the Nobel Prize for Chemistry in 1958 (Brar, 2005:3).

The next great breakthrough was in the 1970s, with the successful synthesis of insulin. In addition, recombinant DNA (rDNA) technology was used to produce human insulin in 1978. This was only the second time that a human gene had been expressed in bacteria (*Escherichia coli*). Somatostatin was the first hormone expressed in bacteria in 1977 (Thayer, 2005:2).

Since 1986, Eli Lilly has employed a recombinant pro-insulin route. This pro-insulin route is the route of choice for manufacturing insulin, because it requires a single fermentation and isolation step. Many producers have created insulin analogues that differ in terms of a few amino acids in order to control their onset and duration of activity (Thayer, 2005:2).

2.2.2 Chemical characteristics

2.2.2.1 Chemical structure

Insulin is a polypeptide hormone, has a relatively simple structure and contains 51 amino acids. This structure consists of two peptide chains (A and B) linked by two interchain disulfide bonds. The structure of pro-insulin can be seen in figure 2.1. Chain A consists of 21 amino acids and chain B of 30 amino acids. Pro-insulin is converted to insulin when the connecting peptide (C) and four basic amino acids are removed with proteolysis (Davis & Granner, 2001:1680).

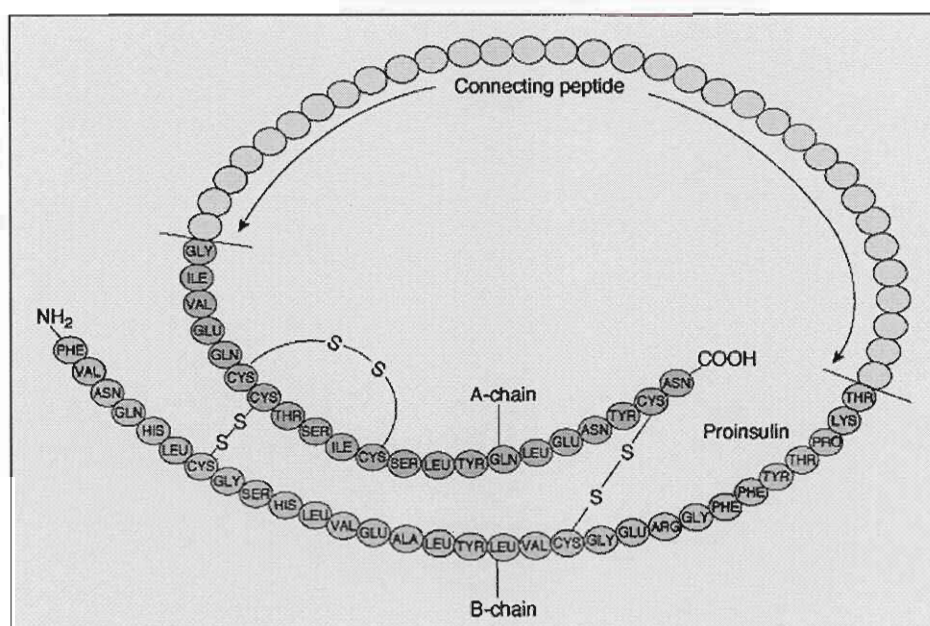


Figure 2.1 Structure of pro-insulin (Lippincott Williams & Wilkins, 2005)

2.2.2.2 Molecular formula and weight

The molecular formula (MF) and molecular weight (MW) of insulin are summarised in table 2.1.

Table 2.1 Molecular formula (MF) and molecular weight (MW) of insulin (Sigma-Aldrich, 2005)

MF	$C_{257}H_{383}N_{65}O_{77}S_6$
MW	5807.57

2.2.3 Physicochemical characteristics

2.2.3.1 Description

Insulin can be described as a white or almost white crystalline powder. In a solution, insulin is a white suspension. This suspension deposits a white sediment and leaves a colourless or almost colourless supernatant liquid when standing. The sediment is readily resuspended by gentle shaking (Lund, 1994:918).

2.2.3.2 Solubility

Insulin is insoluble in water, ethanol, chloroform and ether. On the other hand, it dissolves in dilute solutions of mineral acids, solutions of alkali hydroxides and with degradation (Lund, 1994:920).

2.2.3.3 Isoelectric precipitation and pH changes

When the pH of insulin changes, amorphous or crystalline precipitates are formed, which cause reduced solubility. Small amounts of acidic substances, used in devices for insulin delivery, may result in a decrease in pH, bringing insulin to its isoelectric precipitation zone of pH 4.5 – 6.5 (Brange & Langkjær, 1993:322).

2.2.4 Stability and storage

2.2.4.1 Powder form

Insulin powder must be stored in airtight containers and protected from light. The USP advises storage at a temperature not exceeding 8.0°C, whereas the BP 1988 recommends storage not exceeding -20.0°C. Only the powder form of insulin may be subjected to storage conditions that lead to freezing (Martindale, 1993:283).

2.2.4.2 Solution (injections)

Insulin injections are recommended by both the BP and the USP to be stored in a refrigerator at 2-8°C and not allowed to freeze (Martindale, 1993:283).

2.2.5 Pharmacokinetics

Pharmacokinetics involves the kinetics of drug absorption, distribution and elimination (i.e. excretion and metabolism). The study of pharmacokinetics involves both the development of experimental and theoretical approaches. The experimental aspect involves the development of biological sampling techniques, analytical methods and metabolites, and procedures that facilitate data collection and manipulation. The theoretical aspect, on the other hand, involves

the development of pharmacokinetic models that predict drug disposition after drug administration (Shargel & Yu, 1999:30).

2.2.5.1 Absorption

The absorption of human insulin after subcutaneous administration is the rate-limiting step of insulin activity. Insulin is absorbed directly from the bloodstream when administered via subcutaneous injection. Various sites of injection (abdomen, gluteus, thigh and deltoid) show blood flow differences, leading to variable degrees of insulin absorption. Insulin injected into the abdomen is absorbed twice as fast as from other sites. The abdomen is the preferred site of injection because it is the least susceptible to factors affecting insulin absorption. See table 2.2 for factors affecting insulin absorption.

Table 2.2 Factors affecting insulin absorption (Kroon, 2003)

Factor	Comment
Exercise of injected area	Strenuous exercise of a limb within one hour of injection. Clinically significant for regular insulin.
Local massage	While it is acceptable to press on the injection site to prevent seepage, the site should not be rubbed vigorously or massaged.
Temperature	Heat can increase absorption rate. Cold has the opposite effect.
Site of injection	Insulin is absorbed faster from the abdomen.
Lipohypertrophy	Injection into areas with lipohypertrophy delays insulin absorption.
Jet injectors	Increased absorption rate.
Insulin mixtures	Loss of short-acting action of regular insulin when mixed with Lente; inadequate resuspension of insulin suspensions.

Insulin doses	Larger doses have been known to delay action and duration.
Physical status (soluble vs. suspension)	Suspension insulins must be sufficiently resuspended prior to injection to reduce variability.

Not only the injection site but also local blood flow has an affect on insulin absorption. The change in local blood flow in the tissues will alter the absorption rate of insulin (Kroon, 2003).

2.2.5.2 Distribution

After insulin molecules are absorbed, they have to be distributed through the body. Insulin in the bloodstream is distributed equally between free insulin and insulin bound to IgG antibodies. The presence of insulin antibodies may cause (1) a delayed onset of insulin activity, (2) a reduction of free peak concentration insulin and (3) a prolonged biologic half-life of insulin (Kroon, 2003). Insulin antibodies play an enormous role in the distribution of insulin. For human insulin, the volume of distribution is in the range of 0.26 – 0.36 L/kg (Medtronic, 2004).

2.2.5.3 Metabolism and excretion

Besides its absorption and distribution, the elimination of insulin is also an essential pharmacokinetic process. The majority of insulin is degraded in the liver and kidneys. The liver normally degrades 60% of insulin secreted by the pancreas, and the kidneys between 35 and 45%. This degradation profile is altered when insulin is injected directly into the portal vein. The kidneys are involved in the degradation of insulin and renal dysfunction will therefore reduce the clearance of insulin and prolong its effect. Both endogenous insulin production and exogenous insulin administration showed decreased clearances (Kroon, 2003).

The kidneys are extremely important in the metabolism of insulin. When human insulin was administered subcutaneously, it showed a half-life ($t_{1/2}$) of 1.5 hours, whereas the $t_{1/2}$ was 52 minutes at 0.2 U/kg when given intravenously (Medtronic, 2004).

2.2.6 Pharmacology

2.2.6.1 Indication

Insulin is used for the treatment of the fatal disorder of insulin dependent diabetes mellitus (type 1 diabetes mellitus) (Davis & Granner, 2001:1679). Diabetes mellitus is a chronic metabolic disorder characterised by an absolute or relative deficiency of insulin, which can be associated with peripheral insulin resistance (SAMF, 2001:70).

2.2.6.2 Insulin production

In the human body insulin is produced by the beta cells only. These cells are located in the pancreas, in clusters known as the Islets of Langerhans. The structure of insulin consists of two polypeptide chains which are linked by two sulphur bridges. The production of insulin involves a few intermediate steps, which are illustrated in figure 2.2. A first messenger RNA (ribonucleic acid) transcript is translated into pre-pro-insulin, which is inactive during the protein assembly of insulin. Pre-pro-insulin is then secreted into the endoplasmic reticulum. The N-terminal signal sequence is cut off by means of a post-translational process to form disulfide bridges and pro-insulin (Beta Cell Biology Consortium, 2004-2005; Davis & Granner, 2001:1681-1682).

Pro-insulin is transported in small vesicles to the Golgi complex. The polypeptide is finally cut off at two positions in order to release the dominant C-chain. The final product of the biosynthesis is active insulin, which is packed into secretory granules for storage (Beta Cell Biology Consortium, 2004-2005; Davis & Granner, 2001:1681-1682).

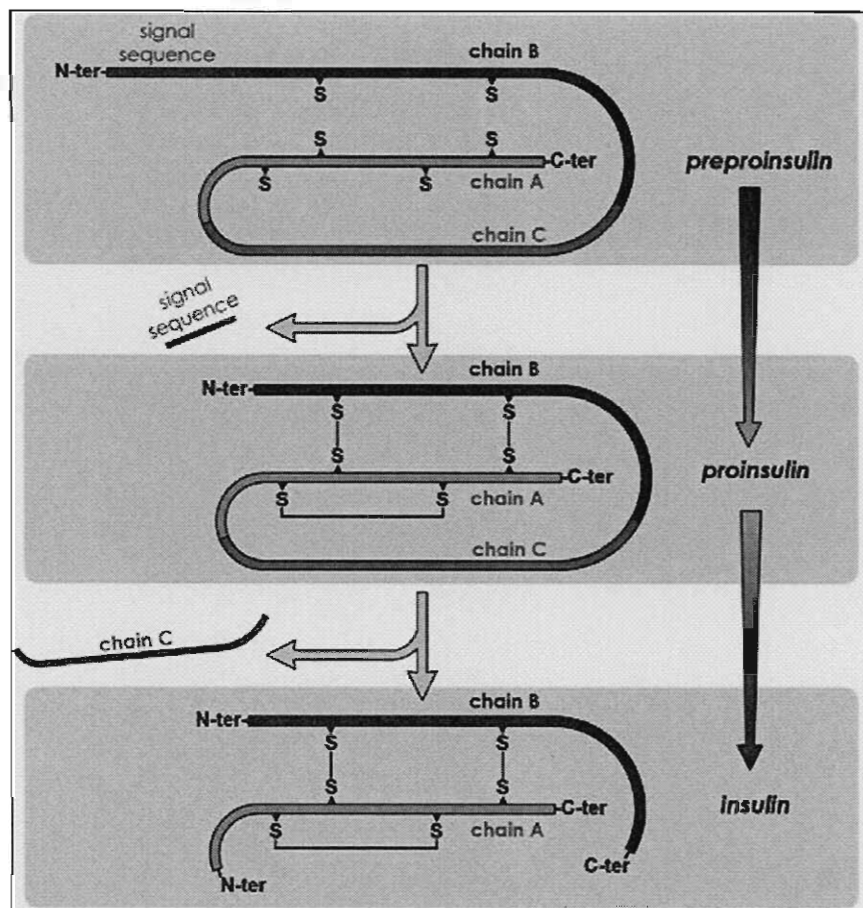


Figure 2.2 Insulin synthesis (Beta Cell Biology Consortium, 2004-2005)

2.2.6.3 Regulation of insulin secretion

The secretion of insulin is triggered by (1) rising blood glucose levels, (2) amino acids, (3) fatty acids and (4) ketone bodies. Glucose enters the beta cells through type 2 glucose transporters (GLUT 2), where it is phosphorylated by glucokinase. This modified glucose, which is trapped within the beta cells, is further metabolised to create the energy molecule ATP (adenosine triphosphate). The process of insulin secretion is illustrated in figure 2.3.

The ATP:ADP (adenosine diphosphate) ratio deactivates the potassium channel that depolarises the membrane, which causes a positive charge inside the beta cell. This causes the calcium channel to open up, allowing calcium ions to flow inward. The increase in intracellular concentrations of calcium leads to the release of insulin from the storing granules, by a process known as exocytosis. When insulin is secreted from the beta cells, it diffuses into nearby blood vessels (Beta Cell Biology Consortium: 2004-2005).

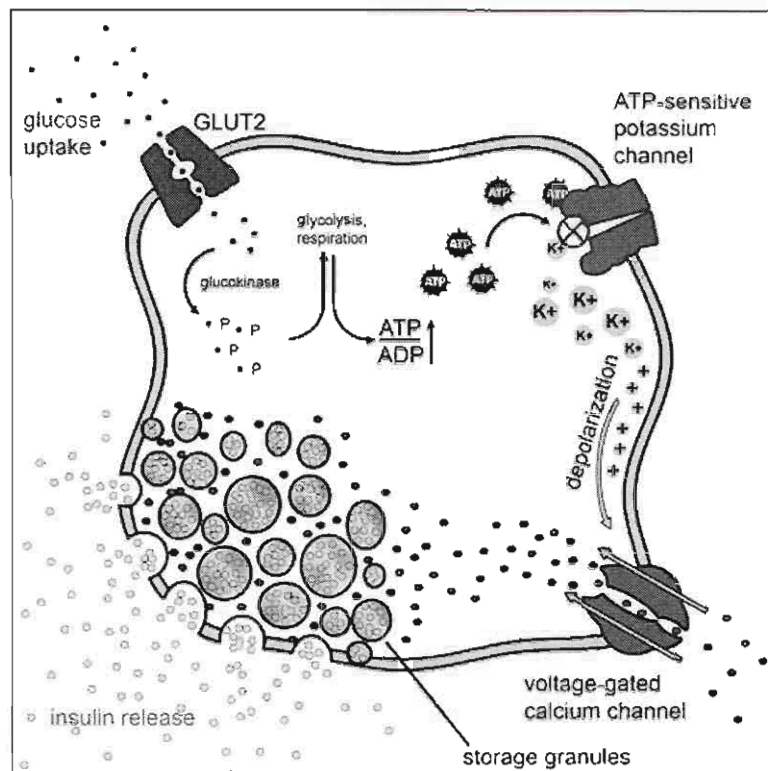


Figure 2.3 Insulin secretion from the beta cell (Beta Cell Biology Consortium, 2004-2005)

2.2.6.4 *Diabetes mellitus and the physiological effects of insulin*

Diabetes mellitus is known as the most important disease of the endocrine pancreas and is characterised by abnormally high levels of glucose in the blood (Karam, 1998:684). The most common forms of diabetes are type 1 diabetes mellitus (known as insulin-dependent diabetes or IDDM) or type 2 diabetes mellitus (known as non-insulin-dependent diabetes or NIDDM). This group of syndromes is characterised by the following symptoms: Hyperglycaemia, metabolic disorder and an increased risk of vascular disease complications (Davis & Granner, 2001:1686).

Type 1 diabetes occurs mostly early in life and sometimes in non-obese adults. This is characterised by low insulin production, high blood glucose, glucose in the urine, excess urine flow and switch to fat metabolism. Circulating insulin is nearly absent and plasma glucagon is high in this catabolic disorder. In this form of diabetes, beta cells fail to respond to all insulinogenic stimuli. Exogenous insulin is therefore required to prevent ketosis as well as to reverse the catabolic state. Type 1 diabetes can be treated with insulin injections, a carefully balanced diet and an active lifestyle (Karam, 1998:684; Davis & Granner, 2001:1687-1689).

Type 2 diabetes occurs mainly in obese adults and occasionally in adolescents. The insulin levels may be normal, but are ineffective due to insulin resistance. Many of the symptoms are the same as for type 1 diabetes, but less severe. Ketoacidosis is prevented by circulating endogenous insulin. This endogenous insulin is often either below normal or insufficient to be able to reduce blood glucose levels due to tissue insensitivity. Type 2 diabetes may be reversed if the patient loses weight and adopts a healthier lifestyle (Karam, 1998:684; Davis & Granner, 2001:1687-1689). In both type 1 and type 2 diabetes mellitus the complications are severe and the disease can be fatal if left untreated.

2.2.6.5 Contraindications

The use of insulin is contra-indicated for patients who suffer from hypoglycaemia, or are allergic to any of the ingredients in the preparation. When a patient has a renal or hepatic impairment, his/her insulin requirements may be reduced (MDR, 2003:717).

2.2.6.6 Drug interactions

Drugs with hypoglycaemic activity, for example monamine oxidase inhibitors and β -adrenergic blockers, may induce insulin requirements. β -adrenergic blockers may mask the symptoms of insulin-induced hypoglycaemia (MDR, 2003:721).

2.2.6.7 Adverse effects of insulin

Patients who take insulin normally suffer from three types of complications: (1) hypoglycaemia, which develops from an excessive insulin effect; (2) immunological toxic effect, from the development of antibodies and (3) lipodystrophy at the injection site (MDR, 2003:722).

2.3 Absorption enhancers for insulin

Insulin is not well-absorbed across the nasal mucosa because it has a high molecular weight and is a hydrophilic substance unless absorption enhancing materials are used. The two absorption enhancer systems investigated in this study are chitosan and pheroid technology. Chitosan is mucoadhesive and has the unique ability to open tight junctions between cells. The pheroid delivery system, on the other hand, improves the entrapment and active transportation of insulin

molecules through various physical barriers. Results and comparisons of these two delivery vehicle systems will be discussed in more detail in the chapters to follow.

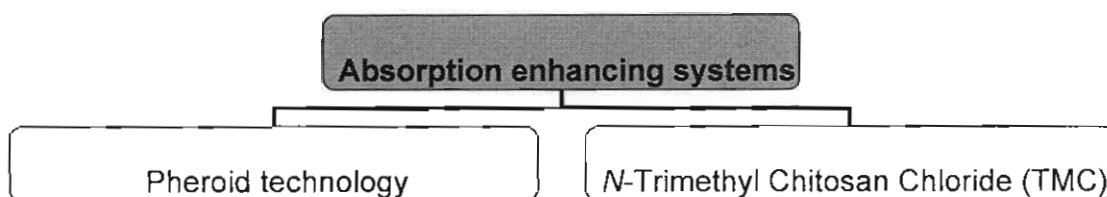


Figure 2.4 Different absorption enhancing systems for insulin

2.4 Pheroid technology as a drug delivery system

2.4.1 The pheroid system

Pheroid technology (previously Emzaloid™) (hereafter referred to as pheroid or pheroids) is a patented system consisting 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 and linolenic acid, as well as oleic acid, emulsified in water saturated with nitrous oxide. Pheroids can entrap, transport and deliver pharmacologically active compounds and other useful molecules (Saunders *et al.*, 1999:99).

2.4.2 Pheroid types, characteristics and functions

There are many barriers to the delivery of drugs. Pheroids entrap drugs and deliver them to target sites in the body. They penetrate keratinised tissue, skin, intestinal lining, nasal epithelium, the vascular system, fungi, bacteria and parasites. Figure 2.5 shows confocal laser scanning micrographs of various formulations of the pheroid delivery system (Grobler, 2004:4).

Although there are many delivery systems, the pheroid is unique in that its components are manipulated in a specific way to ensure its high entrapment capabilities, fast rate of transportation, delivery and stability. The absorption capabilities and drug release characteristics of the pheroid can therefore 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).

Pheroids also contain essential fatty acids that are necessary for many cell functions. Fatty acids cannot be manufactured by human cells and therefore have to be ingested. It has been shown that the Western is often deficient in essential fatty acids. Some of the functions of the fatty acid components of the pheroid system are (1) the maintenance of membrane integrity of cells, (2) energy homeostasis and modulation of the immune system through leukotrienes and prostaglandins and (3) some regulatory aspects of programmed cell death. The pheroid system has inherent therapeutic qualities that give it significant advantages over some of the other delivery systems (Grobler, 2004:4).

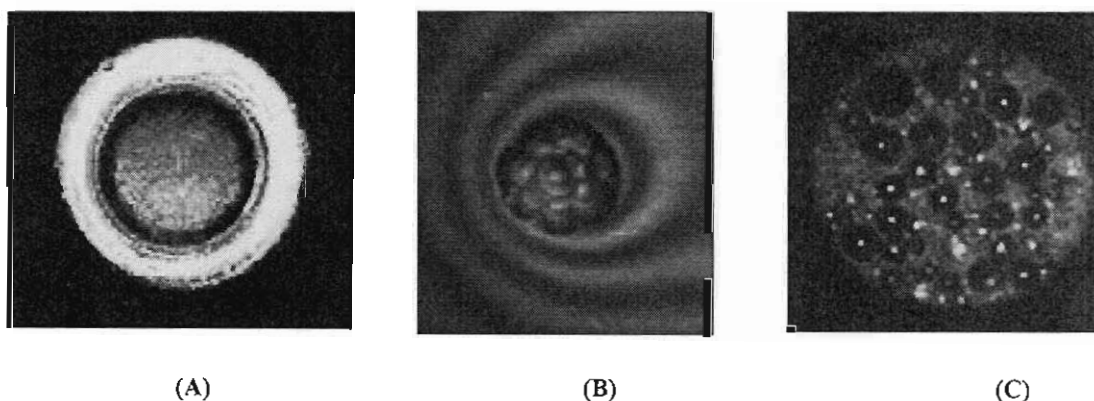


Figure 2.5 Confocal laser scanning microscopy of some of the basic pheroid types. (A) A bilayer membrane vesicle containing Rifampicin. (B) The formation of small pro-pheroids used in oral drug delivery. (C) A reservoir that contains multiple particles of coal tar (Grobler, 2004:5).

2.4.3 Pheroids versus other lipid-based delivery systems

The pheroid system differs significantly from conventional macromolecular carriers such as liposomal delivery systems. Table 2.3 provides a comparison of the differences and key advantages of the pheroid and other lipid-based delivery systems.

Table 2.3 Differences between and advantages of pheroid and other lipid-based delivery systems
(Grobler, 2004:4)

<i>Pheroid technology</i>	<i>Lipid-based delivery systems</i>
Consists mainly of essential fatty acids, a natural and essential ingredient of the human body.	Generally contains 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.
Since it is part of the natural biochemical pathways, the pheroid causes no cytotoxicity and assists with cell maintenance.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body. Liposomal systems may decrease the cytotoxicity of compounds or may cause cytotoxicity.
It is polyphonic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
Due to its composition it is sterically stabilised without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be sterically stabilised. This generally leads to an increase in size and rigidity of the carrier.

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.
It is designed to show a high degree of elasticity and fluidity, with a relatively high phase transition temperature.	Because of the general use of stabilising compounds and cholesterol, liposomal systems lose fluidity and elasticity. The phase transition temperatures of phospholipid groups are higher than that of the essential fatty acids, resulting in a loss of elasticity.
Due to the composition of pheroids, they are able to inhibit the drug efflux mechanism in the intestinal lumen and can thereby enhance the bioavailability of a compound.	Liposomal systems containing this feature have not been described. A separate compound (e.g. Cremophor) is co-administered to achieve the same effect.
The entrapment efficiency of compounds inside the pheroid is high (between 85 and 100%).	Due to the charge and steric limitations of liposomal delivery systems, entrapment efficiencies may be problematic.
The type of pheroid formulated for a specific compound determines the loading capacity of the pheroid.	The loading capacity of most lipid-based delivery systems is dependent on the interior of the intramembrane volume and is therefore limited.
Pheroid microsponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other drug in the sponge spaces. Geographical	Combination therapies are problematic in most liposomal drug delivery systems.

separation of active compounds into different interior spaces minimises drug interactions or interactions between drug compounds.	
Batch-to-batch reproducibility and stability has been proven with existing products containing pheroids, such as the registered product Exorex®.	Large scale manufacturing of other liposomal delivery systems sometimes shows low batch-to-batch reproducibility as well as problems with size control.

2.4.4 Pharmaceutically applicable features of the pheroid system

2.4.4.1 Decreased time to onset of action

Research has indicated that the pheroid delivery system rapidly transverse 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).

2.4.4.2 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 entrapping the active compound in pheroids (Grobler, 2004:9).

2.4.4.3 Reduction of minimum drug concentration

Research conducted with the pheroid system has shown that, for certain active compounds, using as little as 1/40th of the active compound may result in an effective drug plasma concentration. In practice, this characteristic will result in a reduction of the adverse effects on patients as well as major savings in treatment costs (Grobler, 2004:10).

2.4.4.4 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).

2.4.4.5 Reduction in cytotoxicity

Cellular damage caused side-effects of drugs in most instances. The pheroid system has the potential to minimise the cellular damage resulting from membrane damage caused by active compounds. Therefore, by incorporating the drug in the pheroid system, the occurrence of side-effects can be dramatically reduced (Grobler, 2004:11).

2.4.4.6 Immunological responses

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

2.4.5 Therapeutic and preventative uses of pheroid technology

2.4.5.1 Pheroid technology for nasal vaccine delivery

The hypothesis for the nasal delivery of vaccines using pheroid technology 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 absorbed by the microfold cells (M-cells) in the nasal epithelium, which are responsible for the sampling and transportation of antigens to the underlying nasally-associated lymphoid tissue (NALT), germinal centres containing B and T cells, plasma cells and antigen presenting cells (APCs), all of which are involved in the regulation and induction of antigen-specific effector cells that produce the protective humoral and cellular immune responses (Grobler, 2004:6).

2.5 *N*-Trimethyl chitosan chloride (TMC)

As a partially quarternised derivative of chitosan, *N*-trimethyl chitosan chloride (TMC) has enhanced water solubility and superior drug absorption enhancing properties (Kotzé *et al.*, 1999b:254). TMC contributes to its activity as a drug absorption enhancer due to (1) its mucoadhesive characteristics; (2) its decreased intrinsic viscosity; and (3) its reduced transepithelial electrical resistance (TEER) across Caco-2 cell layers (Kotzé *et al.*, 1997a:1199).

2.5.1 Synthesis of TMC

The method of Domrad *et al.* (1986:105) is used for the synthesis of TMC from sieved fractions (<500 μm) of chitosan. Experimental conditions involve the reductive methylation of chitosan with iodomethane in a strong basic environment at 60.0°C for 60 minutes. The quarternised polymer is dissolved in a sodium chloride solution, where the counterion (I^-) is changed to Cl^- (Kotzé *et al.*, 1997a:1197). By repeating the reaction step for the reductive methylation of chitosan several times and under the same conditions, different TMC polymers with varying degrees of quarternisation can be prepared. The polymer is obtained from each reaction step (Hamman & Kotzé, 2001:374).

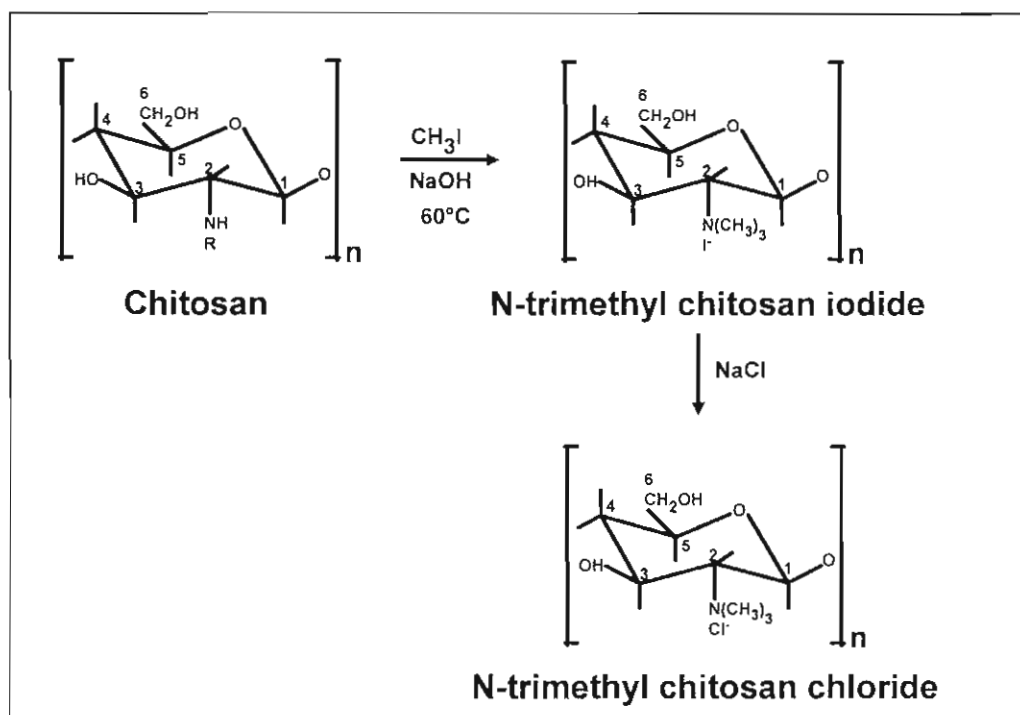


Figure 2.6 Synthesis of *N*-trimethyl chitosan chloride from chitosan by reductive methylation

2.5.2 Physicochemical properties of TMC

TMC with a degree of quaternisation as low as 10% is soluble in water (Kotzé *et al.*, 1998a:39). Compared to chitosan salts, TMC demonstrates highly increased water solubility, especially at neutral and basic pH values (Kotzé *et al.*, 1997b:244).

Because TMC has a decreased intrinsic viscosity compared to chitosan, polymer degradation under reaction conditions in an alkaline medium is indicated. The number of positive charges on the polymer chain is increased during the synthesis of TMC, causing the molecule to expand in solution due to repelling forces between the functional groups (Snyman *et al.*, 2002:145).

Either acidic or basic mediums can be used to prepare TMC solutions of 10%. As a result, TMC is a derivative of chitosan, with superior solubility and basicity, even at low degrees of quaternisation. The replacement of the primary amino group on the C-2 position of chitosan with methylated quaternary amino groups, increases its solubility and basicity (Kotzé *et al.*, 1998a:39). According to Kotzé *et al.* (1999a:273) the degree of quaternisation of the TMC plays an important role in its absorption enhancing activity, especially in neutral environments.

2.5.3 Effect of TMC on the transepithelial electrical resistance (TEER) of intestinal epithelial cells (Caco-2 cell monolayers)

The measurement of TEER is a good indication of the tightness of the tight junctions between cells. The TEER has also been used to predict the paracellular transport of hydrophilic compounds (Kotzé *et al.*, 1998b:149). 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 dependent way compared to the control group. No significant reduction in the resistance of the cells was measured in concentrations of 1% and less (Kotzé *et al.*, 1997a:1199).

Higher degrees of TMC quaternisation proved to be more effective in reducing the TEER of the Caco-2 monolayers in neutral environments than lower degrees of quaternisation. This can be explained by how large a positive charge on the TMC molecule is 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; they are therefore more effective in lowering TEER values (Hamman *et al.*, 2000:37). Decreased TEER values lead to increased transportation of peptide drugs across Caco-2 monolayers (Kotzé *et al.*, 1997b; 250).

2.5.4 Mucoadhesive properties of TMC

The administration of a drug delivery system to an epithelium suitable for the absorption of the active compound is necessary for non-invasive drug delivery. To control and/or increase the residence time at these absorption sites, the use of mucoadhesive polymers as drug carriers has been investigated (Bernkop-Schnürch, 2000:8).

Mucoadhesives are generally known as negatively charged groups and have hydrogen-bonding capabilities. Additional molecular attraction forces could probably develop by electrostatic interactions with the negatively charged mucosal surfaces and positively charged polymers such as chitosan and TMC (Bernkop-Schnürch, 2000:8).

The absorption via the paracellular transportation pathway (through the tight junctions) is enhanced by (1) the mucoadhesive properties and (2) the increased contact time of the active ingredient in a formulation with the absorptive epithelium with chitosan and TMC (Snyman *et al.*, 2002:145).

2.5.5 Effect of TMC on the absorption of hydrophilic model compounds and peptide drugs

The bioavailability of peptide and protein drugs is deficient mainly due to the mucosal membranes' resistance to their penetration. The size and polarity of peptide drugs also limit their diffusion across biological membranes. The absorption of these compounds is limited to the alternative paracellular pathway that is primarily controlled by the tight junctions (Lee *et al.*, 1991:182).

The effect of TMC (degree of quaternisation 12%) on the cumulative transportation 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). An evident accumulation of all the compounds in the acceptor compartments was from incubation with TMC solutions of 1.0 to 2.5% (w/v). The increase in the molecular weight of the compounds leads to a decreased permeability. [^{14}C]-mannitol has the lowest molecular weight, exhibiting the highest permeability, and FD-4 exhibits the lowest permeability. The permeation of these compounds across intestinal epithelial cells is, among other factors, dependent on molecular size. Thus an increase in TMC concentration resulted in an increase in permeability and transportation, with 2.5% (w/v) concentrations of TMC showing the greatest effect (Kotzé *et al.*, 1997a:1200).

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

2.5.6 Proposed mechanism of action of TMC

Tight junctions play a fundamental role in (1) maintaining the selective barrier function of cell membranes and in (2) sealing cells together to form a continuous cell layer. Even small molecules cannot penetrate through this cell layer. The paracellular route occupies only a very small surface area compared to the transcellular route. On the contrary, 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 pH, calcium concentration, osmolarity and c-AMP (Cereijido *et al.*, 1993:18).

Absorption across intestinal epithelia was enhanced by chitosan and TMC polymers through the opening of tight junctions. Cationic macromolecules such as chitosan and TMC can interact with the anionic components of the glycoproteins on the surface of epithelial cells, due to their

positive charge characteristics. In addition, cationic macromolecules displace cations from electronegative sites on cell membranes, thereby disrupting their dimensional stability. The interior of the tight junction channel is known to be hydrated and contains fixed negative sites. If the concentration of certain ions in the pore changes, it could result in a change in tight junction resistance. This change in the concentration will lead to the pore opening with increased paracellular permeability (Artursson *et al.*, 1994:1359).

In order to visualise the transportation pathway across the cell monolayers, confocal laser scanning microscopy was used (Kotzé *et al.*, 1998a:44). The optical cross-sections of cell monolayers, after incubation with a control solution containing only FD-4, showed no evidence of any intracellular or intercellular fluorescence. It was only on top of the monolayers that fluorescence was detected. After 60 minutes 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. Horizontal (XY) images showed similar results. 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 no damage was done to the cell membranes with any TMC polymers (Kotzé *et al.*, 1998a:44).

2.5.7 TMC toxicity studies

The effect of TMC on Caco-2 cell monolayers has been investigated in various studies of the viability of the cells. The viability of Caco-2 cell monolayers was tested in a study by Kotzé *et al.* (1997b:246) after their transportation experiments had been completed. In this study the cell monolayers were incubated 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 without trypan blue were considered to be viable. After prolonged incubation with TMC polymers there was no visible intracellular uptake of trypan blue. This implies that the Caco-2 cell monolayers remained undamaged and functionally intact, and that viability was not affected (Kotzé *et al.*, 1997b:246).

Kotzé *et al.* (1997a:1199) performed similar tests after a separate study of the effect of TMC on the TEER of Caco-2 cell monolayers. Another study by Kotzé *et al.* (1999a:271) was performed after testing the effect of TMC polymers with different degrees of quaternisation on the permeability of Caco-2 cell monolayers. Both studies indicated that there was no visible uptake of trypan blue, which showed 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%. This was done by testing trypan blue exclusion as well as propidium iodide exclusion. All monolayers excluded the trypan blue staining at the end of their experiments, which indicates that cell viability was retained. 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 indicated that the absorption enhancing effects of TMC were not due to possible cytotoxic activities (Thanou *et al.*, 2000:23). For the further investigation of local toxicity, the effect of TMC on the ciliary beating frequency of epithelia has also been tested. Thanou *et al.* (1999:82) showed that TMC solutions of 1.0% (w/v) had minimal influence on the ciliary beating frequency. Low molecular weight TMC polymers (oligomers) inhibited ciliary beating frequency to a lesser extent than high molecular weight TMC (Jordaan, 2001:79).

2.5.8 Effect of the degree of quaternisation of TMC on absorption enhancement

In order to determine the ability and effectiveness of TMC to open tight junctions and regulate permeation through the paracellular transport pathway, it is important to look at the degree of quaternisation of TMC, since that determines the amount and density of the positive charges on the C-2 position of this chitosan derivative (Kotzé *et al.*, 1999a:174). Compared to those with lower degrees of quaternisation, highly quaternised TMC molecules have proven to be more effective in increasing 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 absorption enhancement of [¹⁴C]-mannitol across Caco-2 cell monolayers (Kotzé *et al.*, 1999b:225). Only

highly quaternised polymers enhanced absorption at pH 7.2. At low degrees of quaternisation it was projected that the charge density had not reached the threshold concentration. The attached methyl groups may partially protect the positive charge from significant interaction with the cell membranes or tight junctions. In contrast,, highly quaternised TMC has a much higher proportion of quaternary amino groups that seem 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).

[¹⁴C]-mannitol absorption across Caco-2 monolayers and in rat nasal epithelia by TMC, was enhanced even more by an increased degree of quaternisation (Thanou *et al.*, 2000:22; Hamman *et al.*, 2002:240). This confirms 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 48.0% degree of quaternisation. Hamman *et al.* (2002:241) projected that this was due to steric effects caused by the attached 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.

2.5.9 Effect of the molecular weight of TMC on its absorption-enhancing properties

Chitosans with low and high molecular weight and with varying degrees of deacetylation have been investigated to test their effect on epithelial permeability, using Caco-2 cell monolayers. The molecular weight and degree of deacetylation of chitosan have been found to dictate the absorption-enhancing properties and toxicity to a large extent (Schipper *et al.*, 1996:1689). Chitosans with a high molecular weight (98 000 to 190 000 g/mole) and/or high degree of deacetylation increased epithelial permeability, whereas chitosans with low molecular weight (22 000 g/mole) and low degree of deacetylation lacked absorption-enhancing activity. A high molecular weight and/or high degree of deacetylation will be 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 [¹⁴C]-mannitol across Caco-2 cell monolayers. Both the high and low molecular weight polymers were shown to reduce the TEER across the Caco-2 cell monolayers (Swartz, 2002:69). Only the high molecular weight TMC showed an increase in transport of [¹⁴C]-mannitol across the Caco-2 cell monolayers (Swartz, 2002:80).

2.6 Conclusion

Insulin is one of the most extensively studied molecules in biochemistry. As discussed in this chapter, insulin is an ideal model compound, as it has all the structural features of a large protein. Insulin is extremely suitable for investigating the characteristics, structure and properties of proteins. In an attempt to increase the bioavailability of insulin, a drug delivery system (pheroid technology) and an absorption-enhancer (TMC) have been described and selected for this study.

Two groups of pheroids (microsponges and vesicles) have been chosen, considering their characteristics based on their ability to entrap, transport and deliver drugs, as well as their absorption-enhancing potential. Pheroid technology carries and delivers active compounds through most physiological barriers with reduced membrane damage and minimal side effects, as indicated by research. The advanced absorption-enhancing properties of *N*-trimethyl chitosan chloride (TMC) were also discussed.

Nasal delivery of insulin with pheroid technology and N-trimethyl chitosan chloride (TMC): Experimental design

3.1 Introduction

The choice of an administration route and the dosage form to deliver a drug is extremely important, as these two factors influence drug absorption. Peptides and more specifically insulin have hydrophilic properties and a high molecular weight. These properties contribute to a decrease in transnasal absorption of peptide and protein drugs, which leads to a low bioavailability.

In chapter 1, the nose was discussed, as an ideal administration route for delivering peptide and protein drugs. Many factors (such as the large absorptive surface area, the high vascularity of the nasal mucosa, and bypassing the first pass metabolism) ensure the absorption of these compounds in the nose. However, the abovementioned factors are in most cases not sufficient to establish acceptable plasma concentrations. Therefore, it is necessary to include certain components in the formulation to improve their bioavailability. The absorption-enhancing properties of pheroid technology and TMC were explained in chapter 2. The potential of improving the bioavailability of insulin with these two absorption enhancers was also explained.

In this chapter the characterisation of pheroid vesicles and microsponges with regard to size and morphology will be discussed. The preparation of pheroid vesicles and pheroid microsponges will also be explained in full detail. Insulin was carefully entrapped into the pheroid formulations just before nasal administration in the rats. Blood glucose levels and plasma insulin levels were obtained. The absorption properties of pheroids with and without insulin were compared to the absorption profiles of insulin and TMC administered together.

3.2 *In vivo studies in rats*

3.2.1 *Route of administration*

Nasal drug delivery achieved wide spread interest among scientists as an alternative route for the administration of drugs (especially systemic acting drugs) with poor bioavailability (Chien *et al.*, 1981:1; Hinchcliffe & Illum, 1999:1). In chapter 1 this route was also described as an excellent choice for the delivery of biosensitive and high molecular weight compounds such as proteins and peptides.

There is evidence in the literature that the nasal route has been thoroughly examined, and there is reliable information describing absorption enhancement in the nose. This proves that the nasal route is an excellent choice of a route through which to study the absorption properties of insulin when administered with pheroids and TMC.

In this section, the experimental methods used to study the effects of these compounds will be described. The rat nasal absorption model will be used to examine these absorption promoter systems.

3.2.2 *Animals*

In this study, rats were chosen as the *in vivo* model for the following reasons: (a) They are easy to handle; (b) They are readily available; and (c) They cost relatively little to care for compared to other animals. A specialised surgical procedure, cannulation of the artery *carotis communis*, was carried out to ensure that sufficient blood volumes could be obtained from the same rat at different time intervals. These blood volumes are necessary for analysing the drug content.

The animals used during this study had to comply with the following criteria as summarised in table 3.1, which was in agreement with the criteria in the Animal Research Centre (North-West University):

Table 3.1 Criteria for animals used in this study

Criteria	Results
Specie	Sprague Dawley
Gender	Male
Weight	200–300 grams
Specific requirements	Rats must fast for 18 hours prior to nasal drug administration.
Water provision	Water was supplied to rats <i>ad libitum</i> .

3.2.3 Breeding conditions

Sprague Dawley rats were bred and kept at the Animal Research Centre (North-West University). They were kept under artificial conditions to create an ideal environment for optimum health and growth. Infections with pathogen organisms were minimised, whereas variables were kept constant. Table 3.2 summarises the conditions at the Animal Research Centre under which the rats were bred and kept during this study:

Table 3.2 Conditions at the Animal Research Centre, North-West University, Potchefstroom

Conditions	Recommended value*	Value in Animal Research Centre
Temperature	$19 \pm 2^{\circ}\text{C}$	$21 \pm 2^{\circ}\text{C}$
Relative humidity	$55 \pm 15\%$	$55 \pm 10\%$
Rate of ventilation/air movement	15-20 changes per minute	18 changes per minute
Light intensity	350-400 lux one meter above floor level	350-400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

* Values recommended by the Animal Research Centre (NWU) according to international standards

3.2.4 Feeding the rats

The rats were fed Epol[®] mouse cubes as provided at the Animal Research Centre of North-West University. Food was withdrawn for 18 hours before the initiation of the experiment, but the rats had unlimited access to water.

3.2.5 Anesthesia

The rats were anesthetised with halothane (Fluothane[®], Zebeca SA (Pty) Ltd, Woodmead, RSA) and kept anesthetised for 3-4 hours.

3.2.5.1 Induction of anesthesia

Rats were placed in a closed glass container with a metal grid floor above a cotton wool layer containing liquid halothane. Direct contact between the rat and the halothane in the cotton wool layer was not possible. As soon as the rat lost consciousness it would be removed from the container.

3.2.5.2 Maintenance of anesthesia

The maintenance of anesthesia required special apparatus, consisting of two 5-liter plastic bags, containing respectively 0.6 ml and 1.2 ml halothane in medical oxygen. Mixtures of 2.0% and 4.0% halothane in medical oxygen were used alternatively as required. 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.

3.2.6 Surgical procedures

For this study the *artery carotis communis* was cannulated to obtain several blood samples from the same rat at different time intervals. This is a convenient method which does not take much time and has a low mortality rate.

3.2.6.1 Cannulation of the *artery carotis communis*

The following procedures were performed to obtain blood samples from the rats: Rats were placed prone in a supine position on a heated working surface to prevent hypothermia. The hair in the area of the ventral neck was shaved and the rat's skin disinfected.

Figure 3.1 shows the schematic presentation of the experiment carried out on the rats. A midventral incision of 1.0 cm was made in the neck skin, while a blunt dissection was made between the muscles in the neck to expose the *artery carotis communis*. This artery was lifted out of the operation wound and kept wet with physiological saline at body temperature.

The rostral part of the *artery carotis communis* was ligated with silk and tension was exerted on the artery by taping the loose ends of the ligature in front of the rat. Another ligature was pre-placed loosely on the caudal part of the artery. The artery was temporarily clamped with a mosquito artery clamp, proximal to the loosely pre-placed ligature. A pair of scissors was used to carefully cut a 'V'-shaped incision between the two ligatures in the artery wall.

A sterile fine bore polythene cannula, with an inner diameter of 0.54 mm (Portex Limited, Hythe, Kent, England) was filled with sodium heparin (Heparin Sodium, Fresenius, South Africa) dissolved in sterile saline at a concentration of 5 IU/ml and a temperature of 37.0°C. The cannula was then connected to a syringe, which was guided through the 'V' incision in the artery. The clamp was released and the cannula threaded so that 1.0 cm of the cannula remained inserted in the artery. The loose ligature was tied around the artery with the cannula inside. Another ligature was tied for safety. The syringe and clamp were removed from the cannula to be able to obtain blood samples. The integrity of the cannula was maintained with 5 IU/ml heparin in saline.

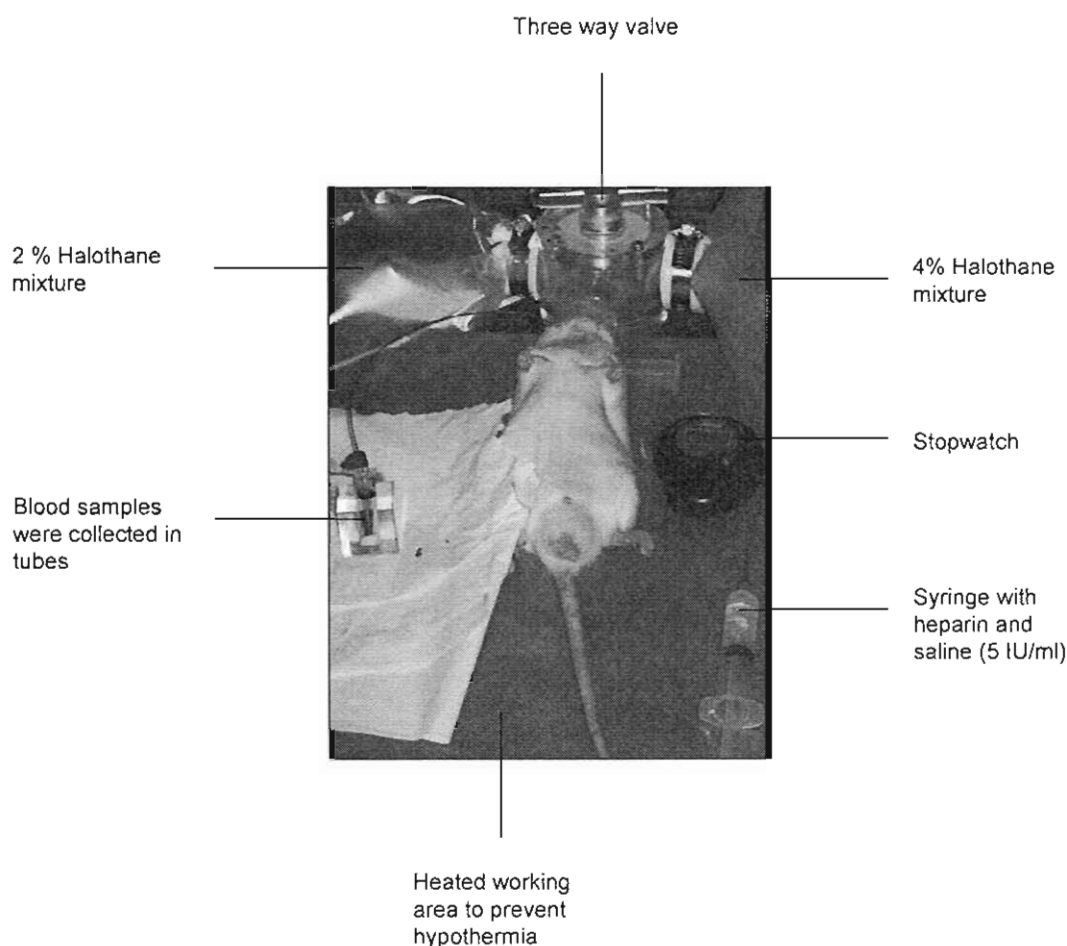


Figure 3.1 Schematic presentation of the anesthetised rat model, used during nasal delivery of insulin

3.2.7 Nasal administration of insulin

Insulin was administered in the rat's nose with respectively pheroid vesicles, pheroid microsponges and TMC polymers in solution, using an Eppendorf® micropipette (10-100 μ l), which is illustrated in figure 3.2. A dose of 4, 8 or 12 IU/kg bodyweight was administered in the left nasal cavity of the rat in a volume of 100 μ l/kg bodyweight. The test solution was administered carefully to ensure that the nasal epithelium was not injured during the administration of the test solutions. Table 3.3 summarises the different nasal formulations administered to the rats.

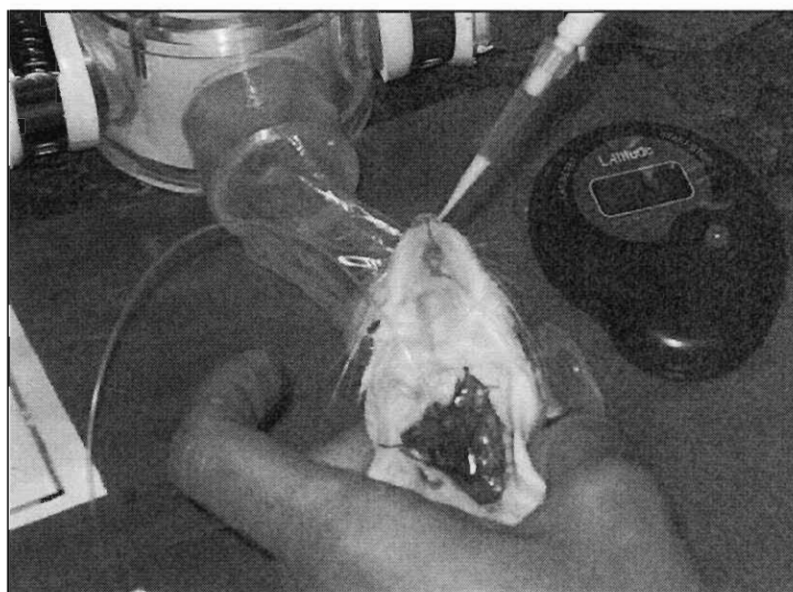


Figure 3.2 Nasal administration to a rat with a micropipette (10-100 μ l)

Table 3.3 Summary of nasal formulations administered to rats

<i>Formulation</i>	<i>Dose of insulin*</i> <i>administered</i>
Control	4 IU/kg
Pheroid vesicles	4 IU/kg
Pheroid microsponges	4 IU/kg
TMC (<i>N</i> -trimethyl chitosan chloride)	4 IU/kg
Control	8 IU/kg
Pheroid vesicles	8 IU/kg
Pheroid microsponges	8 IU/kg
Control	12 IU/kg
Pheroid vesicles	12 IU/kg
Pheroid microsponges	12 IU/kg

* 1 mg insulin human, recombinant, expressed in yeast, is equivalent to 27.5 insulin units (dry base)

3.2.8 Blood sampling

Blood samples of 1000 µl were collected from the *carotis* artery in 1.5 ml Eppendorf® tubes (Merck, RSA). These samples were taken at 0, 5, 10, 15, 30, 60, 120 and 180 minutes after nasal administration of the test solutions. To compensate for the volume of blood lost when taking samples, the rats were given 1.0 ml of heparin in saline (5 IU/ml) at 37°C.

The first few drops of blood appearing from the cannula were discarded before each blood sample was collected, in order to prevent dilution from occurring. The tubes containing blood were stored on crushed ice and centrifuged (Centrifuge 54150, Eppendorf, Germany) at 7000 rpm for 7 minutes. After centrifugation, the plasma was kept on ice for further analysis. The rats were euthanised with CO₂ gas after the completion of the experiments.

3.2.9 Determination of blood glucose

Immediately after each sampling interval, blood glucose levels were measured with a Glucometer® II reflectance meter (Boehringer Ingelheim Pharmaceuticals, Randburg, South Africa). A single drop of blood was put on a haemoglucotest stick (Glucostix®) and was left for 30 seconds before being blotted with soft tissue paper. The glucostick was placed in the Glucometer® II reflectance meter and a mmol/L reading was obtained after 20 seconds.

3.2.10 Determination of plasma insulin concentrations

Plasma insulin levels were determined by the *in vitro* quantitative measurement of human insulin (INS) in plasma, using an immunoradiometric assay (IRMA) method. The BioSource INS-IRMA kit was obtained from Diagnostic Systems Laboratories (Laboratory Specialities, Randburg, South Africa). All samples were assayed using a volume of 50 µl of each plasma sample in duplicate (i.e. 100 µl of each sample) according to the standard procedures and preparations provided with the kit.

Results were expressed in µIU/ml, and converted to a percentage of the base level because different batches of kits were used. Due to technical factors, the plasma insulin concentrations for both 8 and 12 IU/kg bodyweight insulin formulations respectively, were not available.

3.2.10.1 Principles of the immunoradiometric assay (IRMA) method

The INS-IRMA kit used is based on coated-tube separation. The captured antibodies, monoclonal antibodies (Mabs 1), were attached to the lower inner surface of the plastic tube. The addition of calibrators or samples to the tubes showed first low affinity for Mabs 1, where the addition of Mabs 2, the signal antibody labelled with ^{125}I , completed the system and triggered the immunological reaction. The remaining radioactivity bound to the tubes reflects the antigen concentration, after the tubes were washed.

3.2.10.2 Procedure of the immunoradiometric assay (IRMA) method

- ✓ Coated tubes were labelled in duplicate for each calibrator, sample and control. Two normal tubes were labelled for the determination of total counts.
- ✓ Calibrators, samples and controls were briefly vortexed and 50 μl of each was dispensed into the respective tubes.
- ✓ 50 μl of tracer was dispensed into each tube.
- ✓ The racks containing the tubes were shaken gently by hand to release any air-trapped bubbles.
- ✓ The tubes were incubated for 2 hours at room temperature.
- ✓ The content of each tube (except total counts) were aspirated in such a way that the plastic tip of the aspirator reached the bottom of the coated tubes in order to remove all the liquid.
- ✓ The tubes were washed with 2.0 ml of working wash solution (except total counts) and with caution in order to prevent the formation of foam when adding the working wash solution.
- ✓ The content of each tube (except total counts) were aspirated again.
- ✓ The tubes were washed again with 2.0 ml working wash solution (except total counts).
- ✓ The tubes stood upright for two minutes after the last washing and the remaining liquid was aspirated.
- ✓ The tubes were then counted for 60 seconds in a Gamma counter (Packard, Cobra Gamma Counter, United States of America).

3.3 Preparation of pheroid vesicles and pheroid microsponges

Pheroid vesicles and microsponges were prepared at the North-West University (Potchefstroom Campus).

3.3.1 Materials

The following ingredients were used to prepare pheroid vesicles and microsponges: Vitamin F ethyl ester was obtained from Kurt Richer Pharma (Germany); Cremophor RH-40 was obtained from BASF (Germany); Incromega E3322 and E7010SR were obtained from Croda Chemicals (South Africa) and Vitamin E dl-2-tocopherol was obtained from Chempure (South Africa). Purified N₂O water was freshly prepared at the North-West University (South Africa) and amber glass bottles were obtained from Merck (South Africa).

3.3.2 Method

The pheroid vesicles and microsponges were both prepared in the same way. The only difference was the Incromega, which was added to the formula of the pheroid microsponges.

To prepare pheroid vesicles, the materials mentioned above were weighed and mixed together. Firstly, 2.8 g of Vitamin F ethyl ester, 1.0 g of Cremophor RH-40 and 0.2 g of Vitamin E were weighed individually and mixed together in a closed glass container. In the preparation of pheroid microsponges, 0.25 g of Incromega E3322 and 0.25 g of Incromega E7010SR were added to the same ingredients as for pheroid vesicles. All these ingredients were used to create the oil phase of the pheroid vesicle or pheroid microsphere preparations respectively. The mixture was then heated for approximately 80 to 120 seconds in a 900-watt microwave oven until the solution was clear, which ensured the complete mixing of the components of the oil phase.

Secondly, 96.0 g of water saturated with nitrous oxide (N₂O) was placed in a glass beaker, and the N₂O saturated water was heated to $\pm 75.0^{\circ}\text{C}$ on a hotplate. By adding the oil phase to the N₂O saturated water, pheroids were formed. This emulsion preparation was then mixed with a Heidolph DiAx 600 homogeniser at 8000 rpm for 2 minutes until homogenous, and then

transferred to amber glass bottles. After the contents of the amber bottles had reached room temperature, they were stored in a refrigerator at a temperature of $\pm 6.0^{\circ}\text{C}$.

3.3.3 Characterisation

3.3.3.1 Confocal Laser Scanning Microscopy (CLSM)

To establish the approximate size and morphology of the pheroid vesicles and microsponges, confocal laser scanning microscopy were used, namely a PCM 2000 CLSM taken with a Nikon digital camera DXM 1200 (real-time imaging, Nikon, Holland and He/Ne laser with a 60 x ApoPlanar, NA 1.4, oil immersion objective, excitation at 505 nm and oil emission at 568 nm). The vesicles and microsponges were labelled with Nile Red and placed separately on a glass slide, covered with a glass cover slip. Images were obtained through capturing by photomultipliers (see figure 3.3).

3.3.3.2 Particle size analysis

The droplet sizes of the pheroid vesicles and microsponges were measured with a Malvern Mastersizer (Malvern Instruments, United Kingdom). The Malvern Mastersizer was washed with 800 ml water for 30 seconds before it was used. Fresh water was used for the alignment of the laser. The background was measured and a certain volume (2.0 ml) of the sample was added for the measurement of volume sizes.

3.3.4 Results and discussion

As mentioned earlier, CLSM was used to analyse the morphology of the pheroid vesicles and microsponges. In figure 3.3, micrographs A and B illustrate the spherical nature with a homogenous intensity of the pheroid vesicles, whereas micrographs C and D show the sponge-like appearance with a homogenous intensity of the pheroid microsponges. These confocal laser scanning micrographs of the pheroid vesicles and microsponges indicate that insulin, after entrapment, causes neither a significant decrease nor a significant increase in the quantity and size of pheroid vesicles and microsponges compared to the preparations before the entrapment of insulin.

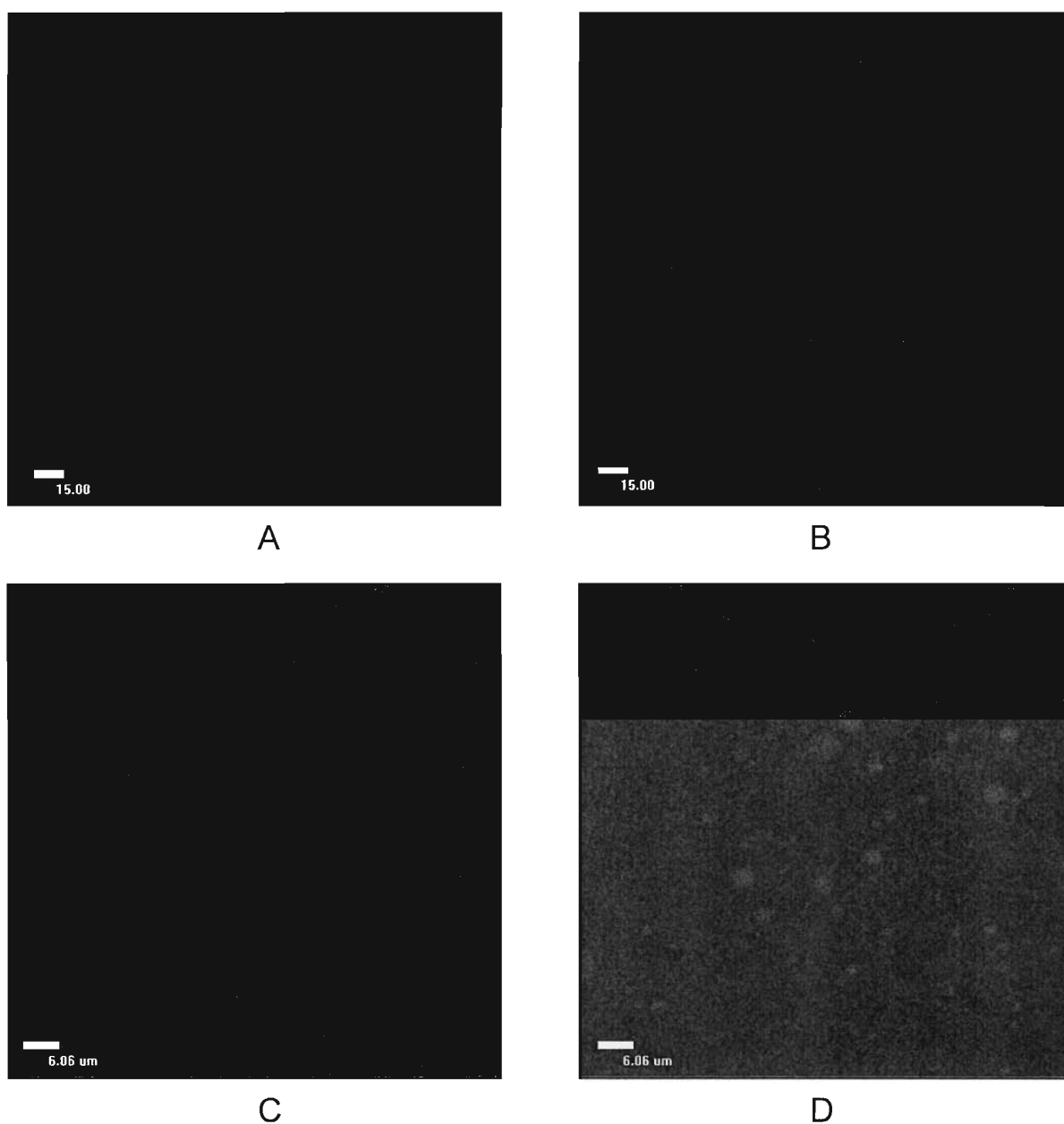


Figure 3.3 Micrographs of (A) pheroid vesicles, (B) pheroid vesicles with insulin, (C) pheroid microsponges and (D) pheroid microsponges with insulin

The results of the particle size analysis of the pheroid vesicles and microsponges are summarised in table 3.4. The Malvern Mastersizer indicated that the particle distribution of pheroid vesicles varied mainly between 0.65 and 5.54 μm before insulin entrapment and between 0.59 and 4.68 μm after. In addition, the approximate size for the pheroid microsponges was found to be between 1.39 and 17.34 μm before insulin entrapment and between 1.82 and 19.49 μm after.

Table 3.4 **The sizes (volume) of pheroid vesicles and pheroid microsponges before and after insulin entrapment**

<i>Parameters</i>	<i>Sizes of pheroid vesicles <u>before</u> insulin entrapment (μm)</i>	<i>Sizes of pheroid vesicles <u>after</u> insulin entrapment (μm)</i>
D (v, 0.1)	0.65	0.59
D (v, 0.5)	1.94	1.71
D (v, 0.9)	5.54	4.68
<i>Parameters</i>	<i>Sizes of pheroid microsponges <u>before</u> insulin entrapment (μm)</i>	<i>Sizes of pheroid microsponges <u>after</u> insulin entrapment (μm)</i>
D (v, 0.1)	1.39	1.82
D (v, 0.5)	5.71	8.25
D (v, 0.9)	17.34	19.49

D (v, 0.5); (v, 0.1); (0.9) are standard “percentile” readings from the analysis.

- D (v, 0.5) → The size at which 50% of the sample is smaller and 50% is larger than this size. This value is also known as the mass median diameter (MMD).
- D (v, 0.1) → The size of particle at which 10% of the sample is smaller than this size.
- D (v, 0.9) → The size of particle at which 90% of the sample is smaller than this size.

3.4 The entrapment of insulin in pheroids

3.4.1 Materials

Insulin (human, recombinant, expressed in yeast) was obtained from Sigma-Aldrich (Kempton Park, South Africa) and had a potency of 27.5 International Units per milligram (dry base). Pheroid vesicles and pheroid microsponges were freshly prepared at the North-West University as described in section 3.3. For the control groups, saline (a 0.9% NaCl-solution) was used.

3.4.2 Method

Table 3.5 shows the quantity of insulin weighed for the preparation of pheroid vesicle and pheroid microsphere formulations.

Table 3.5 Quantity insulin weighed and volume of pheroid vesicles or pheroid microsponges for each dose administered

<i>Dose administered to rats</i>	<i>Quantity of insulin weighed</i>	<i>Volume of pheroid vesicle- or pheroid microsphere solutions</i>
4 IU/kg	7.25 mg	5.0 ml
8 IU/kg	14.50 mg	5.0 ml
12 IU/kg	21.75 mg	5.0 ml

The pheroid preparations were prepared 24 hours before administration and insulin was entrapped one hour before administration. Pheroid preparations were brought to a temperature of 37.0°C in a water bath before the insulin was dissolved and entrapped into the pheroid solutions. These formulations were then slowly shaken for 15 minutes and then kept on ice until being administered to the rats. Final preparations contained the equivalent quantity of insulin of 4 IU/kg, 8 IU/kg and 12 IU/kg bodyweight respectively when administered in a volume of 100 µl/kg bodyweight.

The control solution was prepared in the same way, by dissolving either 7.25 mg, 14.50 mg or 21.75 mg of insulin in 5.0 ml saline solution (0.9%). All solutions were freshly prepared each time on the day of nasal administration.

3.5 Preparation of *N*-trimethyl chitosan chloride (TMC) solution

3.5.1 Materials

Insulin was obtained from Sigma-Aldrich (Kempton Park, South Africa). *N*-trimethyl chitosan chloride (TMC) was a generous gift from the Department of Pharmaceutics at the North-West University, Potchefstroom Campus (South Africa) and was used as received. The degree of quaternisation of TMC was given as 48.0% and the molecular weight was 166 000 g/mole.

3.5.2 Method

For the preparation of a 1.0% (w/v) TMC solution, 50 mg of TMC polymer was dissolved in 5.0 ml distilled water. Insulin (5.80 mg) was added to 2.0 ml of saline solution to obtain an 8 IU/kg insulin solution. Then 1.0 ml of the 1.0% (w/v) TMC solution and 1 ml of the 8 IU/ml insulin solution were mixed together to obtain a final polymer concentration of 0.5% (w/v). When nasally administered at a volume of 100 μ l/kg bodyweight, rats received a dose of 4 IU/kg bodyweight of insulin.

3.6 Conclusion

The bioavailability of intranasally administered insulin is very low, due to the high molecular weight, hydrophilic nature of insulin and the degradation through various enzymes in the nose. Pheroid vesicles, pheroid microsponges and the TMC polymers are all delivery systems, which are expected to improve the absorption and bioavailability of insulin. This chapter described the preparation and characterisation of pheroid vesicles, pheroid microsponges, TMC solutions and the entrapment of insulin in these formulations. The different formulations will be administered to the left nostril of Sprague Dawley rats, based on the *in vivo* experimental procedures described in this chapter. A discussion of the results of the study will be presented in the next chapter.

***Nasal delivery of insulin with
pheroid technology and
N-trimethyl chitosan chloride (TMC):***

Results and Discussion

4.1 Introduction

When insulin (4, 8 and 12 IU/kg bodyweight) is administered nasally to rats with absorption-enhancing systems such as pheroid vesicles, pheroid microsponges and *N*-trimethyl chitosan chloride (TMC), it is expected to result in a decrease in blood glucose levels as well as an increase in the absorption of insulin compared to the baseline measurements. Blood glucose levels were determined with a glucometer and the results were presented in mmol/L, while plasma insulin concentrations were determined *in vitro* with an immunoradiometric assay kit and the results were expressed in μ IU/ml.

The results of both the blood glucose levels and plasma insulin concentrations were converted to percentages (%), since different batches of kits were used during the immunoradiometric analyses. Graphs were drawn, using the blood glucose levels achieved and the plasma insulin concentrations, during the 3-hour sampling periods respectively, to indicate how efficacious the study was.

4.2 Experimental design

The experimental design of this study is presented in figure 4.1, where insulin was nasally administered to rats at three different dosages respectively (4, 8 and 12 IU/kg bodyweight). Each dose of insulin was administered with saline (0.9%) as the control group, pheroid vesicles and

pheroid microsponges. TMC was administered only with insulin at a dose of 4 IU/kg bodyweight. A total of ten groups with six male Sprague Dawley rats in each were used in this study. Blood glucose levels were measured in all the groups, whereas plasma insulin concentrations were determined only in the 4 IU/kg bodyweight insulin formulations due to technical factors.

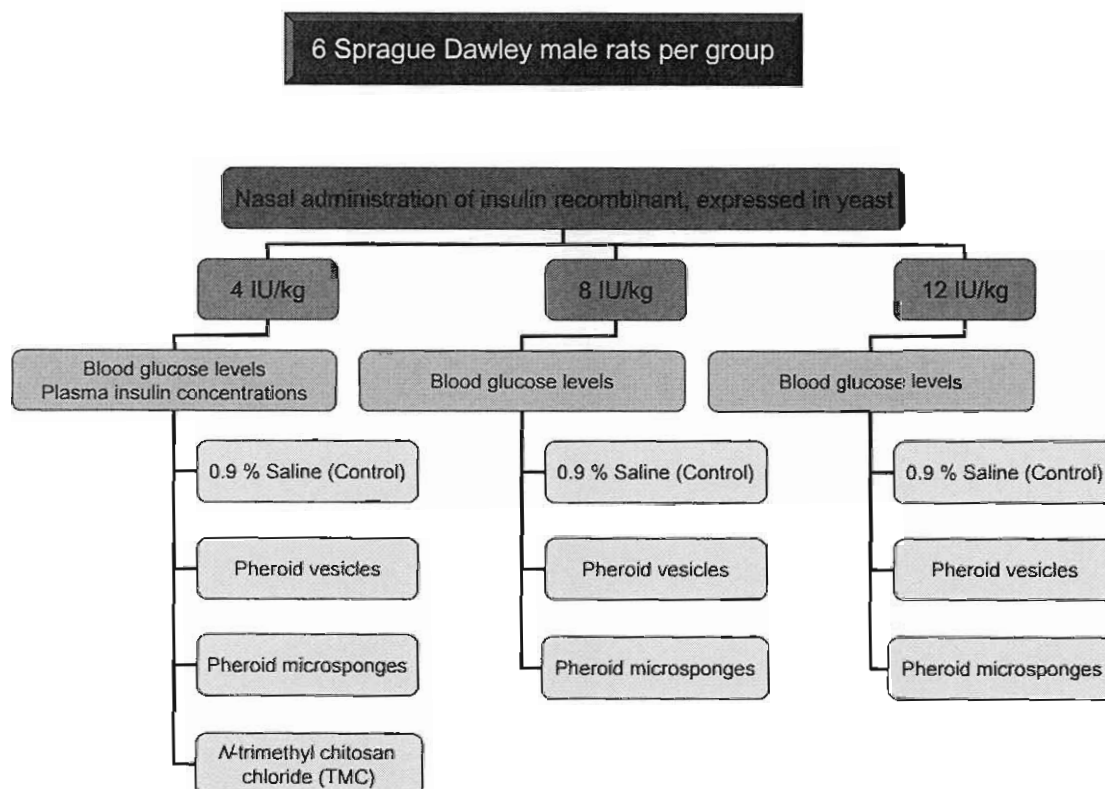


Figure 4.1 Schematic representation of the experimental design in this study

4.3 The effect of pheroid technology and *N*-trimethyl chitosan chloride (TMC) on the nasal adsorption of 4 IU/kg bodyweight insulin

4.3.1 Blood glucose levels

The blood glucose levels obtained after the nasal administration of 4 IU/kg bodyweight insulin to rats, with saline (control), pheroid vesicles, pheroid microsponges and *N*-trimethyl chitosan chloride respectively, are given in table 4.1.

Table 4.1 Blood glucose levels (%) after nasal administration of insulin (4 IU/kg bodyweight) to rats

<i>Blood glucose levels (%)</i>				
<i>Formula</i>	<i>Control</i>	<i>Pheroid vesicles</i>	<i>Pheroid microsponges</i>	<i>TMC</i>
<i>Rats used</i>	n = 5	n = 6	n = 6	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	94.37 ± 8.88	95.72 ± 7.32	111.81 ± 8.44	98.19 ± 11.96
10	93.96 ± 12.05	99.36 ± 8.65	109.14 ± 24.92	98.61 ± 13.47
15	92.80 ± 11.74	113.45 ± 15.93	106.41 ± 11.49	92.78 ± 7.23
30	94.34 ± 9.33	102.89 ± 11.97	97.23 ± 14.91	72.89 ± 12.45
60	104.72 ± 24.42	97.78 ± 24.81	98.26 ± 21.58	50.19 ± 16.01
120	111.67 ± 26.01	110.06 ± 39.98	118.48 ± 36.39	42.30 ± 11.65
180	131.39 ± 18.39	134.51 ± 27.71	123.22 ± 39.85	48.97 ± 14.67

AV → Average blood glucose level

SD → Standard deviation

Figures 4.2 to 4.5 represent the blood glucose levels in % against time (min) after the nasal administration of insulin to rats. Figure 4.6 gives a comparison of the average blood glucose levels after nasal administration of 4 IU/kg bodyweight insulin in different formulations.

The reduction in blood glucose levels of the different formulations 120 minutes after nasal administration of 4 IU/kg bodyweight is illustrated in figure 4.7.

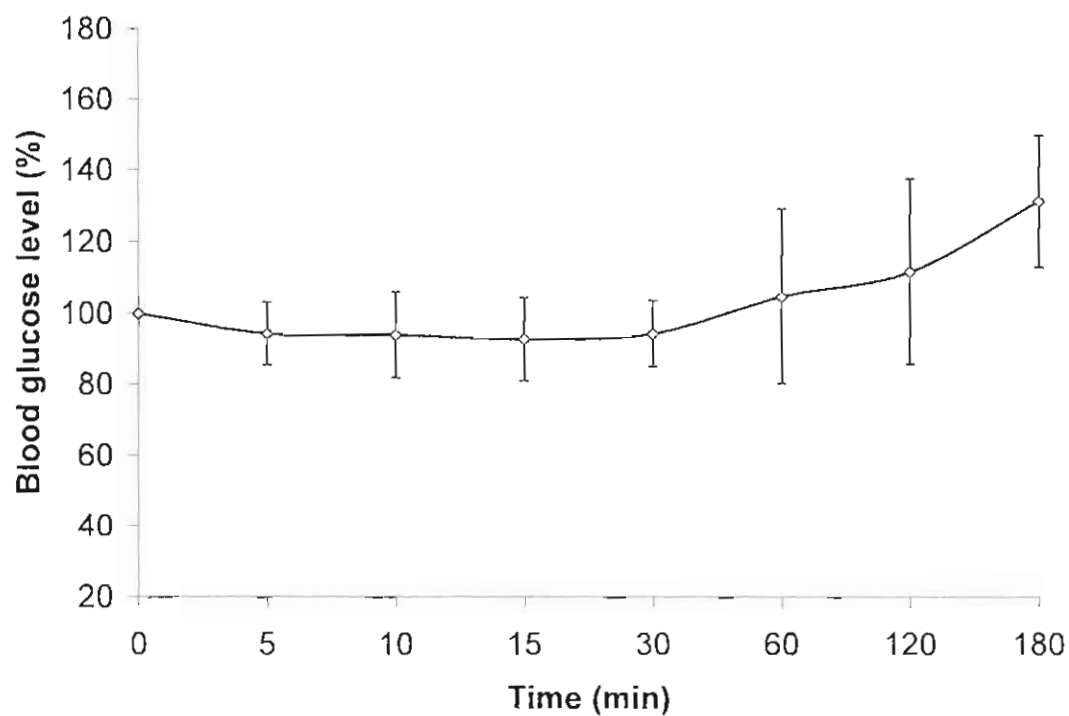


Figure 4.2 Average blood glucose level (n = 5) after nasal administration of insulin (4 IU/kg bodyweight) with saline to rats

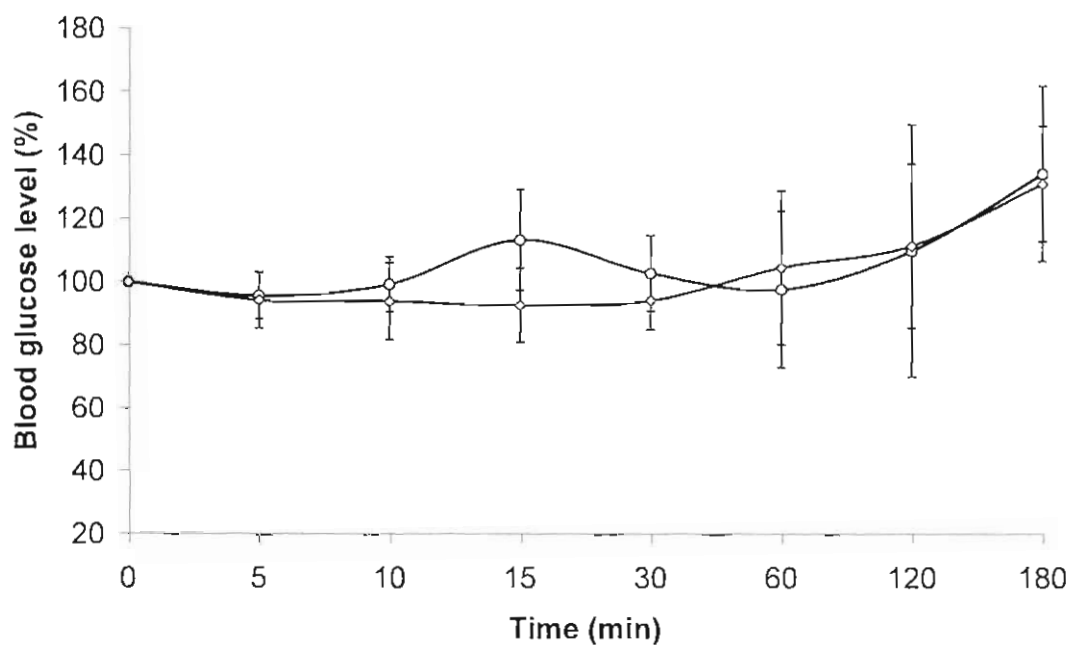


Figure 4.3 Average blood glucose level (n = 6) after nasal administration of insulin to rats (4 IU/kg bodyweight) with pheroid vesicles

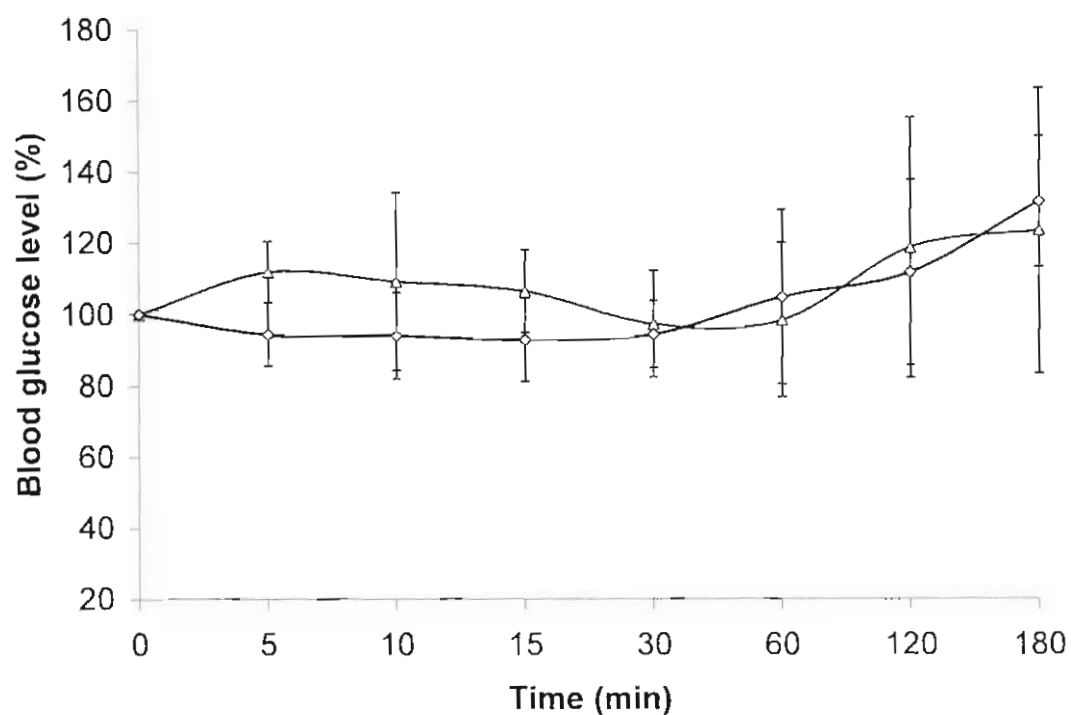


Figure 4.4 Average blood glucose level (n = 6) after nasal administration of insulin to rats (4 IU/kg bodyweight) with pheroid microsponges

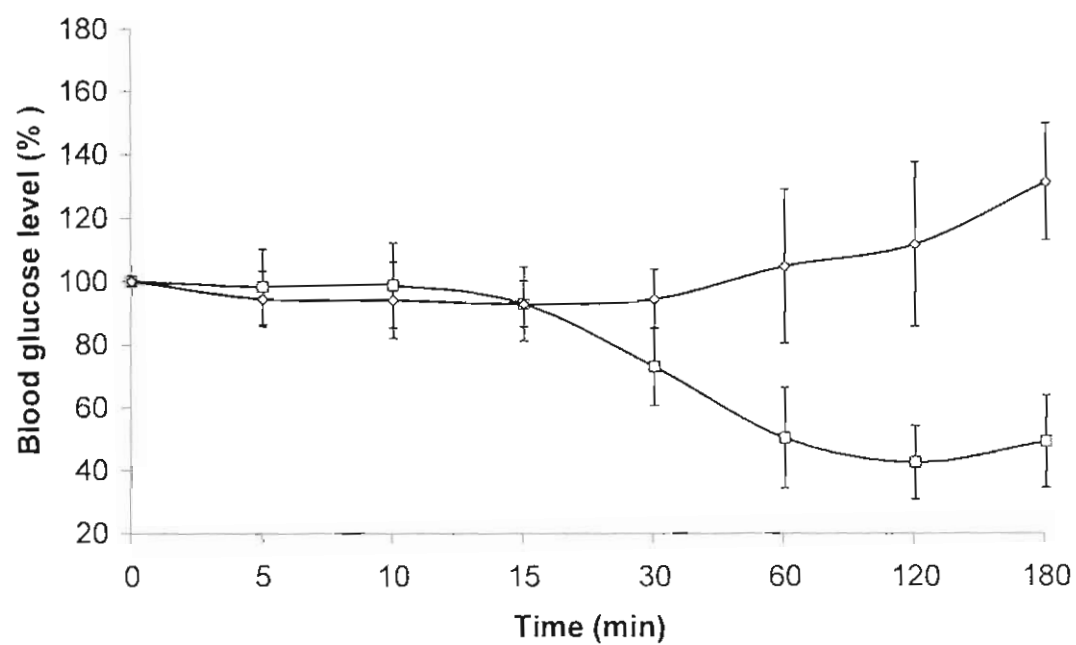


Figure 4.5 Average blood glucose level (n= 5) after nasal administration of insulin to rats (4 IU/kg bodyweight) with TMC

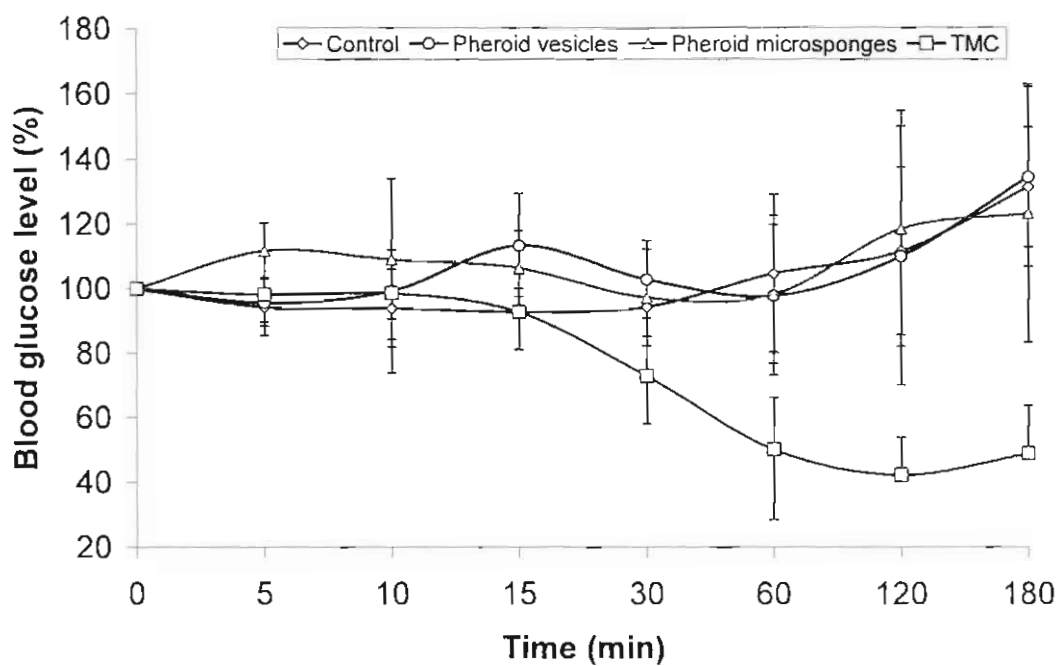


Figure 4.6 Comparison of average blood glucose levels against time (min) after nasal administration of insulin to rats (4 IU/kg bodyweight)

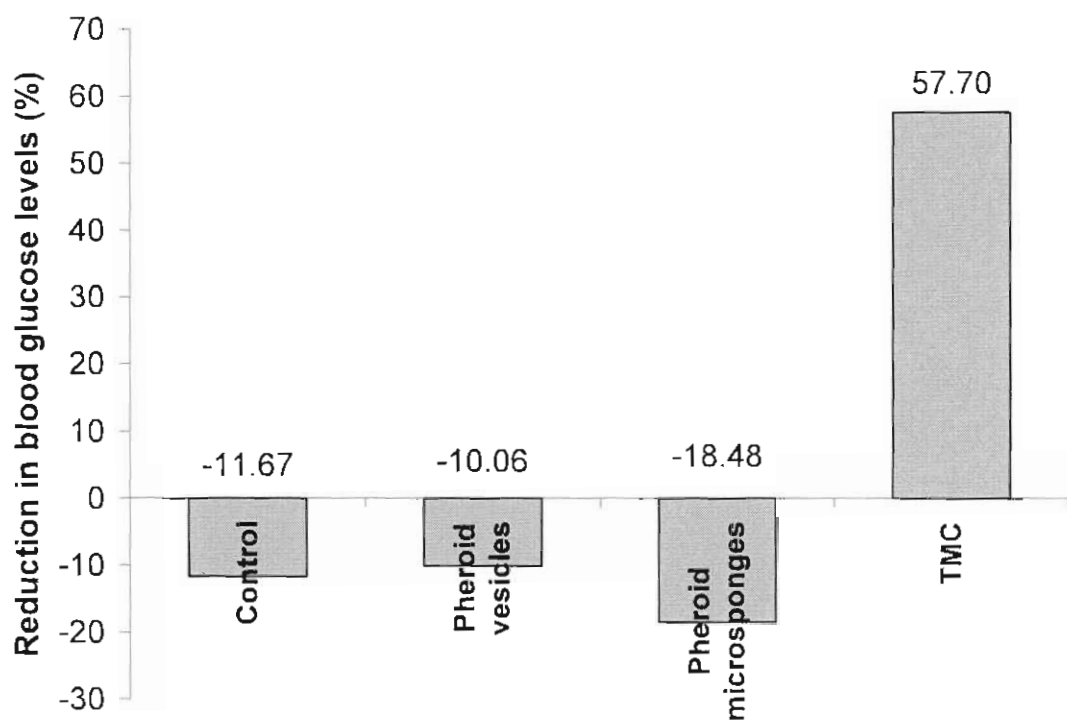


Figure 4.7 The reduction in blood glucose levels 120 minutes after nasal administration of 4 IU/kg bodyweight insulin with different formulations to rats

4.3.2 Plasma insulin concentrations

The plasma insulin concentrations after analysis of the blood samples of 4 IU/kg bodyweight insulin with saline (control), pheroid vesicles, pheroid microsponges and *N*-trimethyl chitosan respectively are given in table 4.2. The results of the plasma insulin concentrations in percentage (%) were also calculated, due to the different batches of kits used.

Table 4.2 Plasma insulin concentration (%) after the nasal administration of insulin (4 IU/kg bodyweight) to rats

<i>Plasma insulin concentrations (%)</i>				
<i>Formula</i>	<i>Control</i>	<i>Pheroid vesicles</i>	<i>Pheroid microsponges</i>	<i>TMC</i>
<i>Rats used</i>	n = 5	n = 6	n = 6	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	59.76 ± 14.11	108.23 ± 21.27	107.41 ± 23.43	100.48 ± 55.23
10	72.44 ± 11.61	98.57 ± 24.31	111.28 ± 34.44	167.98 ± 84.00
15	86.93 ± 37.69	101.12 ± 15.47	119.49 ± 30.52	305.67 ± 80.64
30	89.64 ± 34.95	106.43 ± 24.25	107.05 ± 37.39	427.81 ± 117.79
60	90.07 ± 27.77	105.72 ± 12.74	105.19 ± 20.88	365.63 ± 118.21
120	96.99 ± 25.74	100.95 ± 25.00	111.82 ± 17.47	210.37 ± 71.00
180	71.99 ± 30.99	103.46 ± 16.82	93.45 ± 19.45	130.12 ± 21.90

AV → Average plasma insulin concentration SD → Standard deviation

Figures 4.8 to 4.11 represent the plasma insulin concentrations in % against time (min) after the nasal administration of insulin to rats. The comparison of the average plasma insulin concentrations against time is given in figure 4.12, whereas figure 4.13 illustrates the increase in plasma insulin concentrations 30 minutes after nasal administration of 4 IU/kg bodyweight insulin with the different formulations.

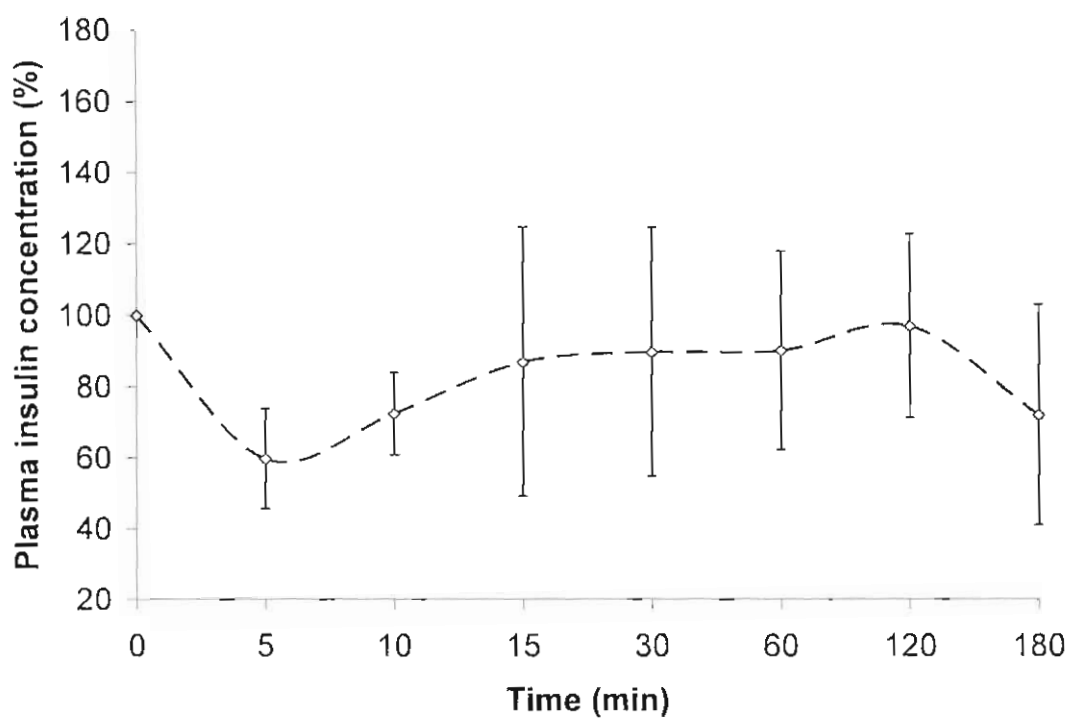


Figure 4.8 Average plasma insulin concentrations ($n = 5$) after nasal administration of insulin (4 IU/kg bodyweight) with saline to rats

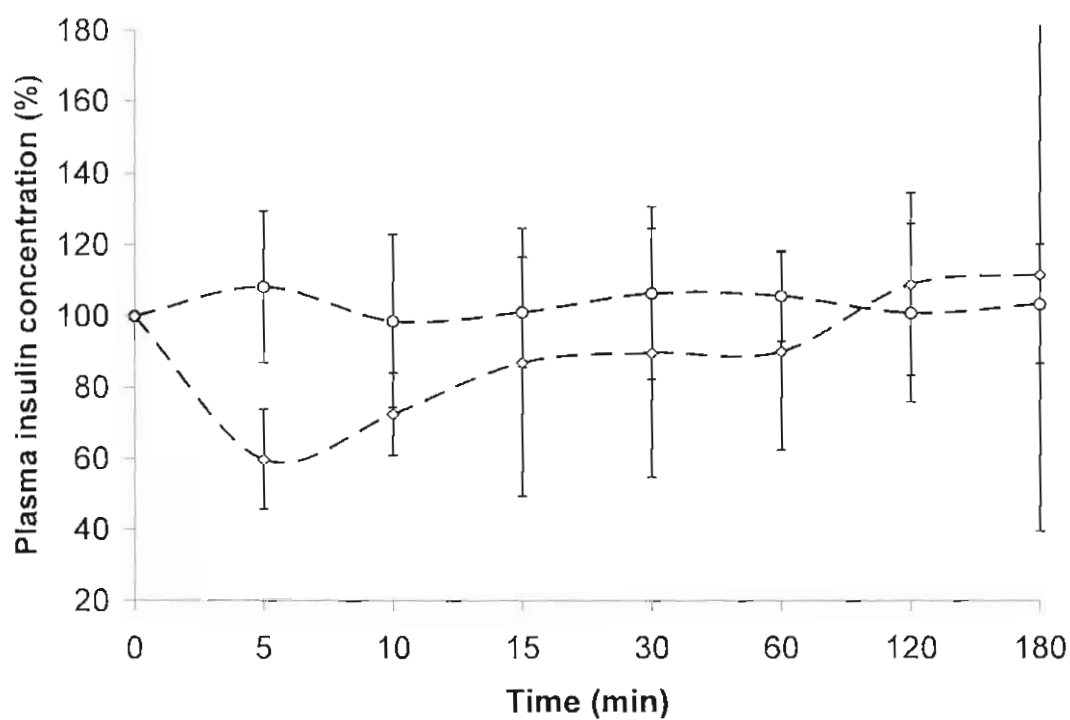


Figure 4.9 Average plasma insulin concentrations ($n = 6$) after nasal administration of insulin (4 IU/kg bodyweight) with pheroid vesicles to rats

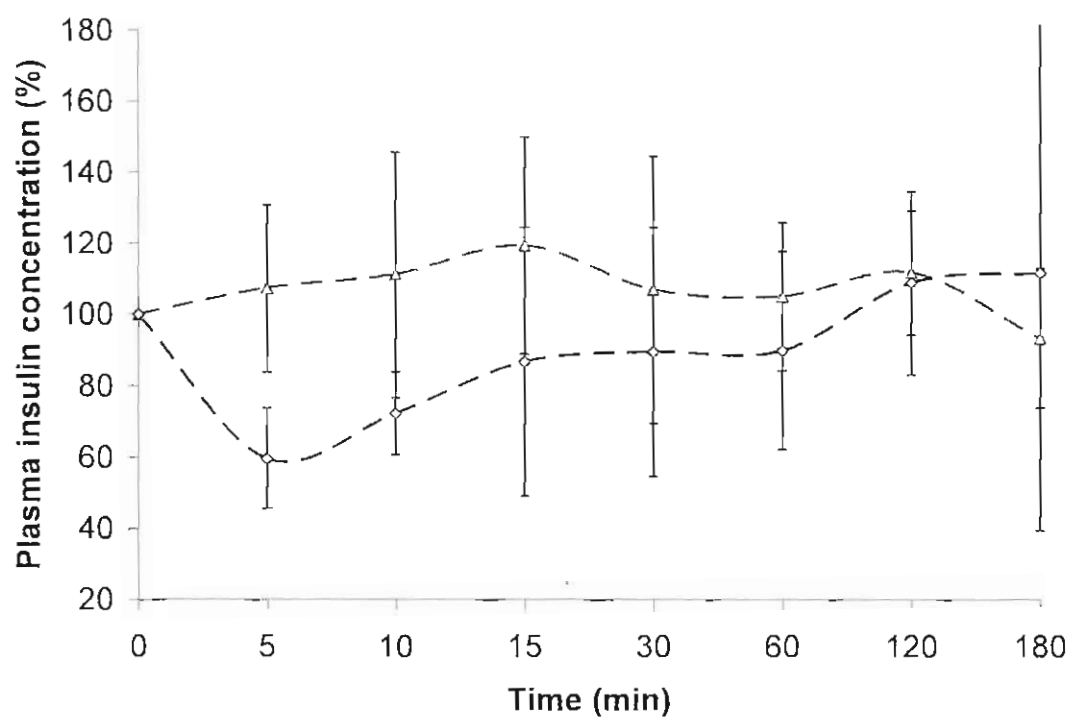


Figure 4.10 Average plasma insulin concentrations ($n = 6$) after nasal administration of insulin (4 IU/kg bodyweight) with pheroid microsponges to rats

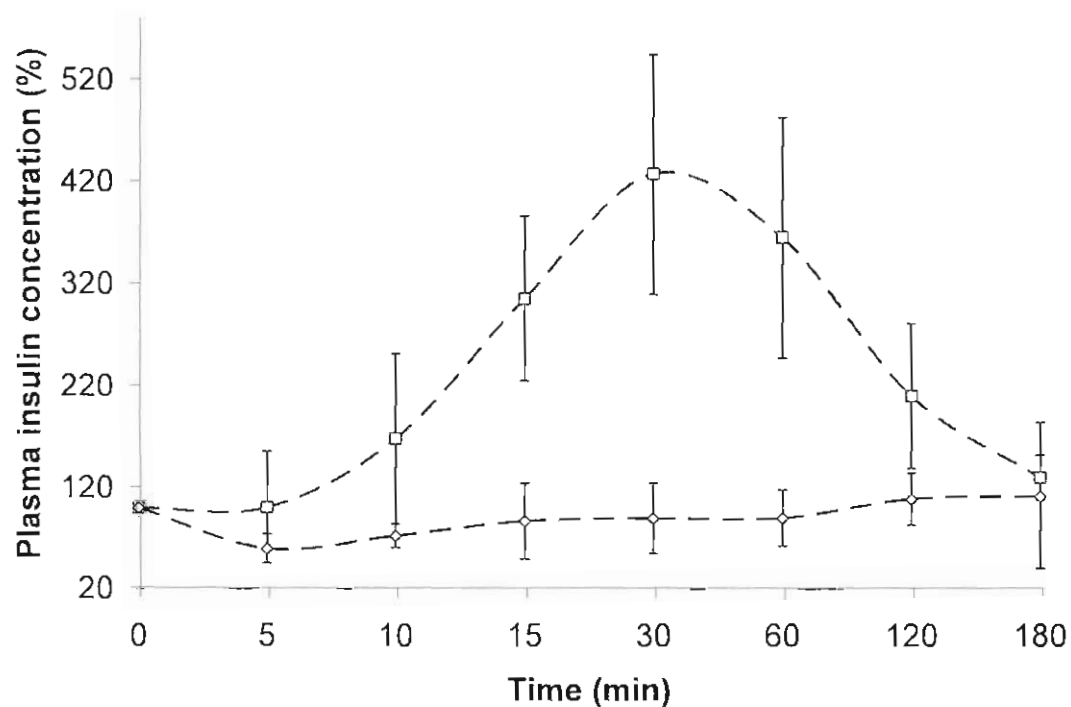


Figure 4.11 Average plasma insulin concentrations ($n = 5$) after nasal administration of insulin (4 IU/kg bodyweight) with TMC to rats

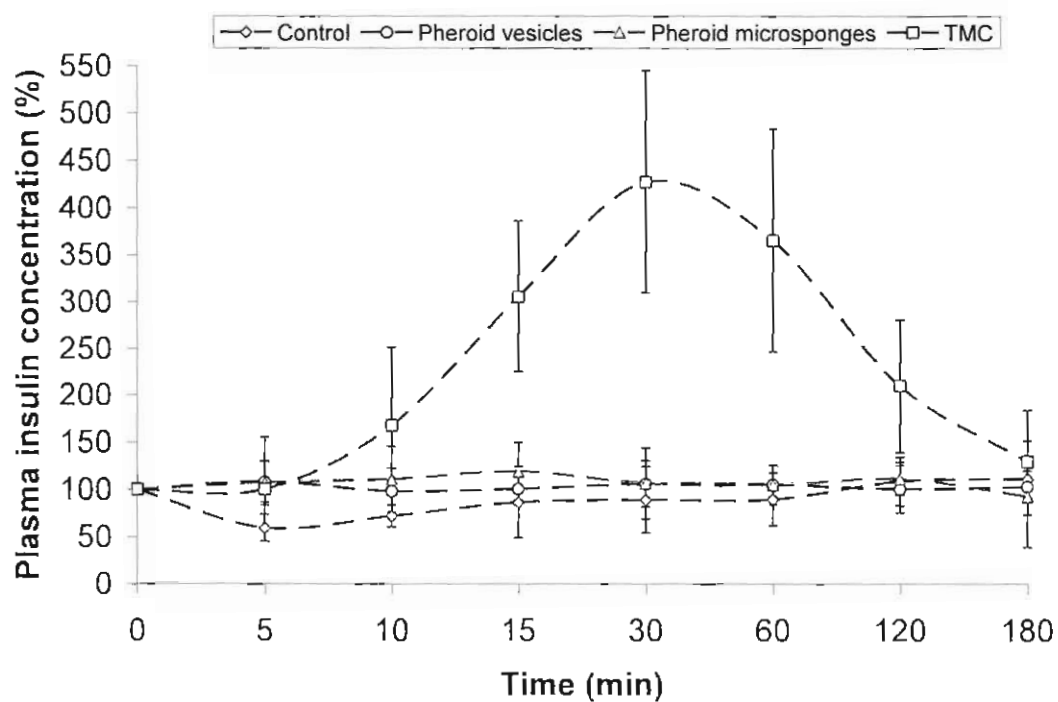


Figure 4.12 Comparison of plasma insulin concentrations against time (min) after nasal administration of insulin (4IU/kg bodyweight) to rats

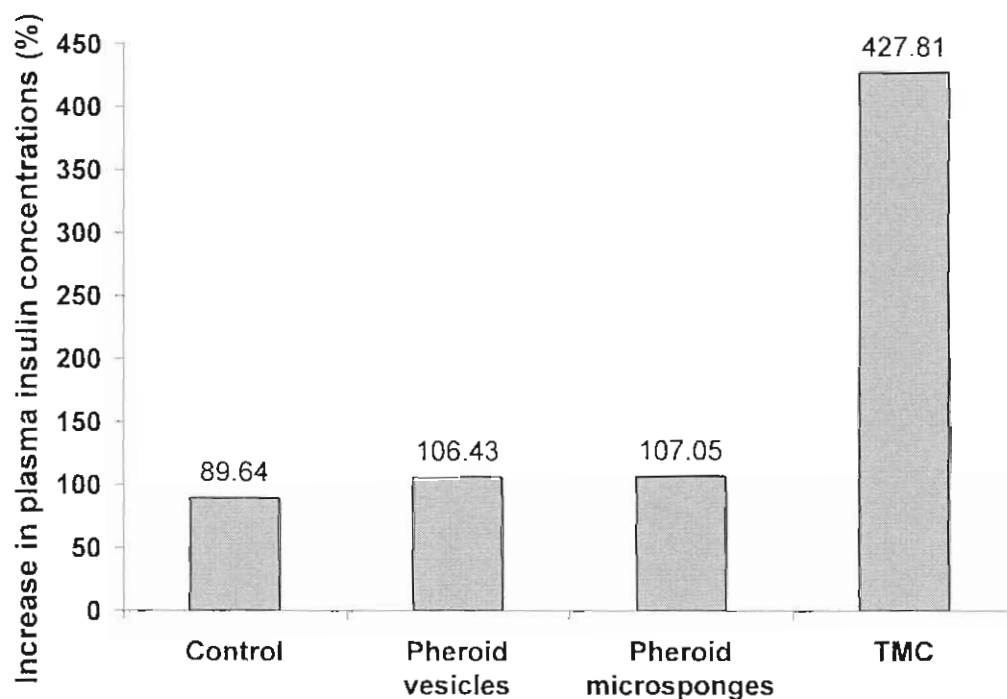


Figure 4.13 The increase in plasma insulin concentrations 30 minutes after nasal administration of 4 IU/kg bodyweight insulin with different formulations

The effects of saline, pheroid vesicles, pheroid microponges and TMC on blood glucose levels after administration of 4 IU/kg bodyweight insulin across the nasal mucosa are shown in figures 4.2 to 4.5 respectively, whereas the plasma insulin concentrations are shown in figures 4.8 to 4.11. The data in terms of the blood glucose levels is summarised in table 4.1 and that regarding plasma insulin concentrations in table 4.2.

As shown in figure 4.3, the blood glucose levels after the nasal administration of 4 IU/kg bodyweight insulin to rats decreased at 60 min, but not significantly when pheroid vesicles were used as an absorption-enhancing system (table 4.1). Similar results were obtained for the plasma insulin concentrations showed in figure 4.9. Compared to the control group, no major differences were observed against time. Thus, the nasal administration of insulin with saline and pheroid vesicles respectively did not lead to a significant change in the blood glucose levels. The plasma insulin concentrations after the administration of the pheroid vesicles and insulin 4 IU/kg bodyweight can possibly be normal levels, maintained in the rats under normal conditions.

There was, however, a slight increase in the plasma insulin concentration of pheroid microsponges with insulin 4 IU/kg bodyweight after 10 minutes (as seen in table 4.2 and figure 4.10). The blood glucose levels did not show a remarkable decrease over time with pheroid microsponges, as seen in figure 4.4, and compared to the control group the effect was more or less the same. Therefore, the pheroid microsponges with insulin and the control group both indicated that there was no significant decrease in blood glucose levels that corresponded with the plasma insulin concentrations.

Figure 4.5 illustrated clearly that the blood glucose levels decreased significantly with the TMC formulation. When compared to pheroid vesicles and pheroid microsponges in figure 4.6, and to other formulations, an extreme decrease in blood glucose levels of TMC can be seen. Nasal administration of insulin with saline, pheroid vesicles and pheroid microsponges failed to reduce the blood glucose levels although a definite decrease was seen with the formulation containing TMC. The minimum blood glucose level, 42.30% of the initial value, was obtained 120 minutes after the administration of the insulin (4 IU/kg bodyweight) formulation containing TMC. Immunoradiometric analysis indicated that TMC showed a remarkable increase in the absorption of insulin (see figure 4.11). The maximum plasma insulin concentration of 427.81% was obtained 30 minutes after nasal administration of TMC, which is illustrated in figure 4.13. It was

possible for the TMC formulation to increase the absorption of insulin significantly, compared to the other formulations with saline, pheroid vesicles and pheroid microsponges. This correlates with the decrease in blood glucose levels (see figures 4.7 and 4.13).

When the blood glucose levels obtained from pheroid vesicle and pheroid microsphere formulations are compared to each other in table 4.1 and figure 4.6, it is clear that similar results were obtained for both. Pheroid vesicles offer a minimum blood glucose effect of 95.72% after 5 minutes, whereas pheroid microsponges shows its lowest point of 97.23% after 30 minutes. However, both these decreases in blood glucose levels are not significant. Pheroid microsponges lead to a slightly better absorption of insulin compared to pheroid vesicles. In figure 4.12 and table 4.2 it can clearly be seen that the maximum effect of pheroid microsponges (119.49%) was obtained 15 minutes after administration whereas pheroid vesicles (106.43%) only achieved maximum point of absorption at 30 minutes after administration. It therefore seems that pheroid microsponges have a faster onset of action and a slightly better absorption of insulin when compared to pheroid vesicles.

The results obtained in this study are not comprehensive; more research and investigations need be done before the assumption can be accepted.

Because the blood glucose levels did not show a significant decrease with both the pheroid vesicle- and pheroid microsphere formulations, it was justifiable to investigate the influence of a higher dose of insulin with these formulations. In the next section, the results in terms of and discussion of insulin at 8 IU/kg bodyweight with pheroid vesicles and pheroid microsponges respectively will be discussed.

4.4 The effect of pheroid technology on the nasal absorption of 8 IU/kg bodyweight insulin

After nasal administration of insulin (4 IU/kg bodyweight) to rats with no significant change in blood glucose levels, it was decided to increase the dose of insulin to 8 IU/kg bodyweight in which a greater decrease in blood glucose levels was anticipated. Only blood glucose levels were monitored and plasma insulin concentrations were not measured. Table 4.3 gives the blood

glucose levels obtained after nasal administration of 8 IU/kg bodyweight insulin with saline (control), pheroid vesicles and pheroid microsponges respectively.

Table 4.3 **The blood glucose levels (%) after nasal administration of insulin (8 IU/kg bodyweight) to rats**

<i>Blood glucose levels (%)</i>			
<i>Formula</i>	<i>Control</i>	<i>Pheroid vesicles</i>	<i>Pheroid microsponges</i>
<i>Rats used</i>	n = 5	n = 6	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	96.78 ± 5.67	97.52 ± 12.23	96.75 ± 14.99
10	88.75 ± 9.35	98.14 ± 11.99	107.34 ± 19.59
15	89.58 ± 10.39	98.66 ± 14.47	103.49 ± 11.57
30	96.46 ± 26.96	99.25 ± 16.90	96.78 ± 15.15
60	92.60 ± 27.05	84.44 ± 23.97	93.13 ± 10.33
120	120.30 ± 31.49	81.78 ± 22.48	88.80 ± 12.31
180	131.28 ± 32.72	82.52 ± 34.42	97.21 ± 18.52

AV → Average blood glucose level SD → Standard deviation

The following graphs (figures 4.14 to 4.16) represent the blood glucose levels as a percentage against time (min) after the nasal administration of insulin (8 IU/kg bodyweight) to rats. Blood glucose levels were measured in mmol/litre and translated to a percentage (%).

Figure 4.17 illustrates the comparison of the results in terms of the average blood glucose levels obtained after the nasal administration of 8 IU/kg bodyweight insulin with the different formulations. Figure 4.18 records whether and how much blood glucose levels reduced 120 minutes after nasal administration of 8 IU/kg insulin with the different formulations.

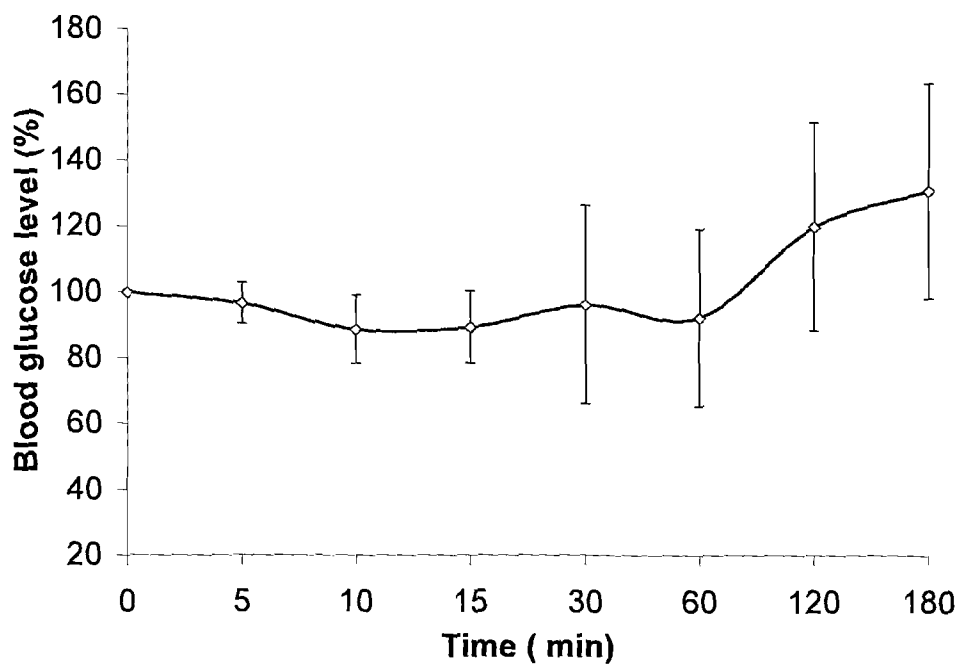


Figure 4.14 Average blood glucose level ($n = 5$) after nasal administration of insulin (8 IU/kg bodyweight) with saline to rats

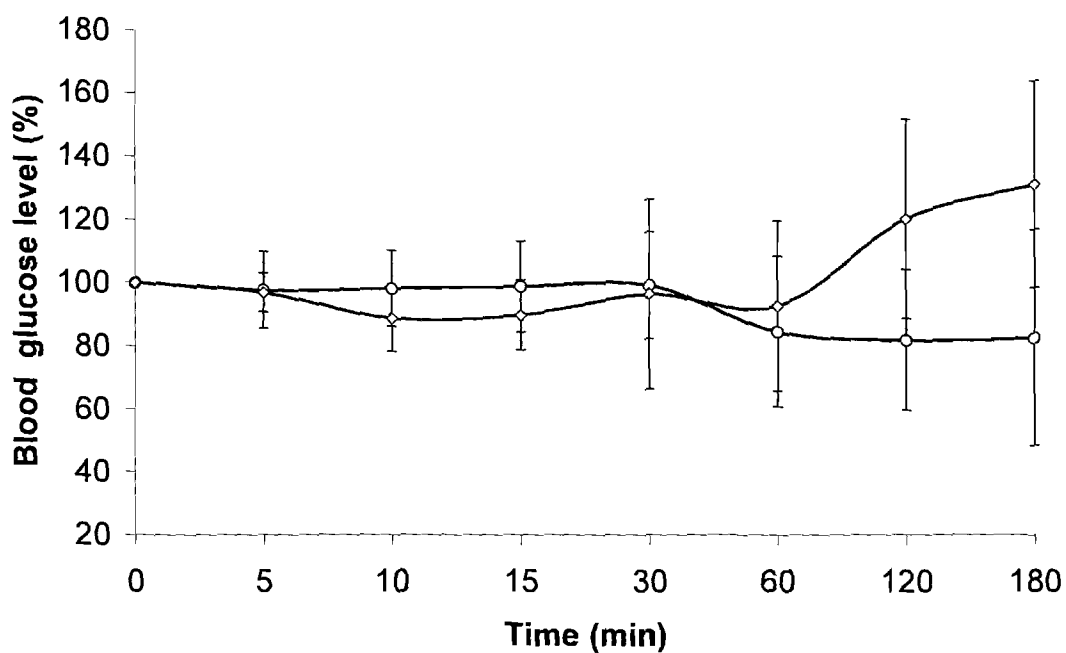


Figure 4.15 Average blood glucose level ($n = 6$) after nasal administration of insulin (8 IU/kg bodyweight) with pheroid vesicles to rats

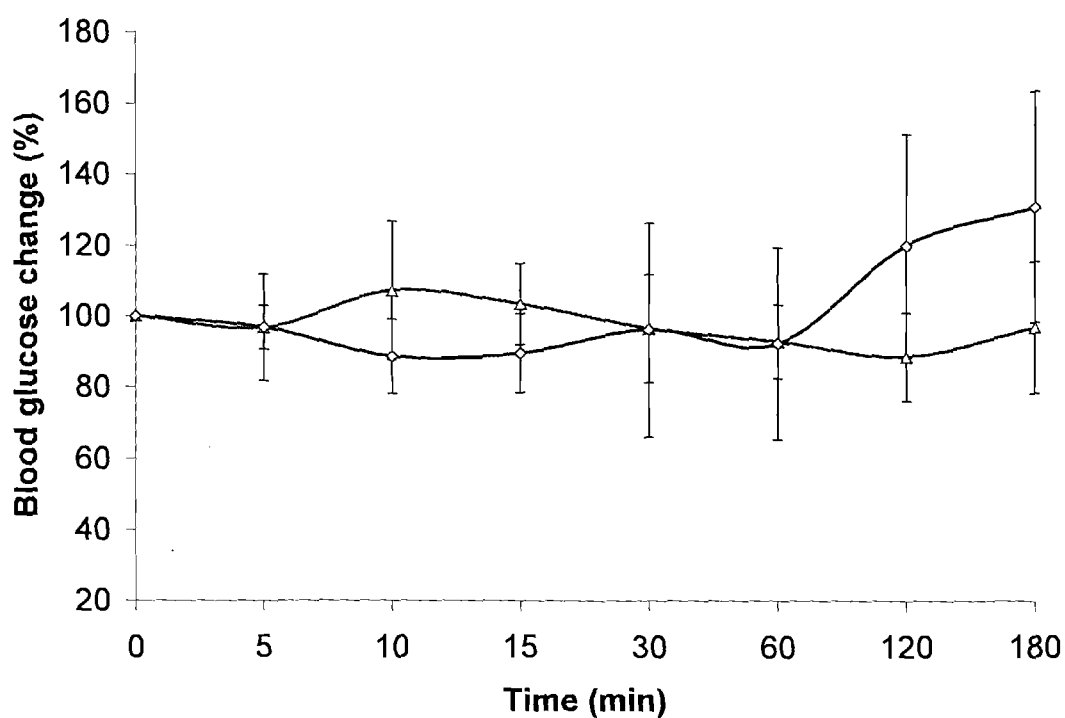


Figure 4.16 Average blood glucose level ($n = 5$) after nasal administration of insulin (8 IU/kg bodyweight) with pheroid microsponges to rats

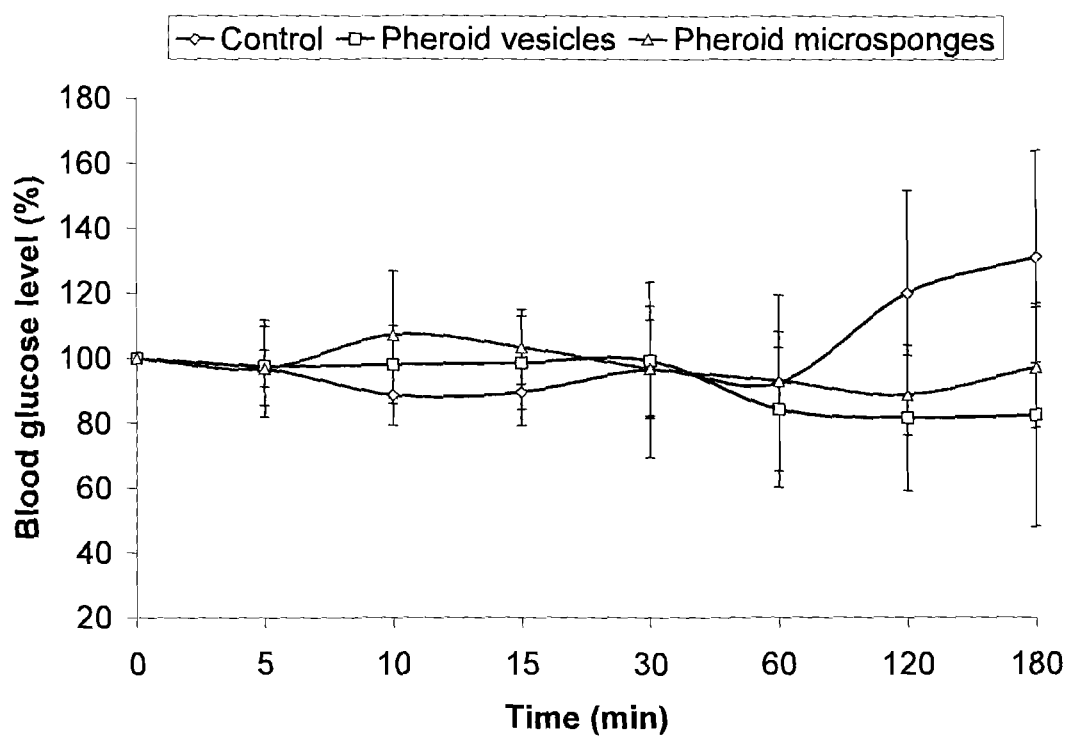


Figure 4.17 Comparison of average blood glucose levels against time (min) after nasal administration of insulin (8 IU/kg bodyweight) to rats

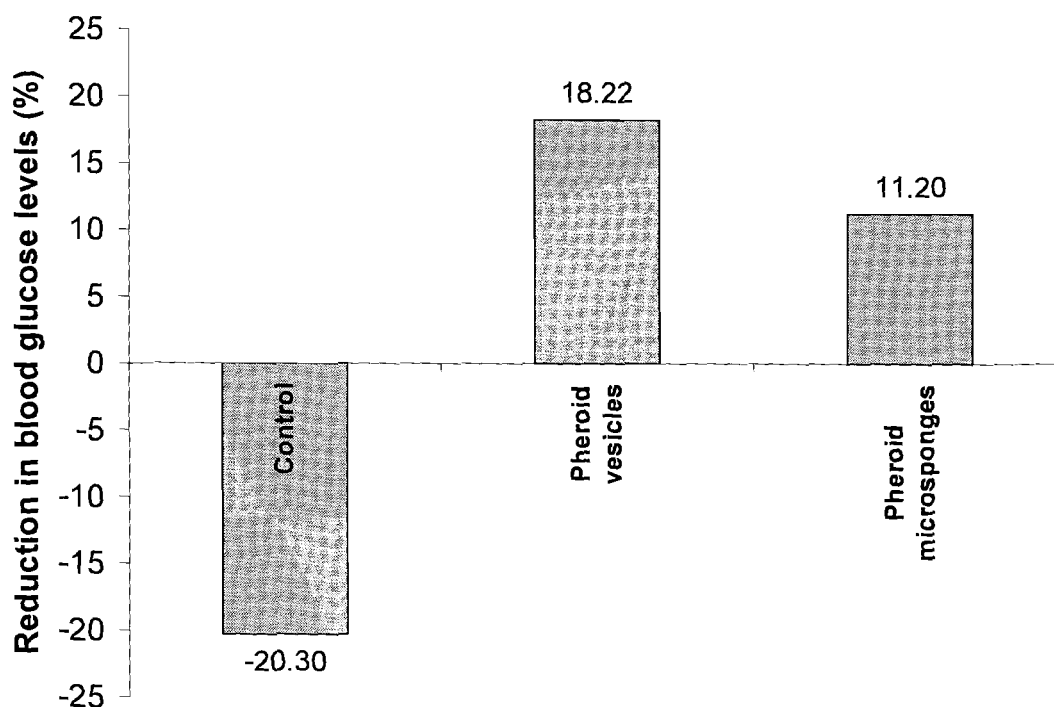


Figure 4.18 The reduction in blood glucose levels 120 minutes after nasal administration of 8 IU/kg bodyweight insulin with different formulations

In figure 4.14 – 4.16 the effects of saline, pheroid vesicles and pheroid microsponges on the blood glucose levels after administration of 8 IU/kg bodyweight insulin are illustrated. These results are summarised in table 4.3.

After the nasal administration of 8 IU/kg bodyweight insulin with pheroid vesicles to rats, blood glucose levels started to decrease at 60 minutes to 84.44 % (see figure 4.15) and continued to decrease, with the minimum blood glucose point of 81.78 % at 120 minutes, whereafter the levels began to increase slightly. Blood glucose levels started to increase with the control group (saline) at 120 minutes after nasal administration, and did not show any significant blood glucose lowering effect (see figure 4.14).

As shown in figure 4.16, the blood glucose level of insulin (8 IU/kg bodyweight) with pheroid microsponges began to decrease slightly at 15 minutes already, and had a maximum effect after nasal administration where blood glucose levels were lowered to 88.80 % of the original value. Compared to the control group, there was a greater decrease in blood glucose levels with insulin

containing pheroid microsponges, but not enough to achieve a definite lowering in the blood glucose levels.

When the effects of blood glucose levels with pheroid vesicles and pheroid microsponges were compared to each other in figure 4.17, it was clear that the formulation of insulin with pheroid vesicles resulted in a greater decrease in blood glucose levels than that with pheroid microsponges. Although insulin with pheroid microsponges presents an initial decrease in blood glucose levels, 30 minutes after nasal administration, whereas pheroid vesicles began to decrease blood glucose after 60 minutes, the maximum effect of pheroid vesicles of 81.78% was obtained 120 minutes after administration. This leads to a faster onset of action for the pheroid microsponges, but there was a much greater decrease in the blood glucose level for the pheroid vesicles. Pheroid vesicles seemed to decrease 16.18% more in terms of blood glucose levels than pheroid microsponges.

Plasma insulin concentrations after an analysis of the blood samples of 8 IU/kg bodyweight insulin were not determined due to technical factors. This is why no data is available.

4.5 The effect of pheroid technology on the nasal absorption of 12 IU/kg bodyweight insulin

After the administration of 8 IU/kg bodyweight insulin to rats with pheroid technology, it was necessary to find out if there would be a greater decrease in blood glucose levels with a higher dosage of insulin. Therefore, it was decided to investigate the effect of 12 IU/kg bodyweight insulin on the blood glucose levels of rats after nasal administration with pheroid vesicles and pheroid microsponges. Only blood glucose levels were monitored and plasma insulin concentrations were not measured.

The data in respect of the blood glucose levels obtained after the nasal administration of 12 IU/kg bodyweight insulin with saline (control), pheroid vesicles and pheroid microsponges respectively is given in table 4.4.

Table 4.4 **Blood glucose levels (%) after the nasal administration of insulin (12 IU/kg bodyweight) to rats**

<i>Blood glucose levels (%)</i>			
<i>Formula</i>	<i>Control</i>	<i>Pheroid vesicles</i>	<i>Pheroid microsponges</i>
<i>Rats used</i>	n = 5	n = 6	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	89.45 ± 3.28	98.31 ± 7.80	91.93 ± 13.72
10	83.36 ± 6.86	94.41 ± 3.81	89.86 ± 9.37
15	81.38 ± 5.69	92.72 ± 8.55	90.90 ± 7.93
30	75.07 ± 9.76	65.82 ± 21.34	75.69 ± 12.97
60	77.98 ± 18.49	54.02 ± 33.61	55.96 ± 20.89
120	88.58 ± 15.83	47.12 ± 27.00	66.34 ± 13.85
180	101.28 ± 28.15	34.71 ± 11.55	72.42 ± 20.39

AV → Average blood glucose level SD → Standard deviation

Figures 4.19 to 4.23 illustrate the blood glucose levels in % against time (min) after nasal administration of insulin (12 IU/kg bodyweight) to rats. Blood glucose levels were measured in mmol/litre and calculated as a percentage (%).

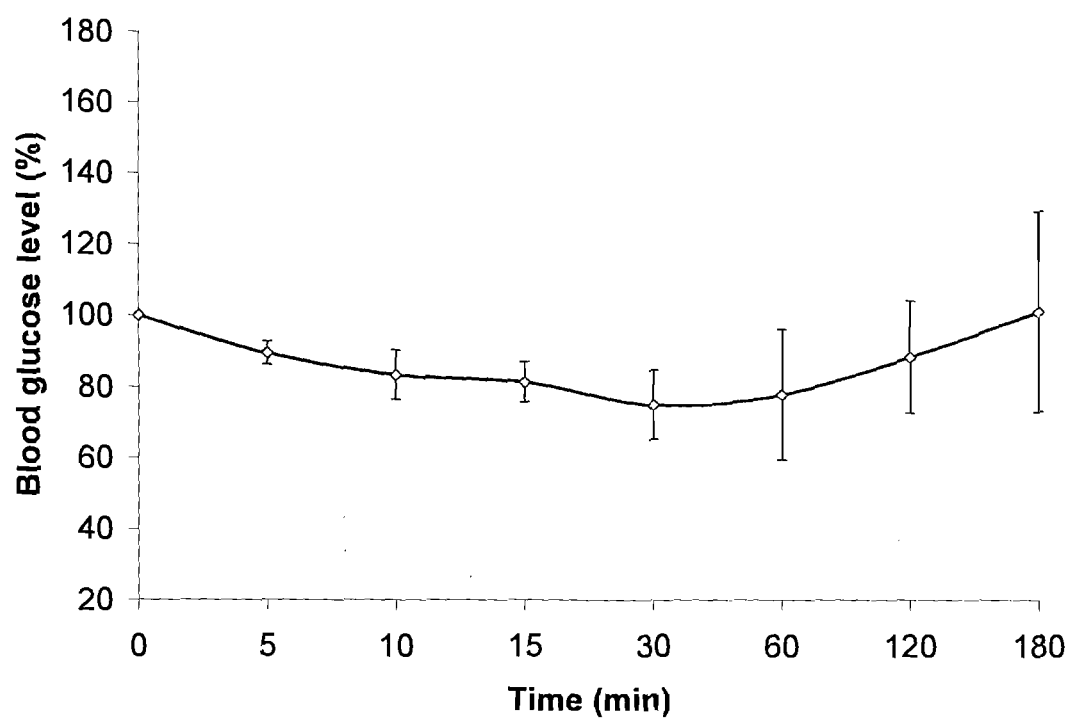


Figure 4.19 Average blood glucose level ($n = 6$) after nasal administration of insulin (12 IU/kg bodyweight) with saline to rats

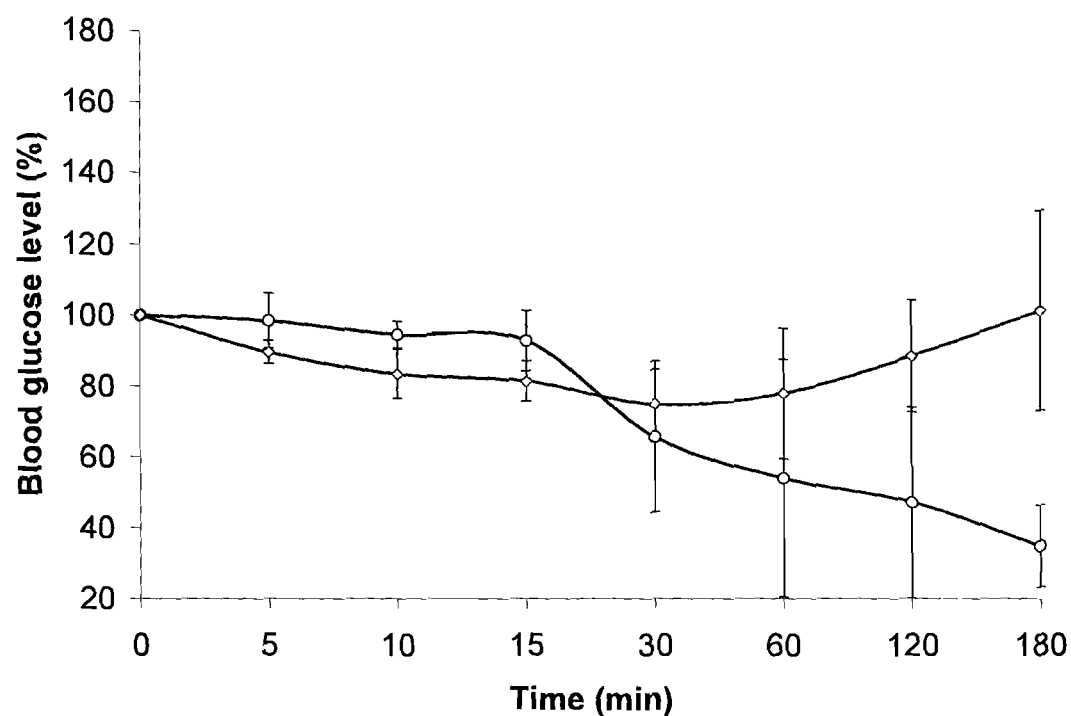


Figure 4.20 Average blood glucose level ($n = 6$) after nasal administration of insulin (12 IU/kg bodyweight) with pheroid vesicles to rats

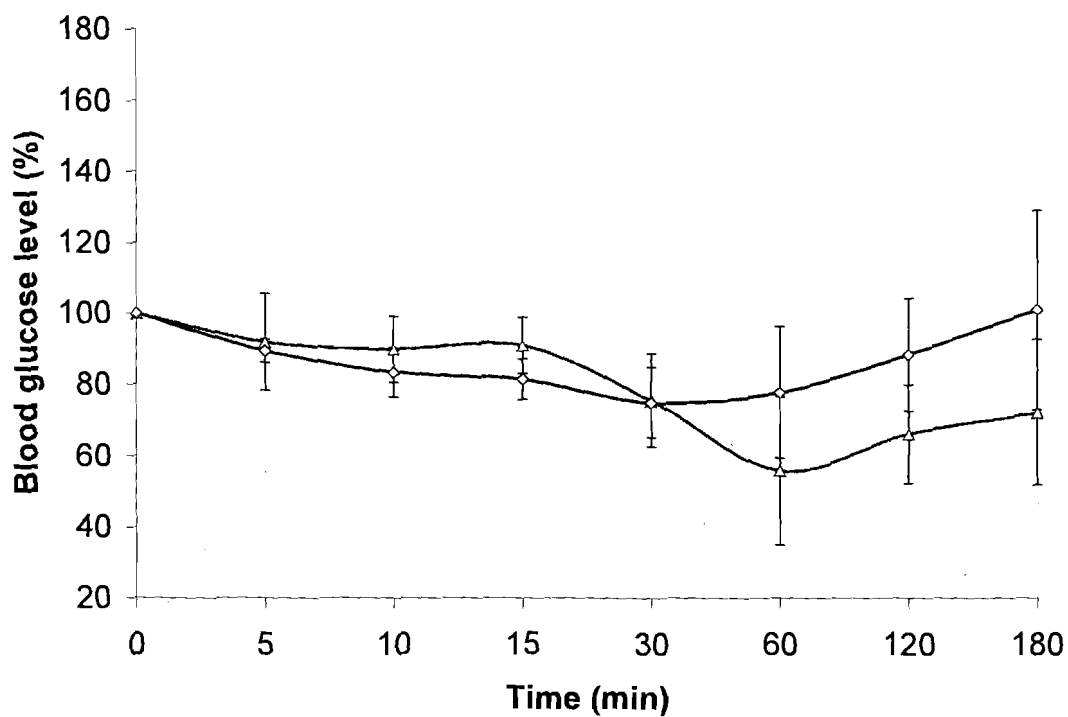


Figure 4.21 Average blood glucose level ($n = 6$) after nasal administration of insulin (12 IU/kg bodyweight) with pheroid microsponges to rats

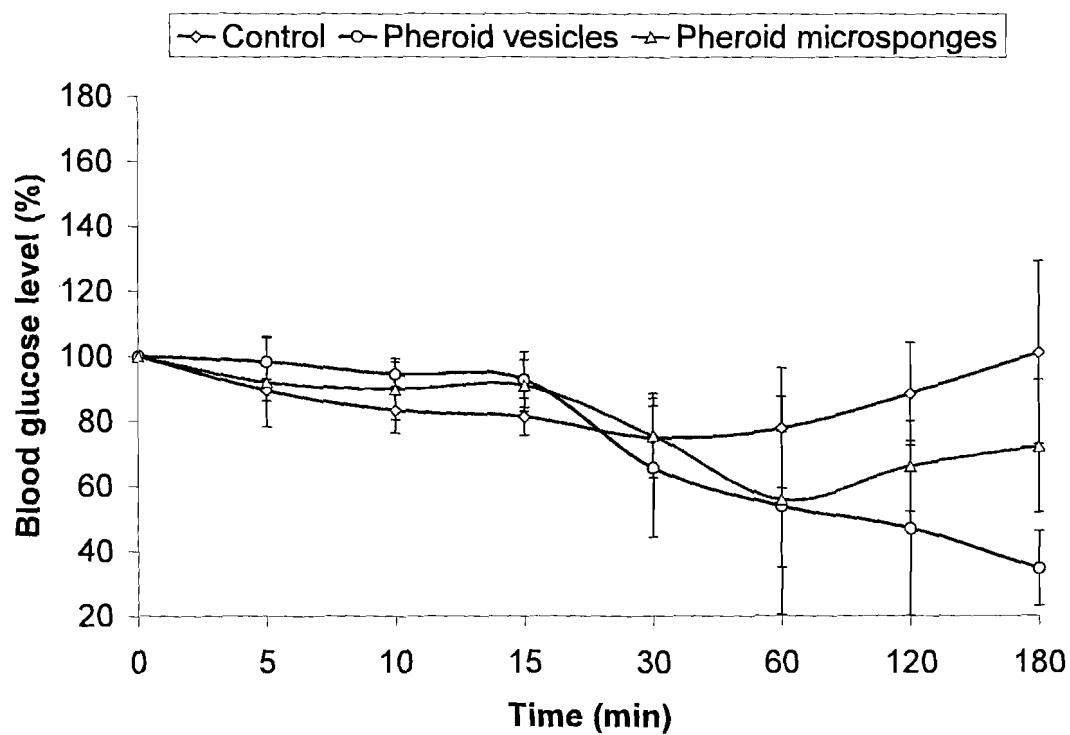


Figure 4.22 Comparison of average blood glucose levels against time (min) after nasal administration of insulin (12 IU/kg bodyweight) to rats

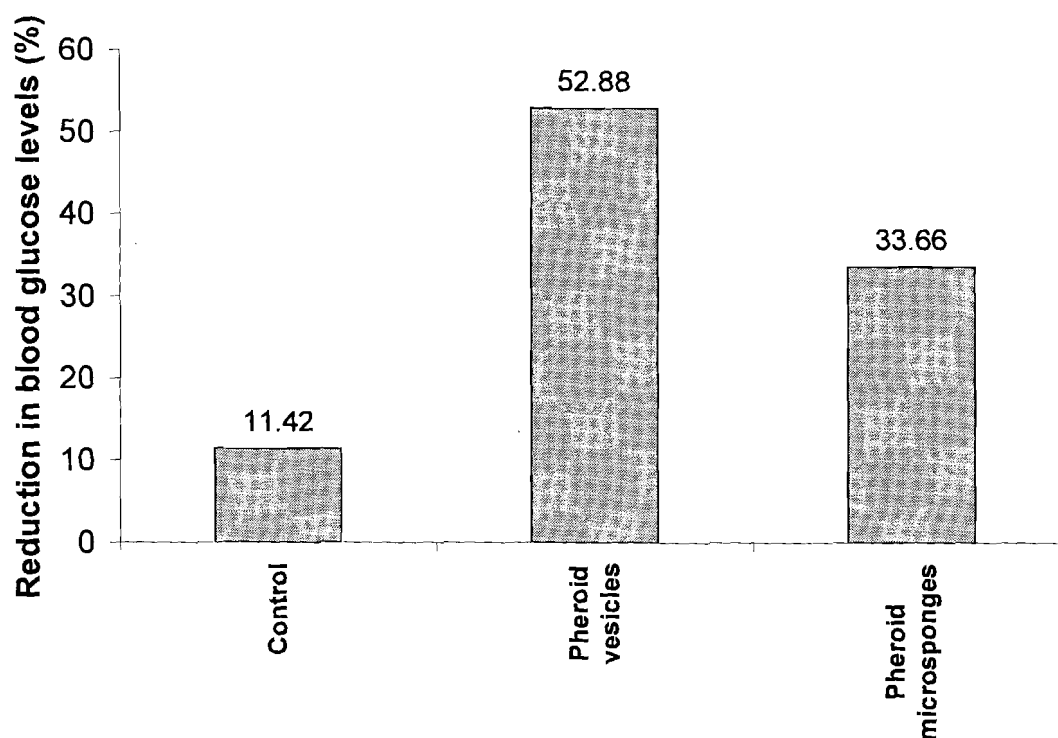


Figure 4.23 The reduction in blood glucose levels 120 minutes after nasal administration of 12 IU/kg bodyweight insulin with different formulations

Figures 4.19 to 4.21 show the decrease in blood glucose levels after the nasal administration of 12 IU/kg bodyweight insulin with saline, pheroid vesicles and pheroid microsponges respectively. The data is summarised in table 4.4.

In figure 4.19 it can be seen clearly that the average blood glucose level began to decrease slightly as early as 5 minutes after nasal administration of 12 IU/kg bodyweight insulin with saline, and that it continued to decrease up to 30 minutes, with 75.07% as the lowest blood glucose point. Thereafter the blood glucose levels started to increase again to 101.28% at 180 minutes. Nasally administered insulin of 12 IU/kg bodyweight with saline therefore does not seem to cause a definite decrease in blood glucose levels.

When pheroid vesicles, containing 12 IU/kg bodyweight of insulin, was administered in the rat's nose, a definite decrease of the blood glucose levels was seen after 30 minutes (65.82%), as shown in figure 4.20. This effect grew, producing an obvious decrease in the blood glucose level and reaching a minimum value of 34.71% of the initial concentration at 180 minutes.

As shown in figure 4.21, the blood glucose levels after nasal administration of 12 IU/kg bodyweight insulin to rats decreased significantly when pheroid microsponges were used as a delivery system. The minimum glucose level of 55.96% of the initial value was obtained 60 minutes after the administration of the insulin formulation containing pheroid microsponges, whereafter it began to increase to 72.42% after 180 minutes.

By comparing the results of nasally administered insulin at 12 IU/kg bodyweight with saline, pheroid vesicles and pheroid microsponges respectively, it can clearly be seen that the insulin formulation containing pheroid vesicles showed a significant lowering in blood glucose levels, as illustrated in figure 4.22. Pheroid microsponges began to decrease the blood glucose level after 5 minutes after nasal administration already and it continued to decrease until 60 minutes, with 55.96% as the lowest point, whereafter the blood glucose level began increasing. Blood glucose levels of the pheroid vesicle formulation also began to decrease after 5 minutes, but continued to decrease until 180 minutes, with a minimum point of 37.71%. It can be clearly seen that pheroid vesicles did not increase the blood glucose levels in the same way as pheroid microsponges did, and therefore had a more lasting effect in decreasing the blood glucose levels.

In the nasal administration of insulin at 12 IU/kg bodyweight it is indicated that the formulation containing pheroid vesicles achieved the maximum effect and also maintained the blood glucose lowering effect for a longer period of time (see figure 4.23).

4.6 Comparison of results obtained

In this section, formulations are compared to each other. For example the control groups (saline) of 4, 8 and 12 IU/kg bodyweight insulin will be compared to each other, as well as pheroid vesicle formulations at 4, 8 and 12 IU/kg bodyweight insulin and finally, pheroid microsphere formulations at 4, 8, and 12 IU/kg bodyweight insulin.

4.6.1 Comparison of saline formulations

The blood glucose levels obtained after the nasal administration of 4, 8 and 12 IU/kg bodyweight insulin respectively with saline to rats are summarised in table 4.5.

Table 4.5 **Blood glucose levels (%) after nasal administration of saline formulations with different dosages insulin (4, 8 and 12 IU/kg bodyweight)**

<i>Blood glucose levels (%)</i>			
<i>Formula</i>	<i>Control 4 IU/kg</i>	<i>Control 8 IU/kg</i>	<i>Control 12 IU/kg</i>
<i>Rats used</i>	n = 5	n = 5	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	94.37 ± 8.88	96.78 ± 5.67	89.45 ± 3.28
10	93.96 ± 12.05	88.75 ± 9.35	83.36 ± 6.86
15	92.80 ± 11.74	89.58 ± 10.39	81.38 ± 5.69
30	94.34 ± 9.33	96.46 ± 26.96	75.07 ± 9.76
60	104.72 ± 24.42	92.60 ± 27.05	77.98 ± 18.49
120	111.67 ± 26.01	120.30 ± 31.49	88.58 ± 15.83
180	131.39 ± 18.39	131.28 ± 32.72	101.28 ± 28.15

AV → Average blood glucose level SD → Standard deviation

Figure 4.24 represents the blood glucose levels graphically against time for the three different insulin formulations (4, 8 and 12 IU/kg bodyweight) with 0.9% saline (control).

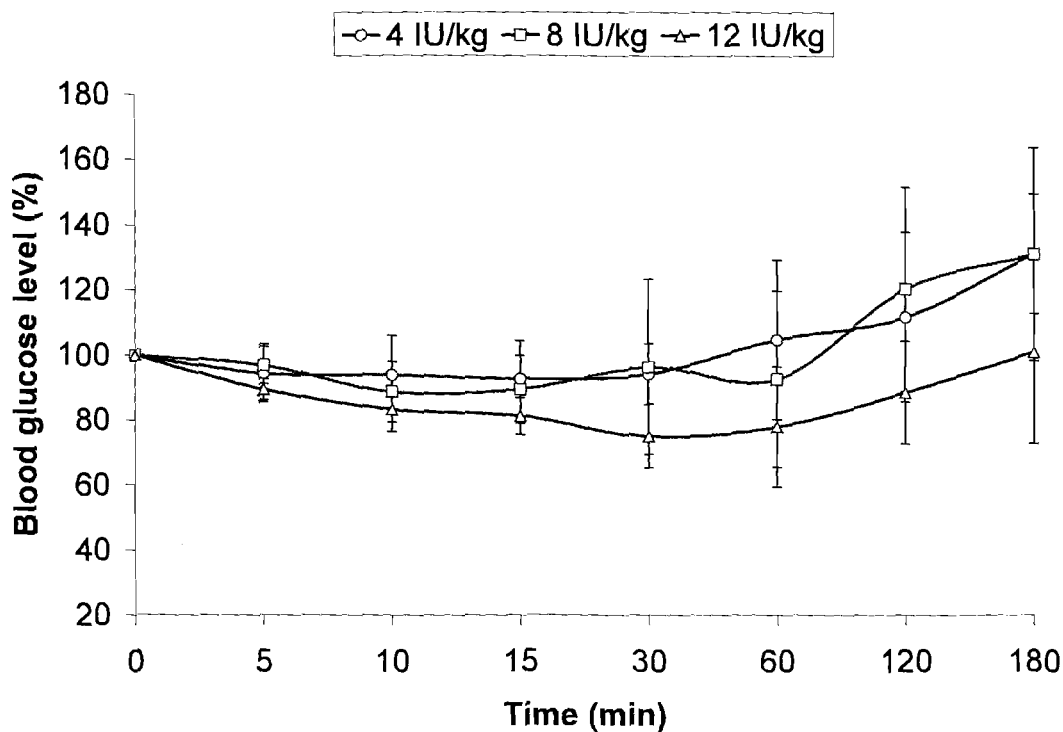


Figure 4.24 Comparison of average blood glucose levels against time (min) after nasal administration of insulin (4, 8 and 12 IU/kg bodyweight) with saline to rats

The data in table 4.5 and figure 4.24 clearly signifies that the saline formulation with 12 IU/kg bodyweight insulin lead to a higher decrease in blood glucose levels compared to the 4 and 8 IU/kg bodyweight insulin formulations. Although a slight blood glucose lowering effect of 75.07% was obtained for the 12 IU/kg bodyweight insulin after 30 minutes, this effect didn't last long and then the blood glucose levels started to increase. The saline formulations (control groups) were not expected to achieve a blood glucose lowering effect, due to the enzymes in the nose that influence the absorption of insulin in the nose (as described in chapter 1).

4.6.2 Comparison of pheroid vesicle formulations

Table 4.6 gives the blood glucose levels obtained after the nasal administration of 4, 8 and 12 IU/kg bodyweight insulin respectively with pheroid vesicles to rats

Table 4.6 Blood glucose levels (%) after nasal administration of pheroid vesicle formulations with different dosages insulin (4, 8 and 12 IU/kg bodyweight)

<i>Blood glucose levels (%)</i>			
<i>Formula</i>	<i>Pheroid vesicles 4 IU/kg</i>	<i>Pheroid vesicles 8 IU/kg</i>	<i>Pheroid vesicles 12 IU/kg</i>
<i>Rats used</i>	n = 6	n = 6	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	95.72 ± 7.32	97.52 ± 12.23	98.31 ± 7.80
10	99.36 ± 8.65	98.14 ± 11.99	94.41 ± 3.81
15	113.45 ± 15.93	98.66 ± 14.47	92.72 ± 8.55
30	102.89 ± 11.97	99.25 ± 16.90	65.82 ± 21.34
60	97.78 ± 24.81	84.44 ± 23.97	54.02 ± 33.61
120	110.06 ± 39.98	81.78 ± 22.48	47.12 ± 27.00
180	134.51 ± 27.71	82.52 ± 34.42	34.71 ± 11.55

AV → Average blood glucose level SD → Standard deviation

Figure 4.25 illustrates the blood glucose levels against time for the three different insulin formulations (4, 8 and 12 IU/kg bodyweight) with pheroid vesicles.

When the pheroid vesicle formulation was administered to rats with a dose of 12 IU/kg bodyweight insulin, a blood glucose lowering effect of 65.82% was obtained after 30 minutes (see figure 4.25 and table 4.6). The 8 IU/kg bodyweight insulin formulation lowered blood glucose levels, with a minimum blood glucose point of 81.78% at 120 minutes after the nasal administration of the formulation. Insulin in a dosage of 4 IU/kg bodyweight in pheroid vesicles had no effect on blood glucose levels. The formulation containing 12 IU/kg bodyweight insulin gave the greatest decrease in blood glucose levels, namely 34.71% at 180 minutes after the nasal administration. This shows clearly that pheroid vesicle formulations containing insulin are definitely dose-dependent when it comes to achieving a significant lowering of the blood glucose levels.

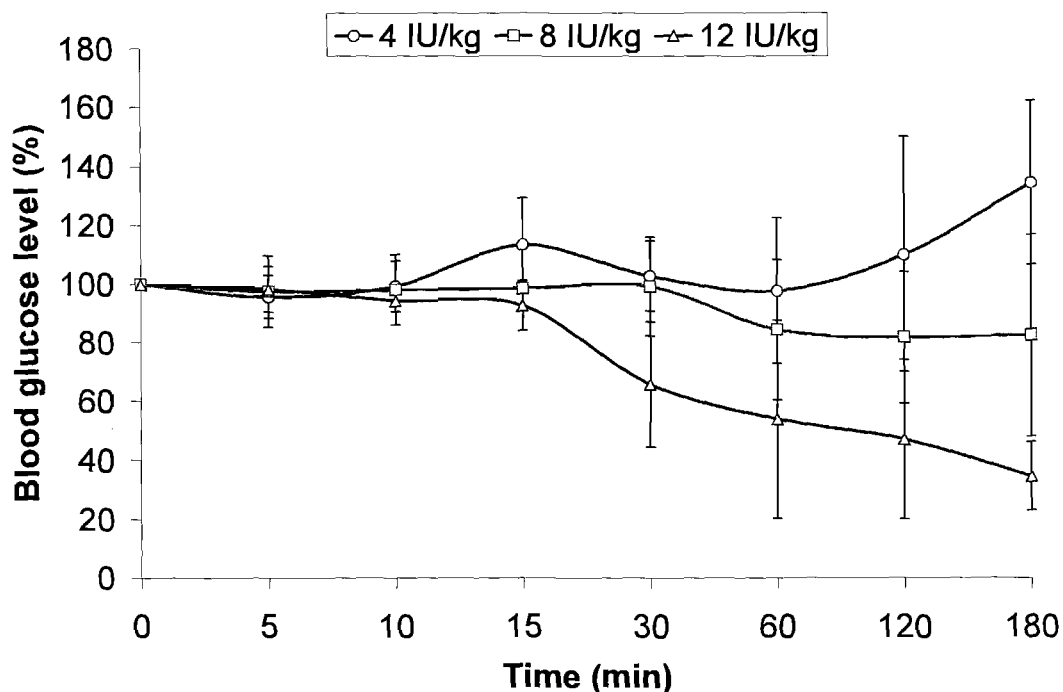


Figure 4.25 Comparison of average blood glucose levels against time (min) after nasal administration of insulin (4, 8 and 12 IU/kg bodyweight) with pheroid vesicles to rats

4.6.3 Comparison of pheroid microspunge formulations

Table 4.7 summarises the blood glucose levels obtained after nasal administration of 4, 8 and 12 IU/kg bodyweight insulin respectively with pheroid microsponges to rats.

Table 4.7 Blood glucose levels (%) after nasal administration of pheroid microspunge formulations with different dosages insulin (4, 8 and 12 IU/kg bodyweight)

<i>Blood glucose levels (%)</i>			
<i>Formula</i>	<i>Pheroid microsponges 4 IU/kg</i>	<i>Pheroid microsponges 8 IU/kg</i>	<i>Pheroid microsponges 12 IU/kg</i>
<i>Rats used</i>	n = 6	n = 5	n = 5
<i>Time (min)</i>	AV±SD	AV±SD	AV±SD
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

5	111.81 ± 8.44	96.75 ± 14.99	91.93 ± 13.72
10	109.14 ± 24.92	107.34 ± 19.59	89.86 ± 9.37
15	106.41 ± 11.49	103.49 ± 11.57	90.90 ± 7.93
30	97.23 ± 14.91	96.78 ± 15.15	75.69 ± 12.97
60	98.26 ± 21.58	93.13 ± 10.33	55.96 ± 20.89
120	118.48 ± 36.39	88.80 ± 12.31	66.34 ± 13.85
180	123.22 ± 39.85	97.21 ± 18.52	72.42 ± 20.39

AV → Average blood glucose level SD → Standard deviation

Figure 4.26 shows blood glucose levels against time for the three different insulin formulations (4, 8 and 12 IU/kg bodyweight) with pheroid microsponges.

The results of pheroid microsphere formulations with different dosages of insulin (4, 8 and 12 IU/kg bodyweight) are given in table 4.7 and are graphically represented in figure 4.26. When administered nasally to rats, the pheroid microsphere formulation containing 12 IU/kg bodyweight insulin achieved a lowering of the blood glucose levels after 30 minutes. The greatest decrease in blood glucose levels, namely 55.96%, was achieved 60 minutes after the nasal administration of the pheroid microsphere formulation containing 12 IU/kg bodyweight insulin.

As shown in figure 4.26, the nasal administration of pheroid microsponges with 8 IU/kg bodyweight insulin to rats did produce lower blood glucose levels, but the change was not significant, i.e. 88.80 % at 120 minutes. The pheroid microsphere formulation containing 4 IU/kg bodyweight insulin did not lower blood glucose levels at all.

Therefore the nasal administrations of pheroid microsphere formulations with insulin are, in the same way as the pheroid vesicle formulations containing insulin, dose-dependent in order to achieve an obvious reduction in blood glucose levels.

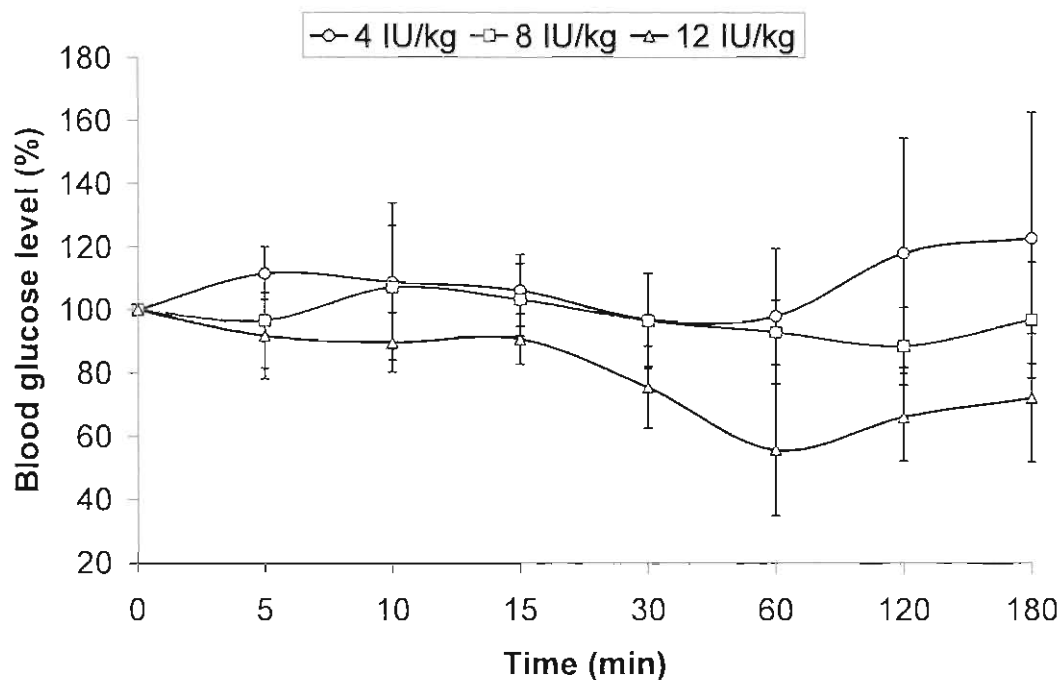


Figure 4.26 Comparison of average blood glucose levels against time (min) after nasal administration of insulin (4, 8 and 12 IU/kg bodyweight) with pheroid microsponges to rats

A comparison of the results in terms of the different dosages of insulin (4, 8 and 12 IU/kg bodyweight) with the different pheroid formulations is illustrated in figure 4.27, which shows clearly that the formulation containing pheroid vesicles at 12 IU/kg bodyweight insulin, achieved the greatest decrease in blood glucose levels, with a reduced blood glucose effect of 52.88%. The second best formulation in lowering blood glucose levels is pheroid microsponges at 12 IU/kg bodyweight with a maximum reduction in blood glucose levels of 33.66%. This is a very important observation and helpful for future studies, although a lot more research still needs to be done.

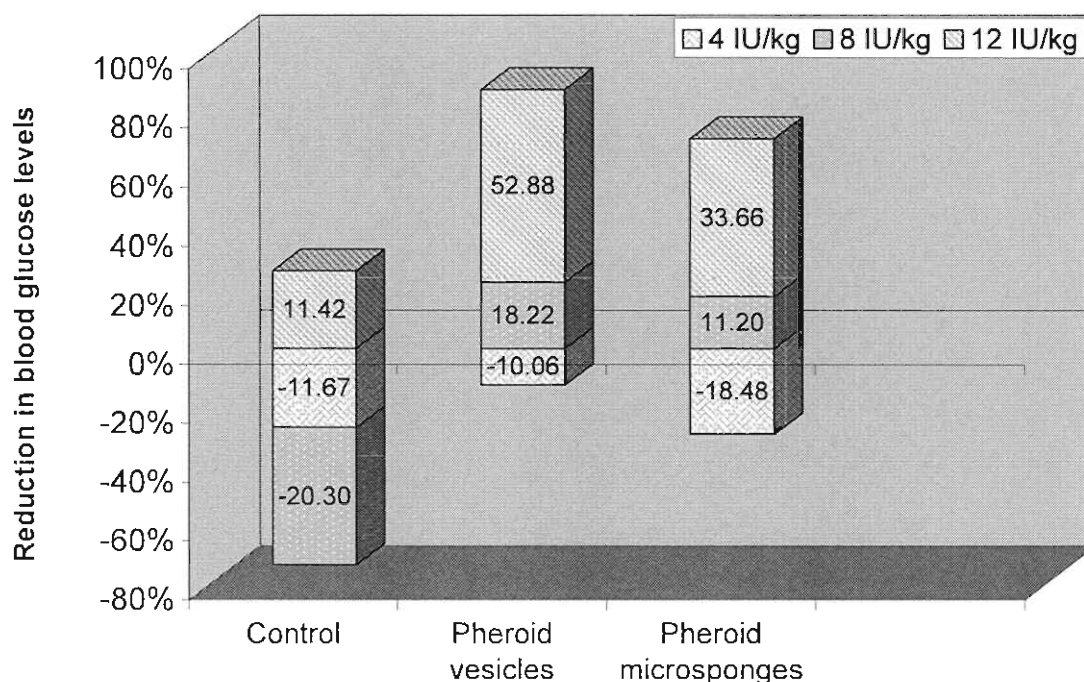


Figure 4.27 The comparison of reduced blood glucose levels at 120 minutes after nasal administration of insulin with different formulations

4.7 Conclusion

The results obtained in this study indicate that absorption-enhancing systems like pheroid vesicles, pheroid microsponges and TMC cause a decrease in the blood glucose levels after the nasal administration of insulin.

The incorporation of absorption-enhancing systems in insulin formulations is essential for the effective absorption of insulin, since these agents are able to modulate nasal epithelial permeability in terms of insulin, or to prolong the residence time of the drug formulation in the nasal cavity. The criteria for absorption-enhancing systems are the following: (a) They should be effective; (b) They should be economically viable; and (c) They should be safe for chronic nasal administration (Hinchcliffe & Illum, 1999:226). However, it has not been proven that pheroid formulations meet the abovementioned requirements of effective absorption-enhancing systems. This is why more research has to be done in order to explain the mechanism of action of pheroid formulations.

This study illustrates clearly that TMC appeared to be the most promising absorption-enhancing system. The best results were achieved with the TMC formulation containing 4 IU/kg bodyweight of insulin, which gave the highest decrease in blood glucose levels and also correlates with the plasma insulin concentrations.

The excellent results in terms of TMC can be explained by two properties of TMC. Firstly, TMC improves the absorption-enhancement of drugs so that they can pass through the membrane due to their adherence to the mucosal surface in the nose, thereby increasing the contact time of the drug with the mucosa, which makes insulin stay in the nasal cavity for a longer time. Secondly, TMC opens tight junctions between epithelial cells, which increases membrane permeability to larger molecular weight peptides and proteins, thus making possible access to the systemic circulation (Artursson *et al.*, 1994:1360).

Summary and future prospects

The nasal delivery of peptide drugs is a very attractive route of administration because of its effective absorption and easy administration, both of which assure greater bioavailability. The nasal route has several characteristics; such as an excellent blood supply, permeability of the nasal epithelium and bypass of first pass metabolism through the liver; these make this route an ideal alternative to the parenteral route.

Insulin is a peptide drug which has a high molecular weight and is barely absorbed via the oral route due to enzymatic degradation. However, it is well known that a peptide drug such as insulin is subjected to the degradation of proteolytic enzymes during passage through the mucosal membrane. That is why and how absorption-enhancing systems such as TMC, pheroid vesicles, and pheroid microsponges can make it possible for such systems to entrap, transport and deliver these molecules effectively. Especially TMC produced a significant absorption of insulin and a definite hypoglycemic effect after *in vivo* nasal administration to rats.

CLSM micrographs indicate that insulin molecules were entrapped in the pheroid vesicles and pheroid microsponges. This fact confirms that pheroid vesicles, pheroid microsponges and TMC have the ability to enhance the nasal absorption of insulin and to lead to a significant hypoglycemic effect.

The results of this study describe the effect of pheroid formulations where insulin was entrapped immediately before being administered to rats. Another approach could be to entrap the peptide drug a day (24 hours) before administration; which could help with the investigation of the stability profile of insulin in pheroid formulations.

Detailed mechanisms of the promoting effect of pheroid formulations on the nasal absorption of insulin must also be investigated in further studies.

On the basis of the experiments done in this study, it would appear that the nasal administration of insulin with an absorption enhancing system may have a useful therapeutic effect. However, further more detailed chronic testing will be required to confirm the safety of these formulations. Possible damage and toxicity to the nasal epithelial cells, caused by pheroid formulations, could be determined with a ciliary beating frequency test.

It is not currently known whether the combination of TMC with pheroid vesicles or microsponges could exhibit a synergistic effect in the nasal absorption of insulin. This matter, too, warrants further investigation.

The development of a safe and effective nasal delivery system for insulin remains a demanding and exciting challenge. The availability of a commercial nasal insulin formulation would be a major breakthrough in the treatment of diabetes mellitus and would help improve the lives of millions of diabetics worldwide.

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