NASAL DELIVERY OF RECOMBINANT HUMAN GROWTH HORMONE WITH PHEROID TECHNOLOGY

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Destiny is not a matter of chance, it is a matter of choice.

It is not a thing to be waited for, it is a thing to be achieved.

William Jennings Bryan

Dedicated to my

parents

Johan and Cobi Steyn

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I would like to express my sincerest appreciation to all of the following people, without whom this study would not have been possible.

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Dewald Steyn
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TABLE OF CONTENTS

TABLES AND FIGURES

TABLES **Page**

FIGURES **Page**

INTRODUCTION AND AIM OF STUDY

Over the past couple of years there has been rapid progress in the develoment and design of safe and effective delivery systems for the administration of protein and peptide drugs. The effective delivery of these type of drugs are not always as simple as one may think, due to various inherent characteristics of these compounds.

Due to the hydrophilic nature and molecular size of peptide and protein drugs, such as recombinant human growth hormone, they are poorly absorbed across mucosal epithelia, both transcellularly and paracellularly. This problem can be overcome by the inclusion of absorption enhancers in peptide and protein drug formulations but this is not necessarily the best method to follow.

This investigation focussed specifically on the evaluation of the ability of the Pheroid™ carrier system to transport recombinant human growth hormone across mucosal epithelia especially when administered via the nasal cavity. The Pheroid™ delivery system is a patented system consisting of a unique submicron emulsion type formulation. The Pheroid™ delivery system, based on Pheroid™ technology, will for ease of reading be called Pheroid(s) only throughout the rest of this dissertation.

The Pheroid carier system is a unique microcolloidal drug delivery system. A Pheroid is a stable structure within a novel therapeutic system which can be manipulated in terms of morphology, structure, size and function. Pheroids consist mainly of plant and essential fatty acids and can entrap, transport and deliver pharmacologically active compounds and other useful substances to the desired site of action.

The specific objectives of this study can be summarised as follows:

- a literature study on Pheroid technology;
- a literature study on chitosan and *N*-trimethyl chitosan chloride;
- a literature study on recombinant human growth hormone (somatropin);
- a literature study on nasal drug administration;
- formulation of a suitable Pheroid carrier;
- entrapment of somatropin in the Pheroid carrier, and
- *in vivo* evaluation of nasal absorption of somatropin in Sprague-Dawley rats.

CHAPTER 1

NASAL ADMINISTRATION OF PEPTIDE DRUGS

1.1 INTRODUCTION

The anatomy and physiology of the nasal passage clearly indicate that nasal administration has potential practical advantages for the introduction of therapeutic peptides into the systemic circulation. The highly vascular nasal mucosa makes rapid absorption of the administered drug possible and furthermore ensures that the drug avoids degradation in the gastrointestinal tract and first-pass metabolism in the liver. Nasal administration and intravenous administration often exibit very similar concentration-time profiles which suggest that a rapid onset of pharmacological activity is possible after nasal administration (Hussain, 1998:41).

1.2 FACTORS THAT SYNERGISTICALLY ENHANCE THE PERMEATION OF NASALLY ADMINISTERED DRUGS

The various factors that synergistically enhance the permeation of nasally administered drugs are:

- a highly vascularized epithelium,
- a porous endothelial membrane and
- a relatively large surface area due to the presence of a large number of microvilli (Cornaz & Buri, 1994:264).

The enzymes present in the nasal cavity and the nasal mucosal lining are the two main barriers for drug permeation (Ugwoke *et al.*, 2001:8).

Despite the presence of these mentioned barriers, it is still possible to deliver a large number of drugs via the nasal cavity such as peptides, proteins, hormones and even vaccines. The ease of nasal administration is an attractive alternative in comparison to the more invasive routes of administration such as injections and will ensure better patient compliance.

1.3 ANATOMY AND PHYSIOLOGY OF THE NOSE

The nasal septum devides the nasal cavity in two symmetrical halves. The septum consist mainly of a central partition of bone and cartilage, each side opens at the face via the nostrils

and connects with the mouth at the nasopharynx. The three main regions of the nasal cavity consist of the nasal vestibule, the olfactory region and the respiratory region. The lateral walls of the nasal cavity include a folded structure which consists of three turbinates, namely the inferior, median and superior and this folded structure enlarges the nasal surface area to about 150 cm^2 (Proctor, 1973:134).

The passages of the main nasal airway are relatively narrow, usually in the region of 1 - 3 mm wide, which enables the nose to carry out its main functions (Proctor, 1982:24).

During inspiration, the inhaled air is warmed and moistened as it passes over the mucosa and this is facilitated by the high blood supply in the nasal epithelium and the fluid secreted by the mucosa. The mucus is also instrumental in cleaning the inhaled air by trapping dust, bacteria and other impurities in the air (Proctor, 1973:135).

A very important point of nasal administration is that first-pass metabolism can be avoided, this phenomenon can be explained by the fact that the submucosal zone of the nasal passage is extremely vascular and this network of veins drain blood from the nasal mucosa directly to the systemic circulation, hence eliminating first-pass metabolism (Mygind *et al.*, 1982:82).

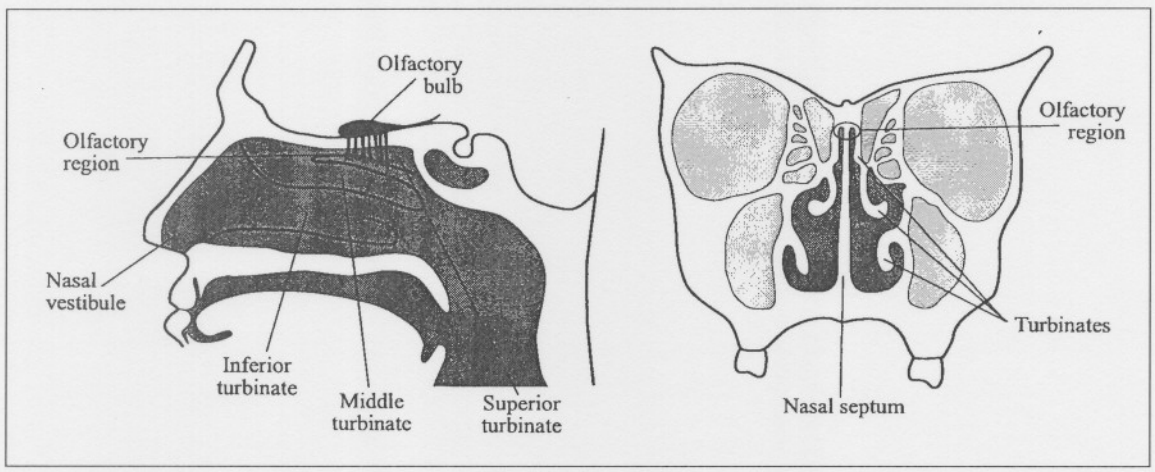


Figure 1.1: Anatomy of the nose. To the left is the lateral wall of the nasal cavity with the olfactory region at the roof of the cavity; just below the cribriform plate of the ethmoid bone. To the right is a cross-section of the nose showing the narrow nasal airway passage and the folds of the turbinates (Mygind *et al.*, 1982:82).

1.3.1 The olfactory region

Another major function of the nose is olfaction and this is accomplished by the olfactory region which is located on the roof of the nasal cavity. The cavity is covered with a mucous membrane and is divided into olfactory and non-olfactory epithelium areas (Geurkink, 1983:125).

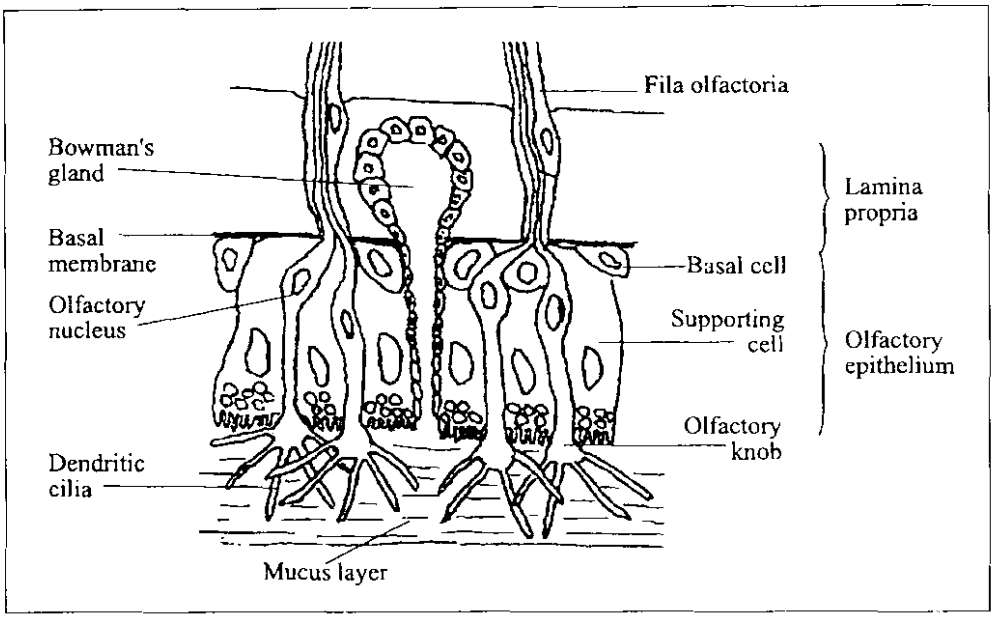


Figure 1.2: The olfactory epithelium of the nasal cavity showing the three main cell types. Modified from Mathison *et al.* (1998:420).

The olfactory region possesses specialized ciliated olfactory nerve cells which is instrumental in smell perception. The central axons of these nerve cells pass through the cribriform plate of the ethmoid and into the olfactory bulb (Ridley *et al.*, 1992:12). The olfactory epithelium has a total surface area of approximately 200 - 400 mm^2 (Baroody, 1999:10).

The opening to the outside environment is called the nasal vestibule and possesses numerous nasal hairs called vibrassae which is responsible for filtering large air-borne particles. The vestibule is very resistant to dehydration, due to its nature, and can withstand insults from noxious substances of the environment which means that the permeation of substances, including drugs, through it is very limited. The region between the nasal vestibule and nasal conchae is called the atrium which is a transitional epithelial region which contain stratified and pseudo-stratified columnar cells with microvilli posteriorly and squamous cells anteriorly. Collectively, the epithelium and lamina propria are called the

respiratory mucus membrane or respiratory mucosa. Drug absorption is optimal in this region (Mygind & Dahl, 1998:83).

1.3.2 The respiratory region

The nasal respiratory epithelium is generally described as a pseudo-stratified ciliated columnar epithelium. Four main types of cells can be found in this region, namely:

- non-ciliated columnar cells;
- ciliated columnar cells;
- basal cells and
- goblet cells as can be seen in figure 1.3.

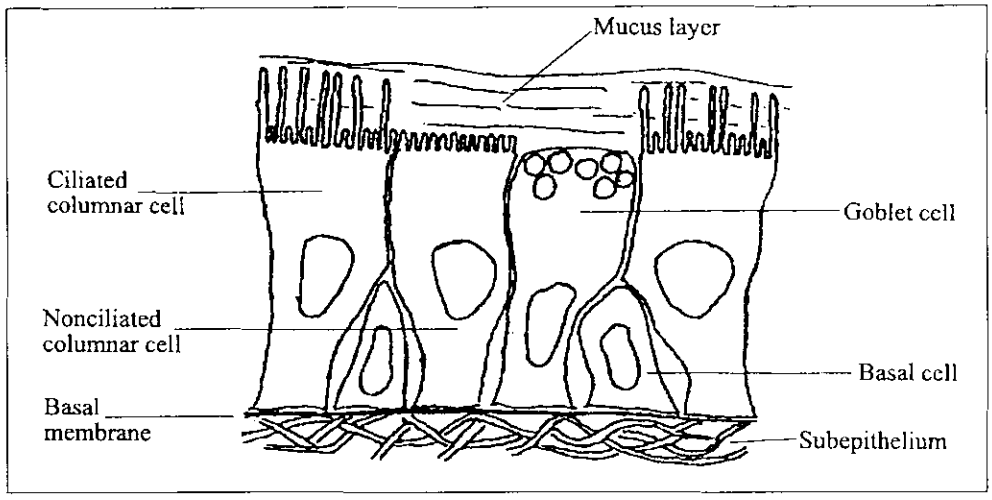


Figure 1.3: The respiratory epithelium of the nasal cavity, showing the four main cell types. Modified from Mathison *et al.* (1998:422).

Neurosecretory cells may also be present. They do not protrude into the airway lumen and can be compared with basal cells (Petruson *et al.*, 1984:579). The number of ciliated cells increase towards the nasopharynx area with a proportionate decrease in non-ciliated cells (Popp & Martin, 1984:430). The importance of ciliated cells for the absorption of drugs across the nasal epithelium are indicated by their high numbers in this area. Microvilli are present in both columnar cell types with numbers in the region of 350 - 400 per cell which dramatically increase the surface area which is one of the main reasons for the high absorptive capacity of the nasal cavity (Mygind, 1975:79). Goblet cells account for 5 - 15% of the total amount of mucosal cells in the turbinates and contain numerous secretory granules filled with mucin which in conjunction with the nasal glands form the mucus layer (Petruson *et al.*, 1984:580).

Basal cells vary greatly in both number and shape and never reach the airway lumen and act mainly as stem cells to replace other epithelial cells (Jahnke, 1972:35).

1.4 MECHANISM OF PERMEATION OF DRUGS VIA THE NASAL CAVITY

The lipophilicity of the administered compound will determine whether it will permeate passively via the paracellular pathway or both passively and actively via the transcellular pathway. The passive transport pathways play the greatest role in nasal drug permeation but there are a few other possible pathways namely transcytosis, carrier mediated transport and transport through intercellular tight junctions.

The effective permeability coefficient $P_{eff.}$ under steady state conditions across excised mucosa can be mathematically expressed as:

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

In this equation $(dc/dt)_{ss}$ represents the time dependent change of concentration in the steady state, A is the permeation area, V is the volume of the receiver compartment and C_D represents the initial concentration in the donor compartment (Long *et al.*, 1996:1193).

1.5 FACTORS AFFECTING THE NASAL PERMEABILITY OF ACTIVE COMPOUNDS

1.5.1 Biological factors

1.5.1.1 Environmental influences

A moderate reduction in the mucociliary clearance can be caused by temperatures in the region of ± 24 °C and it has been observed that the ciliary beat frequency increase proportionately with an increase in temperature which suggest that a linear relationship exist between the increase in temperature and the increase in ciliary beat frequency (Gizurarson, 1993:335).

1.5.1.2 Pathological conditions

Mucociliary disfunction and hypo- or hypersecretions as well as irritation of the nasal mucosa can be associated with diseases such as rhinitis, atropic rhinitis, common cold and nasal

polyposis which in turn may influence drug permeation of the nasal mucosa (Merkus *et al.*, 2001).

1.5.1.3 Physiological influences

The *pH of the nasal cavity* varies between 5.5 - 6.5 in adults. In infants the pH variation is greater with values in the region of 5.0 - 7.0. The best penetration of drug molecules take place when the penetrant molecules exist as unionized species and to achieve this it is essential that the nasal pH has a lower value than that of the drug's pKa (Huang *et al.*, 1985:609).

The nasal cycle or diurnal variation implies that circardian rhythms affect nasal secretions. In various studies it was found that the nasal clearance and secretion rates decrease dramatically during the night thus altering drug permeation (Mygind & Thomsen, 1976:220). The viscocity of the nasal secretions play a major role in the *ciliary beating frequency*. For instance if the sol layer of mucus is too thin the ciliary beating will decrease and if the sol layer is too thick clearance will also be impaired due to the fact that contact with the cilia is lost. These variations affect drug permeation by altering the time of contact between the drug and the mucosa (Mortazavi & Smart, 1994:88).

Solubility of the drug in *nasal secretions* is an important factor to investigate since a drug needs to be solubilized before it can permeate the nasal mucosa. Nasal secretions contain 90% water, 2% mucin, 1% salts and approximately 1% proteins such as albumin, immunoglobulins, lysozyme, lacto ferrin, etc. The rest of the nasal secretions consist mainly of lipids (Kaliner *et al.*, 1984:320).

Blood supply and neuronal regulation is another important point to investigate. The presence of arteriovenous anastomosis and venous sinusoids give the nasal mucosa the distinction of being a highly permeable site. Nasal cycles of congestion and relaxation, caused by an increased blood supply resulting from parasympathetic stimulation and decreased blood supply from sympathetic stimulation respectively, regulate the rise and fall in the amounts of drug permeated (Misawa, 1988:17).

Based on the above it would be relevant to conclude that parasympathetic stimulation can lead to the increased permeability of a compound (Revington *et al.*, 1997:830).

Mucociliary clearance (MCC) and ciliary beating are normal defence mechanisms of the nasal cavity which is responsible for clearing mucus and other substances adhering to the nasal mucosa, such as bacteria and allergens and draining them into the nasopharynx for eventual discharge into the gastrointestinal tract. A nasally administered substance is cleared from the nasal cavity in approximately 21 minutes by means of MCC (Merkus *et al.*, 1998:21).

A reduction in MCC enhances drug permeation due to the increased time of contact between the mucus membrane and the administered drug, the opposite is also applicable and it can be concluded that an increase in MCC will subsequently lead to a decrease in drug permeation (Merkus *et al.*, 1998). Various factors affect the MCC and in turn exert significant effect on drug permeability. The factors include:

- formulation factors such as rheology;
- pathological conditions;
- hormonal changes of the body;
- environmental conditions and
- various drugs (Schipper *et al.*, 1991:810).

1.5.2 Formulation factors

1.5.2.1 Physicochemical properties of administered drug

- **Molecular weight and size**

Drug permeation is determined by a combination of the molecular weight and size of the particles as well as the lipophilicity or hidrophilicity of the specific drug. The bioavailability of compounds with a molecular weight higher than 1 kDa range from 0.5% - 5% and can be directly predicted from knowledge of the molecular weight (Huang & Donovan, 1998:149).

Lipophilic compounds show a direct relationship between the molecular weight and drug permeation and in the case of hydrophilic compounds an inverse relationship can be detected. It can be concluded that the permeation of drugs with a molecular weight of less than 300 Da will not be influenced significantly by the physicochemical properties of the specific drug due to the fact that the molecules will mostly permeate through the aqueous channels of the membrane. On the other hand, for compounds with a molecular weight

higher than 300 Da the rate of permeation is highly sensitive to molecular size (Fischer *et al.*, 1992:552).

*** Solubility**

The solubility of the specific drug is a major factor to consider when determining the absorption of the drug through biological membranes. The relationship between the solubility of a drug and its absorption via the nasal route have not been studied extensively and very little information is available. For increased dissolution a drug should have appropriate aqueous solubility in order to be compattable with the aqueous nature of the nasal secretions (Fischer *et al.*, 1992:553).

• Lipophilicity

Permeation of the specific drug normally increases through the nasal mucosa as the lipophilicity of the drug increase. The lipid domain plays an important role in the barrier function of the nasal mucosa. The mucosa appear to be primarily lipophilic by nature although some hydrophilic characteristics are also present. It is a known fact that excess hydrophilicity will lead to a decrease in the systemic bioavailability of some drugs (Corbo *et al.*, 1989:850).

*** Partition coefficient and pKa**

According to the pH partition theory it is obvious that unionized species are absorbed much better than their ionized counterparts and in the case of nasal absorption the same holds true. A quantitative relationship exists between nasal absorption and the partition coefficient (Jiang *et al.*, 1997:459).

Various studies indicate that with an increase in either the partition coefficient or lipophilicity of a specific drug there would be a significant rise in the concentration of that specific drug in the cerebrospinal fluid. The nasal absorption of weak electrolytes are highly dependent on their degree of ionization. For example the absorption rate of aminopyrine can be increased with an increase in the pH. The rate of absorption deviated substantially with salicylic acid when the pH was increased which would suggest that a different transport pathway, along with the lipoidal pathway, might exist for salicylic acid (Hirai *et al.*, 1981).

Similarly, more than 10% of benzoic acid is absorbed at pH 7.19, where 99% of the drug exist in the ionized form, which indicate that ionized species can also permeate through nasal mucosa (Huang *et al.*, 1985).

Based on the above observations it would be fair to consider partition coefficients as a major factor governing nasal absorption and it suggests that other transport pathways might be of importance for hydrophilic drugs. Figure 1.4 demonstrate a few drug transport pathways across epithelium as described by Ugwoke *et al.* (2000:11).

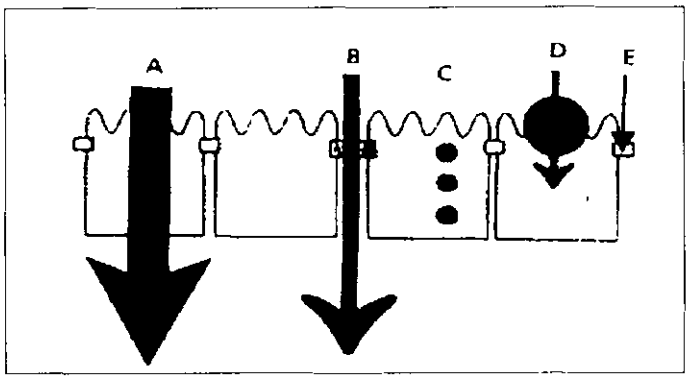


Figure 1.4: Drug transport pathways across nasal epithelium (Ugwoke *et al.*, 2000:11).

1.6 PHYSICOCHEMICAL PROPERTIES OF NASAL FORMULATIONS AFFECTING NASAL PERMEABILITY

1.6.1 pH and mucosal irritancy

The pH of both the nasal surface and the formulation can affect a drug's permeation and the pH of the nasal formulation should be in the range of 4.5 - 6.5 in order to avoid nasal irritation. In addition, to avoid irritation and obtain more efficient drug permeation, the slightly acidic pH also prevents the growth of bacteria and other micro-organisms (Rathbone *et al.*, 1994:38).

1.6.2 Viscosity

The contact time between the drug and the nasal mucosa increase with an increase in viscosity of the formulation thereby increasing the time for permeation. The ciliary beating and mucociliary clearance are also affected by the administration of highly viscous formulations which in turn may also alter the permeability of the administered drug (Ohwaki *et al.*, 1985:551).

1.6.3 Osmolarity

Research has shown that absorption is at its best at a sodium chloride concentration of 0.462 M which can be attributed to the fact that the nasal epithelial mucosa undergoes some structural changes due to dehidration which in turn lead to shrinkage of the mucosa and enhanced absorption and permeation of the specific drug (Ohwaki *et al.*, 1985:551). Isotonic solutions are preferred for nasal administration based on the above observations.

1.6.4 Drug distribution

1.6.4.1 Area of the nasal mucus membrane exposed

The bioavailability of various drugs increases when the test solution is applied to both nostrils instead of just one. This increase in bioavailability suggests that when the area of the mucus membrane exposed to the test solution is increased, the permeation of the administered drug would increase accordingly (Dalton *et al.*, 1987:86).

1.6.4.2 Volume of solution applied

The volume that can be delivered to the nasal cavity ranges between 0.05 - 0.15 ml. Different approaches can be followed in order to use this small volume to its greatest effect such as making use of solubilizers, gelling agents or viscofying agents (Park *et al.*, 2002:148).

1.6.4.3 Dosage form

The simplest and most convenient dosage form for nasal administration is nasal drops but the exact amount delivered cannot be easily quantified and this may often result in an overdose of the patient (Patel & McGarry, 2001:634).

Rapid drainage from the nasal cavity is another problem which is often encountered with the use of nasal drops. In the case of nasal sprays it is recommended that solutions and suspensions are used rather than powder sprays due to the fact that powder sprays may often cause mucosal irritation. Specialized systems such as lipid emulsions, microspheres, liposomes and proliposomes provide prolonged contact between the drug and the mucosal membrane which in turn offer a better chance of permeation for the administered drug (Mitra *et al.*, 2000:129).

1.6.4.4 Device related factors

The *particle size* of the droplet depends mainly on the type of device used to administer the drug solution to the nasal cavity. The ideal particle or droplet size is in the range of 5 - 7 µm because particles in this range will be retained in the nasal cavity and subsequently permeated. Particles larger than 10 µm will be deposited in the upper respiratory tract, whereas particles smaller than 0.5 µm will be exhaled (Huang & Donovan, 1998:150).

The *site and pattern of deposition* is affected by formulation composition, the physical form of the formulation (liquid, viscous, semi-solid, solid), the device used for administration and also the administration technique (Vidgren & Kublik, 1998:163).

The absorption of the administered drug is greatly affected by both the permeability of the site at which the formulation is deposited and the area of nasal cavity exposed. The retention of the drug in the nasal cavity is also greatly affected by these factors (Gonda & Gipps, 1990:71).

1.7 SUMMARY OF FACTORS WHICH AFFECT NASAL PERMEABILITY OF ACTIVE COMPOUNDS

1.7.1 Biological factors

Environmental influences	• Temperature
Pathological conditions	• Mucociliary dysfunction • Hypo-secretion • Hyper-secretion • Irritation of mucosa, caused by rhinitis, colds, polyposis etc.
Physiological influences	• pH of the nasal cavity • Nasal cycle or diurnal variation • Solubility in nasal secretions • Blood supply and neuronal regulation • Mucociliary clearance and ciliary beating frequency

1.7.2 Formulation factors

Physicochemical properties of active compound	• Molecular weight • Molecular size • Solubility • Lipophilicity • Partition coefficient and pKa
Physicochemical properties of the formulation	• pH and mucosal irritancy • Viscosity • Osmolarity
Drug distribution	• Area of nasal mucus membrane exposed • Volume of solution applied • Dosage form

1.7.3 Device related factors

Particle size of the administered droplet	• Type of device
Site and pattern of deposition	• Formulation composition • Physical form of the formulation, e.g. * liquid * viscous * semi-solid * solid
Administration device and technique used	

1.8 ADVANTAGES AND LIMITATIONS OF NASAL DRUG DELIVERY

1.8.1 Advantages of nasal drug delivery

Nasal drug delivery provides a viable alternative for the administration of many pharmaceutical agents. Some of the major advantages offered by the nasal route include:

- Rapid absoprtion, higher bioavailability and lower doses.
- Fast onset of therapeutic action.
- Avoids degradation of drug due to hepatic first pass metabolism.
- Avoids acidic or enzymatic degradation of drug in the gastrointestinal tract.
- No irritation of the gastrointestinal membrane.
- Reduced risk of overdose.
- Self-medication is possible through this route.
- There is a reduced risk of infection due to the fact that this is a non-invasive route of administration.
- Improved patient compliance.
- Reduced risk of infectious disease transmission (Behl *et al.*, 1998:96).

1.8.2 Limitations of nasal drug delivery

- Only 25 - 200 µl of drug solution can be administered into the nasal cavity.
- Compounds with a molecular weight greater than 1 kDa cannot be delivered via this route without the addition of a permeation enhancer.
- Nasal drug delivery is adversely affected by pathological conditions.
- The permeability of drugs are affected by normal defence mechanisms such as mucociliary clearance and ciliary beating.
- Enzymatic barrier to permeability of drugs (Arora *et al.*, 2002:968).

1.9 CONCLUSION

History and past research provide convincing evidence that nasal administration is a viable option to explore in order to improve the absorption of drug molecules. The intranasal route has excellent potential to improve drug delivery due to the large surface area and rich blood supply to this region. Nasal administration has the added benefit of avoiding the first-pass hepatic effect and gastro-intestinal degradation which in turn leads to higher drug plasma concentrations and a shorter time to onset of action. Based on these reasons it is very obvious that nasal administration offers a great alternative for the improved absorption of both peptide and protein drugs.

CHAPTER 2

HUMAN GROWTH HORMONE (hGH) AND SOMATROPIN (rhGH)

2.1 HUMAN GROWTH HORMONE (hGH)

Human growth hormone (hGH) is a long chain amino acid molecule which is produced by the anterior pituitary gland that is located at the base of the brain. hGH has a molecular weight of ± 22 000 Da and is a large fragile protein molecule which act on many different tissues in order to promote a healthy metabolism (Cenegenics Medical Institute, 2005:1).

The main effect accomplished by hGH is performed by a related hormone called Insulin-like growth factor-1 (IGF-1), IGF-1 is released in response to the presence of hGH, mainly by the liver, but also to some extent, by other tissues. hGH can be described as one of the primary hormones of importance for the maintenance of optimal cellular performance (Cenegenics Medical Institute, 2005:1).

2.1.1 Potential benefits of raising human growth hormone levels

Many of the bodily changes associated with aging are due to a progressive decline in the natural levels of hGH and IGF-1. Raising hGH and IGF-1 levels to those associated with younger physiology can delay the age related decline in function of many organs.

Some of the many beneficial effects of modulating hGH and IGF-1 are:

- enhance skin elasticity and thickness;
- decrease in total body fat;
- improve blood flow to the kidneys;
- increase bone mineral density;
- decrease in LDL cholesterol levels;
- increase in HDL cholesterol levels;
- improve healing time;
- improve general energy levels;
- increase lean muscle mass, and
- improve exercise capacity and over-all well-being (Cenegenics Medical Institute, 2005:1).

It should be noted that most of the above-mentioned effects are not immediately experienced and that it could take between 3 - 11 months of therapy before compositional changes, such as fat loss or muscle or bone gain, become apparent (Cenegenics Medical Institute, 2005:1).

It is important that one take into account that hGH is a very large polypeptide hormone which consist of 191 amino-acids in exact sequence and is maintained in a specific three-dimensional shape. The only sources of safe and accurately assembled hGH are those that use recombinant DNA technology which requires precise, elaborate and well monitored manufacturing methods (Cenegenics Medical Institute, 2005:1).

2.1.2 Administration and use of hGH

Human growth hormone is most often used to treat short stature in children due to growth hormone deficiency, chronic renal failure or Turner's syndrome. Human growth hormone is currently administered by daily injections which are both difficult to administer and painfull for the patient and therefore it is important to explore alternative routes of administration (Laursen *et al.*, 1996:313).

The nasal delivery of hGH would offer many advantages in patient compliance due to easier administration and the elimination of injection pain. Another advantage of the nasal administration of hGH is the possibility that the normal endogenous pulsatile hGH secretory pattern may be mimicked more closely compared to subcutaneous injections (Ugwoke, 2001:6).

Most pepide drugs experience one main obstacle after nasal administration, namely a low bioavailability of 1 - 2% due to the high molecular weight, high hydrophilicity and metabolic liability of these compounds (McMartin, 1987:536).

The aim of this study is to make an assessment of the potential of Pheroid technology (Chapter 3) as a nasal delivery method for Somatropin (section 2.2) which is a synthetic 191 amino-acid residue polypeptide with an amino-acid sequence and two internal disulfide bridges identical to that of the major component of human growth hormone (Dollery, 1999:73).

2.2 SOMATROPIN

Somatropin is also known as recombinant human growth hormone or rhGH. Somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is a synthetic hGH with the normal structure of the major component of natural hGH which is produced by the pituitary gland located at the base of the human brain. Somatropin consist of a 191 amino-acid single polypeptide chain with two disulfide linkages between positions 53 and 165 and also between positions 182 and 189 (Sweetman, 2002:1286).

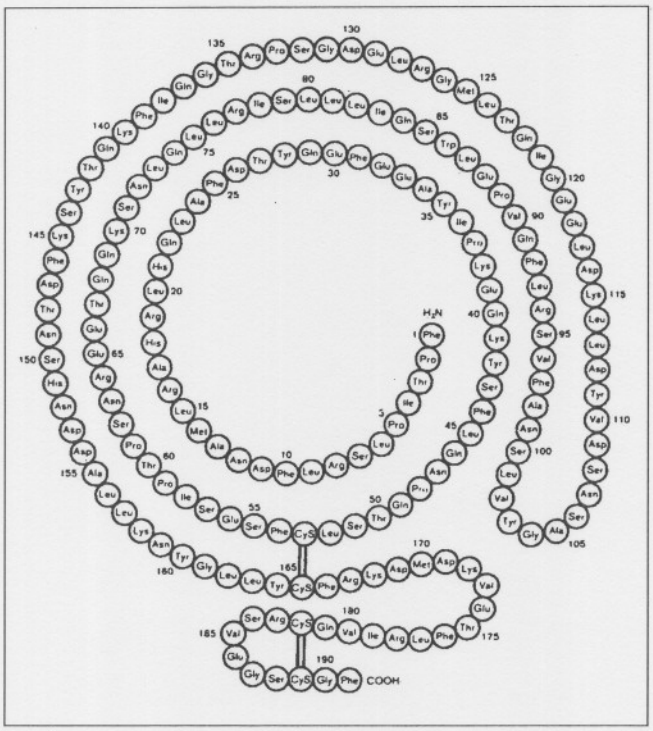


Figure 2.1: Structure of Somatropin (Dollery, 1999:73).

2.2.1 Pharmacology

It is known that Somatropin stimulate soft tissue and skeletal growth by promoting cell division, protein synthesis and the uptake of amino-acids (Thorner, 1985:235). The actions of Somatropin are mediated, predominantly, by hepatic and peripheral insulin-like growth factor-1 (IGF-1) production and as a consequence it has a brief but immediate insulin like effect followed by more significant anti-insulin-like actions such as lipolysis and a decreased glucose utilization (Ihle, 1995:592).

Growth hormone like actions on tissues is mediated via specific GH receptors. The hGH receptor exibit features typical to that of the cytokine receptor family (Ihle, 1995:592).

Growth hormone receptor monomers consist of a single chain containing both the intracellular signal-transducing and ligand-binding domains. Binding of growth hormone promotes receptor dimerization followed by the activation of Janus Kinase-2 (jak-2) with subsequent phosphorylation of STAT (signal transducers and activators of transcription) proteins and the enhancement of target gene transcription (Finidori & Kelly, 1995:16).

The growth hormone receptor is present in osteoblasts and chondrocytes, adipocytes, hepatocytes and in particular in fibroblasts. It is also found in many other tissues such as the gastrointestinal tract and brain but the role of GH in these tissues are not clear at the moment and needs further investigation (Norstedt *et al.*, 1990:81).

The GH receptor expression in humans is absent or very low in fetal tissue and increase progressively during infancy (Norstedt *et al.*, 1990:81).

Receptor expression is reduced by fasting and renal insufficiency and enhanced by insulin and sex steroids. The GH receptor exhibits a short half-life of ± 45 minutes and a few of these receptors are recycled to the cell surface membrane. The expression of specific genes such as somatostatin, growth hormone-releasing hormone, IGF-1, albumin and myosin heavy chains can be directly influenced by GH (Norstedt *et al.*, 1990:81).

Mutations of the gene encoding the GH receptor have been described, particularly involving the extracellular domain of the receptor which exhibit deficient GH binding and in turn do not respond to Somatropin or hGH stimulation which leads to growth failure or the so called Laron type dwarfism (Amselem *et al.*, 1989:991).

2.2.2 Clinical pharmacology

It is well known that Somatropin does not differ significantly from hGH in its metabolic actions (Rosenfeld, 1982:202).

An immediate transient period of hypoglycemia may be observed in growth hormone deficient patients but it does not occur in normal subjects (Wilton & Sietniks, 1987:127).

Two to four hours after administration anti-insulin-like actions are observed which may lead to an increase in serum free-fatty acid levels due to the inhibition of glucose utilization and lipolysis. In growth hormone deficient children this lipolytic effect is reflected by a loss of subcutaneous fat during the early months of growth hormone treatment (Tanner *et al.*, 1977:693).

Growth hormone treatment may provoke hyperinsulinemia without impairment of glucose tolerance in short children who are not growth hormone deficient (Hindmarsh & Brook, 1987:575).

The effects of growth hormone are mediated predominantly by IGF-1 and IGF-2 or the so called insulin-like growth factors or somatomedins. Most body tissues produce these polypeptides, with a molecular weight of approximately 7 500 Da, in response to an increase in growth hormone levels. In growth hormone deficiency the serum IGF levels are low but can rise to normal within a few days of starting Somatropin treatment and is further accompanied by the retention of sodium, phosphate and potassium with a marked increase in the intestinal absorption of calcium. It is important to note that although there is an increase in intestinal absorption of calcium, the serum calcium levels remain unaffected since urinary calcium excretion also increase (Thorner, 1985:236).

The glomerular filtration rate is increased, unless impairment of renal function is already present, due to the restoration of the depleted extracellular fluid volume after Somatropin administration. The response to growth hormone by serum IGF-1 is dose dependent and the effect on IGF-1 by a single dose of growth hormone does not last significantly beyond 24 hours (Jorgensen *et al.*, 1988:39). Central adiposity and insulin resistance are two of the major signs of growth hormone deficiency in adults (Weaver *et al.*, 1995:155).

2.2.3 Pharmacokinetics

Growth hormone can be measured by enzyme-linked immunosorbent assay (ELISA), single-antibody radioimmuno-assay (RIA), double-antibody immunoradiometric assay (IRMA) or immunochemiluminometric assay.

RIA techniques are usually less sensitive than IRMA and a typical working range is in the order of 0.5 - 200 mUl^{-1} for IRMA. ELISA systems can increase the sensitivity 100 fold. It is important to note that these techniques do not distinguish Somatropin from endogenous growth hormone (Reiter *et al.*, 1988:70).

Somatropin is mainly administered by means of a subcutaneous injection or rarely via intramuscular injection where peak serum levels are achieved 2 - 8 hours after injection and return to baseline after 8 - 16 hours (Wilton & Sietniks, 1987:127; Albertsson-Wikland, 1986:95).

There is a considerable variation between individuals with respect to both magnitude and timing of the rise in serum Somatropin levels (Albertsson-Wikland, 1986:95). More consistent serum hormone levels can be obtained with subcutaneous administration with a peak level after 4 - 8 hours after injection and returning to baseline after 11 - 20 hours (Albertsson-Wikland, 1986:96). Although more hormone reaches the systemic circulation after intramuscular injection compared to subcutaneous injection, there are no significant difference in the observed metabolic effects (Jorgensen, 1987:384). Somatropin is not absorbed in an active form from the gastrointestinal tract due to the enzymatic degradation of the active compound.

Somatropin exibit a biphasic clearance curve with a half disappearance time in normal subjects of 9.0 ± 3.5 min. (n = 8) for the first phase over 60 min., and 30.7 ± 10.8 min. (n = 8) for the second phase between 60 and 120 min after intravenous injection (Wilton *et al.,* 1988:117). The metabolic clearance rate ranged from 82 - 139 $ml.min.^{-1}m^{-2}$ body surface area and when given as a subcutaneous injection it was observed that the serum half-life increased to 248 ± 55 min., suggesting a rate-limiting absorption phase. It was further noted that age or sex does not influence the clearance of Somatropin but some medical conditions such as hypothyroidism and diabetes mellitus may well reduce the clearance (Thorner, 1985:236).

The distribution volume for Somatropin and its partitioning within the body are not known and the binding to plasma proteins is highly variable depending on the presence or absence of GH-binding antibodies and specific GH-binding proteins of high and low affinity. The high-affinity GH-binding protein is homologous to that of the extracellular domain of the GH receptor (Baumann & Shaw, 1990:682).

2.2.4 Concentration – effect relationship

The physiological secretion of growth hormone is nocturnal and occurs in a pulsatile manner with a frequency of approximately every 2 - 3 hours. The secretion rate in young men is about 0.6 - 1.5 mg in a 24 hour cycle which is equivalent to 1.3 - 3.0 $IU.24h^{-1}$ (Kowarski, 1971:358). It is suggested that optimal growth is achieved by simulation of the natural

physiological growth hormone pulse frequency and optimum dosing for adult growth hormone deficient patients is best determined by dose titration against clinical characteristics and serum IGF-1 leves (Dollery, 1999:73).

2.2.5 Stability

Somatropin is very unstable when exposed to water, especially at physiological conditions of pH 7.4 and 37 °C. The hormone tend to undergo both aggregation and decomposition in high concentration solutions and at physiological temperatures, resulting in irreversible aggregation, destruction of intact protein, and loss of its biological activity (Buckwalter *et al.*, 1992:360).

Hydration of the release system will also result in hydration of the protein, causing subsequent aggregation through formation of disulfide cross-links or isopeptide bonds and through hydrophobic reactions. Degeneration by unfolding, is expected to be rather rapid (Basitras & Wallace, 1992:9307).

Due to these problems, a prerequisite for the successful development of a Somatropin delivery system is to find a way to stabilize the hormone in solutions and in the controlled release devices. It has been widely reported that Somatropin was much more stable and still in its native structure after being precipitated by a bivalent ion such as zinc or copper (Mitchell, 1995:980).

When redissolved, such bivalent ion-precipitated Somatropin could regain its biological activity. When stabilizing Somatropin by means of bivalent ion precipitation it was found to be useful when a biocompatible oil or a reservoir formed with one to several layers of relatively hydrophobic polymers, such as paraffin or cellulose acetate, was used to develop the controlled release system (Mitchell, 1995:980).

2.2.6 Toxicology

Somatropin does not exibit any toxic effects in animals at doses equal to those used in humans. Extracted human growth hormone and biosynthetic methionyl-growth hormone have shown no mutagenic potential in Ames' bacterial test nor in bone marrow cells of the Chinese hamster (Fryklund, 1986:533).

2.3 THERAPEUTIC USE OF SOMATROPIN / RECOMBINANT HUMAN GROWTH HORMONE

2.3.1 Indications

- Treatment of growth hormone insufficiency or deficiency in children.
- Treatment of growth hormone deficiency in adults.
- Treatment of Turner syndrome.
- Treatment of AIDS-related wasting / cachexia.
- Treatment of growth disturbance in prepubertal children with chronic renal insufficiency (Dollery, 1999:74).

2.3.2 Contra-indications for the use of growth promoting hormones

- Active malignant neoplasm.
- Pregnancy.
- Somatropin should not be used for growth promotion in children with closed epiphyses.
- Proliferative retinopathy.
- Somatropin should be used with caution in patients with diabetes mellitus due to the anti-insulin-like effects of Somatropin and the insulin dosage may also require some adjustment (Dollery, 1999:74).

2.3.3 Special precautions and warnings

- In patients with panhypopituitarism one should monitor the patient closely when using standard replacement therapy.
- During treatment with Somatropin hypothyroidism may develop in some patients and periodic thyroid function tests may be necessary.
- Patients with severe headache, nausea and/or vomiting and visual problems should be advised to undergo a funduscopy for papilledema and if confirmed a diagnosis of benign intracranial hypertension should be considered and Somatropin treatment discontinued if appropriate (Dollery, 1999:740).
- Transient dose-related fluid retention with peripheral oedema may occur and patients may complain about muscle and joint pain.
- It is known that growth hormone has diabetogenic effects but at a high acute dose it has also been associated with hypoglycaemia (Sweetman, 2002:1286).

2.3.4 Recommended dose of Somatropin

A total weekly dose of at least 0.5 - 0.7 $IU.kg^{-1}$ is recommended or a dose of 12 $IU.m^{-2}$, divided into daily or three-times-weekly doses, administered via subcutaneous or intramuscular injection may also be used. The maximum total weekly dose is in the range of 20 - 30 IU and the absolute dose is increased, in line with the patients' growth progress, until the maximum dose is reached.

When growth hormone is administered in divided doses, better results can be achieved by increasing the frequency of injections eg. three times weekly rather than twice weekly. Anti-insulin effects on carbohydrate metabolism, in relation to meals, can be reduced by administering Somatropin in the evening. Patients with symptomatic hypoglycemia should receive at least daily injections of Somatropin when this hypoglycemia is directly associated with a growth hormone deficiency (Preece, 1976:480).

When Somatropin is administered to children, it is important to take into account either the body weight or surface area of the child when determining the total weekly dose. In most cases the standard regimes may be used which is 12 IU per week or when divided doses are preferred 0.3 - 0.5 $IU.kg^{-1}$ twice weekly or even daily are acceptable. In adults with a growth hormone deficiency the recommended starting dose is substantially lower than in children with a minimum of 0.125 $IU.kg^{-1}$ per week with a maximum dose of 0.25 $IU.kg^{-1}$ per week (Dollery, 1999:74).

CHAPTER 3

PHEROID TECHNOLOGY AND CHITOSAN AND *N*-TRIMETHYL CHITOSAN CHLORIDE AS ABSORPTION ENHANCING AGENTS

3.1 INTRODUCTION

As previously mentioned, the Pheroid™ delivery system is a patented system consisting of a unique submicron emulsion type formulation. The Pheroid™ delivery system, based on Pheroid™ technology, will for ease of reading be called Pheroid(s) only throughout the rest of this dissertation.

A Pheroid can be described as a stable structure which is suspended within a novel therapeutic system. The Pheroids can be manipulated in terms of structure, size, morphology and function depending on the type and size of the drug molecules which one want to deliver. Pheroid was first discovered when it was used as a basic formulation which led to the remission of psoriasis. One of the basic ingredients of this first formulation was present in banana peel extract and was later identified as essential fatty acids (Schlebusch, 2002:7). Pheroid consist mainly of plant and essential fatty acids and is able to entrap, transport and deliver pharmacologically active compounds and other useful molecules. It is quite obvious that the Pheroid delivery system can be used in a lot of different formulations and for the delivery of a wide variety of pharmacologically active compounds, therefore it should be quite clear that we need to study all the possibilities of this very unique drug delivery system.

3.2 PHEROID TYPES, CHARACTERISTICS AND FUNCTIONS

There are mainly three different types of Pheroid, namely:

- lipid-bilayer vesicles in nano- and micrometer sizes;
- microsponges, and
- depots containing pro-Pheroids.

The size of the lipid-bilayer vesicles is typically between 80 – 300 nanometer and it should be noted that both the size and shape of the vesicles are reproducible. The size of the microsponges on the other hand usually range between 0.5 and 5.0 μm and the size of the

depots are determined by the amount of pro-Pheroid which is contained within the depot (Schlebusch, 2002:8).

The Pheroid delivery system entraps the pharmacologically active compound and make it possible to create a safer and more effective formulation than one containing the active compound alone (Schlebusch, 2002:8).

Pheroids consist mainly of three phases, namely an aqueous phase, an oil phase and nitrous oxide. The aqueous phase consist mainly of sterile water while the oil phase is a unique combination of essential fatty acids (Grobler, 2004:4). The Pheroid system is unique because of the fact that it's main component, namely essential fatty acids, is manipulated in a specific manner to ensure its remarkably high entrapment capabilities, extremely fast rate of transport, delivery and stability.

Essential fatty acids cannot be manufactured by human cells but is still very necessary to maintain various cell functions which is why the essential fatty acids have to be ingested. It has been shown, however, that western diets often lack these basic essential lipid molecules (Grobler, 2004:4).

The Pheroid system has inherent therapeutic qualities, due to the essential fatty acids which it contain, such as the maintenance of membrane integrity of mammalian cells, modulation of the immune system and energy homeostasis. These characteristics of the Pheroid system affords it significant advantages over other delivery systems (Grobler, 2004:4).

The CLSM micrographs in figure 3.1 show active compounds entrapped in several Pheroid types. Each type has a specific composition.

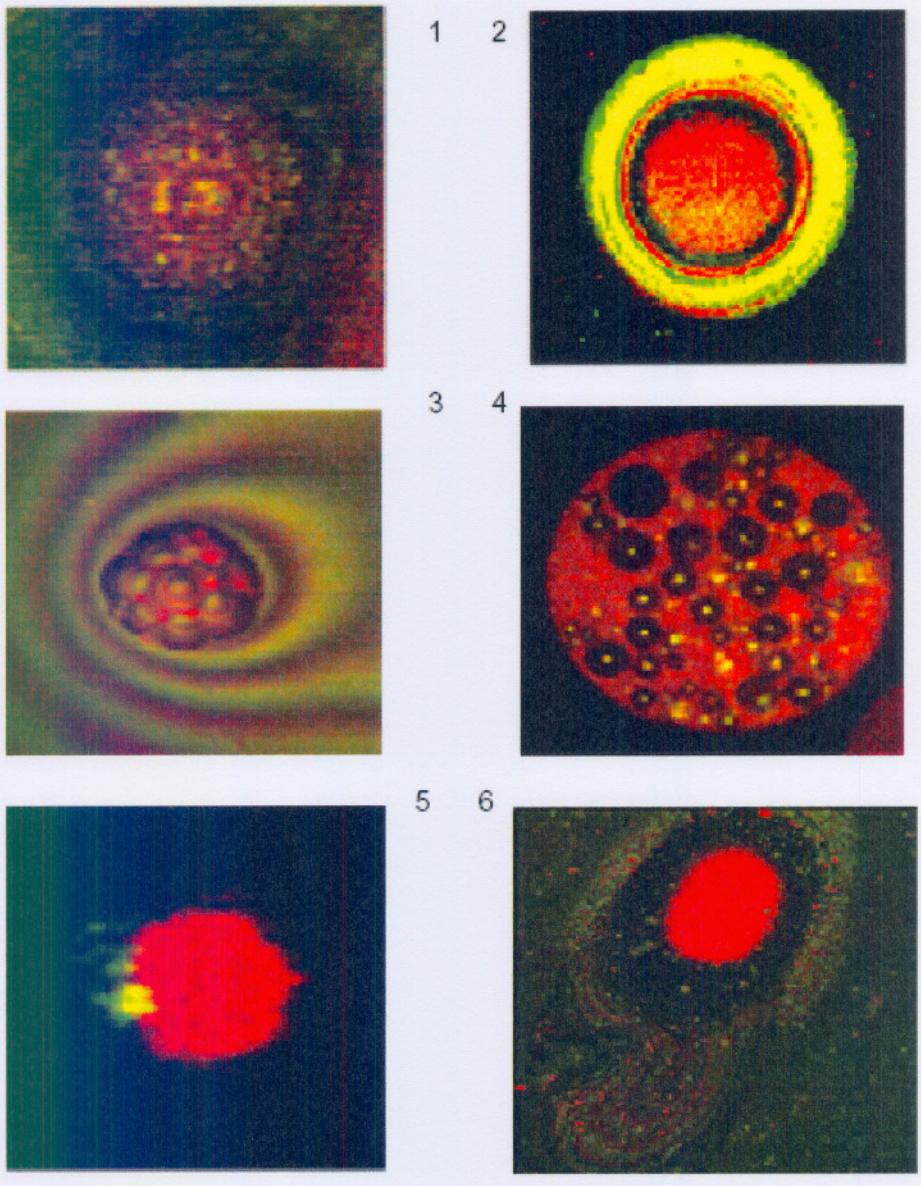


Figure 3.1: Basic Pheroid types: freshly entrapped Rifampicin in a bilayer membrane vesicle (Grobler, 2004:5).

The micrographs in figure 3.1 illustrate some of the basic Pheroid types.

1. A bilayer membrane vesicle with diameter of 100 nm containing Rifampicin.
2. A highly elastic or fluid bilayered vesicle with loose lipid packing, containing the same active compound, Rifampicin.
3. The formation of small pro-Pheroids. The formulation is used for some oral administrations.

4. The reservoir contains multiple particles of coal tar. Reservoirs have large loading capacity to surface area ratios and are good entrappers of insoluble compounds. General size is 1-10 μm.
5. This Pheroid is in the process of entrapping fluorescently labeled water-soluble diclofenac. It is very small (about 30 nm) and the membrane packing is sponge-like.
6. A depot with a hydrophobic core containing pro-Pheroid formulation, a surrounding hydrophilic zone and an outer vesicle-containing zone. Selective addition of fluid results in the release of vesicles from a release zone. The depots are used for sustained release according to a concentration gradient and can range in size from 5 to 100 μm. The sizes of Pheroid reflected above are not all to scale.

3.3 THE PHEROID DELIVERY SYSTEM COMPARED TO OTHER LIPID BASED DELIVERY SYSTEMS

Table 3.1 provides a comparison of the similarities, differences and key advantages of the Pheroid and other lipid-based or liposomal drug delivery systems currently available.

Table 3.1: Key advantages of the Pheroid system compared to lipid-based delivery systems (Grobler, 2004:6).

Pheroid	Lipid-based delivery systems
• Cytokine reactions to Pheroid were very low during a cytokine level study and cannot be regarded as being of any clinical significance.	• Some liposomal formulations have been shown to elicit immune responses.
• Manipulation of the size, charge, lipid composition and membrane packing are easily done in order to optimize the Pheroid system for the specific active compound, thus compatibility and repeatability pose no problems.	• Problems concerning repeatability of liposomal systems have been encountered and some have proven to be difficult if not impossible to overcome.
• A high affinity exist between the Pheroid and cell membranes because of the fact that Pheroid is comprised mainly of fatty acids. Enhanced penetration and delivery are ensured due to the fact that the Pheroid moves through the cell membrane and follow the endosomal sorting mechanisms.	• Problems are experienced with penetration and delivery due to the lack of specific binding and uptake mechanisms by mammalian cells.

Pheroid	Lipid-based delivery systems
• The rate of binding to and uptake of Pheroid by cells are very high and fast, due to the affinity between essential fatty acids contained within the Pheroid and its binding to micro-domains in cell membranes, and can be compared to the rate observed for active transport across the cell membrane.	• A specific cellular binding mechanism has not yet been established for Liposomes.
• Subcellular organelles can be targeted by manipulating the pH or fatty acid content of the Pheroid.	• Targeting of subcellular organelles is very difficult, if at all possible, as a result of the metabolism of the phospholipids.
• The Pheroid causes no cytotoxicity, since it's part of the natural biochemical pathway, and displays rapid clearance and assists with maintenance of cell membrane integrity.	• Cytotoxicity and impaired cell integrity are common problems encountered with substances that enter the body.
• The Pheroid has a unique capability to penetrate a vast array of potential barriers such as keratinised tissue, skin, intestinal lining, the vascular system, fungi, bacteria and parasites.	• Other delivery systems do not demonstrate such versatility.
• The Pheroid has a polyphilic nature which enables it to encapsulate drugs with different solubilities as well as insoluble drugs.	• Other delivery systems are either hydrophilic or lipophilic which limits their use to specific drugs only.
• The Pheroid enhances bioavailability because of its ability to inhibit the drug efflux mechanism in the intestinal lumen.	• No liposomal system has been found to contain this feature and a separate compound such as Cremophor EL is used to achieve the same effect in the patient.
• The interior volume of the Pheroid is very stable although it contains no cholesterol to assist in the maintenance of the vesicles.	• Cholesterol and phospholipids form the basis of most lipid-based delivery systems and it should be noted that cholesterol is essential for the maintenance of the interior of the vesicles.
• An entrapment efficiency of 85 – 100% was encountered in *all* compounds tested.	• Entrapment efficiency can sometimes be very low due to charge and steric limitations of these delivery systems.
• The Pheroid displays high levels of stability in products.	• Other delivery systems often display physical and chemical instability both in products and *in vivo*.

Pheroid	Other lipid-based delivery systems
• Reproducibility has been proven between different batches of products which contain Pheroids.	• Low batch-to-batch reproducibility is often encountered and size control is a very common problem.
• Sterilisation with gamma radiation does not have a negative effect on Pheroid formulations.	• Other delivery systems are sensitive for heat, radiation and chemical sterilisation and filtration is not suitable for formulations which contain large vesicles.
• There are three main types of Pheroid currantly available, namely: ➢ single lamellar vesicles ➢ microsponges ➢ depots (pro-Pheroids)	• A few liposome types have been used such as: ➢ single lamellar vesicles ➢ multi-lamellar vesicles ➢ nanosomes ➢ multi-vesicular vesicles
• The Pheroids can entrap peptides and antibodies and this enables the Pheroid to interact with specific micro-domains on cells in culture.	• Drug targeting is possible with the aid of antibody-containing liposomes.
• The Pheroid is able to protect the drug from opsonization, inactivation and metabolism.	• Some liposomal systems can protect the drug against opsonization and metabolism.
• Pheroid can be formulated as pro-Pheroids.	• Pro-liposomes can also be formulated.
• The reticulo-endothelial system (RES) is passively targetted by the Pheroids with accumulation of the Pheroid in the spleen and liver.	• Liposomal systems also target the RES and also accumulate in the spleen and liver.
• The pharmacokinetics of active compounds have been shown to change with the use of the Pheroid system with a notable decrease in the time needed to achieve maximum blood levels.	• Liposomes have also been shown to change the pharmacodynamics of a wide variety of active compounds.

3.4 KEY CHARACTERISTICS OF THE PHEROID SYSTEM AND PHARMACEUTICAL APPLICABILITY

The Pheroid system exhibits a few key characteristics which make it superior to most, if not all, of the other drug carrier systems currently available on the market.

3.4.1 Increased delivery of active compounds

In vitro and *in vivo* studies have both proven that the percentage of active compound delivered to the site of action can be greatly increased when entrapped in a Pheroid formulation.

Table 3.2 below shows some of the results obtained from membrane diffusion studies which mimic the delivery of active compounds across specific membranes. In this table "PHR" indicate the Pheroid-entrapped product and "COM" represents a comparable commercial product (Grobler, 2004:9).

Table 3.2: Diffusion rates and percentage release per label claim for product tested (Grobler, 2004:9).

Active Agent	% Active/product	Diffusion Rates (µg/cm2/h)	% Release per label claim
Acyclovir PHR	0.5	69.1533	0.1214
Acyclovir COM	0.5	54.0942	0.0952
Miconazole Nitrate PHR	2	389.9238	6.8155
Miconazole Nitrate COM	2	111.2222	1.04662

3.4.2 Decreased time to onset of action

The Pheroid system is capable of rapidly traversing most physiological barriers, as already mentioned, and is able to deliver the active quickly and effectively to the desired site of action. This characteristic of the Pheroid system suggests that it would be possible to deliver the active significantly quicker with this system than with conventional methods which in turn would suggest potentially faster relief from target symptoms (Grobler, 2004:9).

Figure 3.2 illustrates the obtained average plasma levels of Rifampicin for 14 healthy volunteers after oral administration of combination anti-tuberculosis DOTS treatment, with and without the use of Pheroids (Pyriftol and Rifafour respectively).

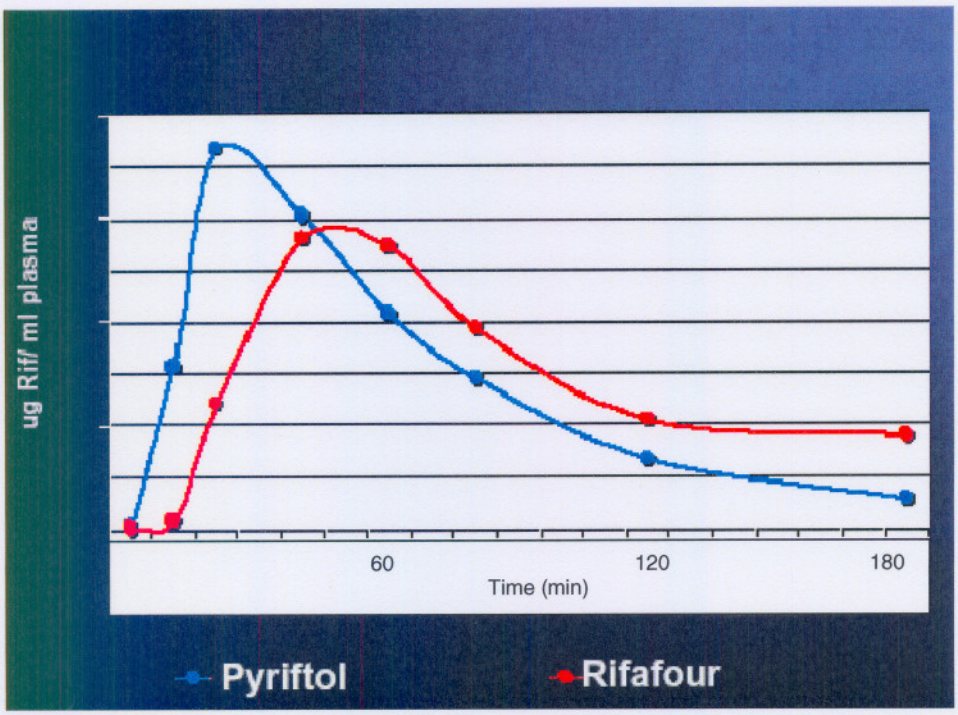


Figure 3.2: The time needed to achieve plasma C_{max} is halved by entrapment in Pheroid when compared to that of one of the preferred comparative products. Pyriftol contained only 60% (400 mg) of the amount of Rifampicin contained in the commercially available Rifafour (600 mg) (Grobler, 2004:9).

3.4.3 Increased therapeutic efficacy

The following samples demonstrate the enhancement of action of anti-infective agents where the active was entrapped in a Pheroid solution as opposed to a few commercially available products. It should be noted that in all cases tested it was obvious that the formulation of an active compound entrapped in a Pheroid solution increased the efficacy of the active as determined by zone inhibition studies (Grobler, 2004:10).

Table 3.3: Zone of inhibition study: Five commercially available products (COM) versus Pheroid (PHR)-formulations of the same active compound (Grobler, 2004:11).

Active Agent	PHR / COM	Dose mg / 5 ml	S. Aureus	P. Aerugin	B. Cereus	E. Coli	A. Niger	C. Albicans
Cloxacillin	PHR	125	30.74	23.96				
Cloxacillin	COM	125	29.45	19.78				
Erythromycin	PHR	250	26.7		29.89			
Erythromycin	COM	250	25.84		27.78			
Ciprofloxacin	PHR	250	33.05			35.78		
Ciprofloxacin	COM	250	30.14			33.40		
Cotrimoxazole	PHR	240	13.95			24.64		
Cotrimoxazole	COM	240	11			22.83		
Itraconazole	PHR	50					16.03	14.28
Itraconazole	COM	50					11.47	10.21
Control			9	9	9	9	9	9

All the above formulations made use of one single Pheroid type. Reformulation of Ciprofloxacin and Erythromycin in micropsonges has since increased efficacy.

3.4.4 Reduction in cytotoxicity

Cellular damage often occurs as a result of exposure to the harmful effects of active ingredients but the Pheroid system has the potential to greatly reduce these harmful effects by enhancing normal cell integrity which in turn will minimise cellular damage (Grobler, 2004:10).

3.4.5 Penetration of tissue, organisms and most known barrier cells

Research has shown that the Pheroid is capable of penetrating a vast array of bariers, commonly found in the human body such as skin, keratinized tissue, vascular walls, subcellular organelles and intestinal epithelium (Grobler, 2004:12).

The Pheroid has also demonstrated its ability to effectively penetrate bacteria, viruses, fungi and parasites and also the capability to deliver drugs to these organisms in order to destroy them. The Pheroid is able to penetrate human skin which means that it is possible to administer active compounds via the topical route. Topical administration focusses the active near the desired site of action which in turn can mean a significant reduction in systemic side effects (Grobler, 2004:12).

3.4.6 Reduction of minimum inhibitory concentration (MIC)

A reduction of the MIC would suggest that it may be possible to use less of the active compound in the Pheroid formulation, in some cases as little as 1/40th of the original dose, and still reach an effective drug plasma concentration. This characteristic in turn would lead to cost savings in product formulation and a reduction of patient side effects caused by the active compound (Grobler, 2004:10).

In figure 3.3 the growth of reference strain H37RV is depicted. Blue indicates Pheroid-entrapped Isoniazid and red indicates free Isoniazid. Growth was determined with the use of radio-active labels in a BACTEC system (Grobler, 2004:10).

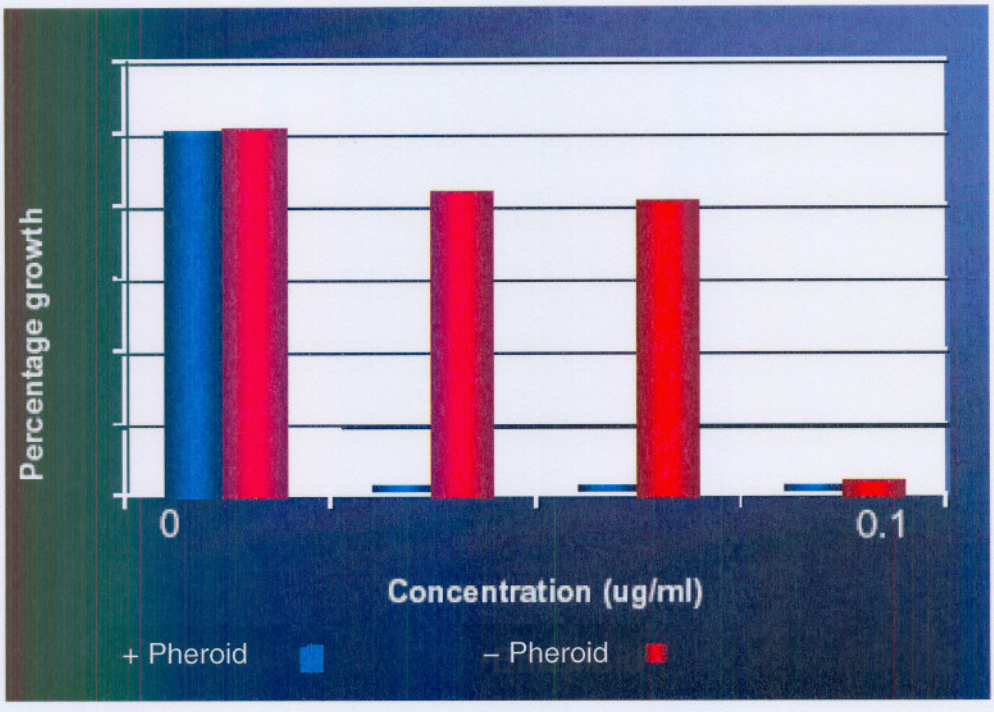


Figure 3.3: *In vitro* inhibition of bacterial growth by INH.

3.4.7 Adaptability and flexibility

The Pheroid system is capable of entrapping a wide variety of drugs with different characteristics such as hydrophilic, lipophilic and even insoluble substances. This characteristic puts the Pheroid system a cut above most other delivery systems since most of them can either carry lipophilic or hydrophilic drugs but not both types and most other systems are unable to carry insoluble drugs. Furthermore with the addition or deletion of selected components of the Pheroid system it is possible to change the structure, size and carrying capacity of the Pheroids which make it even more versatile (Grobler, 2004:7).

3.4.8 Immunological responses

The Pheroid is capable of masking the active compounds, such as peptides or proteins, which in turn reduces the recognition of these compounds by the body's immune system and therefore it would also reduce the occurrence of adverse intolerance and immunologic responses in the patient (Grobler, 2004:12).

3.4.9 Targeting of the treatment area

The Pheroid can be designed in a variety of different structures in order to target specific body systems or organs which means that it is possible to target only the affected area and avoid cellular damage and side effects to other parts of the body (Grobler, 2004:6).

3.4.10 Ability to entrap and transfer genes to cell nuclei

Various experiments conducted on the Pheroid delivery system demonstrated its applicability in both DNA vaccines and gene therapy. *In vitro* studies have demonstrated the effective entrapment of both human and viral DNA, of various lengths, in Pheroid vesicles. Reproducible expression of appropriate proteins was observed after transfection of cells by Pheroid-entrapped genes (Grobler, 2004:13).

3.4.11 Reduction and possible elimination of drug resistance

In vitro studies have demonstrated that by incorporating a drug into a Pheroid vesicle, it is possible to reduce or even eliminate drug resistance. Analysis of bacterial growth of multidrug resistant TB have shown that formulations containing the standard antimicrobial, Rifampicin, obviated pre-existing drug resistance when incorporated in a Pheroid solution. The potential to revive the effectiveness of various antibiotics such as penicillin has

widespread application in the healthcare industry. Figure 3.4 illustrates the effectiveness of Rifampicin when entrapped in a Pheroid solution. The entrapment of Rifampicin in Pheroids resulted in complete bactericidal activity against resistant Mycobacterium isolated from a MDR patient. The free Rifampicin shows no growth inhibition (Grobler, 2004:13).

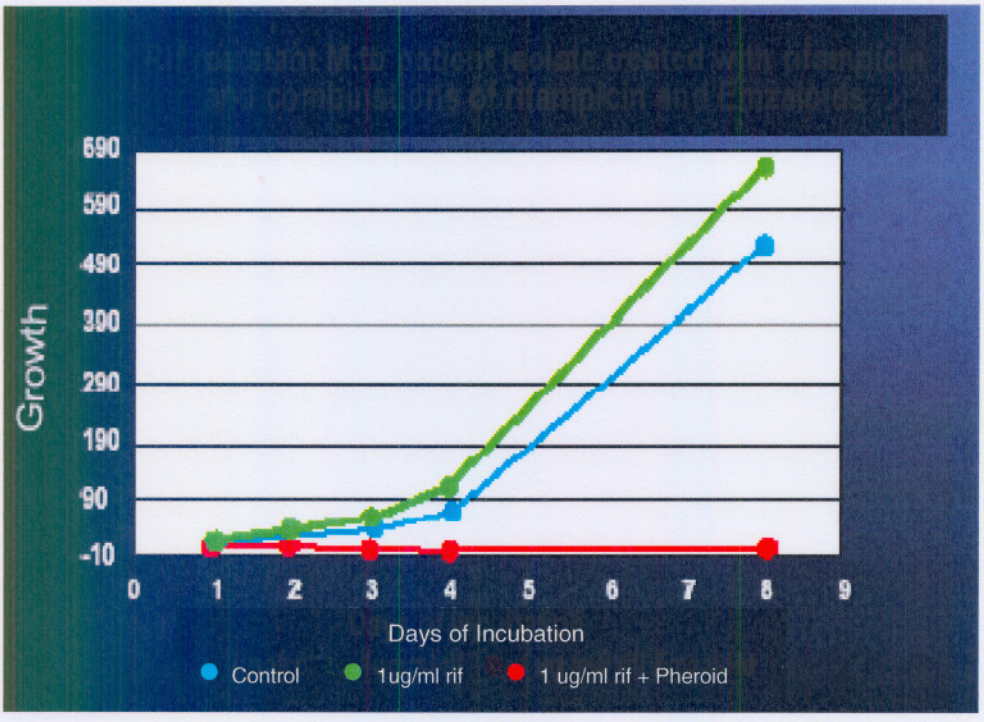


Figure 3.4: Effectiveness of Rifampicin when entrapped in a Pheroid solution (Grobler, 2004:13).

3.5 THERAPEUTIC AND PREVENTATIVE APPLICATIONS OF PHEROID TECHNOLOGY

3.5.1 Therapy of Tuberculosis

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

Previous *in vitro* and *in vivo* studies suggested that the Pheroid delivery system might have significant benefits when used in conjunction with the current TB treatment regime. Due to this aforementioned information, a bioequivalence study was performed to determine whether the Pheroid delivery system would remain as effective in oral administration as it was proven to be in topical applications (Grobler, 2004:14).

The goal of this study was to prove that the use of Pheroid, after administration in the form of a pro-Pheroid solution could:

- increase the availability of the antimicrobials in human plasma;
- enhance absorption of antimicrobials from the gastrointestinal tract without an increase in toxicity;
- increase intracellular concentration of antimicrobials in target cells which are the breeding ground of the tuberculosis bacillus;
- increase the circulatory time of active drugs;
- increase the bactericidal effect of the antimicrobials inside target cells, and
- decrease side effects caused by the antimicrobials (Grobler, 2004:17).

Results of the bio-equivalence study revealed that:

- an increase in plasma levels of these antimicrobials was observed after oral administration which would suggest that the Pheroid-entrapped antimicrobials is better absorbed from the gastrointestinal tract;
- entrapment of the antimicrobials led to an increased cellular response due to an increased rate of absorption;
- the therapeutic concentrations of the drugs were maintained for longer and the circulatory time of the drugs was extended which would indicate that the exposure of the bacillus to the antimicrobials was increased;
- the minimum inhibitory concentration (MIC) was decreased due to the fact that the delivery of the antimicrobials to the target cells was increased by the Pheroid delivery system;
- a lower dosage can be used to obtain similar effects, and
- a decrease in side effects was observed together with an increase in patient compliance. This could help prevent the development of multidrug resistance (Grobler, 2004:17).

3.5.2 Preventative therapies

Historically, vaccination is the only strategy that has led to the elimination of the viral disease, smallpox. An indirect relationship has been observed for vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor, which make it quite obvious that there is an urgent need

for effective vaccine adjuvants to enhance the immunogenicity and immunostimulatory properties of vaccines (Grobler, 2004:17).

3.5.2.1 A peptide-based vaccine: *Hepatitis B*

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

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

3.5.2.2 A virus-based vaccine: *Rabies*

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

3.5.3 Pheroid technology for nasal vaccine delivery

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

The antigen is entrapped in the Pheroid solution and administered. It is absorbed by the microfold cells (M-cells), situated in the nasal epithelium, which are responsible for the sampling of and transporting of antigens to the underlying nasal assosiated lymphoid tissue (NALT), germinal centers containing both B and T cells, plasma cells and antigen presenting

cells (APCs). These cells are involved in the regulation and induction of antigen-specific effector cells, which produce the protective humoral and cellular immune responses (Grobler, 2004:19).

3.6 CONCLUSION

Although there are many similarities between the Pheroid delivery system and other lipid-based delivery systems, there are a few definite advantages which count in the favour of Pheroid technology as conclusively proven by research. Pheroid have infinite possibilities in the pharmaceutical industry due to its versatility as a delivery system. The most significant advantage of the Pheroid system is its capability to greatly enhance the absorption of both peptide and protein drugs. Speeding up the absorption of these drugs will in turn lead to higher plasma levels, faster cellular responses and a significant decrease in the T_{max}.

By using Pheroid technology it is possible to maintain therapeutic concentrations for longer periods of time and lower drug dosages is needed to achieve the MIC which means that there is a much lower incidence of drug related adverse effects. These characteristics of the Pheroid system promotes itself as a great delivery system for both peptide and protein drugs as well as other poorly absorbable drugs. Due to its relatively low manufacturing costs, biocompatibility and low toxicity, this system may hold great potential for the future development of novel drug delivery systems.

3.7 CHITOSAN AND *N*-TRIMETHYL CHITOSAN CHLORIDE AS ABSORPTION ENHANCING AGENTS

3.7.1 Introduction

The history of chitosan and chitosan derivatives dates back to 1859 when Rouget first discussed the deacetylated form of chitosan. Rouget found that when chitin was boiled in a concentrated potassium hydroxide solution, a product was obtained which had quite different characteristics than that of the original chitin from which it was derived. This new product dissolved in dilute iodine and acids, unlike chitin which only stained the solution to a brownish colour (Paul & Sharma, 2000:5).

Chitosan has recently been considered for use in pharmaceutical formulations and drug delivery applications due to its absorption enhancing capabilities, controlled release and bioadhesive properties. This polymer has shown to be both biocompattible and

biodegradable due to the fact that it is synthesised from a naturally occuring source (Dodane & Vilivalam, 1998:246).

3.7.2 Origin and chemical structure

Chitosan is obtained by the deacetylation of chitin, the second most abundant polysaccharide in nature, next to cellulose which is the most abundant. Chitin is mainly found in the exoskeleton of crustaceans, insects and some fungi such as *mucor* and *aspergillus* (Hejazi & Amiji, 2003:151).

Chitin is obtained from the shell wastes of lobster, shrimp, krill and crab which can be considered to be the main commercial sources (Hejazi & Amiji, 2003:151). Chitosan [α (1-4) 2-amino 2 deoxy β-D-glucan] is derived by the alkaline deacetylation of chitin. The chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. The sugar backbone of chitosan consists of β-1.4 linked D-glucosamine with a high degree of *N*-acetylation, a structure very similar to that of cellulose, except that the acetylamino group replaces the hydroxyl group on the C-2 position. Thus, chitosan can be classified as poly (9*N*-acetyl-2-amino-2-deoxy-D-glucopyranose), where the *N*-acetyl-2-amino-2-deoxy-D-glucopyranose units are linked by (1-4)-β-glycosidic bonds (Hejazi & Aiji, 2003:151).

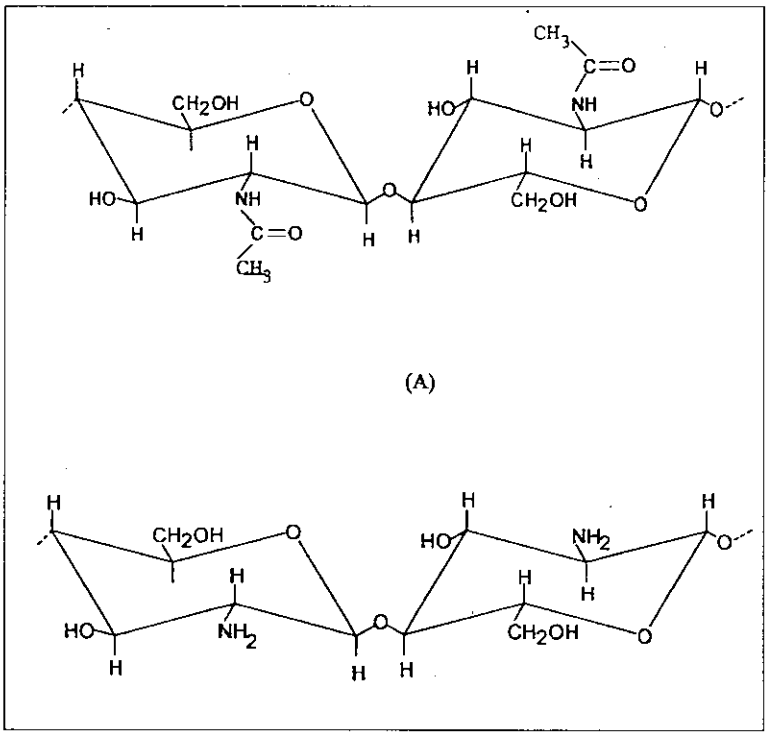


Figure 3.5: Chemical structures of (A) chitin and (B) chitosan (Hejazi & Amiji, 2003:152).

Chitosan is marketed under a variety of forms with different degrees of deacetylation and molecular weights and it is also available as chitosan based salts. It is produced in the form of a solution, flakes, powder, beads and even in a fibre form. Biomedical grade chitosan of high purity is also freely available for biomedical applications such as drug delivery.

The preparation of chitosan can be described in short as follows:

- To prepare chitin, boil crab and shrimp shells in an aqueous sodium hydroxide solution after decalcification in dilute hydrochloric acid and deproteination in a dilute sodium hydroxide solution.
- Deacetylation of the formed chitin is achieved by adding concentrated sodium hydroxide solution in order to render chitosan.
- Figure 3.6 gives a schematic representation of the preparation of chitosan from chitin.

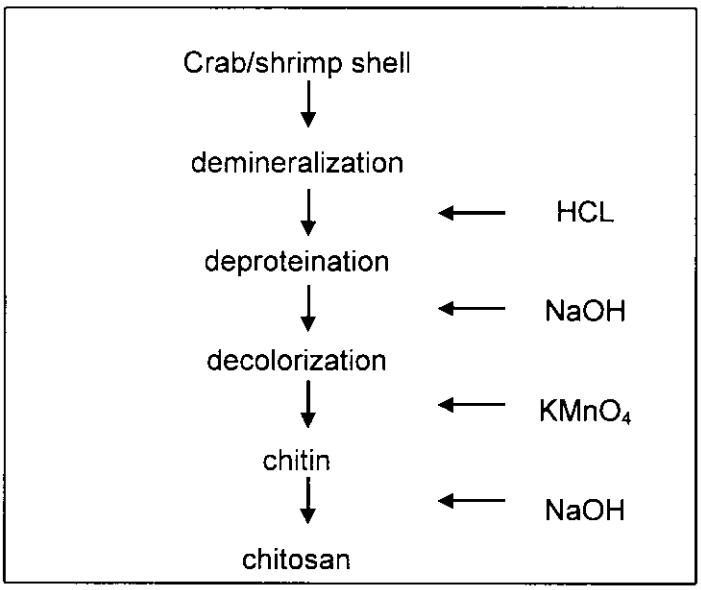


Figure 3.6: Production of chitosan from chitin (Paul & Sharma, 2000:5).

Biomedical grade chitosan is prepared by repeating the deacetylation process. There is a significant difference in purity between pharmaceutical grade and food grade chitosan. Pharmaceutical grade chitosan has a degree of deacetylation greater than 90% in contrast to food grade chitosan with only a 75% degree of deacetylation (Paul & Sharma, 2000:5).

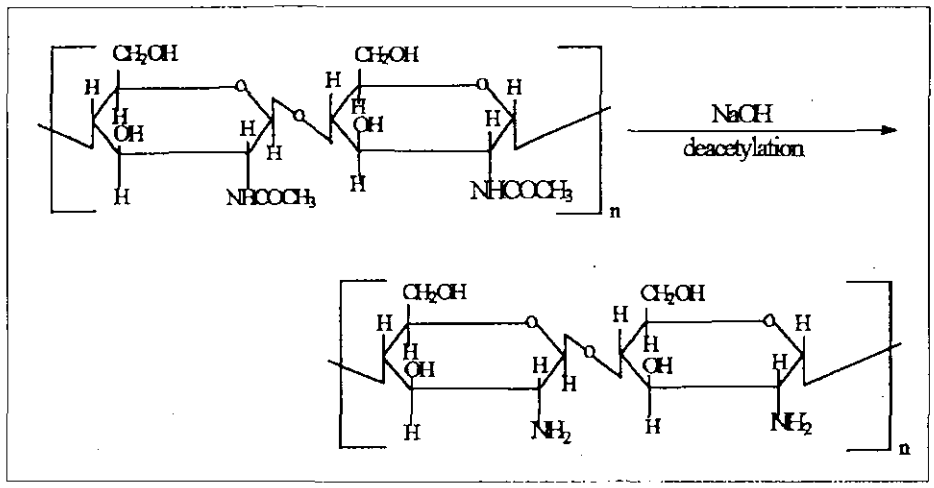


Figure 3.7: Production of deacetylated chitosan (Majeti & Kumar, 2000:3).

3.7.3 Biological and physicochemical properties of chitosan

The term chitosan refers to a large number of polymers which differ greatly in their degree of *N*-deacetylation, which may vary between 40 - 98%, and molecular weight which vary between 50 000 - 2 000 000 Da. The physico-chemical properties are greatly influenced by these two characteristics which in turn have a major effect on the biological properties (Hejazi & Amiji, 2003:152).

Chitosan is considered to be a weak base with a pKa value between 6.2 en 7.0 which renders it insoluble at neutral and alkaline pH values. Chitosan exibits a tendency to form various salts in the presence of both organic and inorganic acids such as hydrochloric acid, acetic acid, glutamic and lactic acid. The amine groups of the polymer are protonated in an acidic medium which results in the formation of a soluble, positively charged polysaccharide that has a high charge density. The degree of deacetylation and the pH of the solution are both instrumental in the determination of the solubility of chitosan salts. Chitosan salts with a high degree of deacetylation, in excess of 85%, are soluble up to a pH of 6.5, whereas salts with a low degree of deacetylation, in the region of 40%, are soluble up to a pH of 9.0 (Hejazi & Amiji, 2003:152).

The viscocity of chitosan solutions is influenced by the degree of deacetylation. Solutions with a high degree of deacetylation exibit a higher viscocity value than solutions with a lower degree of deacetylation. This phenomenon can be attributed to the fact that chitosan exists in different conformations in an aqueous solution depending on the degree of deacetylation.

When chitosan is highly deacetylated the charge repulsion in the molecule increase which leads to a more extended conformation with a relatively flexible chain. However, chitosan has a rod-like or coiled conformation when it has a lower degree of deacetylation which can consequently be attributed to the fact that there is a lower charge density in the polymer chain (Hejazi & Amiji, 2003:152).

In a solution chitosan tend to exist in a quasiglobular conformation which is stabilised by intra- and intermolecular hydrogen bonding. This hydrogen bonding in chitosan chains, which include amine and hydroxyl groups, is responsible for the high viscocity of chitosan solutions. Other factors such as concentration and temperature may also affect the viscocity of chitosan solutions (Hejazi & Amiji, 2003:152).

3.8 BIOPHARMACEUTICAL ORIENTATION

3.8.1 Oral route

The bioavailability of various active compounds has been improved dramatically when incorporated in mucoadhesive dosage forms. This increase in bioavailability can be attributed to the fact that the residence time of drug carriers at the absorption site is prolonged which in turn makes it possible to formulate sustained release dosage forms which incorporates this technology. It is acknowledged that chitosan posses good mucoadhesive properties which imply that it would then most likely lend itself to be implemented in oral dosage forms (Dodane & Vilivalam, 1998:246).

The oral route is the most common, simple, convenient and physiological way of administering traditional active compounds. The oral route does not lend itself to the administration of protein or peptide drugs due to the fact that these drugs are degraded in the gastric environment of the stomach and are in turn poorly absorbed. Chitosan has been thoroughly assesed, and proved effective, as a potential oral delivery vehicle. It has been found that the ingestion of chitosan can effectively reduce the total serum cholesterol, urea and creatinine levels. It has also been found that chitosan increases the serum hemoglobin levels without any clinically relevant symptoms (Paul & Sharma, 2000:7).

3.8.2 Transdermal route

The administration of drugs through the skin has many advantages such as the elimination of first pass metabolism by the liver, no enzymatic degradation, no gastrointestinal effects or

degradation and its not invasive. Chitosan and its derivates can be incorporated in creams, lotions, ointments and patches which makes it extremely versatile and suitable for both membrane and drug reservoirs in transdermal patches (Thacharodi & Roa, 1995:145).

3.8.3 Parenteral route

Formulations containing chitosan and its derivatives were administered both intramuscular and subcutaneously in various studies. These studies concluded that the prepared formulations have the ability to prolong the delivery (extended release) of various drugs and even hormones such as progesterone (Paul & Sharma, 2000:7-8).

3.8.4 Ocular route

The bioadhesive characteristics of chitosan makes it an excellent choice for use in ocular formulations due to the fact that the application intervals are prolonged significantly. Localised activity is of special interest, including antibacterial and antiviral agents, where the use of current formulations are limited due to lacrimal drainage which prevent the delivery of drugs over extended periods of time (Gental *et al.*, 1997:737).

3.8.5 Chitosan implants

Chitosan is a good candidate for incorporation in implants because it is non-toxic, biodegradable and can be sterilised. Chitosan incorporation ensures drug stability, adequate drug storage capacity and even controlled release properties (Muzzarelli & Muzzarelli, 2002:233).

3.8.6 Nasal route

Nasally administered drugs have to be transported over a very small distance before absorption, in comparison to orally administered drugs. Nasally administered drugs are not exposed to extremely low pH values or degrading enzymes, the first pass metabolism is also eliminated by this route. Drugs for nasal administration can be formulated in a chitosan solution or even a powder for inhalation. Chitosan gells may also be a viable option when longer lasting drug release is required. No humoral immune responses against chitosan itself were found after either nasal or subcutaneous administration (Van der Lubben *et al*, 2001:142).

3.8.7 Mechanism of action of chitosan

Chitosan has been shown to have strong mucoadhesive properties and in addition to this there is an interaction between the positively charged amino group at the C-2 position of chitosan and the negatively charged sites on the cell surfaces. These interactions at the tight junctions promote the paracellular transport of large hydrophilic compounds by opening these junctions in the mucosal membranes (Van der Lubben *et al.*, 2001:203).

The interaction with the opening mechanism of the tight junctions can be explained by a decrease in ZO-1 proteins and a change in the cytoskeletal protein F-actin from a filamentous to a globular structure. These characteristics of chitosan and its derivatives emphasize its potential as an absorption enhancer for active compounds via the mucosal paracellular pathways (Junginger & Verhoef, 1998:374).

3.8.8 Safety of chitosan

Studies indicate that chitosan lack irritant or allergic effects and is biocompatible with both healthy and infected skin. In one study chitosan was administered orally to mice and the LD_{50} was found to be in excess of 16 g/kg, which is higher than that of sucrose. Further studies revealed that the salt form, particle size, density, viscocity, molecular weight, degree of deacetylation as well as the pH at which chitosan is used, may influence the properties of this polymer in drug delivery systems. All of these factors must be considered during formulation in order to ensure the safety and efficacy of the drug delivery system (Sinha *et al.*, 2004:2; Dodane & Vilivalam, 1998:250).

3.8.9 Applications of chitosan

Table 3.4 contains some interesting applications of chitosan and its derivatives.

Table 3.4: Applications of chitosan (Paul & Sharma, 2000:6).

FIELD	APPLICATION
Health care	• Contact lenses • Eye bandages • Wound healing ointments and dressings • Anti-cholesterol (hypercholesterolemic agent) • Fat-binding • Surgical sutures • Drug delivery • Ophthalmology • Dentistry • Transportation of cells
Food and beverages	• Food stabiliser • Flavour and tastes • Food packaging • Nutritional additives • Fruit preservation
Agriculture	• Seed treatments (coating of seeds) • Animal feeds • Nematocides and insecticides
Cosmetics and toiletries	• Hair treatment • Skin care • Oral care
Waste and water treatment (clarification)	• Sewage effluents • Drinking water • Recovering metals • Treating food wastes (food processor wastes)
Product separation and recovery (bio-applications)	• Membrane separations • Chromatographic matrix • Immobilisation of enzymes/cells

3.8.10 Chitosan as a drug delivery system

Chitosan has been extensively examined for its potential in the development of controlled release drug delivery systems. Many forms of controlled release formulations have been tested such as tablets, gels, beads, capsules, microspheres and microparticles to name but a few. Nanoparticles have a few added advantages which makes them superior to liposomes and they have a special role in targeted drug delivery. Nanoparticles have a small particle size, a long shelf life and can usually entrap more drug molecules than liposomes (Banerjee *et al.*, 2002:94).

Controlled release technologies emerged during the 1980's and the predictable and reproducible release of a therapeutic agent into a specific environment over an extended period of time, has great merit and infinite possibilities. Controlled release ensures that the therapeutic agent is released in the desired environment with optimal response, prolonged efficacy and minimal side effects. These dosage forms regulate the rate of drug release and in turn reduce the frequency of drug administration which means that greater patient compliance is promoted (Majeti & Kumar, 2000).

Chitosan is non-toxic and easily bioabsorbable, with gel-forming ability at low pH values. This gel-forming character gives chitosan anti-acid and anti-ulcer activity due to its ability to minimise or even prevent drug irritation in the stomach. Chitosan matrix formulations tend to gradually swell and float in an acid medium which make it useful in the treatment of gastro-intestinal reflux disorders (Majeti & Kumar, 2000:14).

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

N-Trimethyl chitosan chloride (TMC) is a derivative of chitosan which is partially quaternised with enhanced water solubility and superior drug absorption promoting properties, especially in neutral and alkaline environments (Kotzé *et al.*, 1999b:254).

TMC displays muco-adhesive characteristics and decreased intrinsic viscocity, and reduces transepithelial electrical resistance (TEER) across Caco-2 cell layers which contributes directly to its activity as a drug absorption enhancer (Kotzé *et al.*, 1997a:1199).

3.9.1 Synthesis of TMC

Domard *et al.* (1986:105) documented a method to synthesize TMC from a sieved fraction of chitosan with particles in the range of 500 µm or less. The experimental method include reductive methylation of chitosan for 60 minutes with the assistance of iodomethane in a strong basic environment at 60 °C. By dissolving the quaternised polymer in a sodium chloride solution, it is possile to exchange the (I^-) counterion with a Cl^- (Kotzé *et al.*, 1997a:1197).

It is possible to prepare different TMC polymers with varying degrees of quaternisation by repeating the step of reductive methylation of chitosan several times under duplicate conditions (Hamman & Kotzé, 2001:374). By repeating the number of reaction steps it is possible to proportionately increase the degree of quaternisation (Hamman & Kotzé, 2001:379).

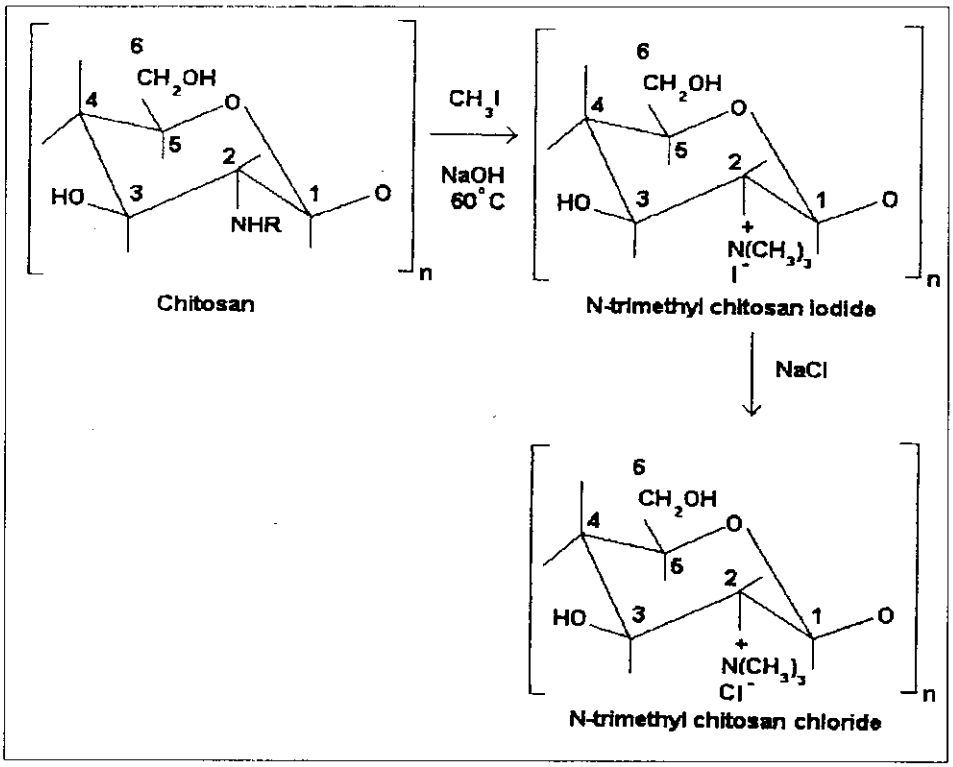


Figure 3.8: Synthesis of *N*-trimethyl chitosan chloride from chitosan by means of reductive methylation (Kotzé *et al.*, 1997a:1197).

Total quaternisation of chitosan is complicated due to the presence of a few acetyl groups on adjoining quaternary amino groups. Additional proof of this occurrence can be found in ^{1}H-

NMR spectra which indicate that a high percentage of the amino groups are still only mono- or dimethylated and can still be protonated in an acidic environment. An increase in basicity has been observed with an increase in the degree of quaternisation and then it becomes soluble at all pH values. This occurs even when the degree of quaternisation is as low as 12 percent (Sieval *et al.*, 1998:158).

3.9.2 Mechanism of action of TMC

TMC has a mechanism of action similar to that of chitosan and the rest of its derivatives. Chitosan's mechanism of action relies primarily on the interaction between the positively charged amino groups on the C-2 position with the negatively charged sites on the cell membranes and tight junctions of target organs in order to promote the opening of these tight junctions (Kotzé *et al.*, 1997a:251).

This interaction can be inhibited by heparin, indicating that the positive charge is essential for the site-binding properties of chitosan. TMC, at all degrees of quaternisation, has positive charges, regardless of the pH value of its surrounding environment (Van der Merwe *et al.*, 2004:232). Confocal laser scanning microscopy (CLSM) was employed to confirm the opening of the tight junctions by TMC in order to allow increased penetration of hydrophilic compounds (Thanou *et al.*, 2001b:122).

3.9.3 Mucoadhesive properties of TMC

TMC polymers posses mucoadhesive properties which can be of great value in order to increase the contact period of the active compound with the epithelium. This increased contact period ensures that greater quantities of the active compound can be absorbed via the paracellular pathway through the tight junctions of the cell membrane (Snyman *et al.*, 2002:145).

The molecular weight of TMC is greatly influenced by the synthesis procedure employed in its production. The molecular weight of TMC polymers have a significant effect on their mucoadhesive properties, up to a value of 100 000 g/mol. Beyond this value there is no visible effect (Snyman *et al.*, 2002:145).

The mucoadhesive properties of TMC are further influenced by the degree of quaternisation. The mucoadhesive properties of TMC decrease proportionally with an increase in the degree of quaternisation. Chitosan salts are superior to TMC polymers as far as mucoadhesion is

concerned. This can be explained by the change in the conformation of the polymers due to interactions between the fixed positive charges on the C-2 position of each polymer. This change in the conformation of the polymer may decrease the flexibility of the molecule which in turn would reduce the interpenetration into the mucus layer and subsequently lead to a decrease in mucoadhesivity (Snyman *et al.*, 2003:67).

3.9.4 The effect of TMC on the transepithelial electrical resistance (TEER) of intestinal epithelial cells (CACO-2)

The measurement of TEER gives a good indication of how narrow the tight junction is between cells and has been used in previous studies to predict the effective paracellular transport of hydrophilic compounds (Kotzé *et al.*, 1998b:149). The effects of two types of TMC polymers on the TEER of Caco-2 cell monolayers were measured by Kotzé *et al.* (1999a:272). The results clearly indicated that both polymers were able to decrease the TEER of the Caco-2 monolayers. Incubation on the apical side of the monolayers with concentrations of the polymers ranging between 1.5 and 2.5% w/v resulted in an obvious decrease in TEER values in comparison with the control group. Polymers with concentrations ranging between 2.0 and 2.5% w/v caused an instantaneous reduction in TEER. This seems to suggest that the reduction of TEER can be considered to be concentration dependent and it is further confirmed by the fact that the greatest reduction in TEER was measured at the highest concentration, namely 2.5% w/v (Kotzé *et al.*, 1999a:272).

Two chitosan salts, namely chitosan hydrochloride and chitosan glutamate, were compared to a TMC polymer with a 12.8% degree of quaternisation and the results revealed that all three compounds were able to reduce the TEER significantly. The decrease in TEER at a concentration of 0.25% w/v, 20 minutes after incubation, was in the following order: Chitosan hydrochloride (71 ± 4% reduction) > chitosan glutamate (56 ± 1% reduction) > TMC polymer (28 ± 1% reduction).

These results indicate that the chitosan salts were much more effective than the TMC polymer at similar weight concentrations (Kotzé *et al.*, 1998:41). Solutions with concentrations greater than 1.5% w/v of the chitosan salts could not be investigated due to its low solubility and high viscocity at a pH value in the region of 6.5. TMC on the otherhand dissolves readily at this pH value in much higher concentrations which consequently caused a furter decrease in TEER values. The difference in effect of these polymers could be

explained in terms of the equivalent weights of each repeating unit in the polymer backone of the respective polymers namely:

- Chitosan hydrochloride - 197.62
- Chitosan glutamate - 308.20
- TMC - 239.80

This information makes it possible to determine the density of the amino groups available for protonation at similair weight concentrations. According to these values, it can be stated that about 50% of the total weight of chitosan glutamate consist of glutamate salt whereas for chitosan hydrochloride the salt part only accounts for about 5 - 10% of the total weight (Kotzé *et al.*, 1998:41).

The slower rate of TEER reduction by TMC can also possibly be attributed to the methyl groups on the C-2 position which may cause steric effects and also partially hide the positive charge on the quaternary amino groups which in turn alters the time needed for interaction with the negatively charged cell membranes and tight junctions. Despite all these negative attributes, it is still possible to achieve similar effects on TEER with TMC polymers, simply by increasing the concentration to between 2 - 2.5% w/v. TMC's better solubility may also compensate, to a lesser extent, for its lower effect at similar weight concentrations as opposed to chitosan salts (Kotzé *et al.*, 1998:41).

3.9.5 Physicochemical properties of TMC

At basic and neutral pH values TMC displayed better solubility properties in comparison to chitosan or chitosan salts (Kotzé *et al.*, 1997b:244). TMC polymers with degrees of quaternisation ranging from 10.37 to 13.06% had pK_a values of 5.9 to 6.0 respectively and a 10% degree of quaternisation was sufficient to ensure good water solubility (Kotzé *et al.*, 1997a:1199).

TMC has a markedly decreased intrinsic viscocity in comparison with chitosan, which indicate polymer degradation under reaction conditions in an alkaline medium. During the synthesis of TMC, the number of positive charges on the polymer chain is increased, this increase causes the molecule to expand in solution due to repelling forces between the functional groups (Snyman *et al.*, 2002:145).

Based on these findings it can be stated that TMC is a chitosan derivative with superior solubility and basicity properties, 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 can contribute to its increased solubility and basicity (Kotzé *et al.*, 1998a:39). The degree of quaternisation plays an important role in the absorption enhancing activity of TMC, especially in neutral environments (Kotzé *et al.*, 1999a:273).

3.9.6 The effect of TMC on absorption enhancement of peptide drugs

[^{14}C]-mannitol- and [^{14}C]-polyethelene glycol are metabolically inert substances which are mostly hydrophilic in nature. Both of these compounds are mainly absorbed through the alternative aqueous paracellular pathways (tight junctions) which promote these substances as a perfect choice for studies which investigate the changes in permeability in paracellular absorption enhancement (Borchard *et al.*, 1996:131).

The effects of TMC, with a degree of quaternisation of 12.28%, chitosan hydrochloride and chitosan glutamate on the permeability of Caco-2 cells at pH 6.20 for the hydrophylic marker [^{14}C]-mannitol are shown in table 3.5 (page 53).

Exposure of the apical side of the monolayers to 0.25% w/v of the polymers resulted in a 34-fold (chitosan hydrochloride), 25-fold (chitosan glutamate) and 11-fold (TMC) increase in the rate of absorption of [^{14}C]-mannitol in comparison to the control group as indicated by the P_{app} values and absorption enhancement ratios (R). Very little change in the rate of absorption was detected when the concentration of the respective polymers were raised to 1.5% w/v. The [^{14}C]-polyethelene glycol rendered results similar to that of [^{14}C]-mannitol (Kotzé *et al.*, 1998:41).

Table 3.5: The effect of TMC, chitosan glutamate and chitosan hydrochloride on the permeability of [^{14}C]-mannitol at a pH of 6.20 (Kotzé, 1998:41).

Marker	Concentration (% w/v)	TMC $P_{app}x10^{-7}$ (cm/s)[a]	R	Chitosan glutamate $P_{app}x10^{-7}$ (cm/s)[a]	R	Chitosan hydrochloride $P_{app}x10^{-7}$ (cm/s)[a]	R
[^{14}C]-mannitol	Control 0.25	0.72 ± 0.08 8.11 ± 0.21[b]	1 11	0.72 ± 0.08 18.25 ± 1.10[b]	1 25	0.72 ± 0.08 24.62 ± 2.13[b]	1 34
	0.50	9.26 ± 0.35[b]	13	14.17 ± 0.45[bc]	20	23.28 ± 1.00[b]	32
	1.00	14.00 ± 0.40[b]	19	20.82 ± 0.30[b]	29	25.56 ± 2.95[b]	36
	1.50	7.52 ± 0.86[b]	10	18.29 ± 1.53[b]	25	26.16 ± 1.86	36
	2.00	12.35 ± 0.43[b]	17	n.d.	n.d.	n.d.	n.d.
	2.50	15.21 ± 1.37[b]	21	n.d.	n.d.	n.d.	n.d.

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

b – Significantly different from control ($p < 0.05$)

c – Significantly different from all other treatments in group ($p < 0.05$)

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

From these results it is evident that TMC is not as effective at similar weight concentrations as chitosan hydrochloride or chitosan glutamate. These results are very similar to those obtained in the TEER studies, discussed earlier in this section, which led the authors to conclude that additional factors play a role in the absorption enhancement mechanism of TMC. This occurrence could most possibly be explained by a combination of the following factors:

- different charge densities;
- the equivalent weight of each repeating unit in the polymer backbone;
- steric effects of the attached methyl groups and
- partial hiding of the positive charge on the quaternary amino groups (Kotzé *et al.*, 1998:43).

In another experiment it was demonstrated that TMC, with a degree of quaternisation of 12%, was able to transport large hydrophilic compounds across Caco-2 cell monolayers. The transport of fluorescein isothiocyanate-labelled dextran (FD-4) (Mw = 4 400 Da) was increased 167, 274 and 373 fold with TMC concentrations of 1.5, 2.0 and 2.5% (w/v) respectively (Kotzé *et al.*, 1997b:1197).

In another study the nasal administration of insulin with chitosan glutamate led to a significant reduction of blood glucose levels in both rats and sheep. A 7-fold increase in the peak plasma insulin levels was observed in the sheep model with the addition of TMC to the nasal insulin formulation (Illum, 2002:1187).

3.9.7 The effect of the degree of quaternisation on the absorption enhancing capability of TMC

The degree of quaternisation of TMC plays a critical role in determining its ability to open the tight junctions in order to increase permeation through the paracellular pathways. The density and amount of positive charges on the C-2 position of TMC is determined by its degree of quaternisation (Kotzé *et al.*, 1999a:274). TMC molecules which are highly quaternised have proven to be more effective than TMC polymers with a lower degree of quaternisation. This can be directly linked to the fact that the highly quaternised TMC molecules causes a greater decrease in the TEER values and an increase in the permeation of hydrophilic drugs (Kotzé *et al.*, 1999b:256).

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

Various studies indicated that the absorption of [^{14}C]-mannitol across Caco-2 cell monolayers increased proportionately with an increase in quaternisation. This would suggest that TMC polymers with a higher degree of quaternisation is more effective for absorption enhancement via the paracellular pathway (Hamman *et al.*, 2002:240). Beyond a 48% degree of quaternisation, there seemed to be no significant increase in the absorption which would suggest that the absorption enhancing effect of TMC is at its best at a 48% degree of quaternisation. This can possibly be attributed to steric effects caused by the methyl groups and changes in the flexibility of TMC polymers with an increase in the degree of quaternisation above the optimum value of 48% (Hamman *et al.*, 2002:241).

3.9.8 The effect of molecular weight on the absorption enhancing properties of TMC

Chitosan polymers with varying degrees of deacetylation and different molecular weights have been investigated for their effect on permeability in intestinal Caco-2 cell monolayers. It has been observed that both the degree of deacetylation and the molecular weight of the polymer play a major role in the absorption enhancing properties and toxicity of that specific polymer (Schipper *et al.*, 1996:1689).

Polymers with a low molecular weight, in the region of 22 000 g/mole, and a low degree of deacetylation lacked absorption enhancing activity, whereas polymers with a high molecular weight, varying between 98 000 and 190 000 g/mole, and/or a high degree of deacetylation increased the epithelial permeability (Schipper *et al.*, 1996:1691).

In another study TMC polymers with varying molecular weights have also been tested for its ability to reduce TEER and to transport [^{14}C]-mannitol across Caco-2 cell monolayers. It was concluded that both the high and low molecular weight polymers was able to reduce the TEER values across Caco-2 monolayers but only the high molecular weight TMC polymer was able to increase the transport of [^{14}C]-mannitol across the Caco-2 cell monolayers (Swartz, 2002:80).

3.9.10 TMC toxicity studies

Various studies have been conducted to determine the effect of TMC on the viability of Caco-2 cell monolayers. The viability of Caco-2 cell monolayers were tested after the completion of transport experiments. The viability test was performed by incubating the cell monolayers apically with a 0.1% trypan blue solution in PBS (0.01 M phosphate-buffered solution, pH 7.4) for a period of 30 minutes, while the basolateral side was incubated in PBS only. The medium was removed from both sides after the 30 minute incuation period has elapsed and the cell monolayers were examined by light microscopy for the exclusion of the marker. Cells which did not contain the trypan blue marker were considered to be viable. After the prolonged incubation with TMC polymers, there was still no visible uptake of trypan blue which implies that the Caco-2 cell monolayers remained undamaged and functionally intact, thus the viability was not affected by TMC (Kotzé *et al.*, 1997b:246).

The viability of Caco-2 cell monolayers were also tested by Thanou *et al.* (2000:18), after incubation with TMC polymers with degrees of quaternisation of 40 and 60% respectively. The viability was tested by both trypan blue and propidium iodide exclusion. The results revealed that the Caco-2 cell monolayers contained no trypan blue staining and only a very small fraction contained propidium iodide. These results indicate that TMC, even at relatively high concentrations of 1.0% w/v, have negligible cytotoxic effects (Thanou *et al.*, 2000:23).

The effect of TMC on the ciliary beat frequency of nasal epithelial cells has also been tested in order to investigate local toxicity. The tests revealed that TMC solutions with a concentration of 1% w/v had minimal influence on the ciliary beat frequency and it was also found that a TMC polymer with a low molecular weight inhibited ciliary beating to a lesser extent than its higher molecular weight counterpart (Jordaan, 2001:79).

3.10 CONCLUSION

The above sections gave a broad overview of chitosan and its quaternary derivative, namely TMC. The extraordinary ability of both chitosan and TMC to enhance the absorption of biologically active compounds has led to great interest in these polymers for the development of bioadhesive dosage forms and other drug delivery systems. The mechanism of action of both chitosan and TMC is based mainly on the opening of the tight junctions between epithelial cells to allow greater paracellular transport of peptide and protein drugs.

These absorption enhancing capabilities were confirmed in studies conducted on Caco-2 cell monolayers where a decrease in TEER was observed as well as an increase in the transport of hydrophilic compounds across these monolayers at neutral pH values. Similar results were obtained in *in vivo* studies. It was also found that the degree of quaternisation of TMC play an important role in its ability to increase the absorption of active compounds. Cytotoxic studies revealed that TMC is safe to use as an absorption enhancer for both peptide and protein drugs.

Based on these characteristics of TMC it would be fair to say that it has great potential as an absorption enhancer and further studies should be undertaken in order to explore its full potential.

CHAPTER 4

EXPERIMENTAL DESIGN AND FORMULATIONS

4.1 INTRODUCTION

Both quaternised chitosan (TMC) and Pheroid delivery systems are great candidates for use as absorption enhancers and mucoadhesive drug carriers. Both of these penetration enhancers have the potential to greatly enhance the absorption of peptide and protein drugs, such as recombinant human growth hormone (rhGH), with the added bonus of minimal cytotoxicity. This chapter describes the experimental procedures performed on male Sprague Dawley rats to evaluate the nasal absorption of rhGH after co-administration with both of these absorption enhancers.

4.2 STUDY DESIGN AND *IN VIVO* MODEL

4.2.1 Route of administration

The nasal route of administration offers many advantages in comparison to other routes of administration for the absorption of peptide and protein drugs. The nasal route is easily accessible, non-invasive, improves patient compliance and avoids first pass metabolism. Based on these reasons the nasal route was chosen as the route of choice for studying the absorption enhancing properties of both TMC and Pheroid technology when administering rhGH.

4.2.2 Experimental animals

Male Sprague Dawley rats with a body weight of between 250 g and 350 g were used as experimental models to study the absorption enhancing effects of TMC and Pheroid solutions. Sprague Dawley rats are readily available, breed successfully and quickly in captivity, and are easy to handle.

Direct administration into the nasal cavity, more precisely the left nostril, was chosen as the *in vivo* experimental procedure for the absorption studies. The experimental procedures are well documented in literature (Cheng *et al.*, 2005:5; Leitner *et al.*, 2004:91; Daugherty *et al.*, 1988:198). An application for the use of Sprague Dawley rats in this study was compiled and was approved by the Ethical Committee of the North-West University (05D18) (Annexure 1). In each experiment six rats were used to ensure that any significant statistical differences

would be detected between the control and experimental groups (Statistical Consultation Services, North-West University). Rats were fasted for a period of 12 hours prior to nasal drug administration but water was supplied *ad libitum.*

4.2.3 Breeding conditions

Male Sprague Dawley rats were bred and kept in a closed, controlled environment at the Animal Research Centre, North-West University in Potchefstroom. The closed and controlled conditions under which the animals were kept ensured an ideal growth environment with an absolute minimum exposure to pathogens which is accomplished by a constant air flow in the closed environment. All the possible variables in the Animal Research Centre were kept constant and the conditions are given in table 4.1 below. Rats were fed with Epol® mice cubes (Epol Pty (Ltd), Pretoria, RSA) which was provided by the Animal Research Centre of the North-West University.

Table 4.1: Conditions under which rats were kept in the closed environment

Condition	Recommended value*	Value in Animal Research Centre
Temperature	19 ± 2 C°	21 ± 2 C°
Relative humidity	55 ± 15%	55 ± 10%
Rate of ventilation / air movement	15 - 20 changes per minute	18 changes per minute
Light intensity	350 - 400 lux one meter above floor level	350 - 400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

*Values recommended according to international standards.

4.3 EXPERIMENTAL DESIGN

Six Sprague Dawley rats were used for each formulation, in accordance with previous literature (Cheng *et al.*, 2005:5; Leitner *et al.*, 2004:91; Daugherty *et al.*, 1988;198) to determine the extent of absorption of rhGH in each treatment group. The experimental design was a parallel design where the test animals were arranged in various test groups and a single treatment was administered to each individual in the different groups. A control group of six animals also received a single dose of rhGH/saline solution each in order to

determine the amount of absorption without any absorption enhancing additive. Nasal administration was performed at a dose of 100 µl/kg bodyweight.

4.3.1 Preparation of experimental formulations

4.3.1.1 Materials

The recombinant human growth hormone (rhGH, 1 mg = 3 IU) was supplied by Warren Chem Chemical Specialities (Midrand, RSA) after the necessary authorization was obtained from the Department of Health (Annexures 2 and 3). *N*-trimethyl chitosan chloride (TMC) was previously synthesised from Primex chitosan raw material with a DA of 97% and a molecular weight of 263 700 g/mole. Two different TMC polymers were used. TMC H-H (High molecular weight with high degree of quaternisation) with a 73.33% degree of quaternisation and a molecular weight of 262 400 g/mole and TMC H-L (High molecular weight with low degree of quaternisation) with a 18.20% degree of quaternisation and a molecular weight of 288 500 g/mole. The TMC polymers and Pheroid formulations were kindly provided by the Department of Pharmaceutics of the North-West University (Potchefstroom). Heparin Sodium 5 000 IU/ml was supplied by Intramed in South Africa. The Immunoradiometric assay (IRMA) kit for the quantitative measurement of human growth hormone was supplied by Biosource (South Africa).

4.3.1.2 Method

A subcutaneous dose of 0.6 IU/kg of rhGH was administered as a reference standard and for the control group and experimental groups a dose of 3.6 IU/kg of rhGH was administered via the nasal cavity. The different experimental formulations used are shown in table 4.2.

Table 4.2: Formulations used for the administration of recombinant human growth hormone.

Formulation	Route of administration	Dose administered
A: Reference Standard	Subcutaneous	0.6 IU/kg
B: Control	Nasal	3.6 IU/kg
C: Pheroid Vesicles	Nasal	3.6 IU/kg
D: Pheroid Microsponges	Nasal	3.6 IU/kg
E: TMC H-H	Nasal	3.6 IU/kg
F: TMC H-L	Nasal	3.6 IU/kg

• Formulation A

For the preparation of the subcutaneous reference, 6 mg of the freeze dried rhGH powder was weighed and dissolved in 10 ml physiological saline solution. This formulation was then shaken for 30 minutes in an IKA VIBRAX VXR basic at 700 MOT/min.

*** Formulation B**

For the control group rhGH in physiological saline solution was prepared. 6 mg of the rhGH powder was weighed and dissolved in 0.5 ml physiological saline. This solution was administered via the nasal cavity at a dose of 100 µl/kg bodyweight.

*** Formulation C**

6 mg of the rhGH powder was weighed and dissolved in 0.5 ml of Pheroid vesicles at 36 °C. The formulation was shaken for 30 minutes in an IKA VIBRAX VXR basic at 700 MOT/min. The particle size of the Pheroid vesicles, prior to entrapment of rhGH, was measured with a Malvern Mastersizer (Malvern Instruments, UK) and it was found that the average particle size was 2.19 µm. After the addition of the rhGH, the particle size increased to 3.01 µm. Two different formulations were prepared, one as described above (rhGH entrapped for 30 minutes) and another formulation which was prepared in the same way but was left to stand in a fridge at 5 °C for a further 24 hours before administration. The purpose of this was to allow further entrapment of rhGH into the Pheroid vesicles.

*** Formulation D**

6 mg of the rhGH powder was weighed and dissolved in 0.5 ml of Pheroid microsponges at 36 °C. The formulation was shaken for 30 minutes in an IKA VIBRAX VXR basic at 700 MOT/min. The particle size of the Pheroid microsponges, before entrapment of rhGH, was 3.32 µm. After the addition of the rhGH, the particle size increased to 4.33 µm. Two different formulations were prepared, one as described above (rhGH entrapped for 30 minutes) and another formulation which was prepared in the same way but was left to stand in a fridge at 5 °C for a further 24 hours before administration. The purpose of this was to allow further entrapment of rhGH into the Pheroid microsponges.

*** Formulation E**

6 mg of the rhGH powder was weighed and dissolved in 0.5 ml of a 0.5% w/v TMC H-H solution (high molecular weight with high degree of quaternisation). The formulation was shaken for 30 minutes to ensure even distribution.

*** Formulation F**

6 mg of the rhGH powder was weighed and dissolved in 0.5 ml of a 0.5% w/v TMC H-L solution (high molecular weight with low degree of quaternisation). The formulation was shaken for 30 minutes to ensure even distribution.

4.3.2 Induction of anaesthesia

For the induction of anaesthesia each rat was placed seperately in a closed glass container with a metal grid floor above a cottonwool layer containing liquid halothane. The concentration of the halothane was 4.0% v/v (provided by Fluothane®, Zebeca SA (Pty) Ltd, Woodmead, RSA). The rats were allowed to inhale the halothane vapours in the container and was then removed upon loss of conciousness. At no time was direct contact possible between the rat and the halothane containing cottonwool layer.

4.3.3 Maintenance of anaesthesia

For the maintenance of anaesthesia two mixtures of halothane and medical oxygen were used alternately as needed. The concentration of these mixtures was 2.0 and 4.0% respectively.

The apparatus for this procedure consisted of two 5 litre plastic bags containing the mixtures of halothane in medical oxygen. Each of these bags were connected to different ends of a three-way valve connector. The remaining end of the three-way valve connector was fitted with a latex rubber sheath which fitted snugly over the head of the rat. This arrangement allowed for easy alternation between the two halothane mixtures by simply rotating the valve. A small amount of soda-lime particles were placed in each plastic bag near the connecting valve to absorb the CO_2 which formed due to the expiration of the rat. Anaesthesia was maintained by connecting the rat to the 2.0% halothane bag, while the 4.0% halothane was used to deepen the anaesthesia when necessary. A constant body temperature of 37 °C was maintained by placing each rat on a small thermal electric blanket for the duration of the experiment. At the end of each experiment euthanasia was performed, before the rat regained conciousness, by deepening the anaesthesia with the 4.0% halothane until breathing ceased.

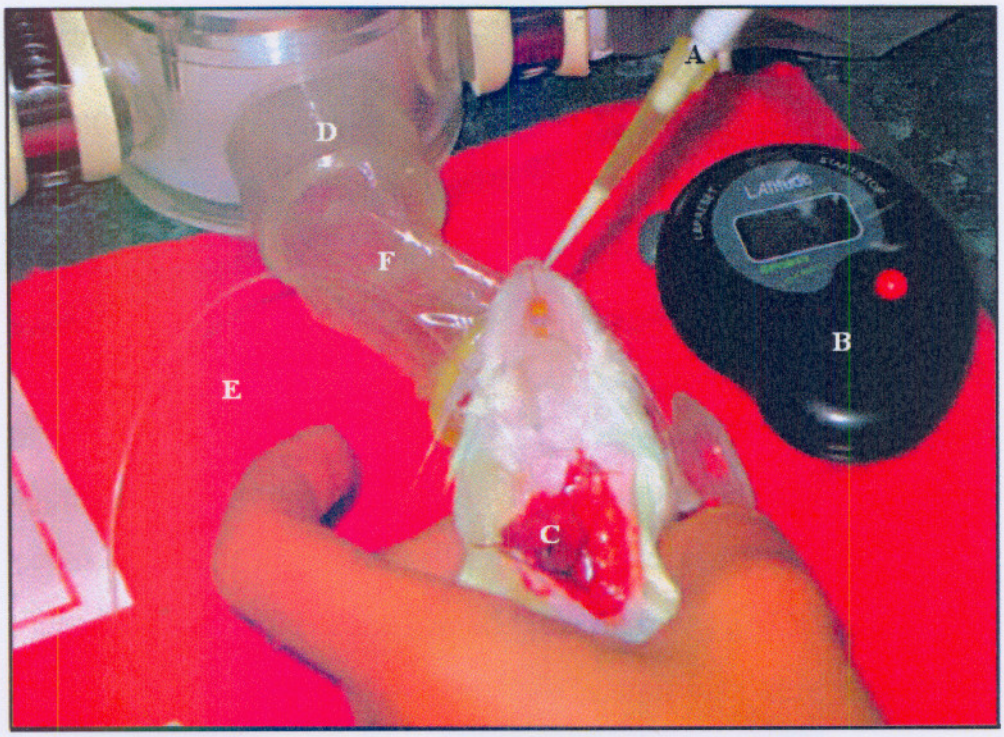
4.4 SURGICAL PROCEDURES

4.4.1 Cannulation of the artery *carotis communis*

All the rats used in the experiments were male Sprague Dawley rats that were fasted for a period of 12 hours prior to the experiments, with water available *ad libitum*. All surgical procedures were carried out while the rats were under anaesthesia, and were performed by a veterinary technician of the Animal Research Centre. The hair was shaved in the ventral neck area of the rat and the exposed skin disinfected. The rat was placed prostrate in a supine position with the head stretched in slight opistotonus. A mid ventral incision of 1 cm in length was made in the exposed neck skin. A blunt dissection was made between the two bilateral *M. stemohyoideus* to the area lateral to the trachea in order to expose the *a. carotis communis* beneath the *M. stemohyoideus*.

The *a. carotis communis* was isolated and lifted out of the operation wound and was kept wet with physiological saline at body temperature. The rostal part of the artery was ligated out with silk and tension was placed on the artery by taping the lose ends of the ligature in front of the rat. Another ligature was preplaced loosely at the caudal part of the artery. The artery was temporarily clamped with a mosquito artery clamp proximal to the loosely preplaced ligature.

A "V" incision was made with a pair of scissors between the two ligatures in the artery wall. A sterile PVC cannula (Fine Bore Polythene tubing, 0.58 mm ID (0.96 mm OD) REF 800/100/200/100, UK) which was filled with a saline-heparin solution at body temperature and connected to a syringe, was guided into the artery. A 5.0% heparin solution was used to avoid blood clotting in the cannula. The cannula was threaded so that ± 1 cm of the cannula was inserted into the artery and the clamp was then released. The loose ligature around the artery with the cannula inside was tied in order to secure the cannula and to prevent blood leakage from the artery. The wound was covered with gauze and wetted with physiological saline which was kept at body temperature to ensure that the artery stays supple.

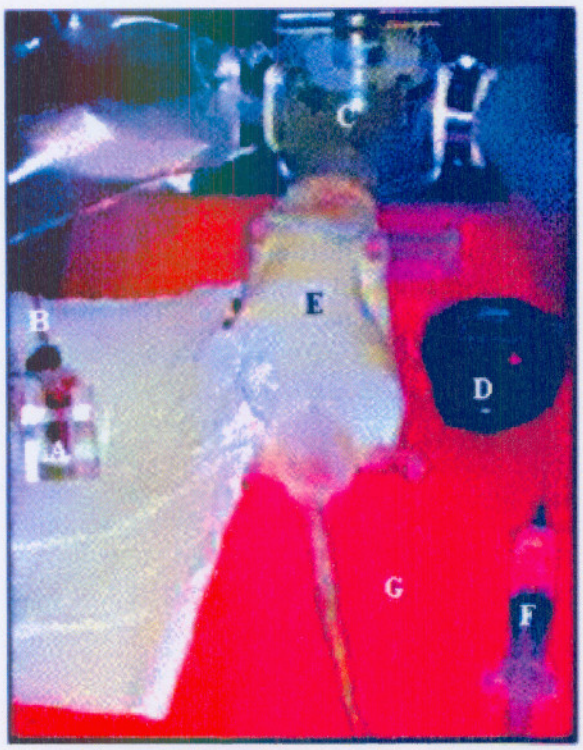


A – Micropipette with test formulation
B – Stopwatch
C – Cannulated *carotis communis*
D – 3-way valve
E – Thermal blanket
F – Rubber sheath

Figure 4.1: Cannulation of the *artery carotis communis*.

4.4.2 Administration of formulations

Between 95 and 105 µl (0.6 IU/kg rhGH) of the subcutaneous formulation was injected directly under the skin in the abdominal region of each rat in the subcutaneous group. Each rat received a single dose of "formulation A". The other formulations, B - F, was administered via the nasal cavity. A single dose of between 25 - 35 µl was administered via the left nostril of each test subject at a dose of 100 µl/kg bodyweight. The final concentration rhGH administered nasally to each rat was 3.6 IU/kg as determined by a simple calculation based on the bodyweight of the rat.



A – Sample holder
B – Cannula
C – 3-way valve
D – Stopwatch
E – Male Sprague Dawley rat
F – Syringe with physiological saline and heparin (5 IU/ml)
G – Thermal blanket

Figure 4.2: Nasal administration of rhGH.

4.4.3 Collection of blood samples

Blood samples with a volume of 800 µl were collected from the cannula in the *carotis communis* into Eppendorf® micro test tubes (Merck, RSA). Samples were collected at different time intevals, the 0 time sample was taken 1 minute prior to administration, thereafter samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes after administration. The collected samples were kept at a temperature of 0 °C with the help of a cold storage box filled with crushed ice. In total 8 samples were collected from each rat over a period of 3 hours. All samples were centrifuged (Eppendorf® centrifuge 5415C) within 20 minutes of collection at 7 000 rpm for 7 minutes. The recovered plasma samples were stored at -70 °C for not more than 3 weeks before all samples were analyzed.

4.5 ANALYSES OF PLASMA rhGH

For the analyses of the collected samples a hGH-IRMA kit was used which was obtained from BIOSOURCE™. This immunoradiometric assay kit can be usd for the *in vitro* quantitative measurement of human growth hormone (hGH) in both serum and plasma samples.

4.5.1 General information of the hGH-IRMA kit

- Proprietory name : BioSource hGH-IRMA
- Catalogue number : KIP1081 (96 tests)
- Manufactured by : BioSource Europe S.A., Rue de l'Industrie, 8, B-1400 Nivelles, Belgium

4.5.2 Principles of the method

This specific immunoradiometric assay is based on coated-tube seperation:

- Mab 1 The capture antibody is attached to both the lower and inner surfaces of the plastic tube. Calibrators or samples added to these tubes will at first show low affinity for Mab 1.
- Mab 2 The signal antibody labelled with ^{125}I. Addition of Mab 2 will complete the system and trigger the immunological reaction. Wash the coated tubes with the supplied wash solution. The remaining fraction of radioactivity, bound to the coated tube, reflects the antigen (rhGH) concentration.

4.5.3 Reagent preparation

- Calibrators Reconstitute zero calibrator with 2.0 ml distilled water.
  Reconstitute other calibrators with 0.5 ml distilled water.
- Controls Reconstitute the controls with 0.5 ml distilled water.
- Working wash solution Prepare an adequate volume of Working wash solution by adding 19 volumes of distilled water to 1 volume of wash solution.
  Homogenize with a magnetic stirrer.
  Discard unused Working wash solution at the end of the day.

4.5.4 Method

4.5.4.1 Handling notes

- Bring all the reagents to room temperature prior to use.
- Thoroughly mix all reagents and samples by means of gentle agitation or swirling.
- Avoid cross-contamination by using a clean disposable pipette tip for the addition of each reagent and sample.

- Adhere to the incubation times.
- Prepare a calibration curve for each run.

4.5.4.2 Procedure

- Label coated tubes in duplicate for
  - calibrator (C)
  - control (CON)
  - sample (S)
- Label 2 normal tubes for the determination of total counts (TC).
- Briefly vortex calibrators, controls and samples and then dispose 50 µl of ^{125}Iodine labelled anti-hGH into each tube, including those for total counts. Shake tube rack gently by hand.
- Incubate at room temperature for 2 hours.
- Decant or aspirate the content of each tube EXCEPT total counts. Remove all the liquid.
- Wash tubes with 3 ml working wash solution EXCEPT total counts. Avoid foaming when adding the wash solution.
- Decant or aspirate content of each tube EXCEPT total counts.
- Wash tubes again with 3 ml working wash solution EXCEPT total counts and decant or aspirate.
- Let the tubes stand upright for 2 minutes and aspirate the remaining drop of liquid.
- Count tubes in a gamma counter for 60 seconds (Auto-Gamma® 5 000 series, Gamma counting system, Packard).

4.6 RESULTS AND DISCUSSION

4.6.1 Introduction

Several studies have been carried out, as mentioned earlier in the text, to improve the absorption of rhGH following nasal administration. The results of these studies indicate that the nasal administration of peptide drugs, such as rhGH, may indeed be an excellent alternative compared to the parenteral route of administration commonly used for peptide drugs intended for a systemic therapeutic effect. The main objective of this study was to compare the absorption enhancing capabilities of TMC H-H, TMC H-L, Pheroid vesicles and Pheroid microsponges following nasal administration in male Sprague Dawley rats.

4.6.2 Subcutaneous administration of rhGH

To give an indication of the relative bioavailability of rhGH, a dose of 0.6 IU/kg was administered subcutaneously in the lower abdominal region. Table 4.3 gives the blood plasma concentrations (μIU/ml) of rhGH obtained after subcutaneous administration with formulation A measured over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. Figure 4.3 shows the resulting rhGH plasma concentration-time profile after subcutaneous injection.

Table 4.3: Plasma rhGH concentrations after subcutaneous administration of rhGH (0.6 IU/kg).

Subcutaneous administration of rhGH									
	Subject Number								
Time (min)	**1**	**2**	**3**	**4**	**5**	**6**	**Average (μIU/ml)**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.95	3.58	2.90	2.13	1.66	4.43	2.61	1.28	0.52
10	2.46	4.64	5.93	2.95	2.65	4.95	3.93	1.44	0.59
15	1.83	5.22	6.51	2.85	3.03	6.45	4.32	2.01	0.82
30	1.55	5.04	5.66	3.02	3.92	9.46	4.78	2.72	1.11
60	0.86	5.57	4.40	2.46	3.89	7.36	4.09	2.29	0.93
120	0.46	2.42	2.68	0.82	2.60	3.32	2.05	1.14	0.47
180	0.34	0.86	0.79	0.52	0.93	0.88	0.72	0.24	0.10

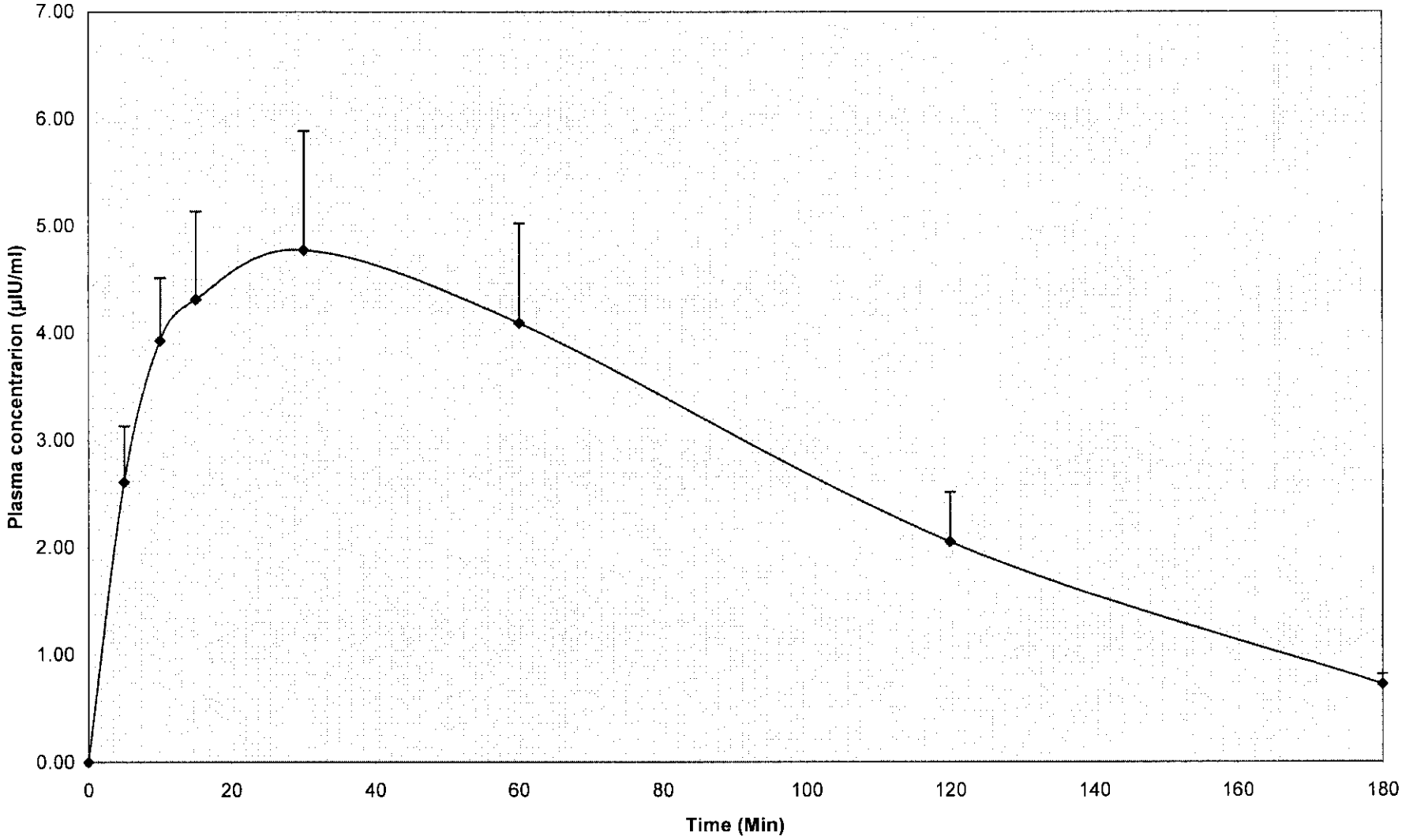


Figure 4.3: Plasma rhGH concentration after subcutaneous administration of rhGH (0.6 IU/kg).

As evident from table 4.3 and figure 4.3 there was no rhGH present in the bloodplasma at time t = 0 minutes. At time t = 5 minutes, 5 minutes after subcutaneous injection, the bloodplasma concentration of rhGH was 2.61 µIU/ml and further increased to 3.93 µIU/ml at time t = 10 minutes. At time t = 15 minutes the plasma concentration reached 4.32 µIU/ml and reached its maximum concentration, namely 4.78 µIU/ml, at time t = 30 minutes. At time t = 60 minutes a slight decrease is observed with a plasma concentration of 4.09 µIU/ml, the concentration continues to decrease with values of 2.05 and 0.72 µIU/ml at time t = 120 and t = 180 minutes respectively. These results are in agreement with the results obained in previous studies for the subcutaneous administration of rhGH.

4.6.3 Control

Table 4.4 contains the results obtained after the nasal administration of formulation B (rhGH/saline solution) over a period of 180 minutes. The blood plasma concentration of rhGH is given in micro-international units per milliliter (µIU/ml). The symbols SD and SE represent the standard deviation and standard error values respectively. These results are graphically depicted in figure 4.4.

Table 4.4: Plasma rhGH concentrations after nasal administration with formulation B (Control, 3.6 IU/kg rhGH).

Control									
	Subject number								
Time (min)	1	2	3	4	5	6	Average (µIU/ml)	SD	SE
0	0.13	0.00	0.12	0.00	0.13	0.00	0.06	0.07	0.03
5	0.00	0.00	0.13	0.00	0.00	0.00	0.02	0.05	0.02
10	0.13	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.02
15	0.00	0.15	0.22	0.22	0.00	0.13	0.12	0.10	0.04
30	0.18	0.00	0.28	0.00	0.00	0.00	0.08	0.12	0.05
60	0.18	0.14	0.11	0.20	0.00	0.00	0.11	0.09	0.04
120	0.26	0.28	0.14	0.22	0.00	0.00	0.15	0.13	0.05
180	0.16	0.28	0.25	0.24	0.12	0.00	0.18	0.10	0.04

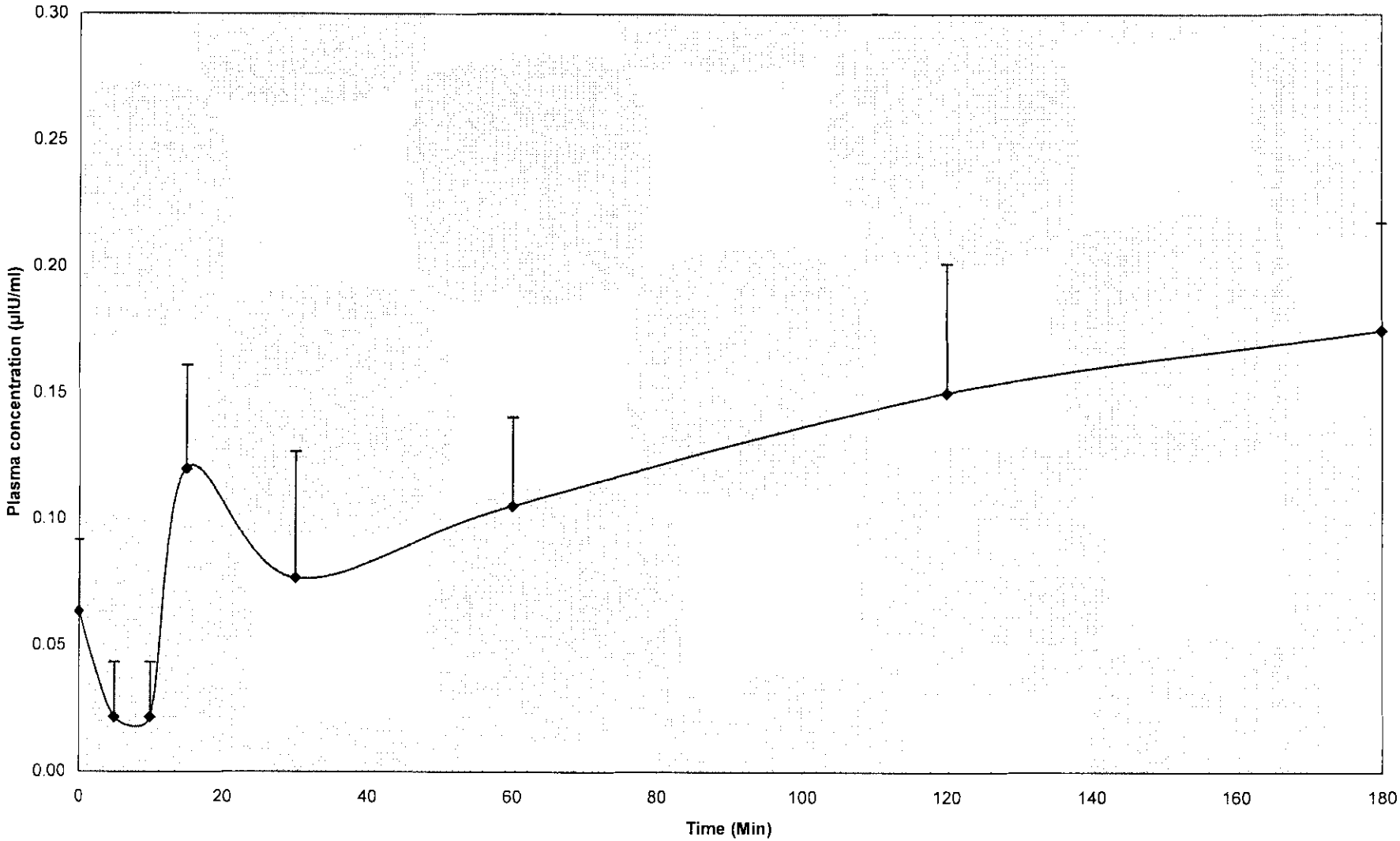


Figure 4.4: Plasma rhGH concentrations after nasal administration with formulation B (Control, 3.6 IU/kg rhGH).

It is evident from table 4.4 and figure 4.4 that there was virtually no absorption of rhGH in the control formulation. These results may be explained by looking at the structure, size and molecular weight of rhGH. The molecular weight of rhGH is 22 000 Da, corresponding to 191 amino acids, which suggest that larger peptide molecules, such as this, need to be administered in conjunction with a penetration enhancer in order to obtain acceptable therapeutic plasma levels of nasally administered rhGH.

4.6.4 Nasal administration of rhGH in Pheroid vesicles (entrapped for 30 minutes; experiment duration 180 minutes)

Table 4.5 gives the plasma rhGH concentrations obtained after the nasal administration of rhGH directly after entrapment in Pheroid vesicles (formulation C). The rhGH plasma concentrations are measured in µIU/ml over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. The obtained results are graphically depicted in figure 4.5 in the form of a concentration-time profile.

Table 4.5: Plasma rhGH concentrations after nasal administration of rhGH in Pheroid vesicles (3.6 IU/kg) entrapped for 30 minutes.

Nasal administration rhGH in Pheroid vesicles entrapped for 30 minutes							
	Subject Number						
Time (min)	1	2	3	4	Average (µIU/ml)	SD	SE
0	0.00	0.17	0.14	0.00	0.08	0.09	0.05
5	0.00	0.28	0.27	0.00	0.14	0.16	0.08
10	0.00	0.13	0.00	0.18	0.08	0.09	0.05
15	0.00	0.19	0.16	0.00	0.09	0.10	0.05
30	0.41	0.41	0.00	0.23	0.26	0.19	0.1
60	0.58	0.74	0.28	0.46	0.52	0.19	0.1
120	3.42	4.75	0.53	3.48	3.05	1.79	0.89
180	3.67	5.90	0.87	6.13	4.14	2.45	1.22

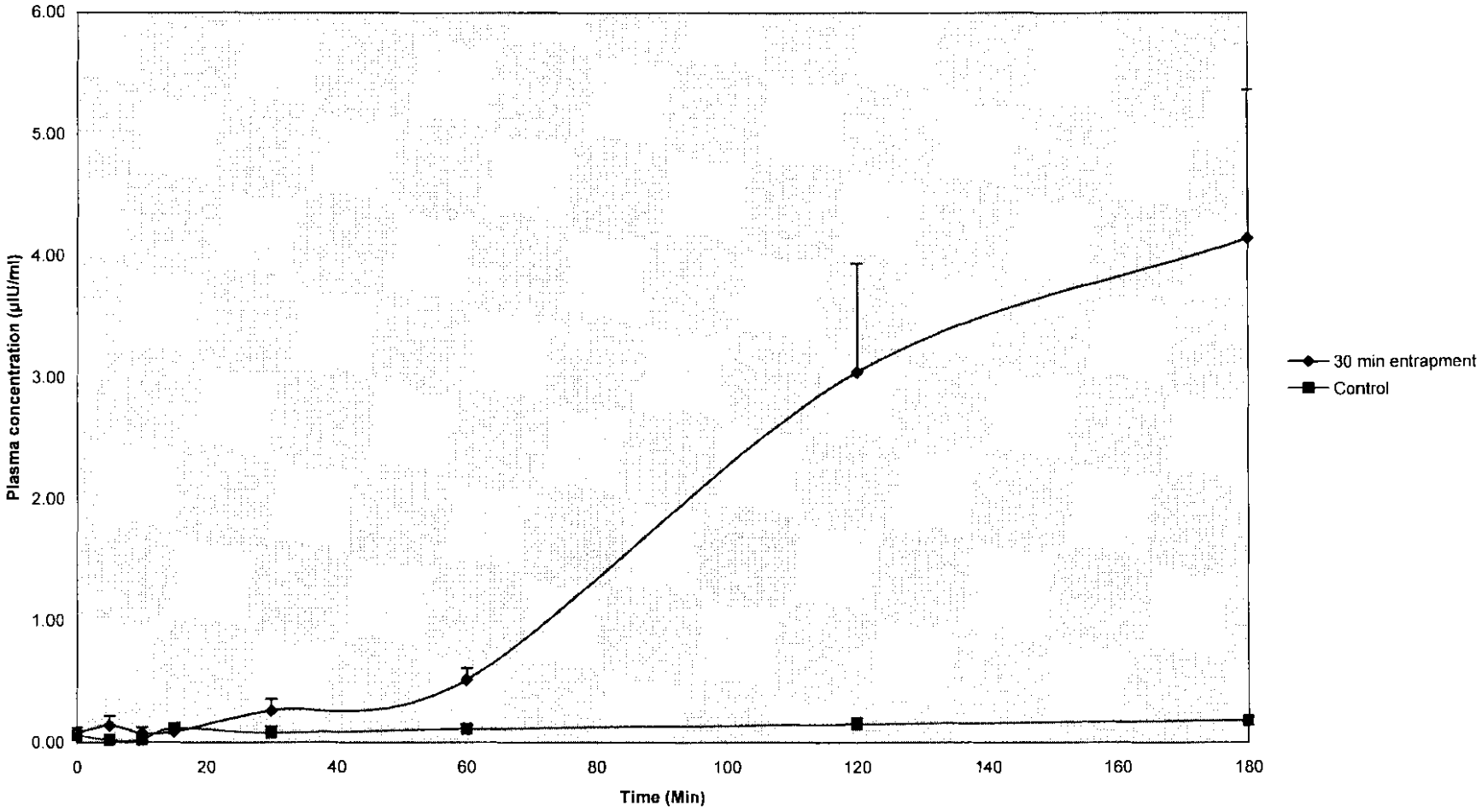


Figure 4.5: Plasma rhGH concentrations after nasal administration with Pheroid vesicles (rhGH 3,6 IU/kg; 30 min, experiment duration 180 min).

In contrast to the control formulation, the nasal administration of rhGH with Pheroid vesicles led to a significant increase in the absorption and plasma concentration of rhGH. At time t = 0 there was virtually no rhGH present in the plasma with an average value of only 0.08 µIU/ml. At time t = 5 minutes the concentration of rhGH was 0.14 µIU/ml and at time t = 10 minutes a value of 0.08 µIU/ml was recorded. This trend continued at time t = 15 minutes with a concentration of 0.09 µIU/ml. At time t = 30 minutes a gradual increase in concentration is observed with a rhGH concentration of 0.26 µIU/ml, this gradual rise in concentration continues to a concentration of 0.52 µIU/ml recorded at time t = 60 minutes. At time t = 120 minutes a significant rise is recorded with a concentration of 3.05 µIU/ml and a further rise to 4.14 µIU/ml at time t = 180 minutes.

It is clear that Pheroid vesicles are able to increase the absorption of nasally administered rhGH. The fact that the concentration of rhGH was still on the increase at time t = 180 minutes, when this experiment was stopped, indicated that this experiment must be conducted over a longer time period.

4.6.5 Nasal administration of rhGH in Pheroid vesicles (24 hours entrapment; experiment duration 180 minutes)

Table 4.6 gives the plasma rhGH concentrations obtained after the nasal administration of rhGH entrapped for 24 hours in Pheroid vesicles (formulation C). The rhGH plasma concentrations are measured in µIU/ml over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. The obtained results are graphically depicted in figure 4.6 in the form of a concentrationtime profile.

Table 4.6: Plasma rhGH concentrations after nasal administration of rhGH in Pheroid vesicles (24 hours entrapment; experiment duration 180 minutes).

Nasal administration of rhGH (24 hours entrapment; experiment duration 180 minutes)									
	Subject number								
Time (min)	1	2	3	4	5	6	Average (µIU/ml)	SD	SE
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.14	0.00	0.02	0.06	0.02
15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.53	0.16	0.82	0.22	0.23	0.18	0.36	0.26	0.11
60	4.09	0.25	5.70	0.71	1.94	1.45	2.36	2.11	0.86
120	8.68	0.53	4.60	0.71	2.18	3.80	3.42	3.05	1.24
180	N/D	0.58	3.64	1.74	2.45	2.32	2.15	1.11	0.50

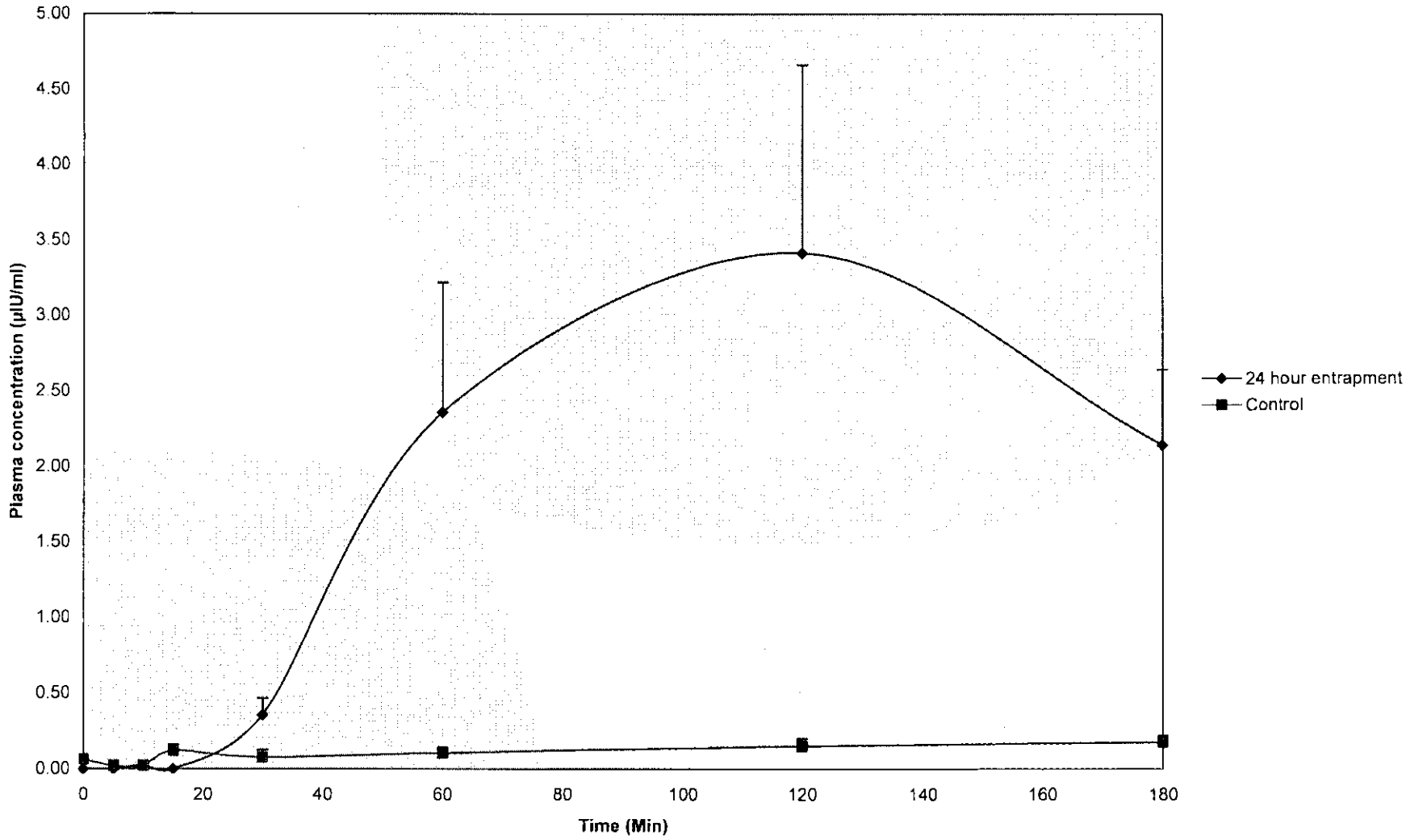


Figure 4.6: Plasma rhGH concentrations after nasal administration with Pheroid vesicles (rhGH 3,6 IU/kg; 24 hour entrapment, experiment duration 180 min).

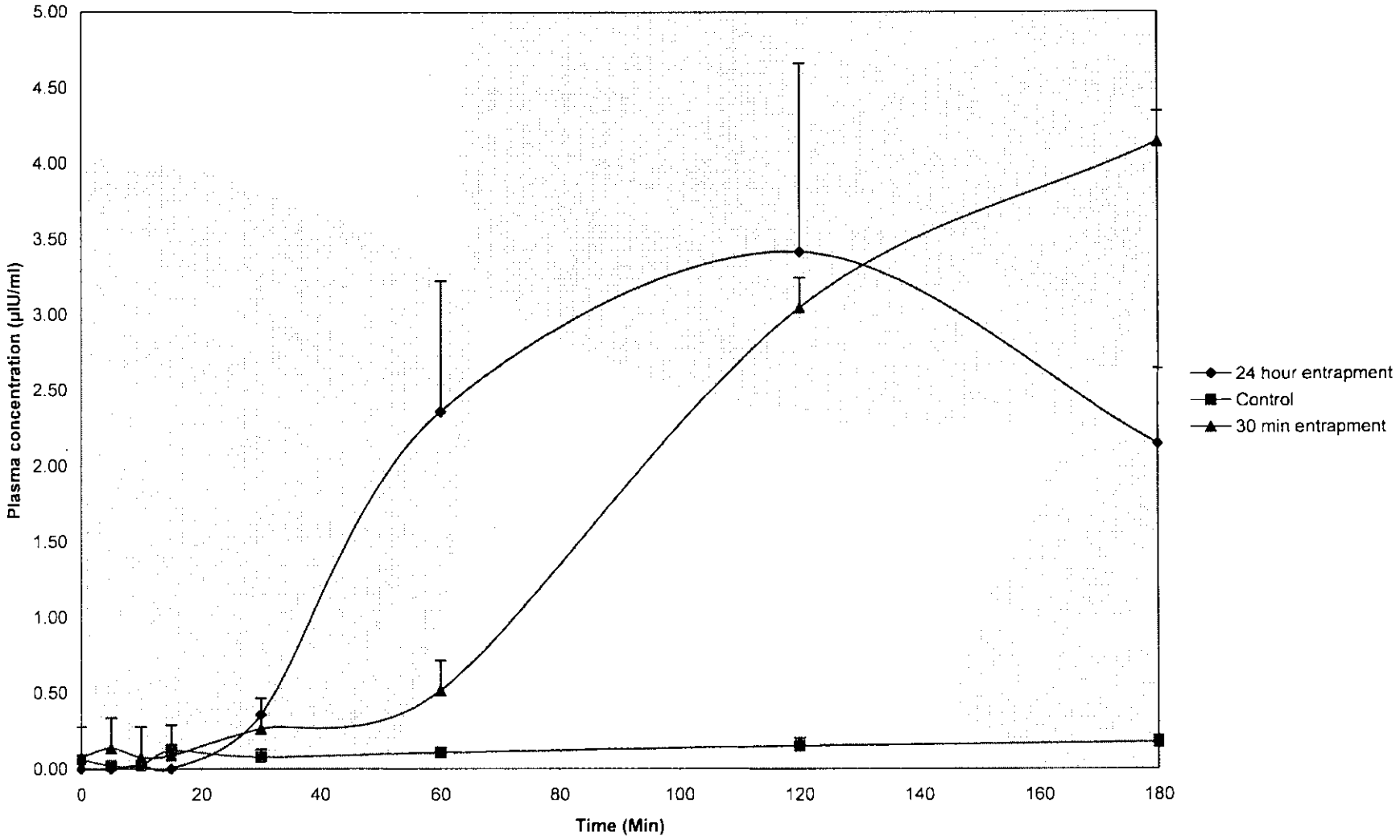


Figure 4.7: Comparison between plasma rhGH concentrations after nasal administration with Pheroid vesicles (rhGH 3,6 IU/kg; 30 min and 24 hour entrapment respectively, experiment duration 180 min).

In contrast to the control formulation, the nasal administration of rhGH, 24 hours after entrapment in Pheroid vesicles, rendered much greater plasma concentrations. At time t = 0 to time t = 15 minutes, there was virtually no rhGH present in the plasma. At time t = 30 minutes a plasma concentration of 0.36 µIU/ml was recorded and at time t = 60 minutes the concentration increased to 2.36 µIU/ml. The maximum concentration was measured at time t = 120 minutes with a value of 3.42 µIU/ml. The last value recorded was 2.15 µIU/ml at time t = 180 minutes.

Again it is very obvious that the rhGH entrapped in Pheroid vesicles are much better absorbed in comparison to the control group. When comparing the administration of rhGH entrapped for 30 minutes in Pheroid vesicles to the rhGH/Pheroid vesicles administered 24 hours after entrapment it is interesting to note that the 24 hour formulation reaches its peak value, namely 3.42 µIU/ml, at time t = 120 minutes while the 30 minutes entrapped formulation's concentration values are still on the increase at time t = 180 minutes.

4.6.6 Nasal administration of rhGH in Pheroid vesicles (24 hours entrapment; experiment duration 5 hours)

Based on the results of the previous Pheroid experiments, this experiment was conducted using the same of rhGH/Pheroid formulation (3.6 IU/kg rhGH) and administered 24 hours after entrapment but blood samples were collected over a 5 hour period instead of the usual 3 hour period.

Table 4.7 gives the rhGH concentrations obtained after administration. Blood samples were taken at 1 hour intervals, namely 1, 2, 3, 4 and 5 hours respectively after administration. The concentration of rhGH in each sample is measured in µIU/ml and the symbols SD and SE represent the standard deviation and standard error values respectively. The results are graphically depicted in figure 4.8.

Table 4.7: Plasma rhGH concentrations after nasal administration of rhGH in Pheroid vesicles (24 hours entrapment; experiment duration 5 hours).

Administration of Pheroid/rhGH vesicles (24 hours entrapment; experiment duration 5 hours)								
	Subject Number							
Time (hours)	1	2	3	4	5	Average (µIU/ml)	SD	SE
0	0.53	0.35	0.29	0.00	0.39	0.31	0.20	0.87
1	0.51	0.63	2.51	N/D	1.78	1.36	0.96	0.5
2	1.79	1.83	6.54	11.37	N/D	5.38	4.57	2.3
3	26.26	43.23	37.65	4.44	N/D	27.90	17.16	8.6
4	N/D	22.06	32.85	42.34	8.20	26.36	14.67	7.3
5	3.16	8.98	N/D	2.56	2.76	4.37	3.09	1.5

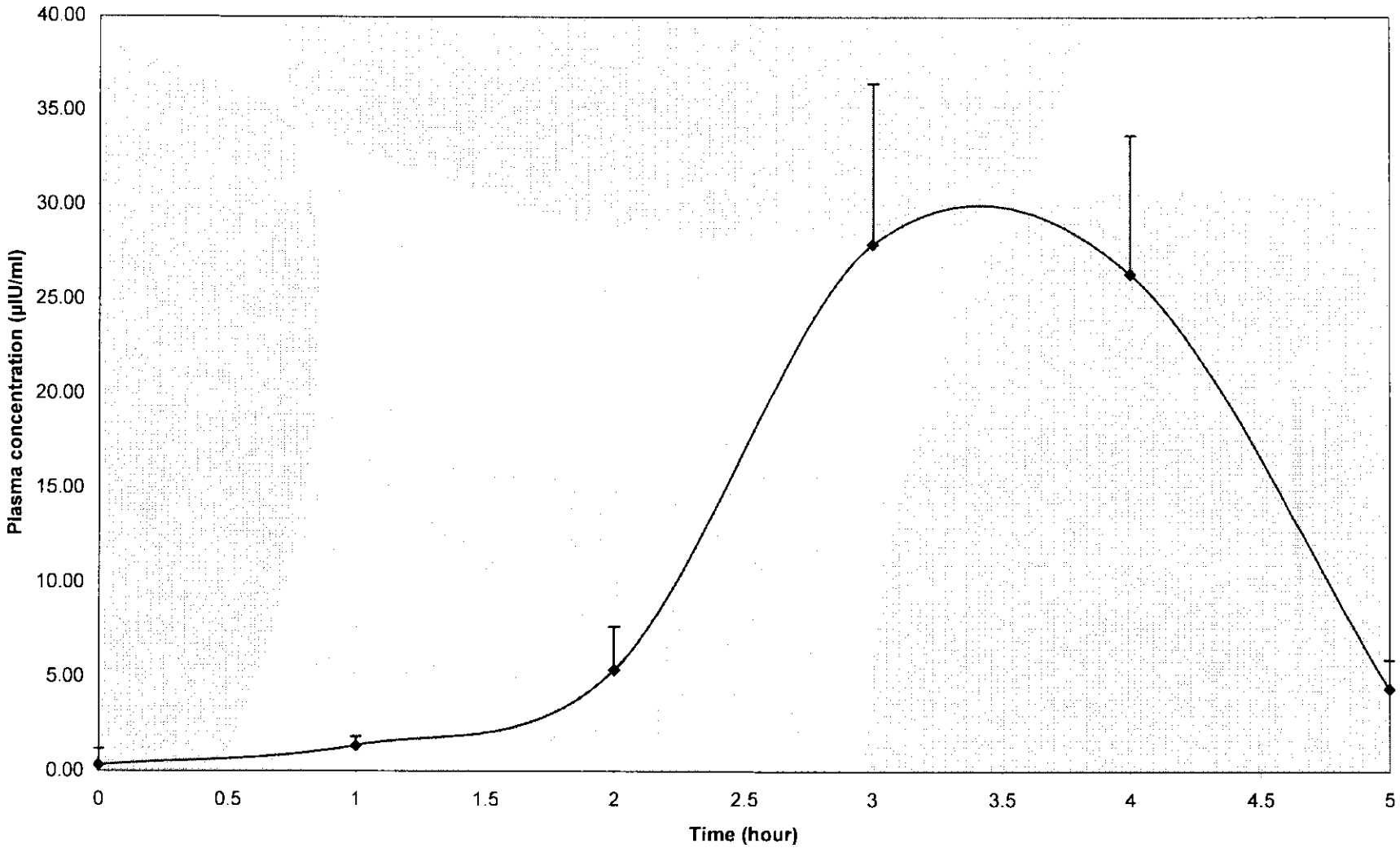


Figure 4.8: Plasma rhGH concentrations after nasal administration of rhGH in Pheroid vesicles (24 hours entrapment, experiment duration 5 hours).

This experiment rendered higher plasma concentrations of rhGH in comparison to the previous experiment with the same formulation, the only difference was that this experiment was conducted over a 5 hour period and subsequently 6 blood samples were collected from each subject instead of the usual 8 samples.

At time t = 0 an average value of 0.31 µIU/ml was recorded and at time t = 1 hour a plasma concentration of 1.36 µIU/ml was measured. The concentration of rhGH continue to rise with a value of 5.38 µIU/ml at time t = 2 hours which is followed with a tremendous increase at time t = 3 hours with a recorded value of 27.90 µIU/ml. The concentration stays fairly constant for the next hour with a recorded value of 26.36 µIU/ml measured at time t = 4 hours. At 5 hours the concentration decreased dramatically with a value of only 4.37 µIU/ml. It is interesting to compare the obtained average maximum concentration values of the 3 hour and 5 hour experiments when considering that the same formulation was used in both experiments. It is unclear what the reason for this major difference in plasma levels, 3 hours after administration, could be. The possibility exist that the short time span between blood sampling in the 3 hour study group may have an impact on this occurrence.

4.6.7 Nasal administration of rhGH with Pheroid microsponges (entrapped for 30 minutes)

Table 4.8 gives the plasma rhGH concentrations obtained after the nasal administration of rhGH entrapped in a Pheroid microsponge solution for 30 minutes. The rhGH plasma concentrations are measured in µIU/ml over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. The obtained results are graphically depicted in figure 4.9 in the form of a concentration-time profile.

Table 4.8: Plasma rhGH concentrations after nasal administration with Pheroid microsponges (rhGH 3.6 IU/kg, entrapped for 30 minutes).

Pheroid microsponges (rhGH entrapped for 30 minutes)								
	Subject number							
Time (min)	1	2	3	4	5	Average (µIU/ml)	SD	SE
0	0.00	0.00	0.00	0.12	0.00	0.02	0.05	0.02
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.54	0.00	0.00	0.11	0.24	0.11
15	0.00	0.00	0.14	0.18	0.00	0.06	0.09	0.04
30	0.00	0.00	0.00	0.20	0.18	0.08	0.10	0.05
60	0.32	0.19	0.21	0.23	0.00	0.19	0.12	0.05
120	0.63	0.72	0.13	N/D	0.15	0.41	0.31	0.16
180	0.73	N/D	0.25	1.62	0.13	0.68	0.68	0.34

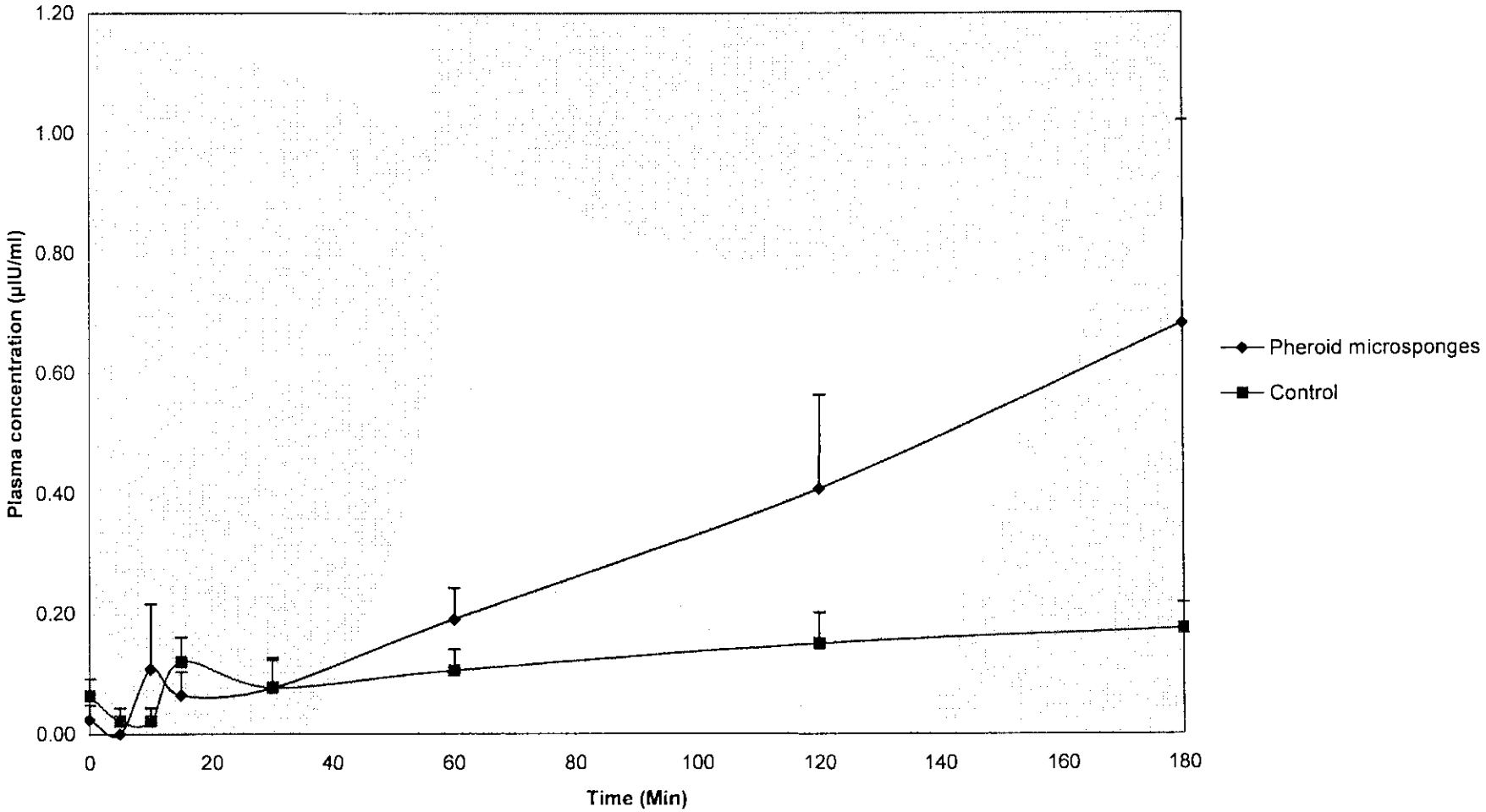


Figure 4.9: Plasma rhGH concentrations after nasal administration with Pheroid microsponges (rhGH 3.6 IU/kg, 30 min entrapment).

In contrast to the control formulation, the nasal administration of rhGH with Pheroid microsponges did show a slight increase in plasma concentrations of rhGH. From time t = 0 tot t = 30 minutes the rhGH concentration in the plasma was very low with values in the range 0.00 - 0.11 µIU/ml. At time t = 60 minutes a small, yet very definite, increase in concentration is observed to a value of 0.19 µIU/ml and the concentration continues to increase and at time t = 120 minutes a value of 0.41 µIU/ml is recorded. The last recorded concentration is 0.68 µIU/ml at time t = 180 minutes which indicate that the concentration is still on the increase at that time. These findings suggest that in order to obtain a more complete concentration time profile, the experiment must be conducted over a longer period of time instead of the usual 180 minutes.

4.6.8 Nasal administration of rhGH with Pheroid microsponges (24 hours entrapment; experiment duration 5 hours)

Based on the results of the previous Pheroid microsponge experiment, this experiment was conducted using the same rhGH/Pheroid microsponge formulation (rhGH 3.6 IU/kg). This formulation was administered 24 hours after entrapment and blood samples were collected over a 5 hour period.

Table 4.9 gives the rhGH plasma concentrations obtained after administration. Blood samples were taken at 1 hour intervals, namely 1, 2, 3, 4 and 5 hours respectively after administration. The concentration of rhGH is measured in µIU/ml and the symbols SD and SE represent the standard deviation and standard error values respectively. The results are graphically depicted in figure 4.10.

Table 4.9: Plasma rhGH concentrations after nasal administration with Pheroid microsponges (24 hours entrapment; experiment duration 5 hours).

Pheroid microsponge (rhGH entrapped for 24 hours; experiment duration 5 hours)								
	Subject Number							
Time (hours)	1	2	3	4	5	Average (µIU/ml)	SD	SE
0	0.08	0.52	0.38	0.13	0.53	0.33	0.21	0.10
1	1.28	2.71	1.83	3.18	0.74	1.95	1.00	0.45
2	2.93	3.67	5.77	1.78	2.1	3.25	1.59	0.71
3	4.3	5.39	3.66	2.32	4.31	4	1.12	0.50
4	2.38	4.38	2.15	N/D	N/D	2.97	1.23	0.71
5	1.64	3.21	1.97	N/D	N/D	2.27	0.83	0.48

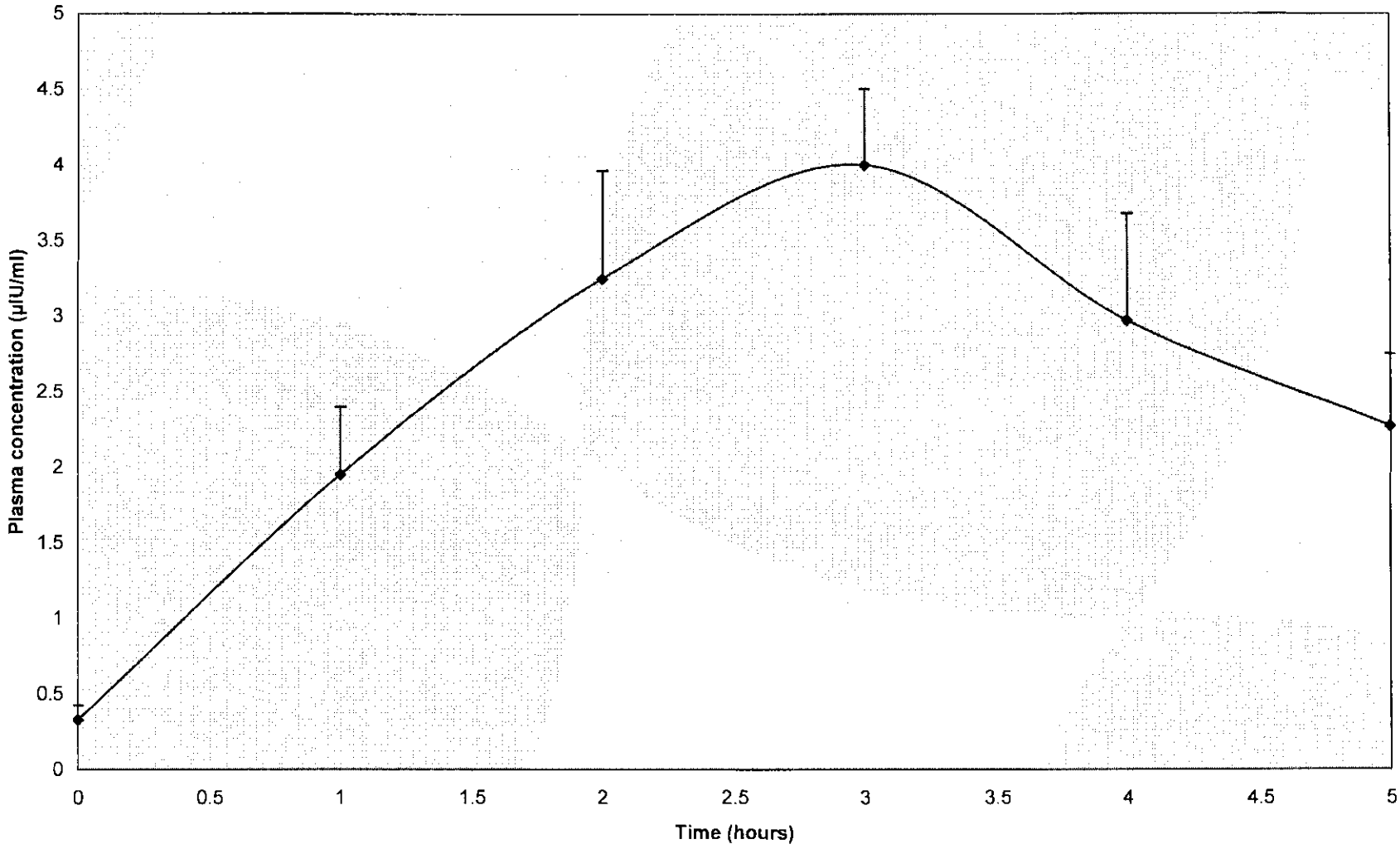


Figure 4.10: Plasma rhGH concentrations after nasal administration with Pheroid microsponges (24 hours entrapment; experiment duration 5 hours).

This 5 hour experiment rendered higher plasma concentrations of rhGH in comparison to the previous 3 hour experiment with the same formulation. At time t = 0 an average value of 0.33 µIU/ml was recorded and at time t = 1 hour a concentration of 1.95 µIU/ml was measured. The concentration continue to rise with a value of 3.25 µIU/ml at time t = 2 hours which is followed with a slight increase to 4.00 µIU/ml at time t = 3 hours. The concentration decrease to 2.97 µIU/ml at time t = 4 hours and falls even further to 1.36 µIU/ml at the final reading where the elapsed time is represented by t = 5 hours. It is very obvious that the Pheroid microsponge formulation did render higher plasma concentrations than the control group, the highest recorded average concentration of 4.00 µIU/ml is already a great improvement and further experiments may render even greater concentrations.

4.6.9 Nasal administration of rhGH in a TMC H-L solution

Table 4.10 gives the plasma rhGH concentrations obtained after nasal administration with a TMC H-L solution. The rhGH plasma concentrations are measured in µIU/ml over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. The results are graphically depicted in figure 4.11.

Table 4.10: Plasma rhGH concentrations after nasal administration with a TMC H-L solution (rhGH 3.6 IU/kg).

Administration of rhGH in TMC H - L solution									
	Subject number								
Time (min)	1	2	3	4	5	6	Average (µIU/ml)	SD	SE
0	0.00	0.00	0.16	0.00	0.00	0.00	0.03	0.07	0.03
5	0.21	0.30	0.53	0.63	0.86	0.26	0.47	0.25	0.10
10	6.90	3.04	4.41	5.74	6.56	3.09	4.96	1.70	0.69
15	27.41	7.76	12.44	10.22	10.91	7.97	12.79	7.38	3.01
30	29.29	34.64	27.43	27.75	17.83	17.42	25.73	6.79	2.77
60	26.68	41.12	28.82	32.09	18.09	35.55	30.39	7.90	3.23
120	16.68	23.24	20.57	15.66	8.88	22.44	17.91	5.37	2.19
180	12.09	13.05	8.22	8.44	4.34	9.63	9.30	3.11	1.27

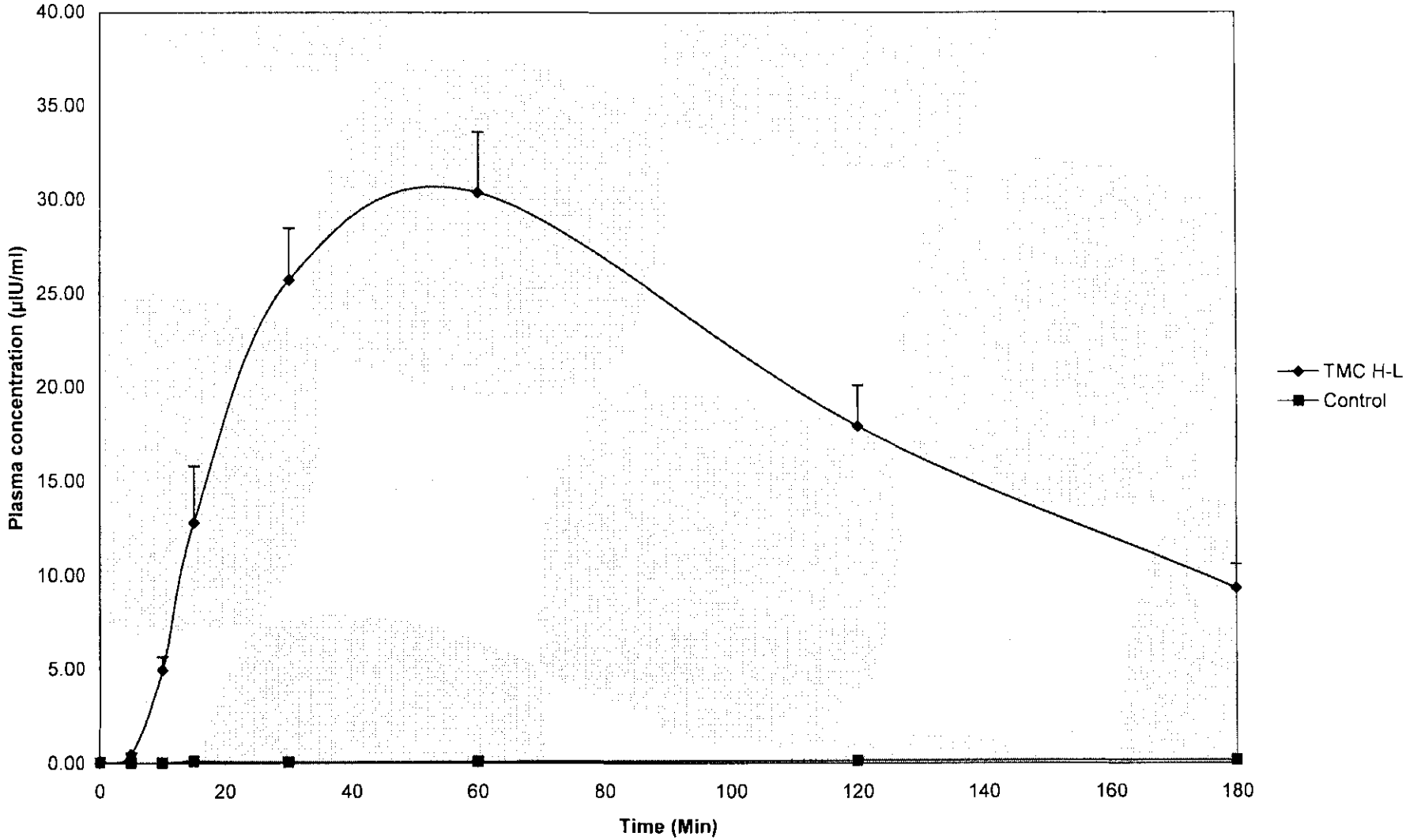


Figure 4.11: Plasma rhGH concentrations after nasal administration with a TMC H-L solution (rhGH 3.6 IU/kg).

In contrast to the control formulation, the nasal administration of rhGH with a TMC H-L solution rendered much higher plasma concentrations.

At time t = 0 minutes the measured plasma concentration of rhGH was 0.03 µIU/ml and at time t = 5 minutes a concentration of 0.47 µIU/ml was recorded. The concentration increased to 4.96 µIU/ml at time t = 10 minutes and at time t = 15 minutes a further increase to 12.79 µIU/ml was observed. At time t = 30 minutes the concentration was already in the mid twenties with a value of 25.73 µIU/ml and still climbing to its maximum recorded concentration of 30.39 µIU/ml at time t = 60 minutes. At time t = 120 minutes, a decrease in the rhGH concentration is observed with a value of 17.91 µIU/ml which is followed by an even lower value of 9.63 µIU/ml at time t = 180 minutes. These results are in agreement with results obtained in previous studies where TMC was also used as an absorption enhancer for several other peptide drugs. TMC act primarily by opening the tight junctions in the cell membrane thereby allowing paracellular transport of the drug molecules. This study with rhGH confirms that TMC is a potent absorption enhancer for peptide drugs.

4.6.10 Nasal administration of rhGH in a TMC H-H solution

Table 4.11 gives the plasma rhGH concentrations obtained after nasal administration with a TMC H-H solution. The rhGH plasma concentrations are measured in µIU/ml over a period of 180 minutes. The symbols SD and SE represent the standard deviation and standard error values respectively. The results are graphically depicted in figure 4.12.

Table 4.11: Plasma rhGH concentrations after nasal administration with a TMC H-H solution (rhGH 3.6 IU/kg).

Administration of rhGH in TMC H-H solution									
	Subject number								
Time (min)	**1**	**2**	**3**	**4**	**5**	**6**	**Average (µIU/ml)**	**SD**	**SE**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.31	0.62	0.80	0.39	0.48	0.23	0.47	0.21	0.09
10	0.31	2.73	4.97	2.20	4.36	0.86	2.57	1.85	0.76
15	4.52	8.52	9.59	3.78	9.33	3.93	6.61	2.81	1.15
30	7.94	13.95	13.56	9.86	18.27	9.74	12.22	3.78	1.54
60	10.13	14.08	15.23	11.93	23.60	21.21	16.03	5.30	2.16
120	5.65	7.94	11.10	17.21	14.59	16.33	12.14	4.69	1.92
180	2.01	4.42	10.53	21.66	9.94	11.19	9.96	6.82	2.78

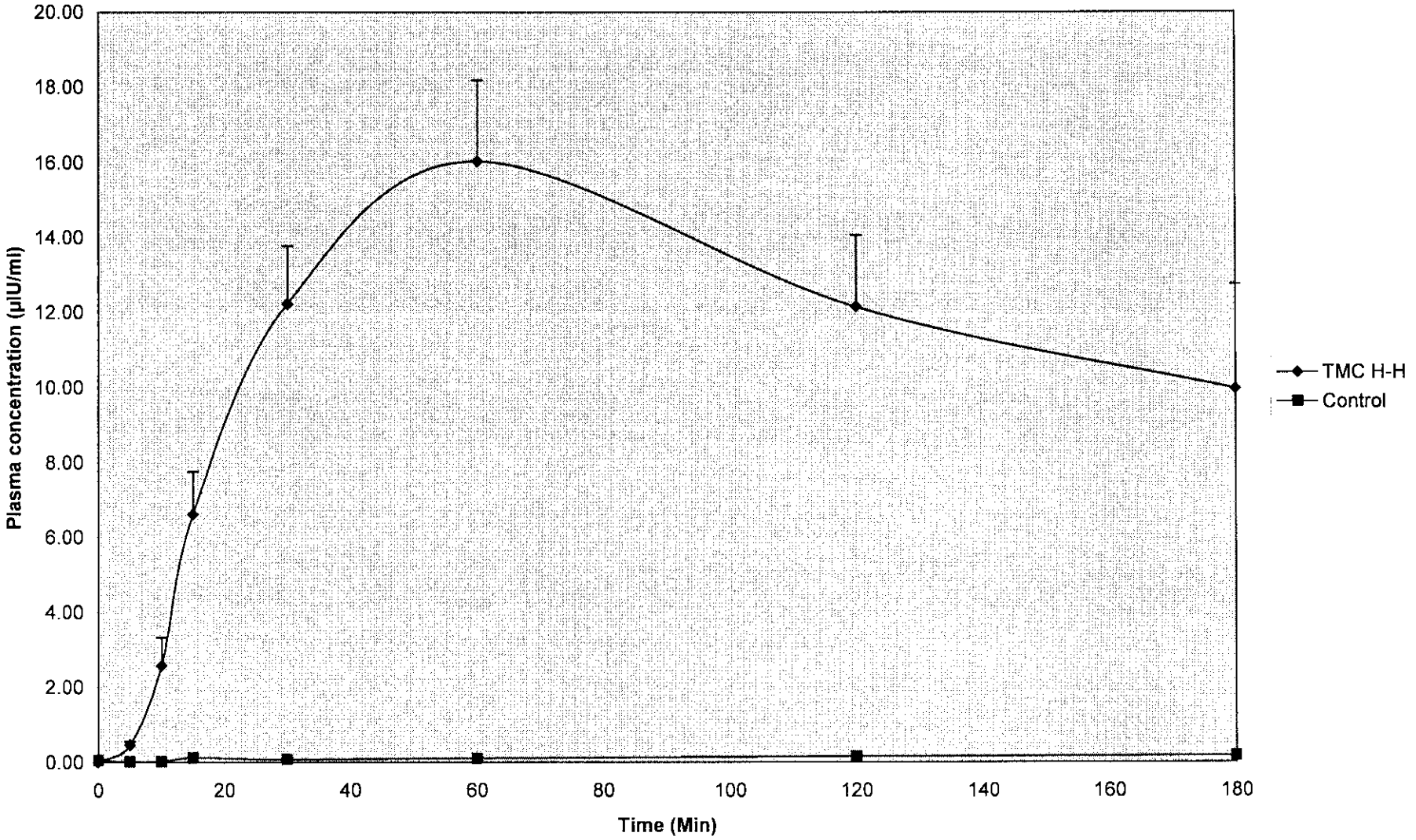


Figure 4.12: Plasma rhGH concentrations after nasal administration with a TMC H-H solution (rhGH 3.6 IU/kg).

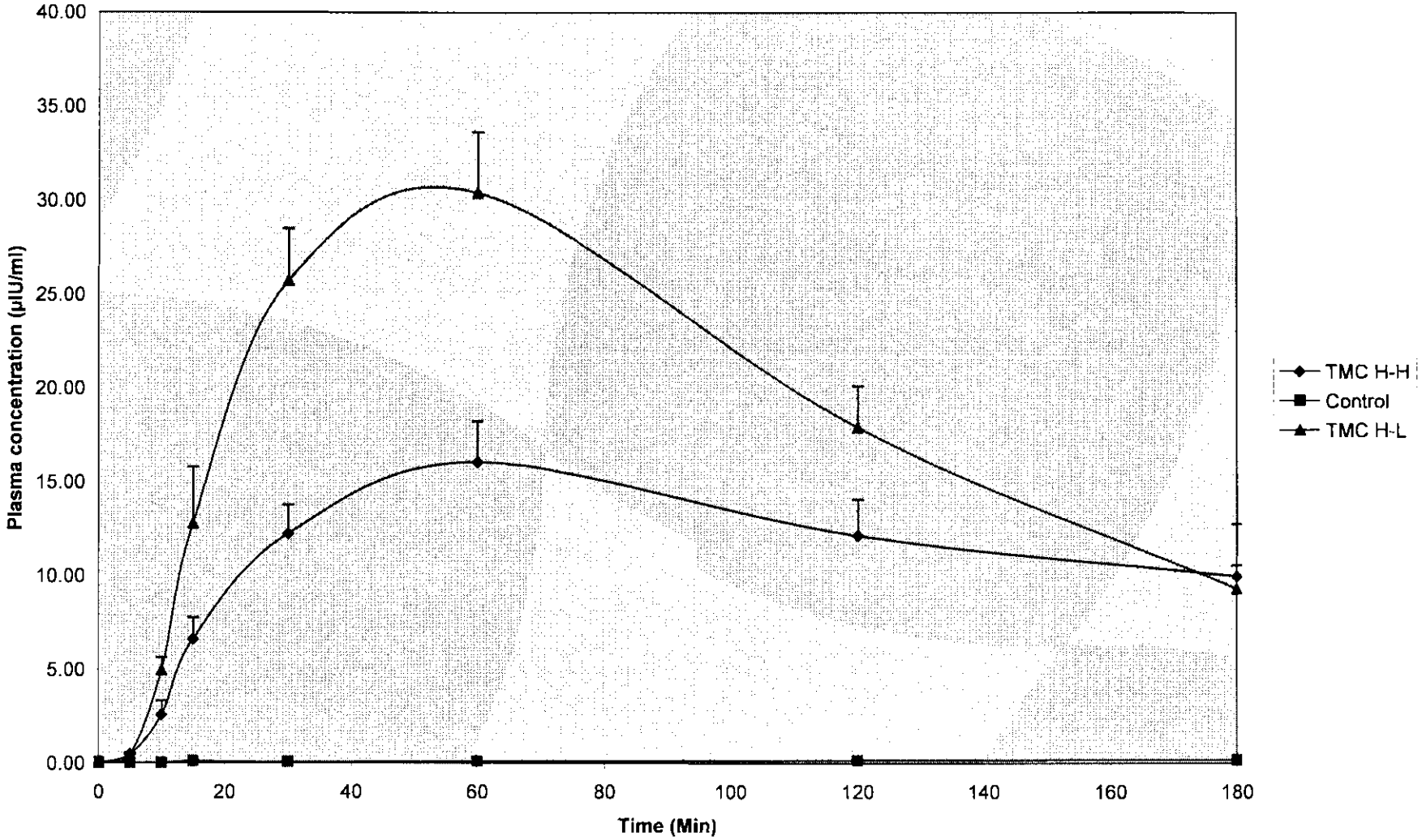


Figure 4.13: Comparison between plasma rhGH concentrations after nasal administration with TMC H-L and TMC H-H solutions respectively (rhGH 3.6 IU/kg).

In contrast to the control formulation, the nasal administration of rhGH with a TMC H-H solution rendered much higher plasma concentrations. At time t = 0 minutes the measured concentration of rhGH was 0 µIU/ml and at time t = 5 minutes a concentration of 0.47 µIU/ml was recorded. The concentration increased to 2.57 µIU/ml at time t = 10 minutes and at time t = 15 minutes a further increase to 6.61 µIU/ml was observed. At time t = 30 minutes the concentration doubled to 12.22 µIU/ml and was still rising to its maximum recorded concentration of 16.03 µIU/ml at time t = 60 minutes. At time t = 120 minutes a decrease in the rhGH concentration is observed with a value of 12.14 µIU/ml and this is followed by an even lower value of 9.96 µIU/ml at time t = 180 minutes.

These results are in agreement with results obtained in previous studies where TMC was also used as an absorption enhancer for several other peptide drugs. It is interesting to note that although the maximum values recorded for TMC H-L and TMC H-H differ significantly, the concentration time profiles are very similar in configuration and both solutions reach its maximum concentration at time t = 60 minutes. TMC H-H seem to open the tight junctions to a lesser extent than TMC H-L which in turn translates to a lower plasma concentration. These results indicate that the degree of quaternisation, 73.33% for TMC H-H and 18.20% for TMC H-L, play and important role in the absorption enhancing properties of TMC when used to administer large peptide drug molecules.

4.6.11 Comparison of obtained results

The goal of this study was to determine the effectiveness of both of the TMC formulations as well as the Pheroid vesicle and Pheroid microsponge formulations to act as absorption enhancers for rhGH.

Since the administered dose was the same in all of the above formulations (rhGH 3.6 IU/Kg) it would be fair to link the effectiveness of each formulation directly to the maximum average rhGH plasma concentration obtained with each formulation. Table 4.12 gives the maximum average plasma concentrations of rhGH obtained for each formulation in comparison to the control formulation.

Table 4.12: Maximum rhGH plasma concentrations.

Formulation	Time of maximum response	Concentration (µIU/ml)
Control	-	0.18
Pheroid vesicles	3 h	27.90
Pheroid microsponges	3 h	4.0
TMC H-H	60 min	16.03
TMC H-L	60 min	30.39

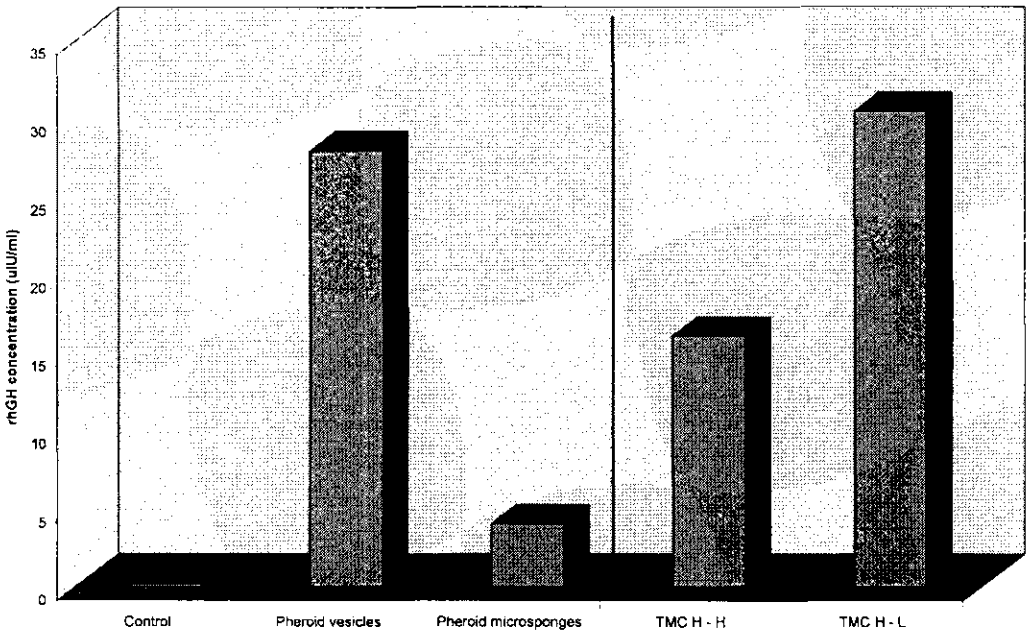


Figure 4.14: Maximum rhGH plasma concentrations.

The difference in the effectiveness between the TMC and Pheroid formulations can be linked to their different mechanisms of action.

The Pheroid formulations act by transporting the entrapped drug molecules via the transcellular pathways in contrast to the paracellular pathways which is employed by the TMC formulations for drug permeation. TMC facilitates paracellular permeation of drug molecules by opening the tight junctions between cells.

The difference in the T_{max} values can be attributed to the difference in the pathways employed for permeation. It is evident from the obtained results that the paracellular pathway

has the least resistance to drug permeation which explains why the TMC formulations have reached their T_{max} values much quicker than the Pheroid formulations.

4.7 CONCLUSION

Based on the obtained results it is clear that TMC H-L is the most effective absorption enhancer followed by the Pheroid vesicles and then TMC H-H in the 3rd place. The Pheroid microsponges was the least effective of all the test formulations. Both of the TMC formulations rendered very promising results, the TMC H-L formulation achieved an average maximum rhGH plasma concentration of 30.39 µIU/ml and the TMC H-H achieved a concentration of 16.03 µIU/ml. Both of the formulations reached their peak values only 60 minutes after administration with a very definite decline in plasma concentration 180 minutes after administration. In the 5 hour experiment with the Pheroid vesicles, it became quite evident that the average maximum rhGH plasma concentration is only reached after 3 hours, it achieved a very respectable concentration of 27.90 µIU/ml, with a gradual decline in concentration towards the end of the experiment. The Pheroid vesicles performed much better than the Pheroid microsponges which only managed to achieve a maximum concentration of 4.0 µIU/ml. Both the Pheroid microsponges and Pheroid vesicles reached their maximum concentrations at 3 hours after administration with a gradual decrease in concentration towards the 5 hour mark. The control formulation, rhGH in saline, clearly shows that virtually no absorption of rhGH took place without the aid of an absorption enhancer. The obtained results indicate that absorption enhancement of rhGH did take place with all 4 test formulations.

SUMMARY AND FUTURE PROSPECTS

Various studies have proven that large peptide and protein drugs, with a molecular weight greater than 1 000 Da, are not easily absorbed via the most popular delivery routes. This low rate of absorption can be attributed to a few inherent characteristics of these drugs such as:

- high molecular weight;
- polarity and charge density;
- instability due to pH fluctuations and
- low lipophilicity.

The effective absorption of these drugs are also complicated by various other factors which include:

- low permeability and bioavailability;
- rapid clearance and
- enzymatic degradation.

To achieve therapeutic levels of these compounds it is essential to overcome all of the above mentioned challenges. This may be accomplished either by exploring different routes of administration or by adding penetration enhancers to the existing formulations. A combination of both of these alternatives may also be a feasible option. It is clear that the development of a pharmaceutically acceptable delivery system for peptide and/or protein drugs is much more complicated than meets the eye.

The nasal route offers a few unique advantages, in comparison to other routes of administration, for the delivery of both peptide and protein drugs. One very important advantage is the fact that the effect of first pass metabolism is completely eliminated when administering a drug via the nasal cavity which in turn translates to potentially higher plasma concentrations of that specific drug. Another advantage of nasal administration is that it may improve patient compliance when considering that the parenteral route is the most common route of administration for peptide and protein drugs which is very invasive and traumatising for some patients.

To improve the nasal absorption of rhGH, a protein drug, several absorption enhancers was considered for the *in vivo* experiments which was carried out on male Sprague Dawley rats.

The rhGH was entrapped in Pheroid vesicles and Pheroid microsponges to establish their effectiveness as absorption enhancers for rhGH. Two other formulations were also prepared for this study, namely TMC H-H and TMC H-L. The concentration of both of these polymer formulations was 0.5% w/v. All four of the above formulations contained rhGH (3.6 IU/Kg) and was administered directly into the left nostril of each test subject by means of a micropipette. A dose of 25 - 35 µl was administered to each test subject. The appropriate dose was calculated specifically for each subject based on the fasted bodyweight of the test animal (100 µl/kg bodyweight).

The fasted bodyweight varied between 250 - 350 g. Blood samples were collected at various time intervals. Most of the experiments was carried out over a 3 hour period and a few was conducted over 5 hours. The blood samples were centrifuged and the plasma was subsequently removed and stored at - 70 °C. The plasma samples were analyzed with a rhGH-IRMA kit, obtained from BioSource® to determine the rhGH content of each sample.

The results obtained in this study clearly indicate that TMC H-L, and to a lesser extent TMC H-H, is the most effective absorption enhancers when compared to the other formulations included in this study. The effectiveness of TMC can most probably be attributed to its superior mucoadhesive properties and its ability to open tight junstions in order to facilitate absorption of the administered rhGH through the paracellular pathway. The Pheroid vesicles came in at a close second place to TMC H-L. The Pheroid microsponges did improve the absorption of rhGH but to a lesser extent than the other test formulations.

It is clear that Pheroid technology has great potential as an absorption enhancer and further studies should be conducted to exploit this system's full potential. Chitosan and TMC polymers are well known polymers and numerous researchers have already confirmed its astounding absorption enhancing capabilities. This study once again confirmed the effectiveness of TMC derivatives as a potent absorption enhancer.

RECOMMENDATIONS

A few recommendations to consider for future studies on Pheroid technology:

- Further studies should be conducted to determine the exact mechanism of action of Pheroids. These studies should be conducted on a cellular and molecular level.
- Complete toxicity studies should be performed on all of the Pheroid formulations.
- The stability profile of different peptide and protein drugs, entrapped in Pheroid formulations, should be investigated.
- The differences in absorption efficacy, onset and duration of effect between Pheroid vesicles and Pheroid microsponges should be investigated in more detail.
- The effect of different concentrations of rhGH in combination with these absorption enhancers should be evaluated.
- In order to effectively administer nasal Pheroid formulations it is essential to develop a suitable and effective administration device with zero interaction with any of the Pheroid components.

ABSTRACT

Previous studies have demonstrated that chitosan and its derivative, *N*-trimethyl chitosan chloride (TMC) are effective and safe absorption enhancers to improve mucosal delivery of macromolecular drugs, including peptide and protein drugs. Pheroid™ technology has proven in the past to be an effective delivery system for numerous therapeutic substances. Pheroids™ can entrap, transport and deliver large amounts of drugs, including peptide and protein drugs.

This study investigated the possibility to effectively administer and enhance the absorption of the macromolecular protein drug, recombinant human growth hormone, via the nasal cavity with the aid of a few different absorption enhancers. Two different TMC formulations, namely TMC H-L and TMC H-H are evaluated and compared to two Pheroid™ formulations, namely Pheroid™ vesicles and Pheroid™ microsponges.

The research strategy was to administer recombinant human growth hormone (3.6 IU/kg) in a physiological saline solution and in combination with the two different Pheroid™ and TMC formulations via the nasal cavity. *In vivo* studies were conducted on fasted male Sprague-Dawley rats. The *artery carotis communis* was cannulated for the collection of blood samples at various predetermined time intervals. The first blood sample was collected at time t = 0, directly before administration, and thereafter at the various predetermined time intervals. Blood samples were collected and centrifuged within 20 minutes after collection and the recovered plasma was stored at - 70 °C until analysis. Analysis were done with the aid of a rhGH-IRMA kit.

All of the test formulations did increase the recorded plasma concentration of rhGH in comparison to the control formulation. The greatest increase in absorption was obtained with the TMC H-L formulation followed by the Pheroid™ vesicles in second place and TMC H-H in third. The Pheroid™ microsponge formulation was the least effective.

It can be concluded that Pheroid™ technology has great potential as an absorption enhancer for both peptide and protein drugs and that further studies must be conducted in order to discover its full potential. TMC has once again proved itself as one of the greatest absorption enhancers currently available and will be of great value for many years to come.

Key words: Recombinant human growth hormone (rhGH), Pheroid™ vesicles, Pheroid™ microsponges, *N*-trimethyl chitosan chloride (TMC), nasal administration.

UITTREKSEL

In vorige studies is bewys dat chitosan en sy derivaat, *N*-trimetiel chitosan chloried (TMC), doeltreffende en veilige absorpsiebevorderaars is wat die aflewering van groot makromolekulêre geneesmiddels soos peptiede en proteïene oor mukusmembrane kan verbeter. Vorige studies het ook bewys dat Pheroid™ tegnologie oor die vermoë beskik om 'n groot verskeidenheid terapeutiese stowwe, veilig en effektief af te lewer. Pheroid™ kan groot hoeveelhede geneesmiddels, insluitende proteïen- en peptiedgeneesmiddels, enkapsuleer, vervoer en aflewer.

Hierdie studie het die moontlikheid ondersoek om makromolekulêre proteïengeneesmiddels soos byvoorbeeld rekombinante menslike groeihormoon via die nasale toedieningsroete af te lewer. Die effektiwiteit van twee gewilde absorpsiebevorderaars en 'n paar variante daarvan is ondersoek en met mekaar vergelyk. Daar is twee TMC formulerings voorberei, naamlik TMC H-L en TMC H-H wat elk rekombinante menslike groeihormoon bevat (3.6 IU/kg). Daar is verder ook twee Pheroid™ formulerings saamgestel, naamlik Pheroid™ mikrodruppeltjies en Pheroid™ mikrosponsies wat dieselfde dosis rekombinante menslike groeihormoon as die TMC formulerings bevat.

Die voorbereide toetsformulerings en 'n kontrole formulering, rekombinante menslike groeihormoon (3.6 IU/kg) in 'n fisiologiese soutoplossing, is intranasaal toegedien. *In vivo* studies is uitgevoer op manlike Sprague-Dawley rotte wat gevas is voor die aanvang van die eksperimentele werk. Bloedmonsters is met behulp van 'n kanule geneem wat in die *carotis communis* arterie ingeplant is. Bloedmonsters is stiptelik op verskeie voorafbepaalde tydsintervalle geneem. Die eerste bloedmonster is op tyd t = 0 geneem, direk voor toediening, en daarna op die voorafbepaalde tydsintervalle. Alle bloedmonsters was binne 'n maksimum tydperk van 20 minute na versameling gesentrifugeer en die plasma wat sodoende verkry is, was gestoor by - 70 °C tot by uitvoer van die analise. Die analise was met behulp van 'n rhGH-IRMA analisepakket uitgevoer.

Al die toetsformulerings het 'n verhoogde plasmakonsentrasie van rekombinante menslike groeihormoon teweeg gebring in vergelyking met die kontrolegroep. Die TMC H-L-formulering het die beste gevaar met die Pheroid™ mikrodruppeltjie formulering kort op sy hakke. Die TMC H-H formulering het ook uitstekende resultate gelewer, terwyl die Pheroid™ mikrosponsie formulering die laagste plasmakonsentrasie van al die toetsformulerings opgelewer het.

Dit is uit die resultate baie duidelik dat Pheroid™ tegnologie groot moontlikhede inhou en dat daar meer omvattende eksperimente uitgevoer moet word om die volle potensiaal van hierdie tegnologie te ontsluit.

TMC het weereens uitstekende resultate opgelewer en dit is duidelik dat dit 'n onmisbare plek inneem as absorpsiebevorderaar vir beide peptied en proteïen geneesmiddels en dit sal vir nog baie jare van groot waarde wees.

Sleutelwoorde: Rekombinante menslike groeihormoon (rhGH), Pheroid™ mikrodruppeltjies, Pheroid™ mikrosponsies, *N*-trimetiel chitosan chloried (TMC), nasale toediening.

ANNEXURES

ANNEXURE 1

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

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

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

4 July 2005

Dear Prof Kotze

APPROVAL FOR EXPERIMENTING WITH HUMAN SUBJECTS

Your application for project "*Enhancement of somatotropin after nasal and peroral administration with Emzaloid™ technology*" has been approved with reference number 05D18. Please quote this number in all correspondence regarding your project. According to a decision by the Senate (4 November 1992, Art. 9.13.2) approval of a project is valid for a period of five years. There after you have to re-apply.

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

Wishing you every success

Yours sincerely

PROF NT MALAN
CHAIRMAN

ANNEXURE 2

DEPARTMENT OF HEALTH
Republic of South Africa

FAX COVER SHEET

TO: **SCHOOL OF PHARMACY**

FOR ATTENTION: **PROF. AF KOTZE**

FAX NO: 018 2992248

NO OF PAGES: 1 (Including this one:) DATE: 13/01/2006

MEDICINE REGULATORY AUTHORITY
INSPECTORATE & LAWENFORCEMENT

SUBJECT: **AUTHORIZATION TO IMPORT SAMPLES FOR EXPERIMENTAL/RESEARCH PURPOSES ;PROJECT 05D18**

MESSAGE:
Your request dated 13/01/2006 refers :
Authorization is hereby granted to WARREN CHEM SPECIALITIES (MR JD STEYN) to import 40 mg sample of rHGH (RECOMBINANT HUMAN GROWTH HORMONE)
for experimental purposes on project study 05D18 purposes in terms of section 15(1) of the Medicines and Related Substances Control Act, (Act 101 of 1965)

Importation of the sample of this product is allowed on a ounce off basis

Kind Regards,

MEDICINE REGULATORY AFFAIRS
LAW ENFORCEMENT
DIRECTOR GENERAL

FROM: **PE BEKKER**
FAX NO: **012 312 0914** **E-MAIL bekkep@health.gov.za**
TEL: **082 4416003/ 012 312 0023**

ANNEXURE 3

ANHUI ANKE BIOTECHNOLOGY CO., LTD.

ADD: AnkeBio Building, 669 Changjiang Road, West, Hefei, Anhui 230088, China
TEL:+86-551-5319890, 5318811 FAX:+86-551-5319895
e-mail:ankebio@mail.hf.ah.cn http://www.ankebio.com

CERTIFICATE OF ANALYSIS

Product : SOMATROPIN/RECOMBINANT HUMAN GROWTH HORMONE FOR INJ.
Batch No.: 20051211 Report Date: 20051227
Quantity: 15 IU/vial Mfg. Date: 2005. 12. 11
Test Basis: EP4th. Exp. Date: 2007. 12 (STORAGE:2-8℃)

Items of Test	Standard	Results of Analysis
CHARACTERS	A WHITE FREEZE-DRIED POWDER	CONFORMED
IDENTIFICATION	POSITIVE	CONFORMED
RELATED PROTEINS (HPLC/RP)	≤ 10%	CONFORMED
DIMER & RELATED SUBSTANCES OF HIGHER MOLECULAR MASS (HPLC/SEC)	≤ 6.0%	CONFORMED
ISOELECTRIC FOCUSING (IEF)	UNIQUE BAND OF IP≈ 5.2	CONFORMED
BACTERIAL ENDOTOXIN	≤ 5.0 EU/mg rHGH	< 5.0 EU/mg rHGH
WATER CONTENT	≤ 3.0%	CONFORMED
TEST FOR PYROGENS	CONFORMED	CONFORMED
TEST FOR STERILITY	CONFORMED	CONFORMED
ASSAY	89.0% - 105.0%	CONFORMED

CONCLUSION: CONFORMS TO EP4th.

NOTE: 1mg rHGH ≈ 3IU

Issued by manufacturer
ANHUI ANKE BIOTECHNOLOGY CO., LTD
安徽安科生物工程股份有限公司
Signature: _____
Authorized Signature

Warren Chem Specialiti
(PTY) LTD
P.O. BOX 29069
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The greatest pleasure in life is doing what people say you cannot do.

Walter Bagehot